Isabela Corina Santos Fortunato

Bachelor Degree in Human Biology

Unveiling mechanotransduction during collective cell migration

Dissertation to obtain the Master of Science Degree in Molecular Genetic and Biomedicine

Supervisor: Doctor Cláudio Franco, Instituto de Medicina Molecular

Júri:
Presidente: Professora Doutora Alexandra Fernandes
Arguente: Professor Doutor Nuno Santos
Orientador: Doutor Cláudio Franco

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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"… the trick is to combine intellectual humility with limitless ambition."

(Floyd Romesberg)

Abstract

Collective cell migration (CCM) describes the coordinated movement and behavior of a group of cells. It relies heavily on cell-cell communication and microenvironmental interactions and is essential for embryonic morphogenesis, angiogenesis, wound repair and cancer invasion. CCM depends on physical forces applied directly onto adherent junctions (AJs). AJs are composed by a dynamic protein complex including core members of the cadherin and catenin families, and additional partners such as vinculin and actin regulators. Efficient mechanotransduction and collective polarization in CCM is directly dependent on the intricate connectivity between junctional proteins and the actin cytoskeleton. Although α-catenin is essential in cadherin-dependent mechanobiology, it remains unclear whether there are also α-catenin cadherin-independent functions in CCM. Moreover, despite the firm link between actin polymerization and mechanotransduction in CCM, little is known on the mechanisms recruiting and activating the Arp2/3 complex at AJs during CCM.

In our lab, we established a method to evaluate cell coordination during CCM using front-rear polarity axis of groups of endothelial cells (ECs). We used this methodology to investigate α-catenin cadherin-independent functions and the mechanisms recruiting Arp2/3 complex at AJs in ECs undergoing CCM. We found that the role of α-catenin in CCM depends primarily on its binding to the cadherin complex, suggesting that cadherin-independent functions of α-catenin is absent in ECs. Additionally, we confirmed that Arp2/3 complex is essential for CCM and collective axial polarity. Mechanistically, we present initial evidences that Arp2/3 complex is recruited specifically to AJs by vinculin.

Altogether, our work shows that cadherin-dependent mechanotransduction relies on Arp2/3 complex activity and highlights its function as novel regulator of axial polarity in ECs. Future insights on mechanobiology could provide new clues to design therapies to prevent cancer invasion.

Keywords

Collective cell migration, adherens junctions, mechanotransduction, catenins, vinculin, Arp2/3 complex

Resumo

Migração coletiva das células (MCC) descreve o movimento coordenado e o comportamento de um grupo de células. MCC depende da comunicação célula-célula e das interações com o microambiente e é essencial para morfogénese, angiogénese, reparo de feridas e invasão de cancro. MCC depende de forças físicas aplicadas nas junções aderentes (JAs). JAs são compostas por um complexo proteico que inclui membros das famílias das caderinas e cateninas e parceiros adicionais, tais como vinculina e reguladores de actina. Eficiências na mecanotransdução e na polaridade coletiva na MCC dependem da conectividade entre as proteínas das JAs e o citoesqueleto de actina. Embora a α-catenina seja essencial para a mecanobiologia dependente de caderina, não está claro se existem funções independentes na MCC. Adicionalmente, apesar da forte ligação entre a polimerização de actina e a mecanotransdução na MCC, pouco se sabe sobre os mecanismos de recrutamento e activação do complexo Arp2/3 para as JAs durante MCC.

No nosso laboratório, estabelecemos um método que usa os eixos de polaridade frente-trás para avaliar a coordenação celular durante MCC de células endoteliais (CEs). Usámos esta metodologia para investigar funções da α-catenina independentes de caderina e mecanismos de recrutamento do complexo Arp2/3 para as JAs durante MCC de CEs. Descobrimos que o papel da α-catenina na MCC depende da sua ligação ao complexo da caderina, sugerindo que as funções da α-catenina independentes de caderina estão ausentes nas CEs. Confirmámos também que o complexo Arp2/3 é essencial para MCC e polaridade axial coletiva, sendo recrutado especificamente para as JAs pela vinculina.

Este estudo mostra que a mecanotransdução dependente de caderina requer a actividade do complexo Arp2/3 e destaca a sua função como um novo regulador da polaridade axial em CEs. Estudos futuros em mecanobiologia poderão fornecer novas pistas para desenvolver terapias de modo a prevenir a invasão de cancro.

Palavras-chave

Migração coletiva das células, junções aderentes, mecanotransdução, cateninas, vinculina, complexo Arp2/3

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Abbreviations

AJs	Adherens junctions
ARHGAP24	Rho GTPase activating protein 24
ARHGAP35	Rho GTPase activating protein 35
ARHGEF2	Rho/Rac guanine nucleotide exchange factor 2
ARHGEF40	Rho guanine nucleotide exchange factor 40
BSA	Bovine serum albumin
CCM	Collective cell migration
Cdc42	Cell division control protein 42 homolog
CIL	Contact inhibition of locomotion
DMSO	Dimethyl sulphoxide
ECM	Extracellular matrix
ECs	Endothelial cells
EMT	Epithelial-to-mesenchymal transition
E-Cadherin	Epithelial cadherin
FAs	Focal adhesions
FAKs	Focal adhesion kinases
F-Actin	Filamentous actin
GAPs	GTPase-activating proteins
GDP	Guanosine diphosphate
GEFs	Guanine-nucleotide exchange factors
GTP	Guanosine triphosphate
G-Actin	Globular actin
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cell
KD	Knockdown
MDCK	Madin-Darby canine kidney cells
MI	Middle I domain
MII	Middle II domain
MIII	Middle III domain
MS1	Mile sven 1
MTOC	Microtubule-organizing center

NM-MII	Nonmuscle myosin II
NPFs	Nucleation promoting factors
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI	Polarity index
PVDF	Polyvinylidene difluoride
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
ROCKs	Rho-associated protein kinases
SCM	Single cell migration
TBS	Tris-buffered saline
VASP	Vasodilator-stimulated phosphoprotein
Vav2	Vav guanine nucleotide exchange factor 2
VEGF	Vascular endothelial growth factor
VE-Cadherin	Vascular endothelial cadherin
VH1	Vinculin homologous 1 domain
VH2	Vinculin homologous 2 domain
VH3	Vinculin homologous 3 domain
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
Wnt	Wingless/Integrated

I. Introduction

A. Single and collective cell migration

The ability of cells to migrate is essential for physiological processes, such as tissue morphogenesis, wound repair, immune function and angiogenesis, but also pathological conditions, such as cancer invasion and metastasis (Friedl and Gilmour, 2009). Cells can migrate individually or collectively.

Single cell migration (SCM) describes the motility of cells as individual independent entities. It has been widely studied and the molecular mechanisms through which individual cells move are very well defined (Ridley et al., 2003; Petrie et al., 2009; Bear and Haugh, 2014). SCM is based on the establishment of a front-to-rear polarity axis, defined by the presence of chemical gradients or substrate-bound ligands (Duchek and Rorth, 2001; Duchek et al., 2001; Huang et al., 2005). Physical constraints in the cell microenvironment can also prevent cells from moving in all directions (Le Digabel et al., 2010).

Collective cell migration (CCM) describes the coordinated movement and behavior of a group of cells. In CCM, cells use similar mechanisms as in SCM, however supracellular mechanisms leads to coordination of movements and polarities. Although a group of cells have a lower instant velocity, their movement is more persistent, promoting the coverage of longer distances more efficiently than isolated cells (Mayor and Etienne-Manneville, 2016). Mechanisms behind CCM are being broadly studied in recent years. CCM depends on cell-cell communication and environmental interactions leading ultimately to coordination and cooperation between migrating cells. There are two types of CCM: (1) epithelial collective migration; and (2) mesenchymal collective migration. The first one depends on stable cell-cell interactions while the second one relies on transient cell-cell interactions (Figure I.1) (Theveneau et al., 2010; Scarpa et al., 2015). Nevertheless, both types of collective migration need cell-cell interactions, for cell's connection but also for cell communication, which combined ultimately regulate the properties of individual cell motility and protrusion behavior within the cluster of cells (Theveneau et al., 2010; Weber et al., 2012; Cai et al., 2014; Davis et al., 2015). Besides cell-cell communication, both types of CCM require an interaction with extracellular matrix (ECM) to sense and respond to cues in the surrounding microenvironment. This interaction is important for a normal collective tissue guidance.

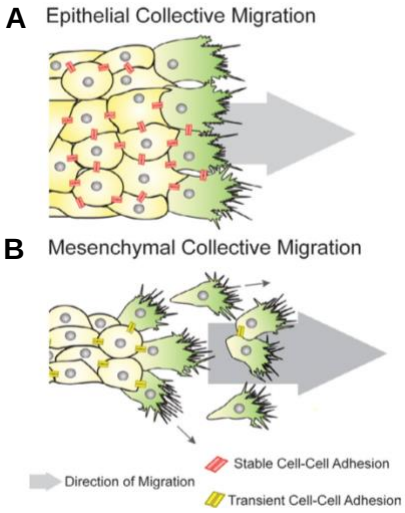


Figure I.1- Epithelial and mesenchymal collective migration. (A) Epithelial cells migrate as a solid group keeping their cell-cell interactions stable and a well-established polarization that will promote protrusions formation in order to maintain directionality. (B) Mesenchymal cells migrate collectively but with transient cell-cell interactions which could result in constant redirection of protrusion formation.
(Adapted from Scarpa and Mayor, 2016)

a) Mechanisms underlying CCM

The mechanisms underlying the migratory capacity of each cell undergoing either collective or single cell migration are mostly the same. One of the key features for directed migration is the establishment of a front-rear polarity axis that, ultimately, dictate the migratory behavior of each cell. Front-to-rear polarization occurs due to signaling cascades, such as the small GTPase proteins of the Rho family (Figure I.2). Rac1, RhoA and Cdc42 are the most renowned protein members of the Rho family. These small GTPase proteins have an inactive state, bound to GDP, and promoted by GTPase-activating proteins (GAPs), and an active state, bound to GTP, and promoted by guanine-nucleotide exchange factors (GEFs). Spatial and temporal balance between Rac1, RhoA and Cdc42 activities ensures directional cell migration by a close-fitting control of cytoskeleton remodeling. At the front of the cell, active Rac1 and Cdc42 are responsible for inducing actin cytoskeleton polymerization and remodeling that promotes formation of membrane protrusions, such as lamellipodia and filopodia. At the rear of the cell, a different Rho pathway involving RhoA and Rho-kinases (ROCKs) promote actomyosin contraction by stress fibers formation (Zegers and Friedl, 2014). Cdc42 can also regulate polarity by promoting asymmetric distribution of organelles, proteins and lipids at the plasma membrane and cytoplasm. Notably, this internal polarity can be easily observed by the localization of the Golgi apparatus and the microtubule-organizing center (MTOC) in front of the nucleus, towards the direction of migration in some cell types, such as in endothelial cells (ECs) (Ridley et al., 2003). This distribution establishes a front-rear polarity plane in cells, which we designated as axial polarity.

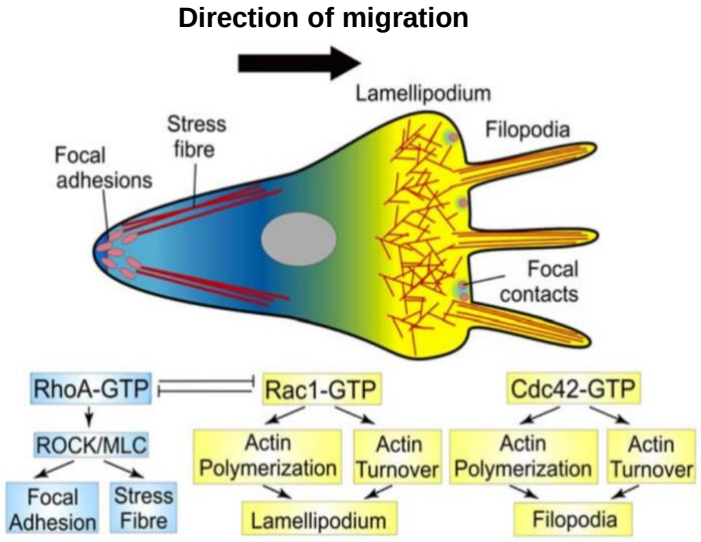


Figure I.2- Rho GTPases spatial distribution. A front-rear gradient is defined during cell migration. At the front, active Rac1 and Cdc42 (yellow) induce the formation of cell membrane protrusions, such as lamellipodia and filopodia. At the rear, active RhoA and ROCKs (blue) mediate cell contraction by promoting stress fibers formation. This gradient is established and maintained because Rac1 and RhoA antagonize each other's activity.
(Adapted from Mayor and Carmona-Fontaine, 2010)

Even if the molecular mechanisms regulating cellular motility are the same in single and collective migration, signals originating from cell-cell contacts in clusters of cells feedback to those mechanisms and modulate the formation of lamellipodia and filopodia, and thus regulate single cell migration features.

Extensive work in this field have unveiled some of the general rules necessary for CCM: (1) Exposure to a mutual symmetry breaking event and, consequently, polarization, simultaneously, towards the same direction; (2) Migration must be in the same direction at a similar speed to avoid collective disruption; (3) Presence of cell-cell communications and coordinative response with the microenvironment (4) Solid cell-cell interactions to ensure group integrity and allow biochemical and mechanical coupling between adjacent cells (Figure I.3) (Mayor and Etienne-Manneville, 2016). Moreover, two types of subpopulations co-exist in CCM: (1) leader cells, which are the ones that not only sense and explore the microenvironment but also define speed and contribute to directed migration of all cells in the migrating population; and (2) follower cells, which are the ones located at the back of the leaders, not in direct contact with a free-edge. However, during migration in complex microenvironments, leaders and followers can exchange positions and functions during collective migration (Jakobsson et al., 2010). Since leaders are localized at the migration front, they are able to interact with ECM, soluble factors and neighbor cells. Due to directional cues provided by ECM fibers (Ventre et al., 2012), cell-ECM interactions promote cytoskeletal rearrangement, structural reorganization and morphological polarization (Etienne-Manneville and Hall, 2001). Leader cells are also capable of sensing environmental soluble factors which will stimulate chemotaxis of the entire migrating group. For example, in vascular biology, the vascular endothelial growth factor (VEGF) is the

main chemotactic factor for leader cells, named endothelial tip cells, promoting its directional migration (Lamalice et al., 2007).

Although most of studies relay on processes at the front of the cell group, follower cells are also being pointed as essential for a proper collective migration behavior. On one hand, follower cells can regulate leader's polarization and, consequently, the cell cluster polarization (Abercrombie et al., 1953). On the other hand, follower cells also play an important role in chemotaxis (Cai and Montell, 2014) and sensing gradients of soluble factors (Theveneau et al., 2010; Malet-Engra et al., 2015).

