2022

The Role of EHD2 in Endothelial Cells

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Document Type

Thesis

Degree Name

M.S.

Department

Biological Sciences

First Advisor

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Keywords

EHD2, Endothelial cells, Biophysics

Subject Categories

Biochemistry, Biophysics, and Structural Biology | Biology | Biophysics | Cell and Developmental Biology | Cell Biology

The Role of EHD2 in Endothelial Cells

A Thesis

Presented to

the Faculty of the College of Natural Sciences and Mathematics

University of Denver

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

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November 2022

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Title: The Role of EHD2 in Endothelial Cells
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Chapter One Introduction

Blood vessels allow for the transport of oxygen, hormones, sugar, and other necessities to cells, and facilitate the removal of carbon dioxide and cellular waste away from cells[1]. The formation of blood vessels is a crucial step in the development of all mammals. This process of creating blood vessels requires two pathways. First, vasculogenesis, the de novo creation of blood vessels via differentiation of mesodermal cells to angioblasts[2]. Second, angiogenesis, the sprouting of new vessels from existing vessels. Angiogenesis is the major developmental program that controls blood vessel density and location[3]. Any negative perturbation in angiogenic signaling suggests embryonic lethality, underscoring the requirement and conservation of this process.

Correct formation of blood vessels is vital to health. Cancer, hemorrhages, tissue regeneration, wound healing and other diseases leverage aspects of blood vessel growth [4]. Understanding how blood vessels develop and are altered over time can provide valuable insight into the etiology of cardiovascular disease (CVD)[5]. Cardiovascular disease is the primary cause of death in the United States according to the NIH[6]. Cardiovascular disease is an umbrella term to describe strokes, heart failure, hypertension, and other issues in the vasculature that lead to almost one-third of deaths worldwide, according to a study from 2017[7]. Risk factors for cardiovascular disease build over time. Understanding how to

improve angiogenesis in aging populations by targeting the molecular controls of angiogenesis could provide relief to people struggling with CVD. This targeted approach

to treatment could assist in the repair and replacement of damaged blood vessels, leading to a lowered rate of mortality in CVD[5].

In tumor formation the prevention of angiogenesis is key to preventing growth in solid mass tumors and metastasizing from occurring[8,9]. Cancer is the second leading cause of death worldwide. Investigations researching the impact of regulators of angiogenesis on solid tumors, found that lowering signals for growth of blood vessels slows tumor growth[10,11]. Another study linking blood vessels and tumor development reported that tumors promote angiogenic processes; however, this growth is less-well controlled as the vasculature formed is often dysmorphic and leaky compared to physiological angiogenesis[12]. This increased growth of blood vessels relies on two strategies: genetic changes and increased hypoxia in the surrounding tissues[13]. Current angiogenesis targeted cancer therapy is often found to have little effect because of the heterogeneity of vessel types within in a single tumor[13]. A better understanding of how to prevent growth even in areas that have the switch turned on may lead to better preventative care[13].

Regulation of Angiogenesis

Angiogenesis is highly influenced by Vascular Endothelial Growth Factor (VEGF) ligand and its cognate receptor, Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)[14]. VEGF is a required signaling factor of the development of blood vessels as mice deleted for a single copy of VEGF are nonviable[14]. The VEGF ligand is released from the surrounding cells due to a state of hypoxia where it binds VEGFR2 that are

exclusively expressed on endothelial cells[15]. VEGFR2, also known as FLT-1(FM's-like tyrosine kinase-1), is a receptor tyrosine kinase (RTK) and has been shown to activate a MAPK pathway[16]. This pathway suggests changes in the endothelial cell (EC) where the binding occurs and suggests different changes in the surrounding cells.

VEGFR2 signaling suggests the differentiation of endothelial cells into tip cells and stalk cells[17]. To control the location and density of blood vessels only cells that are exposed to the highest amount of VEGF is released are able to respond to this signal to prevent sheets of blood vessels from forming. To do this the ECs designate a tip cell, a cell that will lead migration, and multiple stalk cells, a set of cells unable to respond to VEGF but are pulled along by the tip cell and will make up the vessel. The release of VEGF from the surrounding cells triggers the response of the tip cell[17]. When VEGF binds to VEGFR2 and the tip cell pulls in the transmembrane protein's extracellular domain of Notch (NECD) from the surrounding cells after it binds to DLL4. The surrounding, or stalk cells, then have the intracellular domain of Notch enters the nucleus. This causes the surrounding cells to remove VEGFR2 from the surrounding cells. This cascades down the rest of the original vessel using Jag1 and Notch to trigger the Notch Intracellular Domain (NICD) into the nucleus to prevent excess tip cell formation. The tip then starts to migrate up the gradient pulling along the stalk cells to stabilize the vessel releasing necessary support structures as the cells move into the hypoxic area[14].

Junctional regulation

Junctions allow cells to bind together and form monolayers. Junctional proteins prevent fluid leakage from occurring between blood vessels, surrounding tissues, and bind endothelial cells to other cell types. ECs need to be connected to surrounding tissue, even when facing pressure and tension changes, by binding tightly to other ECs and the surrounding tissue. There are three main types of junctions found in cells: tight junctions, adherent junctions, and desmosomes[18]. Adherent junctions are made of cadherin proteins that vary by cell type[19]. VE-cadherin or cadherin 5 is only expressed in endothelial tissue[20]. These cadherin groups are stabilized by alpha-catenin, beta-catenin, and p120 preventing blood from leaking from blood vessels and causing strokes and hemorrhages[21]. VE-cadherin is a calcium-dependent adhesion molecule that forms a hexamer on the cell membrane[22]. VE-cad formation starts in cells when cadherin diffuses around the cell forming clusters and binding to another cell's VE-cad in filopodia[23,24]. This clustering allows for actin and its regulator to start increasing the amount of VE-cad and expand the contact between the two cells. Cyclical AMP increases the amount of VE-cad at the cell border and induced cell tension reduction[19]. Alpha-catenin and beta-catenin work together to regulate VE-cadherin and actin dynamics. Actin bundling and stress fibers work as a negative feedback loop and decrease the amount of VE-cad at the junction[25]. VEGF signal is transduced through VE-cad to the Akt – pathway that is connected to Jag1 and helps maintain mature vasculature. VE-cad promotes stability and stops[25]. VE-cad responds when tension increases in endothelial cells[25].

Tension and adhesion in dynamic tissues is an important topic for the migration of cells in development and cell-cell interaction in development. This migration of sheets of cells relies on cell-cell contact for signaling and actin contractions to move the cell in a specific direction. The remodeling of these junctions must be tightly regulated by applied force and tension to signal when to break and tighten junctional stability. Endocytosis of Cadherin is usually a sign of junctional inhibition [24,26–2925]. The VE-cadherin increase and loss of tension allow for lumen formation and allow for the movement of blood in arteries[21,24,30]. VE-cadherin is selectively inhibited in migratory tip cells [28]. VEGF decreases the amount of VE-cadherin during elongation, trigging junctional-associated intermittent lamellipodia under actin complexes[28]. Junction-associated intermittent lamellipodia acts as a migrating force during wound-healing assays for HUVECs[28]. This elongation does not lower the amount of VE-cadherin, but the relative concentration of VE-cadherin at junctions that match the Mouse retinal vasculature data[29]. The migratory front has a lower concentration than the perivenous and venous architecture[28]. A nonfunctional VE-cadherin and latrunculin B have a similar effect on ZO-1 junctions in Intersegmental vessels showing that VE-cadherin and actin polymerization work together to shape cells [24]. This acts as a stabilizing force for stalk cells during tip stalk signaling. For cells to migrate adhesion junctions are endocytosed when Moy II is increased in a migrating tissue during germband extension [31].There is a distinct lack of Epithelial cadherin (E-cadherin) at the plasma membrane where only two cells touch but the stabilizing force of E-cadherin after the high-tension ratcheting motion is shown to

stabilize the new cell border [27]. The artificial increase of stable tension by overexpressing E-cadherin in embryos halts movement and migration that is used to change cell position[32].

EHD2 and Caveolae

Epsin15 homology domain protein 2 (EHD2) is a protein connected to Caveolin 1 (Cav1) and is enriched in endothelial cells. Cav1 is a hair pin like dimer found on the plasma membrane of many cells but is enriched in skeletal muscle and ECs[33]. Cav1 has been known to passively internalize proteins and form pit like invagination. EHD2 has been called a dynamin like protein and linked to mechanical tension and Cav1 at the plasma membrane[34]. This protein exists at the plasma membrane as an oligomer ring at the neck of Cav1 pits and as dimer that can form with itself and EHD1 and EHD4 in cells[34]. While a lot of the research based in adipose and other cell types focus on endocytosis and the movement of Cav1 pits recycling, some of the research focus on endothelial cells links Cav1 and EHD2 to the modulation of membrane tension when monolayers are under stress[35,36]. This is a significant focus for EHD2 and Cav1 because there has been some connection between junctions and actin movements in epithelial cells and in cancerous cells[10,37].This is role of EHD2 may be more important and a more accurate role for EHD2 because of the enrichment of EHD2 in endothelial cells versus the roles it may play in interacting with the rest the EH domain family found in other cell types.

EHD2 domains are KPFxxxNPF, the p-loop, coil-coil domain, nuclear localization sequence, EH domain, and EF-hand domain[38,39]. The purpose of the KPFxxxNPF domain is to attach the plasma membrane[40]. This sequence could help explain the presence of EHD2 on the plasma membrane and why EHD2 is located near Cav1. Although EHD2 does not have a Cav1 localization sequence; the presence of this domain could be what keeps these two proteins in close proximity. The p-loop is the site of phosphorylation in EHD2. Two mutants have been studied in the past that create a constitutively active and a dominant negative form that influences the activity, functions, and localization of EHD2[37]. The presence of the nuclear localization sequence led to the discovery that EHD2 has an effect on the regulation of KLF4 and KLF7 in response to membrane tension and osmotic stress[39]. The EH domain is named for the homology of Epsin15's N-terminal domain and contrary to Epsin15, a protein used by clathrin-endocytosis and scission, the domain is located on the C-terminus of the EHD2[34]. This domain is why EHD2 is thought to enhance Cav1's ability to undergo fission and fusion to the cell membrane. However, Cav1 pits undergo fission and fusion without the presence of EHD2 with a slight increase in frequency. These domains link to possible functions of EHD2, but many studies are unable to give a clear function of these domains and why EHD2 still contains them.

