UNIVERSITY OF CALIFORNIA, MERCED

The RhoUV Family of Atypical RhoGTPases is a Possible Regulator of Endoderm Morphogenesis

A Thesis submitted in partial satisfaction of the requirements for the degree of Master of Science

in

Quantitative and Systems Biology

by

Leesa Annalyn Strasser

Committee in charge:

Professor Xuecai Ge, Chair

Professor Chris Amemiya

Professor Laura Beaster-Jones

Professor Stephanie Woo, Advisor

The Thesis of Leesa Strasser is approved, and it is acceptable in quality and form for publication electronically:

Xuecai Ge, Chair

Chris Amemiya

Laura Beaster-Jones

Stephanie Woo

University of California, Merced

2023

Dedication

I dedicate this to my husband, Steven, who was with me at the very beginning of this journey and supported me throughout. I also dedicate this to my family for their continuous encouragement.

Table of Contents

List of Abbreviations

EMT	Epithelial to Mesenchymal Transition
EVL	Enveloping Layer
GAP	Guanine Activating Protein
GDI	Guanine Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine Exchange Factor
GRB2	Growth Factor Receptor-Bound Protein 2
GTP	Guanosine Triphosphate
HH	Hamburger-Hamilton
JNK	Jun N-terminal Kinases
NF	Nieuwkoop and Faber
MET	Mesenchymal to Epithelial Transition
MDCK	Madin-Darby Canine Kidney Cells
PAK	P21 Activated Kinase
qPCR	Quantitative Polymerase Chain Reaction
RHO	Ras homologous protein
T-ALL	T-Cell Acute Lymphoblastic Leukemia
TNBC	Triple Negative Breast Cancer

List of Tables

List of Figures

Acknowledgments

I would like to acknowledge my advisor, Dr. Stephanie Woo for allowing me to be her first graduate student at UC Merced. I thank Dr. Woo for her guidance and encouragement during my graduate studies. I also thank members of the Woo and Materna labs, especially Jesselynn LaBelle and Gloria Ligunas, for the many times of assistance, helpful conversations, and laughs. I would like to acknowledge Dr. Chris Amemiya for the beginning of my real research lab experience as a technician in his lab. I also would like to acknowledge Dr. Laura Beaster-Jones as being a great role model for pedagogy in the biology field. Lastly, I would like to acknowledge Dr. Xuecai Ge for being my committee chair and providing helpful comments.

Abstract

The RhoUV Family of Atypical RhoGTPases is a Possible Regulator of Endoderm Morphogenesis

Leesa Annalyn Strasser

Master of Science in Quantitative and Systems Biology

University of California, Merced

Advisor: Stephanie Woo

RhoU and RhoV are Rho GTPases a part of the Ras superfamily and are atypical due to fast-cycling of GTP/GDP, making them constitutively active. Their unique N and C terminals regulate their submembrane localization for cellular activity. This along with their fast-cycling nature categorizes them into their own subfamily. Rho GTPases are well known for their regulation of cell migration, polarity, apoptosis, proliferation and many other processes depending on effector protein interaction and behave in a switch-like mechanism. Over the last 20 years, studies have been connecting RhoV and RhoU to regulating the cytoskeleton due to their involvement with adhesion protein localization and induction of migratory protrusions. Our lab has found that the Rho GTPase RhoV is dynamically expressed in the zebrafish endoderm; *rhov* expression is high during gastrulation and low during mesenchymal to epithelial transition (MET). The endoderm is an essential germ layer that will form the epithelial layer of organs such as the respiratory tract, gut, and intestines. How MET is triggered in endodermal cells is still unknown. RhoV is a Rho GTPase whose role has not been investigated within the endodermal cell migration to sheet formation. Here, we used CRISPR-generated RhoV mutants and *rhov* overexpression to investigate the role of RhoV in the mesenchymal to epithelial transition in zebrafish endoderm. This work, in addition to past studies investigating RhoU and RhoV in development provides compelling reasoning into how RhoU and RhoV may be regulating endodermal cell migration in zebrafish.

Chapter 1 Atypical Rho GTPases RhoU and RhoV in Development and Disease

1.1 Regulation of Rho GTPases

Rho GTPases are a family within the Ras superfamily of small GTPases (Hodge and Ridley, 2020) and are involved in several processes including cytoskeleton reorganization, cell proliferation, apoptosis, adhesion, and motility (Ridley 2001). The Rho family proteins are characterized by a rho-specific insert within the GTPase domain (Freeman et al. 1996). Like other G proteins, Rho GTPases act as molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state. Rho GTPases are activated by GEFs (guanine nucleotide exchange factors), which trigger the exchange of GDP for GTP. In the active state, Rho proteins undergo a conformational shift in the switch regions to allow specific effectors to bind, activating downstream signals for various cell processes (Zegers and Friedl, 2014; Mosaddeghzadeh and Ahmadian, 2021). Rho GTPases are inactivated by GAPs (GTPase-activating proteins), which trigger the hydrolysis of GTP to GDP (Ridley 2001). In addition to GTP/GDP binding, Rho GTPases are also regulated by subcellular localization. Most Rho proteins are post-translationally prenylated, which allows them to be localized to membranes where activating GEFs and downstream effectors are located. GDIs (guanine dissociation inhibitors) prevent non-specific activation of Rho GTPases by binding and masking these lipid modifications, sequestering the Rho proteins in the cytoplasm and away from membranes (Spiering et al. 2011; Mosaddeghzadeh and Ahmadian, 2021). The best characterized Rho GTPases are Rac1, RhoA, and Cdc42. These are particularly well known for their regulation of actin dynamics and cell migration. However, the functions of other Rho GTPases have recently become better appreciated.

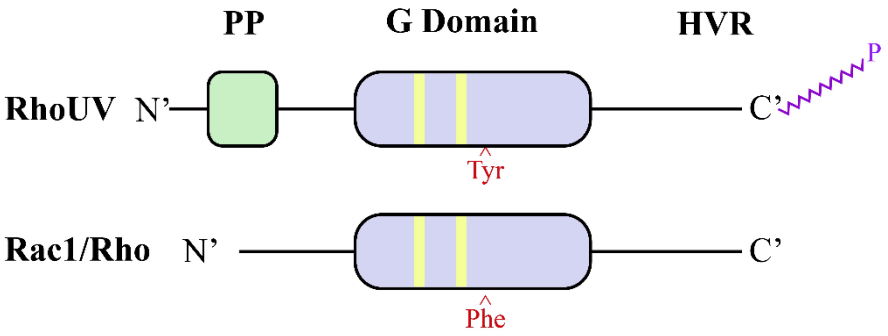


Figure 1.1.1 Structure of RhoUV and Typical RhoGTPase. The structure of Rho GTPases consists of conserved G Domain containing the switch mechanism sequences in yellow. RhoUV is distinguished from typical Rho GTPases by their additional polyproline rich sequence and palmitoylated C-terminal. The fast-cycling nature of RhoUV may be due to the Tyrosine at the codon site of Phenylalanine in Rac1.

1.2 Structure and Function of RhoU and RhoV

Rho proteins are divided into different subfamilies based on homology. RhoV (also known as Chp or Wrch2) and RhoU (also known as Wrch1) belong to a subfamily of Rho GTPases distinguished by their high rate of GTP exchange and distinct N and C termini. The RhoUV subfamily is derived from Cdc42 and share 57% and 52% sequence identity with Cdc42, respectively, and is conserved among mammals (Chenette et al. 2005; Boureux et al. 2007).

RhoU and RhoV differ from other Rho GTPases in several ways. First, they have a unique N-terminal polyproline domain that can bind SH3 domain-containing adaptor proteins. In addition, while RhoUV proteins are post-translationally lipid modified on their C-terminal ends like other Rho GTPases, they are S-palmitoylated rather than prenylated and, more strikingly, this S-palmitoylation is reversible. As a consequence, it is thought that membrane localization and thus activity of RhoU and RhoV is regulated not by Rho-GDI binding but by the presence or absence of S-palmitoylation (Berzat et al. 2005; Chenette et al. 2006). Consistent with this idea, the C-terminus of RhoV was demonstrated to be required for RhoV's ability to induce lamellipodia and localize to the golgi (Aronheim et al. 1998).

The structure and regulation of the RhoUV GTPase domain also contributes to their unique characteristics. RhoU along with RhoD and RhoF have historically been considered "atypical" Rho GTPases due to their significantly faster rates of GTP/GDP exchange, which is thought to render them more active than "typical" Rho GTPases and less reliant on regulation by GEFs and GAPs. GTP/GDP exchange rates have not been measured for RhoV but are also assumed

to be elevated due to sequence similarity with RhoU (Aspenström 2022). The exact mechanism of this "fast cycling" is unknown but may be due to differences in a key amino acid residue. In Rac1, a phenylalanine to leucine mutation at position 28 leads to a faster GTP/GDP exchange rate and produces gain of function effects when expressed in cells (Jaiswal et al. 2013; Saras et al. 2004; Shutes et al. 2004). Atypical Rho GTPases like RhoU and RhoV have a tyrosine rather than a phenylalanine at this same position, suggesting that this residue contributes to the rate of GTP/GDP cycling (Reinstein et al. 1991; Aspenström, 2020). Because fast-cycling Rho GTPases are thought to be constitutively active, it has been suggested that these atypical GTPases are not regulated by GAPs, GEFs, or GDIs (Aspenström 2022). However, RhoGDI-3 was identified as a regulator of RhoV activity in mammalian cells and is speculated to act as a chaperone for RhoV's localization to cellular compartments (Mokhtar et al. 2021). Furthermore, both RhoU and RhoV have been shown to interact with β-Pix (also known as Arhgef7), which is a known Rho-GEF (Tay et al. 2010; Dickover et al. 2014).