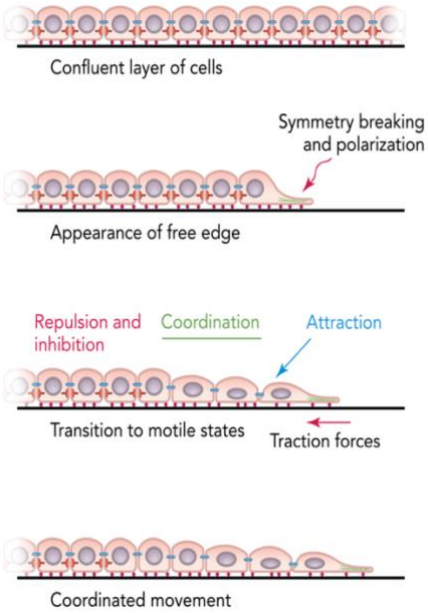


Figure I.3- Collective cell migration mechanisms. (A) Low or null migration is present when cells are on confluent monolayer due to symmetrically inhibitions. However, when a free space appears, which is an example of a symmetry breaking event, cells start to polarize towards the free edge. Then, this influences the inhibitory mechanisms and promotes cells transition to a motile state. Therefore, leader cells generate, sense and apply traction forces to their neighbors which will induce a coordinate movement of the group.
(Adapted from Vedula et al., 2013)

B. Importance of cell-matrix and cell-cell interactions in CCM

During collective migration, the mechanical and chemical coupling between cells and their microenvironment influence cell behavior and modify axial polarity. A cell cluster is hierarchically defined by leaders and followers. Leaders sense the mechanical and chemical cues that induce migration and then influence followers via mechanical coupling (Scarpa et al., 2015). In migrating collectives, cells not only generate forces that creates motion between them and the surface, called traction forces, but also adjust, sense and transduce the mechanical properties to their neighbors. Leader cells produce stronger and most of traction forces that drag follower cells behind (Caussinus et al., 2008), however weaker forces are also generated in cells far form the leading edge. Due to this unbalanced distribution of traction forces in the migrating collective, the cell cluster is under high tensile stress and forces. (Trepat

et al., 2009; Tambe et al., 2011). Nevertheless, even if leaders are capable of producing high traction forces, this is not sufficient to pull all the monolayer behind. Thus, normal CCM relies on mechanical waves that propagate from the front to the rear of the monolayer where leaders communicate those mechanical and polarity information to the followers. Leader and follower cells continuously perceive and transmit signals, aligning their traction and frictional forces (e.g., shear stress) with average velocity of the group in order to maintain coordinated behavior and, consequently, the collective migration process (Serra-Picamal et al., 2012). The relative strength and orientation of the different local forces are sensed and interpreted by individual cells, which through mechanisms not totally understood, leads to coordination of forces at the supracellular scale, ensuring the collective orientated movement (Zaritsky et al., 2015). Two types of adhesions complexes play crucial roles in mechanosensing and mechanotransduction in CCM: (1) cell-ECM interactions at focal adhesions, which enable cells to exert traction on the ECM to promote protrusive motility; (2) cell-cell interactions at adherens junctions, which are responsible for cohesion and force transmission within the group of cells (Trepat et al., 2009; Tambe et al., 2011; Ventre et al., 2012).

a) Cell-matrix interactions: focal adhesions

Most of cell types move through a 3D microenvironment by making adhesive structures with the ECM called focal adhesions (FAs). FAs are dynamic protein complexes that connect cell's actin cytoskeleton to the ECM. FAs are also responsible for the traction forces exerted on ECM, allowing the propagation of internal myosin-mediated contractile forces to the external environment, which promote cell propulsion. Besides anchoring the cells, FAs function as sensors for cells to be informed about ECM characteristics, which is crucial for translation of cytoskeletal forces into motion and behavior (Riveline et al., 2001; Geiger et al., 2009). Comparing to immotile ones, migrating cells have decreased FA's stability, manifested by a constant formation of new FAs and breakage of old ones, resulting in constant turnover of these adhesions. FAs association with the ECM involve mostly integrins receptors, which are formed by heterodimers composed by an α- and a β-subunit. Within the cell, integrin's intracellular domain binds to actin filaments mediated by anchoring proteins like talin, α-actinin, filamin and tensin. Additionally, other intracellular proteins are associated and regulate the connection between the integrin complex and the actin cytoskeleton, such as focal adhesion kinases (FAKs) and vinculin (Figure I.4A) (Berrier and Yamada, 2007; Geiger et al., 2001). Talins, talin1 and talin2, are key proteins in FAs assembly due to its unique functions in formation and maintenance of these cell-ECM interactions (Figure I.4B). Besides linking integrins to actin filaments, talins also play a role on integrins activation (Zhang et al., 2008; Geiger et al., 2009). Mechanical forces generated by actin polymerization or contraction increase talin's association with FAs. Interestingly, vinculin also engages with stretched talin, which helps keeping talin in an open conformation and stabilizes FAs (Atherton et al., 2016). Furthermore, vinculin binding to actin filaments and α-actinin enhances stress fibers formation and FAs growth. Thus, talin and vinculin are proteins that enables FAs to respond to intracellular forces generated

by actomyosin contractility and to sense ECM stiffness (Kumar et al., 2016; Grashoff et al., 2010). Therefore, FAs function as mechanical sensors.

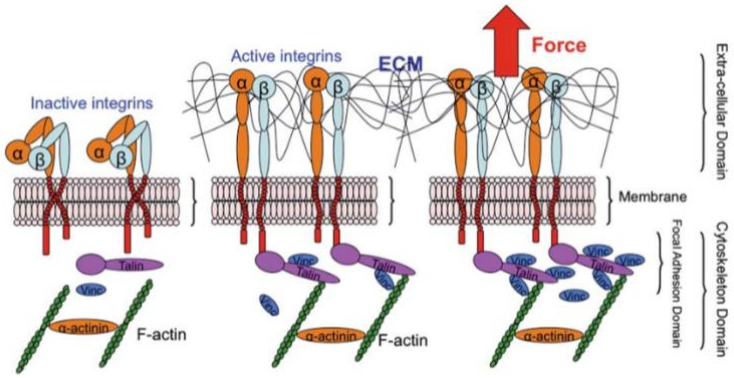


Figure I.4- Cell-ECM adhesion complex. When cells receive a stimulus to migrate, integrins are activated by vinculin/talin protein complex. This leads to ECM-integrin-cytoskeleton linkages in focal adhesions and, consequently, formation of traction forces.
(Adapted from Honarmandi et al., 2011)

b) Cell-cell interactions: adherens junctions

When cells are migrating as a group, they are connected by adhesive structures, such as adherens junctions (AJs) which have a tight association with actin cytoskeleton. AJs not only are crucial to maintain the collective integrity but also is required to the chemotactic gradient interpretation within the group. The intricate connectivity between AJs and the actin cytoskeleton is the driving force underlying efficient mechanotransduction, collective polarization and CCM (Friedl and Gilmour, 2009). Depending on the mode AJs associate with actin filaments, these junctions emerge in a linear or punctate organization typically in epithelial and fibroblast cells, respectively.

In order to preserve cohesive migration of all cells, the migrating cluster need a dynamic control of cell-cell interactions. For instance, AJs face a continuous actomysion-driven retrograde flow along lateral sides of adjacent migrating cells, which its movement ends near the cell rear (Figure I.5). When AJs reach the cell rear, cadherin-mediated interactions are dissociated and internalized. Subsequently, cadherins are recycled towards the leading edge and new AJs formation occurs at the front of lateral contacts. As AJs are dynamic, this makes them malleable intercellular contacts that maintain the mechanical strength in the migrating collective (Peglion et al., 2014).

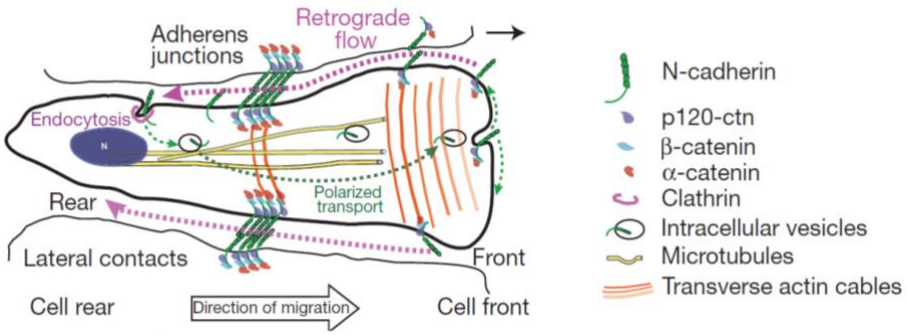


Figure I.5- Retrograde flow in AJs. Dynamic AJs and its maintenance is crucial to a proper migration. The mechanism behind these two needed characteristics relies on actomyosin cables align from basal to apical end of lateral AJs moving together in a retrograde flow (purple dashed line).
(Adapted from Peglion et al., 2014)

AJs are composed by a protein complex containing several junctional proteins, such as cadherins, β-catenin, α-catenin and vinculin (Figure I.6A). Different types of classical cadherins where found in AJs from diverse cell types (e.g., E-cadherin is epithelial, whereas VE-cadherin is endothelial specific). Classical cadherins are transmembrane glycoproteins containing an extracellular domain, a transmembranar domain and a cytoplasmic domain. While the extracellular domain is responsible for mediating Ca^{2+}-dependent homophilic interactions with the neighbor's cadherin molecule, the cytoplasmic domain binds to members of the catenin protein family (e.g., β-catenin and p120-catenin) (Takeichi, 2011; Gloushankova et al., 2017). p120-catenin is crucial for cell-cell adhesion stability by retaining cadherin at the cell membrane. β-catenin is the intermediate linker of cadherin to α-catenin and, in addition to its role in AJs, it works as transcription factor for canonical Wnt signaling. α-catenin, in turn, associates AJs to the actin cytoskeleton and promotes AJs protein clustering (Maiden and Hardin, 2011).

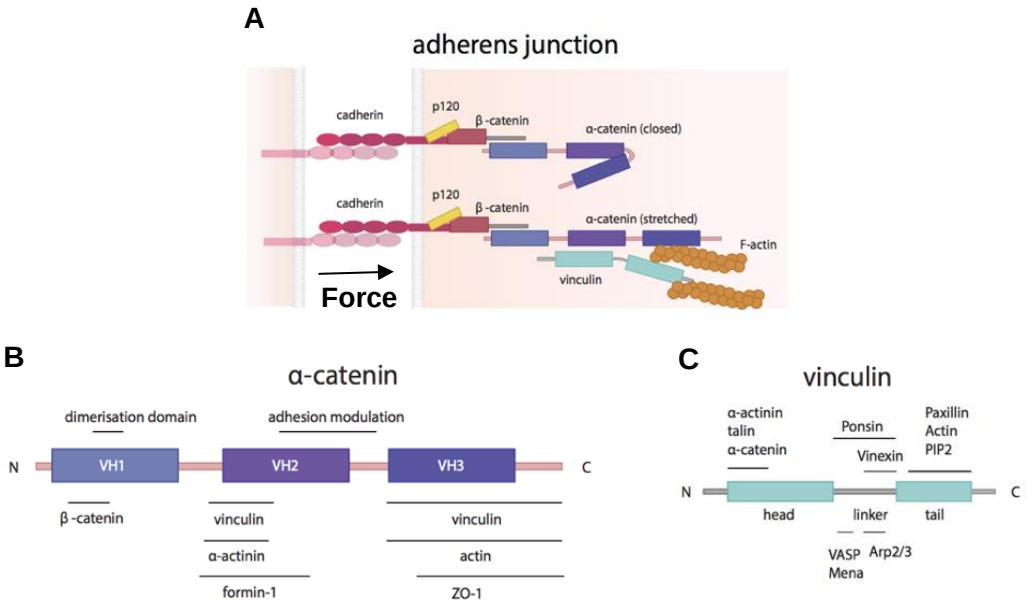


Figure I.6- Cell-cell adhesion complex and major proteins. (A) Adherens junctions components when α-catenin is on its closed (up) and stretched (down) conformation. (B) Schematic representation of most relevant α-catenin domains. (C) Schematic representation of most relevant vinculin domains.
(Adapted from De Pascalis and Etienne-Manneville, 2017)

i. α-Catenin

α-Catenin is a highly-conserved protein in eukaryotic kingdom. In mammals, there are three main α-catenins: (1) αE-catenin, mostly common in epithelial tissue; (2) αN-catenin, specifically present in neural tissues; (3) αT-catenin, primarily expressed in heart tissue. Human αE-catenin, usually simply called as α-catenin, is a 102 kDa protein encoded by the CTNNA1 gene and is composed by 906 amino acids (Kobielak and Fuchs, 2004). Although, α-catenin is a homodimer in solution, it links AJs and actin cytoskeleton as a monomer. α-catenin dimers exist in the cytoplasm and form an equilibrium with α-catenin monomers present in junctions (Rangarajan and Izard, 2013). Molecularly, α-catenin is divided in three main domains called vinculin homologous 1, 2 and 3 (VH1, VH2, and VH3) (Figure I.6B). VH1 is closer to the N-terminal and has the β-catenin binding domain. VH3 is closer to the C-terminal and has the binding domain for actin filaments. VH2 is located in the middle part of α-catenin and has three sub regions called middle I, II and III (MI, MII and MIII) that contain binding sites for actin-binding proteins, such as vinculin and α-actinin (Mayor and Etienne-Manneville, 2016). It is known that α-catenin functions as a sensor of mechanical forces. When α-catenin links β-catenin, via VH1 domain, the affinity of α-catenin for actin filaments decreases. However, when a mechanical force is applied, this inhibition is overcome. Tension-dependent conformational changes in α-catenin results in its binding to actin filaments, which stabilizes adhesive clusters and initiates vinculin recruitment to AJs. α-catenin's MI domain is contains the vinculin binding site, while MII and MIII domains interact with MI domain, masking the vinculin binding site. Force application onto α-catenin molecule destabilizes interactions between its MI domain and MII-MIII domains, resulting in MI domain unfolding, leading to the α-catenin "open" conformation. This leads to a high affinity for vinculin to bind MI domain and stabilize AJs providing additional linkages to actin filaments (Figure I.4A). Therefore, α-catenin is force-sensitive protein in which the ability to bind actin filaments is modified by tension on its molecular structure and the presence or recruitment of binding partners (Mayor and Etienne-Manneville, 2016; Kang et al., 2017). Thus, AJs function as mechanical sensors.

ii. Vinculin

Vinculin is a monomeric 117 kDa cytoplasmic protein which is present at cell adhesion complexes, both cell-cell and cell-ECM, and its recruitment is force-dependent. Vinculin binds to several partners, such as talin, Arp2/3 complex, paxilin, α-catenin, and filamentous actin (Goldmann, 2016). Vinculin is a bipolar protein containing a head and a tail domain (Vh and Vt, respectively) separated by a short proline-rich linker (Figure I.6C). α-catenin binds to vinculin through Vh domain while actin filaments bind to Vt domain, similarly to talin. Vh domain is mainly associated with adhesion strength enhancement while Vt domain is related with force enrichment by transmitting them and mediating myosin contractility (Atherton et al., 2016; Dumbauld et al., 2013; Dumbauld et al., 2010). Like α-catenin, vinculin activity is tension-regulated via its own conformation. Vinculin has an auto-inhibitory state, or closed conformation, when Vh and Vt have a high-affinity intramolecular interaction. However, when forces are applied to cells, vinculin links to actin filaments enabling the stretch of the protein which will

lead to Vh and Vt dissociation and consequent switch to an open, or active, conformation (De Pascalis and Etienne-Manneville, 2017; Spanjaard and Rooij, 2013). Since vinculin only exposes its binding sites in order to link to the adhesion complexes in a force-dependent manner, it is considered one of the key proteins in mechanotransduction (Atherton et al., 2016).

C. Mechanosensing and mechanotransduction during CCM

During cell migration, cells sense changes in the physical environment and are capable of translating those mechanic stimuli into biochemical signals (Figure I.7). These two processes are called mechanosensing and mechanotransduction, respectively (Jaalouck and Lammerding, 2009). Throughout migration, cells are exposed to different forces, such as tensile forces, compressive forces and frictional forces. Besides these, migrating collectives also apply traction forces to the extracellular environment (Pandya et al., 2017).

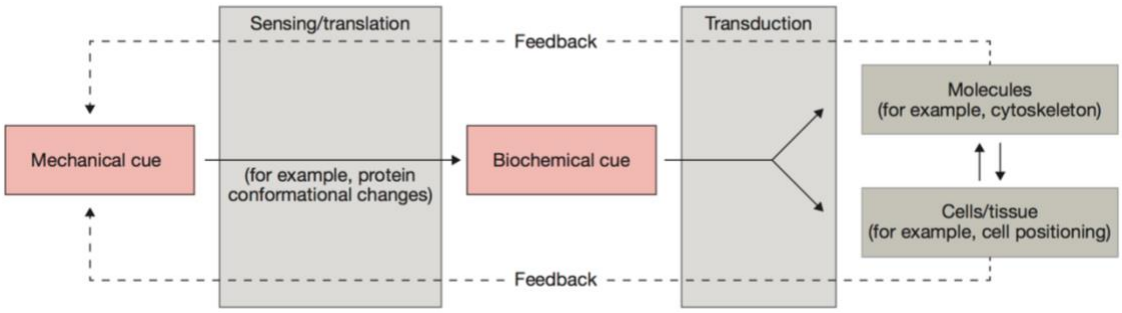


Figure I.7- Mechanical and biochemical signals interference. Mechanical cues are perceived (mechanosensing) and transduced into a biochemical signal (mechanotransduction), initiating a force response at the molecular level or cell/tissue level. In turn, these force responses can regulate the primary mechanosensing process by feedback on the mechanical properties.
(Adapted from Petridou et al., 2017)

Physical properties are perceived by cells through several molecular complexes, including FAs and AJs. Conformational changes of key proteins, called mechanotransducers, are force-induced and it is what mediates the mechanosensing process, at the molecular level. Mechanotransducers undergo a conformational change upon stretching, exposing new protein-interaction domains. This exposure will induce biochemical signaling that can modulate the strength of adhesion. Thus, mechanotransducers are capable of bridging transmembranar adhesion receptors (integrins in FAs and cadherins in AJs) to the actin cytoskeleton and are submitted to forces exerted between the actomyosin contractile network and the extracellular microenvironment. In AJs, α-catenin undergoes a conformational change that exposes its intramolecular interaction domain, enabling vinculin binding (Figure I.8). This results in an increase in junctional stability (Yonemura et al., 2010). Additionally, α-catenin interaction with actin filaments is tension-dependent and, consequently, links external mechanical forces to the cytoskeleton (Buckley et al., 2014). Therefore, α-catenin functions as a mechanosensor protein, mediating tensional force-induced AJs maturation. In FAs, talin is the major mechanosensor protein, playing a crucial role

in mechanotransduction. Talin senses the forces derived from FAs and ECM stiffness by changing its folding state and, then, promoting the link of integrins to the actin filaments (Figure I.8). Besides talin, integrin itself also seems to have a role as mechosensor due to its increase in binding affinity to ECM in response to tensional force (Kong et al., 2009). FAs and AJs are not only very similar – in structure, cytoskeleton connection, mechanosensing mechanisms and mechanotransduction pathways – but also influence each other by regulating their formation localizations in a bidirectional crosstalk (Han and de Rooij, 2016).