These domains found in EHD2 are similar to the Epsin15 homology domain family of proteins that are built quite similar to EHD2 and can be found acting as dimerization partners, but they are more often found in other cells types and in varying

amounts[34]. EHD1 and EHD4 have both been linked to Cav1 and endocytosis in both other cell types and in endothelial cells[41]. EHD3 is a regulator of vesicles leading to control of apoptosis within many cell types[42]. While there are highly conserved regions between EHD2 and the rest of the family, there seems to be a difference in the function of EHD2 compared to the rest of the EH domain family proteins.

Cav1 has been linked to the function of EHD2 in endothelial due to the proximity between the two proteins. Cav1 is a favorite protein among endocytosis investigators, lipid raft researchers, and membrane tension investigators. Cav1 basic structure changes the curvature of the membrane through its dimer hairpin design that causes a change in curvature in the membrane of cells to form pits. This change in membrane curvature and linkage to cholesterol makes Cav1 a prime candidate for non-clathrin-based endocytosis. There are many studies linking Cav1 and Pascin2, dynamin, and different Rabs[43,44]. This interaction led to the idea that Cav1 helps with active and controlled endocytosis although there is no clear link to what moves these receptors and internalized proteins to the Cav1 pits[45]. This leaves doubt if the true purpose of Cav1 is that of endocytosis, especially when Cav1 pits are upregulated in skeleton muscles, neurons, and endothelial cells[26,45,46]. These cell types undergo a lot of changes in pressure and tension and are in constant flux[26]. Lipid raft researchers looking at the type of phospholipids and fats that are influenced by the shape and binding of cholesterol leads people to think that this protein could lead to a better understanding of the formation and movement of lipid rafts[47]. The theory that makes the most sense is that Cav1 pits regulate and form in the

preparation for changes in membrane tension[8]. This hypothesis must be considered when the type of cells that upregulate Cav1 are cells that are bound tightly and required to change due the minor or massive shifts in tension, pressure, or movement. They used SEM microscopy to look at the bundling of actin with Cav1, with FMNL2, and with cavin1. There was a straightening of actin in the cells that lost Cav1 and cavin1 that is indicative of an increase in the tension of the cell[26]. In Zebrafish, FMNL 3 localizes at junctions during lumen formation, and increases of stable and polymerizing actin stabilize Endothelial cell junctions [49]. This is shown by removing the actin polymerization catalyst of FML3 through the deletion of the C- terminal domain and the inability of actin bundles to appear at lumens [49].

If the movement of Cav1 pits into the cells is a by-product of the overall change in the tension or pressure on a cell it could mean that the endocytosis that occurs is not the main purpose of Cav1 but a reaction to something else. Of the studies that look into the Cav1 outside the membrane tension and endocytosis, there are several that examines Cav1's role in the control of Rac1 and the changes in actin and junction stability due to the removal of v1[50,51]. This paper demonstrates the increase of Rac1 due to the loss of Cav1 leading to Rac1's inability to be degraded leading to an increase in the amount of migration in epithelial cells[43,52]. In the mechanical stress study, Cav1 acts as an anchor to phosphorylated EHD2 preventing EHD2'smovement into the nucleus[39]. If Cav1 and EHD2 are linked and in a similar location, this could mean that EHD2 may have a similar function in the control of Rac1, actin, and junctional stability.

Regulation of the actin cytoskeleton in the endothelial cells

Rac1, RhoA, and CDC42 are at their most basic a group of proteins that influence cell movement, migration, and intercellular binding through a signal cascade that suggests modulations in the actin cytoskeleton[53]. Rac1 is known to act within the first 30 minutes of HUVEC movement and movement through the lamellipodia of cells[53]. RhoA is known to act more long-term in migration studies and controls actin stress fiber creation and junctional stability[53,54]. This helps regulate myosin, a motor protein that binds to actin[55]. CDC42 is known for filopodial actin formations[56]. These three proteins interpret outside signals and through a signal cascade act on actin, myosin, and cadherin[57]. While many studies aim to define clear roles for these pathways. It is hard to separate out specific functions with no overlap between the proteins that are impacted and what these downstream proteins control, all three proteins lead to alterations in actin function and junctions to modulate and control the movement of a cell[54,58]. This makes searching for direct and specific controls for each protein a challenge and because these proteins interact and antagonistically inhibit each other determining the specific pathway requires multiple checks and tests to make sure the protein is only impacting one pathway directly. RhoA works with RhoA-associated kinases (ROCK)[59]. These kinases act on the downstream targets of RhoA including actin, myosin, cadherin, and CDC42[58]. Rac1 works through p21-associated kinases (PAK) to control the downstream targets like actin RhoA and CDC42[60]. RhoA, Rac1, and CDC42 are all required for the correct development of vessels and lead to embryonic lethality in mice when removed on a

global scale[61]. RhoA is activated by the release of VEGF from the surround cells. This activates ROCK and moves this protein from the cytoplasm to the plasma membrane, leading to the phosphorylation of MyoII[61,62]. Rac1 and RhoA are also known to have an antagonistic effect on each other, though RhoA deficiency does not lead to embryonic lethality when removed specifically from mice[61,63].

The research on the connection between Rac1 and Cav1 suggests the possibility that EHD2 acts in a comparable manner to Cav1 and that is why they are in the same place at the same time. While the study linking loss of Cav1 with an increase in Rac1 is based on endothelial cell culture work, there are a few studies that use breast cancer tissue and tumors to link loss of EHD2 to a decrease in E-cadherin and increase in Rac1[10,50]. These studies are in a different cell type so there is no reason to believe that HUVECs will be the same, but it is a clue to what EHD2 may be doing in endothelial cells[10]. This data matches the results of what occurs when Cav1 is removed from endothelial cells[50]. Though the link is tenuous, it is a good starting hypothesis to investigate how EHD2 may affect actin structures and cadherin stability in endothelial cells. This study may lead to a better understanding of how junction stability and migration are controlled during angiogenesis and what the possible long-term effects of overactive migration and weaker junctions mean for development, disease, and a possible new player in the actin and junctional stability pathway.

The purpose of my research was to untangle the role of EHD2 in the Rac1 and Cav1 interaction. Because Cav1 is shown to lead the ubiquitination of Rac1, the line of

research was showing if EHD2 had a role in Rac1 regulation, which had been shown to occur in breast cancer tissue. However, failing to produce comparable results while finding an increase in long-term migration in endothelial cells, suggests the idea that EHD2 may have an impact on the RhoA pathway. This suggests the hypothesis that EHD2 controls a part of the Rho A pathway. This hypothesis is supported by the loss of filamentous actin and an increase in serrated junctions in cells that have an excess of EHD2. When EHD2 is knocked down, there is an increase in actin, and the junctions over-stabilize. This change is not just seen in cells, but when the EHD2 gene is knocked out in fish, there is a visible change in the junctions that are comparable to what occurs in the EHD2si endothelial cells.

Chapter Two Methods

Cell Culture

Cell culture is maintained in 37°C incubator with 5% CO_2 piped in and double distilled water to maintain humidity. The HUVECs come from Protocell and are split three times using the protocol used by the Kushner lab three time to 20 plates of 70-90% confluence and then resuspended in FBS and frozen in the -80 for a slow freeze and placed the liquid nitrogen tank making 20-25 1mL cryotubes[64]. All HUVECS are grown in EGM2 medium from a company. Heka 293 cells were from Thermosphere and propagated as previously described in Webb, et al.

Cell passaging was performed as follows. If the passage and confluency are above 70% confluence and below passage eight, remove EGM2, add 4mL of dPBS, remove dPBS and repeat twice. Then add trypsin to the plate (1mL for a 10 cm dish and .5 for a 6cm dish), and returned to the incubator for 1 minute or until the HUVECs are detached from the bottom of the dish. Once cells are detached from the plate an equal amount of DMEM (Dulbecco's Modified Medium) containing 10% FBS was added to quench the trypsin. Push the remaining cells off the dish, and move the resuspended cells to a new 15mL conical tube. Spin at 5000xg for 2.5 minutes. Remove DMEM and trypsin mixture off the top of the cells and wash the pellet with dPBS once. Gently resuspend in EGM2 or

DMEM, depending on the cell type, for replating into multiple plates. When splitting the most one should split from 1 plate at 80% confluency should be four plates. This procedure was a starting point for most other procedures.

Fixation

The cells were removed from the EGM2 and put in 1mL 4% PFA (Paraformaldehyde) (dPBS base) for 10 minutes. Washed three times in TBST and moved to 1mL of 0.1% triton-X for 10 minutes and then moved to 2% BSA (Bovine Serum Albumin) in TBST for thirty minutes to an hour. After removing the BSA, add the primary antibody in the correct concentration and leave overnight in the four-degree fridge. Then move the slide to the shaker to rotate at 30 for an hour and then wash with TBST three times. After this, move the slide into secondary at a 1:1000 dilution concentration for all, unless specified otherwise, for 2 hours at room temperature or overnight in the 4°C fridge. The slide is then washed at least 3 more times.

Mounting slides

Two methods were used to image my cells. The first being mounted coverslips and the other being coverslip attached to a 3.5mm TC dish. One mounting method is to use flouromount to mount a cover slip on a slide and sealing the coverslip with any clear quick dry nail polish from any store. Another is making imaging dishes by punching multiple holes in 35 mm TC dish to create a large opening and using optical adhesive to attach the coverslip to the bottom of the dish and cure for 10minutes in the UV box. This lab used the gel imaging box covered in saran wrap to cure the dishes. These dishes do

not need to be mounted with flouromount. Both coverslips are soaked in 70% ethanol and dried and then treated with UV light for 3 minutes before cells are mounted. Most of the cells are plated directly on glass which does increase the tension the cells are under.

siRNA Knockdowns.