1.3 Signaling Effectors of RhoU and RhoV

Downstream effectors have been identified for both RhoU and RhoV, most belonging to the p21-activated kinase (PAK) family of serine/threonine kinases. PAK proteins have well-characterized roles in regulating cytoskeleton dynamics, cell adhesion, and apoptosis (Hofmann et al. 2004; Kumar et al. 2017).

In a yeast two-hybrid assay, RhoV was shown to bind to PAK1, PAK2, PAK4, and PAK6 (Shepelev and Korobko, 2012; Aspenström et al. 2004; Aronheim et al. 1998; Weisz Hubsman et al. 2007). RhoU has been shown to bind to PAK4 (Dart et al. 2015). PAK1 is known to regulate cell adhesion by forming a multiprotein complex with β -PIX and Rac1. In zebrafish embryos, RhoV was shown to interact with both PAK1 and β -PIX to control the localization of the adherens junction proteins E-cadherin and β-catenin, while RhoU was also shown to work with PAK1 and β -PIX to localize N-cadherin and the cell adhesion molecule Alcama (Tay et al. 2010; Dickover et al. 2014).

RhoV has also been linked to growth factor receptor signaling. By immunoprecipitation, RhoV was shown to directly bind to GRB2, an SH3 domain-containing adapter protein that functions downstream of the epidermal growth factor receptor (EGFR). Disrupting the binding between RhoV and GRB2 in breast cancer cells inhibited EGF-dependent migration, which also required PAK1 and Jun Kinase 1/2 (JNK1/2) (Jin et al. 2023). The JNK pathway has been linked to RhoV during induction of apoptosis in PC12 and HEK298 cells (Shepelev et al. 2011). By immunoprecipitation, RhoV has been shown to bind to

N-WASP, MLK3, Pak1B, and Par6 (Aspenström et al. 2004), but the functional significance of these interactions has not been investigated.
Similar to RhoV, RhoU has been shown to interact with PAK1 and JNK1 — in this case, to induce cytoskeleton rearrangements to form filopodia (Tao et al. 2001). RhoU has also been linked to EGRFR signaling through GRB2, which was shown to activate JNK/AP1-dependent transcription and cell motility (Zhang et al. 2011). RhoU and PAK4 are highly expressed in metastatic breast cancer cells; PAK4 was shown to prevent RhoU degradation and both PAK4 and RhoU were shown to promote focal adhesion disassembly and cell migration (Dart et al. 2015). In Madin-Darbin canine kidney (MDCK) cells, RhoU was shown to interact with the cell polarity protein Par6 to facilitate tight junction formation and epithelial morphogenesis (Brady et al. 2009).

1.4 RhoU and RhoV in Development

1.4.1 Early development

RhoU and RhoV have been shown to be expressed during early development in several vertebrate embryos including chick, frog, mouse and zebrafish. Both RhoU and RhoV are Wnt responsive through noncanonical and canonical signaling, respectively. RhoU is transcriptionally regulated by Wnt-1, independent of β-catenin, and phenocopies the effect of Wnt overexpression on cellular transformation (Schiavone et al. 2009; Tao et al. 2001). In chick embryos, *cRhoV* is expressed in the primitive streak and Hensen's node at Hamburger-Hamilton (HH) stage 5 (Notarnicola et al. 2008). Its expression becomes restricted to the posterior end of the primitive streak by HH 7 and expands to neural folds and undifferentiated epithelial somites by HH8 (Notarnicola et al. 2008). *cRhoU* expression is similar to *cRhoV*, beginning expression at HH 5 in the primitive streak and Hensen's node, however it is also present in the prospective anterior neural plate (Notarnicola et al. 2008). In *Xenopus* embryos, RhoV is expressed strongly in the dorsal marginal zone at Nieuwkoop and Faber (NF) stage 10, where it functions downstream of canonical Wnt signaling in neural induction (Guémar et al. 2007). RhoU expression is also expressed at NF stage 10 within the dorsal marginal zone, neural plate border and pharyngeal arches (Chen et al. 2005). In mouse embryos, *Rhov* is faintly expressed between E1-E2.5. *Rhou* is expressed in the neural ectoderm between E8.5 – 9.75 and expression increases into adult stages (Blake et al. 2021). In zebrafish embryos, *rhov* is broadly expressed from 50-80% epiboly, after which its expression slowly decreases (Thisse et al. 2001). Zebrafish possess two *rhou* genes, *rhoua* and *rhoub*. Both are expressed at the 1-4 somite stage in the neural plate, however *rhoub* is also present in paraxial mesoderm (Thisse et al. 2001).

Functional studies of RhoU and RhoV during early embryonic stages is relatively understudied. In zebrafish, RhoV has been linked to epiboly — the process by which cells spread over and eventually cover the yolk. In zebrafish development, epibolic movements coincide with the specifications of germ layers. Knockdown of *rhov* expression by injection of antisense morpholino oligonucleotides resulted in zebrafish embryos being unable to complete epiboly, leading to embryonic lethality by 24 hours post-fertilization (Tay et al. 2010). These epiboly defects were shown to be due primarily to mislocalization of E-cadherin and β-catenin away from adherens junctions via a mechanism that also required β-pix and PAK1 (Tay et al. 2010).

RhoUV proteins have also been implicated in the establishment of apicobasal polarity in differentiating epithelial cells. In *C. elegans*, loss of the RhoU/V ortholog CHW-1 resulted in uniform distribution of Wnt receptors in vulval precursor cells, leading to an inability to apicobasal polarity (Kidd III et al. 2015). During polarization of MDCK cells, RhoU was shown to localize to apical and basolateral membranes, bind to the polarity protein Par6, and negatively regulate tight junction assembly (Brady et al. 2009).

1.4.2 Organ Formation

Cardiovascular System

RhoU and RhoV have been shown to be expressed in the developing cardiovascular system of both mouse and zebrafish embryos. In zebrafish, *rhoua* is expressed in the heart tube during the pharyngula stages Prim 15 to 25 (Thisse et al. 2001). In mice, RNA sequencing data showed *Rhou* expression in the heart and pericardium from E9 to adult stages, and *Rhov* was detected at low levels in the heart and pericardium from E12.5 to adult (Blake et al. 2021). In terms of function, *rhoua* knockdown in zebrafish embryos resulted in abnormalities in the atrioventricular canal as well as the aberrant cardiac looping. These defects were due to the mislocalization and downregulation of adhesion proteins N-cadherin and Alcama in cardiomyocytes. This study also showed that, similar to *rhov*, *rhoua* signals through *arhgef7b* (β-pix) and *pak1* to regulate cell adhesion (Dickover et al. 2014). RhoUV proteins have also been shown to regulated adhesion signaling in endothelial cells. RhoV was shown to promote focal adhesion formation while RhoU is associated with focal adhesion disassembly (Aspenström et al. 2004; Ory et al. 2007). Interestingly, the latter observation is in contrast with reports in Hela cells showing RhoU drives focal adhesion assembly through phosphorylated myosin (Chuang et al. 2007), suggesting the function of RhoU and RhoV may be cell-type specific.

Gastrointestinal Tract

Both RhoU and RhoV have been shown to be expressed in the developing gastrointestinal tract of chick and mouse embryos. In gastrointestinal organs, the inner epithelial layers are derived from the endoderm while the surrounding smooth muscle is derived from the mesoderm. In chick embryos, *cRhoV* is expressed in the endoderm-derived layers of the foregut, caudal hindgut, gizzard and cloaca (Notarnicola et al. 2008). *cRhoU* is broadly expressed throughout the mesoderm-derived layers of the entire GI tract except for the colon (Notarnicola et al. 2008). In mouse embryos, RhoU is expressed in the foregut epithelium; however its expression decreases once the epithelium develops into multiple layers (Loebel et al. 2011). *Rhov* is also detected in the mouse gut from E15 to adult, however its function has not been investigated (Blake et al. 2021).

Rhou-deficient embryonic stem cells exhibited decreased expression of endoderm markers, indicating that RhoU facilitates endoderm differentiation. Knockdown of *Rhou* in mouse embryos resulted in a collapsed foregut and irregular thickness of the epithelium. Additionally, these *Rhou*-deficient mice exhibited decreased F-actin and α-tubulin levels within the apical domain of the endodermal cells. However, this study did not observe any defects in apicobasal polarity or E-cadherin localization, in contrast to what was observed in zebrafish cardiomyocytes (Dickover et al. 2014).

