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Vinculin-Arp2/3 interaction antagonises branched actin to control single and collective cell behaviours

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Thèse de doctorat



Vinculin-Arp2/3 interaction antagonises branched actin to control single and collective cell behaviours

Thèse de doctorat de l'Institut Polytechnique de Paris
préparée à École polytechnique

École doctorale n°626 École doctorale de l'Institut Polytechnique de Paris, (EDIPP)
Spécialité de doctorat : Biologie Cellulaire

Thèse présentée et soutenue à Palaiseau, le 30-08-2023, par

JOHN JAMES

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Abstract

Development, growth and maintenance of tissues are emergent properties arising from individual cell behaviour. Cell behaviour is finely tuned by a multitude of regulatory pathways in response to stimuli received from their microenvironment. Physical forces are sensed at cell-substrate contacts called focal adhesions (FAs) and cell-cell contacts called adherens junctions (AJs) which connect the exterior of a cell to its actin cytoskeleton. In response to force sensing, the actin cytoskeleton is remodelled to regulate complex cell behaviours such as proliferation, migration and cell-junction maintenance that are under the control of branched actin. During cancer progression, these three processes are deregulated. Vinculin (VCL), described as a tumour suppressor, is a structural and mechanotransductory protein present in both FAs and AJs. In addition to reinforcing the link between the actin cytoskeleton and adhesive structures, VCL is likely to play a second regulatory role on the actin cytoskeleton by interacting with the branched actin nucleator Arp2/3. The goal of this project was to determine the effects of VCL on branched actin, and ultimately cell behaviour. To this end, we used CRISPR-Cas9 genome editing techniques to perturb this interaction.

We began studying the effects of the interaction on individual cell behaviour by expressing a VCL 811-881 peptide in Parental MCF10A cells. We found that the peptide binds the canonical Arp2/3 complex, and cells expressing the peptide are able to migrate more persistently, spread over a larger area, make larger lamellipodia and continue to proliferate at high cell densities. All these phenotypes indicate that Arp2/3 activity is increased in these cells. VCL knockout (VCL^{-/-}) cell lines and a mutant cell line where VCL cannot bind Arp2/3 (VCL P878A-KI) both behave similarly. TIRF-SIM imaging revealed that the actin network assembly rate was increased in VCL^{-/-}, VCL P878A-KI and VCL 811-881 expressing cells compared to Parental MCF10A. Together, this demonstrates that the function of the VCL-Arp2/3 interaction is to antagonize generation of branched actin networks in the lamellipodium, and that the VCL 811-881 peptide acts as a dominant negative of VCL function.

To understand the role of the VCL-Arp2/3 interaction on collective cell behaviour, we

first studied AJs which not only hold cells together but also allow them to pass on mechanical signals. We found that the VCL controls Arp2/3 recruitment to AJs and cell-cell junction stability. Once AJs were mature, Parental MCF10A cells were able to organise long-range transcellular actin networks coordinated across multiple cells. During hypotonic unjamming, these cells migrate collectively as domains constrained by the long-range actin network. VCL P878A cells are not constrained by any long-range transcellular actin network and migrate more collectively while VCL^{-/-} cells develop a very short-range actin network leading to reduced collective migration. Our results indicate that Arp2/3 activity has to be finely regulated at AJs by VCL to form this long range network and regulate collective migration.

Thus, we have established as a novel role for VCL in regulating the actin cytoskeleton through a direct interaction that antagonizes Arp2/3. Perturbation of this interaction leads to several phenotypes characteristic of cancer cells – increased proliferation, increased persistence of migration, perturbation of cell junctions and misregulation of collective migration. Since Arp2/3 activity is known to be upregulated in several cancer types, our results provide a potential mechanism for vinculin's role as a tumour suppressor.

Summary in French

Le développement, la croissance et le maintien des tissus sont des propriétés découlant du comportement individuel des cellules. Ce comportement est finement contrôlé par de multiples voies de régulation en réponse aux stimuli environnementaux. Les forces sont détectées au niveau des adhésions focales (FAs), et des jonctions adhérentes (AJs), des structures d'adhérence qui relient l'environnement d'une cellule à son cytosquelette d'actine. En réponse à ces forces, le cytosquelette est remodelé et régule des comportements cellulaires complexes comme la prolifération, migration et maintien des jonctions cellulaires sous le contrôle de l'actine branchée. Ces processus sont dérégulés durant la progression des cancers. La vinculine (VCL), décrite comme suppresseur de tumeur, est une protéine structurale et mécanotransductrice aux FAs et les AJs. En plus de renforcer le lien entre le cytosquelette d'actine et les structures d'adhérence, VCL pourrait jouer un rôle dans la régulation du cytosquelette d'actine en interagissant avec Arp2/3, le nucléateur d'actine branchée. Le but de ce projet était de déterminer les effets de VCL sur l'actine branchée et le comportement des cellules. Pour cela, nous avons utilisé des techniques d'édition du génome afin de perturber cette interaction.

Nous avons étudié les effets de l'interaction VCL-Arp2/3 sur le comportement cellulaire individuel en exprimant un peptide VCL 811-881 dans les cellules parentales MCF10A. Nous avons constaté que ce peptide se lie au complexe Arp2/3 canonique, et que les cellules l'exprimant sont capables de migrer de manière plus persistante, de s'étendre sur une plus grande surface, de former des lamellipodes plus grands et de continuer à proliférer à de fortes densités cellulaires. Ces phénotypes indiquent une activité d'Arp2/3 augmentée. On retrouve ces phénotypes avec des lignées cellulaires KO de VCL (VCL^{-/-}) et une lignée cellulaire mutante dans laquelle VCL ne se lie pas à Arp2/3 (VCL P878A-KI). L'imagerie TIRF-SIM a révélé que la vitesse d'assemblage du réseau d'actine est augmentée dans les cellules VCL^{-/-}, VCL P878A-KI ou exprimant le peptide VCL 811-881 par rapport aux cellules parentales MCF10A. Ensemble, ces résultats démontrent que l'interaction VCL-Arp2/3 antagonise la formation des réseaux d'actine branchée dans le lamellipode, et que VCL 811-881 agit comme un dominant

négatif de VCL.

Pour comprendre le rôle de l'interaction VCL-Arp2/3 sur les comportements cellulaires collectifs, nous avons d'abord étudié les AJs qui maintiennent les cellules ensemble et leur permettent de transmettre des signaux mécaniques. Nous avons trouvé que VCL contrôle le recrutement d'Arp2/3 aux AJs et la stabilité des jonctions cellule-cellule. Une fois les AJs matures, les cellules parentales MCF10A peuvent organiser des réseaux d'actine transcellulaires sur de longues distances et coordonnés entre plusieurs cellules. En condition d'unjamming hypotonique, ces cellules migrent collectivement au sein de domaines restreints par ce réseau d'actine transcellulaire. Les cellules VCL P878A ne développent pas ce réseau et migrent de manière plus collective, alors que les cellules VCL^{-/-} migrent moins collectivement et développent un réseau à très courte distance. Nos résultats indiquent donc que l'activité d'Arp2/3 doit être finement régulée aux AJs pour former ce réseau et contrôler les migrations collectives des cellules.

Ainsi, nous montrons un nouveau rôle pour VCL dans la régulation de l'actine branchée par son interaction directe qui antagonise Arp2/3. La perturbation de cette interaction entraîne plusieurs phénotypes caractéristiques des cellules cancéreuses : une prolifération et persistance de migration accrue, une perturbation des jonctions cellulaires et une dérégulation des migrations collectives. Étant donné que l'activité d'Arp2/3 est surexprimée dans plusieurs types de cancer, nos résultats expliquent comment VCL pourrait agir comme suppresseur de tumeur.

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1 Introduction

1.1 Cell behaviour in tissue growth and maintenance

Over the course of a lifetime, each of our organs has to be grown and be maintained. We all start from single celled embryo which divides to give rise to all the cells that comprise our body. During development these cells differentiate and migrate to organise the different tissues in the right parts of the body. In a fully grown organism, these tissues are maintained over decades, by continuous cycles of proliferation, migration and death. Ultimately all these processes are accomplished by individual cells behaving cooperatively to give rise to the emergent properties of tissues.

A well studied example of tissue maintenance arising out of cell behaviour is the epithelial layer of the small intestine. Cells are arranged in crypts and villi with stem cells in the crypt proliferating continuously to replenish the tissue. The daughter cells differentiate and migrate towards the apex of villi where cell adhesions are disassembled and cells extrude out of the tissue (Fig1.1). These processes are regulated by a gradients of different signalling pathways[1] across the tissue and can even be perturbed by applying external mechanical forces on the tissue[2].

Collective regulation of individual cells can be achieved either through biochemical or mechanical signalling. Cells generate signals by secreting diffusible molecules, expressing surface ligands or by applying forces on their surroundings. Surrounding cells can then regulate their behaviour by sensing and responding to these signals. For instance, EGF is growth factor which is secreted at the intestinal crypt and it induces proliferation in stem cells which express the EGF receptor[1]. On the other hand, increase in tissue stiffness can lead to changes in cell behaviour like increased proliferation and migration[4].

In this section we will look at 3 essential processes for tissue maintenance - prolifer-

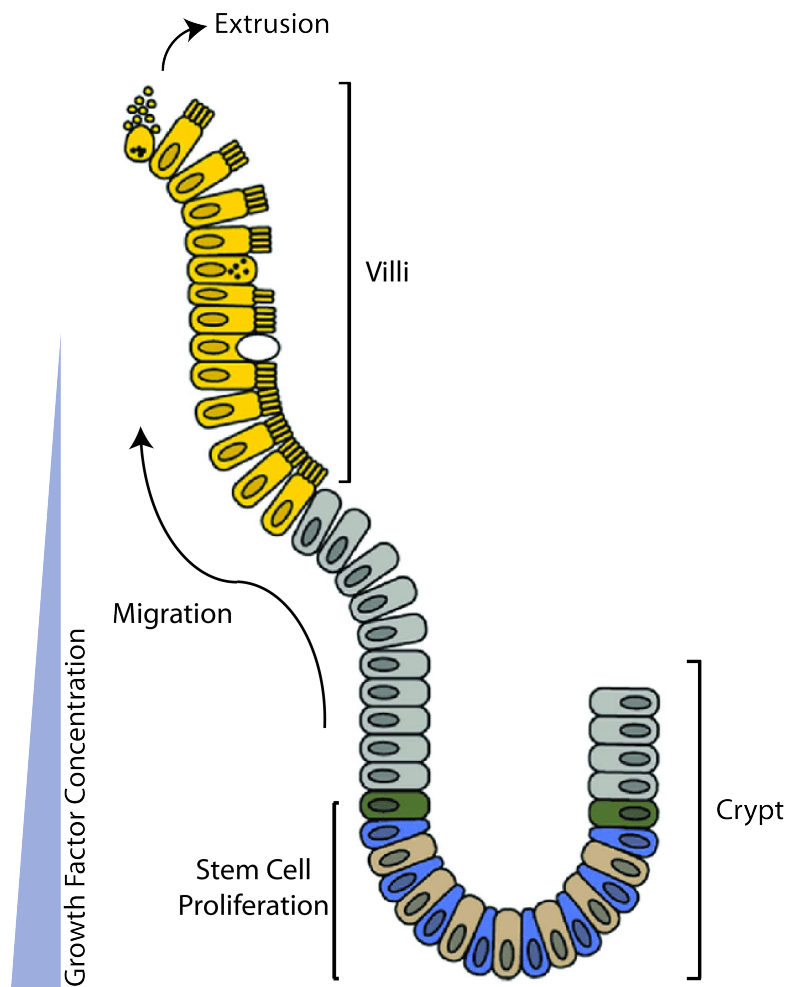


Figure 1.1: Tissue Maintenance in Small Intestine - Tissues are maintained by continuous cycles of proliferation migration and cell death. In the small intestine stem cells in crypts proliferate, daughter cells migrate to the villi and at the apex disassemble adhesions and extrude from the tissue. Adapted from [3]

ation which gives rise to the constituent cells of the tissue, migration which assures these cells arrive at the place they should be, and cell adhesions which hold all of this together. We will also look at some reductive model systems which allow us to study the minutia of their regulation.

1.1.1 Proliferation

The human body comprising around $3 \cdot 10^{13}$ cells, develops from a single-celled embryo. It is estimated that 10^{16} cell division events take place of the course of a human life. After cleavage of the embryo, the daughter cells continue to grow and divide

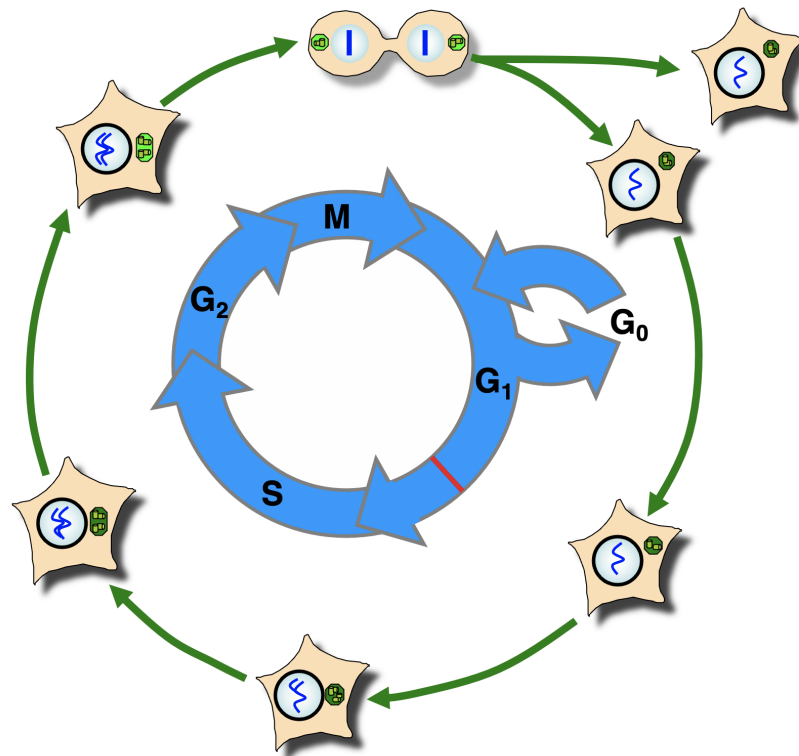


Figure 1.2: Cell Cycle Progression - Cells proliferate by going through the phases of the cell cycle. The G₁-S checkpoint is particularly important for cells to decide to reenter into the cell cycle and begin to divide. Adapted from [7]

giving rise to exponential proliferation. As the cells proliferate, they progressively differentiate and give rise to the different types of cells in the body. This continues in the developing organism as tissues grow until cells differentiate terminally and lose the ability to divide[5].

Proliferation of cells is regulated by the cell cycle [6](Fig1.2). In preparation to divide, a cell first synthesises the proteins and nucleotides required for DNA replication during a growth phase(G₁). The cell then replicates its DNA in the synthesis(S) phase after which it undergoes another growth phase(G₂) during which it creates the machinery required to divide. Finally the cell divides into 2 during the mitosis(M) phase and the daughter cells can either restart the cycle or enter a period of relative homeostasis called the G₀ phase. From this G₀ phase, cell can reenter the G₁ phase by to repeat the cycle of division or alternatively, they may experience senescence and die.

Progression through the cell cycle is controlled by different cyclin proteins which are expressed at different stages of the cell cycle[8]. Each cyclin can bind to its respective

cyclin dependent kinase which phosphorylates other proteins required to progress the cell cycle. Transitions between cycle phases are tightly controlled by molecular checkpoints, to coordinate the process across the entire cell. For instance, passing the G1-S checkpoint is essential for the cell to reenter into the cell cycle and replication of DNA. Cells cross the G1-S checkpoint only in optimal conditions for proliferation, like presence of growth factors and availability of space[9]. The G2-M transition is another checkpoint that is only passed when all the DNA in a cell has been replicated[10]. This checkpoint can prevent a loss of large portions of DNA in a daughter cell.

Cell lines in culture are often immortalised to allow them to continue proliferating indefinitely. These cells only stop dividing once they have filled the space available to them. This arrest of proliferation when they are surrounded by other cells was termed contact inhibition of proliferation. Contact inhibition, as the name suggests is regulated by cell-cell contacts but can also be dependent on environmental cues like growth factor concentration[11]. This collective regulation of proliferation is referred to as density dependent control of proliferation.

More recent studies have aimed to understand the coupled processes of proliferation and differentiation in tissue formation by growing organoids. For instance, the breast cell line MCF10A when grown in appropriate conditions of growth factor, ECM availability and substrate stiffness forms acini which are a model system for the mammary gland[12]. A single cell can proliferate and differentiate to create this multi-cellular spherical structure. Remarkably, cells in these organoids show properties of cells in tissues like deposition of basement membrane proteins, cell death to form an empty lumen and development of apico-basal polarity[13].

Misregulated proliferation can lead to diseases most common among which is cancer. A tumor is formed when cells disregard environmental cues and start proliferating uncontrollably. This perturbation of signalling pathways can occur multiple ways[14]. For instance, upregulation of an oncogene or even a mutation in a tumor-suppressor gene which renders it inactive can lead to indiscriminate proliferation. Cancer progression is usually a multi-hit process, driven by perturbation in a series of pathways. Once a tumour is formed cancerous cells can then secrete growth factors to induce growth of blood vessels in the surrounding tissue[15]. Eventually cells from the tumor can invade into these blood vessel and move to different parts of the body in process termed metastasis. Another essential process, misregulation of which leads to metastasis is cell migration.

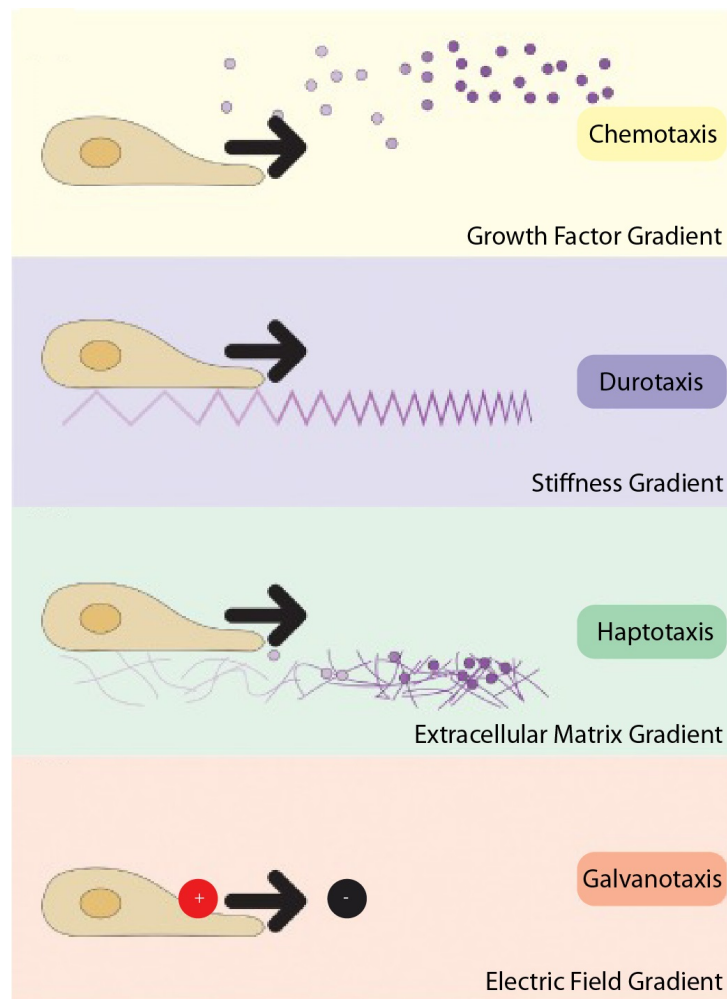


Figure 1.3: Directional Cell Migration - Cells orient their movement using migratory cues like growth factor concentration, stiffness of the substrate, extracellular matrix composition and electric field. Adapted from [17]

1.1.2 Cell migration

Cell migration is important for many processes in development and tissue homeostasis. Cell migration begins with the creation of a protrusion at the cell membrane to move the cell forward. There are two main types of cell migration that exist, which differ in the mode of formation of the protrusion. Amoeboid cell migration involves the formation of blebs due to hydrostatic pressure from the cytoplasm [16], while mesenchymal migration utilizes pushing forces created by actin polymerisation to create lamellipodia or filopodia. In the latter case, once the protrusions are formed, they attach to the substrate and cells utilize these adhesions as anchor points to pull themselves forward.

Individual cell migration is seen both in developing and mature tissues. For instance, during development neural crest cells differentiate and migrate to different part of the body to become melanocytes[18]. An example of individual cell migration in mature tissues can be observed in the immune system. Leukocytes express surface receptors which allow them to recognise gradients of diffuse signalling molecules like chemokines[19]. This allows them to hone in on the sites of inflammation in the body. Similarly, cells at a wound secrete growth factors which can be sensed by fibroblasts to hone in to sites of injury[20]. These fibroblasts then proliferate and secrete extracellular matrix proteins to facilitate wound healing.

In culture, individual cells tend to migrate randomly, but they can become highly directional in response to gradients in the environment. The directionality of migration can be controlled by both biochemical as well as mechanical cues. As we have seen, cells tend to move towards a gradient of signalling molecules in a process called chemotaxis. These gradients can be in the form of diffuse molecules which attach to receptors on the cell surface, or molecules of the extracellular matrix which guide migrating cells by contact in a process term haptotaxis[21]. Cells in culture also move on gradients of stiffness to conditions optimal for their respective tissues[22] and even respond an external electrical field[23]. Whether these processes, termed durotaxis and galvanotaxis respectively, are relevant to cell migration in-vivo is yet to be established (Fig 1.3).

Collective migration involves a group of cells moving together, often held together by cell junctions. As most cells exist as collectives attached to and surrounded by other cells, this process is both common and essential. Collective migration plays a critical role during development of the embryo to bring cells to the correct position they have to occupy for normal development. The most well studied of these is during gastrulation when migration of cell collectives allows an unordered embryo to differentiate into three ordered layers. During this migration, cells are ordered by physical forces applied by follower cells on leaders[24]. Cells in mature tissues as well are constantly moving collectively. In addition to the aforementioned example of the small intestine, collective migration has been observed in tissues such as egg chamber in *Drosophila* where live imaging is feasible[25].

Wound healing experiments have allowed us to study in vitro, how cells coordinate collective migration. It is not only the cells which directly perceive the wound that migrate into the available space. Even cells behind the leading edge create cryptic lamellipodia under the cells ahead of them to migrate towards the wound[26]. This implies that cells pass on the information of an open wound to followers that do not

directly experience the space to migrate into- and indeed waves of activation of the migration promoter Erk have been observed emanating from the wound[27].

Since cells in the body are packed into tissues, jamming and unjamming of monolayers has been used a more physiologically relevant alternative to study collective migration[28]. Cells in culture divide and migrate until they fill the space available to them. This contact based inhibition of migration is called jamming and can be reversed by treating cells with different conditions including hypotonic medium. As cells unjam and begin moving again, their migration is collective, restricted within domains[29].

Unregulated migration during development can lead to a variety of disorders ranging from loss of viability of the embryo to neuronal migration disorders(NMD)[30]. Misregulation of migration in immune cells has been shown to contribute to the onset of arthritis[31] and uncontrolled migration from a tumor can lead to metastasis. For these cells to migrate across a tissue, they have to repeatedly form and break of adhesions to the extracellular matrix and adjacent cells.

1.1.3 Cell adhesions

Cell are attached to the substrate and to adjacent cells by cell adhesions. Cell substrate adhesions utilise integrins whose extracellular domain binds to extracellular matrix proteins like fibronectin, collagen and laminin. The intracellular domain of integrins can then bind to several molecules which can serve as structural links or signalling molecules[32]. Cell-substrate adhesions can be generally classified into 2 types - hemidesmosomes which connect the adhesion to intermediate filaments in the cell[33] and focal adhesions which connect to the actin cytoskeleton. These adhesions differ in their isoform of integrins and the constituent proteins that are recruited downstream.

Cell-cell adhesions, called cell junctions connect adjacent cells not only keeping the tissue intact but also allowing cells to communicate. Epithelial tissues contain 4 main types of cell junctions. Tight junctions at the apical surface of cells create a tight barrier preventing invasion of external particles (Fig 1.4). At tight junctions, the extracellular link between cell can be made by a variety of proteins most commonly occludins and claudins, whose intracellular domains bind to the ZO proteins which then link to the actin cytoskeleton[34]. A second type of cell junctions, gap junctions act as gates in the membrane between adjacent cells allowing for the passage of signalling molecules.