Knockdown used a predesigned siRNA ehd2 (s26959), cav1 (s2446), and Scram from ThermoSci. siRNA was resuspended in 250 microliters of nuclease-free water. The concentration of siRNA is 20μM stock. The concentration is diluted to by adding 5 microliters to 120 microliters E2 buffer resuspended cells. The cells are then shocked by the neon system and moved back to 10 cm plates with EGM. The neon electroporation system and protocol come from Invitrogen. The E2 buffer is 250 mM sucrose and 1mM $MgCl_2$ in dPBS. The settings used for all HUVEC experiments are 1350V, 30-width, and 1 pulse. Experiments were rerun if the system shorted due to bubbles in the sample.

Monolayers

Every monolayer represented in this paper was moved to an imaging dishes 14-24 hours before imaging. All monolayers are at least 70% confluent, most were 90% or higher. They were all fixed according to the fixation protocol above.

Single cells

Single-cell assays (no cells are touching it) investigated the amount of actin (Integrated density of fluorescence normalized to the average of the wild-type cells imaged at the same date) in the cell that has been allowed to freely move for at least 12 hours. These cells were added to cover slips 14-24 hrs before fixing. These were then

immediately moved to secondary after blocking in BSA overnight and imaged the next day.

Wound healing assay

Migration assay, IDIBI well plates with partition, fixed 8.5 hours after partition removal. Migration assays used cells that were added to the IDIBI partition plates 18-24hrs. At this point, each condition lifts the partition, and half the dishes are fixed. These other half dishes are monitored every 30 minutes for at least eight hours or until both sides touch in only condition. For this migration assay, the cells only traveled for 8.5 hours until they were fixed.

Spread assay

Spreading assay was used to determine Rac1 behavior and begins by adding the cells to the dish with media and/or drugs in it and then replacing dishes in the incubator and waiting 45 minutes. At 45 minutes, at which point most Rac1 activity is done, and fix the cells. After blocking, add secondary to the cells for 2 hours or overnight. And then image.

EGTA Junction Disruption Study

The EGTA study uses EGTA at the final molarity of 250 μM to dissolve junctions at the edge of cells for an hour. To make sure that all junctions are dissolved, a time course was done for an hour with time points at 0 minutes, 15 minutes, 30 minutes, 45 minutes, and 1 hour. After an hour EGTA was removed from the cells and then another time course was set for 20 minutes, 45 minutes and 1 hour, and 1 hour and 15 minutes.

These cells were fixed, according to the above protocol, and the measurements of the percentage of straight and serrated junctions and if all junctions are dissolved were taken.

G-actin Fixation

G-actin/ F- actin is like the actin amount experiment in that the cells are dropped on the cover slip 18-24 hrs before fixation. They are then washed in dPBS, and put in 100% acetone for 15 minutes. They are then soaked in GC protein was in dPBS for an hour at room temperature, blocked in 2% BSA based in dPBS, and then it was placed in GC primary antibody in BSA overnight. This was washed three times in PBS and moved to secondary for two hours and then further washed in PBS. At no point should this experiment use a stronger detergent than acetone because it could allow for the release of globular actin and change the amounts and ratio in the cell.

Antibodies

Antibodies used for this project were DLL4 (goat) 1:1000 ThermoSci PA546974, EHD2 (rabbit)1:2000 ThermoSci PA561497, Cav1 (rabbit)1:20,000 ThermoSci PA1064, VE-cad(mouse)1:1000 14-1449-82, VE-cad (goat)1:2000 R&D AF938, Rac1(rabbit) 1:500 ThermoSci PA1091, RhoA(mouse) 1:250 Santa Cruz sc-418, and Anti-GC (rabbit)1:1000 sigma HPA019855-25UL. The Secondaries used (1:1000 cell culture, 1:250 in fish) were 488,555,647- phalloidin, Alexa flour 488- anti-rabbit, anti-mouse, Alexa flour 647- anti-rabbit, anti-goat, anti-mouse, and Alexa flour 555- anti-rabbit, anti-goat, anti-mouse.

Drugs

Drugs used were a ROCK inhibitor Y27632- 688001-500UG, CN04 or RhoA inhibitor 1 from cytoskeleton, NSC rac1 inhibitor, CN02 or Rac1 activator from cytoskeleton, IPTG from Goldbio, Src inhibitor1 Selleck Chem S6567, Cycloheximide sigma C7698-1GS, and Blebbistatin sigma B0560-1MG. The concentration was as followed: blebbistatin was used 10µM, Y27632 was used at 10µM, ITPG was used at concentration of .5mM, CNO2 was 100ng/mL, and NSC was 10 µM, Cycloheximide 10µg/ml.

Adenovirus generation and Use

Adenovirus constructs (tagRFP-EHD2 and Emerald-Clathrin) were created as previously described.[24] In brief, constructs were introduced via Gibson Assembly into pShuttle-CMV. pShuttle-CMV plasmids were then digested overnight with MssI (Thermo Fisher Scientific, IVGN0244) and purified via gel extraction. Linearized pShuttle-CMV plasmids were transformed into the final viral backbone using electrocompetent AdEasier-1 cells. Successful incorporation of the pShuttle-CMV construct into AdEasier-1 cells confirmed via digestion with PacI (Thermo Fisher Scientific, IVGN0184). 5000 ng plasmid was then digested at 37°C overnight, then 85°C for 10 min, and transfected in a 3:1 polyethyleneimine (PEI, Sigma Aldrich, 408747): DNA ratio into 70% confluent HEK 293A cells (Thermo Fisher Scientific, R70507).

Over the course of 2–4 weeks, fluorescent cells became swollen and burst or budded off the plate. Once approximately 50% of the cells had lifted off of the plate, cells were

removed and centrifuged at 500x *g* for 5 min in a 15 ml conical tube. The cells were resuspended in 1 ml DPBS (Genesee Scientific, 25-508B). Cells were then lysed by 3 consecutive quick freeze-thaw cycles in liquid nitrogen, spun down for 5 min at 500x *g*, and the supernatant was added to two 70% confluent T-75 flasks. Propagation continued and the collection was repeated for infection of 10 15 cm dishes. After collection, 8 ml viral supernatant was collected and combined with 4.4 g CsCl (Sigma Aldrich, 289329) in 10 ml DPBS. The solution was overlaid with mineral oil and spun at 100 000xg 100°C for 18 h. The viral fraction was collected with a syringe and stored in a 1:1 ratio with a storage buffer containing 10 mM Tris, pH 8.0, 100 mM NaCl, 0.1 percent BSA, and 50% glycerol.

Western Blot Protocol

Western blot protocol uses cells that have either been drugged, have had a protein knocked down, or have been exposed to adenoviruses according to the protocols above. These cells are either placed in 150 microliters of Ripa or Cell lysis buffer based on if this is looking into protein amounts or into phosphorylation states of RhoA and Rac1. The cell lysis buffer is based on the concentrations found in the Cytoskeleton RhoA measurement kit: the recipe is as follows 1.25mL of 1M Tris, 0.25mL of 1M MgCl2, 12.5 mL of 1M NaCl, .5mL of Ipegal and 10.5 mL of dH_2O. This buffer is added to plates that have been washed in cool dPBS once and has been completely removed from the dish. The buffer is spread with the cell scraper and then pipetted off the dish and into the

Eppendorf tube and put on ice for ten minutes and then spun down for ten minutes at 11000xg.

These lysates are then measured using the BCA kit from ThermoSci and adding 5 microliters of either lysis buffer or sample to 45 of lysis buffer and adding 20 microliter BCA reagent B to .98ml of BCA reagent A. Add 950microliters to each sample and the blank. Incubate for 30 minutes at 37^0C and measure the OD at 562, according to instructions supplied by Thermofischer. This OD is normalized according to the equation provided by my lab. The OD measurement was used to normalize the amount between samples.90microliters of 6x lamelli is added to 10microliters of 1MDTT on the day samples are set up to run for western. SDS page gels were made in a 1.5 mm mold and ranged from 12% to 9%. All gels were run at 150 volts on a Bio-Rad system using the running buffer, transferred at 80 volts for an hour and fifteen minutes, then blocked for 30 minutes and soaked in primary overnight. The secondary was anti-mouse or anti-rabbit HRP and was soaked for at least 2 hours. ECL was applied directly after mixing the blots that were imaged on a black background.

Protein Purification

Protein purification starts with inserting the correct sequence into a pGEX backbone or purchasing the correct plasmid on AddGene and moving those plasmids into chemically competent NiCo cells. The GST-RhoA binding domain came from AddGene and GST-Pak1 (rac1 binding domain) from AddGene. These plasmids were then screened for and confirmed via digest and sequencing from QuintaraBio.

Protein A beads/resin from Goldbio, LB, and TB made in the lab. A stock of IPTG was stored at a 1M concentration from Goldbio. Coomassie was made in lab and De-stain was made with 40% acetone, 10% methanol, and 50% water.

After confirming that the sequence is correct, the night before growing the protein make a 30mL LB plus 1x antibiotic culture. This culture is added to 900 mL of TB and 100 mL TB salts and 1x antibiotics and grown in-between 2 hours and -4 hours. Checking OD at 600nm at 1 hour and every 30 minutes after this till the OD reaches .4 stopping the growth phase before .6 OD. At this point take a pre-ITPG sample (1mL) and add ITPG to a final concentration of 0.5mM(500microliters) and let the culture shake at 37 degrees, 250 rpm for 3 hours. Take a post-expression sample. Spin the samples down for 10 min and remove the LB and freeze. Split the culture between four bottles making sure the bottles are of equal weight and then spin at 4,000xg for 10 minutes and put immediately on ice. Remove LB and resuspend in 20mL of ice-cold PBS because the plasmids use a GST tag and spin at 4000xg for 10 minutes remove dPBS and place in freezer.

At this point resuspend pre- and post-samples in 100 microliters 1x Lamelli plus DTT and boil for 5 minutes. Make a 12% SDS page gel and run 50 microliters of each sample for about an hour, waiting for the ladder to reach bottom of the gel. Place gel in Coomassie overnight. Add de-stain in the morning, after Coomassie has been removed from the gel and it has been rinsed in dH2O. The post should have a huge band at the correct size of the protein.