Central Nervous System

RhoU and RhoV expression in the developing in the central nervous system has been reported for chick, mouse, and zebrafish embryos. In chick embryos, *cRhoU* expression is present in the neural tube and the optic vesicles (Notarnicola et al. 2008). In mouse, *Rhou* and *Rhov* are expressed in the central nervous system starting at E8 and E10, respectively, until adulthood (Blake et al. 2021). *Rhou* was also reported to exhibit significant expression in the spinal cord and trigeminal ganglion (Tao et al. 2001). In zebrafish, *rhov* is expressed first in the neural plate then the brain and retina (Thisse et al. 2001; Tay et al. 2010). *Rhoua* is mostly expressed in the brain while *rhoub* is present in the otic vesicle, retina, and spinal cord (Thisse et al. 2001).

1.4.3 Neural Crest Cells

Most Rho GTPases are known for their involvement in cell migration promoting both actin-driven protrusion and myosin-based contraction as well as the cellular signaling pathways the provide directionality during migration. During development, the neural crest cells undergo an epithelial-to-mesenchymal transition to migrate out of the neural tube and into several locations within the embryo as the differentiate into a wide variety of cell types. Both RhoU and RhoV have been demonstrated to play roles in neural crest induction and

migration. RhoU has been shown to be expressed in the migrating neural crest cells in both *Xenopus* and chick embryos (Fort et al. 2011; Notarnicola et al. 2008). In *Xenopus*, overexpression of RhoU promoted extensive lamellipodial protrusions in migrating neural crest cells. Both overexpression and knockout of RhoU inhibited proper cranial neural crest migration, indicating a balanced level of RhoU activity is required for optimal migration behavior (Fort et al. 2011). Rather than neural crest cell migration, RhoV has been linked to induction of neural crest progenitors. In *Xenopus* embryos, RhoV is initially expressed lateral to the neural plate in a region corresponding to the future neural crest progenitor domain. However, RhoV is no longer expressed once neural crest cells start migrating. Overexpression of RhoV led to expansion of the neural crest region while loss of RhoV function led to reduced expression of neural crest marker genes: Slug, Sox9, Sox10, and Twist (Guémar et al. 2007). RhoV expression was shown to be under canonical Wnt signaling, indicated by the lateral expansion of RhoV expression in the cranial neural crest region in response to Wnt overexpression (Guémar et al. 2007). Interestingly, in chick embryos, *cRhoV* expression in the neural folds overlaps with Wnt6, a known neural crest inducer (Garcia-Castro et al. 2002), suggesting that the role of RhoV in neural crest induction may be conserved.

Neural crest cells are not the only cell type in which RhoU and RhoV regulate migratory behaviors. In endothelial cells, filopodial protrusions can be induced by RhoU and RhoV, and lamellipodial protrusions can be induced by RhoV (Aspenström et al. 2004). RhoU has also been shown to increase migration and adhesion of osteoclast precursor cells (Brazier et al. 2009).

1.5 RhoU and RhoV in Disease

Many Rho GTPases are upregulated in tumors, including RhoU and RhoV (Haga and Ridley, 2016). A study which looked at expression of Rho GTPases in lung adenocarcinoma tumors found that RhoV was significantly overexpressed compared to all other Rho genes (Chen et al. 2021). This study also showed that RhoV silencing decreased cell proliferation markers while RhoV overexpression increased proliferation. RhoV was also identified as overexpressed in triple-negative breast cancer (TNBC), and its expression increased with cancer stage progression (Jin et al. 2023). RhoV may promote metastasis of tumor cells. Expression of an over-activated RhoV mutant promoted cell migration while RhoV knockdown decreased migration of breast cancer cells. In lung adenocarcinoma cells the overexpression of RhoV caused a decrease in E-cadherin and an increase of N-cadherin expression; this “cadherin switch” is a hallmark of epithelial-to-mesenchymal transition (Zhang et al. 2021).

Increased RhoU expression has been reported in several cancers including prostate cancer, breast cancer, and T-cell acute lymphoblastic leukemia (T-ALL) (De Piano et al. 2020; Dart et al. 2015; Bhavsar et al. 2013). Depletion of RhoU in T-ALL cell lines displayed a decrease in migration and an overall round morphology indicative of reduced adhesion (Bhavsar et al. 2013). RhoU has also been shown to promote cell adhesion turnover and cell migration in cancer cells through increased phosphorylation of the focal adhesion protein paxillin (De Piano et al. 2020; Dart et al. 2015). While these studies have linked RhoU overexpression to different cancers, RhoU downregulation has been linked to colorectal cancer (Slaymi et al. 2019). In mice, loss of RhoU induced hyperplasia of the gut epithelium and inhibited apoptosis, therefore allowing intestinal cells to over-proliferate (Slaymi et al. 2019). RhoU is highly expressed in human colon and small intestine and was found to maintain gut homeostasis, which is also dependent on Wnt signaling (Jeong et al. 2012; Krausova and Korinek, 2014; Schatoff et al. 2017). Since RhoU has been shown to be Wnt responsive in other contexts (Schiavone et al. 2009; Tao et al. 2001), it's possible that RhoU may act downstream from Wnt/β-catenin signaling during gut homeostasis.

In addition to cancer, RhoU and RhoV have been shown to be involved in infectious diseases. RhoV was shown to promote flavivirus infection and enhance viral entry of the Zika virus (Luu et al. 2021). This study is consistent with other reports of Rho GTPases promoting cytoskeleton rearrangements that make the cell more accessible for viral entry (Barocchi et al. 2005; Van den Broeke et al. 2014).

1.6 Conclusion

RhoU and RhoV research has increased in recent years due to their connections with different cancers. However, their importance in development is equally compelling and merits more investigation. One underappreciated aspect of RhoUV proteins may be their specialized functions, especially during development. Although similar in structure, RhoU and RhoV exhibit obvious differences in their spatiotemporal expression patterns. RhoV expression is often more restricted in terms of developmental time points and cell and tissue types while RhoU is often expressed more broadly; an example is the different expression patterns of RhoU and RhoV in the chick gastrointestinal tract (Notarnicola et al. 2008). RhoV also appears closely associated with progenitor cells, while RhoU is more involved in cell differentiation; for example, RhoV expression was observed in developing neural crest cells, while RhoU expression was detected once these cells started migrating (Notarnicola et al. 2008).

Several lines of evidence also point to a role for RhoU and RhoV in epithelial-to-mesenchymal transitions (EMT) and the counterpart mesenchymal to

epithelial transitions (MET). They can regulate the levels and localization of E- and N-cadherin. They also play important roles in neural crest cells, which prominently undergo EMT during their development. Finally, both RhoU and RhoV can promote tumor development and migration of cancer cells. Cancer metastasis involves cycles of EMT/MET as cells disseminate, invade other tissues, and establish secondary tumors. The contribution of RhoU and RhoV to EMT/MET is a potentially impactful area for future investigation.

Table 1.1 RhoUV functions during development

	Organism, Cell type	**Function**	**References**
RhoU	Zebrafish, cardiomyocytes	*Localization of adhesion proteins, N-cadherin and Alcama*	Dickover et al. 2014
	Mice, foregut endoderm	*Differentiation and cytoskeleton organization of F-actin in endoderm*	Loebel et al. 2011
	Xenopus, neural crest	*Induces migration*	Fort et al. 2011
RhoV	Zebrafish, EVL	*Localization of E-cadherin and β-catenin at cellular junctions*	Tay et al. 2010
	Xenopus, neural crest	*Induces expression of neural progenitors*	Guémar et al. 2007

Table 1.2 Associations of RhoUV with diseases

	Disease	**Description**	**References**
RhoU	Colorectal Cancer	*Maintains homeostasis of small intestine cell population by promoting apoptosis*	Slaymi et al. 2019
	Breast Cancer	*Overexpression promotes cell migration and promotes focal adhesion disassembly*	Dart et al. 2015
	Prostate Cancer	*Overexpression and proposed to promote cell migration and cell adhesion turnover*	De Piano et al. 2020
	T-cell acute lymphoblastic leukemia	*Overexpressed; knockouts showed its requirement for T-ALL cells migration, adhesion and chemotaxis*	Bhavsar et al. 2013
RhoV	Lung adenocarcinoma Cancer	*Overexpression promotes cell migration and metastasis; increased cell proliferation markers; decrease in E-cadherin and increase in N-cadherin*	Zhang et al. 2021; Chen et al. 2021
	Triple Negative Breast Cancer	*Overexpression promotes metastasis*	Jin et al. 2023

Chapter 2 Endoderm Development and Cell Migration

2.1 Endoderm Specification and Morphogenesis

Organogenesis is a fascinating topic in developmental biology, involving several cell types, migration, and organization in forming tissues and body structure. Organ tissues are derived from the three germ layers that develop out of gastrulation: ectoderm, mesoderm, and endoderm. Ectoderm will form the spine, neural crest, brain, and skin; mesoderm will give rise to somites, muscle, blood and cardiovascular system; and endoderm will give rise to the gastrointestinal and respiratory tracts (Zorn and Wells, 2009). Endoderm specification is highly conserved and occurs before the onset of gastrulation. All vertebrates require nodal signaling to promote mesoderm and endoderm for proper patterning and development. The concentration of nodal signaling, promoted by Wnt/β-catenin pathway, distinguishes mesoderm and endoderm as distinct cell populations, which is conserved among fish, frog, mouse and human (Zorn and Wells, 2009). In zebrafish the two nodal genes: *squint* and *cyclops*, members of the TGF-β family of ligands, are essential for endoderm specification (Feldman et al. 1998). High levels of Nodal promote expression of endoderm markers such as Foxa2, Sox17, Eomesoderm and GATA4-6 (Zorn and Wells, 2009). Nodal is regulated by its antagonist, Lefty, that targets ligand-receptor interactions distinguishing mesoderm from endoderm (Meno et al. 1999). During gastrulation, endodermal cells will undergo an epithelial to mesenchymal transition that allows them to move inward through synchronous ingression and form the inner-most layer of the embryo (Zorn and Wells, 2009; Nowotschin et al. 2019). Once gastrulation is complete, endodermal cells will undergo a mesenchymal to epithelial transition (MET) to form coherent epithelial layers for the future gut tube and other organs. In zebrafish, these transitions are accompanied by changes in migration directionality. Immediately after internalization, endodermal cells exhibit "random walk" migration, which allows the spreading of cells over the yolk surface, then later migrate directionally and collectively as they undergo MET (Pézeron et al. 2008). While these changes in endodermal cell migration behavior have been linked to cytoskeleton actin dynamics, regulated by Nodal and Rac1 signaling (Woo et al. 2012), we still lack knowledge regarding how these cells initiate MET.