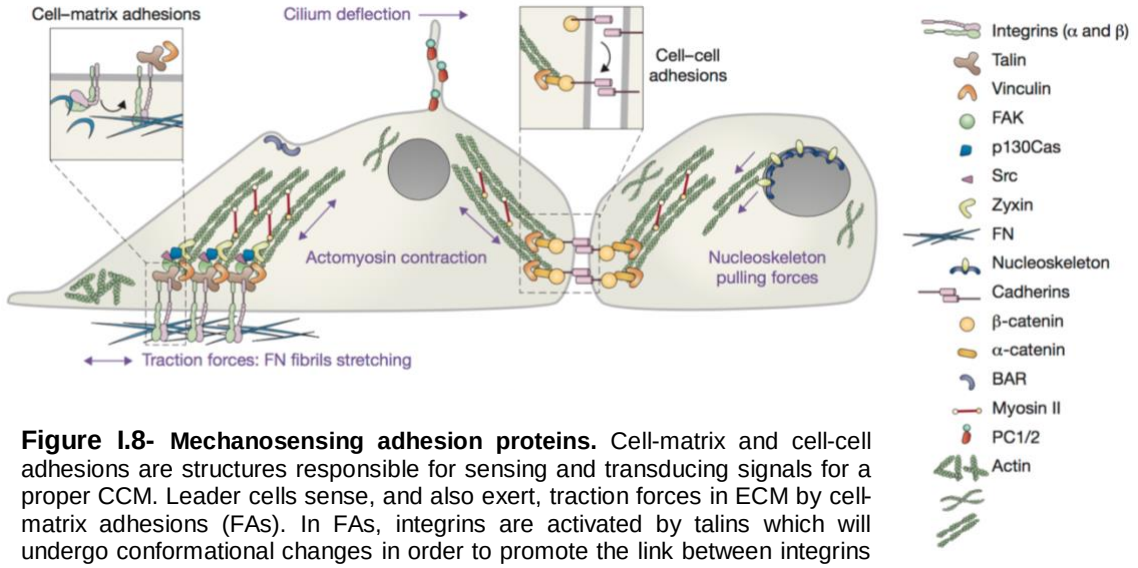


Figure I.8- Mechanosensing adhesion proteins. Cell-matrix and cell-cell adhesions are structures responsible for sensing and transducing signals for a proper CCM. Leader cells sense, and also exert, traction forces in ECM by cell-matrix adhesions (FAs). In FAs, integrins are activated by talins which will undergo conformational changes in order to promote the link between integrins and the actin filaments. Force propagation between cells is mediated by actomyosin cables. These forces are transmitted to other leaders and to followers by cell-cell adhesions (AJs). In AJs, α-catenin will also pass through conformational changes and enable the association of cadherin with actin. In addition to talin and α-catenin, others proteins such as p130Cas, integrin and Src also have mechanosensing functions.
(Adapted from Petridou et al., 2017)

Due to these adhesion complexes, leader cells sense the migratory substrate and communicate the mechanical information to their followers through actomyosin contractility (Ng et al., 2012). Intracellular pulling forces of leaders leads to relocalization of the tumour-supressor protein merlin from cell-cell interactions to the cytoplasm. This promotes polarized Rac1 activation at the leading edge of the monolayer and lamellipodia formation (Das et al., 2015). Intercellular pulling forces in followers spread the lamellipodia development mediated by merlin/Rac1 at the multicellular level (Das et al., 2015). Moreover, RhoA signaling is needed at the rear of the cells to generate actomyosin-mediated forces towards the substrate (Pandya et al., 2017). These forces will support force transmission from leaders to followers through cellular actomyosin coupling, facilitating CCM. Thus, Rho family proteins are spatiotemporally regulated to respond appropriately to different mechanical stresses and have important roles in mechanotransduction (Lessey et al., 2012). Accordingly, GEFs, proteins that activate small GTPases (e.g., Vav2, ARHGEF2 and ARHGEF40), and GAPs, proteins that deactivate small GTPase (e.g., ARHGAP24 and ARHGAP35), are also regulated during mechanotransduction and are actively involved in actin cytoskeletal remodeling in mechanoresponses (Ohashi et al., 2017).

Continuous reciprocal communication between cells and their ECM is established as migrating collectives modify the ECM. In return, changes in matrix physical properties will determine how cells migrate (Pandya et al., 2017). Therefore, cells need to have a tight regulation of adhesion molecules, actomyosin activity and specific transcription for a proper CCM.

In collectively migrating cells, when forces are applied into AJs and FAs, small GTPases are activated spatiotemporally in the cells. Each GTPase seems to have distinct roles regarding actin cytoskeleton. Rac1 is responsible for lamellipodia formation. In addition, Cdc42 activates WAVE and WASP and, subsequently, Arp2/3 complex which increases actin polymerization and lamellipodia formation. RhoA not only regulates stress fiber formation but also assembly and adhesion of actomyosin (Lambrechts et al., 2004). Therefore, Rho GTPases are key regulators of mechanosensing and mechanotransduction in CCM by regulating actin cytoskeleton dynamics.

a) Actin cytoskeletal remodeling

Three main polymers compose cytoskeleton: actin filaments, microtubules and intermediate filaments. These polymers provide structure, organization, mechanics and shape to eukaryotic cells. All these structures are organized into networks that are altered in response to external forces but also can resist to deformation. Thus, these polymers are capable of rearrange and maintain the integrity of intracellular compartments (Fletcher and Mullins, 2010). The architecture of the networks is controlled by many regulatory proteins, such as: (1) nucleation-promoting factors, which start filament formation; (2) capping proteins, which end filament growth; (3) polymerases, which induce sustained and faster filament growth; (4) depolymerization factors, which disassemble filaments; and (5) cross-linkers, which organize and reinforce order networks structures (Fletcher and Mullins, 2010). The activity of these regulatory proteins is affected by internal or external mechanical forces. Additionally, these forces also disturb the local organization of filaments in the networks. The major differences between the three polymers are: (1) polarity; (2) type of associated molecular motors; (3) assembly dynamics; and (4) mechanical stiffness (Fletcher and Mullins, 2010).

Motile cells have morphological features (Figure I.9), such as membranar protrusions, that are formed and maintained by the local actin cytoskeleton dynamics, which in turn are regulated by actin-binding proteins (ABPs) (Revenu et al., 2004). Lamellipodia are composed by branched actin and is an example of the membranar protrusions that are formed in cells during migration (Figure I.9). These membranar extensions are used to explore if the microenvironment is suitable and, then, to decide the direction of migration. Local actin filaments assembly and disassembly regulate dynamics of lamellipodia formation and retraction. Actin depolymerization regulates the remodeling dynamics of actin's network and the cycle extension-retraction of lamellipodia (Tang and Gerlach, 2017). This indicates that cytoskeleton remodeling is essential for migration.

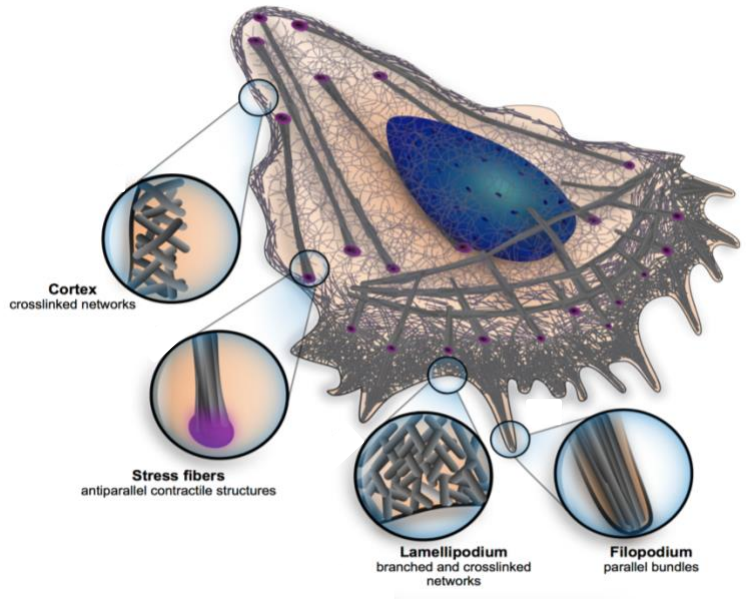


Figure I.9- Actin architecture. Schematic representation of different actin architectures in the cell. Architectural specificities are highlighted in the zoom regions: (1) Cortex, crosslinked networks in lateral and rear of the cell periphery; (2) Stress fibers, antiparallel contractile structures which are important for cell-cell and cell-ECM contacts; (3) Lamellipodium, branched and crosslinked networks in the front of cell, which is important for mechanosensing; (4) Filopodium, parallel bundles in the front of the cell which also plays a role in mechanosensing.
(Adapted from Blanchoin et al., 2014)

Actin is one of the most highly conserved protein. Monomeric globular form of actin (G-actin) is polymerized to form and increase the polymeric form, known as filamentous actin (F-actin) (Pollard and Cooper, 2009). Nucleating factors are necessary for actin polymerization and produces two types of actin filaments: (1) linear actin filaments, which are mostly formin-dependent; and (2) branched actin filaments, which are Arp2/3-dependent. Arp2/3 complex is a protein complex composed by seven-subunits. The activity of this complex is regulated by nucleation promoting factors, including neuronal Wiskott-Aldrich syndrome protein (N-WASP) and WASP-family verprolin-homologous protein (WAVE). Upon a specific pro-migratory stimulus, small GTPases bind to the GTP-binding domain of N-WASP and WAVE activating them and promoting Arp2/3 complex-mediated actin filament branching (Malinova and Huveneers, 2017). An organized network of actin filaments associates with motor proteins (e.g., myosins family) or with other actin filaments creating the actin cytoskeleton. Actin cytoskeleton generates directed forces that drive cell shape modifications and control cellular components organization. Actin cytoskeleton also controls transduction of mechanical signals and produce intracellular forces. These forces are crucial for muscle contraction, organelle movement, cell signaling, cell motility, formation and maintenance of cell junctions, for instance (Pollard and Cooper, 2009; Fletcher and Mullins, 2010).

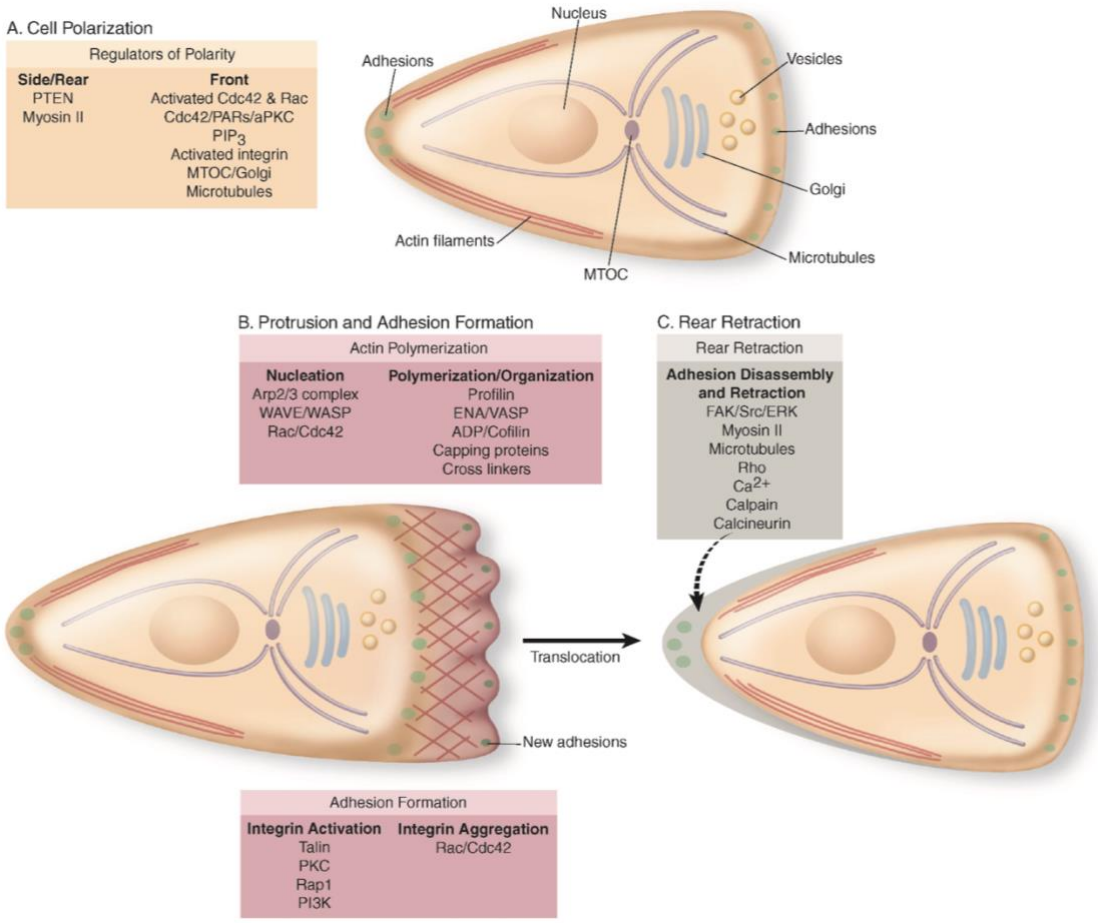


Figure I.10- Major mechanisms underlying cell migration. When a symmetry breaking event appears, cells induce cell polarization via Rho GTPases family, mainly by Cdc42 protein. Then, adhesion complexes, AJs and FAs, are created and maturated by actin cytoskeleton organization. At the same time, cytoskeleton remodeling promotes membrane protrusions and rear cell retraction which will lead to cell translocation on the migratory substrate.
(Adapted from Ridley et al., 2003)

In conclusion, CCM is a complex multicellular process that involves several processes – symmetry breaking event, front-to-rear polarity establishment, leader and follower cell organization, cytoskeleton remodeling, turnover of AJ and FA complexes, and mechanotransduction of forces at cell-cell and cell-ECM interfaces – that requires tight continuous coordination and integration of biochemical and biomechanical information at the supracellular level (Figure I.10).

II. Goals

Recent findings from a project in our lab interconnect a chemical organizer of morphogenesis, non-canonical Wnt signaling, with a physical organizer of collective behavior, junctional mechanotransduction, in the regulation of vascular morphogenesis (Carvalho et al., in revision). During that project, two interesting results were observed:

1- VE-cadherin KD and α-catenin KD had different axial polarity phenotypes in ECs, even if both KDs led to an abnormal collective migration behavior. This suggests that α-catenin could have a cadherin-independent function in collective cell polarity of ECs;

2- Vinculin KD also led to deficient CCM and impaired collective axial polarity. Efimova and Svitkina studies showed that Arp2/3 complex associates with AJs (Efimova and Svitkina, 2018). Since vinculin has an Arp2/3-binding domain, we hypothesize that tension-dependent activation of vinculin could be recruiting the Arp2/3 complex to play a role in collective cell polarity at AJs.

Thus, this thesis will focus on investigating two specific questions:

a. Does α-catenin have a cadherin-independent function in mechanotransduction during epithelial CCM?
b. What are the vinculin downstream signalling events that can promote mechanotransduction in CCM?

III. Material and Methods

A. Cell culture

All cells were routinely cultured following the manufacturer's guidelines, in filter-cap T75 flasks Nunclon ∆ surface treatment (VWR international, LLC) and cultured at 37ºC and 5% CO_2 to ensure a stable environment for optimal cell growth. Human umbilical vein endothelial cells – HUVECs (C2519A, Lonza) – and immortalized HUVECs (CI-HUVECs) were cultured with complete medium EGM-2 Bulletkit (CC-3162, Lonza) supplemented with 1% penicillin/streptomycin (#15140122, Gibco). Mile sven 1 (MS1) and bEnd5 were cultured with complete medium DMEM with high glucose and pyruvate (#41966-029, Gibco) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (#10500-064, Gibco). MCF10a cells were cultured with complete medium DMEM/F-12 with L-glutamine and 15mM HEPES (#21331-020, Gibco) supplemented with 5% horse serum (#16050-122, Thermo Scientific), 20ng/ml epidermal growth factor human – EGF (E9644, Sigma-Aldrich) –, 0.5µg/ml hydrocortisone (H0888, Sigma-Aldrich), 100ng/ml cholera toxin (C8052, Sigma-Aldrich), 10µg/ml insulin (I1882, Sigma-Aldrich) and 1% penicillin/streptomycin. When passaging cells for experiments, cells were washed twice in sterile PBS (137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4, pH7.4). Then, for HUVECs, CI-HUVECs, MS1 and bEnd5, cells were incubated for 3-5min in trypsin/EDTA (#15400054, Gibco) or in TrypLE™ Express (#12605-028, Gibco) at 37ºC, 5% CO_2. For MCF10a, this incubation was for 15-25min. When 95% of the cells detached, complete medium was added to each flask to inhibit the activity of the trypsin/EDTA or TrypLE™ Express and the cell suspension was transferred into a falcon tube. To maximize the amount of cells collected, all the flasks were washed again with complete medium, which was added to the cell suspension gathered previously. HUVECs, CI-HUVECs, MS1 and bEnd5 cells were then centrifuged at 115g for 5min at room temperature and MCF10a at 300g for 5min. The pellet was re-suspended in fresh complete medium. The cell concentration present in the suspension was determined using a Neubauer Chamber Cell Counting (Hirschmann EM Techcolor). All cells were then seeded on the desired culture vessels at 200.000–300.000 cells/mL and placed in the incubator. All experiments with HUVECs were performed between passages 3 and 5.