Only if this is confirmed should the rest of the procedure be followed. The pellet collected post IPTG is resuspended in 30mL of dPBS with 100 microliters of proteinase inhibitors, and 1 mg/mL of lysozyme. This resuspension then sits on ice for 30 minutes and is sonicated (5s on and 20s off for 5 minutes) three times with a cool-down period of 5 minutes in between, while on ice. This is spun down at 12000xg for 10 minutes. The supernatant is moved to a new 50mL conical, remove 1 ml (for next western) then loaded with 1mL of beads and rocked in the fridge for 2 hours. Spin this down at 500 for 1 minute, move the beads to a 15mL conical and wash in PBS five times. Rolling for 15 minutes and spinning down at 500g for a 1minute. Remove excess liquid and put in a 4°C fridge. After the western is done, throw excess supernatant.

At this point add 10 microliters of 6x lamelli + DTT to 50 microliters of supernatant and add 20 microliters of 1x Lamelli + DTT to 20 microliters of beads and boil for 5 minutes. Make a 12% SDS page gel and run 50 microliters of each sample for about an hour, waiting for the ladder to reach the bottom of the gel. Place gel in Coomassie overnight. Add de-stain in the morning, after Coomassie has been removed from the gel and it has been rinsed in dH2O. both should have a big band, but the beads should have only had a huge band at the correct size of the protein. This means that the correct protein has been expressed and attached to beads. Add an equal amount of glycerol to the beads, mix thoroughly and freeze at -20 or -80 °C. This should preserve the protein for at least a year.

Protein Pulldown

Protein pulldowns were performed with cells in a 10 cm dish with about 70-90% confluence. These cells were washed with cold dPBS and moved into cell lysis buffer based on the recipe from Cytoskeleton. The protein amount between the samples is normalized and a sample of background is taken (at 5x) then to all samples add 20 microliters of Pak1 or 50 microliters of RBD beads (these beads come from the protein purification stage) and bring the total volume to 500 microliters. All samples are rotated at 4 °C for at least 4 hours. After this, the supernatant is removed, and the beads resuspended in 50 microliters of 1x lamelli plus DTT and run western. The two gels one is a background sample which will be tested with RhoA or Rac1, and GAPDH (to check that protein levels are the same between samples), and the other is phosphorylated RhoA or Rac1. This will follow the western blot protocol above.

Cloning

Cloning allows for the creation of fluorescently tagged proteins, proteins that are altered, and many other properties. Most start with primers that will copy the gene of interest from a gene block from IDT.The primers used are: T72A Q5 primers; ΔNLS Q5 primers- GGCAGCTTCACAGGTAGCTGATGATGTAAGC, CAGCTACCTGTGAAGCTGCCTGTCATC. These genes of interest with a Gibson overhang. The PCR mixture for cloning into a backbone can use SuperFi master mix or using Rediload, 10x PFU buffer, dNTPs, and PFU. The backbone is cut using restriction enzymes, EcoR123 and Xho1. Most restriction digest is done in AnzaRed buffer. These

are placed together with 1-2 microliters of backbone and 4-3 microliters of the insert into a Gibson reaction tube (15 microliters) with the overall total being 20 microliters. This Gibson (3 microliters) is transformed into 90 microliters of chemically competent top ten cells. These are plated and grown overnight at 37 °C . Colonies are picked and mini-prepped according to the protocol by Zymo. Mini-preps are digested with restriction digests and run on a 1% agarose gel made with TAE and using Ethidium bromide to visualize DNA. Then confirmed via Sanger sequence from QuintaraBio.

Q5 mutagenesis kit from NE biolabs was used to alter existing plasmids. Using this method, tagRFP-EHD2-CA based on paper, tagRFP-EHD2-DN based on paper, and TagRFP-EHD2-NLS based on paper were created.

Zebrafish Staining

Fixation protocols used for zebrafish are as follows. Fish were collected at 24-27 hours post fertilization (hpf) fish, 48-51 hpf fish, and 72-75 hpf fish. If drugs were used, they were applied at 23.5hpf and kept on till 4 8hpf. The living fish are added to 4%PFA overnight at 4 °C and rotating. Then moved to 100% MeOH till all samples are collected. All MeOH samples are rehydrated and moved from 100% to 75% MeOH to 50% MeOH to 25% MeOH and then to TBST and washed 4 times. The fish are then moved to .5% triton for 12 hours in a 4°C rotating and blocked in 2% BSA with TBST and moved to primary VE-cad 1:1000 rabbit anti-zebrafish for 24 hours and then washed 4 times in TBST and then moved to 1:250 secondary anti-rabbit 647. The protocol was adapted from the Belting lab and the VE-cad antibody was soaked in wild-type fish to prevent

non-specific binding before being used in experiments. These fixed fish were then embedded in 70% agarose and positioned to be imaged in 20x objective and 40x objective. No 405 laser was used, as Hoechst was not used to look at DNA to prevent background noise when imaging whole fish.

Imaging Live Zebrafish

Movie protocols for imaging fish at 24hpf begin with embedding the fish and adding 1x tricaine on top of hardened agarose-containing fish that has been moved into position. Then move the fish to the confocal microscope. At this point, the microscope should be on and on the 40x objective and the correct laser to view the fluorophore, in this specific case it was KDRL: mCherry viewed with the epi-Texas Red and the 555 lasers. Orient the fish to view 3 vessels and set an image to be gathered every 3 minutes for 3 hours. At this point, the fish is between 25-29 hours. Repeat up to three times in a single session. The fish for this experiment were set up on different days in the same week and if drugs were applied, they were applied thirty minutes before imaging started and never removed, similar to the treatment of the fixed fish or live fish imaged at 48hpf.

Zebrafish breeds

Breeds used for imaging were KDRL: GFP; Ehd2b-/-, KDRL: mCHerry; Ehd2b-/-, KDRL: mCHerry; KDRL: mCHerry; Ehd2b-/-, KDRL: GFP, KDRL: mCHerry, KDRL: mCHerry; KDRL: mCHerry, KDRL: mCHerry. The ehd2b knock was generated in a KDRL: GFP background through CRISPR/cas9 injection at the single cell stage by Amelia Webb. The rest were generated through breeding schemes and screened for

correct fluorescent protein expression and knockout of the correct gene through PCR and visualization. All fish were set up in individual tanks either one male to one female or two males to three females, one the night before and released at 10:30-11:15 am the next day.

Genotyping

The genotyping protocols start with cutting fish tails and end with the sequence confirmed via QuintaraBio Sanger sequencing. Removal of fish tails happens when the fish are at least 2 months old and can be done any time after that. Clipping a small piece from the tail occurs after the fish is under 1x Tricaine and in the system water. After which the fish is briefly removed from the water when it can no longer swim, with a spoon, and a piece of the outermost tail is cut with a razor blade. The fish is moved quickly into a numbered tank and used the spoon is to move clean water by the fish. The tailpiece is moved into a correspondingly numbered PCR tube. The fish is moved back to the shelf in the numbered tank to be fed and wait for the results. Lysis protocol uses NaOH, EDTA, Tris-HCl, and the fishtail to run samples on. Combining 2.23 microliters of 10M NaOH, 8.93 microliters of 20mM EDTA, and 892.86 microliters of distilled water into an Eppendorf tube makes a lysis buffer. Add 15 microliters of the lysis buffer to each PCR tube containing a tail clipping and move to the PCR machine to run the program at 95°C for one hour. After the hour is complete immediately add 15 microliters of 40mM Tris-HCl to each tube containing lysis buffer to stop the reaction. Each PCR only used 3microliters of DNA for each fish. Primers used for genotyping are. And they

used this program to create the sequence for gel extraction. PME backbone was used to provide a platform to perform Sanger sequencing. These backbones were integrated with the genomic sequence obtained from the fish. The plasmid was transformed into bacteria and then sent for sequencing to validate the deletion of EHD2b in zebrafish. The sequencing of all plasmids was verified by sanger sequencing done by QuintaraBio and the primers they provided or our own primers. This along with an in-situ hybridization was done by former master's student Amelia Webb to show that EHD2b is the expressed orthologue in the vessels of zebrafish instead of EHD2a.

Chapter Three Results

The first goal of this study was to prove that EHD2 and Cav1 colocalize in endothelial cells (fig. 1A). Because these two proteins are in similar areas the thought is that similar functions within the cell. To test this idea, a spreading assay was used to compare how quickly cells cover an area after being added to a dish. Comparing the knockdown of EHD2 and Cav1 in a spreading assay is a straightforward way to determine if the ablation of EHD2 is comparable to the ablation of Cav1. However, the Cav1 knockdown has a substantial increase in area over a brief period of time compared to both the Scramble condition and the EHD2si condition (fig. 1B, C). The original thought was that EHD2 would work in a comparable manner to Cav1 and the EHD2 knockdown would show a similar increase in area. To continue down this line of thought, a wound-healing migration assay was done[10]. This assay would allow for a more long-term study of migration over at least an eight-hour period. This experiment indicates that the EHD2 knockdown migrates at a faster rate than scramble condition cells. Cav1 has a massive increase in migration compared to the scramble conditions and EHD2 knockdown (fig. 1D, E). It should be noted that cells without Cav1 and EHD2 have a spreading rate and migration rate analogous to the EHD2 knockdown (fig. 1D, E). These results indicate that EHD2 has a role in long term migration that is independent of the cav1 Rac1 pathway.