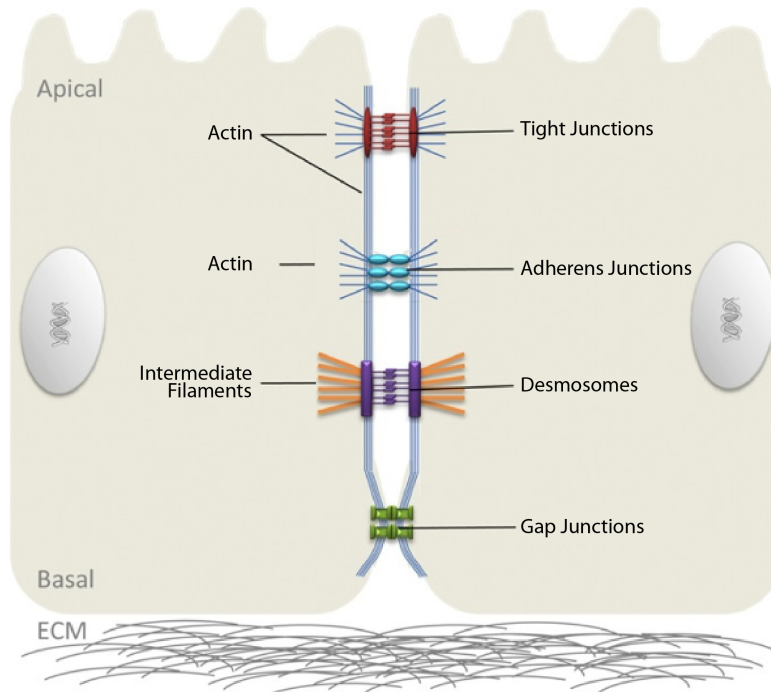


Figure 1.4: Types of Cell Junctions - Epithelial monolayers have four main types of cell junctions - tight junctions, adherens junctions, desmosomes and gap junctions. Adapted from [37]

Gap junctions are made by connexons which polymerise in a circular manner, to create a channel in the middle[35]. Desmosomes connect the intermediate filaments of adjacent cells[36] while adherens junctions connect their actin cytoskeleton.

Cell adhesions play a critical role both in maintaining structure of the tissue. They can become stronger when tension is increased across the junction, acting as catch bonds[38]. This is achieved through a combination of intrinsic properties of the chemical bonds between junctional proteins, as well as feedback loops that recruit junction strengthening proteins on application of tension by the actin cytoskeleton. Primary among these proteins is vinculin which can then recruit other junctional proteins through downstream signalling pathways. This role of vinculin, being a core part of the project, is explored in further detail in Section 1.3.3.

Cell adhesions also function as mechanotransductive complexes, converting mechanical signals to biochemical ones. Focal adhesions allow cells not only to sense the stiffness of the substrate but also to apply forces on the substrate which is required for processes such as migration. Cells also respond to the stiffness of the substrate by regulating processes like migration and proliferation[22]. Detached cells without adhesions tend to die in a process called anoikis[39]. Connecting the actin cytoskeleton of adjacent cells allows adherens junctions to sense and generate forces across the tissue. This mechanotransduction is important to establish collective behaviours of cells like density dependent control of proliferation. Contact inhibition of migration as well, is dependent on adherens junctions and downregulation of adherens junction proteins results in defects in wound healing[40].

The stability of adherens junctions also has an important role to play in tissue organisation. Loss of cell junction stability leads to an increase in cell junction rearrangement events, and the number of cell-contact rearrangement events has been shown to be a good measure of how jammed cell monolayers are[41]. The rearrangement of cell junctions has been proposed to be the driving force behind tissue remodelling during development[42]. For instance, increasing junctional tension in drosophila wings during development leads to larger size of the wing[43].

Genetic defects in cell adhesion proteins have been linked with a wide variety of disorders ranging from minor disorders of the skin[44] to schizophrenia[45]. The initial stages of metastasis during cancer progression also involve the breaking of cell junctions, before the metastatic cells can start to migrate. Cell adhesions are important structurally as well to regulate proliferation and migration to prevent cancer progression. All these processes, migration, proliferation and cell adhesion maintenance are controlled by the actin cytoskeleton.

1.2 The Actin Cytoskeleton in cell behaviour

The actin cytoskeleton is the scaffold that gives a cell its shape and allows it generate forces. Actin is ubiquitously expressed in every cell in the body. In fact, actin homologues make up the cytoskeleton of all organisms archaea to eukaryotes[46]. Reorganisation of the actin cytoskeleton is essential for processes like migration which require both a change in cell shape as well as the generation of physical force. Recent evidence shows that it even serves as a sensor for environment signals to signal for proliferation. In this section we will see how the see how the actin cytoskeleton is reorganised to give rise to cell behaviour.

1.2.1 Actin polymerisation

Actin is 42kDa globular protein. Actin has 3 main isoforms[47], α -actin expressed only in muscles, β -actin which is enriched in linear actin fibres and γ -actin which is seen diffuse in the cytoplasm. Actin monomers have a role to play in transcriptional regulation in complex with other proteins but we will focus here on its role in the cytoskeleton.

Globular actin(G-actin) monomers can polymerise to form long actin filaments(f-actin)[48]. G-actin can polymerise spontaneously at physiological conditions but most of the g-actin in the cell is bound by actin sequestering proteins which sterically inhibit polymerisation. Since actin dimers are unstable, the formation of a trimer becomes the limiting step for actin polymerisation to occur. Once the nucleus for polymerisation is formed, actin monomers can be added to either end to form long filaments. An actin filament is asymmetric having a barbed(+) and pointed(-) end with a difference in critical concentrations for polymerisation at the barbed and pointed end. Most g-actin is bound to ATP and hydrolysed to ADP once part of the filament. This hydrolysis is more efficient at the barbed end[49] further adding to the asymmetrical polymerisation of actin. Actin polymerisation being more efficient at the barbed end allows cells to grow actin filaments directionally (Fig 1.5). In-vivo, the initial nucleus for polymerisation is formed by nucleation factors which stabilise the actin dimer and elongation of the filament is regulated by elongation factors and capping proteins.

The localisation of nucleation factors defines the state of the actin cytoskeleton in the different compartments of the cell[50]. The two main nucleation factors which initiate actin filaments are formins and Arp2/3 (Fig 1.6). Formins are a family proteins which simultaneously bind actin and can form homodimers with their FH2 domains. This brings the 2 actin molecules together, stabilising the actin dimer to start linear actin

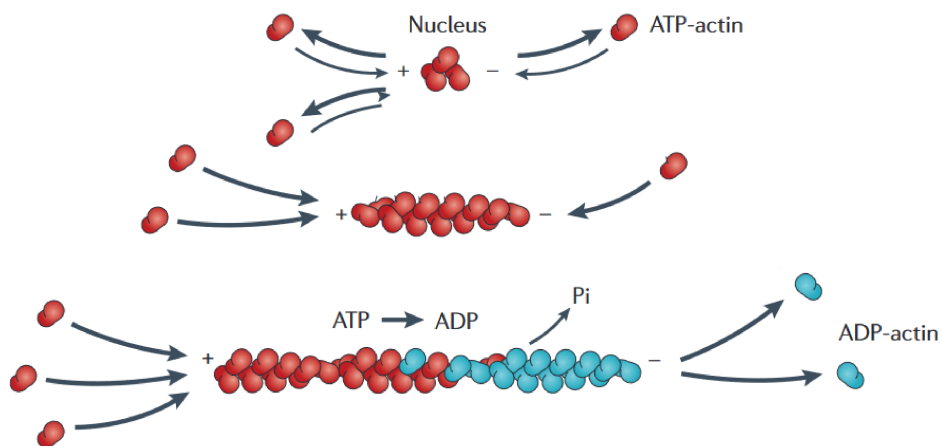


Figure 1.5: Spontaneous Actin Polymerisation: The limiting step of actin polymerisation is formation of the trimer. Once the trimer is formed actin monomer can be added to either end of the filament at different rates. Adapted from [53]

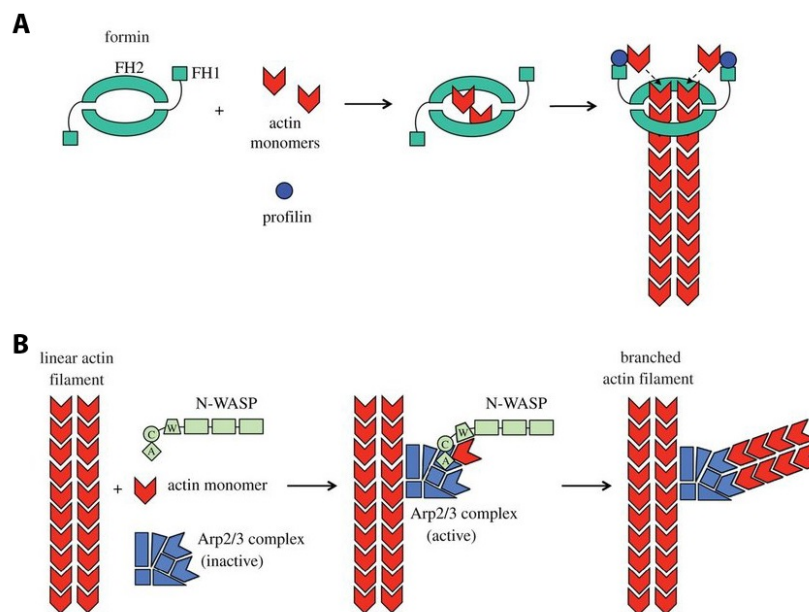


Figure 1.6: NF-mediated Actin Polymerisation - Cells nucleate actin network from nucleation factors. Formins can begin de-novo actin polymerisation(A) into linear filaments while Arp2/3 creates branches in an existing actin filament(B). Adapted from [54]

polymerisation[51]. On the other hand, Arp2/3 is a protein complex which binds to an existing actin filament and creates a branch. Its core subunits Arp2 and Arp3 mimic an actin dimer and the WH2 domain of associated proteins like N-WASP, delivers an actin monomer to initiate a branch[52]. The function and regulation of Arp2/3 being a core part of the project, is explored in further detail in Section 1.2.1.

Polymerisation, once initiated can be accelerated by elongation factors, which bind to actin filaments and have additional g-actin binding sites(WH2 motif) to deliver actin monomers to the polymerising filament. These include proteins of the ENA/Vasp family[55] and some formins(mDia)[56, 57]. F-actin severing proteins like cofilin can also promote polymerisation by providing a new barbed end to begin polymerisation[58]. Actin capping proteins can bind to the barbed end of an actin filament to prevent further polymerisation[59].

1.2.2 Arp2/3: Structure and Function

Arp2/3 is the protein complex a cell uses to create branched actin[60]. It is a 7 subunit complex, with 2 core subunits ARP2 and ARP3 which are structurally similar to actin. In the cytoplasm, Arp2/3 exists in an inactive state [61] where these subunits are held apart from each other. The other 5 regulatory subunits, ARPC 1-5 function to activate Arp2/3 in the right cellular context. ARP2 and ARP3 are structurally similar to actin and when ARP2/3 is in the activated conformation associated with an actin filament, they mimics a barbed end of an f-actin filament to which actin monomers can be added[52].

Branched actin networks are established by the activation of Arp2/3 and stabilised by cortactin (Fig 1.7). Activation of Arp2/3 is mediated by Nucleation Promoting Factors(NPFs). Cells have a number of NPFs each of which is present in a specific context where Arp2/3 activity is required. These include nWasp[62] and WAVE in lamellipodia, WASH at endosomes[63], and WHAMM [64] and JMY[65] at the golgi. NPFs bind Arp2/3 through their CA domains and create conformational changes in Arp2/3 for activation. NPFs also contain WH2 domains which deliver actin to Arp2/3 for polymerisation. Recent evidence has shown that the human Arp2/3 complex requires binding of 2 NPFs for activation. The first NPF binds to the ARP2-ARPC1 interface changing converting the complex into a "short-pitch" conformation of the complex to reveal the barbed end of ARP3. A second NPF can bind ARP3 and deliver an actin monomer to begin polymerisation[66]. The NPFs themselves are activated by unmasking of the WH2 motif- for instance, WAVE is activated in lamellipodia by Rac1

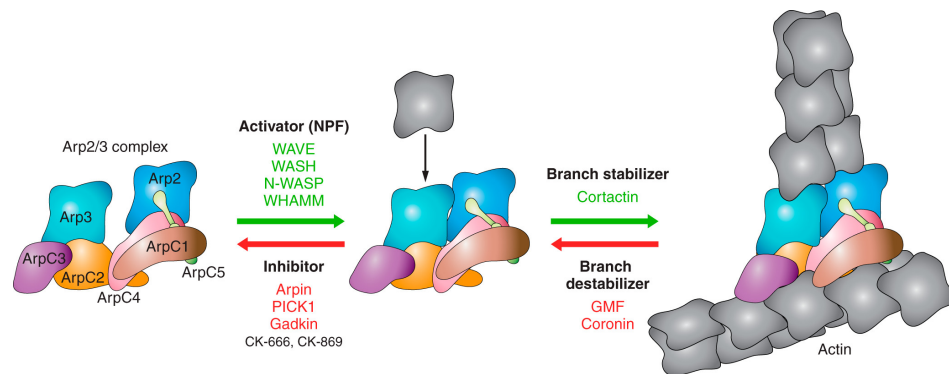


Figure 1.7: Arp2/3 Regulation - Branched actin networks can be upregulated by nucleation promoting factors and branch stabilisers or downregulated by Arp2/3 inhibitors and branch destabilisers. Figure from [77]

GTPase[67]. Once the branch has been created, cortactin binds Arp2/3 in a branch releasing the NPF[68] allowing for elongation[69, 70]. Cortactin remains bound to the Arp2/3 at the actin branch and prevents it from debranching[71].

On the other hand, downregulation of branched actin networks can be achieved by inhibiting Arp2/3 activators or by debranching existing networks (Fig 1.7). Arpin prevents WAVE activity by competitively binding to Arp2/3 in lamellipodia[72] and GADKIN[73] and PICK1[74] antagonise Arp2/3 at golgi and endosomes respectively. Coronin1B[75] has been shown to debranch actin networks in-vitro and can also inhibit Arp2/3-mediated nucleation[71] in lamellipodia. Branched actin networks can also be disassembled by cofilin-like proteins called GMFs which bind to Arp2 in a branch and lead to debranching[76].

Arp2/3 can also be regulated by subunit specificity and by post translational modifications of its subunits. ARPC1 and ARPC5 and ARP3 all have 2 isoforms. ARPC1B and ARPC5L polymerise actin faster in lamellipodia[78] and these networks are better stabilised by cortactin than networks made by their isoforms ARPC1A and ARPC5. ArpC5L has been shown to impact the accumulation of the elongation factor MENA leading to differential lamellipodia morphology in ARPC5 and ARPC5L knockout cell lines[79]. The oxidation of Arp2/3 complexes that contain ARP3B and not ARP3 by MICAL2 is another protein which enhances branched actin network disassembly[80]. Phosphorylation of ARP2 at T237 and T238 by TNIK is essential for Arp2/3 activity [81] and phosphorylation of ARPC1B and ARP3 by pathogens has been also shown to decrease activity[82].

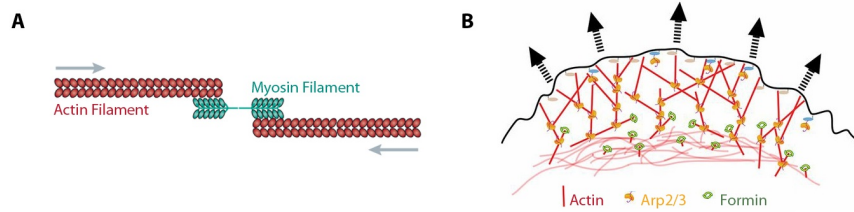


Figure 1.8: Linear and Branched Actin Networks - Contractile linear actomyosin bundles can create pulling force while polymerising actin in branched networks can create pushing forces. Adapted from [87, 89]

1.2.3 Branched and Linear Actin Networks

Branched actin networks are used by the cell to create pushing forces. Branched actin networks are generated by Arp2/3 which binds to an existing actin filament and initiates a new branch. Since the daughter filament can itself be a substrate for Arp2/3, this gives rise to an exponentially expanding network[83]. Polymerisation of actin onto these branches can then be supported by formins or Vasp. This rapid growth of the branched network can create pushing forces (Fig 1.8) essential for processes including lamellipodia formation[84], cell spreading and cell junction formation.

On the other hand, linear networks of actin can produce both pushing and pulling force. Linear networks of uniform polarity have + ends of individual filaments facing the same direction held together by actin bundling proteins like fascin. These fibres can create pushing forces by rapid polymerisation of actin in the presence of elongation factors like Vasp. These pushing forces are vital to the formation of filopodia[85] during normal cell migration as well as invadopodia during cancer progression[86].

Linear networks of actin made of anti-parallel actin fibres held together by myosin can create pulling forces[87]. These linear networks are of non-uniform polarity having the barbed ends of individual filaments facing opposite directions. Myosin dimers can attach to 2 actin filaments and walk in opposite direction along either filament to create a net contractile force across the bundle (Fig 1.8). Transverse arcs of these linear networks are found at the base of lamellipodia following the disassembly of branched networks by actin severing proteins cofilin and GMF and the subsequent capture of the resulting actin strands can be captured by bundling proteins like α -actinin and myosin[88]. Contractile acto myosin bundles are also found in stress fibres which create the contractile forces required to pull the cell forward during migration.

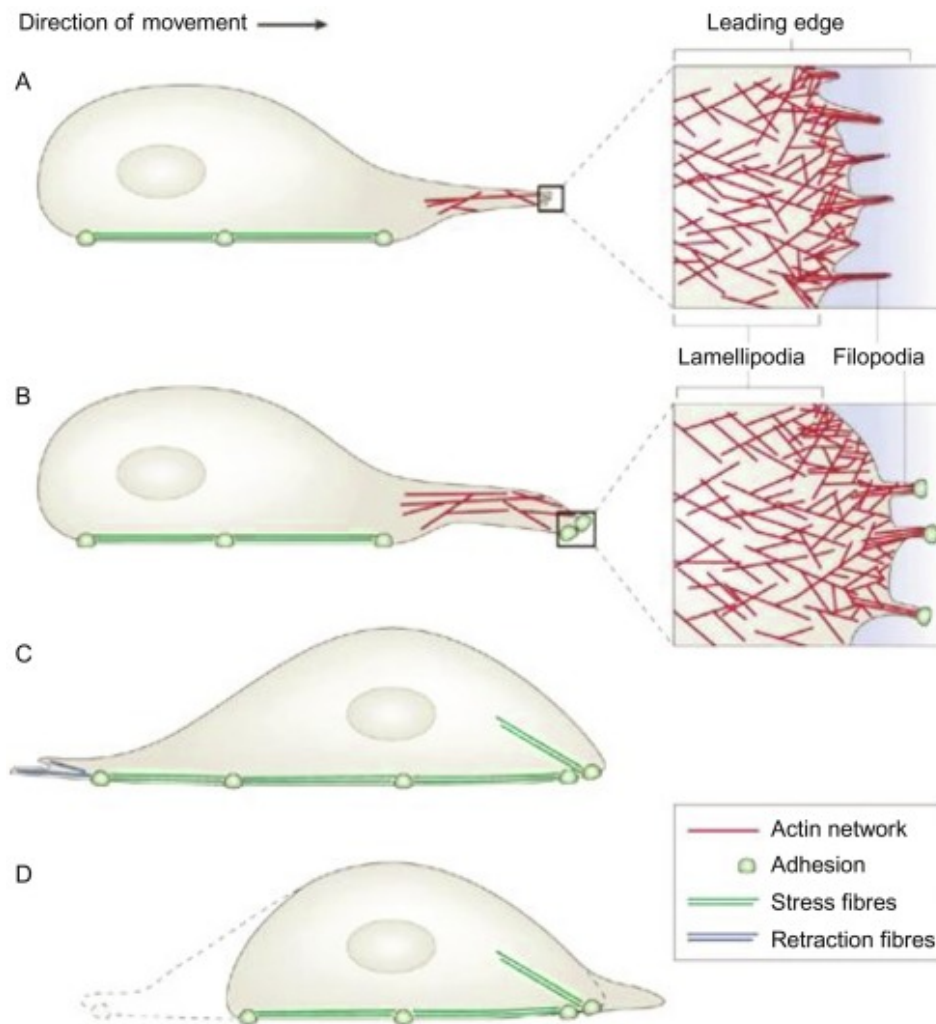


Figure 1.9: Single cell migration - Cells migrate by creating actin rich protrusions like lamellipodia and filopodia. Once these protrusion attach the actin cytoskeleton to its substrate, the cell can pull itself forward. Figure from [90]

1.2.4 Single Cell Migration

Individual cells migrate by creating protrusions in the plasma membrane, anchoring these protrusions to the substrate and then pulling themselves forward (Fig 1.9). This process requires generation of both pushing forces to create the initial protrusion as well as contractile forces to pull the cell. Both of these forces are generated by the actin cytoskeleton, the former by polymerisation and the latter by myosin induced contraction.

Migrating cells create finger like protrusion called filopodia, made by polymerisation

of linear actin bundles to feel the substrate surrounding them. They can then create wide protrusions called lamellipodia which are supported by Arp2/3-mediated branched actin networks to drive migration persistently towards the migratory cue. Migrating cells are polarised by increased RAC1 activity at the leading edge and increased RhoA activity at the retracting edge[91]. Lamellipodial protrusions are generated by activation of the Rac-Wave-Arp2/3 pathway towards the direction of migration while at the retracting edge, RhoA activity leads to the inhibition of Rac1 activity through the ROCK kinase. Protrusion formation and attachment to the substrate are intimately coupled at the leading edge, while RhoA contributes to the disassembly of adhesions at the retracting edge.

At the leading edge, Arp2/3 is activated by WAVE which localises to membrane in lamellipodia by interacting with a membrane lipid PIP3[92]. When the protrusion comes in contact with the substrate, transmembrane integrins attach to the extracellular matrix. Integrins can dimerise to cluster and recruit several proteins, including focal adhesion kinase (FAK), that together form nascent adhesions. WAVE is further activated by Rac which is in turn activated by phosphorylation by FAK[93]. Together this forms a positive feedback loop allowing for persistent polymerisation of actin, on adhesion to the substrate, and ultimately protrusion extension and cell migration. Arp2/3 activity has been consistently correlated with an increase persistence of cell migration. Inactivating Arpin, ArpC1A and CYFIP2, all negative regulators of cortical branched actin enhances persistence[94, 95], while downregulating ARPC2, ARPC1B[96] or HSBP1, a component of the Arp2/3 activating WASH complex, leads to a decrease in persistence. Regulation of the Arp2/3 complex has also been shown to be essential for haptotaxis[97] of cells in culture.

As actin continues to polymerise at the leading edge, the pressure against the cell membrane creates a flow of actin toward the back of the lamellipodia called retrograde flow. As nascent adhesions develop, they can hold actin bundles in places, slowing the retrograde flow, allowing for polymerisation push the membrane and extend the protrusion. This theory is called the molecular clutch[98] Fig(1.10), with nascent adhesions being the clutch connecting the force producing actin cytoskeleton to the integrin bound substrate. The retrograde flow then contributes to the rearrangement of branched actin networks into stress fibres. Actin polymerisation at focal adhesions by formins and Vasp also contribute to the formation of contractile stress fibres[88] that then allow the cell to pull the rest of itself along with the protrusion.