B. siRNA transfection

In order to silence the expression of genes of interest, a set of ON-TARGET human and mouse siRNAs were purchased from Dharmacon (see Table III.1). Briefly, all cells were seeded the day before the transfection to reach 60-70% confluence and were then transfected with 25nM of siRNA using the DharmaFECT 1 reagent (Dharmacon, GE Healthcare) following the Dharmacon's siRNA transfection protocol. 24h after transfection the culture medium was replaced by fresh complete medium and cells were kept under culture conditions up until 72h post-transfection and then processed for live-imaging, immunofluorescence, protein or RNA extraction.

Table III.1: List of siRNAs.

Name	Specie	Brand	Catalog Number	Sequence
Control siRNA	na	Dharmacon	D-001810-01-05	UGGUUUACAUGUCGACUAA
siActr3	Human	Dharmacon	J-012077-08	GGAAUUGAGUGGUGGUAGA
siCDH5 (1)	Human	Dharmacon	J-003641-07	GAGCCCAGGUCAUUAUCAA
siCDH5 (2)	Human	Dharmacon	J-003641-09	UGACGUGGAUUACGACUUC
siCTNNA1	Human	Dharmacon	J-010505-06	GAUGGUAUCUUGAAGUUGA
siCTNNB1	Human	Dharmacon	J-003482-11	GCGUUUGGCUGAACCAUCA
siTLN1	Human	Dharmacon	J-012949-07	UCAAUCAGCUCAUCACUAU
siVCL (1)	Human	Dharmacon	J-009288-05	UGAGAUAAUUCGUGUGUGUUA
siVCL (2)	Human	Dharmacon	J-009288-06	GAGCGAAUCCCAACCAUAA
Control siRNA	na	GeneCust	SI-91-0101-05 - A06001	UUCUCCGAACGUGUCACGUTT ACGUGACACGUUCGGAGAATT
siCdh5	Mouse	Dharmacon	J-041968-11	GAAAAUGGCUUGUCGAAUU
siCtnna1	Mouse	Dharmacon	J-048960-11	GGGCAACGCUGGACGUAAA
siCtnnb1	Mouse	Dharmacon	J-040628-08	AAGCUGACCUGAUGGAGUU

C. Site directed mutagenesis

Site directed mutagenesis was performed in αCat-Vinc-HA lentiviral plasmids in order to inhibit the Arp2/3 complex ligation to vinculin. For this, polymerase chain reaction (PCR) was executed with Herculase II fusion DNA polymerase (HPA600675, Soquimica) following the manufacturer's guidelines and a temperature gradient from 65ºC to 55ºC in the step of plasmid amplification. To produce αCat-Vinc-HA lentiviral plasmid, point mutation consisted in substituting a proline to an alanine (described by Kris A. DeMali et al., 2002) in the position 57 of vinculin's fusion protein sequence in the plasmid using forward primer 5' CCAGGCCCCCACCAGCAGAAGAGAAGGATG 3' and reverse primer 5' CATCCTTCTCTTCTGCTGGTGGGGGCCTGG 3' (primers designed based on http://www.bioinformatics.org/primerx/). PCR product was then digested 3h at 37ºC using restriction enzyme DpnI (R0176S, New England Biolabs) to eliminate original plasmids. Digested PCR product was transformed into 50uL chemically competent Stbl3 *Escherichia coli* cells (C7373-03, Life Technologies) and then plasmid concentration was increased using GeneJET Plasmid Miniprep Kit (K0503, Bioportugal) following the manufacturer's guidelines. Then, Sanger sequencing (GATC Biotech) confirmed point mutations in the plasmids.

D. Viral production and transduction

Replication-incompetent lentiviruses were produced by transient transfection of HEK293T of pLX303 lentiviral expression vector co-transfected with the viral packaging vector Δ8.9 and the viral envelope vector VSVG. Medium was replaced with fresh culture medium 6h post transfection. 48h after medium replacement, lentiviral particles were concentrated from supernatant by ultracentrifugation at 90000g for 1h30 and re-suspended in 0.1% BSA PBS. Seeded HUVECs were transduced 24h post-transfection with varying concentrations of lentiviral plasmids containing αCat-Vinc-HA, EGFPC1/GgVcl

1-1066, EGFPC1/GgVcl 1-1066 T12 mutant and αCat-Vinc-HA (vinculin P57A) protein sequences (see Table III.2). 24h after viral transduction the culture medium was replaced by fresh complete medium and cells were kept under culture conditions up until 48h post-transduction and then processed for immunofluorescence or imaging.

Table III.2: List of constructs and fusion protein sequences.

Name	Construct information
αCat-Vinc-HA	Commercial sequence (cloned in pUC57, General Biosytems) inserted into lentiviral backbone (pLX303, #25897 Addgene)
αCat-Vinc-HA_mut(vinculin P57A)	Commercial sequence (cloned in pUC57, General Biosytems) with P57A mutation inserted into lentiviral backbone (pLX303, #25897 Addgene)
EGFPC1/GgVcl 1-1066	Commercial sequence (#46265 Addgene) inserted into lentiviral backbone (pLX303, #25897 Addgene)

E. Scratch-wound assay and drug treatments

To assess functional collective cell behavior properties (*i.e.*, polarity and migration), as well as morphological features of *in vitro* cultured cells, we used the scratch-wound assay. The wound was created by scratching the surface of a well-plate or a microscopy glass slide containing a monolayer of adherent cells with a 200µL pipette tip. The culture medium was then replaced by fresh complete medium after 5-10min and cells were allowed to migrate under optimal physiological conditions. When appropriate, Ck-666 drug (SML0006, Sigma-Aldrich), an Arp2/3 complex inhibitor, was added to the medium at a final concentration of 200µM. For immunofluorescence staining experiments, cells migrated for 5h before being fixed. For live imaging experiments HUVECs were the only cell line used and migration was followed up to 16h. Imaging was performed using a Zeiss Cell Observer SD (Carl Zeiss) equipped with an EC Plan-Neofluar 10x NA 0.3 PH1. To track individual cells within the monolayer more efficiently using the cell nuclei as reference. Images of the scratch front were acquired at multiple positions every 10min. Analysis of migration, including cell velocity and straightness was performed using FIJI TrackMate plug in and Chemotaxis and Migration Tool (free software from Ibidi).

F. RNA extraction and quantitative Real-Time PCR

RNA extraction was performed from HUVECs and MS1 cells seeded on 12-well plates using the RNeasy Mini Kit (Qiagen) as described by the manufacturer's protocol. RNA concentration was quantified using NanoDrop 1000 (Thermo Scientific) and adjusted equally, followed by DNase I digestion (EN0523, Thermo Scientific) and cDNA synthesis (Superscript IV First-Strand Synthesis System, Invitrogen). cDNA samples were then diluted in RNAse/DNAse-free water for the subsequent quantitative real-time PCR (RT-qPCR) reactions. RT-qPCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems) following the standard program of the system previously mentioned. For each reaction, 5µL of cDNA was combined with 10µL of Power SYBR Green PCR Master Mix, 4.5µL of RNAse/DNAse free

water and 0.5µL of 4µM primers pool (Forward+Reverse) (see Table III.3) in a MicroAmp Fast Optical 96-well Reaction Plate (Applied Biosystems). The expression levels of each sample duplicate were then normalized to GAPDH and the $2^{-\Delta\Delta T}$ method was used to calculate relative alterations in gene expression.

Table III.3: List of qPCR primers.

Primer	Specie	Forward Sequence	Reverse Sequence
ACTR3	Human	CTGTAGATGCCCGGCTGAAA	TATCGCTGCATGTGGTGTGT
CDH5	Human	TCTCCGCAATAGACAAGGACA	TGGTATGCTCCCGGTCAAAC
CTNNA1	Human	GGACCTGCTTTCGGAGTACATG	CTGAAACGTGGTCCATGACAGC
CTNNB1	Human	CACAAGCAGAGTGCTGAAGGTG	GATTCCTGAGAGTCCAAAGACAG
GAPDH	Human	GTCAAGGCTGAGAACGGGAA	TGGACTCCACGACGTACTCA
TLN1	Human	TTGGAGATGCCAGCAAGCGACT	CCAGTTCTGTGGCTGCCTGATT
VCL	Human	TGAGCAAGCACAGCGGTGGATT	TCGGTCACACTTGGCGAGAAGA
Cdh5	Mouse	GAACGAGGACAGCAACTTCACC	GTTAGCGTGCTGGTTCCAGTCA
Ctnna1	Mouse	GTCCGAATGTCTGCAAGCCAGT	GCCAGTTTACTCTGCGGCTTTG
Ctnnb1	Mouse	GTTCGCCTTCATTATGGACTGCC	ATAGCACCCTGTTCCCGCAAAG
Gapdh	Mouse	GTGCAGTGCCAGCCTCGTCC	GCCACTGCAAATGGCAGCCC

G. Protein extraction and Western Blotting

Protein extraction was performed from HUVECs and MS1 cells seeded on 6-well plates which were lysed in 150µL of RIPA buffer (50mM Tris/HCl pH7.5, 1% NP-40, 150mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS in H_2O) supplemented with phosphatase and proteinase inhibitors cocktail (1:100, #10085973 Fischer Scientific). Adherent cells were then detached from the plate with a cell scrapper and the cell lysates were gathered and transferred into an ice cold eppendorf tube. The cell lysates were then centrifuged at maximum speed for 10min at 4ºC and the supernatants collected into a new eppendorf tube. Protein concentration was quantified using the BCA protein assay kit (Pierce) following the guidelines recommended by the manufacturer. The Multimode microplate reader, Infinite M200 (Tecan), was used for spectrophotometric measurement of protein with the i-control™ software. For western blotting, protein samples were normalized up to 25µL and combined with a mixture of 2x Laemmli Sample Buffer (#161-0747, Bio-rad Laboratories) with 450mM DTT (D0632, Sigma-Aldrich). Protein samples were then incubated at 70ºC in a Dry Block Thermostat (Grant Instruments, Ltd) for 10min. Protein samples were loaded and separated on a 4-15% Mini-PROTEAN® TGX™ gel (#456-1084, BioRad) along with 5µL of protein ladder (Full-Range RPN800E, GE Healthcare Rainbow Molecular Weight Markers), first at 50V for 5min and then at 100V for 1-2h in SDS-PAGE running buffer (10x SDS-PAGE: 250mM Tris, 1.92M glycine, 1% SDS, pH8.3). Gels were then transferred onto a polyvinylidene difluoride (PVDF) membrane (#IPVH00010, Merck Milipore) with Mini Trans-Blot® Electrophoretic Transfer Cell (Biorad) following the manufacturer's guidelines. After transfer, blotted membranes were incubated in Ponceau Red to assess transfer quality and then washed in TBS-T (50mM Tris/HCl, 150mM NaCl, 0.1% Tween-20, pH7.5). Then membranes were incubated in blocking buffer containing 3% BSA (Bovine Serum Albumin, MB04602, Nzytech) in TBS-T for 1h at room

temperature (RT), followed by an overnight incubation at 4ºC with the primary antibodies diluted in the same blocking buffer (see Table III.4). On the following day membranes were washed 3 times for 5min in TBS-T and incubated in blocking buffer containing the secondary horseradish peroxidase (HRP) conjugated antibodies for 1h at RT. Before revelation membranes were washed again 3 times in TBS-T for 5min and then incubated in ECL™ Western Blotting Detection Reagent (RPN2209, GE Healthcare) following the manufacturer's protocol. Protein bands were visualized in Chemidoc XRS+ and relative protein quantities were measured using the Image Lab software, both from Bio-Rad Laboratories. All results were normalized to tubulin levels.

H. Immunofluorescence

Immunofluorescence of *in vitro* cultured cells were seeded on 24-well plates with glass coverslips previously coated with 0.2% gelatin in sterile water (G1393, Sigma-Aldrich). After the scratch-wound assay (described above), cells were fixed in 1% paraformaldehyde (PFA) supplemented with 1M $MgCl_2$ and 1M $CaCl_2$ (1µL/2mL) in PBS for 30min at RT. Cells were then washed with PBS to remove the remaining PFA and the immunostaining protocol initiated. When the PBS was removed, cells were blocked and permeabilized with blocking solution containing 3% BSA in PBS-T (PBS with 0.1% Triton X-100) for 30min at RT. Then cells were incubated for 2h at RT with the primary antibodies diluted in the blocking solution (see Table III.4) and washed 3 x 15min washes in PBS-T. Afterwards, cells were incubated in blocking solution containing the secondary fluorophore conjugated antibodies for 1h at RT in the dark, followed again by 3 washes of 15min in PBS-T. Finally, cells were incubated with 1x DAPI (D1306, Molecular Probes by Life Technologies) diluted in PBS-T for 5min in the dark. Coverslips were then mounted on microscopy glass slides using Mowiol DABCO (Sigma-Aldrich). For polarity quantification, a tile-scan spanning the entire region of the scratch was acquired on a motorized inverted widefield fluorescence microscope, Zeiss Axiovert 200M (Carl Zeiss) equipped with the Metamorph software with an EC Plan-NeoFluar 40x NA 0.75 dry objective.

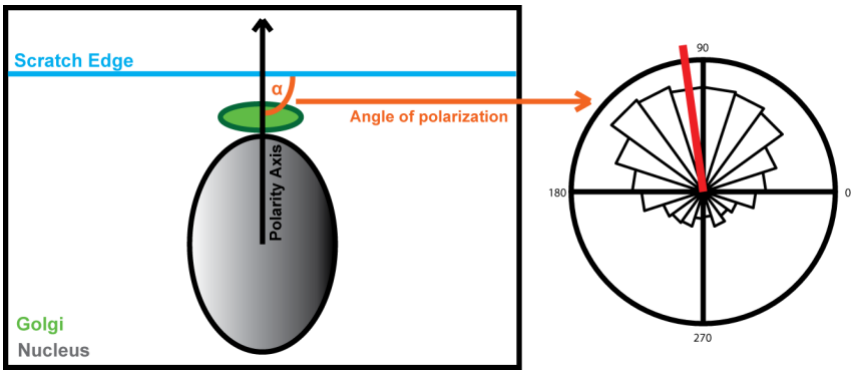
Table III.4: List of primary and secondary antibodies.

Name	Brand	Type	Cat. Number	Host	IF	WB
Anti-α-catenin	Sigma-Aldrich	Primary	C2081	Rabbit	1/200	1/1000
Anti-α-tubulin	Sigma-Aldrich	Primary	T6199	Mouse	–	1/2000
Anti-GM130	BD Biosciences	Primary	610823	Mouse	1/400	–
Anti-Golph4	Abcam	Primary	ab28049	Rabbit	1/400	–
Anti-HA tag	BioLegend	Primary	901513	Mouse	1/100	–
Anti-p34/ARPC2	Merck Millipore	Primary	07-227	Rabbit	1/100	–
Anti-VE-cadherin	R&D	Primary	AF938	Goat	–	1/400
Anti-VE-cadherin	Santa Cruz Biotechnologies	Primary	sc-6458	Goat	1/100	1/1000
Anti-vinculin	Sigma-Aldrich	Primary	V9264	Mouse	1/400	–
Anti-γ-tubulin	Sigma-Aldrich	Primary	T6557	Mouse	–	1/2000
Donkey anti-goat Alexa 647	Thermo Fisher Scientific	Secondary	A21447	Donkey	1/400	–
Donkey anti-goat HRP	Bethyl	Secondary	A50-201P	Donkey	–	1/5000

Donkey anti-mouse Alexa 488	Thermo Fisher Scientific	Secondary	A21202	Donkey	1/400	–
Donkey anti-rabbit Alexa 568	Thermo Fisher Scientific	Secondary	A10042	Donkey	1/400	–
Goat anti-rabbit HRP	Life Technologies	Secondary	G-21234	Goat	–	1/5000
Sheep anti-mouse HRP	GE Healthcare	Secondary	NA931V	Sheep	–	1/5000

I. Polarity analysis

To quantify cell polarity, tile-scan images of cells stained with Golgi (Golph4 or GM130) and nuclear (DAPI) markers were processed on Adobe Photoshop. This process aimed to separate first (leaders) to fourth (followers) rows. Afterwards, each set of images was imported and analyzed in MATLAB using a modified version of a polarity analysis script kindly provided by Anne-Clémence Vion and Holger Gerhardt. Briefly, after segmenting each channel corresponding to the Golgi and nuclear staining, the centroid of each organelle was determined and a vector connecting the center of the nucleus to the center of its corresponding Golgi apparatus was drawn. The Golgi-nucleus assignment was done automatically minimizing the distance between all the possible couples. The polarity of each cell was defined as the angle between the vector and the scratch line. An angular histogram showing the angle distribution and the direction of migration was then generated. Circular statistic was performed using the Circular Statistic Toolbox. To test for circular uniformity, we applied the Rayleigh test, yielding a p-value indicating the likelihood of the distribution to be uniformly distributed around the circle. The polarity index was calculated as the length of mean resultant vector for a given angular distribution (Figure III.1). Polarity index (PI) vary between 0 and 1, with 0 corresponding to random distribution and 1 corresponding to all cells polarized in the same direction. PI indicates the collective orientation strength of the cell monolayer.



$$Polarity\ Index = \sqrt{\left(\frac{1}{N}\sum_{1}^{N} cos \propto\right)^2 + \left(\frac{1}{N}\sum_{1}^{N} sin \propto\right)^2}$$

Figure III.1- Equation for polarity index. Cell polarity was defined as the angle (α) between the scratch edge and the cell polarity axis, defined by the vector drawn from the center of the cell nucleus to the center of the Golgi apparatus. The polarity index was calculated according to the formula and it was used as a measure for collective polarization.