Actin

Because actin is central to a cell's ability to migrate, this next set of experiments investigated changes in the amount of filamentous actin (f-actin) in a cell. When looking

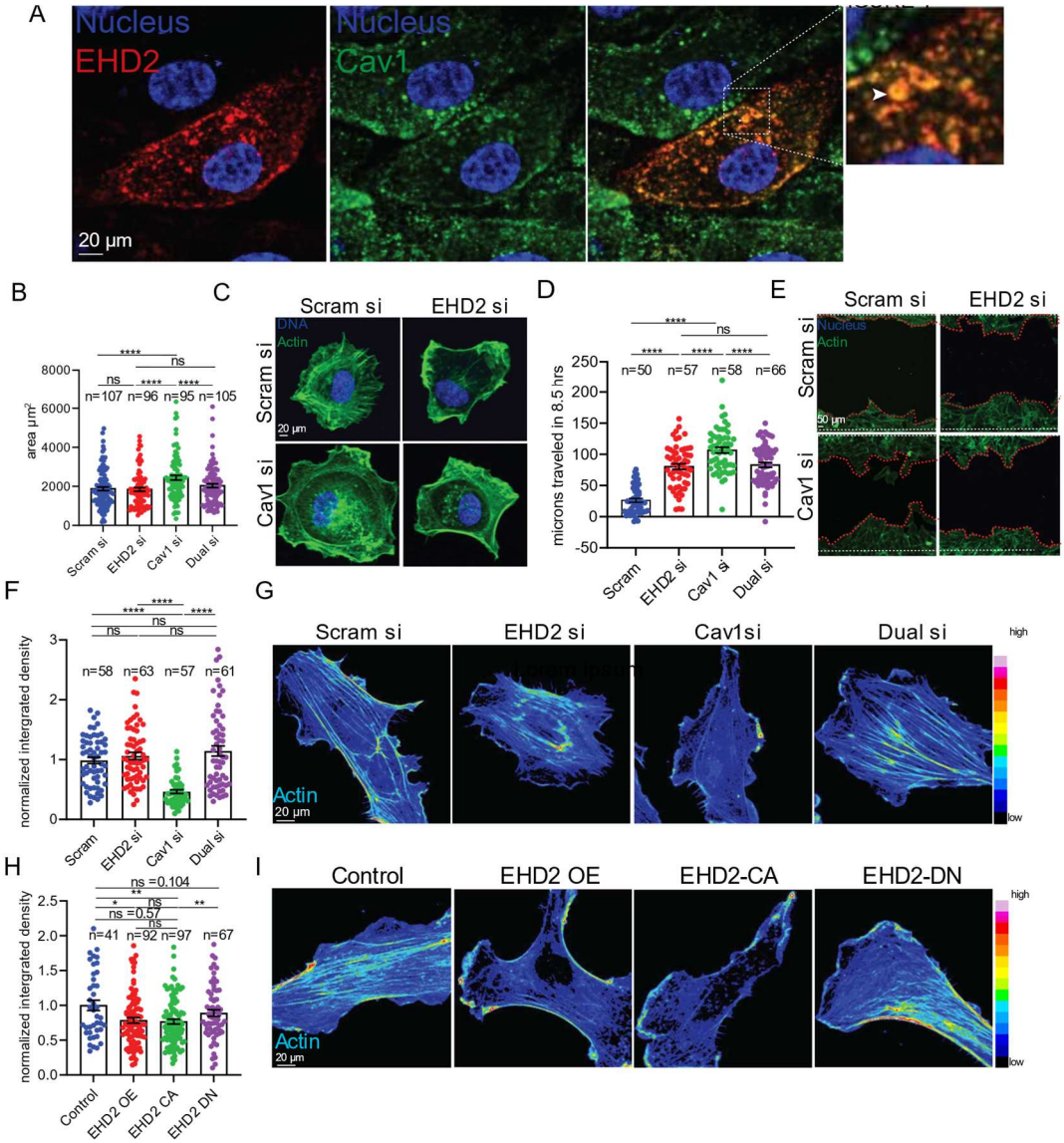

Figure 1 (A) Image of HUVECs Stained with Cav1 and Treated with tagRFP-EHD2. (B, C) The quantification of a spreading assay and the representative images of the knockdown conditions measured in μm^2. this experiment was repeated three time (n=107, 96,95, 105). (D, E) a scratch wound assay post 8.5 hours and the graph measuring distanced traveled in µm. Only one reputation with the distance measured n=50,57,56,66 times. (F, H) Pseudo colored rainbow representative images to high changes in actin, where the highest amounts are white and the lowest amounts are black. (G, I) the quantification of the amount of filamentous actin in those cells with three repetitions of those experiments and n=58,63,57,61 and 41,92,97,67.

into the amount of f-actin in the cell, the loss of EHD2 did not seem to impact the fluorescence intensity of the actin. The Cav1 knockdown has a huge decrease in the amount of actin filaments formed. Because the loss of EHD2 did not seem to have a huge effect on actin, excess EHD2 was added to cells. Conversely, when EHD2 is overexpressed in HUVECs there is a 25% decrease in fluorescent filamentous actin. This decrease is also observed in the cells expressing EHD2 constitutively active (EHD2-CA). However, the EHD2 dominant negative (EHD2-DN) does not show a significant decrease from cells that do not overexpress EHD2. This set of experiments proves that EHD2 does have an impact on actin's ability to form filaments, and this ability is present regardless of its phosphorylation state.

Because Rac1 is a known actin effector molecule, some of the above experiments were repeated with the addition of NSC, a Rac1 inhibitor, and CN02, a Rac1 activator. The spreading assay was repeated with these new conditions. Examining these results indicates that EHD2 was not responsive to the Rac1 inhibitor treatment (sup. fig. 1A, B). This is the opposite of what is seen in Cav1 where the activator of Rac1 has little effect but the NSC does rescue the Cav1 phenotype. This CN02 acts very similarly to Cav1 when measuring the filamentous in the cell, but this does not look like the over-expression of EHD2. These findings suggest that Rac1 is not be the only molecule accounting for the for the differences between EHD2 and Cav1, and that there are other molecules that impact actin contractility.

Given that contractility-based modulations have been shown to counteract Rac1 activity, we next tested if EHD2 KD cells were responsive to perturbations in RhoA-

mediated contractility. In control cells treated with the RhoA inhibitor, y27632, or the

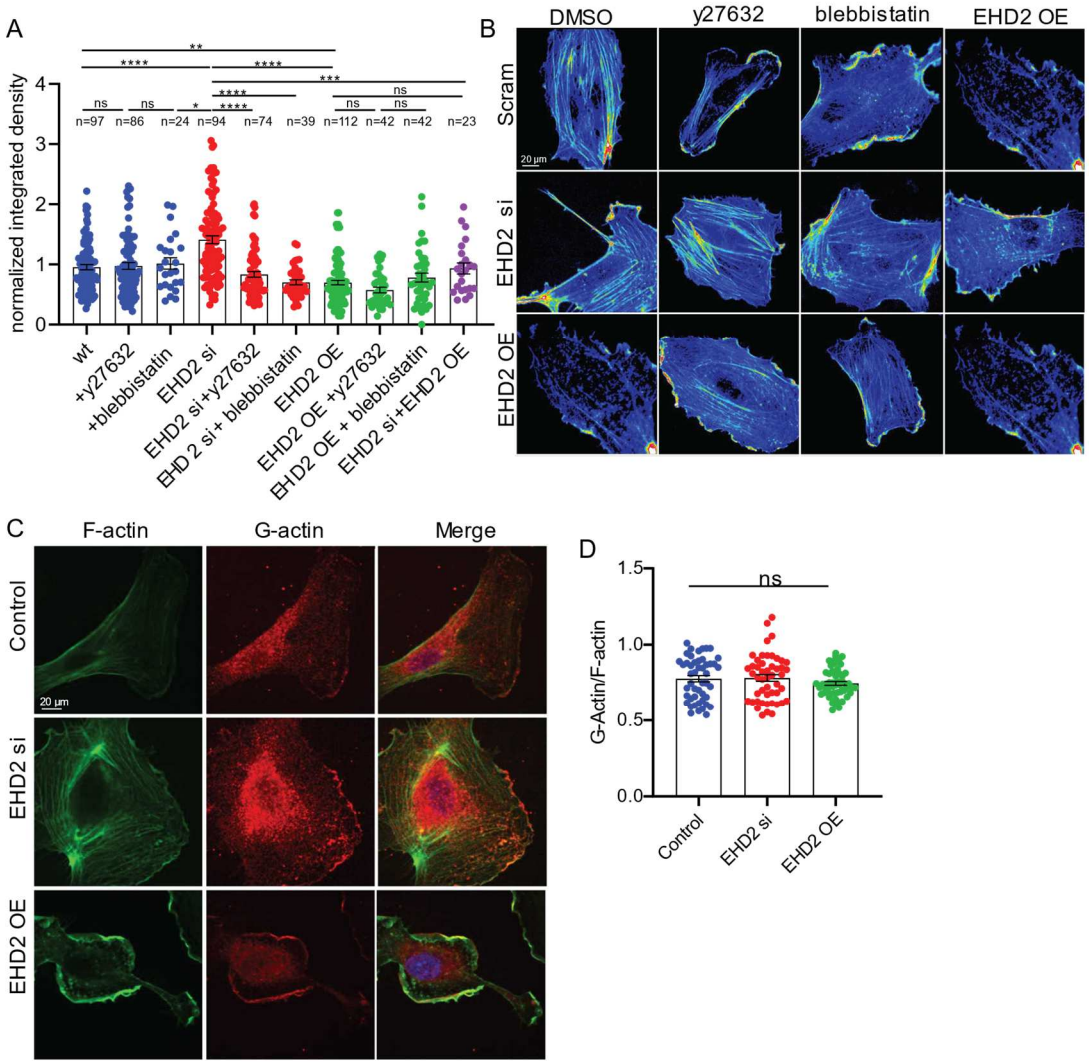

Figure 2 (A, B) a quantification and representative images of the application of ROCK inhibitors and Myosin inhibitors to HUVECs. This was replicated three times with an n=97, 86, 24, 94, 74, 39,112, 43, 43, 24. (C, D) The representatives are pseudo colored rainbow to highlight the differences in actin amounts. The representative and quantification of g-actin and f-actin ratio. This experiment was replicated three times and the n= 48, 49,50.

myosin inhibitor, blebbistatin, did not produce differences in actin content (fig. 2A). When comparing the treatment of cells with y27632 and the overexpression of EHD2, there is a similar loss of intensity. This comparison highlights that the pathway disrupted by EHD2 must be something that controls ROCK. While EHD2si did lead to an increase in the brightness of the actin, the intensity decreases in the presence of y27632 and

blebbistatin. Using the EHD2-tagRFP adeno virus to rescue endogenous EHD2 in the EHD2 knockdown indicates that EHD2-tagRFP can replace endogenous EHD2 in function and reduce the filamentous actin in cells (fig. 2A). This result demonstrates that this may be a RhoA phenotype.

Investigating if there are any changes in the availability of globular actin(g-actin) may give more insight into how quickly these changes in actin are occurring. This increase in filamentous actin (f-actin) should alter the amount of globular actin(g-actin) in the cell as there is usually an amount of free actin and actin that has formed fibers. If there is an increase in the amount of F-actin then there may be a decrease in the amount of g-actin. However, there is an only slight change in the ratio between g-actin and f-actin between the conditions. This lack of results in the three cell types suggests the possibility that EHD2 may alter actin amounts in the nucleus.