2.2 Cell Migration: Cytoskeleton and Protrusions

Cell migration begins with the protrusion of the cell's plasma membrane in the direction of migration, often referred to as the leading edge. Rho GTPases facilitate membrane protrusion by interacting with proteins and enzymes that regulate the extension and retraction of the membrane. The most common

mechanism of membrane protrusion is through the formation of lamellipodia and filopodia by actin polymerization promoted by the Rho GTPases Rac1 and Cdc42. Once leading edge protrusion occurs, translocation of the cell body requires myosin-driven contraction of actin filaments at the rear of the cell; this process is controlled by the Rho GTPase RhoA.

A less studied mechanism of migration involves the protrusion of membrane blebs. As opposed to lamellipodia and filopodia, blebs are devoid of actin filaments. Instead, blebs are formed at sites of decreased cytoskeleton integrity, which allows hydrostatic pressure from the cytosol to expand the plasma membrane outwards into a bleb (Diz-Muñoz et al. 2010). RhoA and Rac1 have been shown to be activated during blebbing, with Rac1 associated closely with the actin polymerization needed for bleb retraction (Kardash et al. 2010). It is hypothesized that RhoA initiates this interaction, however the regulation of the blebbing mechanism remains to be fully understood (Ridley et al. 2011). It has been largely observed that cancerous cells and cells undergoing apoptosis utilize the blebbing mechanism (Coleman et al. 2001). In addition to these instances, cells undergoing rapid migratory transitions have been observed utilizing blebs (Charras and Paluch, 2008).

2.3 Mesenchymal to Epithelial Transition

Mesenchymal to Epithelial Transition (MET) is a highly conserved cellular process that occurs throughout the animal kingdom during many biological processes (Solnica-Krezel, 2005; Rodriguez-Boulan, 2014; Pei et al. 2018). MET is associated with differentiating cells, reprogramming, metabolic switching, and contributing to cell fate. Additionally, it is a crucial movement to establish epithelial sheets and contribute to the overall organization of tissue layers (Yoshida et al. 2001). Indicated by its name, a cell undergoing MET transitions from a mesenchymal-like cell to an epithelial-like cell to establish coherent epithelial layers. Cells in the mesenchymal state are migratory and express high levels of N-cadherin, which make them loosely adhered to the extracellular matrix. As cells undergo the transition into the epithelial state, N-cadherin expression decreases as E-cadherin expression increases, promoting strong adhesion at cell-cell junctions and highly organized apicobasal polarity (Yoshida et al. 2001). Although MET is an essential process for cell organization,

it is still unclear how MET is regulated, specifically during zebrafish endoderm development.

Chapter 3 Overexpression of Rho GTPase, *rhov*, disrupts migration of zebrafish endodermal cells

Abstract

The endoderm is an essential germ layer that will form the epithelial layer of organs such as the respiratory tract, gut, and intestines. In zebrafish and other vertebrates, endodermal cells are first migratory during gastrulation and then undergo a mesenchymal to epithelial transition (MET) during organ formation. How MET is triggered in endodermal cells is still unknown. Our lab has found that the Rho GTPase RhoV is dynamically expressed in the zebrafish endoderm; *rhov* expression is high during gastrulation and low during MET. RhoV is a Rho GTPase whose role has not been investigated within the endodermal cell migration to sheet formation. Here, we used CRISPR-generated RhoV mutants and *rhov* overexpression to investigate the role of RhoV in the mesenchymal to epithelial transition in zebrafish endoderm.

3.1 RhoV is a Potential Regulator of Endoderm Cell Migration

3.1.1 RhoV is differentially expressed during endoderm differentiation

To identify candidate genes that function during endodermal MET, our lab previously performed bulk RNA sequencing of endodermal cells collected at 8 hours post-fertilization (hpf), during mid-gastrulation when cells are undergoing mesenchymal-type migration, and at 12 hpf, when gastrulation is complete and cells are undergoing MET. Interestingly, we observed that *rhov* is highly expressed at 8 hpf then downregulated at 12 hpf. These observations were confirmed by qPCR (Fig. 3.1.1A). To determine whether this downregulation of *rhov* expression is necessary for normal development, we overexpressed *rhov* by injecting embryos with *rhov* mRNA. At 2 dpf, *rhov* mRNA-injected larvae had shorter body lengths than controls (Fig. 3.1.1B-C). These results suggest that the overexpression of *rhov* causes disturbances in normal development, possibly in the endoderm's ability to form completely.

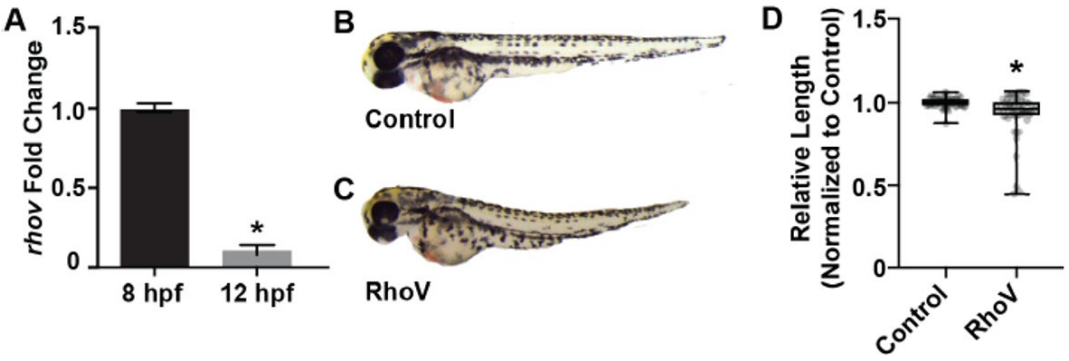


Figure 3.1.1. Overexpression of RhoV causes shorter body length.
A) Quantification of changes in endodermal *rhov* expression before (8 hpf) and during (12 hpf) MET. Hpf, hours post-fertilization. **B-C)** 2 day post-fertilization (dpf) zebrafish embryos injected with mRNA for Palmitoylated-tagRFP (control, B) or RhoV-tagRFP (C). **D)** Box plot of body lengths measured at 2 dpf of control embryos (n=60) and RhoV-overexpressing embryos (n=60). *p<0.05 by unpaired t-test.

3.1.2 Overexpression of rhov decreases endodermal cell migration velocity and directionality

To determine whether *rhov* overexpression affects endodermal cell migration, I performed time-lapse confocal microscopy. Embryos were injected at the one cell stage with mRNA encoding a red fluorescent protein fused to either *rhov* (*rhov*-tagRFP) or a membrane-targeting palmitoylation motif (palm-tagRFP) as the control. Endodermal cells were labeled with a transgene that drives expression of GFP under the endoderm-specific *sox17* promoter (*Tg(sox17:GFP)*) (Mizoguchi, 2008). Time lapse images were acquired by confocal microscopy during the mesenchymal stage of endoderm development (from 7-8.5 hpf). I used the Manual Tracking plug-in in ImageJ to measure two different parameters of cell migration: instantaneous migration velocity and directional persistence. Instantaneous velocity is calculated as the distance the cell moved from one frame to the next. Directional persistence is a measure of migration directionality. It is calculated as the cell's net distance traveled divided by the total distance traveled; values closer to 1 indicates the cells are moving with higher directionality in a straighter line.

I found that during the mesenchymal stage of migration, there was no statistically significant difference in either velocity or directional persistence between control and *rhov* overexpressing cells (Fig. 3.1.2A-B). This result is consistent with our observation that *rhov* is already highly expressed during this time.

I then imaged endodermal cells overexpressing *rhov* closer to the start of MET (8.5-11 hpf), when *rhov* expression decreases, to determine whether prolonged expression impacts normal endoderm development (Fig. 3.1.2A-B). Although there is no statistically significant difference between control and *rhov*-overexpressing cells in the current data (n=3 embryos), there is a trend toward slower migration velocity (p = 0.21) and decreased directional persistence (p = 0.28). However, power calculations suggest that a larger sample size is needed. Using the formula in Equation 1, I can calculate the sample size required to obtain significant data. Table 1 shows the numerical values I used based on the data I have obtained so far and determines that a significant sample size for velocity migration requires 13 embryos and directional persistence ratio requires 6 embryos.