J. Statistical analysis

Comparisons between two experimental groups were analyzed with unpaired parametric t test, while multiple comparisons between more than two experimental groups were assessed with one-way ANOVA. All statistical analysis was performed using GraphPad Prism 7 and Matlab (Mathworks).

IV. Results

A. Cadherin-independent roles of catenins in mechanotransduction:

a) α-catenin/β-catenin seems to regulate collective cell polarity independently of VE-cadherin in HUVECs

Adherens junctions are essential for cell-cell mechanotransduction during collective cell migration (Ladoux et al., 2015). To study the possible cadherin-independent functions of α-catenin in CCM, we used the well described *in vitro* model for collective cell migration, the scratch wound assay. First, we performed a 16h time-lapse imaging of HUVECs, using previously validated siRNAs against VE-cadherin and α-catenin. We found that both siRNAs led to a significant decrease in cell migration straightness (Figure IV.1A and Figure IV.1B) compared to control cells. This was not surprising, as force transmission and cell migration are directly influenced by junction integrity. Additionally, abrogating adherens junctions also led to a significant increase in cell velocity (Figure IV.1A and Figure IV.1B), which could be explained by the sudden loss of cell-cell adhesion and tension, causing a shift from collective to single cell migration. As expected, α-catenin depletion also led to a loss of axial polarity (PI=0.049). However, VE-cadherin knockdown (KD) cells were able to remain oriented (PI=0.203), resembling control cells (PI=0.326) (Figure IV.1C). These are in accordance with previous results obtained in the laboratory. Altogether, these data suggest that α-catenin might have a cadherin-independent function in axial polarity during collective cell migration.

We started by analyzing junction's morphology and localization of key adherens junction proteins by immunofluorescence in the absence of VE-cadherin and α-catenin (Figure IV.2A) in HUVECs. siRNA-mediated KD of either α-catenin or VE-cadherin led to a marked disruption of adherens junctions, with a significant decrease in VE-cadherin and α-catenin at cell-cell interfaces. Then, we measured KD efficiency of both siRNAs by measuring mRNA and protein levels (Figure IV.2B, Figure IV.2C and Figure IV.2D). We found that both siRNAs efficiently decreased levels of mRNA in HUVECs. However, at the protein, we found that α-catenin depletion was strongly effective (~90%), but the KD efficiency of VE-cadherin protein was strong yet not total (Figure IV.2D), reaching a 60-70% decrease. We further checked whether α-catenin was still expressed in the absence of VE-cadherin, and found that mRNA levels were ~40% decreased, whereas protein levels were reduced by ~60% in comparison with control samples (Figure IV.2B, Figure IV.2C and Figure IV.2D). VE-cadherin mRNA and protein levels were identical in α-catenin KD and in control cells (Figure IV.2B, Figure IV.2C and Figure IV.2D). β-catenin mRNA levels were also kept normal in both VE-cadherin and α-catenin KDs (Figure IV.2B). These results show that in VE-cadherin KD, both α-catenin and β-catenin are still present, reinforcing the possibility of a cadherin-independent function in HUVECs.

α-catenin binds to the cadherin complex through β-catenin. Thus, we next thought to test if α-catenin would function alone or together with β-catenin to regulate collective cell polarization and CCM. First, we characterized junction's morphology in the absence of β-catenin. KD of β-catenin led to a complete disruption of adherens junctions (Figure IV.3A). Looking at protein levels, β-catenin was ~70%

depleted (Figure IV.3B and Figure IV.3C). Although, α-catenin and VE-cadherin mRNA expression levels were unaltered in β-catenin KD cells (Figure IV.3D). Second, we analyzed axial polarity patterns in β-catenin KD cells. Analogous to α-catenin KD cells, cells lacking β-catenin displayed a loss of collective polarization (PI=0.092) (Figure IV.3E). Altogether, these results support the hypothesis that β-catenin and α-catenin function as a complex to regulate axial polarity during collective cell migration, independently of their cadherin-binding function.

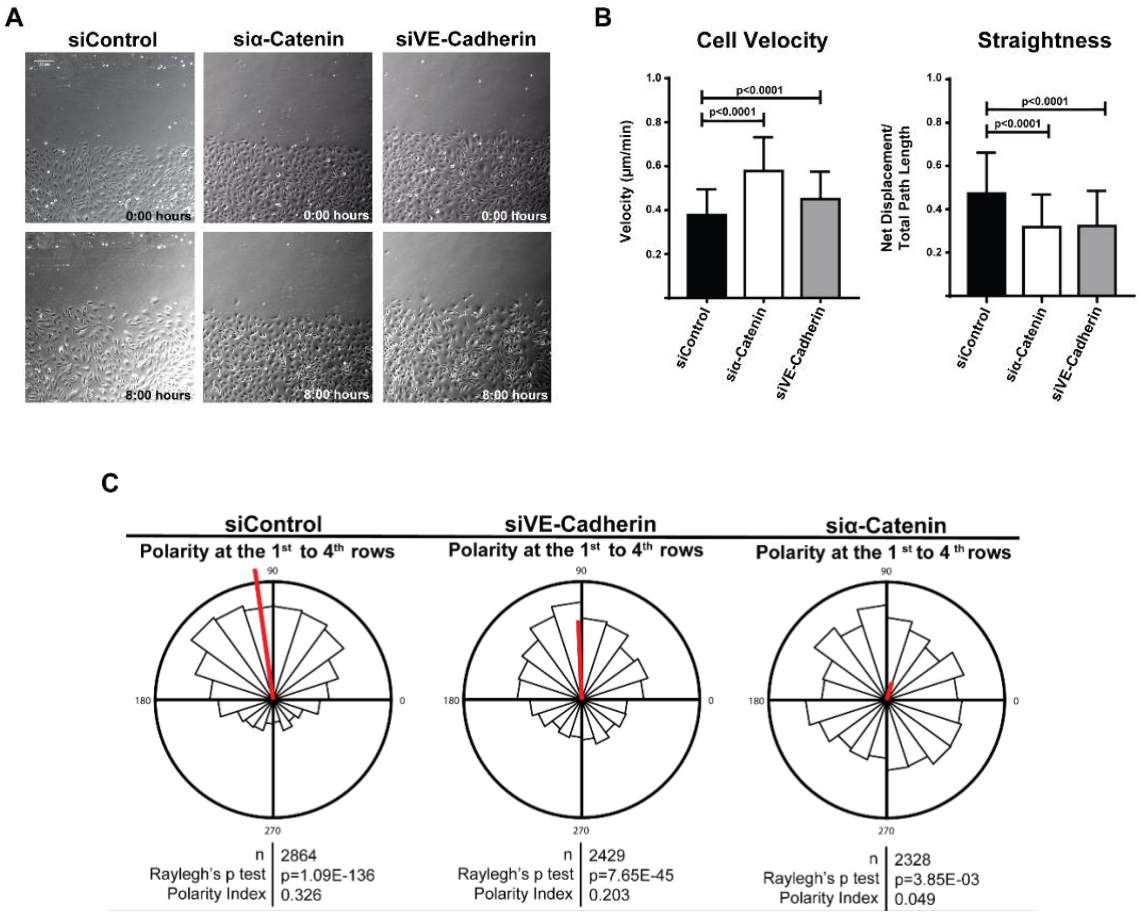


Figure IV.1- α-catenin seems to have a cadherin-independent function in axial polarity during collective cell migration in HUVECs. (A) Example of HUVECs at 0 hours and 8 hours after migration from siControl, siα-catenin and siVE-cadherin transfected cells. (B) Quantification of cell velocity and straightness from siControl (n=4), siα-catenin (n=1) and siVE-cadherin (n=2) transfected cells during 16h of migration. Data are mean ± SD, p-values from multiple comparisons in one-way ANOVA. (C) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl, siα-catenin and siVE-cadherin transfected cells (n=2).

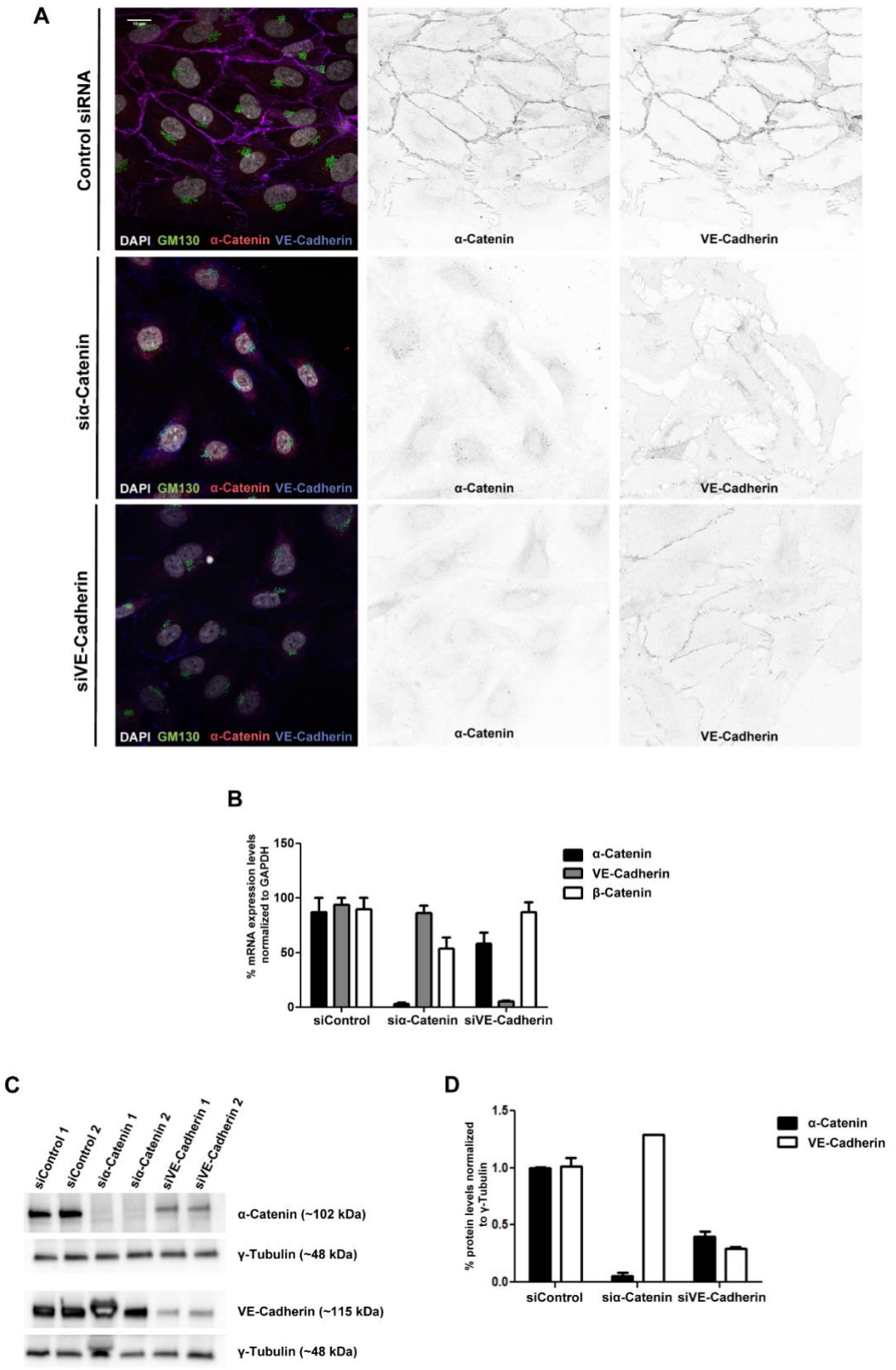


Figure IV.2- Analysis of VE-cadherin and α-catenin siRNAs efficiencies at mRNA and protein level. (A) Example of HUVECs labeled for nuclei (DAPI), α-catenin and VE-cadherin from siControl, siα-catenin and siVE-cadherin transfected cells. (B) Quantification of α-catenin, VE-cadherin and β-catenin mRNA levels normalized to GAPDH from siControl, siα-catenin and siVE-cadherin transfected cells (n=1). (C) Immunoblot of α-catenin, VE-cadherin and γ-tubulin from siControl, siα-catenin and siVE-cadherin transfected cells (n=1). (D) Quantification of α-catenin and VE-cadherin protein levels normalized to γ-tubulin from siControl, siα-catenin and siVE-cadherin transfected cells (n=1).

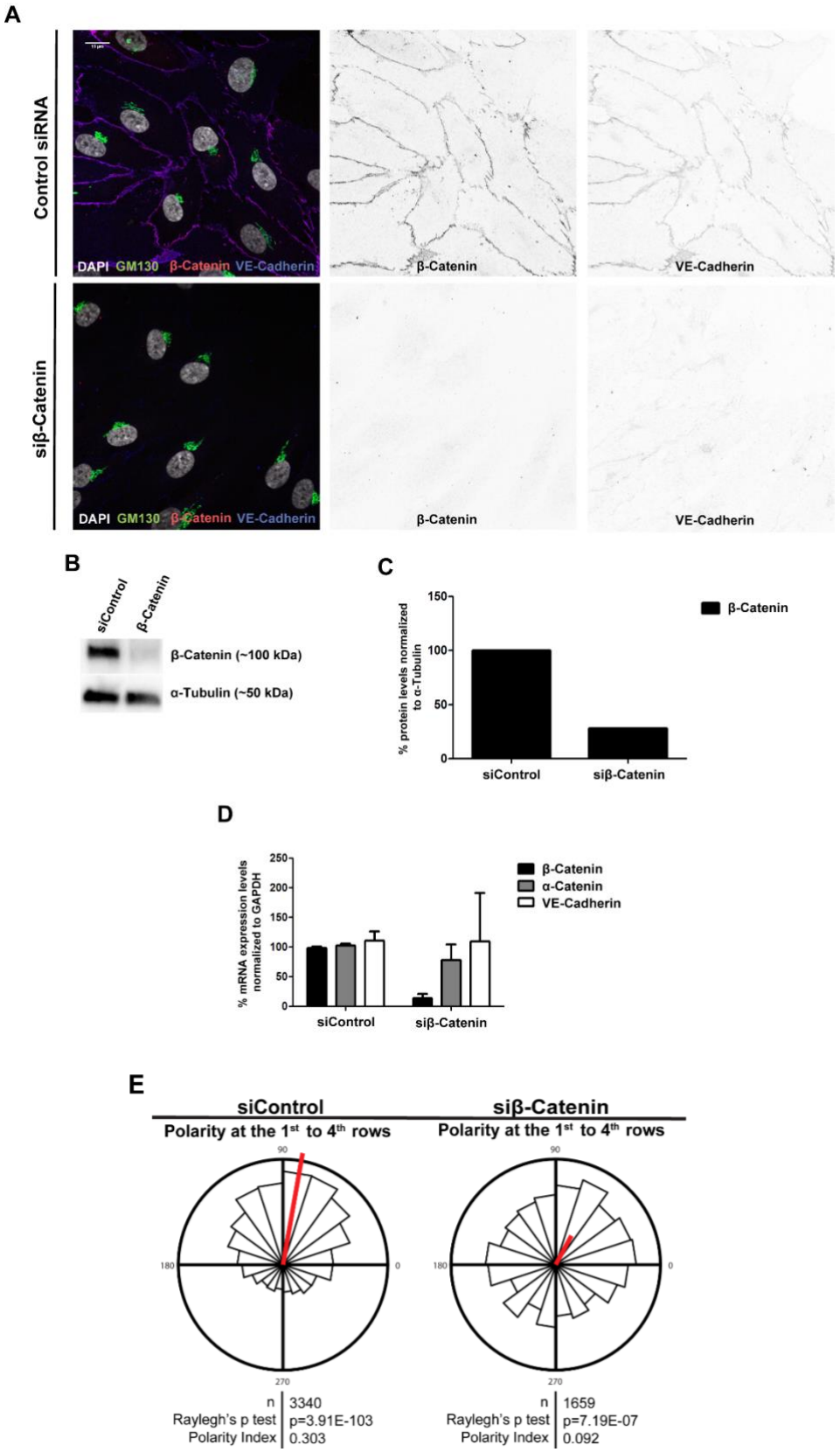


Figure IV.3- β-catenin together with α-catenin seems to have a role in cadherin-independent function in HUVECs. (A) Example of HUVECs labeled for nuclei (DAPI), β-catenin and VE-cadherin from siControl and siβ-catenin transfected cells. (B) Immunoblot of β-catenin and α-tubulin from siControl and siβ-catenin transfected cells (n=1). (C) Quantification of β-catenin protein levels normalized to α-tubulin from siControl and siβ-catenin transfected cells (n=1). (D) Quantification of β-catenin, α-catenin and VE-cadherin mRNA levels normalized to GAPDH from siControl and siβ-catenin transfected cells (n=1). (E) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl and siβ-catenin transfected cells (n=2).

b) Cadherin-independent function of catenins is absent in MS1 cells

To understand which specific domain of α-catenin could be involved in this cadherin-independent function of catenins, we aimed to create an immortalized α-catenin knockout cell line that would behave the same way as HUVECs regarding collective polarity and CCM. Hence, we analyzed axial polarity in four different types of cells: (1) MCF10a, epithelial cells from human mammary glands; (2) CI-HUVECs, immortalized HUVECs; (3) bEND5, endothelial cells from mouse blood-brain barrier; and (4) MS1, endothelial cells from mouse pancreas. In MCF10a, the Golgi apparatus was always positioned behind the nuclei during collective cell migration, with a mean ~270º and partially oriented PI (PI=0.194) (Figure IV.4). CI-HUVECs and bEND5 had a PI closer to 0 (PI=0.135 and PI=0.063, respectively), meaning these cells were unable to polarize after 5h of migration (Figure IV.4). MS1 cells showed a PI=0.292 that is similar to the PI observed in HUVECs (Figure IV.4). Thus, these results showed that MS1 cell line presented a similar behavior as HUVECs during CCM.