Junctions

RhoA is known to strengthen junctions, so investigating if there is an alteration in how junctions are forming may give more insight into how EHD2 is impacting RhoA. Testing how EHD2 changed VE-cadherin (VE-cad) junctional integrity, the first measure of VE-cad junctions taken was the relative fluorescence intensity of the VE-cad found at the edge of cells with EHD2, without EHD2, with excess EHD2. Both experimental conditions demonstrated less VE-cad at the border of cells compared to controls (fig. 3A). Because this change in fluorescence does not give any concrete information, the quality of the junctions was observed. Looking at cells and determining if a junction is straight or serrated corresponds to stabilized and non-stabilized junctions, respectively

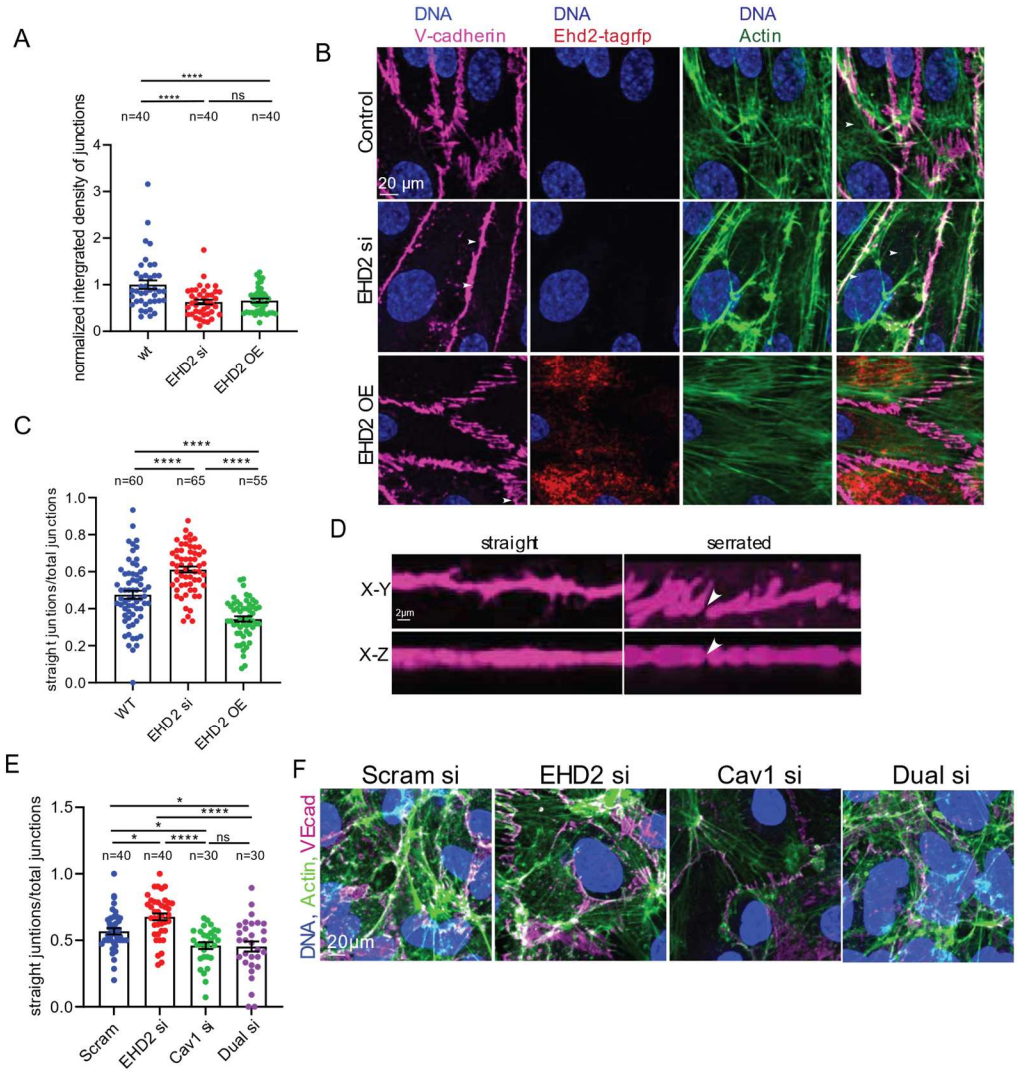

Figure 3 (A, B) comparison the fluorescence of VE-cadherin at the junctions of cells in the control, the EHD2 knockdown, and the adeno-tagRFP-EHD2 that was repeated three times (n=40,40,40). (C) A measurement the percentage of straight junctions. This experiment was repeated 3 times (n=60,65, 55). (D) A look straight and serrated junctions at a top down and axial view. (E, F) The quantification and representation of the number of straight junctions with EHD2si, Cav1si, and Dualsi, repeated three times

(fig. 3D). Our results show a significant difference between EHD2si and the control. The EHD2si show a higher percentage of straight junctions compared to the control. EHD2 over expression had a decrease in the percentage of straight junctions (fig. 3C). This demonstrates that EHD2 destabilizes junctions. When looking at the knockdown series and comparing the percentage of straight junctions with and without EHD2 and Cav1 there is a significant difference between the conditions (fig. 3E). Knocking down Cav1 in

cell indicates a similar impact as a EHD2 over expression but not as significant as the over expression. The dual knockdown indicates a recovery to the control cells. This pattern tracks with what occurs in single cells.

Given that the EHD2 knockout seemed to increase RhoA-based contractility, we tested if limiting contractility could rescue EHD2 loss of function junctional perturbations. To test if the phenotype matches a RhoA deficiency, y27632 and

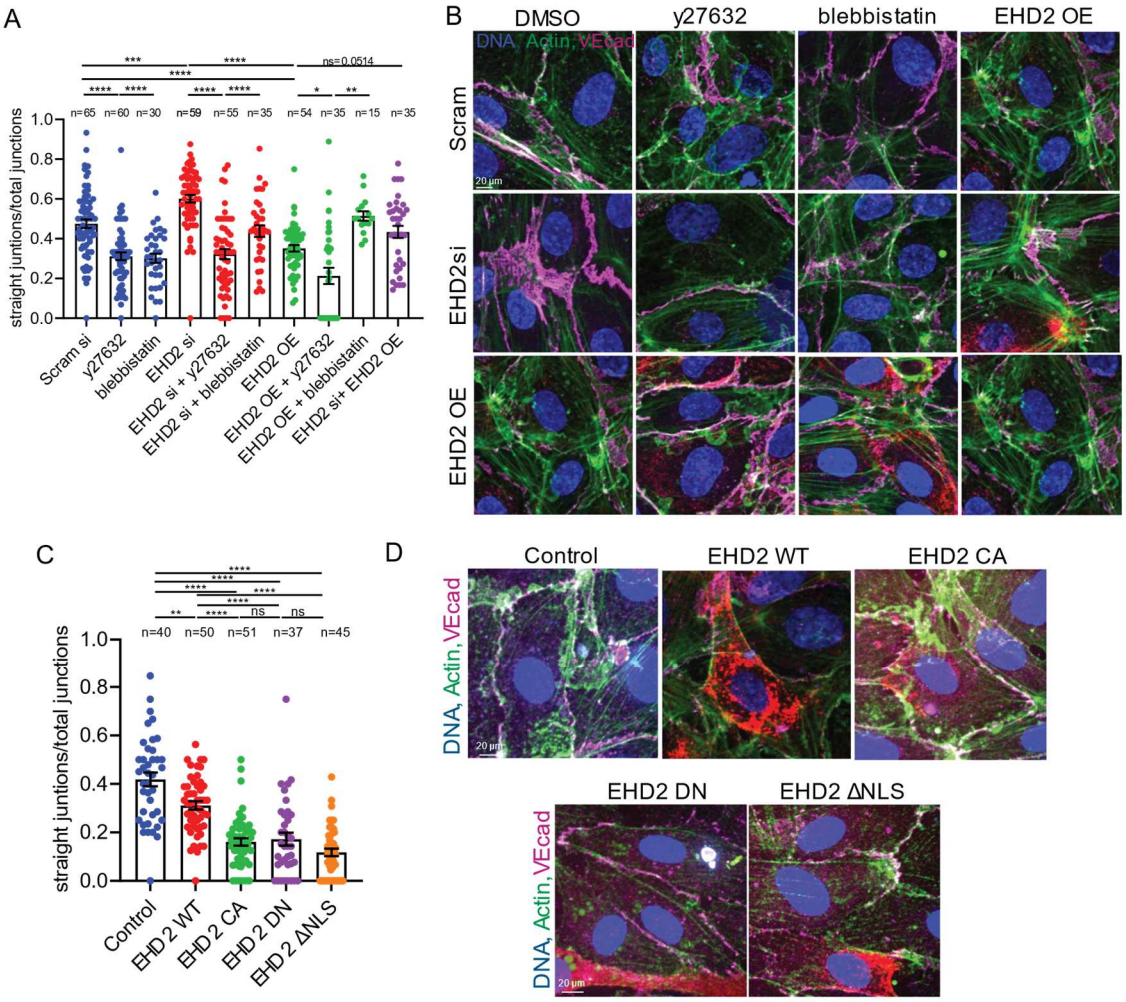

Figure 4 (A, B) a comparison of EHD2, 10µM y27632, a ROCK inhibitor, and 10µM blebbistatin, a Myosin 2 inhibitor on the formation of junctions in a monolayer of HUVECs this was repeated three times with a. (C, D) A comparison of junctions when different forms of EHD2 are added to HUVEC cells.

blebbistatin were added to all conditions. These drugged conditions mimic the loss of RhoA that is replicated when EHD2 is overexpressed. When the EHD2 knockdown was exposed to y27632, the impact of the ROCK inhibitor was more than return junctions to a wild-type percentage but similar to the effect of when the ROCK inhibitor was only applied. Blebbistatin treatment to the knockdown condition is more pf a return to normal than y27632(fig. 4A, B). But this statement is also true when looking at the EHD2 overexpression treated with blebbistatin. When one is able to have a cell return to normal function after the removal of a protein using something like a knockdown with either add excess protein, or a virus that will create the missing protein it is referred to as rescue experiment. When the EHD2si condition, the removal of the protein, is exposed to the adeno-EHD2-tagRFP, the addition of replacement protein, the cell begins to recover or act like wild-type cells (fig. 4A, B). This indicates that the missing protien can be replaced with the adeno version to some extant. Though the recovery is not a full recovery and this could be due to adeno-EHD2-tagRFP is still overexpressing EHD2 and this could lead to minor defects. The phenotype that occurs when EHD2 is overexpressed, also occurs when the RhoA pathway is inhibited.