Regulation of protrusions occurs through regulation of Arp2/3, its NPF activators and its inhibitors. Cells treated with CK666, an Arp2/3 inhibitor[99] can no longer produce

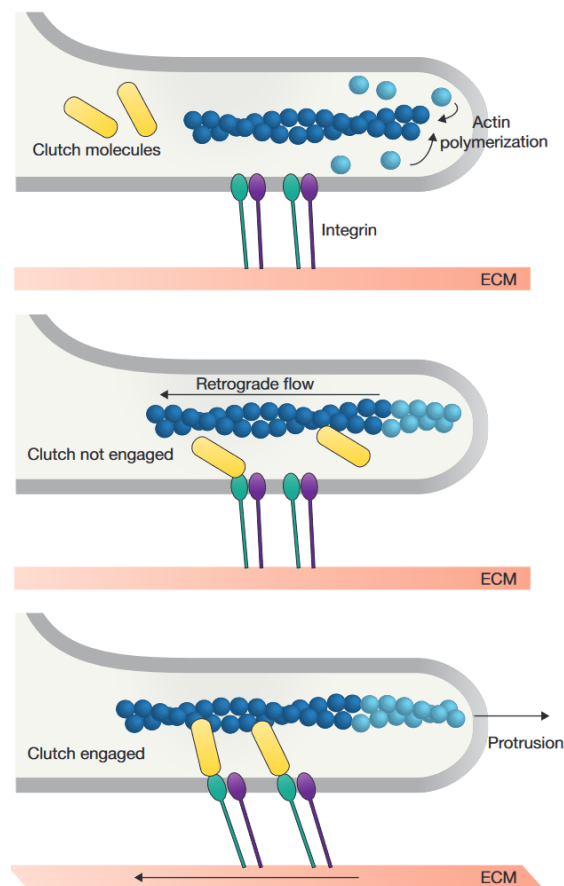


Figure 1.10: Molecular Clutch - Polymerisation of actin at the leading edge of the protrusion, pushing against the membrane leads to a retrograde flow of actin. Once the actin filament is attached to the substrate by the molecular clutch, actin polymerisation can push out the membrane to create a protrusion. Figure from [98]

lamellipodia and thus have defects in migration and wound closure. The Arp2/3 inhibitor Arpin is an inhibitor of cell migration[100]and without Arpin, lamellipodia protrude for longer, and as a consequence, sustain active directional migration. The myosin inhibitor blebbistatin also has inhibitory effects on migration and wound healing as cells cannot generate contractile forces to pull themselves forward[101]. Notably, several studies show that upregulation of branched networks through increased expression of Arp2/3 or its activators is associated with cancer progression in patients[77], potentially by allowing cells to migrate and metastasise. Additionally, one would expect that the coupling of polymerisation to attachment could be regulated by Arp2/3 interacting with nascent adhesion proteins such as vinculin. We will explore this possibility further in the results section.

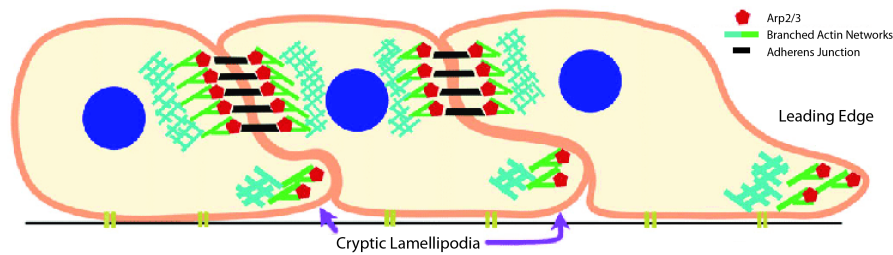


Figure 1.11: Dual role of Arp2/3 in collective migration - Arp2/3 contributes to collective migration not only by creating lamellipodia in cells at and behind the leading edge, but also by holding together cell junctions. Adapted from [102]

1.2.5 Collective Cell Migration

Collective migration combines two processes that involve the actin cytoskeleton- the migratory capabilities of each cell and the collective behaviours organised by cell junctions. Cell junctions contribute to collectivity by functioning as both a structural link between cells and a mechanism for signalling to control directionality of movement.

The actin cytoskeleton plays an important role in collective migration by contributing to the assembly and stability of these adherens junctions. Wound healing experiments in cell culture as well as in *Drosophila* wings have revealed a contractile actin band [103] at the leading edge organised by Rho activity [104]. This actin band is composed of actin bundles in each cell connected to each other at adherens junctions. This actin band constrains migration and cutting it by laser ablation has been shown to release a cell to become a leader cell [105]. These leaders can pull follower cells with them depending on the strength. Thus adherens junctions are important to structurally hold cells together and disrupting adherens junctions by depleting calcium ions from the medium results in a loss of collective migration [106]. In addition adherens junctions also contribute to collectivity by being mechanotransductive structures that direct migration. Knocking out the primary mechanosensor at adherens junctions, α -catenin, results in the loss of lamellipodia in follower cells [107]. Recent reports have shown a second mode of guidance, by follower cells. α -catenin based mechanosensing is important to maintain directionality of collective cell migration by allowing follower cells to guide their leaders [24] during zebrafish gastrulation. Several other cell junction proteins like PTEN, ZHFX3 [108] and Merlin [109] have been shown to coordinate collective migration as well.

The actin cytoskeleton is, of course, plays a second important role for collective migration due to its involvement in the migration of individual cells. Establishment of polarity of

migration relies on a gradient of increasing RAC1 activity at the protruding edge and increased RhoA activity at the retracting edge[110]. In wound healing experiments, cells at the leading edge create lamellipodia as they sense the area to migrate into. Although it is not entirely clear how the signal of the open wound is passed on, cells behind the wound create cryptic lamellipodia under the cells in front of them and migrate in the direction of the wound (Fig 1.11). Waves of increase in Erk activity, a promoter of cell migration, have been observed emanating from the wound across a large number of follower cells[27].

1.2.6 Cell-Cycle Progression

Cell migration and cell-proliferation seem to be inherently linked. Cells that migrate more persistently tend to transition into S phase more consistently. Recent work in our lab has shown that this link is mediated by branched actin networks[96]. Disrupting branched networks by downregulating Arp2/3 and Rac with inhibitors, and by decreasing Arp2/3 subunit expression through siRNA treatment, both result in decreased cell cycle progression. Overactivation of RAC1 using a P29S mutant has been shown to upregulate proliferation of cells in culture as well as in melanomas in mice[111]. On the other hand, treatment with siRNA against Arpin as well as expression of dominantly active mutant of Rac results in increased cell cycle progression. Thus branched actin acts as an integrator for biochemical and mechanical stimuli to signal the cell to pass the G1-S checkpoint. This signal is dependent on Coronin1B but its exact mechanism is still unclear.

Lamellopodin, another protein enriched in lamellipodia has also been implicated in this coupling. Lamellopodin stimulates cyclin expression and thus cell proliferation[112]. Additionally it has been reported that knocking down ARPC2 leads to DNA damage and premature mitotic exit[113]. Thus branched actin may also regulate cell proliferation through a role in mitotic spindle organisation perturbation of which leads to cellular senescence.

1.3 Vinculin: A mechanotransducer at cell adhesions

1.3.1 Focal Adhesion and Adherens Junctions

The actin cytoskeleton is attached to the environment at cell adhesions. At cell-matrix contacts actin fibres are connected to the extracellular matrix by focal adhesions while actin fibres of one cell are connected to actin fibres in another through adherens junctions. These structures are capable of mechanotransduction, allowing cells to sense and respond to the stiffness of the substrate or to forces applied by adjacent cells.

Focal adhesions are complex structures formed on maturation of nascent adhesions. At a nascent adhesion, transmembrane integrins bind to the extracellular matrix. The intracellular domain of integrins can bind talin, and talin can simultaneously bind to integrins as well as actin completing the structural core of the chain that connects the extracellular matrix to the cytoskeleton. The maturation of nascent adhesions is mediated by Rho which phosphorylates ROCK and Dia which promote actomyosin contractility[115]. Talin functions as a mechanotransducer as it experiences conformational changes when pulling forces are applied on it by the the actin cytoskeleton[116, 117]. A multitude of other proteins can then be recruited by these core components which contribute to maturation or disassembly of the adhesion[118].

Adherens junctions are similar structures at cell-cell contacts. At an adherens junction, cells are held together by dimerisation of extracellular domains of cadherins from adjacent cells. The intracellular domains of cadherins can bind β -catenin. β -catenin can bind to α -catenin which can then bind and actin to complete the connection between cytoskeletons of two cells[119]. α -catenin, like talin, functions as a mechanosensor as it is stretched by actin contractility[120, 121]. Again, this core can recruit a multitude

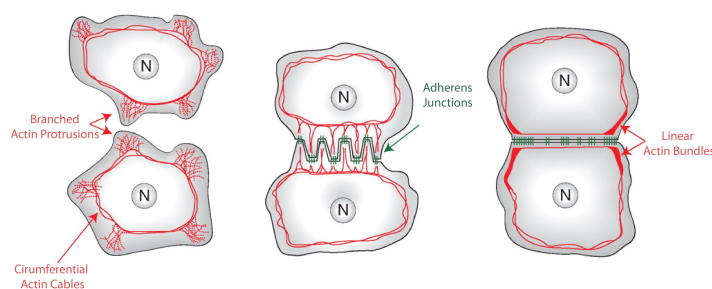


Figure 1.12: Adherens junction maturation - At early junction Arp2/3 mediated protrusion push against the junction to keep the cell membranes together. At mature junctions, linear actin bundles are arranged parallel to the junction. Adapted from [114]

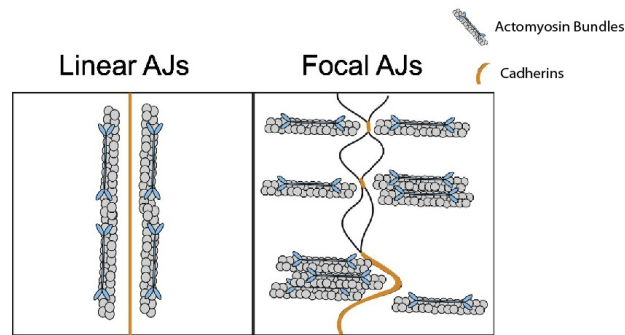


Figure 1.13: Types of adherens junctions - At classical adherens junction, actin bundles are arranged parallel to the surface of contact while at focal adherens junction, actin bundles are perpendicular to the cell-cell contact. Adapted from [128]

of proteins which are together called the cadhesome[122].

The actin cytoskeleton plays a critical role in the formation and maintenance of adherens junctions. Adherens junctions are initially formed by filopodia-like structures from either cell, interlocking with each other resulting in an actin network arranged perpendicular to the axis of the cell-cell contact (Fig 1.12)[114]. Creation of branched actin networks then pushes the cell membrane to fill in the area between these finger like projections. Arp2/3 recruitment is required to form stable adherens junctions[123] and cells expressing a dominant negative WCA domain which leads to downregulation of Arp2/3 activity are unable to form these junctions[124]. Downregulation of WAVE leads to a decrease in recruitment of E-cadherin[125] and a delay in the assembly of adherens junctions[126] as well. When the junction matures, most of this branched actin network is rearranged to form linear networks parallel to the junction (Fig 1.12). However recent evidence does suggest that Arp2/3 mediated branched actin networks continue to push against these mature junctions to keep them intact[127].

The distribution of adherens junction along a cell-cell contact can vary. The classical view is that adherens junctions are formed all along a cell-cell contact and the actin fibres are arranged parallel to the junctions. However, in contractile monolayers and in collectively migrating cells[129], the adherens junctions are punctate along the cell-cell contact and actin fibres are arranged perpendicular to the axis of contact (Fig 1.13). These junctions are called focal adherens junctions and share proteins several proteins like Mena and zyxin, with the focal adhesions[130].

1.3.2 Mechanosensing at cell adhesions

Focal adhesions and adherens junction are the key points of contact through which the actin cytoskeleton senses and applies forces on its surroundings. Mechanotransductory proteins convert these mechanical signals into biochemical signalling pathways that are initiated at cell adhesions. In response the actin cytoskeleton can be remodelled to control cell behaviour. Actomyosin contractility at cell adhesions has been shown to control various processes like migration and proliferation as well as reinforcement of the adhesion.

Density dependent regulation of cell proliferation is mediated through adherens junctions. This is achieved through activation of Hippo signalling[131], which is an essential tumor-suppressor pathway. Hippo signalling is mediated by YAP, a transcription factor which can increase proliferation and has been shown to cause tumors in mice[132]. At adherens junctions α -catenin forms a complex with YAP sequestering it away from the nucleus. Since cells have more adherens junctions at high cell densities, the amount of YAP in the nucleus is reduced leading to reduced proliferation.

The Wnt pathway is another signalling cascade that allows regulation of proliferation by adherens junctions[133]. β -catenin is a junctional protein that can also function as a transcription factor. At the junction, it interacts with E-cadherin and α -catenin simultaneously to form a structural part of the complex. In the nucleus, β -catenin functions as a member in the Wnt signalling pathway. In the absence of cell-cell adhesions, it localises in the nucleus to increase expression of genes like c-myc and cyclin D1 which increase proliferation. In fact, the Wnt/ β -catenin pathway is known as one of the drivers of EMT in cancer[134].

Focal adhesions can also have an effect on cell behaviour, as cells react differently on substrates of different stiffness. Cells tend to spread more and migrate faster on substrates of higher stiffness as the actin cytoskeleton is able to apply more contractile forces before the substrate deforms. Cells migrate toward regions of their optimal substrate stiffness in a process termed durotaxis. Durotaxis has been shown to be dependent on Arp2/3 mediated branched actin networks as well as actomyosin contractility[22]. Substrate sensing can effect the transcriptional profile of cells as well, thus regulating their behaviour. For instance, mechanosensitive expression of lamellipodin promotes cyclin expression and cell proliferation[112].

Vinculin is a protein that functions as a mechanotransducer at both focal adhesions and adherens junctions, since its recruitment by primary adhesion mechanosensors,

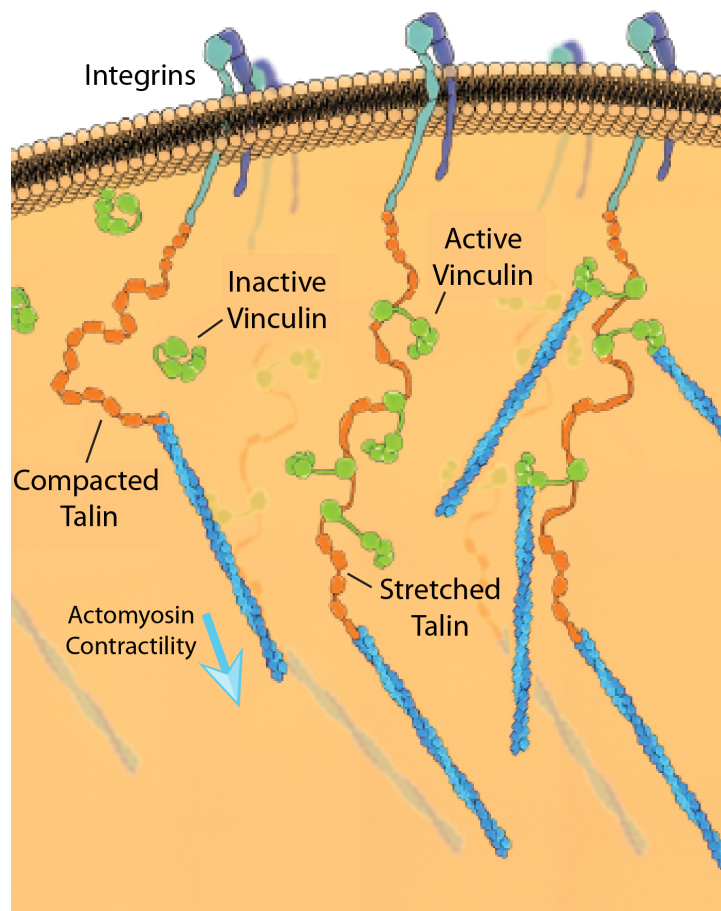


Figure 1.14: Vinculin is recruited to focal adhesions when a pulling force is applied to talin by the actin cytoskeleton. Vinculin can simultaneously bind talin and actin filaments, thus reinforcing the adhesion. Adapted from [135]

talins and α -catenin is force-dependent (Fig 1.14). In vitro studies have shown that forces applied by actomyosin contractility increase the vinculin-talin association[136]. Stretching of talin can reveal 3 cryptic vinculin binding sites that recruit vinculin to focal adhesions[116] and stretching the cell substratum has been shown to increase vinculin recruitment to mature focal adhesions[137]. Force-dependent conformational changes to α -catenin also promote its binding to vinculin[121] and vinculin recruitment to cell junctions has been shown to be dependent on both tension at the junction and myosin contractility[138, 139]. Thus, vinculin is the first protein recruited to focal adhesions and adherens junction in response to force.

1.3.3 Vinculin: Structure and Function

Vinculin is 116kDa protein which is very conserved across the animal kingdom[140]. In humans, it is expressed from unique gene with no homologues. Alternate splicing of this genes results in 2 main isoforms, 1 on which, metavinculin, is only expressed in smooth muscle. We will deal here with the more common isoform which is expressed in all cells[141]. Vinculin functions both as a structural link as well as a signalling hub conserved between both focal adhesions and adherens junctions. Its role and dynamics at focal adhesions are very well studied while its adherens junctions its function is less well understood.

In the cytoplasm, vinculin in cytoplasm exists in an auto-inhibited state with its head domain bound to its tail[142]. Vinculin is recruited to focal adhesions and is activated at the adhesion[143]. At the focal adhesions, the vinculin head domain binds to vinculin binding sites(VBS) in talin[144]/ α -actinin[145] and vinculin tail binds to actin[146]. Removing the physical link between the vinculin head and tail domains decreases the strength of focal adhesions despite both domains being recruited to the adhesion[147] and mutations that prevent the head-tail interaction increase the residency time of vinculin at the focal adhesion[148]. Thus activation of vinculin reinforces the link between cell adhesion and actin cytoskeleton to strengthen the adhesion (Fig 1.14).

It was first proposed that activation requires dual binding of the head and tail of vinculin to their respective ligands[149]. The vinculin tail has 2 actin binding sites[150] 1 of which is available in its closed conformation[151]. However, it seems that actin binding is not essential for activation since isolated VBSs from talin and α -actinin are sufficient to activate vinculin in-vitro[152]. Moreover, mutating the actin binding sites of the vinculin tail (I997A and V1001A), in vivo, did not have an effect on vinculin activation[153]. Concomitantly, binding of the vinculin tail to membrane lipid PIP2[154] promotes activation by increasing its affinity for binding to talin[155] but not actin[156].

The actin binding function of the vinculin tail plays an additional role in the maturation of focal adhesions by facilitating bundling of actin fibres. Once vinculin is in its active conformation, binding of the vinculin tail to actin promotes dimerisation of the tail domain[157] thus leading to bundling of actin filaments[158, 159]. In-vitro experiments have shown that vinculin can also reorganise branched actin filaments into linear bundles[160].

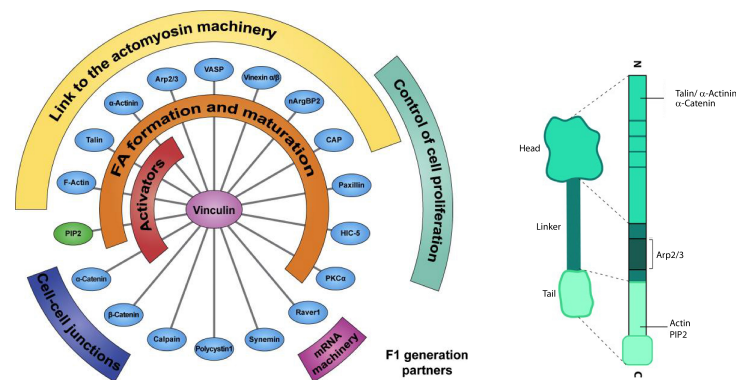


Figure 1.15: Vinculin is an adaptor molecule with 18 known binding partners implicated in several cellular processes. Not all of these interactions are well studied. A few of the interaction important to this study are highlighted here. Adapted from [161, 162]

In vitro, vinculin also has a direct effect on actin polymerisation. In vitro, high concentrations of the vinculin tail have been shown to initiate actin polymerisation[163]. Vinculin can cap actin filaments at the barbed end inhibiting polymerisation[164, 165]. Like other actin capping proteins, vinculin is capable of nucleating capped filaments of actin with a free pointed end for polymerisation[164] as well. Whether this function of vinculin has a role to play at focal adhesions is unclear.

Vinculin regulates focal adhesion maturation not only by serving as a structural link but also by functioning as signalling hub binding different proteins. Vinculin binding to talin keeps talin stretched leading to a feed back loop that strengthens the junctions[136]. The vinculin tail also binds paxillin[166] and the vinculin linker binds vinexin[167, 168] which are both both important for the maturation of focal adhesions.

Early evidence of vinculin's role at cell junctions comes from vinculin knockout mice which had abnormal intercalated disc in their heart[169]. Initially it seemed that α -catenin and vinculin may compete for the same binding site on β -catenin[170]. However, α -catenin deficient mice did not have vinculin in their intercalated discs[171] and it was later shown that α -catenin recruits vinculin to cell junctions in a force dependent manner[120, 121]. This vinculin- α -catenin interaction differs from its interaction with talin[172] and it has been shown that β -catenin was required to recruit vinculin[173]. Taken together it seems that vinculin is recruited to adherens junctions by a combination of α - and β -catenin. Other proteins like α -Actinin-4[138] and EPLIN[139] have been shown to be important for vinculin recruitment as well.

Vinculin plays an important structural role at cell junctions as well. It has been shown

that depletion of vinculin decreases E-cadherin-mediated adhesion strength[174] and that the perturbation of the α -catenin-vinculin interaction also leads to a decrease cadherin-mediated traction forces[175, 176]. Vinculin is required to maintain α -catenin at the junction and perturbation of the interaction leads to loss of stability of the junction as well decrease collectivity during migration[177]. vinculin- α -catenin interaction is required to maintain cell junction stability. Together, these studies make it clear that vinculin is essential for adherens junction strength and maturation. However, the mechanisms of how vinculin strengthens junctions are less well understood than its role at focal adhesion due to the fact that adherens junction form at the apical surface of cell monolayers and are thus more difficult to image.

Vinculin is also regulated by post-translational modifications. Phosphorylation of vinculin Y1065, mediated by Src Kinases, facilitates binding of talin[178] and F-actin[179], and so affects vinculin retention at focal adhesions[180, 181]. This in turn affects the transmission of contractile forces[178] and perturbs focal adhesion maturation[180], binding strength[179] and cell spreading[182]. At adherens junctions, forces applied to E-cadherin, activate Abelson tyrosine kinase to phosphorylate vinculin at Y822, which marks vinculin at adherens junctions but not focal adhesions[183]. Y822 phosphorylation can be reversed by SHF2 in a force dependent manner[184] and it has been hypothesised that it may promote activation of vinculin[185]. Interestingly, a Y822C mutation is common in uterine cancer and has been associated with differential binding of proteins like paxillin. Cells with this mutation proliferate more and migrate faster into a wound. A nonphosphorylatable mutant Y822F on the other hand increases focal adhesion formation, cell spreading and migration but has no effect on proliferation[186].

Vinculin functions as an adaptor protein capable of interacting with 18 different proteins (Fig 1.15). The functions of many of these interactions are not entire clearly. For instance, vinculin can bind Raver1 which is a protein that can bind in turn bind vinculin mRNA[187] suggesting a possible feedback loop to regulate vinculin transcription in response to substrate stiffness. Protein kinase c phosphorylates serines 1033 and 1045[188], and vinculin can bind other focal adhesion proteins like ponsin[189] and HIC5[190] but the functions of these interactions are not known. The most interesting for us, is the capability of the vinculin linker region to bind proteins that promote actin polymerisation. The elongation factor Vasp[191] as well as the branched actin nucleator Arp2/3[192] associate with the linker region but its effect on their activity is not known.

1.3.4 Relevance to Cancer

Mechanical properties of a tissue can influence cancer progression. Changes to the mechanical properties of tissues are brought about by the uncontrolled proliferation of cells. Unregulated growth of a cell mass can produce outward stresses leading to cells invading surrounding tissues[193], as well as inwards stress leading to ECM remodelling[194]. Together these give rise to a dense fibrous tissue which can further become a niche for generation of invasive cancer cells[195]. In fact, mammographic density is consistently associated with an increased risk of breast cancer[196] and tamoxifen, a breast cancer drug has been shown to reduce breast density[197]. Positive association between digestive tract tumors and cystic fibrosis [198] as well as pancreatic cancer and chronic pancreatitis[199], also suggest that perturbation of mechanical properties of tissues leads to cancer progression.

Tissues with altered mechanical properties are more prone to cancer since mechanosignalling of the constituent cells is altered. ECM stiffening by increasing collagen cross-linking was enough to induce the invasion of oncogene-initiated epithelium[200] in mice. Fibrosis also increased the invasive potential of premalignant breast cancer cell in mice through the Yap/Taz signalling[201]. Apart from ECM remodelling, intrinsic actomyosin contractility of a mass of cells can increase tissue stiffness and the exertion of pressure by surrounding cells can lead to β -catenin induced tumor growth[202, 203].

As we have seen, vinculin plays a key part in mechanotransduction at focal adhesions and adherens junction, and unsurprisingly it is found to be mutated in several cancers types. Vinculin mutations are present most commonly in epithelial tissues – 8.6% of uterine cancers, 6% of lung cancers, 3% of gastrointestinal cancers and 3% of skin cancers. Although vinculin has CNV loss in 83% of glioblastomas, it is unclear whether this is a driving mutation as these cancers have characteristic large deletions in chr10. Mutations in vinculin are relatively more common in metastasized tumours (5% compared to 1% in primary)[204, 205] suggesting that the roles of vinculin in cell migration and cell adhesion are disrupted during metastasis.