We next tested mouse siRNA against α-catenin and VE-cadherin at mRNA and protein level. VE-cadherin mRNA and protein levels were not changed compared to control in α-catenin depleted cells (Figure IV.5A, Figure IV.5B and Figure IV.5C). The same was observed regarding α-catenin levels in VE-cadherin KD cells (Figure IV.5A, Figure IV.5B and Figure IV.5C). Expression levels of β-catenin mRNA also remained unaltered in the absence of both VE-cadherin or α-catenin (Figure IV.5A). In addition, we analyzed junction's morphology in α-catenin and VE-cadherin MS1 depleted cells. Adherens junctions were completely disrupted in both KDs (Figure IV.5D).

Then, we analyzed the collective polarity phenotype of α-catenin, β-catenin and VE-cadherin KD in MS1 cells. Depletion of α-catenin and β-catenin resulted in loss of axial polarity (PI=0.145 and PI=0.094, respectively) (Figure IV.6). However, the absence of VE-cadherin also led to randomized axial polarity (PI=0.070) (Figure IV.6), which was not in agreement with the previously results obtained using HUVECs. These contradictory results could have two different explanations: (1) catenins independent-function of cadherin may not be conserved in MS1 cell line; or (2) residual VE-cadherin complex in HUVECs could be sufficient to collectively polarize cells, due to a lower VE-cadherin KD efficiency (Figure IV.2D).

To distinguish between these two hypotheses, we first try to enhance the VE-cadherin KD efficiency. We used a combination of two different siRNAs to increase VE-cadherin depletion. The combination of siRNAs led to a substantial decrease in VE-cadherin protein expression (~98% reduction) when compared to single siRNA (~93% reduction) (Figure IV.7A and Figure IV.7B). Interestingly, under these experimental conditions, collective cell polarity of VE-cadherin KD cells was randomized (PI=0.132) (Figure IV.7C). Therefore, we conclude that the original hypothesis of cadherin-independent function of catenins in collective polarity was not correct, and that catenins necessarily need to be in complex with VE-cadherin to promote collective polarity. Yet, it also reveals that collective polarity is established using a relatively low pool of VE-cadherin in cells.

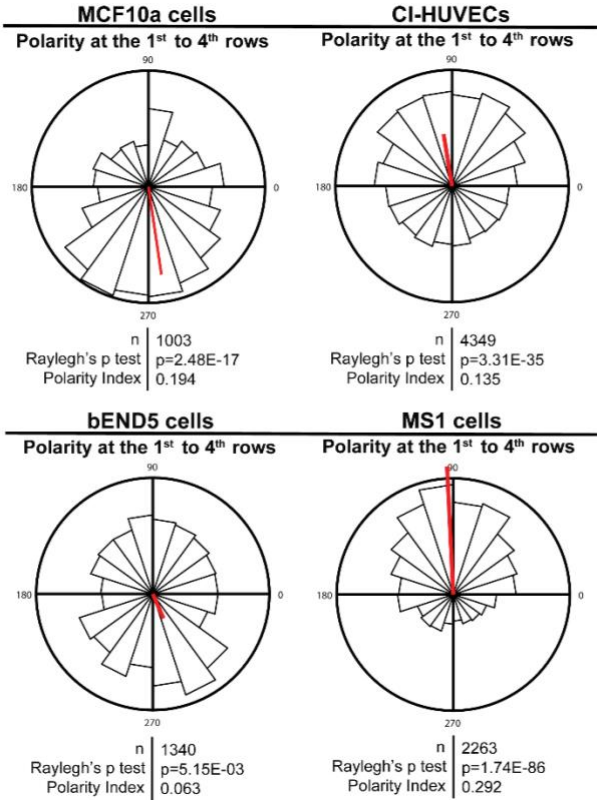


Figure IV.4- MS1 cells have the same axial polarity behavior as HUVECs. Angular histograms showing the distribution of polarization angles of 1st to 4th rows from wild type MCF10a cells (n=2), wild type CI-HUVECs (n=2), wild type bEND5 (n=1) and wild type MS1 cells (n=1).

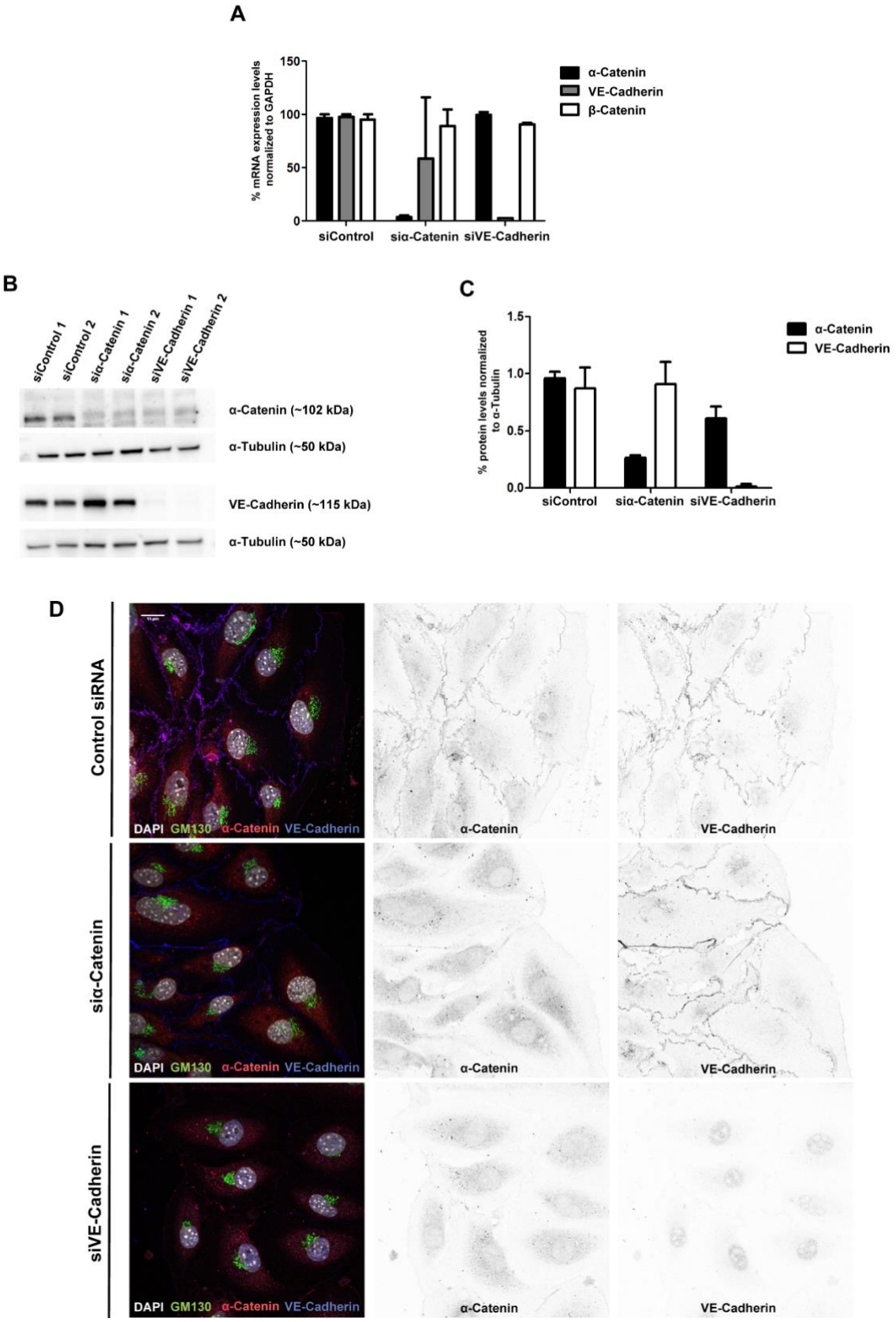


Figure IV.5- In the absence of VE-cadherin α-catenin is still present in MS1. (A) Quantification of α-catenin, VE-cadherin and β-catenin mRNA levels normalized to Gapdh from siControl, siα-catenin and siVE-cadherin transfected cells (n=1). (B) Immunoblot of α-catenin, VE-cadherin and γ-tubulin from siControl, siα-catenin and siVE-cadherin transfected cells (n=1). (C) Quantification of α-catenin and VE-cadherin protein levels normalized to γ-tubulin from siControl, siα-catenin and siVE-cadherin transfected cells (n=1). (D) Example of MS1 cells labeled for nuclei (DAPI), α-catenin and VE-cadherin from siControl, siα-catenin and siVE-cadherin transfected cells.

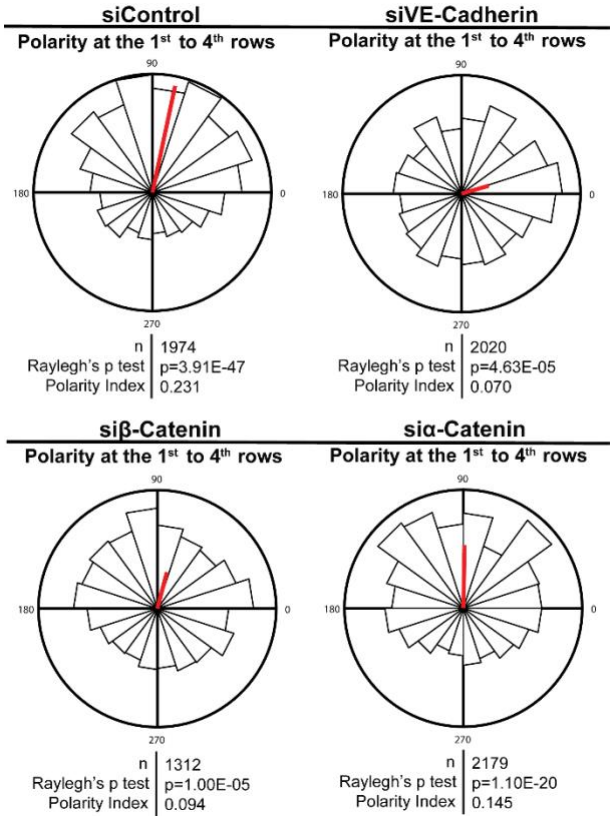


Figure IV.6- Catenins-independent function of cadherin seems to be absent in MS1 cells. Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl (n=2), siα-catenin (n=2), siVE-cadherin (n=2) and siβ-catenin (n=1).

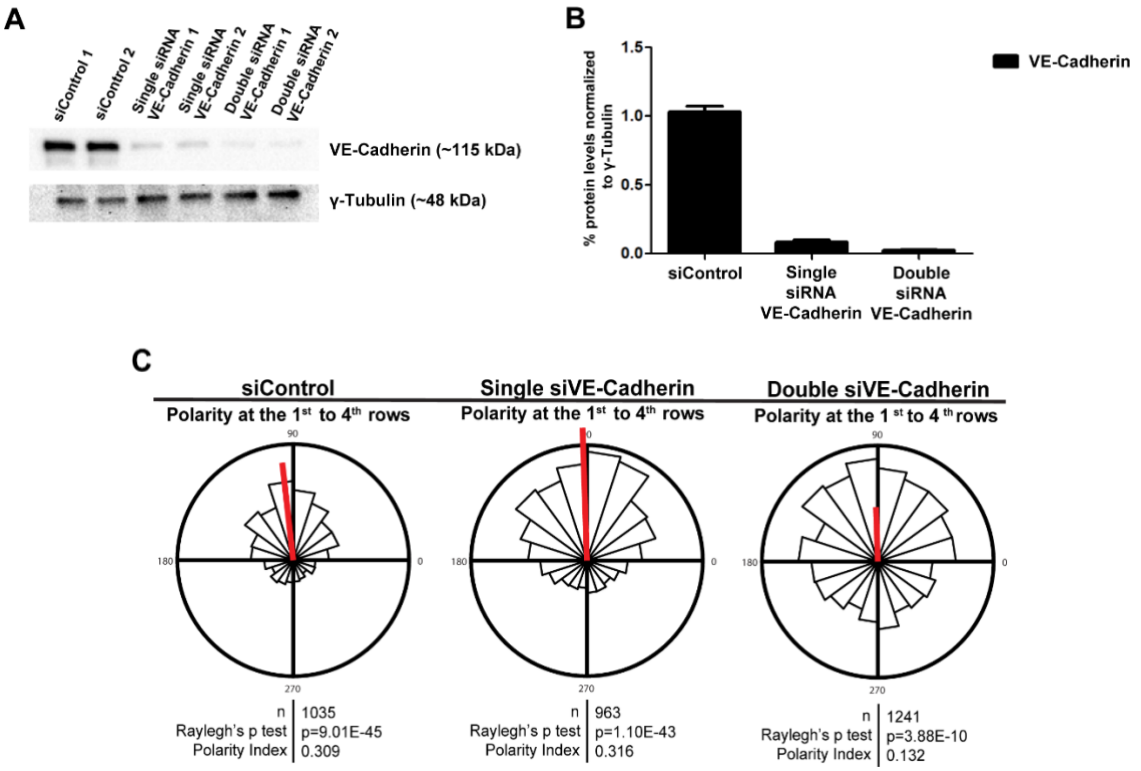


Figure IV.7- Increased VE-cadherin KD efficiency abolished collective cell polarity. (A) Immunoblot of VE-cadherin and γ-tubulin from siControl, siVE-cadherin with single siRNA and siVE-cadherin with double siRNA transfected cells (n=1). (B) Quantification of VE-cadherin protein levels normalized to γ-tubulin from siControl, siVE-cadherin with single siRNA and siVE-cadherin with double siRNA transfected cells (n=1). (C) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl, siVE-cadherin with single siRNA and siVE-cadherin with double siRNA transfected cells (n=1).

B. Role of vinculin and Arp2/3 complex in CCM

a) Vinculin is essential for collective cell behavior

Vinculin is a mechanotransduction protein that simultaneously binds to α-catenin and to filamentous actin, and that participates in junction's stabilization and cell-cell force transmission (Goldmann, 2016). However, the biological function of vinculin at adherens junctions has been a theme of controversy. Despite being present at high-tension junctions in several model organisms, vinculin is dispensable for zebrafish and fruitfly normal development (Han et al., 2017; Alatortsev et al., 1997). However, its absence during mouse embryonic development results in lethal cardiovascular and neuronal defects (Xu et al., 1998). One main controversy on the role of vinculin at AJs is linked to its dual function in cells. Vinculin has identified functions simultaneously both at AJs and FAs. Moreover, vinculin was shown to be important for force transmission in MDCK cells, its relevance for collective cell migration and coordination of cell polarities was yet to be established.

To address vinculin's function in CCM, we decided to analyze collective cell behavior in the absence of vinculin. First, we confirmed the efficiency of vinculin KD by looking at mRNA and protein levels (Figure IV.8A, Figure IV.8B and Figure IV.8C). Then, performing a 16h time-lapse imaging, vinculin KD led to issues in cell migration straightness, however cell velocity was unaffected (Figure IV.8D). As expected, vinculin depleted cells also showed loss of cell coordination (PI=0.170) in comparison to control cells (PI=0.283) (Figure IV.8E). This phenotype could be rescued by re-expression of a chicken vinculin full-length construct (PI= 0.242) (Figure IV.9B). To distinguish the specific effects of vinculin at AJs and FAs, we depleted talin1, a key protein for FA assembly. Talin1 KD efficiency, measure by mRNA levels, had a reduction of ~95% (Figure IV.10B). Immunofluorescence showed a significant reduction in size and number of FAs when talin1 was depleted (Figure IV.10A). Vinculin mRNA levels remain unaffected in the absence of talin1 (Figure IV.10B). Interestingly, talin1 KD resulted in polarity indexes (PI=0.258) similar to control cells (PI=0.309) (Figure IV.10C), suggesting that FAs are not essential for coordination of cell polarities during CCM.