Looking into the role of the EHD2 means looking into the role of phosphorylation and how the specific domains of EHD2 impact its role. While the overexpression of EHD2 suggests a decrease in straight junctions, the EHD2- constitutively active (CA), EHD2- dominant negative (DN), and EHD2- delta nuclear localization sequence (ΔNLS) has an even more significant decrease in straight junctions (fig. 4C, D). This significant decrease could be due to the fact that, unlike the wild-type EHD2, an adenovirus, these

constructs are shocked into to the cells. However, this suggests that there are not significant differences between the domains in EHD2 in this context.

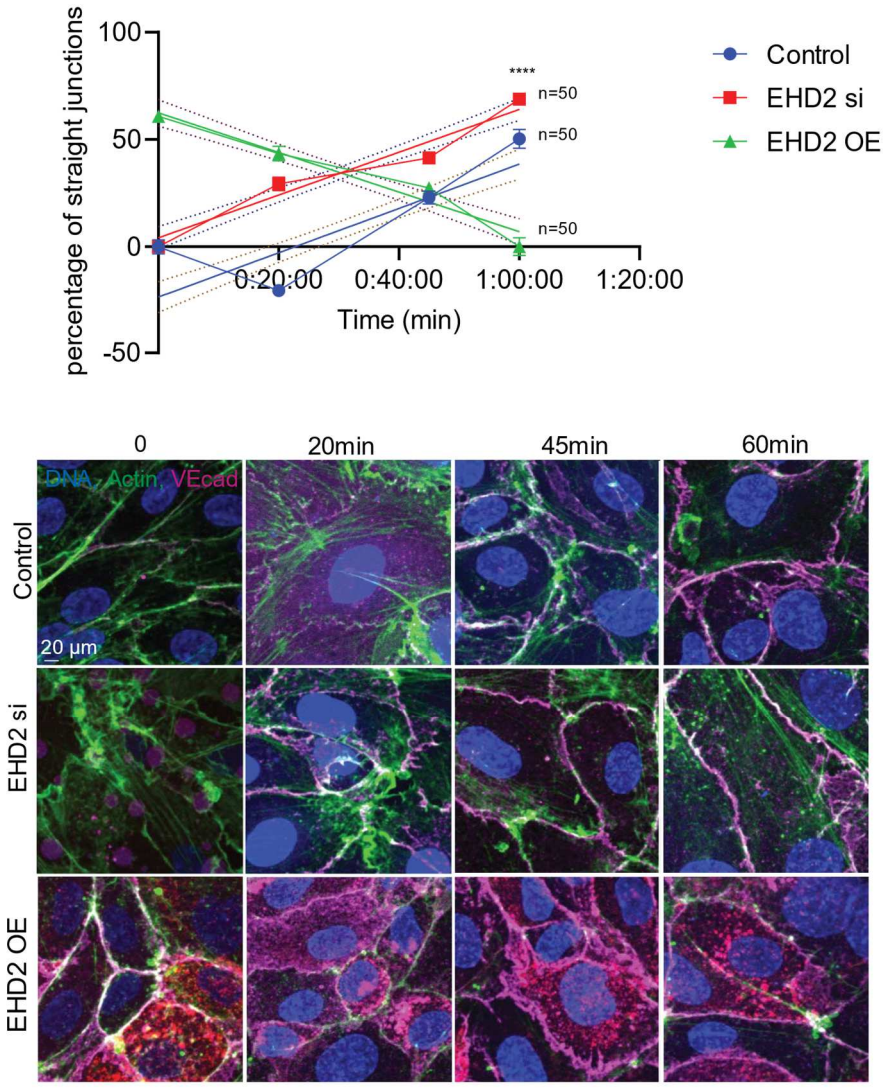

Figure 5 (A, B) A graph that looks into the changes in straight junctions over time after the removal of 250µM EGTA and the representative images that show the increasing number of straight junctions over an hour. This experiment was repeated twice and the n=50 for all conditions

Junctions are incredibly dynamic in nature. To better understand if these junctions behaved differently upon formation; have less movement, have a quicker turnover rate, or if upon disassembly these junctions recovered at different rates; EGTA was used to

dissolve these junctions and look at how they recover. Treating monolayers with EGTA will remove VE-cad from the borders of cells and destroy most of the junctions in wild-type cells in about 30 minutes to an hour (sup 3). For this study, EGTA was added to monolayers and after an hour was removed. At each time point, you can see that cells without EHD2 recovered faster than their wild-type counterparts. Unexpectedly in the overexpression, the amount of junctions starts quite high, and as more time passes in the presence of calcium more junctions deteriorated. This indicates that removing EHD2 allows straight junctions to form at faster rates than the control monolayer.

Animal Modeling

To determine if any of what was occurring in 2D cell culture had any real impact in an organismal model, animal models have been relied on heavily to determine if the loss of a single protein can have any effect on the health of an individual. Using Zebrafish (Danio rerio) animal models to test for physiological effects of loss of EHD2, there were observed defects in the formation of Inter Somatic Vessels (ISVs). While there were no lethal side effects of the loss of EHD2, consistent with reports in mice[35], we observed that at 48 hours, fish without EHD2 tended towards thinner vessels. When searching for explanations for the thinness of EHD2 KO, it is likely that there were fewer cells traveling during migration, and there were fewer nuclei in these vessels. This trend continued and the fish do not recover in overall width or number of nuclei per vessel. This led us to look at VE-cad in zebrafish at 48 hours and there appeared to be straighter junctions in more places in the EHD2 knockdown. This demonstrates that EHD2 can alter the formation of blood vessels in fish.

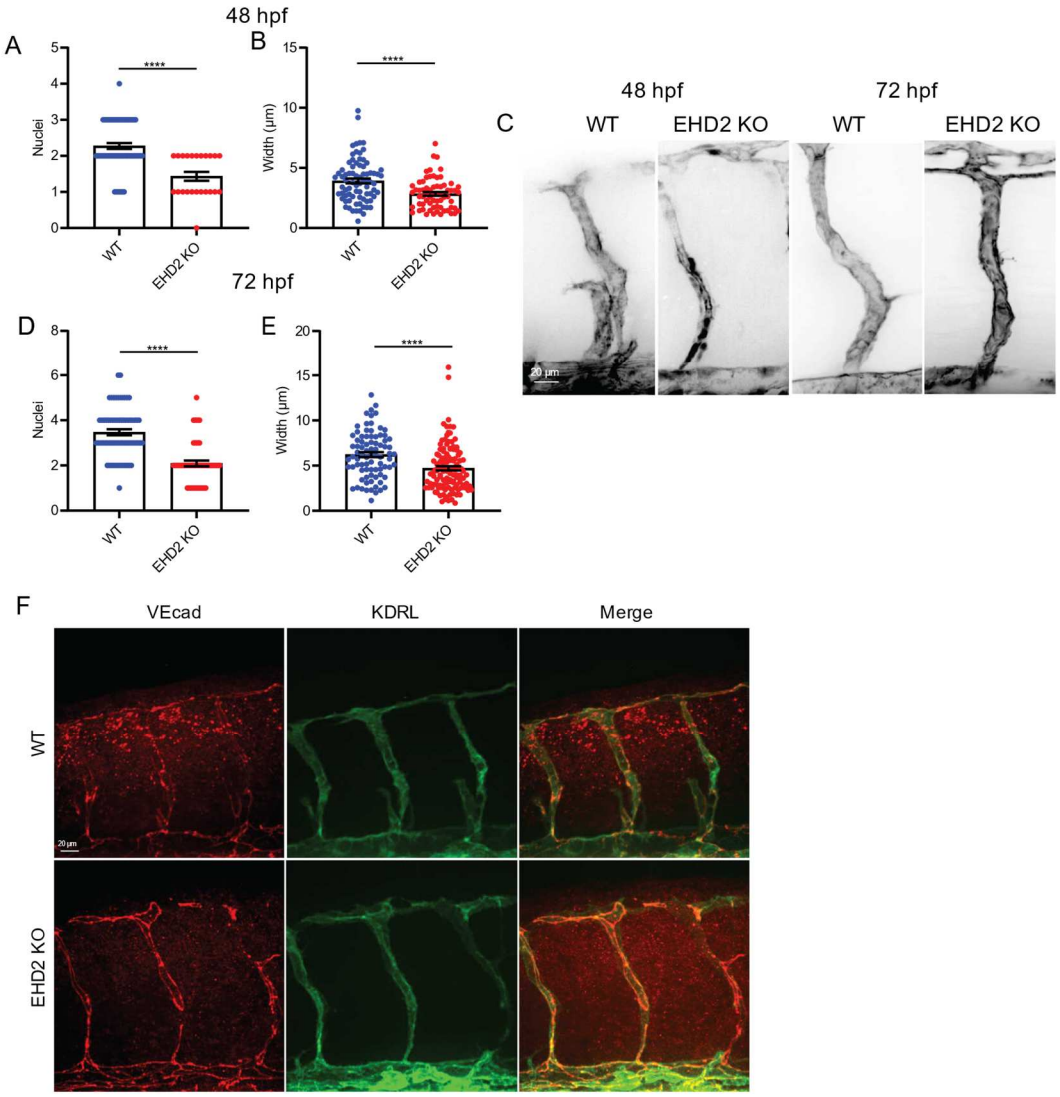

Figure 6 (A, B) comparison of width of ISVs and number of nuclei at 48hpf. (C, D) A comparison of width of ISVs and number of nuclei at 72 hpf (B, D). Whole zebrafish that have been stained with an anti - zebrafish antibody for CDH5(red) in a KDRL: mCherry (Green) fish

Chapter Four Discussion and Conclusion

The first unexpected finding was that Cav1 and EHD2 seem to have separate roles in actin-based contractility pathways. Cav1 removal seems to limit actin filament formation and junction stabilization, which is the opposite of what occurred when EHD2 was removed. If these two proteins were linked for their proximity, however, they do not seem to perform the same tasks within the cell. These proteins both show a role in contractility. Cav1's role matches the literature and behaves similarly to CN02, but EHD2's role matches the inhibition of RhoA. The difference between the spreading assay and the wounding healing indicates that while EHD2 does impact a protein in the migration pathway, it is a protein that works in opposition to Rac1. RhoA is a known antagonist of Rac1. Ehd2's role is based on tension modulation through actin filament formation and breakdown and the ability to destabilize junctions. The junction destabilization is acting in a RhoA dependent manner, which is outside what most literature says about EHD2. This ability to prevent RhoA function matches what is seen when ROCK inhibitors are applied to the monolayers. This change indicates that EHD2 is not a Rac1 control protein as previously stated in Yang, et al.