Vinculin was first reported as a potential tumor suppressor[206] in an initial study where cells transfected with antisense-vinculin cDNA displayed increased colony sizes in soft agar. Since then, numerous studies have explored phenotypes of cells in culture that point toward vinculin being a tumor-suppressor. Knockout of vinculin in fibroblast cell lines resulted in an increase in the number of cells migrating into a wound[169, 207, 208] and invading through pores in Boyden chamber assays[169].

Knockout cells showed a decrease in capability to attach to the substrate[169, 209] as well. Vinculin knockout cells were also shown to be less prone to cell death, specifically anoikis, which occurs when cells are not attached.[210]. Together these results show that vinculin decreases cell proliferation, increases attachment, decreases migration, decreases invasion and increases anoikis, all indicators that it is a tumor suppressor.

However, since different tissues have different mechanical properties and vinculin itself has several different functions, its role in cancer progression is likely tissue specific. Recent studies have shown that loss of vinculin is correlated with metastasis and poor prognosis in colorectal cancer[211]. In contrast, vinculin overexpression is correlated with negative prognosis in gastric[212] and ovarian[213] cancers. In support of this unexpected result, a couple of in-vitro reports [214, 215] do suggest that downregulation of vinculin leads to decreased speed and persistence of migration, which should prevent metastasis.

Notably, it has been reported that vinculin interacts with Arp2/3. As we have seen, Arp2/3 is a branched actin nucleator and it has been reported to be upregulated in several cancer types. The velocity of retrograde flow of actin in lamellipodia is increased in vinculin knockouts[216] suggesting that vinculin somehow upregulates actin polymerisation. This increase in actin polymerisation could explain the phenotypes of increased migration and proliferation observed earlier in vinculin knockouts. It is tempting to hypothesise that this vinculin-Arp2/3 interaction could explain consistently the tumor-suppressor role of vinculin.

1.3.5 Vinculin-Arp2/3 interaction

The vinculin-Arp2/3 interaction was first described in 2002[192]. Immunoprecipitating vinculin pulled down ARPC2 from platelet lysates. The interaction was increased in conditions associated with high Arp2/3 activity including EGF stimulation, fibronectin induced spreading of cells as well as expression of a constitutively active Rac mutant. Concomitantly, the interaction was abolished when cells were treated with an inhibitor of PI3K(a Rac-Wave-Arp2/3 activator) as well as when a dominant negative Rac mutant was expressed, together suggesting that vinculin binds to activated Arp2/3. The interaction was also increased in presence of PIP2 and abolished upon dephosphorylation of vinculin(Y100 and Y1065) [217] indicating that active vinculin was responsible for binding. The binding region on vinculin was narrowed down to a small peptide 850-881 in the linker region and 2 mutations P878A and P876A were shown to abolish binding.

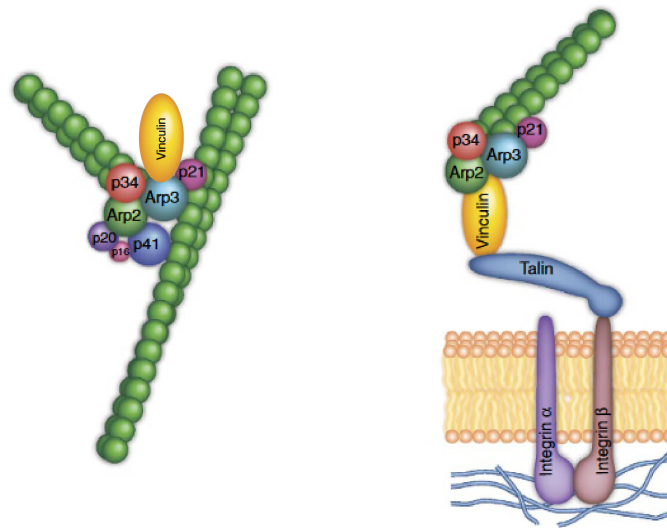


Figure 1.16: Proposed modes of the vinculin-Arp2/3 interaction - Vinculin was first shown to bind to activated Arp2/3. A second study found that vinculin binds to a subset of Arp2/3 subunits to form hybrid complexes. Adapted from [218]

The vinculin-Arp2/3 interaction was reported again in 2014[218] in lysates from chicken gizzard. Mass spectrometric analysis showed the presence of hybrid Arp2/3 complexes. Vinculin was associated with the subset of Arp2/3 subunits- ARP2, ARP3 and ARPC2 along with either α -actinin or ARPC3. Concomitantly, the amounts of the hybrid complexes pulled down was increased by that downregulating ARPC1B which is a subunit excluded from the hybrid complex. The authors speculate that this could be a mechanism for de-novo polymerisation of actin by ARP2/3, however the function of the complex is still unsolved.

In the initial study, the authors opine that the pools of Arp2/3 and vinculin bound to each other are likely very small compared to the total amounts of protein present in the cell. ARPC2 was found co-localised with vinculin only at early adhesions and not in mature focal adhesions. As a result, abolishing the interaction by expressing vinculin with a P878A mutation decreased cell spreading but did not have any obvious effect on either cell invasion or adhesion. As expected, the amounts of hybrid complex pulled down in the second study, were very low and they have a decreased stability compared to the canonical Arp2/3.

Together these results suggest a quite transient interaction between vinculin and Arp2/3 which has proved difficult to detect. As a result, no further investigations into

interaction have been reported, leaving several open questions about the structure and function of the complex. It is possible that the two studies analyse different pools of vinculin-Arp2/3 complexes (Fig 1.16) and it is unclear whether vinculin binds to Arp2/3 only to form the hybrid complex or whether it can bind canonical Arp2/3. It also remains unclear what effect the vinculin interaction has, on the ability of Arp2/3 to propagate branched actin networks in cells.

2 Objectives of the Thesis

As we have seen, the Arp2/3 complex and branched actin are vital for creating protrusions during migration and cell junction formation. Branched actin also serves as an integrator of environmental signals required for cells to proliferate. Vinculin is a protein essential for the attachment of these protrusions and for the maturation of adhesions both to the substrate and to other cells. These adhesions play a vital role in mechanosensing to regulate both migration and proliferation. A vinculin-Arp2/3 interaction has been reported but information about its effects on branched actin networks and on cell behaviour is extremely limited. Here, we chose to study the role of this interaction on various cell behaviours that depend on branched actin - single cell migration, collective cell migration, cell proliferation and cell junction stability.

To investigate the function of vinculin on Arp2/3, an important parameter was to be able to isolate it from vinculin's structural function at focal adhesions and adherens junctions, which is to link receptors to the actin cytoskeleton. To this end we will employ several genome editing techniques to create mutant cells where the functions of vinculin and its Arp2/3 interaction are perturbed. We chose MCF10A cells as a model system as they are untransformed human breast cells and as such should behave in a physiologically relevant manner. A vinculin linker peptide comprising amino acids 811-881 of vinculin has been shown to interact with Arp2/3 and a P878A mutation in this linker abrogates this binding[192]. We began by using TALEN-based genome editing to constitutively express these WT and mutant linkers in MCF10A cells. CrispR-Cas9 genome editing allowed us take another step forward and introduce biallelic mutations in the genome of MCF10A cells, to create cell lines in which endogenous vinculin is either knocked out or mutated at the P878 residue. In the latter case, vinculin loses its ability to bind Arp2/3 while in the former, the cell loses both this function of vinculin as well as its ability to reinforce cell adhesions. This will allow us to delineate cellular behaviours into those that depend on vinculin-mediated adhesion reinforcement and those that are dependant solely on the vinculin-Arp2/3 interaction.

As markers for Arp2/3 activity, we will utilise several behaviours that have been previously shown to be dependent on branched actin. The pushing forces created by branching network are essential for several processes including lamellipodial formation and single cell migration[72]. Branched actin plays a role in signalling the cell to pass the G1-S checkpoint[96]. Thus, we will look at phenotypes of our mutant cell lines in assays studying these processes.

The role of vinculin at adherens junctions is less well understood compared to its role at cell-substrate adhesions. These mutant cell lines will allow us to decipher further the effects of vinculin on Arp2/3 at adherens junctions. We will look at the recruitment of Arp2/3 and the organisation of the actin cytoskeleton at adherens junctions. One would expect that any changes in cell-cell junction dynamics would lead to an effect on cell behaviours like ability to maintain cell-cell junctions.

Since vinculin is involved in single cell migration and maintaining cell junctions, we will study the effect of vinculin and its interaction with Arp2/3 on the collectivity of migration in 3 different contexts: 1) The collectivity across a leading edge of migrating cells, all of which experience the space to migrate into; 2) how vinculin participates in the propagation of the migratory signal to cells behind the leading edge, which cannot directly feel this space; 3) on collective migration of cells in confluent mature monolayers during hypotonic unjamming.

Vinculin Antagonizes Branched Actin in Controlling Membrane Protrusion, Cell Migration and Cell Cycle Progression

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Abstract

Vinculin is a mechanotransducer that reinforces cell adhesions to linear arrays of actin filaments upon myosin-mediated contractility. Both adhesions to the substratum and neighboring cells, however, originate from Arp2/3-nucleated branched actin networks. Vinculin interacts with the Arp2/3 complex, but the role of this interaction is incompletely understood. Here we compared the phenotypes of vinculin knock-out (KO) cells with that of knock-in (KI) cells, where the point mutation P878A that impairs the Arp2/3 interaction is introduced in the two vinculin alleles of MCF10A mammary epithelial cells. The interaction of vinculin with the canonical Arp2/3 complex inhibits actin polymerization at membrane protrusions and migration persistence of single cells. In cell monolayers, vinculin plays a role in recruiting Arp2/3 at cell-cell junctions and through this interaction controls the decision to enter a new cell cycle as a function of cell density.

INTRODUCTION

Actin dynamics control cell shape, adhesion and migration (Pollard and Cooper, 2009). Actin filaments form linear and branched arrays (Clainche and Carlier, 2008). Whereas branched actin arrays generate pushing forces through the Arp2/3 complex, linear arrays can generate pulling forces through myosin-mediated contractility (Pollard, 2016; Garrido-Casado et al., 2021). Cell migration requires a combination of forces to drive protrusion of the plasma membrane and pull on the substratum (Blanchoin et al., 2014).

The RAC1-WAVE-Arp2/3 pathway drives membrane protrusions (Papalazarou and Machesky, 2021; Bieling and Rottner, 2023). This pathway is embedded in positive feedback loops that sustain the membrane protrusion at the front edge of a migrating cell and renders the migration persistent (Krause and Gautreau, 2014). Signaling from branched actin at the cell cortex is also critical for cells to progress into the cell cycle by impinging on tumor suppressor genes controlling the G1/S transition (Molinie et al., 2019). Various Arp2/3 complexes coexist in the same cells through the combinatorial assembly of paralogous subunits (Pizarro-Cerdá et al., 2016). For example, ARPC1B-containing Arp2/3 complexes are more efficient at nucleating branched actin and at forming stable branched junctions than ARPC1A-containing Arp2/3 complexes (Abella et al., 2016). ARPC1B-containing Arp2/3 complexes, but not the ones containing ARPC1A, generate cortical branched actin that drives persistent migration and delivers the signal for cell cycle progression (Molinie et al., 2019).

Vinculin senses forces exerted in cell adhesions and responds to these forces by reinforcing the linear arrays of actin filaments attached to cell adhesions (Bays and DeMali, 2017). Vinculin is composed of a head that interacts with adhesion proteins and a tail that binds to actin filaments (Humphries et al., 2007; Clainche et al., 2010). This ability of vinculin to link actin filaments to adhesion sites is inhibited by an intra-molecular interaction masking relevant binding sites of the head and the tail (Atherton et al., 2016). In adhesions to the extracellular matrix (ECM), vinculin is recruited to cryptic binding sites in talin that are exposed upon stretching due to myosin-mediated contractility (Rio et al., 2009; Ciobanasu et al., 2014; Yao et al., 2014a). Vinculin is similarly recruited to a cryptic binding site of α -catenin at cell-cell adhesions upon myosin-mediated contractility of actin filaments (Yao et al., 2014b; Seddiki et al., 2018; Vigouroux et al., 2020). Force-dependent recruitment of vinculin results in activation of its function as a linker and thereby reinforces the cytoskeletal attachment of cell adhesions. In the process, vinculin mechanotransduction transmits signals, since vinculin-depleted cells

exhibit enhanced proliferation, survival and anchorage-independent growth (Fernández et al., 1993; Subauste et al., 2004; DeWane et al., 2022).

The cytoskeletal reinforcement function of vinculin is in line with the fact that cell adhesions mature over time. Adhesions to the ECM at focal complexes at the edge of membrane protrusions mature into focal adhesions (FAs) as the leading edge moves forward and myosin motors exert contractility on newly formed adhesions (DePasquale and Izzard, 1991; Alexandrova et al., 2008; Thievensen et al., 2013). Similarly, E-Cadherin based adherens junctions (AJs) form initial interdigitations that mature into straight cell-cell adhesions, as contractility develops (Kovacs et al., 2002; Leerberg et al., 2014; Li et al., 2020, 2021). Vinculin was shown *in vitro* to remodel branched actin networks into bundles (Boujemaa-Paterski et al., 2020). In this context, the reported interaction of vinculin with the Arp2/3, the major player in protrusion formation, is particularly intriguing.

Vinculin binds to the Arp2/3 complex through the linker that connects its head to its tail (DeMali et al., 2002). The Arp2/3 site is not masked by the head-to-tail intramolecular interaction, suggesting that the interaction should be independent from vinculin activation. Subsequently, 'vinculin-Arp2/3 hybrid complexes' were purified from chicken smooth muscles (Chorev et al., 2014). These hybrid complexes lack subunits of the canonical Arp2/3, namely ARPC1, ARPC4 and ARPC5 subunits. These observations suggest the existence of constitutive vinculin-Arp2/3 complexes. However, the interaction of vinculin with Arp2/3 is regulated by EGF stimulation, RAC1 activity and Src-dependent phosphorylation of vinculin on tyrosine residues that are located far away from the Arp2/3 binding site, but which contributes to vinculin activation (DeMali et al., 2002; Zhang et al., 2004; Moese et al., 2007; Auernheimer et al., 2015). The point mutation P878A in vinculin was shown to impair its interaction with Arp2/3 and re-expression of this mutant in vinculin-null mouse embryonic fibroblasts (MEFs) does not rescue defective protrusions and spreading of these cells, unlike wild type vinculin (DeMali et al., 2002). Together these data suggested that vinculin was required to activate the Arp2/3 and form membrane protrusions.

Here we used epithelial cells to examine the role of the vinculin-Arp2/3 interaction. We were able to distinguish the specific subset of functions that the vinculin-Arp2/3 interaction endows compared with the more general role of vinculin in the cytoskeletal reinforcement of cell adhesions. We found that vinculin antagonizes Arp2/3-dependent branched actin in the control of membrane protrusion, cell migration and cell cycle progression.

RESULTS

The Vinculin Linker Enhances Membrane Protrusion and Migration Persistence through Arp2/3 Binding

We first wanted to examine the interaction of vinculin with the Arp2/3 complex in the MCF10A cell line, where we have characterized the role of the Arp2/3 complex in cell migration (Molinie et al., 2019; Simanov et al., 2021). MCF10A cells are human mammary epithelial cells, which are immortalized but not transformed (Soule et al., 1990). Importantly this cell line is diploid for the most part of its genome (Worsham et al., 2005; Kadota et al., 2010). We found that antibiotic-selected MCF10A cells down-regulated expression of tagged full length vinculin in an increasing number of cells over time, but not when the construct was limited to the vinculin linker that separates the head and the tail and which contains the Arp2/3 binding site. We obtained stable MCF10A lines expressing the GFP tagged linker of vinculin (amino acids 811-881) or its derivative containing the point mutation P878A that was characterized by DeMali, Burridge and collaborators to impair Arp2/3 binding (DeMali et al., 2002). When mutated, the linker indeed bound much less efficiently to the Arp2/3 complex despite an expression level similar to the wild type (Fig. 1a). Interestingly, the immunoprecipitate of the vinculin linker contained the Arp2/3 subunit ARPC1B, which is not present in the vinculin-Arp2/3 hybrid complexes that were purified from tissues (Chorev et al., 2014), suggesting that in our cell system, vinculin binds to the canonical Arp2/3 complex.

We observed that MCF10A cells expressing the wild type linker, but not its P878A derivative, exhibited extensive membrane protrusions at their periphery (Fig.1b, Supplementary Movie 1). Cells expressing the vinculin linker were significantly more spread (Fig.1c) and migrated more persistently than controls (Fig.1d,e), two read-outs of cortical polymerization of branched actin. Decreased cell speed and mean square displacement (MSD) were associated with this increased persistence of cell migration (Supplementary Fig.1). Decreased speed and MSD are often associated with increased cortical branched actin in MCF10A cells, for example when RAC1 is activated by mutation or when the Arp2/3 inhibitory protein is down-regulated, but to a variable extent (Molinie et al., 2019). The only parameter of cell migration that systematically correlates with increased polymerization of cortical branched actin is migration persistence (Dang et al., 2013; Molinie et al., 2019; Simanov et al., 2021).

We then examined membrane protrusions that power cell migration. Following mCherry-actin expression, we were able to measure lengths of protrusions, operationally defined as the distance between the protrusion edge and the first transverse arc at the base of

protrusions (Fig.1f). Protrusions were longer and protruded 1.7-fold faster when cells expressed the wild type vinculin linker, but not the P878A derivative (Fig.1g). Fluorescent actin also allowed us to image the rearward flow of the cytoskeleton using Total Internal Reflection Fluorescence – Structured Illumination Microscopy (TIRF-SIM, Supplementary Movie 2). We found that the rearward flow, in reference to the substratum, was also significantly increased in cells expressing the vinculin linker and that translated into a 2-fold faster actin assembly rate, in reference to the leading edge (Fig.1j,k). In the different cell lines, protrusion efficiency, that is the ratio of protrusion speed to actin assembly rate, was similar, indicating that protrusion speed scaled with actin assembly rate (Fig.1l). These results suggest either that vinculin activates the Arp2/3 complex through the linker domain or that vinculin inhibits Arp2/3 and the linker behaves as a dominant-negative fragment. We produced and purified the vinculin linker in its wild type and P878A form. In vitro, these proteins did not modify kinetics of spontaneous actin polymerization, nor Arp2/3-mediated branching nucleation (Supplementary Figure 2), indicating that the vinculin linker requires additional factors or post-translational modifications that are only present in cells to regulate the Arp2/3.

A Knock-In of the Point Mutation that Impairs Arp2/3 Binding Phenocopies Vinculin Knock-Out

The two alternative hypotheses prompted us to generate knock-outs of the *VCL* gene that encodes vinculin. To this end, we transfected MCF10A cells with purified Cas9 and a guide RNA (gRNA) that targets the beginning of the Open Reading Frame (ORF) in the first exon of the *VCL* gene. Cas9-mediated double strand breaks (DSBs) are frequently repaired by the error-prone non-homologous end joining (NHEJ) mechanism (Jasin and Haber, 2016). This method using purified Cas9 and gRNA was sufficiently efficient to identify KO clones without selection by a Western blot-based screen (Fig.2a). We selected two KO clones with different frameshifts within the two alleles of *VCL* (Fig.2b), for further characterization. As expected, focal adhesions (FAs) of these two clones were not stained by vinculin antibodies (Fig.2c,d). Paxillin-stained FAs were significantly more elongated, by 60 % on average, in vinculin KO compared with parental cells (Fig.2e).

To specifically examine the role of the Arp2/3 interaction, we designed a strategy based on homology-directed recombination (HDR) to introduce the P878A point mutation in the endogenous *VCL* gene of MCF10A cells. We transfected MCF10A cells with 3 plasmids and a long repair oligonucleotide that provides the P878A mutation together with a PvuII restriction

site. One of the plasmids provided Cas9, the second plasmid encoded a gRNA that allows Cas9 to cut the *VCL* gene close to the P878 codon and the third one encoded a gRNA that targets the *ATP1A1* gene. *ATP1A1* encodes the ubiquitous and essential sodium-potassium ion pump, which is the target of the drug ouabain. The gRNA targets the ATP1A1 region that is directly recognized by ouabain. Upon ouabain treatment, only cells that have repaired the *ATP1A1* DSB by NHEJ, so as to introduce an in-frame indel produce an ion pump that is both functional and insensitive to ouabain (Agudelo et al., 2017). The ouabain selection is more efficient than more classical antibiotic selection of transfected plasmids, because it ensures that Cas9-mediated DSBs were efficiently produced in the selected cells, and not only that the selected plasmid was present. The DSBs in *VCL* are often repaired by NHEJ, but can also be repaired by HDR using the provided oligonucleotide as a template. After extensive screening of clones by PvuII restriction of the PCR amplified genomic region, we managed to isolate the desired knock-in (KI) clone, where the P878A mutation was introduced in both alleles of *VCL* (Fig.2f,g). P878A vinculin properly localized to FAs (Fig.2h,i) and did not impact elongation of FAs (Fig.2j), suggesting it is functional and that Arp2/3 binding is not required for the FA-related functions of vinculin.

We then evaluated KO and KI clones for their ability to migrate and to produce membrane protrusions. Like the MCF10A clone that expressed the vinculin linker, KO clones exhibited more persistent trajectories than parental cells (Fig.3a,b, Supplementary Movie 3). They were also 20 % more spread than parental cells and this effect was significant (Fig.3c). The KI clone that expresses the vinculin containing the P878A substitution displayed a similar, but even more pronounced phenotype of increased persistence and spreading than the KO clones that expressed no vinculin (Fig.3c-f). This suggests that the vinculin-Arp2/3 interaction is critical for these functions. We then examined membrane protrusions by transiently transfecting our KO and KI clones with mCherry-actin. The length of membrane protrusions was increased in both KO and KI clones compared with parental cells (Fig.3g,h). The increase was, however, significantly higher in KO than in KI cells for this parameter. When protrusion speed was examined, both KO and KI cells behaved similarly. They displayed 1.6-fold increased protrusion speed compared with parental cells (Fig.3i,j, Supplementary Movie 4). KO and KI cells also displayed 1.5 and 1.3-fold increased rearward flow, respectively, compared with parental cells (Fig.3k). This translated into increased assembly rate in both KO and KI cells compared with parental cells (Fig.3l). Protrusion efficiency was not significantly different in KO compared with parental cells, but was slightly increased in KI cells (Fig.3m).

The vinculin linker is thus a dominant negative fragment of vinculin, since the phenotype that its expression induces is similar to that of vinculin KO. The interaction of vinculin with Arp2/3 appears critical for the negative regulation that vinculin exerts on membrane protrusion and migration persistence.

Vinculin Controls Stability of Cell Junctions and Efficiency of Collective Migration

Since vinculin plays a key role to stabilize cell-cell junctions (Bays and DeMali, 2017), we used our cell system to examine the potential role of vinculin-Arp2/3 interaction in junction stability. We verified that KO cells displayed no vinculin staining at cell-cell junctions (Fig.4a,b). P878A vinculin of KI cells was recruited as efficiently as the wild type protein in parental cells at α -catenin positive junctions (Fig.4c,d). Because on 2D substrates such as Petri dishes or glass coverslips, cell-cell interactions were not obviously affected in KO cells, we decided to embed cells into collagen gels to examine cell-cell interactions. In this 3D setting, the behavior of KO cells and parental cells was markedly different. Parental cells rarely dissociated when they met and formed multicellular slugs (Fig.4e, Supplementary Movie 5). In contrast, interactions between KO cells appeared not to engage them in a multicellular behavior. KI cells did not present the asocial behavior of KO cells and formed multicellular slugs like parental cells (Fig.4f, Supplementary Movie 6). From movies, we were able to count the number of events, where a cell disengaged from cell-cell interactions after having met another cell or dissociating from slugs, per unit of time. The counts were then converted into frequencies expressed in Hertz. In this 3D setting, the frequency of junction disassembly was about 10-fold higher in KO than parental cells (Fig.4g). We verified that E-Cadherin expression was not down-regulated in KO cells and that E-Cadherin was properly recruited at cell-cell junctions in KO cells (Supplementary Fig.3). Vinculin thus regulates the stability of junctions, but not E-Cadherin-dependent cell-cell adhesion. In contrast, in KI cells, the quantification revealed that cell-cell junctions were significantly more stable than that of parental cells, dissociating 2.6-fold less (Fig.4g).