To further confirm the specific requirement of vinculin in AJs for CCM, we decided to investigate if we could rescue the vinculin KD phenotype by lentiviral overexpression of a fusion protein comprised of the N-terminal domain (β-catenin binding domain) of α-catenin fused to the C-terminal domain of vinculin (actin binding domain) (Figure IV.9C). This construct will bring vinculin's actin binding domain only to AJs and not to FAs (Maddugoda et al., 2007). Remarkably, we observed a rescue in the coordination of cell polarities of vinculin KD HUVECs (PI= 0.217) (Figure IV.9D). Taken together, these results suggest that mechanotransduction and collective cell behavior rely mostly on the pool of vinculin at AJs.

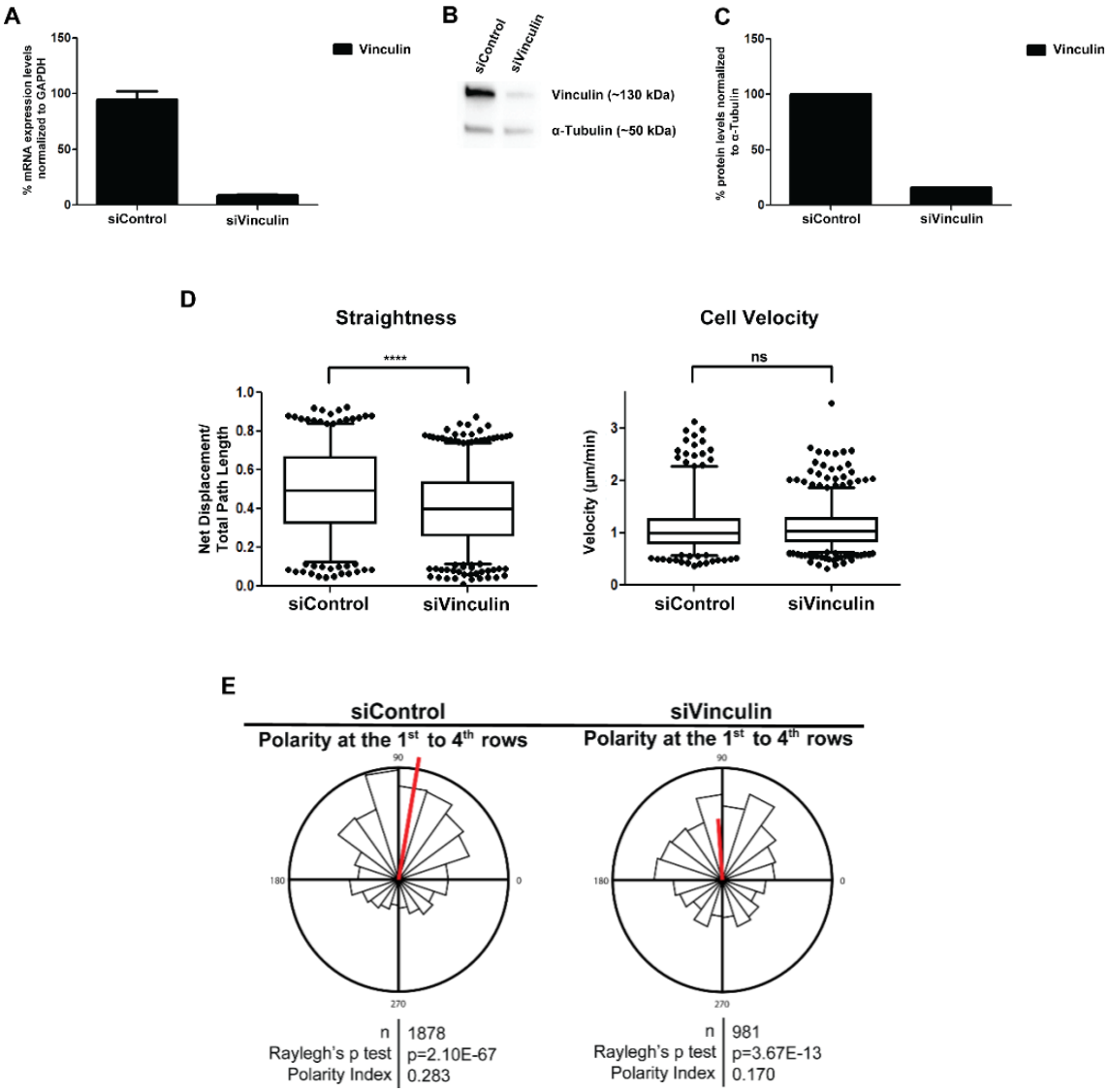


Figure IV.8- Collective cell behavior and collective cell polarity is lost when vinculin is depleted. (A) Quantification of vinculin mRNA levels normalized to GAPDH from siControl and siVinculin transfected cells (n=1). (B) Immunoblot of vinculin and α-tubulin from siControl and siVinculin transfected cells (n=1). (C) Quantification of vinculin protein levels normalized to α-tubulin from siControl and siVinculin transfected cells cells (n=1). (D) Quantification of cell velocity and straightness from siControl (n=1) and siVinculin (n=1) transfected cells during 16h of migration. Data are mean ± SD, p-values from multiple comparisons in one-way ANOVA. (E) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl (n=2) and siVinculin (n=1).

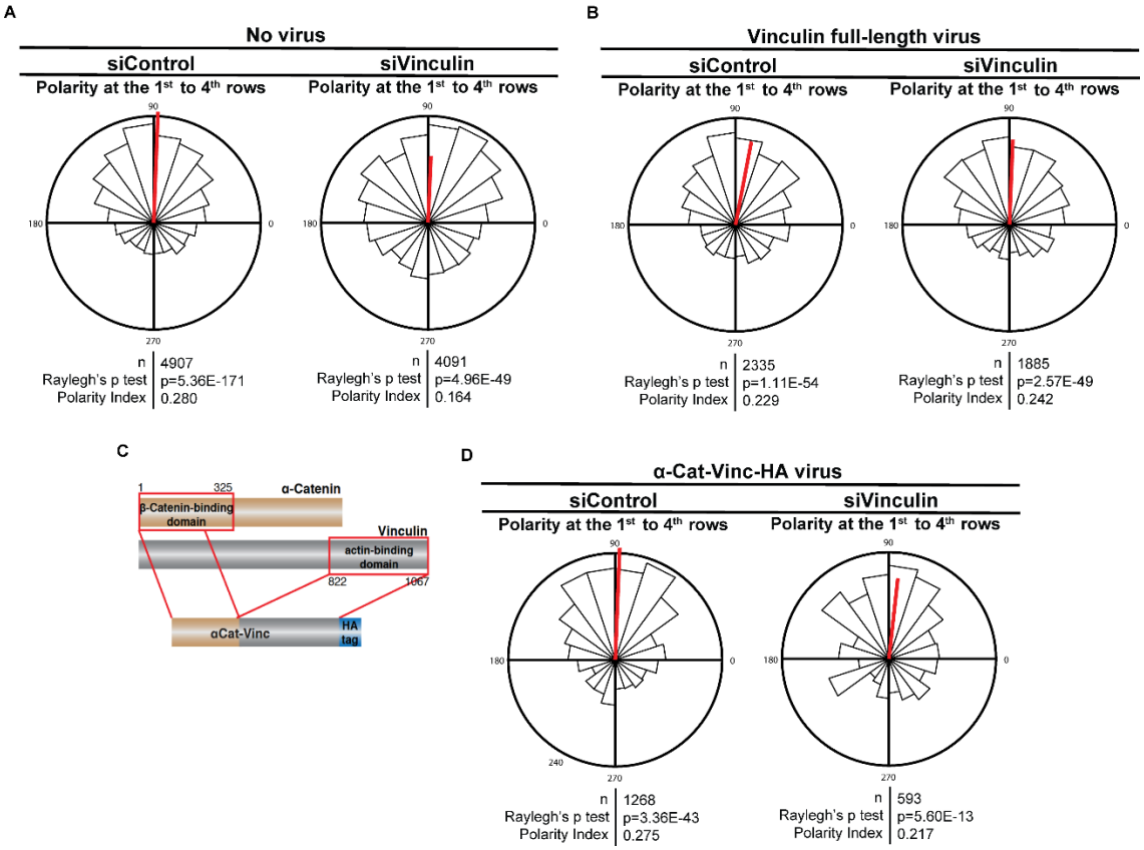


Figure IV.9- Axial polarity is rescued when vinculin is restored in the system. (A) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl and siVinculin without infection (n=3). (B) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl and siVinculin infected with vinculin full-length virus (n=2). (C) Diagram showing the molecular structure of the αCat-Vinc construct. αCat-Vinc-HA is a fusion protein containing the β-catenin-binding domain of α-catenin (brown) fused with the actin-binding domain of vinculin (gray) and the HA tag (blue). (D) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl and siVinculin infected with α-Cat-Vinc-HA virus (n=1).

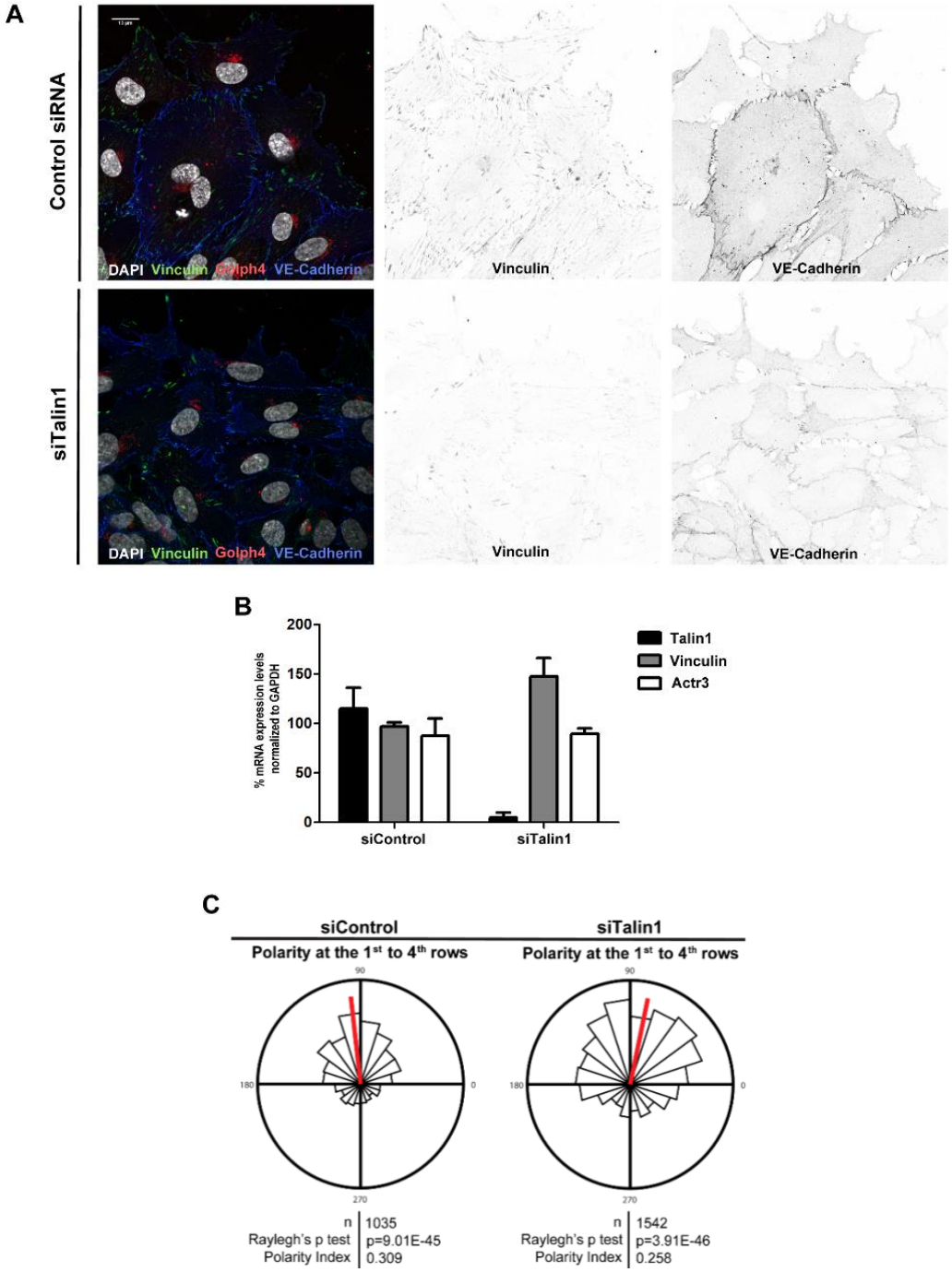


Figure IV.10- Talin1 depletion does not compromise collective cell polarity. (A) Example of HUVECs labeled for nuclei (DAPI), vinculin and VE-cadherin from siControl and siTalin1 transfected cells. (B) Quantification of talin1 mRNA levels normalized to GAPDH from siControl and siTalin1 transfected cells (n=1). (C) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl and siTalin1 transfected cells (n=1).

b) Vinculin recruits Arp2/3 complex to promote mechanocoupling at AJs

The ability of the α-catenin/vinculin fusion protein to rescue vinculin KD phenotype also suggests that vinculin C-terminal domain is important for mechanotransduction and coordination of cell polarities within a group of cells in CCM. Vinculin C-terminus is important to establish firm links with actin cytoskeleton, however, it also contains additional binding sites for other partners, including several regulators of actin dynamics, such as vinexin, VASP and Arp2/3 complex (see Figure I.7B). Recently, Arp2/3 was shown to be associated with adherens junctions, and that the primary link between AJs and the actin cytoskeleton is established through branched actin (Efimova and Svitkina, 2018). Therefore, we proposed that vinculin could mediate Arp2/3 recruitment to AJs, leading to polymerization of branched actin, and firm connection between AJs and cortical actin. To test this hypothesis, we first tested if Arp2/3 complex would be important for CCM and collective polarity in HUVECs. We knocked-down Actr3, one essential subunit of the Arp2/3 complex, that is required for its assembly and function. Actr3 KD HUVECs showed decreased coordination of cell polarities (PI=0.185) in comparison with the control (PI=0.283) (Figure IV.11A), and similar to vinculin KD cells (Figure IV.8E). We also inhibited Arp2/3 complex using Ck-666, a molecule which binds to Arp2/3 complex and prevents its switch to active conformation and stabilizes its inactive form. This pharmacological inhibition also led to a loss of cell polarities (PI=0.058) in comparison with the DMSO control (PI=0.344) (Figure IV.11B). Together, this suggests that Arp2/3 complex confirms an important role in axial polarity during CCM.

Then, to test directly the involvement of Arp2/3 in vinculin junctional activity, we infected HUVECs with an α-catenin/vinculin fusion protein that contains a point mutation on the proline-rich Arp2/3 binding-domain of vinculin (P57A). Unlike previous constructs, the mutated α-catenin/vinculin fusion protein failed to rescue randomized cell polarity associated with vinculin depletion (PI=0.172) (Figure IV.12). Thus, these results suggest that vinculin might recruit Arp2/3 to adherens junctions to promote efficient mechanotransduction and, consequently, coordinated collective cell behavior.

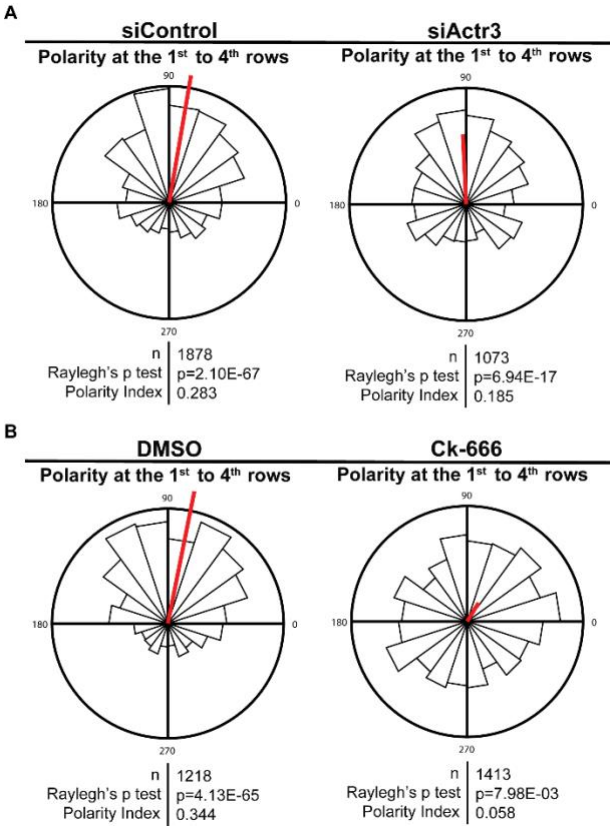


Figure IV.11- Axial polarity is affected when Arp2/3 complex is depleted with siRNA or pharmacologically inhibited. (A) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl (n=2) and siActr3 (n=1) transfected cells. (B) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from HUVECs treated with DMSO and Ck-666 (n=1).