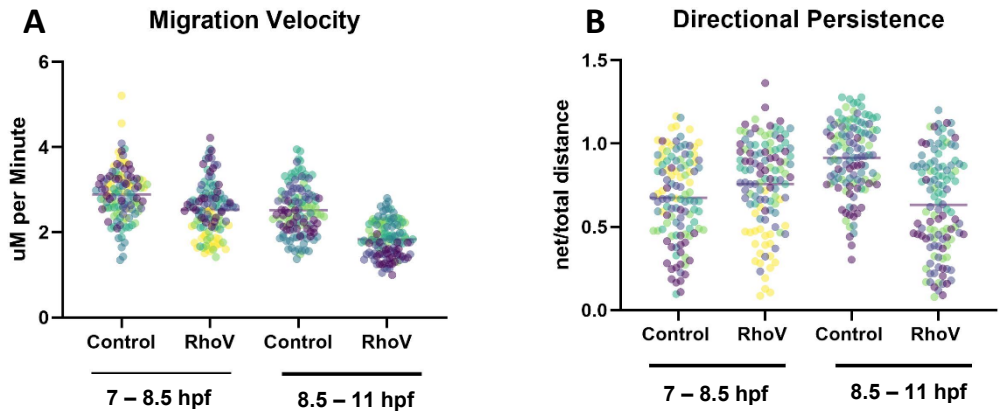


Figure 3.1.2. Effects of RhoV overexpression on migration of endodermal cells. Endodermal cells from *Tg(sox17:GFP)* embryos injected with Palm-tagRFP (control) or Rhov-tagRFP (RhoV, right) were tracked for 90 minutes starting at 7 hpf or 150 minutes starting at 8.5 hpf. A-B) Beeswarm plots of migration velocity (A) and directional persistence (B). Dots represent individual cells. Colors represent each embryo. Horizontal bars represent average. At 7 – 8.5 hpf, n = 5 embryos for each condition. At 8.5 – 11 hpf, n = 3 embryos for each condition.

***Equation* 1**

$$n = \frac{(Z_\alpha + Z_\beta)^2 \times 2(\sigma)^2}{(\mu_T - \mu_C)^2}$$

Table 3.1 Numerical values for determining sample size. Values used in Equation 1 to calculate sample size needed to achieve statistical significance. Z scores are predetermined values. Standard deviation and mean values were obtained from data shown in Figure 3.1.2 above. Sample size, n, is rounded up to a whole number representing the number of embryos to image. Grayed out values are either constant or not essential for the formula.

		Z_α	Z_β	σ	μ	n
Velocity Migration	**Control (C)**	0.05	0.84	0.532	2.312	13
	Rhov (T)	0.05	0.84	0.446	1.711	
Directional Persistence	**Control (C)**	0.05	0.84	0.137	0.844	6
	Rhov (T)	0.05	0.84	0.284	0.616	

3.1.3 *rhov* overexpressing embryos display a lack of uniform cell distribution

Although we did not observe any statistically significant changes in cell migration parameters, we did notice that embryos overexpressing *rhov* exhibited irregular disbursement of the endodermal cells across the embryo. At 10 hpf, or bud stage, endodermal cells begin to converge at the dorsal side of the embryo. At this stage the endoderm cell population displays a regular spacing surrounding the notochord. However, *rhov* overexpressing embryos displayed a lack in regular cell distribution (Fig. 3.1.3). In *rhov* overexpressed embryos 8 out of 12 total displayed some level of irregular spacing, while the control had 3 out of 10 total (data not shown). This lack in regular cell distribution can possibly contribute to a defect in epithelial sheet formation.

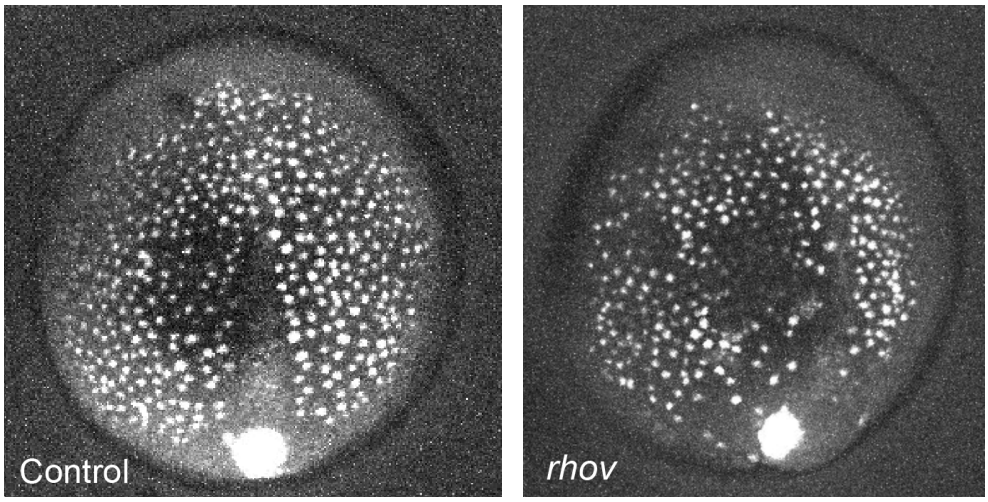


Figure 3.1.3. Overexpression of RhoV causes irregular cell distribution. Representative images of *Tg(sox17:GFP)* embryos injected with Palm-tagRFP (control, left) or Rhov-tagRFP (rhov, right) at bud stage (10 hpf). Dorsal views, anterior is towards the top.

3.1.4 Transplant experiment to confirm phenotype in overexpressed *rhov* endodermal migration

Although overexpression of *rhov* resulted in a change in endodermal cell distribution, we cannot determine if these effects are specific to the endoderm as *rhov* was globally overexpressed. To confirm that the effects of *rhov* overexpression are autonomous to endodermal cells, I performed transplant experiments. In these experiments, donor endodermal cells were generated by overexpressing the transcription factor *sox32* (Kikuchi et al. 2001) either alone or in combination with *rhov* overexpression. These donor cells were then transplanted to host embryos lacking endogenous endoderm via injection of an antisense morpholino to knock down *sox32*. Unfortunately, these experiments are technically challenging, and I was unable to collect enough data points to draw any conclusions.

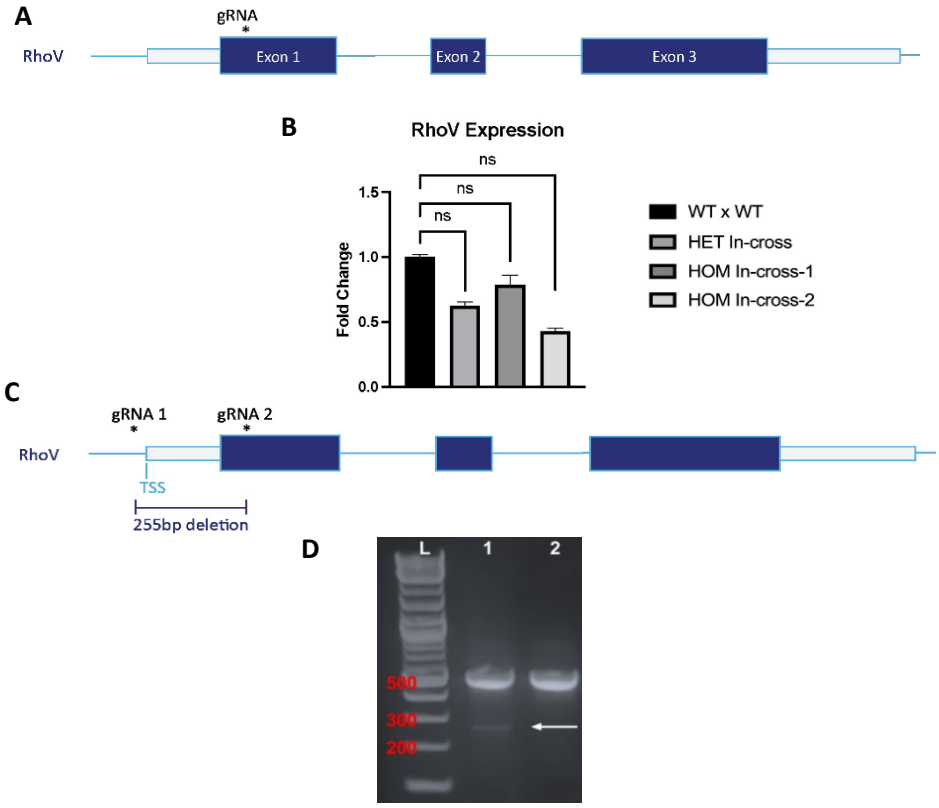
3.1.5 Determining the role of RhoV in endoderm development through deletion mutants

We observed that *rhov* is highly expressed in the endoderm at 8 hpf, suggesting that it may function during mesenchymal-type migration. For understanding the role of *rhov* in endodermal cell migration, we created loss of function *rhov* mutants using CRISPR-Cas9. The first mutant allele I recovered was generated using a guide RNA (gRNA) targeting a region within Exon 1 (Fig. 3.1.5A). This allele was predicted to result in a premature stop codon, yet mutant

embryos did not exhibit any loss of *rhov* expression as measured by qPCR (Fig. 3.1.5B). Therefore, I generated a second mutant allele in which the transcription start site (TSS) was deleted ($rhov^{\Delta TSS}$).