The actin amounts when treated with a ROCK inhibitor and Myosin inhibitor were mostly as expected. The EHD2 knockdown was rescued when exposed to both inhibitors. Though the knockdown was rescued through the adenovirus, the overall amount of actin

was lower than expected. This may mean EHD2 has a dosage-dependent effect and the amount of EHD2 available for that needs to be readily controlled. This has implications of the dominant negative version of EHD2 that does not seem connected to the plasma membrane or located near Cav1(sup fig. 2D). This form of EHD2 may have other roles. Though unexpected, the g-actin and f-actin amounts could have been modulated over a twenty-four-hour period to appear similar to normal, in a way that was not affected by EHD2. Because the time between the onset of conditions and when these were fixed, could account for a possible translational or proteasomal change to either increase the amount of g-actin in the cell or remove the excess.

The connection between actin and junctions is well-known in most cell types. ROCK inhibitors and myosin inhibitors had a greater effect on junctions than they did on actin. This change in junctions looks remarkably similar to EHD2 overexpression and though it matched expectations for the hypothesis, it was different from the changes in action. This could be due to the specific protein being EHD2 targeting.

The brief look into how domains impact the EHD2 is perplexing because there are two options: one EHD2's overall impact is controlled and prevented by the domains shown, which is unlikely, or the adding of plasmids to cells through transduction is disrupting. The wild-type areas I show form the same dishes but in different areas, but this may explain the stark difference compared to the full-length EHD2 overexpression which uses adeno virus to enter the cell which is a softer entry. This could mean that none of these domains impact junctions much differently than EHD2 as a whole protein which

leads the Epsin 15 homology domain, the EF domain, and the KPFxxxNPF sequence as modulators of junctions.

Zebrafish modelling indicates results that are in line with immobilized junctions. The vessels are thinner because fewer cells can be pulled along to become stalk cells. Fewer nuclei are in those vessels because there are fewer cells. In spite of these changes, the fish are still viable. This is an interesting thread to follow up on, and raises the question of what are the other defects in this line of fish associated with overly stable junctions?

While the results of this study strongly suggests that EHD2 affects Rho A, it is not conclusive on its own. Further study, including a protein pulldown for phosphorylated Rho A, is required to prove conclusively that EHD2 impacts Rho A.

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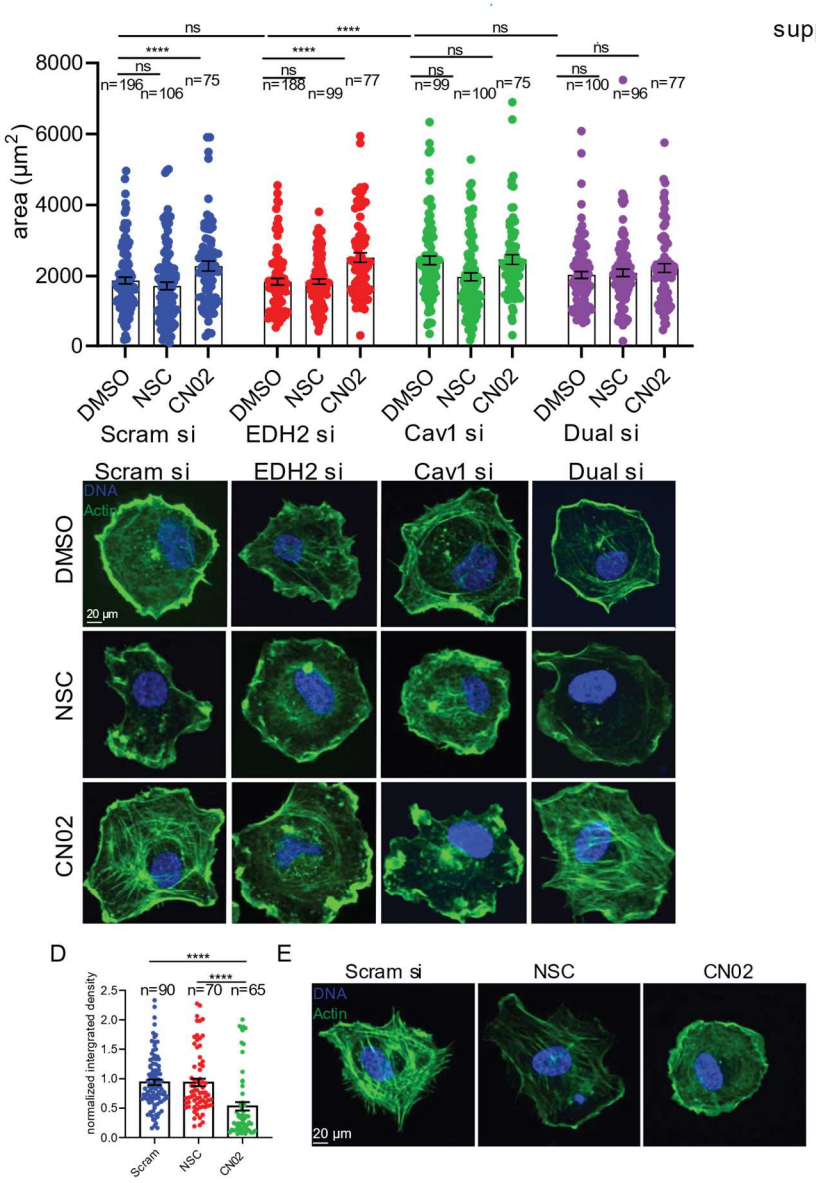
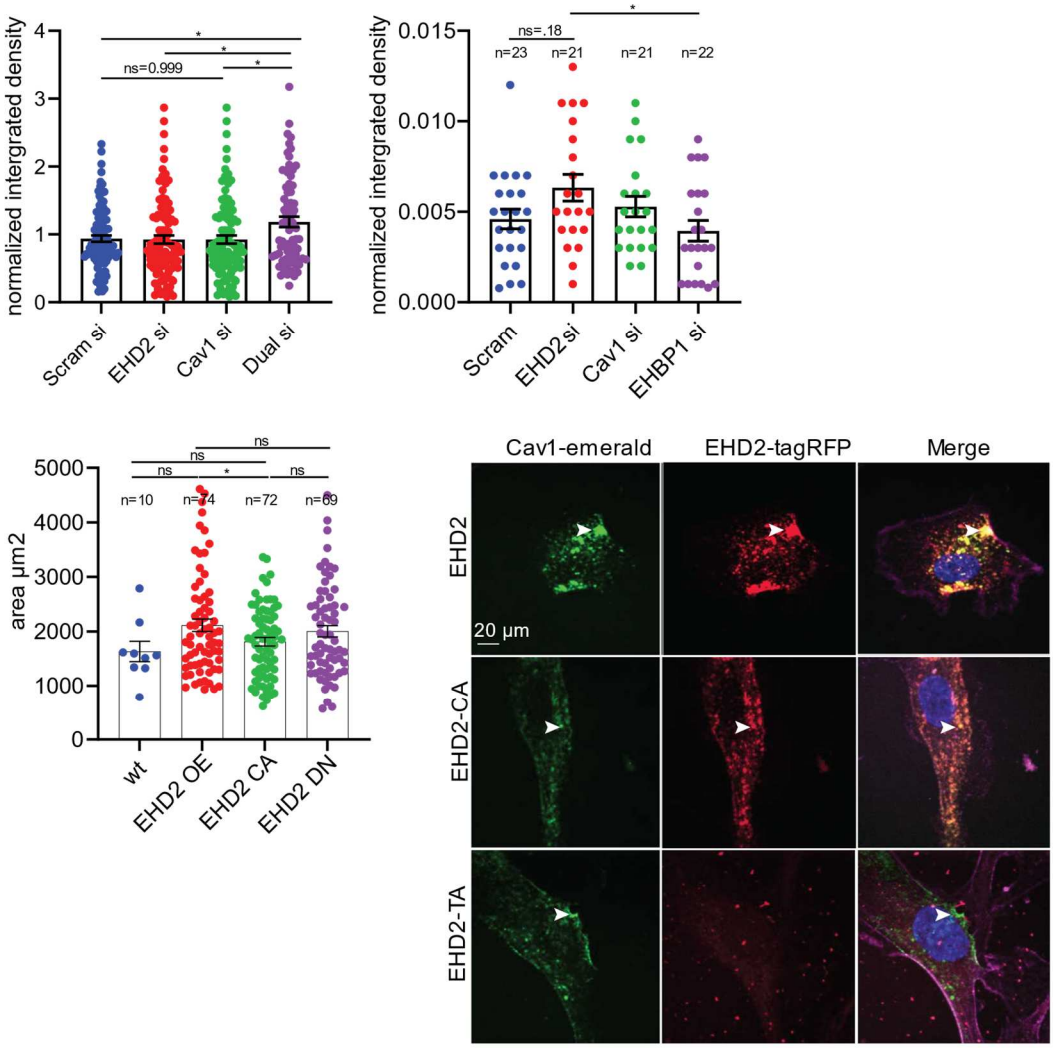
Appendix

supplemental figure1



Supplemental Figure 1 quantification and representative images of a spreading assays using NSC and CNO2(A, B). and a measure in the change in actin when NSC and CN02 were used (D, E)



Supplemental Figure 2 a measure of actin in cells during spreading(A). the measure of actin amounts in a fibrin bead assay after 4 days(B). the area of cells with the plasmids containing EHD2- CA and EHD2-DN looking at changes in area after 24 hrs. (C). The localization of EHD2 and Cav1 based on the its phosphorylation state(D).