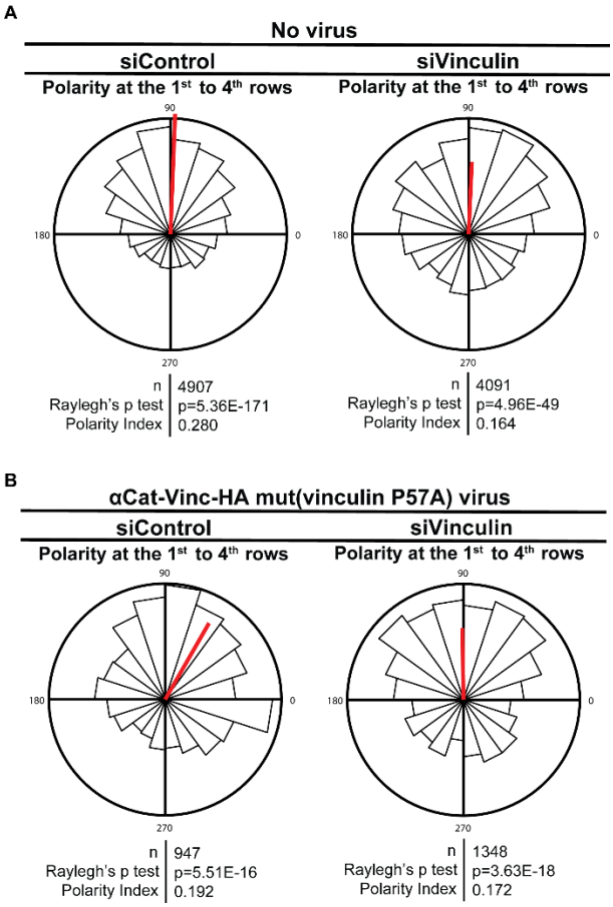


Figure IV.12- Arp2/3 complex seems to be recruited to adherens junctions by vinculin. (A) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl and siVinculin without infection (n=3). (B) Angular histograms showing the distribution of polarization angles of 1st to 4th rows from siControl and siVinculin infected with αCat-Vinc-HA mut(vinculin P57A) virus (n=1).

V. Discussion and Conclusion

In this study, we focused on two aspects of mechanobiology and collective cell behavior dependent on AJs integrity.

On one hand, we found that one of the most important proteins for mechanotransduction, α-catenin, only has a cadherin-dependent function in ECs. Previous studies already showed that cytoplasmic α-catenin regulates actin dynamics independently of cadherin in mammalian cells (Benjamin et al., 2010) and that its depletion results in cell migration and proliferation defects without affecting AJs (Vasioukhin et al., 2000). Further studies found that this cadherin-independent function of α-catenin in regulating actin polymerization was linked to its C-terminal actin binding domain (Hansen et al., 2013). However, how cytoplasmic α-catenin regulates cytoskeleton remodeling and organization required for cell behavior during collective cell migration remains unclear. These unknown mechanisms led to more recent studies where, for the first time, was shown that the N-terminal region of α-catenin is important to establish and stabilize front-rear polarity during cell migration in neural crest cells (Vassilev et al., 2017). Nevertheless, in ECs the mechanism underlying this independent function of α-catenin had never been described. In this study, we hypothesized that α-catenin could have a cadherin-independent role in ECs based on the unexpected well-polarized phenotype that we initially obtained upon VE-cadherin depletion. Although in our first results we showed that in the absence of VE-cadherin ECs remained polarized (Figure IV.1C), even when their collective migratory behavior was abnormal (Figure IV.1A and Figure IV.1B), later experiments revealed this phenotype was only due to residual VE-cadherin protein, due to incomplete depletion (Figure IV.7C). In other words, when ECs retained ~30% of VE-cadherin (Figure IV.2C and Figure IV.2D), the remaining α-catenin still manages to bind to AJs and this pool is sufficient to ensure normal collective cell polarization. Yet, at the same time, depleting ~70% of VE-cadherin is also enough to interfere negatively with collective cell migration, significantly decreasing cell straightness and increasing cell velocity (Figure IV.1B). This is not surprising, especially considering that many signals are required for proper collective cell migration (see Introduction). Therefore, it is reasonable to assume that even maintaining one of these signals intact, in this case Golgi positioning, the migratory behavior of cells will still be affected. Following this train of thought, this set of data also supports the idea that collective cell migration is more sensitive to AJs disruption compared to reorganization of Golgi/MTOC polarity, which only entails the internal reorganization of cellular components. Remarkably, when we increased VE-cadherin KD efficiency using double siRNA, protein levels only differed ~5% compared to the single siRNA transfection (Figure IV.7A and Figure IV.7B) and, in this last case, ECs were still able to maintain relatively normal collective cell polarization (Figure IV.7C). These results, on the other hand, suggest that the threshold required for correct Golgi positioning in the absence of VE-cadherin in ECs is actually relatively low, between 2%-7% of protein expression levels.

Additionally, our results showed that when β-catenin was depleted there was a loss of collective cell polarization (Figure IV.3C), most likely caused by the destruction of AJs, rather than due to a

possible α-catenin/β-catenin cadherin-independent role. Altogether, these results indicate that our initial hypothesis that α-catenin could be involved in a cadherin-independent function was actually being camouflaged by a residual pool of cadherin and, to our current understanding, does not exist in ECs. However, what is the biological relevance of this specific cadherin-independent function of α-catenin in neural crest cells is still unknown. Neural crest cells migrate collectively only after undergoing epithelial-to-mesenchymal transition (EMT). In contrast, ECs have a more classic collective migration behavior, remaining epithelial in nature throughout the entire process. Usually, collective cell migration studies focus on tissues where cell-cell and cell-matrix adhesions play a major role in collective organization (Szabó et al., 2016). However, and contrarily to those systems, collective migration of mesenchymal cells is associated with independent cell movement and relies on other modes of cell interaction, such as co-attraction and contact inhibition of locomotion (Szabó et al., 2016). Thus, future studies should evaluate whether this cadherin-independent function of α-catenin in cell polarity is a conserved feature among other cell types, or if, on the contrary, it is restricted to mesenchymal cells. Furthermore, even though it has been shown that, in humans, cancer cells invasiveness increases upon α-catenin depletion (Benjamin and Nelson, 2008), no studies addressed a potential cadherin-independent function in this pathologic scenario. Therefore, we are convinced that future studies addressing these issues could provide novel clues to design therapies to prevent cancer invasion.

On the other hand, in this study, we also uncovered that the Arp2/3 complex has a function on collective cell polarity after being recruited specifically to AJs by vinculin. Vinculin is one of the major components of FAs (see Introduction) and previous studies already showed this protein has a transient interaction stimulated by matrix adhesion and by growth factor treatment with the Arp2/3 complex (DeMali et al., 2002). Additionally, these studies reported that the interaction between these proteins only occurs at new sites of cell-matrix adhesion, allowing the attachment of this structure with the actin nucleating machinery that drives cell protrusions (DeMali et al., 2002). This interaction requires the simultaneous activation of vinculin that shifts to an open conformation and the Arp2/3 complex, to promote cell spreading and lamellipodia extension (DeMali et al., 2002). Moreover, Arp2/3 complex has also been described as a possible regulator of Golgi polarity organization and conservation during cell migration (Magdalena et al., 2003). Despite these findings, it has never been shown that this protein complex has a role in collective cell polarity associated with AJs and, consequently, mechanotransduction. Our data revealed that in the absence of vinculin, cells lose the ability to migrate and polarize correctly (Figure IV.8D and Figure IV.8E), which could be restored by overexpressing either vinculin full-length or an α-catenin-vinculin fusion protein (Figure IV.9). Since vinculin is also a key player in AJs and is essential to stabilize cell-cell adhesions to promote efficient mechanotransduction (see Introduction), our results are in agreement with previously described functions of Vinculin and support its relevance in cell polarity. Similar to vinculin depletion, KD of Actr3 – a subunit of the Arp2/3 complex – or pharmacological inhibition of Arp2/3 also led to randomized Golgi polarization (Figure IV.11). Curiously, when we attempted to restore cell polarity in vinculin-depleted HUVECs by overexpressing an α-catenin-vinculin fusion protein with a point mutation on the Arp2/3 binding domain of vinculin, we failed to rescue the phenotype, unlike the remaining constructs (Figure IV.12). Thus, we propose that

Arp2/3 complex is recruited to AJs by vinculin to play a role in Golgi polarization and CCM. Further experiments are required to investigate the spatial localization of the Arp2/3 complex and its colocalization with vinculin at AJs.

To determine whether the function of the vinculin-Arp2/3 complex in CCM and collective polarity was independent from FAs, we depleted talin1, one of the most important proteins of FAs. While we observed a reduction in the number of FAs by immunofluorescence using vinculin as a marker (Figure IV.10A), cell polarization remained unaffected in the absence of talin1 (Figure IV.10B). These results suggest that FAs seem to be dispensable for Golgi polarization and CCM, and thus vinculin's function in collective polarity is centralized at AJs. These results are still preliminary, because although the number of FAs in the absence of talin1 is much lower than the control there is still a significant amount of vinculin-positive FAs in cells (Figure IV.10A). This may be due to compensatory mechanisms mediated by talin2, another cell-matrix adhesion protein. Considering this, it is important to confirm the effects on cell polarity in the absence of both talin1 and talin2, or through other strategies, to completely abrogate the formation of FAs and eliminate their potential contribution to Golgi reorientation. Nevertheless, the ability of the α-catenin-vinculin fusion protein to rescue vinculin KD phenotype strongly supports the AJ centric hypothesis.

Previous studies already identified Arp2/3 complex as a regulator of F-actin assembly around AJs and that its inhibition in ECs leads to adhesion defects (Taha et al., 2014). Dynamic actin assembly via WAVE-Arp2/3 was related to junctional integrity, contractile tension and recruitment of myosin to junctional structures for stability of tension at AJs (Verma et al., 2012). Arp2/3 complex was also associated with efficient extension and formation of AJs mediated by N-WASP activation (Verma et al., 2004). Remarkably, different cadherin complexes have different actin organizations around junctions due to the activity of different nucleation promoting factors (NPFs), such as WAVE and N-WASP, which activate the Arp2/3 complex. Thus, this suggests that ECs should use a NPF to activate Arp2/3 complex for a proper mechanotransduction at AJs during CCM. Another important actin regulator that has been linked to junctional stability is non-muscle myosin II (NM-MII). Specifically, inhibition of its functions leads to junctional disassembly, and an abrogation of mechanotransduction properties (Shewan at al., 2005; Ivanov et al., 2007). Indeed, it is thought that RhoA-mediated NM-MII activation, and formation of contractile actomyosin cables, is important for vinculin recruitment and junctional strength (Yamada and Nelson, 2007). Yet, it is not clear how these two different actin organizations co-exist at adherens junctions and specific mechanisms leading to the recruitment and activation of the two key regulators of junctional dynamics, Arp2/3 and NM-MII.

A recent study found that AJs preferentially interact with branched actin networks (Efimova and Svitkina, 2018). Also, contrarily to early ideas that AJs are directly linked to actomyosin contractile networks, this study demonstrated that AJs interacts with NM-MII, a key component of actomyosin filaments, via non-contractile branched actin networks. In addition, contractile actin provides base-filaments for Arp2/3 complex-dependent nucleation and also physically cooperates with branched actin networks to promote force transmission. Therefore, Efimova and Svitkina propose that branched

networks have important functional implications in constantly pushing cells against each other at AJs. Considering these novel insights, it seems reasonable to assume that cadherin-dependent mechanotransduction should rely on a structural bridge between Arp2/3-dependent branched actin and actomyosin filaments. Thus, recruitment and activation of Arp2/3 to AJs would be essential for mechanotransduction during CCM. Given the mechanosensitive role of vinculin at AJs and its known Arp2/3-binding activity, we hypothesize that vinculin could be important for Arp2/3 recruitment to AJs. Our results points to that direction, and provides the first mechanistic insight into a molecular pathway regulating the formation of branched actin at AJs.

Considering our results, we propose a novel model for the role of vinculin and Arp2/3 complex mechanotransduction in CCM. In low force transmission, AJs are not challenged, and thus α-catenin remains in a closed conformation, preventing the recruitment of vinculin and, consequently, the recruitment of the Arp2/3 complex to AJs. On the other hand, when force is transmitted between cells, vinculin is recruited to AJs via mechanical opening of α-catenin, and concomitant exposure of the vinculin-binding site, which will recruit Arp2/3 complex to cell-cell adhesions. Arp2/3 activity will promote the formation of branched actin that is required for efficient mechanotransduction (Figure V.1). However, more studies investigating Arp2/3 complex activation and interaction with AJs are needed to further clarify this pathway. Finally, it would be interesting to evaluate if this mechanism involving Arp2/3 complex could be preserved and relevant for cadherin-independent mechanotransduction present in neural crest cells.

The relationship between mechanical and biochemical signaling are not yet fully understood in many developmental systems. For example, cilia from multiciliated cells of mucociliary epithelia are known to acquire planar polarity due to flow-induced shear forces (Mitchell et al., 2007). Another example is the inner and outer positions of the embryonic blastocyst that appears to be determined by cell fate specification due to the connection between cell positioning and differential cell contractility (Maître et al., 2016). However, in both examples, involvement of authentic mechanosensing and transduction still requires corroboration. Moreover, in recent years, mechanobiology-inspired anticancer drugs have been developed, targeting mechanosensing components and transduction signaling pathways (e.g., integrins, FAK and contractile machinery) (Chaudhuri et al., 2018). However, pathological mechanosensing and mechanotransduction mechanisms still warrant further investigation. For example, cancer cells acquire resistance against mechanical cues, but the mechanisms underlying this ability remain to be comprehended and could facilitate the development of new mechanobiology-inspired therapies for cancer treatment. Additionally, reproducing *in vitro* the mechanical complexity of the *in vivo* microenvironment could potentially improve the current mechanobiology-inspired therapeutic strategies and also develop new ones (Chaudhuri et al., 2018). Thus, several mechanisms remain uncovered in the mechanobiology field that ought to be addressed in future studies. We strongly believe that finding these unknown mechanisms will bring novel insights to both embryo development and pathologic conditions, such as cancer invasion.

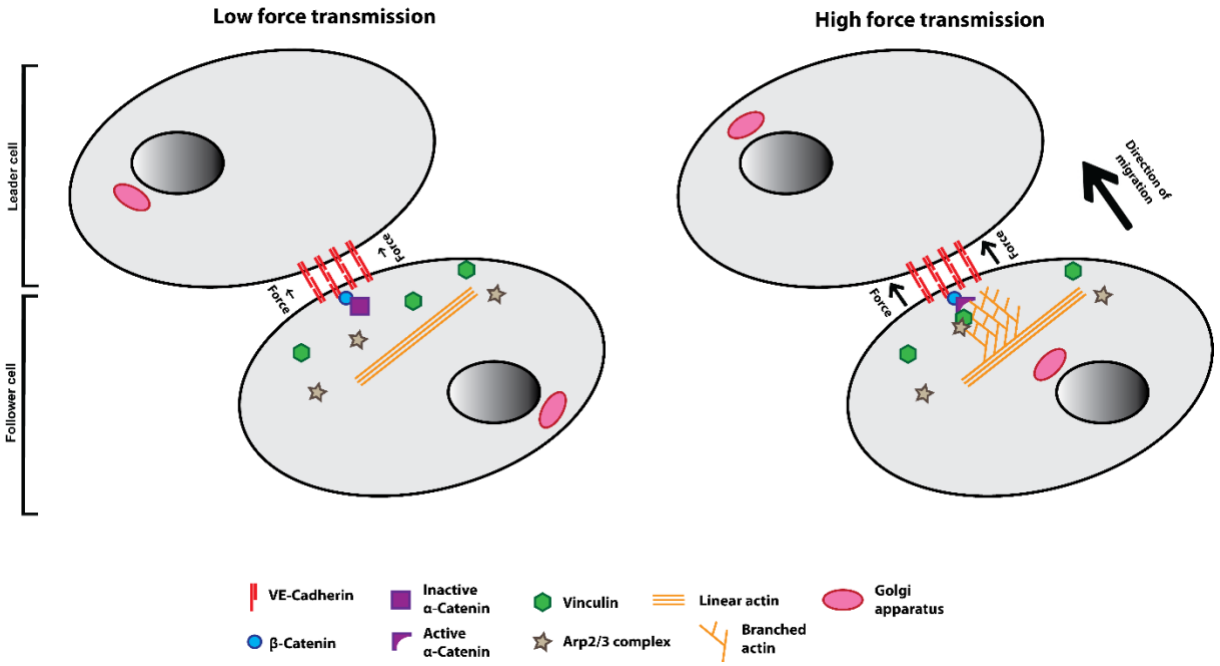


Figure V.1- Working model for the role of Arp2/3 complex in mechanotransduction. When a symmetric breaking event occurs, traction forces are generated with the ECM and propagated between the cell cluster. This will stimulate the recruitment of vinculin to AJs through the mechanical activation of α-catenin. Arp2/3 complex is then recruited to AJs by vinculin and promote the formation of branched actin which will mechanocouple with the actomysion. This ligation of the cadherin-complex with the actomyosin via branched actin seems to be essential for a proper mechanotransduction.

VI. References

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