To make the $rhov^{\Delta TSS}$ mutation, I co-injected guide RNA pairs that targeted two sites in *rhov* genomic locus for a set of double stranded breaks. The first site is immediately upstream of the transcription start site and the second cut site is within exon 1 (Fig. 3.1.5C). Excision of the region between the two sites facilitated the removal of the TSS, and PCR spanning the excised region in injected F_0 embryos confirms this deletion (Fig. 3.1.5D). This deletion has now been raised to F_2 heterozygous generation.

Figure 3.1.5. Generating RhoV mutations with CRISPR/Cas. A,C) Schematics of the *rhov* genomic locus showing approximate locations of guide RNAs (gRNA, asterisks) used to create the first (A) and second (C) *rhov* mutant alleles. Lines represent intergenic or intronic regions. Boxes represent exons; light blue boxes represent untranslated regions; dark blue boxes represent coding sequence. TSS, transcription start site. **B)** Quantification of *rhov* expression in embryos from in-crosses of wild type parents (WT) or parents heterozygous (HET) or homozygous (HOM) for the rhov allele shown in (A). HOM-1 and HOM-2 were two different pairs. Data is represented as fold change normalized to WT. P-value for each comparison: $p = 0.01$ by nonparametric Students-t-test. **D)** Representative gel image showing PCR amplification of the genomic region depicted in (C) from embryos co-injected with gRNAs 1 and 2 or uninjected controls. Arrow points to a smaller band corresponding to a 255 bp deletion compared to wild-type.



3.1.6 In situ hybridization of RhoV during MET in endodermal cell migration

Previous studies have shown *rhov* expression by in situ hybridization between the stages spanning from 50% epiboly to long pec at incremental stages (Thisse et al. 2001; Tay et al. 2010). However, expression of *rhov* between 50% epiboly and 1-4-somite stages remain uninvestigated. Although we showed by RNA sequencing and qPCR that expression of RhoV decreases during 8 and 12 hpf, these data do not provide spatial information. Spatiotemporal expression can change within a small timeframe, therefore it is important that *rhov* expression is revealed between 50% and early somite stages, when endoderm undergoes MET. I attempted to perform whole mount in situ hybridization for RhoV at 75% epiboly (8 hpf), 90% epiboly (9 hpf), bud stage (10 hpf), and 2-somite stage (12 hpf). While a control sox32 probe successfully labeled endodermal cells at these stages, staining with the RhoV probe was inconclusive.

3.2 Methods

Zebrafish Husbandry and Use

Zebrafish were handled under the IUCUC and AALAC animal care guidelines. Fish lines, *Tg(sox17:GFP)* and wildtype, were used for the described experiments. Embryos collected from crosses were stored in egg water at 28°C to 32°C.

RhoV Mutants: Overexpression and Deletion

Tg(sox17:GFP) were used for RhoV overexpression experiments, injected with RhoV-tagRFP mRNA or Palmitoylated-tagRFP for microscopy. RhoV-tagRFP plasmid was constructed from a Gibson assembly (Gibson et al. 2009) of a plasmid containing tagRFP sequence and a plasmid containing the RhoV sequence. The expected sequence was confirmed through cloning and sequencing. RhoV-tagRFP mRNA was prepared with SP6 mMessage mMachine transcription kit (Thermo Fisher Scientific). Injection molds were made with 2% agarose in egg water. RhoV-tagRFP mRNA was injected at 500pg in one-cell stage embryos. Injected mRNA was confirmed through visual RFP fluorescence in *Tg(sox17:GFP)* embryos. Control embryos were injected with Palmitoylated-tagRFP at 500pg at one-cell stage.

RhoV deletion mutants were generated in *Tg(sox17:GFP)* through CRISPR deletion of the transcription start site. A guide upstream of the 5' UTR and in Exon 1 was co-injected with Cas9 at 100pg each. F0s were confirmed through PCR genotyping and grown to F2 heterozygous.

Microscopy and Imaging

Endodermal cells were imaged using a spinning disk confocal microscope. Embryos for live imaging were grown to desired stage (70% epiboly), dechorionated, and embedded in 1% low-melt agarose in 0.3X Ringers in a glass bottom 35mm petri dish. Using fluorescent imaging at 30x magnification, Z-stacks were taken over the span of 90 minutes for "early" videos and 150 minutes for "late" videos, every minute, at 2um depths to observe control (Palmitoylated-tagRFP) and overexpressed *rhov* endodermal cells in *Tg(sox17:GFP)*. Images of fixed embryos were taken at 10x with 2um Z-stacks, embedded the same as described above.

For cell distribution assays, *rhov* overexpressing embryos were fixed imaging at 10 hpf (bud stage) in 4% paraformaldehyde at 4°C overnight, washed in 1xPBS the next morning and manually dechorionated. Fixed embryos were embedded in 1% agarose in glass-bottom 35 mm petri dishes for imaging.

Image processing was done through ImageJ and data was analyzed through GraphPad Prism 9. Cell tracking to measure distance traveled and velocity was done through ImageJ Manual Cell tracker plug-in. Manual Cell

tracker calculates the cell velocity per frame which was calculated as an average. Directional persistence was calculated by dividing the net distance traveled by the total distance traveled. Unpaired T-tests were used to determine statistical significance.

Transplants

In this experiment, *rhov* and *sox32* overexpressed *Tg(sox17:GFP)* embryos were used as the donor embryo with wildtype as the host. In the donor embryo, RhoV was injected at 500pg along with Sox32 at 500pg at one cell stage. Wildtype embryos were injected with Sox32 MO at 1:4 dilution. After injection, embryos were grown in egg water with methylene blue at 28°C – 32°C until desired stage, approximately oblong (3.5 hours) and then manually dechorionated in 0.3X Ringers. The transplant mold is 2% agarose in 0.3X Ringers, filled with 0.3X Ringers to cover the embryos.

While using the transplantation apparatus, there are some key points to consider for a successful transplant. The tube that connects the needle holder to the syringe must be absent of any bubbles (having bubbles inhibits your control). The limiting step in this experiment is the transplantation of the right amount of cells. The transplantation needle (World Precision Instruments Glass 1.0MM 4 IN) must be broken at a slant with a clean razor. The diameter of the needle should be able to easily pull up a cell at a time, however it is better to be almost two cells in diameter than too thin. If the needle is too thin, the cells will shear while getting pulled through. Go to the cells above the margin close to the edge of the embryo, only to pull up one cell deep to avoid pulling up yolk cells. If the yolk is sucked up with the cells, it is possible to transfer that DNA to the host embryo. For example, transplanting yolk cells along with the endoderm from the *Tg(sox17:GFP)* will allow your host to fully express GFP. Use the apparatus to draw up roughly 10 cells and transfer to the host at around the same region (above the margin). As you release the cells into the host, remove the needle before the last couple cells are released, this prevents flooding the host embryo with buffer. To read more about this technique, go to ZFIN cellular methods.

In situ hybridization

In situ RhoV probe was created from 10ug of plasmid PCS2-GFP-rhov linearized separately with BsrG1 and BspE1 incubated at 37°C for 2 hours followed by phenol chloroform DNA extraction and resuspended in 20uL of H2O. 5uL of linearized RhoV DNA was used in a Digoxigenin labelling transcription kit. Followed by ethanol precipitation clean up and resuspended in 50uL Depc-H2O and stored as 1ug per 10uL in Hybridization buffer (100X). The probe was used at 1ug/mL for in situ. Sox32 probe was synthesized using Sox32 plasmid digested with ClaI enzyme followed by the steps mentioned above.

Embryos at 8hpf, 12hpf, and 24 hpf were fixed in 4% PFA at 4°C overnight and rinsed in 1X PBS the following day. Embryos were dechorionated and gradually changed to 100% methanol at room temperature, then placed in -20°C for at least 2 hours and up to several months. The following steps used a 24-well plate using 1 mL of each solution. To easily transport embryos from each well, embryos were transferred to a small basket made from a 2mL collection tube with the bottom cut off and adhered mesh fabric to the bottom using a hot plate. The following steps followed as previously described from Thisse et al. 2007.

3.3 Discussion and Conclusions

Here, I sought to determine the role of RhoV in zebrafish endoderm development. Our data shows that *rhov* expression changes as endodermal cells undergo MET — it is high at 8 hpf, when cells are highly migratory prior to MET, and low at 12 hpf, as cells are undergoing MET. I showed that global overexpression of RhoV affects endodermal cell migration as well as the cell distribution during this same time window. When *rhov* was overexpressed, endodermal cell migration was not significantly affected; although I observed a slight slowing of migration velocity and loss of directionality at 12 hpf, this was not statistically significant. These results suggest that downregulation of *rhov* alone is not sufficient to drive endodermal MET but may require an additional regulator. One potential candidate is RhoU which is in the same family of Rho GTPases as RhoV. In mice, RhoU was shown to be required for the differentiation of endodermal cells into foregut (Loebel et al. 2011). Furthermore, in zebrafish, *rhoub* expression dramatically increases between 75% epiboly and about 1-4 somite stage (White et al. 2017), the same time window as when endodermal cells undergo MET. On the other hand, I also observed that RhoV overexpression resulted in noticeably irregular spacing between endodermal cells. This result suggests that RhoV downregulation is needed for the correct migration pattern of endodermal cells. Additional work is needed to clarify the role of RhoV downregulation in the endoderm at the end of gastrulation..