We then decided to analyze collective migration of MCF10A, KO and KI cells in a wound healing assay obtained by lifting an insert that initially constrained the monolayer. Phase contrast images revealed that the monolayer edge of KO cells progressed significantly faster than MCF10A therefore covered faster the cell-free area (Fig.5a, b). KI cells although slower than KO were faster than MCF10A. When the images were analyzed by Particle Image Velocimetry (PIV) that associates a displacement vector to different (x,y) positions over time.

Cell speed was increased in KO cells and this increase was transmitted further backwards, away from the edge, when compared with MCF10A (Fig.5c). The local order parameter, which reflects the local alignment of displacement vectors was also transmitted further backwards when compared with MCF10A (Fig.5d). The distance to which the local order parameter as well as speed of migration were transmitted were increased in the KI compared to MCF10A although not to the level of KO. Thus, collective migration depends both on the mechanical linker function of and the vinculin-Arp2/3 interaction with a combinatorial effect seen in KO.

Vinculin Recruits Arp2/3 at Adherens Junctions and Restricts Cell Cycle Progression through Arp2/3 Binding

We then analyzed Arp2/3 recruitment by immunofluorescence at E-Cadherin positive AJs of KO and KI cells. In parental MCF10A cells, Arp2/3 was enriched at AJs, 6 h after plating, the amount of junctional Arp2/3 reached a maximum 1 day after plating and declined to residual amount after 3 days (Fig.6a). Vinculin was present at AJs throughout junction maturation, even if it peaked 1 day after plating like Arp2/3 (Supplementary Fig.4). KO cells displayed reduced Arp2/3 staining at AJs compared with parental cells (Fig.6b). This was especially striking one day after cell plating, when no junctional Arp2/3 was detected. 6 h after plating, a low amount of Arp2/3 was detected at AJs of KO cells, but KO cells still recruited significantly less Arp2/3 than parental cells (Fig.6c). In KI cells, Arp2/3 recruitment at AJs was increased 6 h after plating compared with parental cells, and even more so compared with KO cells. Yet, 1 day after plating, Arp2/3 recruitment at AJs of KI cells was dramatically reduced compared with parental cells, as in KO cells. 3 days after plating, Arp2/3 at AJs is minimum in all 3 types of cells.

This kinetic analysis thus reveals two distinct phases of Arp2/3 recruitment at AJs. The early recruitment, at 6 h, is dependent on the presence of vinculin but independent of its direct interaction with Arp2/3. Later recruitment, after one day, depends on the ability of vinculin to interact with the Arp2/3. Therefore, the vinculin-Arp2/3 interaction appears to retain Arp2/3 at AJs after an initial interaction independent recruitment of Arp2/3. Stable MCF10A cell lines expressing GFP fusions with Arp2/3 subunits that are not part of the vinculin-Arp2/3 hybrid complexes revealed a GFP signal enriched at cell-cell junctions, 1 day after cell plating (Supplementary Fig.5), thus confirming that vinculin interacts with the canonical Arp2/3 complex in MCF10A cells.

Untransformed cells stop proliferating when confluent. This phenomenon called contact inhibition can be more precisely referred to as density dependence of cell cycle progression, since cells do not stop proliferating as soon as they touch each other, but rather enter less and less frequently into a new cell cycle, as the cell culture becomes denser. We observed that KO and KI cells reached a ~30 % higher saturation density than parental MCF10A cells (Fig.7a, Supplementary Fig.6). We then measured the number of cycling cells by estimating the % of cells incorporating EdU, an analog of thymidine incorporated in DNA during S phase. The % of cycling cells steadily decreased as a function of the cell density. KO cells behaved like parental cells, with a high cycling rate at low density and a low cycling rate at high density. Yet, at an intermediate density, KO cells were significantly more prone to enter into a new cell cycle than parental cells (Fig.7b). A similar behavior was observed for KI cells (Fig.7c), suggesting that the mere presence of vinculin was not sufficient to control contact inhibition, vinculin should also be able to interact with the Arp2/3. To confirm this point, we analyzed density dependence of cell cycle progression MCF10A cells expressing the vinculin linker. Cells expressing the dominant-negative construct exhibited significantly increased cycling even at high cell density (Fig. 7d). In contrast, the P878A mutation in the linker abolished this increased cycling, thus reinforcing the idea that vinculin controls cell cycle progression through its ability to interact with the Arp2/3 complex. When the Arp2/3 inhibitory compound CK-666 was incubated with parental cells, cell cycle progression was inhibited in a dose-dependent manner (Fig.7e). MCF10A cells expressing the dominant-negative vinculin linker also displayed a dose-dependent inhibition of cell cycle progression, but required more CK-666 to achieve the same level of inhibition. Cell cycle progression thus appears to be inhibited by vinculin through its effect on Arp2/3 activity, in a manner similar to membrane protrusion and persistence of single cell migration.

DISCUSSION

The vinculin-Arp2/3 interaction was first assumed to be a transient regulated interaction that involved the canonical Arp2/3 complex (DeMali et al., 2002). However, alternative assemblies of so-called vinculin-Arp2/3 hybrid complexes have then been discovered (Chorev et al., 2014). Since Arp2/3 subunits analyzed in vinculin immunoprecipitates by DeMali belonged to both canonical and hybrid complexes, it was not known whether the two modes of binding existed or if the vinculin-Arp2/3 interaction only involved assembly of hybrid complexes. We unambiguously detected subunits that belong to the canonical Arp2/3 complex, but not to hybrid complexes, in vinculin immunoprecipitates or at cell-cell junctions at a time when the vinculin-Arp2/3 interaction is responsible for Arp2/3 recruitment. Therefore, even if these experiments do not rule out that some vinculin-Arp2/3 hybrid complexes exist in MCF10A cells, they show that the interaction of vinculin with the canonical Arp2/3 complex does exist, as suggested by the original reference. We also favor the interpretation that the roles of the vinculin-Arp2/3 interaction we report here are due to an interaction of vinculin with the canonical Arp2/3 complexes, because these functions in membrane protrusion, migration persistence and cell cycle progression were previously ascribed to the canonical Arp2/3 complex (Wu et al., 2012; Suraneni et al., 2012; Molinie et al., 2019).

Our strategy to compare the phenotypes of KO and KI cells allowed us to distinguish the cytoskeletal linker function of vinculin that only depends on the presence of vinculin, from the vinculin functions that required both vinculin and its interaction with Arp2/3 (Fig.8). Among the Arp2/3-dependent functions of vinculin in MCF10A cells, we found an inhibition of membrane protrusion and cell spreading. It was reported in the original article mapping the Arp2/3 binding site on vinculin that vinculin KO mouse embryonic fibroblasts (MEFs) had impaired lamellipodia and cell spreading (DeMali et al., 2002). These two phenotypes were rescued by the expression of WT vinculin, but not by the P878A derivative. Our two studies thus implicate the same functions, but in opposite directions.

The increased migration persistence we observed in MCF10A KO is similarly in contrast to the decreased persistence observed upon vinculin depletion in MEF KO cells or siRNA-treated mammary carcinoma MDA-MB-231 cells, using different assays (Thievessen et al., 2015; Rahman et al., 2016; Lee et al., 2019). Even if migration phenotypes of vinculin depleted cells strongly depends on cell types and precise assay conditions, in particular 2D vs. 3D conditions (Fernández et al., 1993; Mierke et al., 2010; Thievessen et al., 2015), it is clear that untransformed epithelial cells MCF10A cells behave in a significantly different manner than

previously examined cell types. Another surprising observation came from the examination of the clutch that connects actin polymerization at the leading edge to cell adhesions to the substratum. In fibroblasts, vinculin has been implicated in mediating the clutch that connects the retrograde flow of actin to cell adhesions to the substratum (Thievensen et al., 2013; Hirata et al., 2014). In our vinculin KO MCF10A cells, however, the increased actin assembly rate at the protrusion edge translates into effective protrusions, with no slippage, suggesting that the clutch is mediated by other proteins than vinculin in epithelial cells.

Our epithelial cell system allowed us to examine the role of vinculin at cell-cell adhesions. We found that vinculin KO MCF10A cells had dramatically decreased stability of AJs when cells were embedded into soft 3D collagen gels, highlighting the interplay between adhesions to the substratum and to neighboring cells. Vinculin belongs to both cell adhesions, the specific incorporation into cell-cell adhesions being determined by Abl-mediated phosphorylation of Y822 (Bays et al., 2014). Decreased cell-cell adhesion upon vinculin KO was recently observed in mouse 4T1 breast cancer line (DeWane et al., 2022) and is in line with the aberrant AJs between cardiac myocytes reported in heart-specific KO of vinculin in mice (Zemljic-Harpf et al., 2007). Vinculin is an essential component of cell-cell junctions that allows myosin-dependent tensile forces to develop mature junctions (Twiss et al., 2012). KI cells do not exhibit unstable AJs and even exhibit more stable AJs than parental cells. Increased branched actin at early cell-cell junctions might explain increased junction stability of KI cells, if we assume that branched actin is remodeled in linear arrays for myosin-mediated contractility. Indeed branched actin is a poor substrate for myosin motors (Muresan et al., 2022), but GMF and coronin proteins debranch actin networks of lamellipodia (Cai et al., 2008; Haynes et al., 2015) and might play a similar role at cell-cell junctions.

In 2D wound healing assay of MCF10A cells, vinculin KO cells were more efficient at closing the wound than parental cells, as previously reported using 4T1 cells (DeWane et al., 2022). KO MCF10A cells exhibited fast and directional migration towards the wound and transmitted the signal further back in the monolayer, indicating that the mechanotransduction of E-Cadherin dependent cell adhesions that vinculin provides (Duc et al., 2010) is rather inhibitory and not essential to this transmission. These phenotypes were also observed with a lesser intensity in vinculin is present but its ability to interact with the Arp2/3 impaired, indicating that both the vinculin-Arp2/3 interaction and the structural role of vinculin are important for collective migration.

Cadherins at AJs were found to be associated with branched actin, which pushes membranes from neighboring cells against one another to initiate cell-cell adhesion or repair unzipped membranes due to excessive tension (Efimova and Svitkina, 2018; Li et al., 2020, 2021; Senju et al., 2023). We found that the vinculin-Arp2/3 interaction was essential to recruit the Arp2/3 complex at AJs, but not at an early time point, 6 h after cell plating, where Arp2/3 recruitment depends on vinculin but not direct binding. These results show that vinculin plays an essential role in Arp2/3 recruitment at AJs, but that there are also other ways to recruit it. The α -catenin molecule, which recruits vinculin at AJs, also binds to the Arp2/3 and inhibits its, but this involves a free form of α -catenin that is not bound to E-cadherin and β -catenin (Drees et al., 2005; Benjamin et al., 2010). The nucleation promoting factors, WAVE and N-WASP, recruit Arp2/3 and induce polymerization of branched actin at AJs (Kovacs et al., 2002; Verma et al., 2012; Rajput et al., 2013). Cortactin, which is now recognized as an Arp2/3 stabilizer of the branched junction of actin filaments (Gautreau et al., 2022), is also critical for Arp2/3 localization at AJs (Helwani et al., 2004; Han et al., 2014). These proteins are obvious candidates for the early vinculin-independent recruitment of Arp2/3 at AJs we observed here. However, their implication is difficult to test, since they also have a critical role in the formation and maintenance of AJs, the very structure where we would like to assess Arp2/3 recruitment.

Vinculin was found to regulate cell cycle progression as a function of cell density and the cell density reached at saturation. This increased proliferation was observed in both KO and KI cells, indicating that this vinculin function strictly depends on its ability to interact with the Arp2/3. We previously established that cell cycle progression in untransformed cells depends on cortical branched actin (Molinie et al., 2019). In fact, the Arp2/3-dependent functions of vinculin uncovered here, membrane protrusions, persistence of single cell migration and cell cycle progression, were previously shown to depend on the RAC1-WAVE-Arp2/3 pathway (Dang et al., 2013; Molinie et al., 2019; Simanov et al., 2021). Increased protrusions, increased persistence and increased cycling observed in KO and KI MCF10A cells are phenotypes, which are all associated with increased branched actin, showing that the vinculin-Arp2/3 interaction should antagonize the formation of branched actin. Nevertheless we were unable to show that the vinculin linker that binds the Arp2/3 in the cell was inhibiting the Arp2/3 complex in vitro, in the pyrene-actin assay, suggesting that additional factors or post-translational modifications might be required in the cell. Future work should be aimed at deciphering the precise molecular mechanisms by which vinculin antagonizes the nucleation of branched actin networks by the Arp2/3 complex.

METHODS

Cell Culture and Drugs

MCF10A cells were grown in DMEM/F12 medium supplemented with 5% horse serum, 20 ng/mL epidermal growth factor, 10 μ g/mL insulin, 100 ng/mL cholera toxin, 500 ng/mL hydrocortisone and 100U/mL penicillin. Medium and supplements were from Life Technologies and Sigma. Cells were incubated at 37°C in 5% CO₂. Cells were trypsinised (12605010, Gibco) and sub-cultured every 3 days. CK-666 (182515, Sigma) was used for Arp2/3 inhibition as stated.

Plasmids, Transfection and Isolation of Stable Cell Lines

GFP tagged proteins were expressed from a home-made vector, MXS AAVS1L SA2A Puro bGHpA EF1Flag GFP Blue SV40pA AAVS1R built using the MXS building blocks (Molinie et al. 2018). Vinculin linker WT, P878A, ARPC1A, ARPC1B, ARPC5, ARPC5 were inserted into this plasmid in place of the Blue cassette using FseI and AscI restriction sites. The P878A mutation was generated from the WT plasmid using the Quikchange Lightning mutagenesis kit (Agilent) and primers (CCTAGGCCTCCACCAGCAGAGGAAAAGGATG, GTAGGAAAAGGAGACGACCACCTCCGGATCC).

Transfections of MCF10A cells were performed using Lipofectamine 3000 (Invitrogen). To obtain stable cell lines, MXS AAVS1 vectors were co-transfected with two TALEN constructs (Addgene #59025 and 59026) inducing a double strand break at the AAVS1 locus (González et al., 2014). Cells were selected with 1 μ g/mL puromycin (ant-pr-1, Invivogen) and pooled if the expression was homogeneous or cloned otherwise.

Genome Editing

To generate vinculin KO lines, MCF10A cells were transfected with a sgRNA (ATCGTGCGCGTATGAAACAC) targeting nucleotides 7 to 16 of the VCL coding sequence, corresponding to amino acids 3 to 9 of the vinculin protein, along with the purified Cas9 protein using the Lipofectamine CrisprMax kit (#CMAX00001, ThermoFisher). Cells were then diluted and seeded in 96-well plate at 1 cell/well. Wells containing two or more clones were not analyzed. 130 clones were screened by dot blot using anti-vinculin antibodies at 1:1000

dilution. Cells with minimal signal on dot blots were further screened using Western blot, immunostaining and sequencing to derive characterized KO clones.

To characterize the VCL mutations on the vinculin gene, base pairs 14-494 were amplified by PCR using DreamTaq (EP0702, ThermoFisher) for 32 cycles with annealing temperature 58°C (TCTGTCTCTTCGCCGGTTC, AGCCTTTTTCATGACTGCTCC) and the PCR product was sequenced. When several sequences overlapped, PCR products were cloned into a blunt vector using the Zero Blunt PCR cloning kit (#K270040, ThermoFisher) to sequence the 2 alleles independently.

To obtain the vinculin KI line, MCF10A cells were cotransfected by electroporation with a Cas9 expressing plasmid (CMV hSpCas9 bGH pA), a plasmid expressing the ATP1A1 sgRNA (Agudelo et al., 2017), a pRG2(-GG) plasmid expressing the VCL sgRNA (GCCTCCACCACCAGAGGAAA) and a single-stranded 87 bp repair oligonucleotide (base pairs 110878-110964 in the VCL gene). Colonies resistant to ouabain (0.5 µM, Sigma 03125) were cloned using dilution and screened by PCR using DreamTaq (EP0702, ThermoFisher) for 32 cycles with annealing temperature 52°C (GGTGACGATCGAAAAAC, TATTGGCAACACAGGAACC) followed by PvuII restriction.

Antibodies

Antibodies used for immunostaining and western blots were anti-vinculin (#V9131, Sigma), anti- α -catenin (#C2081, Sigma), anti-E-cadherin (#MABT26, Merck), anti-Paxillin (#GTX125891, GeneTex), anti-ARPC2 (#07-227-I, Millipore), anti-ARPC1B – (#HPA004832, Sigma), anti-ARPC3 – (#HPA006550, Sigma). For immunostaining, secondary antibodies for anti-mouse-647 (#A21236, Life technologies) and anti-rabbit-405 (#A34556, Life technologies) were used along with Acti-Stain 555 (Cytoskeleton).

Immunoprecipitation and Western Blot

Two 15-cm dishes of MCF10A cells were lysed 1 day after seeding and scraped off in (50mM HEPES pH7.7, 10mM EDTA, 50mM KCl, 1mM MgCl₂ and 1%NP-40) supplemented with a protease inhibitor cocktail (Roche, 1:10000). Cell lysates were centrifuged at 10,000g for 10 min. The supernatant was incubated at 4°C for 1 h with GFP-Trap Agarose beads

(Chromotek) and washed 4 times. Lysates and immunoprecipitates were analyzed by Western blot.

Immunofluorescence and Image Analysis

For imaging individual cells, 15,000 cells (1.5×10^6 cells for imaging monolayers) were plated on fibronectin (10 $\mu\text{g}/\text{mL}$, F1141, Sigma) coated coverslips (22 mm) and fixed after 1 day (unless otherwise stated) with 4% PFA for 15 min. Cells were permeabilized in 0.2% Triton X-100 and blocked in 10% FBS in PBS. Cells were stained in a 1:200 dilution of first primary and then secondary antibodies along with Acti-stain 555. Coverslips were mounted in Dako mounting medium and imaged using an SP8 laser-scanning confocal microscope (Leica). Images were analyzed using FIJI.

To measure cell spreading, 15,000 cells were plated on fibronectin coated coverslips (22 mm), and were fixed and stained with Acti-Stain555, 1 day after seeding. Cells were imaged on an SP8 laser-scanning confocal microscope (Leica) and area was quantified on images by thresholding (Triangle algorithm) in Fiji.

To quantify vinculin recruitment at AJs and FAs, the thresholded α -catenin or paxillin staining, respectively was used to generate a mask in which fluorescence intensity of vinculin was quantified. Lengths of focal adhesions were manually measured using paxillin staining. To quantify Arp2/3 and vinculin enrichment at AJs, a line was manually drawn along the junction labeled with anti-E-cadherin. Width of the line was then increased iteratively to measure total fluorescence intensity at increasing distances from the junction. Intensity at a distance n from the junction corresponded to (total intensity of line width $_n$) – (total intensity of line width $_{n-1}$). Values were finally normalized to the average fluorescence intensity at a line $\approx 12 \mu\text{m}$ from the junction, corresponding to intensity in the cytoplasm. Enrichment of E-cadherin at AJs was measured similarly using phalloidin staining as a reference for cell-cell contacts.

Live Cell Imaging

For 2D cell migration assays, 15,000 cells were seeded 1 day prior to imaging on microslides (#80826, Ibidi) coated with fibronectin. For 3D migration of cells embedded in a collagen matrix, 15,000 cells were plated into microchambers (#80826, Ibidi) on a 3.5 mg/ml Collagen Type1 (#354236, Corning) matrix in DMEM:F12 supplemented with FBS (10%).

After cells attached, another layer of collagen was added on top and cells were incubated in culture medium for 1 day before imaging. Cells were imaged on an AxioObserver Z1 microscope (Zeiss) equipped with an Orca-R2 CCD camera (Hamamatsu) and controlled by the AxioVision software (Zeiss). Images were acquired at 5 min (2D migration) or 10 min intervals (migration in 3D collagen matrix) for 24h. Cell-cell junction disassembly events were counted manually at each time point. The analysis of migration persistence was performed as previously described (Gorelik and Gautreau, 2014).

For TIRF-SIM imaging, 100,000 cells were plated 1 day prior to imaging on glass-bottom dishes (P35G-0.170-14-C, MatTek Corp) coated with fibronectin. Cells were transfected immediately with an mCherry-Actin plasmid (Romero et al., 2011) using jetPrime (PolyPlus, 101000015), the media was changed after 6h and cells were imaged 1day post-seeding. To image actin flows in lamellipodia, images were acquired at 2 s intervals for 2 min using 3 phase shifted angles, each with 3 fringe patterns, on the Deltavision OMX SR (GE Healthcare). High resolution images were reconstructed and 2-color images were aligned using softWoRx (AppliedPrecision). Kymographs were generated in FIJI using manually drawn lines that followed the direction of actin retrograde flow and the Multi Kymograph tool. Protrusion speed and rearward flow are given by the $|\tan^{-1}|$ of the angle made by them with the time axis in kymographs. The actin assembly rate is the sum of lamellipodia protrusion speed and rearward flow.

Cell Cycle and Proliferation

To quantify saturation density, 2×10^6 cells were plated on fibronectin coated cover slips in 6-well plates. Cells were fixed 4 days after seeding and nuclei were stained with DAPI. To perform the EdU incorporation assay, cells were seeded on fibronectin-coated coverslips (12 mm) for 1 day. Cells were incubated with 10 μ M EdU for 1 h prior to fixation in 4% PFA for 15 min and permeabilized in 1% Triton X-100 for 5 min. EdU was labeled with the Alexa Fluor 488 Click-iT EdU Imaging Kit (#C10337, Thermo Fisher Scientific) according to the manufacturer instructions and nuclei were labeled with DAPI (Thermo Fisher Scientific). Images were acquired on an inverted microscope (Olympus IX83) using a 20x objective (NA 0.5) equipped with an Orca-Flash4.0 V3 camera (Hamamatsu), controlled by Micro-Manager 2.0 and analyzed using a custom script in FIJI to count DAPI- or EdU-positive cells. The

percentage of cells in S-phase was scored as the ratio of EdU-positive nuclei/DAPI-stained nuclei in segmented images. For each condition, at least 5000 cells were counted.

Statistical Analysis

For all t-tests, populations were first tested for Gaussian distribution using a Shapiro-Wilk Test with a α -value of 0.05. If both populations were Gaussian, difference between means was tested using Welch's T-test and if one or both of the populations were non-Gaussian, the difference between means was tested using a Mann-Whitney U test. focal adhesion size, protrusion speed and frequency of cell junction disassembly were tested with a one-tailed distribution as the lower limit of measurement was close to a lower bound of 0. All other t-test assumed a two-tailed distribution. Analysis of migration persistence was performed as previously described (Gorelik and Gautreau, 2014). Migration persistence. Exponential decay and plateau fit ($y = (1 - b) * e^{-\frac{t}{a}} + b$) were performed for all individual cells. Coefficients were then compared using one-way ANOVA. Statistical analysis was performed in R using linear mixed-effect models to take into account the resampling of the same statistical unit as previously described (Polesskaya et al., 2022).