Endoderm derived organ formation is still yet to be fully understood. In many animals, endodermal cells undergo MET once gastrulation ends and organ formation begins (Nowotschin et al. 2019). Our data suggest that RhoV is a possible regulator of endodermal MET. Notably, RhoV is expressed in the endodermal layers of the foregut in chick and mouse (Blake et al. 2021; Notarnicola et al. 2008). However, specific roles for RhoV in the gut remain unknown. RhoV may be facilitating cell-cell adhesion. MET is often marked by an increase in E-cadherin expression accompanied by a decrease in N-cadherin (Loh et al. 2019). Previous reports have linked RhoV to E-cadherin localization in zebrafish epiboly (Tay et al. 2010) and to increased N-cadherin expression in

tumor cells (Zhang et al. 2021). I propose a model where high expression of *rhov* during gastrulation induces the loss of E-cadherin, which allows cells to become mesenchymal, while the downregulation of *rhov* at the end of gastrulation allows cells to become epithelial and adhere into an epithelial sheet. An increase in RhoU expression may further facilitate endoderm differentiation and epithelial tissue formation.

References

Ahmad Mokhtar AMb, Ahmed SBM, Darling NJ, Harris M, Mott HR, Owen D. 2021. A complete survey of RhoGDI targets reveals novel interactions with atypical small GTPases. Biochemistry (Easton). 60(19):1533-51.

Aronheim A, Broder YC, Cohen A, Fritsch A, Belisle B, Abo A. 1998. Chp, a homologue of the GTPase Cdc42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton. Current Biology. 8(20):1125-8.

Aspenström P. 2022. The role of fast-cycling atypical RHO GTPases in cancer. Cancers. 14(8):1961.

Aspenström P. 2020. Fast-cycling rho GTPases. Small GTPases. 11(4):248-55.

Aspenström P, Fransson A, Saras J. 2004. Rho GTPases have diverse effects on the organization of the actin filament system. Biochemical Journal. 377(Pt 2):327-37.

Barocchi MA, Masignani V, Rappuoli R. 2005. Opinion: Cell entry machines: A common theme in nature? Nat Rev Microbiol. 3(4):349-58.

Berzat AC, Buss JE, Chenette EJ, Weinbaum CA, Shutes A, Der CJ, Minden A, Cox AD. 2005. Transforming activity of the rho family GTPase, wrch-1, a wnt-regulated Cdc42 homolog, is dependent on a novel carboxyl-terminal palmitoylation motif. The Journal of Biological Chemistry. 280(38):33055-65.

Bhavsar PJ, Infante E, Khwaja A, Ridley AJ. 2013. Analysis of rho GTPase expression in T-ALL identifies RhoU as a target for notch involved in T-ALL cell migration. Oncogene. 32(2):198-208.

Blake JA, Baldarelli R, Kadin JA, Richardson JE, Smith CL, Bult CJ; Mouse Genome Database Group. 2021. Mouse Genome Database (MGD): Knowledgebase for mouse-human comparative biology. Nucleic Acids Res. 2021 Jan 8;49(D1):D981-D987.

Boureux A, Vignal E, Faure S, Fort P. 2007. Evolution of the rho family of ras-like GTPases in eukaryotes. Molecular Biology Evolution. 24(1):203-16.

Brady DC, Alan JK, Madigan JP, Fanning AS, Cox AD. 2009. The transforming rho family GTPase wrch-1 disrupts epithelial cell tight junctions and epithelial morphogenesis. Molecular and Cellular Biology. 29(4):1035.

Brazier H, Pawlak G, Vives V, Blangy A. 2009. The rho GTPase Wrch1 regulates osteoclast precursor adhesion and migration. Int J Biochem Cell Biol. 41(6):1391-401.

Charras G, Paluch E. 2008. Blebs lead the way: How to migrate without lamellipodia. Nat Rev Mol Cell Biol. 9(9):730-6.

Chen H, Xia R, Jiang L, Zhou Y, Xu H, Peng W, Yao C, Zhou G, Zhang Y, Xia H, et al. 2021. Overexpression of RhoV promotes the progression and EGFR-TKI resistance of lung adenocarcinoma. Front Oncol. 11.

Chen J, Voigt J, Gilchrist M, Papalopulu N, Amaya E. 2005. Identification of novel genes affecting mesoderm formation and morphogenesis through an enhanced large scale functional screen in xenopus. Mech Dev. 122(3):307-31.

Chenette EJ, Abo A, Der CJ. 2005. Critical and distinct roles of amino- and carboxyl-terminal sequences in regulation of the biological activity of the chp atypical rho GTPase. The Journal of Biological Chemistry. 280(14):13784-92.

Chuang Y, Valster A, Coniglio SJ, Backer JM, Symons M. 2007. The atypical rho family GTPase wrch-1 regulates focal adhesion formation and cell migration. Journal of Cell Science. 120(11):1927-34.

Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. 2001. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I. Nat Cell Biol. 3(4):339-45.

Dart AE, Box GM, Court W, Gale ME, Brown JP, Pinder SE, Eccles SA, Wells CM. 2015. PAK4 promotes kinase-independent stabilization of RhoU to modulate cell adhesion. The Journal of Cell Biology. 211(4):863-79.

De Piano M, Manuelli V, Zadra G, Otte J, Edqvist PD, Pontén F, Nowinski S, Niaouris A, Grigoriadis A, Loda M, et al. 2020. Lipogenic signalling modulates prostate cancer cell adhesion and migration via modification of rho GTPases. Oncogene. 39(18):3666-79.

Dickover M, Hegarty JM, Ly K, Lopez D, Yang H, Zhang R, Tedeschi N, Hsiai TK, Chi NC. 2014. The atypical rho GTPase, RhoU, regulates cell-adhesion molecules during cardiac morphogenesis. Developmental Biology. 389(2):182-91.

Diz-Muñoz A, Krieg M, Bergert M, Ibarlucea-Benitez I, Muller DJ, Paluch E, Heisenberg C. 2010. Control of directed cell migration in vivo by membrane-to-cortex attachment. PLoS Biology. 8(11):e1000544.

Feldman B, Gates MA, Egan ES, Dougan ST, Rennebeck G, Sirotkin HI, Schier AF, Talbot WS. 1998. Zebrafish organizer development and germ-layer formation require nodal-related signals. Nature. 395(6698):181-5.

Fort P, Guémar L, Vignal E, Morin N, Notarnicola C, de Santa Barbara P, Faure S. 2011. Activity of the RhoU/Wrch1 GTPase is critical for cranial neural crest cell migration. Dev Biol. 350(2):451-63.

Freeman JL, Abo A, Lambeth JD. 1996. Rac "insert region" is a novel effector region that is implicated in the activation of NADPH oxidase, but not PAK65. J Biol Chem. 271(33):19794-801.

García-Castro MI, Marcelle C, Bronner-Fraser M. 2002. Ectodermal wnt function as a neural crest inducer. Science. 297(5582):848-51.

Gibson DG, Young L, Chuang R, Venter JC, Hutchison CA, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods. 6(5):343-5.

Guémar L, de Santa Barbara P, Vignal E, Maurel B, Fort P, Faure S. 2007. The small GTPase RhoV is an essential regulator of neural crest induction in xenopus. Developmental Biology. 310(1):113-28.

Haga RB, Ridley AJ. 2016. Rho GTPases: Regulation and roles in cancer cell biology. Small GTPases. 7(4):207-21.

Hodge RG, Ridley AJ. 2020. Regulation and functions of RhoU and RhoV. Small GTPases. 11(1):8-15.

Hofmann C, Shepelev M, Chernoff J. 2004. The genetics of pak. J Cell Sci. 117(19):4343-54.

Jaiswal M, Dvorsky R, Ahmadian MR. 2013. Deciphering the molecular and functional basis of dbl family proteins. The Journal of Biological Chemistry. 288(6):4486-500.

Jeong W, Yoon J, Park J, Lee S, Lee S, Kaduwal S, Kim H, Yoon J, Choi K. 2012. Ras stabilization through aberrant activation of wnt/β-catenin signaling promotes intestinal tumorigenesis. Science Signaling. 5(219):ra30.

Jin M, Gong Y, Ji P, Hu X, Shao Z. 2023. In vivo CRISPR screens identify RhoV as a pro-metastasis factor of triple-negative breast cancer. Cancer Science. 114(6):2375.

Kardash E, Reichman-Fried M, Maître J, Boldajipour B, Papusheva E, Messerschmidt E, Heisenberg C, Raz E. 2010. A role for rho GTPases and cell-cell adhesion in single-cell motility in vivo. Nat Cell Biol. 12(1):47-11.

Kidd 3, Ambrose R, Muñiz-Medina V, Der CJ, Cox AD, Reiner DJ. 2015. The C. elegans chp/wrch ortholog CHW-1 contributes to LIN-18/ryk and LIN-17/frizzled signaling in cell polarity. PLoS ONE. 10(7):e0133226.