Acknowledgments

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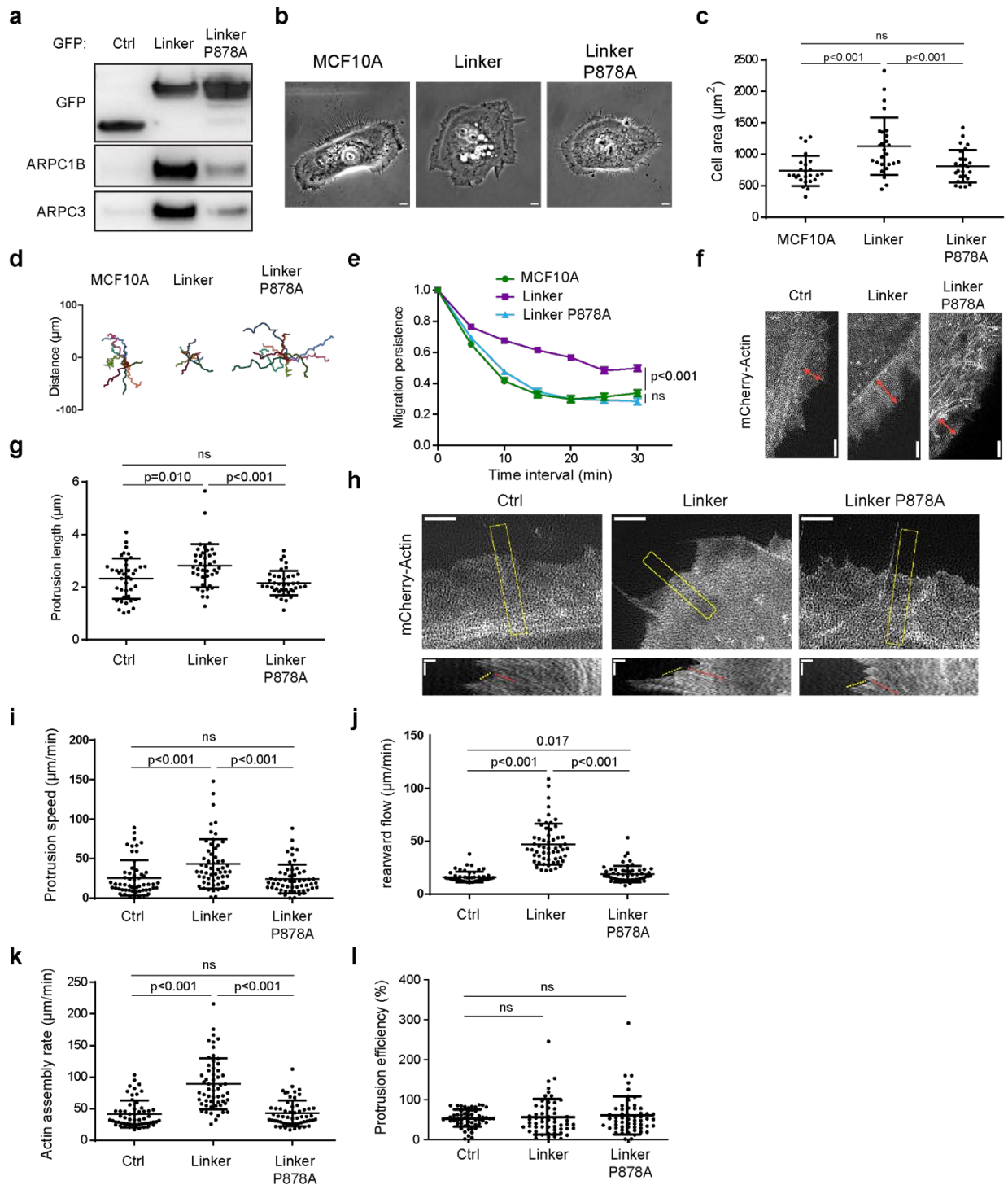


Figure 1. Expression of the vinculin linker that binds to the Arp2/3 complex increases actin polymerization and membrane protrusion. **a** The Arp2/3 complex co-immunoprecipitates with the vinculin linker (amino acids 811-881). MCF10A cells stably expressing GFP, the GFP tagged linker in a WT or P878A form were lysed and subjected to GFP immunoprecipitation and Western blot analysis. N=3, 1 representative experiment shown.

b Phase contrast images of the same cell lines. Scale bar = 5 μm . **c** Cell area of the cell lines. Mean \pm SD, n=25, t-test. N=3, 1 representative experiment shown. **d, e** Single cell migration. Cell trajectories (**d**) and migration persistence (**e**). Mean \pm SD, n=10, linear mixed effect model. N=3, 1 representative experiment shown. **f, g** Membrane protrusions of the stable MCF10A cell lines expressing the vinculin linker transiently transfected with mCherry-actin. TIRF-SIM images of mCherry-actin (**f**) and quantification of protrusion length (**g**). Double-headed arrows in red indicate the length of lamellipodia. Scale bar 2 μm . Mean \pm SD, n=40, t-test. N=3 with similar results, pooled measurements from the 3 independent repeats are plotted. **h** Kymographs (bottom panels, scale bars 0.4 μm horizontal, 40 s vertical) were generated along a line centered in the region boxed in yellow in the TIRF SIM movie (scale bar 2 μm). Dashed and yellow lines indicate protrusion speed (**i**) and rearward flow (**j**), respectively. Actin assembly rate (**k**) is the sum of protrusion speed and rearward flow. Protrusion efficiency (**l**) is the ratio of protrusion speed to actin assembly rate. Mean \pm SD, n= 40, t-test. N=3 with similar results, pooled measurements from the 3 independent repeats are plotted.

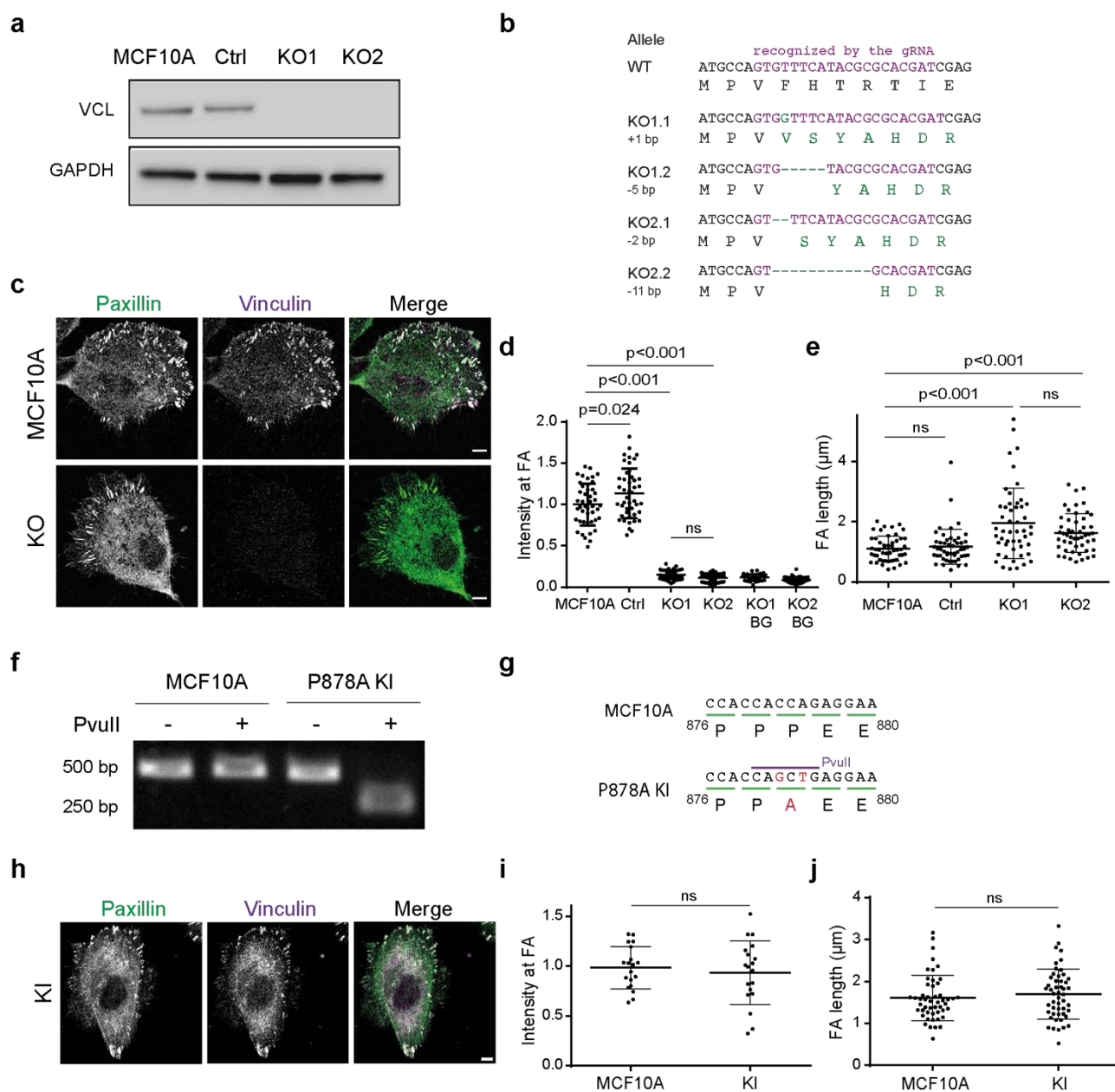


Figure 2. Characterization of *VCL* knock-out and knock-in cell lines. **a** Parental MCF10A, and isolated clones transfected with *VCL* targeting gRNA or a non-targeting gRNA were analyzed by Western blot. **b** Sequences of the two alleles in each KO cell line. All mutations induce a frameshift and thus a premature stop codon in the ORF. **c** Staining of vinculin and paxillin in parental and KO cells. Scale bar = 5 μ m. **d** Quantification of vinculin staining in focal adhesions (FAs) and normalization by the intensity of parental cells. BG refers to the background in the non-FA cytoplasm. Mean \pm SD, n=45, t-test. N=3 with similar results, pooled measurements from the 3 independent repeats are plotted. **e** Quantification of length of FAs.

Mean \pm SD, n=45, t-test. N=3 with similar results, pooled measurements from the 3 independent repeats are plotted. **f** Genome analysis of the KI. Part of the *VCL* ORF containing the P878A mutation was amplified by PCR and digested with PvuII restriction enzyme. Agarose gel electrophoresis of digested or undigested PCR fragment. **g** Sequencing of the genome amplified PCR fragment confirmed the presence of the P878A mutation on the two alleles and the introduction of the PvuII restriction site in the genome of the KI line. **h** Staining of vinculin and paxillin in the KI cells. Scale bar = 5 μ m. **i** Quantification of vinculin staining in focal adhesions (FAs) and normalization by the intensity of parental cells. Mean \pm SD, n=19, t-test N=3 with similar results, pooled measurements from the 3 independent repeats are plotted. **j** Quantification of length of FAs. Mean \pm SD, n=50, t-test N=3 with similar results, pooled measurements from the 3 independent repeats are plotted.

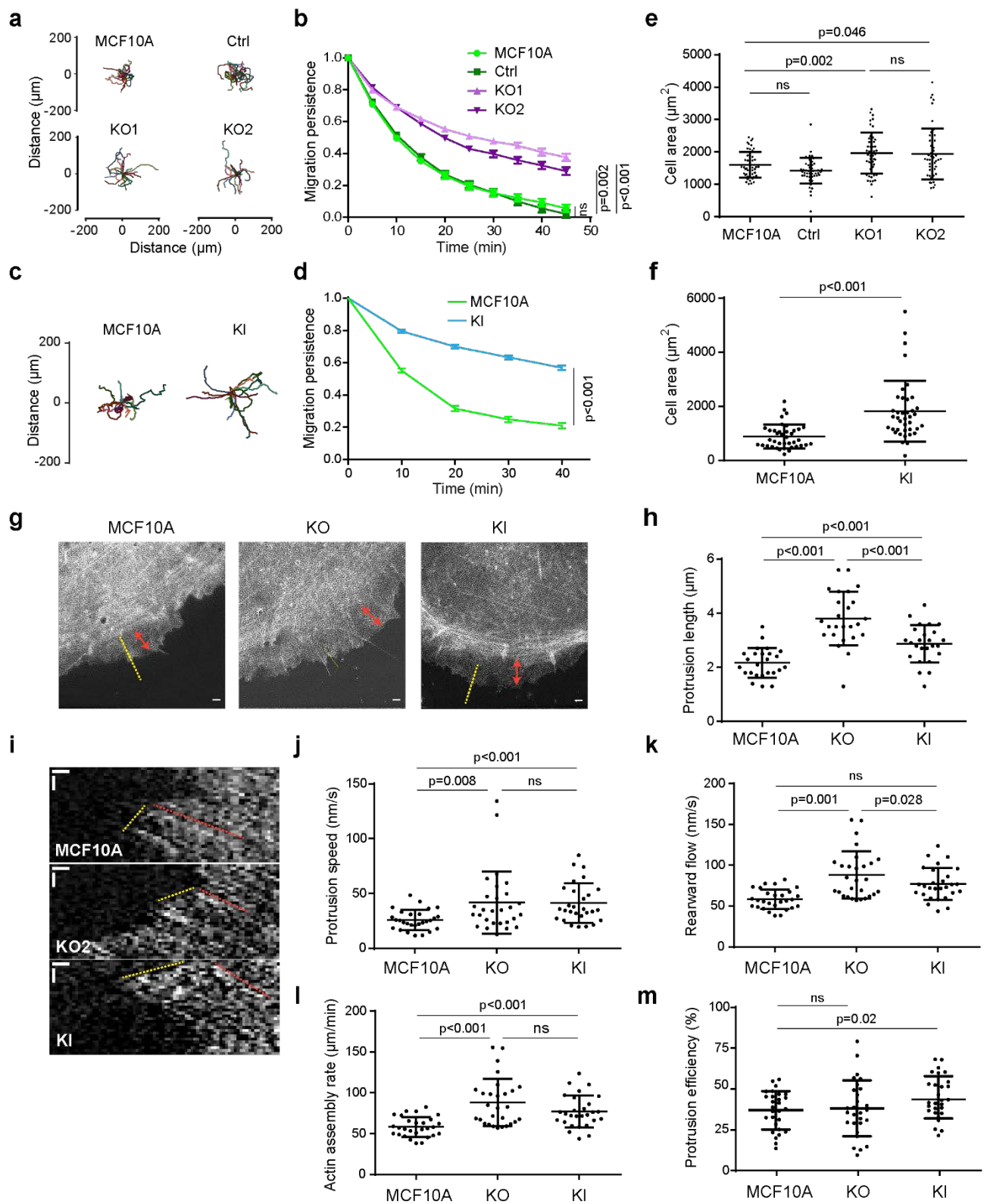


Figure 3. Vinculin controls membrane protrusion, cell spreading and migration persistence through its interaction with the Arp2/3 complex. a-d Single cell migration of KO and KI cells. Cell trajectories (**a, c**) and migration persistence (**b, d**). Mean \pm SD, $n=74$ in

b and $n=35$ in **d** linear mixed effect model. $N=3$, 1 representative experiment shown. **e, f** Cell area of KO and KI lines. Mean \pm SD, $n=50$ in **e** and $n=39$ in **f**, t-test. $N=3$, 1 representative experiment shown. **g, h** Membrane protrusions of the stable MCF10A cell lines expressing the vinculin linker transiently transfected with mCherry-actin. TIRF-SIM images of mCherry-actin (**g**) and quantification of protrusion length (**h**). Double-headed arrows in red indicate the length of lamellipodia and the dashed yellow line indicates the position of kymograph analysis. Double-headed red arrows measure lamellipodia length (**h**). Scale bar 1 μm . Mean \pm SD, $n=25$, t-test. $N=3$ with similar results, pooled measurements from the 3 independent repeats are plotted. **i-k** Kymograph analysis of KO and KI lines drawn along the yellow lines depicted in panel **g**. Protrusion speed (**j**) is measured from yellow lines and rearward flow from red lines (**k**). Actin assembly rate (**k**) is the sum of protrusion speed and rearward flow. Protrusion efficiency (**l**) is the ratio of protrusion speed to actin assembly rate. Mean \pm SD, $n=29$, t-test. $N=3$ with similar results, pooled measurements from the 3 independent repeats are plotted.

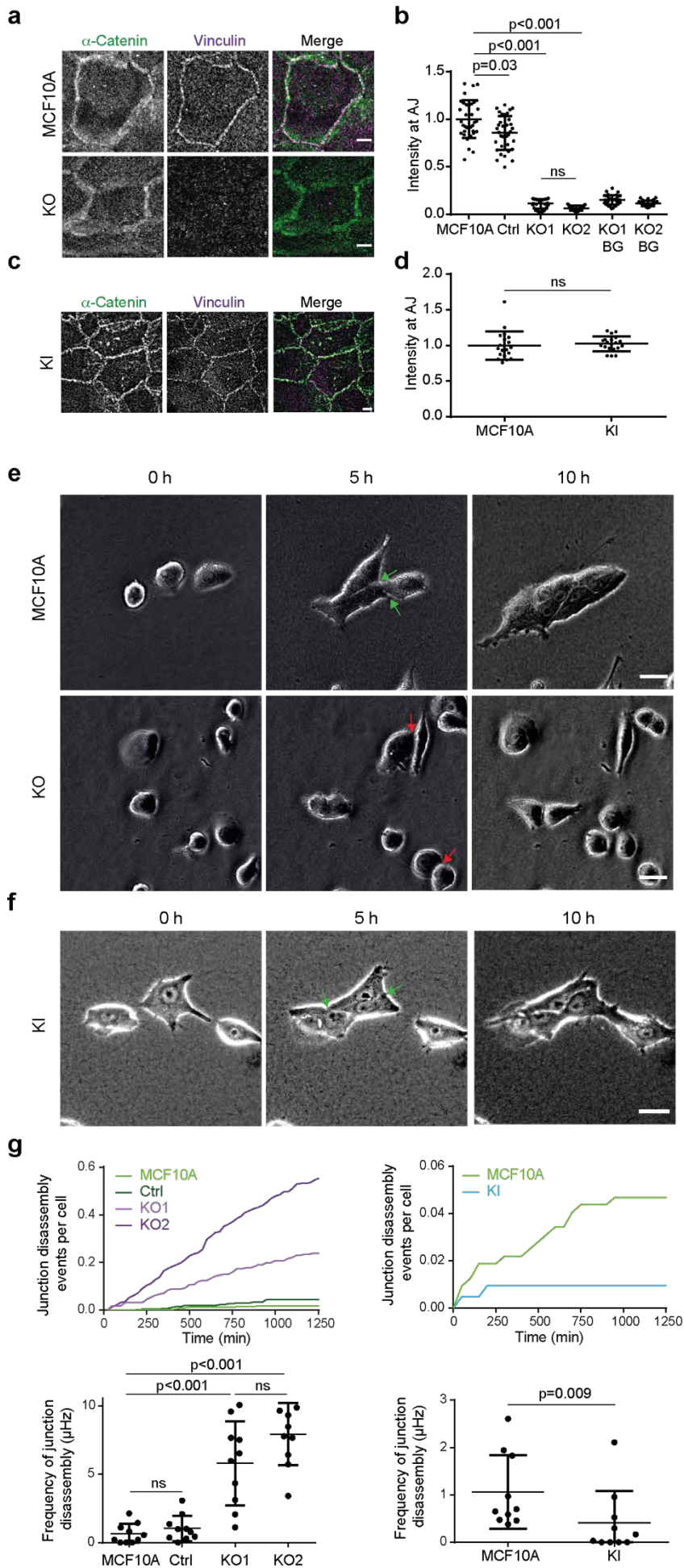


Figure 4. Vinculin stabilizes cell-cell junctions. a-d Presence of vinculin at cell-cell junctions in KO and KI cells. Staining of vinculin and α -catenin (**a, c**) and quantification of intensity at cell-cell junctions (**b,d**). Max z-projection from confocal microscopy. Scale bar 5 μ m. Mean \pm SD, n=37 for **b**, n= 20 for **d**, t-test. N=3 with similar results, pooled measurements from 3 independent repeats are plotted. **e,f** Time-lapse imaging of KO (**e**) and KI (**f**) cells in 3D collagen gels by phase contrast. Green arrows point at cell-cell junctions that were present at 5 h and that did not disassemble at 10 h, red arrows point at cell-cell junctions that were present at 5 h and that were disassembled at 10 h. Scale bars 50 μ m in a, 25 μ m in b. **g** Quantification of cell-cell junction disassembly events per cell and frequency of cell-cell junction disassembly. Mean \pm SD, n=10, t-test. N=3 with similar results, pooled measurements from 3 independent repeats are plotted.

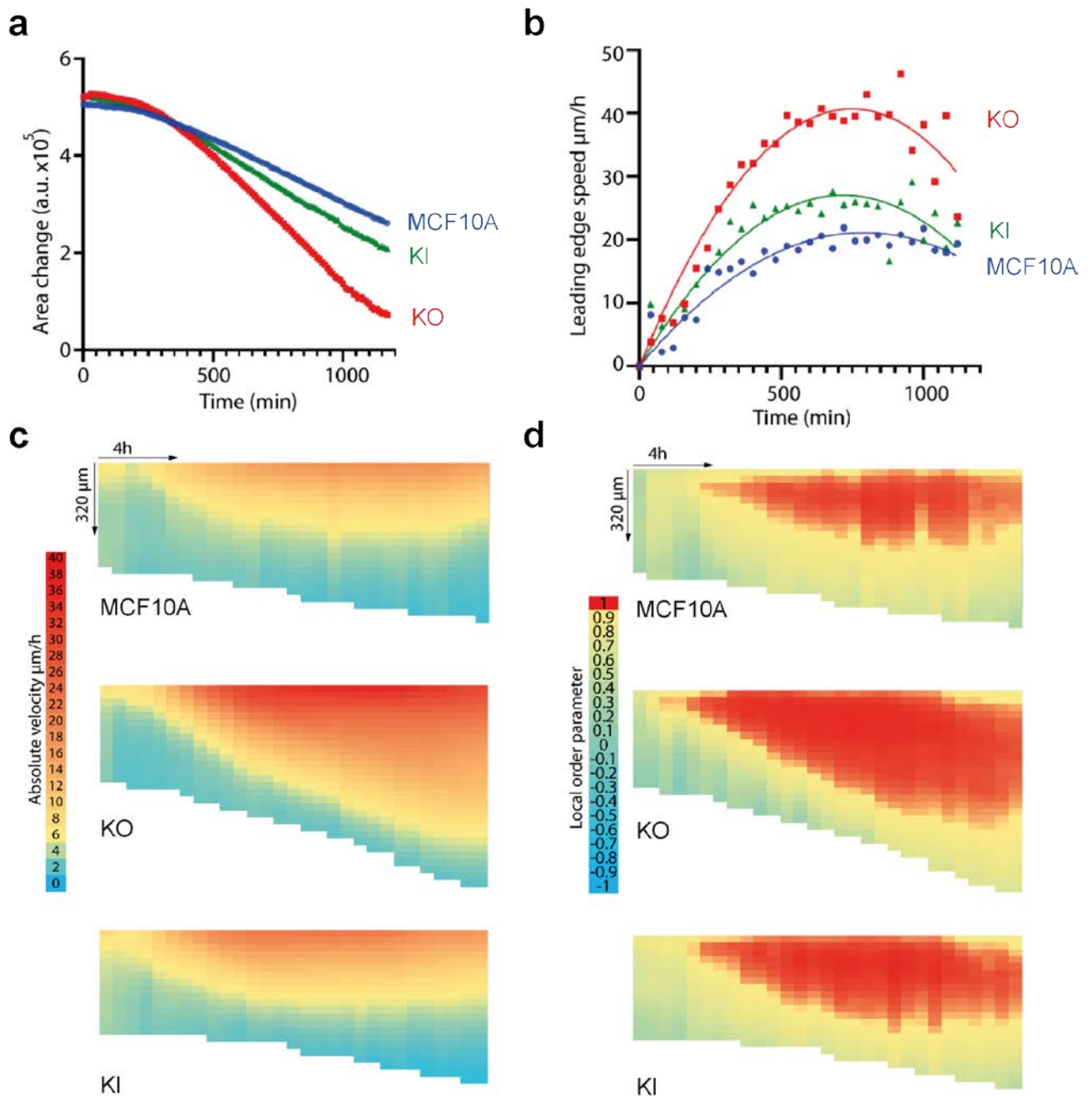


Figure 5. Vinculin controls collective migration upon wound healing. Collective migration of MCF10A, KO and KI cells over the wound was imaged by phase contrast over time and analyzed by Particle Image Velocimetry. **a** Quantification of free area. **b** Quantification of leading edge speed. **c** Heat map representation of velocity (length of displacement vectors). **d** Heat map representation of velocity (cosine of angles between adjacent displacement vectors). $N=3$, 1 representative experiment shown.