Kikuchi Y, Agathon A, Alexander J, Thisse C, Waldron S, Yelon D, Thisse B, Stainier DY. 2001a. Casanova encodes a novel sox-related protein necessary

and sufficient for early endoderm formation in zebrafish. Genes Dev. 15(12):1493-505.

Kikuchi Y, Agathon A, Alexander J, Thisse C, Waldron S, Yelon D, Thisse B, Stainier DY. 2001b. Casanova encodes a novel sox-related protein necessary and sufficient for early endoderm formation in zebrafish. Genes Dev. 15(12):1493-505.

Krausova M, Korinek V. 2014. Wnt signaling in adult intestinal stem cells and cancer. Cell Signal. 26(3):570-9.

Kumar R, Sanawar R, Li X, Li F. 2017. Structure, biochemistry, and biology of PAK kinases. Gene. 605:20-31.

Loebel DAF, Studdert JB, Power M, Radziewic T, Jones V, Coultas L, Jackson Y, Rao RS, Steiner K, Fossat N, et al. 2011. Rhou maintains the epithelial architecture and facilitates differentiation of the foregut endoderm. Development (Cambridge). 138(20):4511-22.

Loh C, Chai JY, Tang TF, Wong WF, Sethi G, Shanmugam MK, Chong PP, Looi CY. 2019. The E-cadherin and N-cadherin switch in epithelial-to-mesenchymal transition: Signaling, therapeutic implications, and challenges. Cells. 8(10):1118. doi: 10.3390/cells8101118.

Luu AP, Yao Z, Ramachandran S, Azzopardi SA, Miles LA, Schneider WM, Hoffmann H, Bozzacco L, Garcia G, Gong D, et al. 2021. A CRISPR activation screen identifies an atypical rho GTPase that enhances zika viral entry. Viruses. 13(11).

Meno C, Gritsman K, Ohishi S, Ohfuji Y, Heckscher E, Mochida K, Shimono A, Kondoh H, Talbot WS, Robertson EJ, et al. 1999. Mouse Lefty2 and zebrafish antivin are feedback inhibitors of nodal signaling during vertebrate gastrulation. Mol Cell. 4(3):287-98.

Mitin N, Roberts PJ, Chenette EJ, Der CJ. 2012. Posttranslational lipid modification of rho family small GTPases. Methods Mol Biol. 827:87-95.

Mizoguchi T, Verkade H, Heath JK, Kuroiwa A, Kikuchi Y. 2008. Sdf1/Cxcr4 signaling controls the dorsal migration of endodermal cells during zebrafish gastrulation. Development (Cambridge). 135(15):2521-9.

Mosaddeghzadeh N, Ahmadian MR. 2021. The RHO family GTPases: Mechanisms of regulation and signaling. Cells (Basel, Switzerland). 10(7):1831.

Notarnicola C, Le Guen L, Fort P, Faure S, De Santa Barbara P. 2008. Dynamic expression patterns of RhoV/chp and RhoU/wrch during chicken embryonic development. Developmental Dynamics. 237(4):1165-71.

Nowotschin S, Hadjantonakis A, Campbell K. 2019. The endoderm: A divergent cell lineage with many commonalities. Development. 146(11).

Ory S, Brazier H, Blangy A. 2007. Identification of a bipartite focal adhesion localization signal in RhoU/wrch-1, a rho family GTPase that regulates cell adhesion and migration. Biol Cell. 99(12):701-16.

Pei D, Shu X, Gassama-Diagne A, Thiery JP. 2019. Mesenchymal–epithelial transition in development and reprogramming. Nat Cell Biol. 21(1):44.

Pertz O, Hodgson L, Klemke RL, Hahn KM. 2006. Spatiotemporal dynamics of RhoA activity in migrating cells. Nature. 440(7087):1069-72.

Pézeron G, Mourrain P, Courty S, Ghislain J, Becker TS, Rosa FM, David NB. 2008. Live analysis of endodermal layer formation identifies random walk as a novel gastrulation movement. Current Biology. 18(4):276-81.

Reinstein J, Schlichting I, Frech M, Goody RS, Wittinghofer A. 1991. p21 with a phenylalanine 28----leucine mutation reacts normally with the GTPase activating protein GAP but nevertheless has transforming properties. J Biol Chem. 266(26):17700-6.

Ridley AJ. 2001. Rho GTPases and cell migration. J Cell Sci. 114(Pt 15):2713-22.

Ridley A. 2011. Life at the leading edge. Cell. 145(7):1012-22.

Ridley AJ. 2015. Rho GTPase signalling in cell migration. Curr Opin Cell Biol. 36:103-12.

Rodriguez-Boulan E, Macara IG. 2014. Organization and execution of the epithelial polarity programme. Nature Reviews. Molecular Cell Biology. 15(4):225-42.

Saras J, Wollberg P, Aspenström P. 2004. Wrch1 is a GTPase-deficient Cdc42-like protein with unusual binding characteristics and cellular effects. Experimental Cell Research. 299(2):356-69.

Schatoff EM, Leach BI, Dow LE. 2017. Wnt signaling and colorectal cancer. Curr Colorectal Cancer Rep. 13(2):101-10.

Schiavone D, Dewilde S, Vallania F, Turkson J, Di Cunto F, Poli V. 2009. The RhoU/Wrch1 rho GTPase gene is a common transcriptional target of both the gp130/STAT3 and wnt-1 pathways. Biochem J. 421(2):283-92.

Shepelev MV, Korobko IV. 2012. Pak6 protein kinase is a novel effector of an atypical rho family GTPase chp/RhoV. Biochemistry Moscow. 77(1):26-32.

Shepelev MV, Chernoff J, Korobko IV. 2011. Rho family GTPase chp/RhoV induces PC12 apoptotic cell death via JNK activation. Small GTPases. 2(1):17-26.

Shutes A, Berzat AC, Cox AD, Der CJ. 2004. Atypical mechanism of regulation of the wrch-1 rho family small GTPase. Current Biology. 14(22):2052-6.

Slaymi C, Vignal E, Crès G, Roux P, Blangy A, Raynaud P, Fort P. 2019. The atypical RhoU/Wrch1 rho GTPase controls cell proliferation and apoptosis in the gut epithelium. Biology of the Cell. 111(5):121-41.

Solnica-Krezel L. 2005. Conserved patterns of cell movements during vertebrate gastrulation. Current Biology. 15(6):R213-28.

Spiering D, Hodgson L. 2011. Dynamics of the rho-family small GTPases in actin regulation and motility. Cell Adh Migr. 5(2):170-80.

Tao W, Pennica D, Xu L, Kalejta RF, Levine AJ. 2001. Wrch-1, a novel member of the rho gene family that is regulated by wnt-1. Genes Dev. 15(14):1796-807.

Tay HG, Ng YW, Manser E. 2010. A vertebrate-specific chp-PAK-PIX pathway maintains E-cadherin at adherens junctions during zebrafish epiboly. PloS One. 5(4):e10125.

Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X.Q. and Thisse, C. (2001) Expression of the zebrafish genome during embryogenesis (NIH R01 RR15402). ZFIN Direct Data Submission (http://zfin.org).

Van den Broeke C, Jacob T, Favoreel HW. 2014. Rho'ing in and out of cells: Viral interactions with rho GTPase signaling. Small GTPases. 5:e28318.

Vasquez CG, de la Serna EL, Dunn AR. 2021. How cells tell up from down and stick together to construct multicellular tissues - interplay between apicobasal polarity and cell-cell adhesion. J Cell Sci. 134(21):jcs248757. doi: 10.1242/jcs.248757. Epub 2021 Oct 29.

Weisz Hubsman M, Volinsky N, Manser E, Yablonski D, Aronheim A. 2007. Autophosphorylation-dependent degradation of Pak1, triggered by the rho-family GTPase, chp. Biochemical Journal. 404(3):487-97.

White RJ, Collins JE, Sealy IM, Wali N, Dooley CM, Digby Z, Stemple DL, Murphy DN, Billis K, Hourlier T, et al. 2017. A high-resolution mRNA expression time course of embryonic development in zebrafish. Elife. 6:10.7554/eLife.30860.

Woo S, Housley MP, Weiner OD, Stainier DYR. 2012. Nodal signaling regulates endodermal cell motility and actin dynamics via Rac1 and Prex1. The Journal of Cell Biology. 198(5):941-52.

Yoshida K, Inouye K. 2001. Myosin II-dependent cylindrical protrusions induced by quinine in dictyosteliam: Antagonizing effects on actin polymerization at the leading edge. Journal of Cell Science. 114:2155-65.

Zegers MM, Friedl P. 2014. Rho GTPases in collective cell migration. Small GTPases. 5(3).

Zhang D, Jiang Q, Ge X, Shi Y, Ye T, Mi Y, Xie T, Li Q, Ye Q. 2021. RHOV promotes lung adenocarcinoma cell growth and metastasis through JNK/c-jun pathway. International Journal of Biological Sciences. 17(10):2622-32.

Zhang J, Koenig A, Young C, Billadeau DD. 2011. GRB2 couples RhoU to epidermal growth factor receptor signalling and cell migration. Molecular Biology of the Cell. 22(12):2119-30.

Zorn AM, Wells JM. 2009. Vertebrate endoderm development and organ formation. Annual Review of Cell and Developmental. 25(1):221-51.