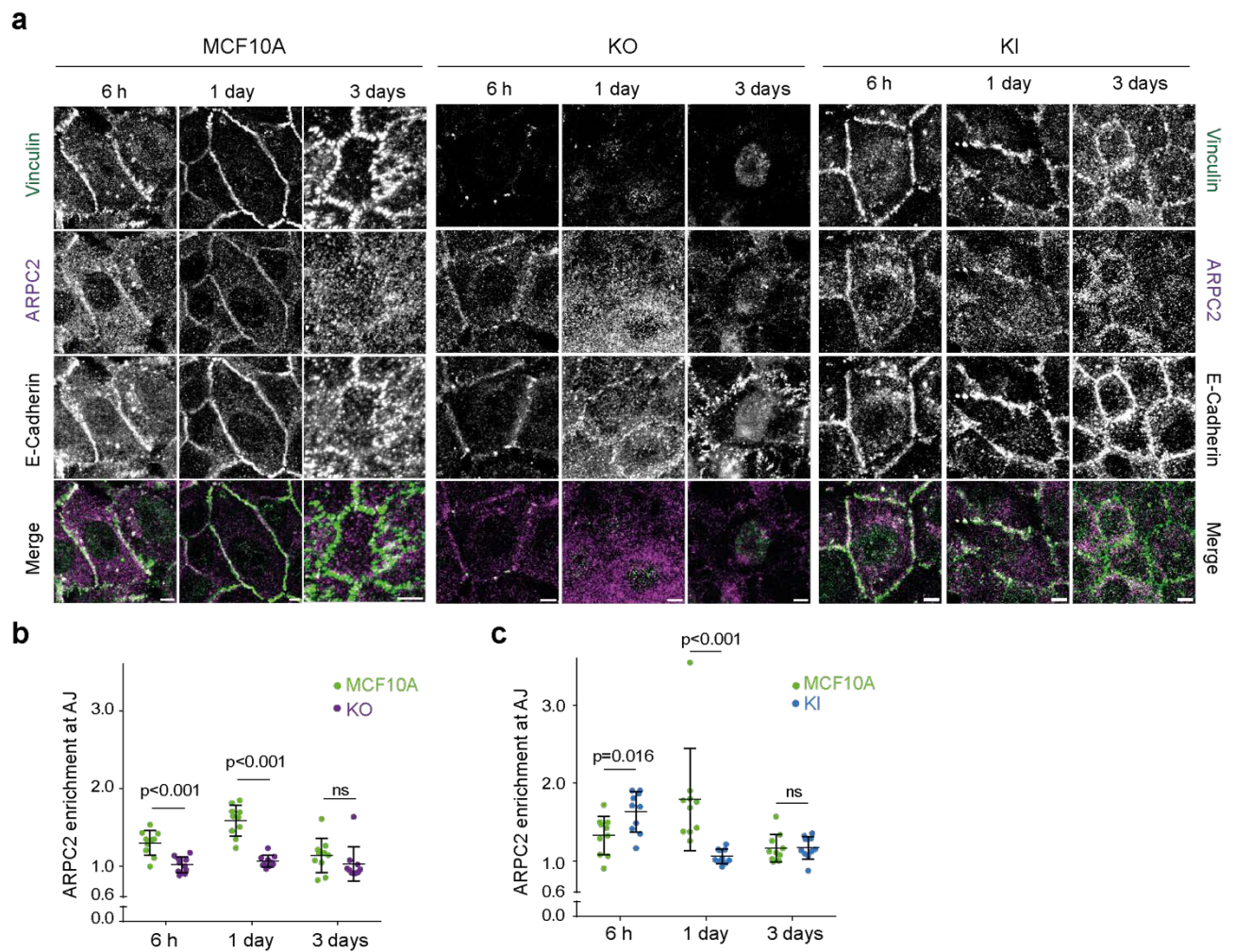


Figure 6. Vinculin retains Arp2/3 at cell-cell junctions. **a** Staining of vinculin, ARPC2 and E-cadherin 6 h, 1 day or 3 days after plating. Scale bar 5 μ m. Max z-projection from confocal microscopy. **b,c** Quantification of ARPC2 enrichment at adherens junctions in KO (**b**) and KI (**c**) cells. Mean \pm SD, n=10, t-test. N=3, 1 representative experiment shown.

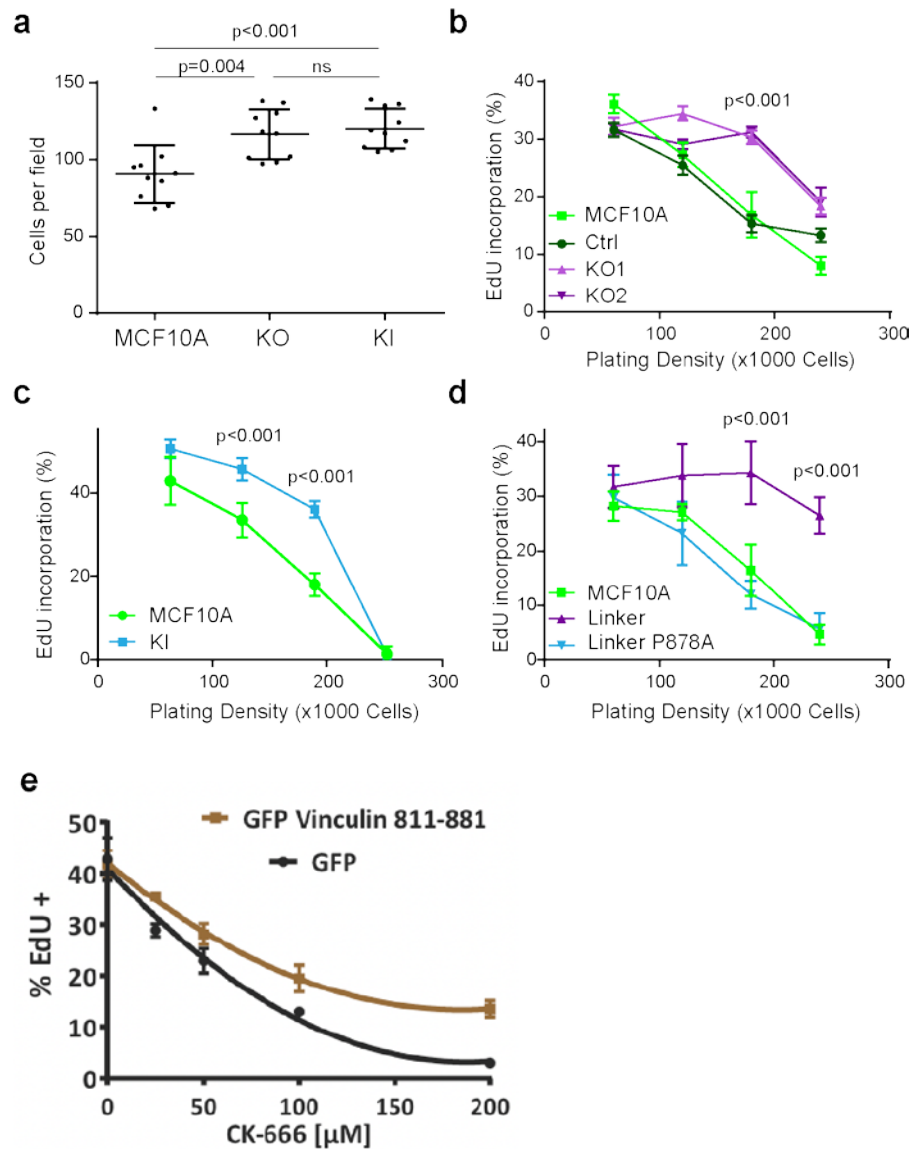


Figure 7. Vinculin controls cell cycle progression through its interaction with the Arp2/3 complex. **a** Saturation density of KO and KI cells. Mean \pm SD, $n=10$, t-test. $N=3$, 1 representative experiment shown. **b-d** Cell cycle progression of KO (**b**), KI (**c**) and linker expressing MCF10A cells (**d**). Percentage of cells incorporating EdU is represented as a function of cell density. Mean \pm SD, $n=8$ fields of views with more than 5000 cells in total, t-test. $N=3$, 1 representative experiment shown. p-values are shown only when both KO are different from both controls in **a**, and when the linker is significantly different from parental MCF10A in **d**. **e** Cell cycle progression of MCF10A cells expressing the vinculin linker treated with increasing doses of the Arp2/3 inhibitory compound CK-666.

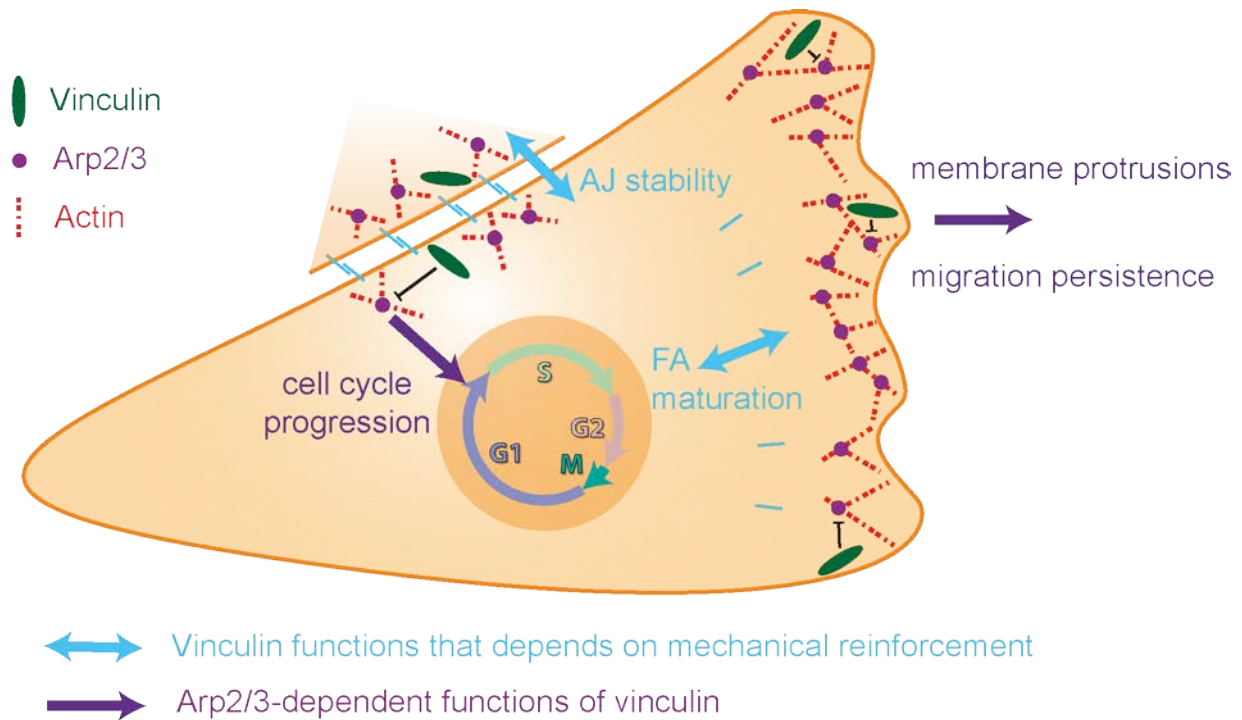


Figure 8. Vinculin controls cell migration and cell cycle progression through its ability to interact with the Arp2/3 complex. The vinculin-Arp2/3 interaction antagonizes actin polymerization in membrane protrusion and inhibits migration persistence of single cells. The actin reinforcement that vinculin provides stabilizes cell-cell junctions. Subsequent vinculin-dependent recruitment of Arp2/3 at cell-cell junctions contributes to collective migration and signals density-dependent inhibition of cell cycle progression.

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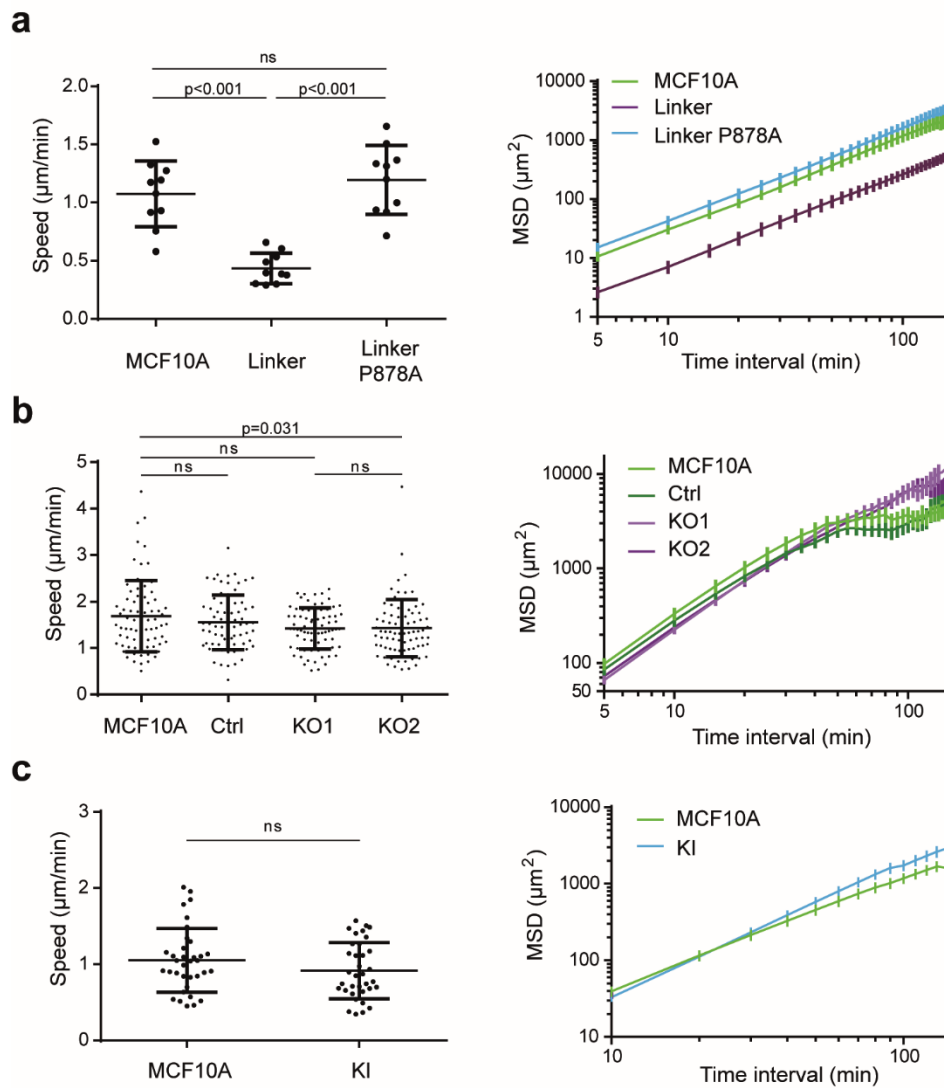
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SUPPLEMENTARY INFORMATION

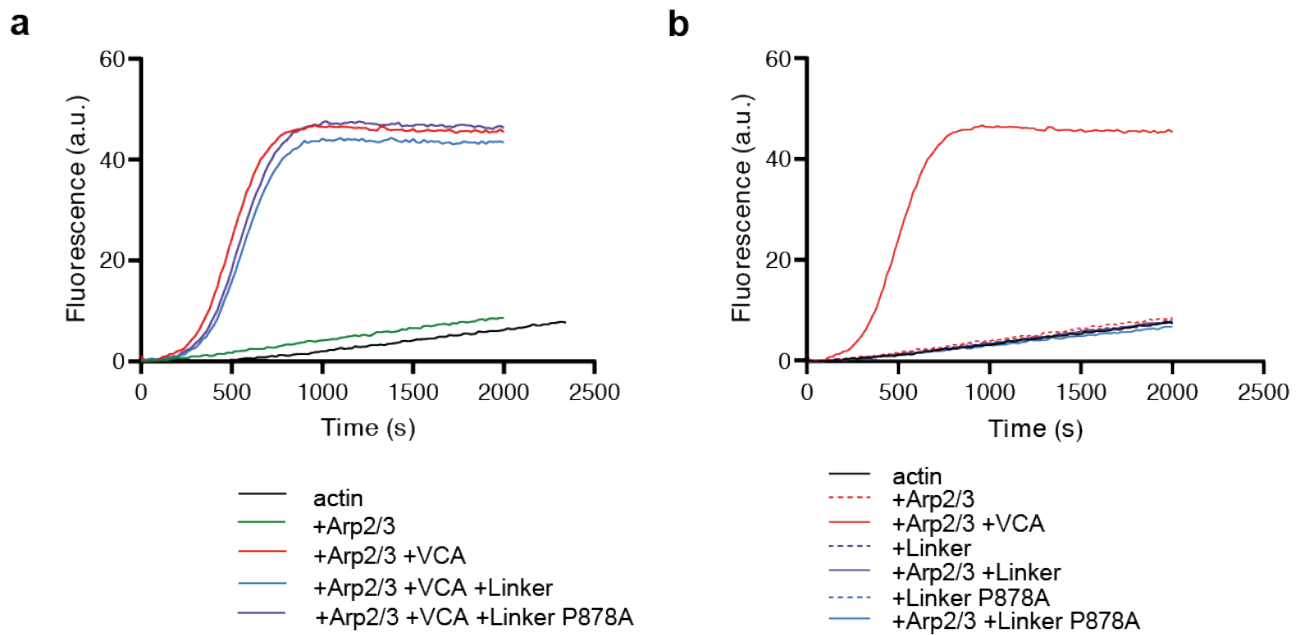
Supplementary Figures 2

Legends to Supplementary Movies 8

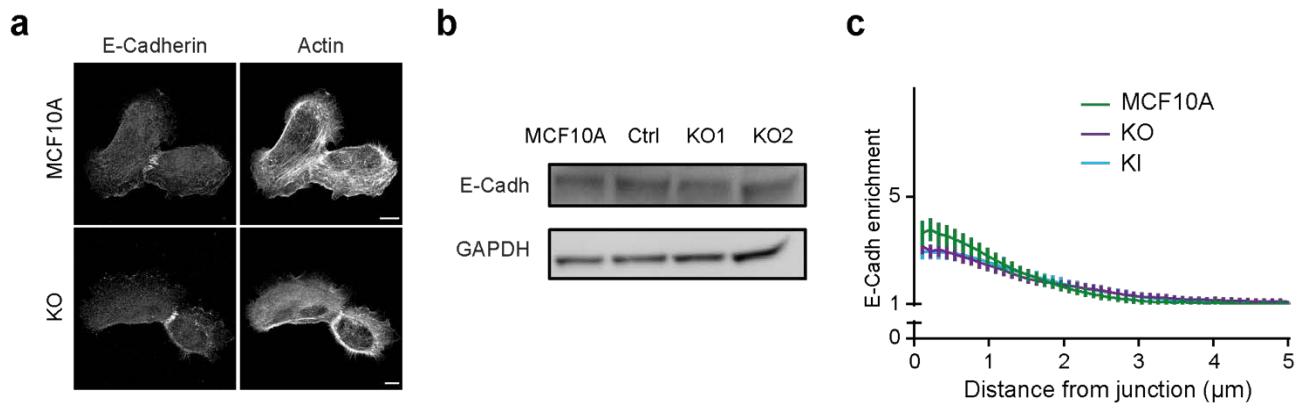
SUPPLEMENTARY FIGURES



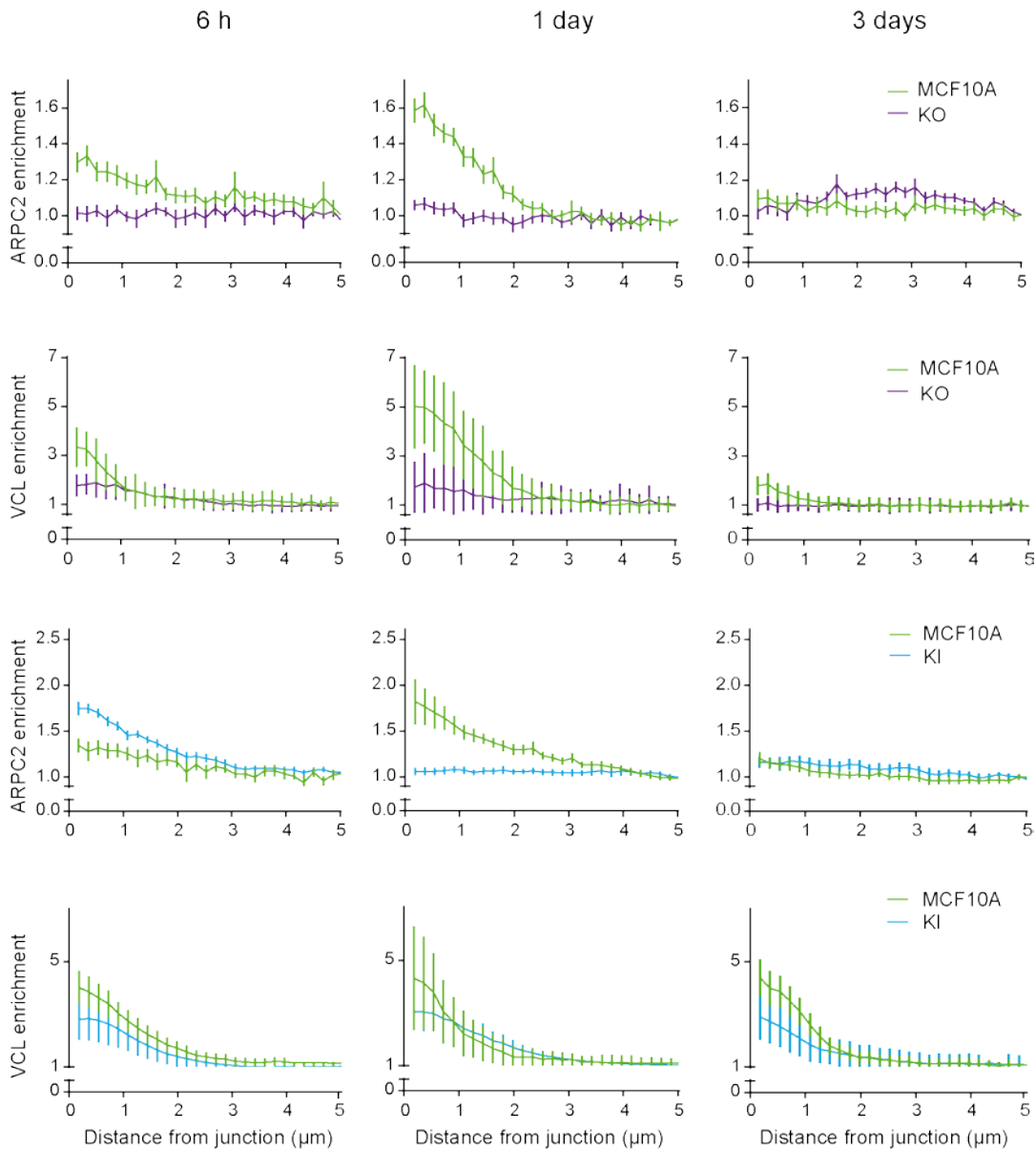
Supplementary Figure 1. Speed and Mean Square Displacement (MSD). **a** MCF10A cells expressing the vinculin linker (n=10). **b** KO cells (n=74). **c** KI cells (n=35). Mean \pm SD, t-test, N=3, 1 representative experiment shown.



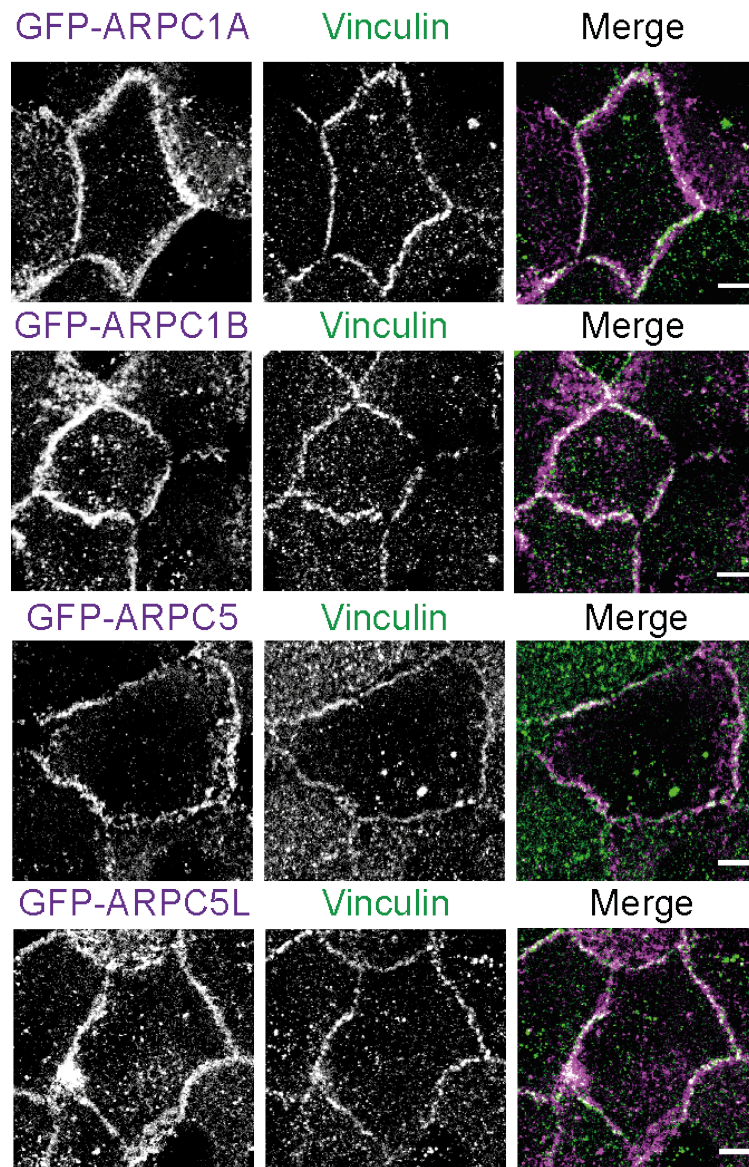
Supplementary Figure 2. Pyrene-actin polymerization assays. **a** The vinculin linker does not regulate Arp2/3 activity in vitro. **b** The vinculin linker does not affect actin polymerization in vitro. Conditions: 1.5 μM of 10% pyrenyl-labelled actin, 20 nM Arp2/3, 250 nM VCA and 1 μM vinculin linker or P878A linker as indicated. All these curves were acquired the same day and the Arp2/3 + VCA is replotted in the two panels for comparison.



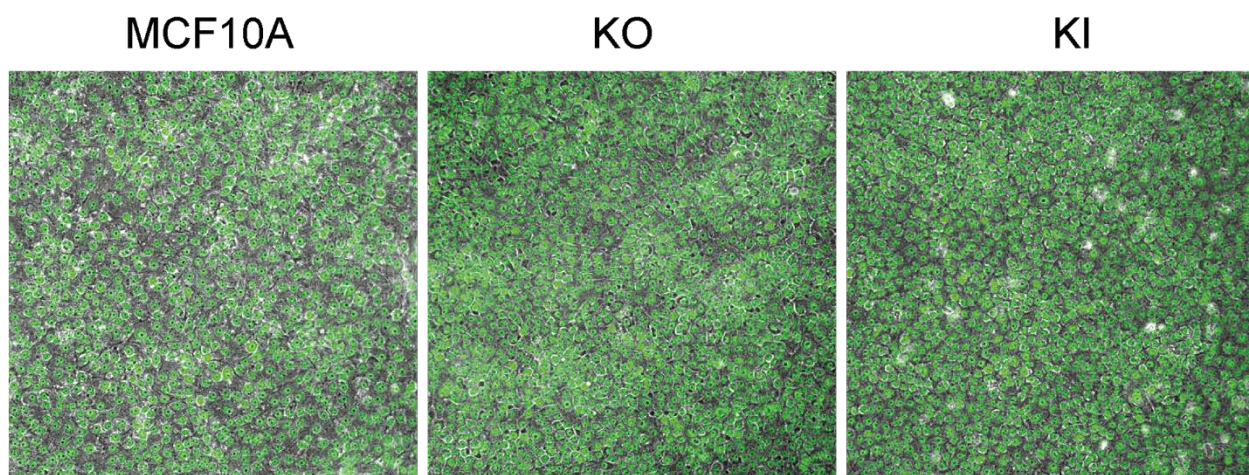
Supplementary Figure 3. E-Cadherin in KO and KI clones. (a) Staining of E-cadherin and F-actin using phalloidin of parental and KO cells, 1 day after plating on glass coverslips. (b) Western blot analysis of KO clones. (c) Enrichment of E-Cadherin at cell-cell junctions of parental, KO and KI cell monolayers, 1 day after plating. Mean \pm SD at each distance are plotted. N=3, 1 representative experiment shown.



Supplementary Figure 4. Enrichment of ARPC2 and vinculin at cell-cell junctions of KO and KI cells. Normalized intensity of staining plotted against distance from junctions, 6 hours, 1 day and 3 days after plating. Mean \pm SD at each distance are plotted. $n=10$, $N=3$, 1 representative experiment shown.



Supplementary Figure 5. Localisation of various Arp2/3 subunits at cell-cell junctions. Stable MCF10A clones expressing the indicated GFP fusion proteins were fixed 1 day after plating and stained with vinculin antibodies. Max z-projection from confocal microscopy. Scale bars 5 μm .



Supplementary Figure 5. Saturation density of KO and KI cells. DAPI staining (green) was overlaid onto phase contrast images.

LEGENDS TO SUPPLEMENTARY MOVIES

Supplementary Movie 1, related to figure 1d. Single cell migration of MCF10A cells expressing the vinculin linker. Cell tracks are superimposed on phase contrast images. Scale bar: 25 μm .

Supplementary Movie 2, related to figure 1h. Actin dynamics at membrane protrusions of MCF10A cells expressing the vinculin linker. Stable MCF10A cells expressing the WT or the P878A linker were transiently transfected with a plasmid encoding mCherry-actin. TIRF-SIM. Scale bar: 1 μm .

Supplementary Movie 3, related to figure 3. Single cell migration of KO and KI cells. Cell tracks are superimposed on phase contrast images. Scale bar: 25 μm .

Supplementary Movie 4, related to figure 1d. Actin dynamics at membrane protrusions of KO and KI cells. MCF10A, KO2 and KI cells were transiently transfected with a plasmid encoding mCherry-actin. TIRF-SIM. Scale bars 5 μm (left), 1 μm (right).

Supplementary Movie 5, related to figure 6a. MCF10A and KO2 cells were sandwiched between two collagen gels and imaged with phase contrast optics. Scale bar: 50 μm .

Supplementary Movie 6, related to figure 6b. MCF10A and KI cells were sandwiched between two collagen gels and imaged with phase contrast optics. Scale bar: 50 μm .

Vinculin-Arp2/3 interaction coordinates collective migration and actin organisation at adherens junctions

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Abstract

Collective cell migration is organised by adherens junctions which serve as both structural links between actin cytoskeleton of adjacent cells as well mechanotransductory structures allowing cells to transmit mechanical signals. Vinculin is a protein of focal adhesions and adherens junctions which contributes to junction maturation by binding to actin and the junction, bundling actin and antagonising branching of actin fibres. Here we have analysed the collective migration and junctional actin organisation of 2 vinculin mutant cell lines - vinculin KO in which all vinculin function is lost and vinculin P878A-KI in which only the vinculin-Arp2/3 interaction and subsequent antagonism of branched actin is perturbed. We found that the vinculin-Arp2/3 interaction is required to form apical contractile bundles. Vinculin is also essential to organise these bundles into a long-range network of transcellular actin fibres, coordinated across multiple cells found in MCF10A monolayers and acini. When connected by the long-range actin network, the first row of cells healing a wound migrate collectively and create coordinated lamellipodia. Vinculin KO cells, which are connected by a short-range actin network migrate less collectively during hypotonic unjamming. Thus, we have shown the vinculin-Arp2/3 interaction at cell-cell junctions plays a key role in organising collective migration.

INTRODUCTION

Collective migration is an important process in development and tissue maintenance. Collective migration is result of coordination between two processes governed by the actin cytoskeleton - individual cells are able to migrate by utilising pushing forces created by Arp2/3-mediated branched actin polymerisation to extend protrusions called lamellipodia. To coordinate migration, cells can communicate through structures which connect the actin cytoskeleton, adherens junctions

Adherens junctions (AJs) specifically, not only connect actin cytoskeletons of adjacent cells but also serve as mechanosensory structures. Disrupting the connection between cells by the depleting calcium ions required for cadherin-cadherin interactions has been shown to lead to loss collectivity (Jain et al. 2020). On the other, α -catenin, the primary mechanosensory molecule at AJs is required to order zebrafish mesendodermal cells where leader cells are guided by mechanical forces from their followers (Ozawa et al. 2020). The classical view of an adherens junctions have the actin cytoskeleton parallel to the surface of contact between cells and cadherins are uniformly distributed along this surface. However, recent evidences with higher resolution imaging show that both branched and linear actin networks coexist at the cell junctions and that cadherin puncta are in fact not entirely uniform along the cell-cell contact (Li et al. 2019). Focal adherens junctions (FAJs) are a subset AJs found in contractile monolayers and collectively migrating cells, characterised by the actin fibres being arranged perpendicular to the surface of contact between cells (Malinova et al., 2017). These junctions display a more punctate distribution of cadherins along the cell-cell contact with separate FAJs arranged radially along the cell boundary (Huveneers et al., 2012). FAJs are termed as such because they contain proteins like zyxin, MENA and TES (Oldenburg et al., 2015) which were initially observed at cell-substrate contacts called focal adhesions.

Vinculin is a mechanotransductory protein recruited to both FAs and AJs. Vinculin in the cytoplasm exists in an auto-inhibited conformation with its head domain bound to its tail and activated when recruited to cell adhesions (Chen et al. 2005). At an adherens junction β -catenin and α -catenin together connect the transmembrane cadherins to the actin cytoskeleton (Meng et al. 2009). The vinculin head domain binds to cryptic binding sites in α -catenin, which are revealed when the acto-myosin network applies contractile forces on the adhesive structures (Yonemura et al. 2010, Yao et al. 2014). The tail domain can then bind further actin filaments

and dimerise to bundle the actin network (Johnson et al. 2000, Shen et al. 2010), thus reinforcing the adhesion.

We previously showed that vinculin antagonises branched networks in the cell by binding to the Arp2/3 complex, the nucleator of branched actin filaments that generate pushing forces against cellular membrane. These pushing forces are essential for collective migration as they contribute to both lamellipodia formation and AJs maintenance. VCL^{-/-} cells which do not express vinculin and VCL P878A-KI cells which express a mutant vinculin that cannot bind Arp2/3 both show increased branched actin activity. Vinculin is required to recruit and retain Arp2/3 at adherens junctions and during wound healing, the vinculin-Arp2/3 interaction impacts the transmission of information regarding presence of the wound, from the leading edge to cells further away from wound.

Here we analysed the degree of collectivity in VCL^{-/-} and VCL P878A-KI cell lines, at leading edge of migration to allow us to distinguish the structural role of the vinculin-Arp2/3 interaction at adherens junctions, unaffected by defects in the transmission of this signal. We found defects in actin organisation at adherens junctions both during migration of a leading edge as well as in mature monolayers. We found that this defect in actin organisation was correlated with defects in collectivity of migration during hypotonic unjamming.

RESULTS

Vinculin-Arp2/3 interaction controls coordination of lamellipodia at the migration front

Parental MCF10A and vinculin KO monolayers were allowed to grow constrained by a plastic insert and were allowed to migrate after removal of the insert. To isolate the role of vinculin in reinforcing cell-cell junctions, we studied the degree of collectivity only across the first row of collectively migrating cells. In this case, the role of vinculin mechanotransduction transmitting the information to cells behind the wound is minimised as all the cells analysed received the same information about the availability of space to migrate into. Unlike other cell lines, the migration front of MCF10A cells is not organised by leader cells. We found that the first row of Parental MCF10A cells migrated uniformly into the free space (Fig. 1a). In comparison, many individual cells spread out from the first row of vinculin KO cells, suggesting a lack of coordination between migrating cells (Fig 1b). To quantify this, a line that follows the first row of cells was drawn. We calculated autocorrelation of angles along this line for distance intervals corresponding to one average cell size. This reflects the ability of cells to migrate in a coordinated manner. As shown in Fig 1d, migration of Parental MCF10A cells was more correlated compared to vinculin KO cells.

In parental MCF10A cells, multiple adjacent cells extend lamellipodia in the same direction, just as if the lamellipodium of one cell was fused to that of the next cell. In vinculin KO cells, the orientation of lamellipodia was less uniform, and each cell is extended by a well-defined lamellipodium (Fig. 1b). To quantify these properties of lamellipodia, we analysed the autocorrelation of angles of the migration front for distance intervals at the subcellular scale, which reflects uniformity of the lamellipodium over one cell distance maximum (Fig 1c). At this scale, we found that autocorrelation of angles of lamellipodia was lower for vinculin KO cells than for Parental MCF10A cells (Fig. 1c), showing that formation of lamellipodia in vinculin KO cells is less correlated than in parental MCF10A cells. Together this suggests that vinculin is required to induce coordinated lamellipodial protrusion and collective migration of first row of cells.

Lamellipodia are generated by the rapid polymerisation of actin networks mediated by Arp2/3. To investigate whether the vinculin-antagonized Arp2/3 activity coordinates lamellipodia extension over multiple cells, we performed similar analysis in the vinculin P878A KI cell line that carries the bi-allelic P878A mutation that prevents vinculin to bind Arp2/3 (James et al., in prep.). We found that the first row of vinculin P878A KI cells was

similarly uniform to that of Parental MCF10A cells (Fig 1e). Indeed, coordinated migration of parental MCF10 and vinculin P878A KI cells was similar because the curves of autocorrelation of angles of the first row of cells was similar for both cell lines (Fig. 1g). However, these curves for distance intervals at subcellular scales were higher for parental MCF10A cells compared to vinculin P878A KI cells (fig 1f), indicating a lack of coordination of lamellipodia extension between vinculin P878A KI cells. Taken together, these results suggest that the structural role of vinculin is important to keep cells together and collectively migrate. Moreover, the vinculin-Arp2/3 interaction is required for the coordination of lamellipodia between cell neighbours.

Because vinculin antagonises Arp2/3 activity (James et al., in prep.), we expected to see a difference in actin organisation during collective migration. Staining of actin in Parental MCF10A cells revealed a long-range network of linear actin bundles in the first row of cells migrating cells, parallel to the migration front (Fig 1h). Those actin bundles extend from cell to cell across cell-cell junctions, perpendicularly to the plane of contact between cells. Thus, this network shares similarities with the previously described transcellular actin fibres (TAFs) (Cochet-Escartin et al., 2014). These TAFs were found in the first row of cells facing the wound even prior to cells starting migration (Fig 1i). TAFs cross cell boundaries within punctate structures enriched in E-cadherin, reminiscent to FAJs (Red arrows in Fig 1i). Thus, FAJs at either end of the cell are connected by the same actin fibre (Yellow arrows in Fig 1i), in a similar manner to actin bundles attached to AJs in drosophila wings (Lopez-Gay et al. 2020). TAFs were not present in either vinculin KO or vinculin P878A KI cells, in which no vinculin-Arp2/3 interaction occurs (Fig 1h). This indicates that the antagonistic activity of vinculin on Arp2/3 is required to form TAFs, and suggest that TAFs are responsible for the coordination of lamellipodia protrusion between two cell neighbours.

Vinculin is required to coordinate transcellular actin fibres over long ranges

Parental MCF10A cells, being non-transformed human breast cells, provide a physiologically relevant model system to study the formation of TAFs at AJs. Z-stacks confocal imaging covering the whole height of phalloidin-stained monolayers allowed us to visualise actin networks at cell boundaries (middle of z-stacks), and actin networks at the apical face (Top of z-stacks, Fig 2a). The height of the monolayer being non-uniform across large distances necessitated imaging at small z-intervals (0.2um) followed by deconvolution and z-projection to visualise the apical actin network. As in migrating monolayers, we observed in monolayers of Parental MCF10A cells a network of TAFs that are coordinated across many

cell boundaries. Topographical maps showed that these TAFs follows the apical face of cells as they undulate along with the the height of the monolayer across the field (Fig 2a, right panel). We found a punctate localisation of vinculin and MENA, both FAJ proteins (Oldenburg et al. 2015) along the TAFs at the intersection of two cells (Fig 1b, c). This indicates that TAFs also cross cell boundaries in monolayers within FAJs, and directly connect FAJs at either end of a cell. We could not observe TAFs 1 day after seeding cells, but only in mature monolayers, 3 days post seeding (Fig 2d). Since TAFs are enriched in phospho-myosin (Fig 2e), TAFs are contractile fibres. We also examined actin staining in mammary organoids grown from Parental MCF10A cells, called acini and which are a model system for mammary glands, retaining a spherical shape forming a lumen by cell apoptosis in the centre (Underwood et al. 2006). Imaging the actin network at high resolution proved challenging in these large structures. However, we could observe faint actin bundles, which cross cell boundaries labelled with E-cadherin, and coordinated across multiple cells, indicating they are TAFs (Fig 2f).

In vinculin KO cells, immunostaining of MENA showed that FAJs still assembled (Fig 3b). However, FAJs were twice more elongated than FAJs in parental MCF10A cells (Fig 3b, 3c). Interestingly, the apical actin network is disorganised in in the interior of vinculin KO cells in a monolayer. As a result, FAJs at either end of a given cell are no more directly connected, but rather connected to the disorganised apical actin network (Fig 3b). Over long ranges, TAFs appeared thus less organised in vinculin KO cells than in parental MCF10A cells (Fig 3d). We counted the number of cells connected by a straight TAF experiencing a maximum direction change of 30°. In Parental MCF10A cells, TAFs connect up to 10 consecutive cells with a median of 4 cells connected (Fig 3e). In contrast, most TAFs only connect 2 adjacent vinculin KO cells. Using a persistence coefficient of TAFs calculated as the average number of cells connected by a TAF across a field, we found that TAFs connect 1.8-fold more cells in Parental MCF10A than in vinculin KO cells (Fig 3f). Thus, Parental MCF10A cells are able to form a long range TAF network while VCL^{-/-} cells for a short range TAF network. This highlights the role of vinculin to coordinate TAFs network over longer ranges.

Arp2/3 activity is finely tuned to form transcellular actin bundles

Next, we wanted to study the effect of Arp2/3 on formation of TAFs . We first used the Arp2/3 inhibitor CK666 to analyse the state of the apical actin network. When grown in the presence of low concentrations (6.25mM) of CK-666, Parental MCF10A monolayers displayed shorter TAFs than untreated monolayers (Fig 4a). The distribution of the number of cells

connected by a TAF was shifted toward 2 adjacent cells (Fig 4b), as for vinculin KO cells grown without CK666 (Fig 3e). As a result, the TAF persistence coefficient was reduced by 1.7-fold, so that only two cells were directly connected by the same TAF (Fig 4D). At a higher CK666 concentration that inhibits branched actin formation in MCF10A cells, 50mM, (Molinié et al., 2019), the apical actin cytoskeleton was completely disorganised (Fig 4A) and no actin bundles perpendicular to the plane of cell-cell contact was observed. Thus, formation as well as coordination of TAFs over long ranges requires Arp2/3 activity.

We next raised the possibility that increased Arp2/3 activity would enhance formation or coordination of TAFs. To upregulate the RAC1-WAVE-Arp2/3 pathway, we used a cell line that stably expresses RAC-Q61L, a constitutively active of mutant RAC1. Surprisingly, actin staining was only localised at cell boundaries in those monolayers and no TAFs were found at the apical surface (Fig 4E). We also examined the organization of the apical actin network in the vinculin P878A KI cell line, which also display an increased Arp2/3 activity as a consequence of the disruption of vinculin-Arp2/3 interaction (James et al., *in prep.*). As for monolayers of RAC-Q61L expressing cells, no apical actin bundles were formed in monolayers of vinculin P878A KI cells (Fig 4F), showing that the antagonism activity of Arp2/3 by vinculin plays an important role in apical actin organisation. Thus, upregulation of Arp2/3 activity also impairs in the formation of TAF networks. Taken together, these results show that Arp2/3 activity has to be finely regulated to connect cells by TAFs over large distances.

Vinculin-Arp2/3 interaction controls collectivity of migration during hypotonic unjamming

MCF10A monolayers are a well-characterized model of collective migration (Palamidessi et al. 2019; Malinverno et al. 2017). Indeed, adding hypotonic media to jammed MCF10A monolayers induced reawakened cell motility with increased properties of collective migration (Malinverno et al., 2017). To investigate the role Arp2/3 regulation by vinculin in collective migration of cells in MCF10A monolayers, we first sought to characterise dynamics of TAFs networks during unjamming. Actin staining after addition of hypotonic media on 3-day-old monolayers shows that the long-ranged network of TAFs were not disrupted at the apical face of parental MCF10A cells (Fig 5a). Three hours after hypotonic media addition, distributions of the number of cells connected by a TAF remained unchanged (Fig 5c) compared to untreated monolayers (fig 2e), with 4 cells directly connected together (Fig 5d).

Similarly, the short-range network of TAFs in monolayers of vinculin KO cells remained also unchanged after addition of hypotonic media (Fig 5 b,c,d).

We next addressed the role of vinculin in regulating collective migration in unjamming monolayers in the same conditions. We used Particle Image Velocimetry (PIV) to analyse monolayer unjamming in hypotonic conditions for MCF10A, vinculin KO and P878A KI cells. The resultant displacement vectors are plotted on phase contrast images. In line with previous reports (Malinverno 2017), this analysis reveals the domains in which parental MCF10A cells migrate collectively (Fig 5e). In vinculin KO cells, the overall correlation of displacement vectors appeared reduced compared to Parental MCF10A cells (Fig 5e). For parental MCF10A and vinculin KO cells, vorticity maps can be derived from these displacement vector and regions of extremes of vorticity indicate boundaries of domains. The distance between extremes of vorticity was smaller in vinculin KO cells compared to Parental MCF10A cells (Fig 5f) indicating that vinculin KO cells have smaller domains of collective migration than parental MCF10A cells. The coefficient of exponential decay of correlation of displacement vector over distance gives a measure of the distance over which cell movement is coordinated. During on unjamming, we found that the correlation distance of Parental MCF10A cells increases from around 2 cell length to 5 cell lengths (60 μ m), 3 hours after addition of hypotonic media. Vinculin KO have their correlation length decreased by 33% to 3 cell lengths. Thus vinculin KO cells are less collective than MCF10A cells, indicating that vinculin is required to maintain collectivity during hypotonic unjamming. For vinculin P878A-KI cells, we found a 2-fold increase in correlation distance compared to Parental MCF10A cells, to around 10 cells lengths. Together, these results suggest that the junctional reinforcement role of vinculin, independent of Arp2/3, plays an important role in coordinating collective migration. This role overrides the effect of the vinculin-Arp2/3 interaction that tends to decrease collectivity.

DISCUSSION

In a previous study we had established that vinculin antagonises Arp2/3 activity (James et al. in prep.) As a result, persistence of individual cell migration, which mainly depends on the balance between activating and inactivating feedback loops of Arp2/3 (Krause and Gautreau 2014), is increased in both vinculin KO and vinculin P878A-KI cells compared to Parental MCF10A cells. Here we show that vinculin also regulates collective cell migration. However, vinculin KO and vinculin P878A-KI cells behave differently from each other depending on the context of collective migration. We have used assays and analysis for collectivity of migration which are unbiased by the speed of migration and thus unbiased by the difference in ability of the cell lines to migrate individually. Hence, we believe that this work succeeded in isolating the effect of vinculin on collective migration in the context of adherens junctions rather than focal adhesions.

Previous studies have shown that Arp2/3 is required to push the cell membrane of adjacent cells together to maintain adherens junctions (Li et al. 2019). Thus, it comes as no surprise that RAC Q61L expressing cells which have high Arp2/3 activity, have a defect in actin organisation. The fact the VCL P878A-KI cells like RAC Q61L cells have no TAFs supports our previous findings that the vinculin-Arp2/3 interaction antagonises branched actin activity at cell-cell junctions. Vinculin has been previously implicated in the bundling of actin fibres by dimerization of its tail domain. The reorganisation of branched networks to linear networks occurs both during migration, as nascent adhesions mature into focal adhesions, as well as during adherens junction maturation. Vinculin, being conserved in both these structures and having the ability actin as well as antagonise Arp2/3, is the prime candidate to explain this reorganisation. The lack of coordination of the transcellular fibres in vinculin KO cells can be explained as a result of disrupting these functions. However, whether the direct vinculin-Arp2/3 binding contributes to bundling of fibres is still unclear as the VCL P878A-KI cells do not form fibres that could be a substrate for bundling.

We have established here, a system of 3 cell lines having different actin organisation at the apical surface - Parental MCF10A cells have a long-range network, vinculin KO cells have a short-range network and vinculin P878A-KI cells have no transcellular network. In wound healing assays, this apical network seems to have an effect on directionality of lamellipodia as cells without the transcellular network do not coordinate the extension of their lamellipodium. As one would expect, Parental MCF10A with the long-range network migrate more

collectively than vinculin KO cells with a short-range network. Vinculin P878A-KI cells on the other hand, migrate much more collectively in the absence of the network. Together, these results suggest that the transcellular actin network when present coordinates collectivity and overrides other mechanisms that govern collective migration.

METHODS

Cell culture, cell lines and reagents

MCF10A cells were cultured in DMEM:F12 GlutaMax (31331028, Gibco) supplemented with horse serum (5%, H1270, Sigma), EGF (20ng/ml, AF-100-15, Peprotech), hydrocortisone (0.5ug/ml, H0888-1G, Sigma), insulin (0.1%, I9278, Sigma), cholera toxin (0.1ug/ml, C8052, Sigma), Pen-Strep (1%, 15140122, Gibco). Cells were trypsinised (12605010, Gibco) and sub-cultured every 3 days. VCL^{-/-} and VCL P878A-KI cell lines were generated by CrispR-Cas9 based genome editing (James et al., in prep) and RacQ61L was stably expressed using plasmids reported previously (Molinie et al 2019). For CK666 treatment, cells were allowed to adhere for 6 hours in media without CK666 (182515, Sigma) before adding the drug.

Immunofluorescent staining and antibodies

Cells were permeabilized for 5mins in either 0.2% Triton X-100 for E-cadherin staining or 100% Ethanol at -20°C for all other staining, followed by blocking in 10% FBS in PBS. Primary antibodies against vinculin (#V9131, Sigma), E-cadherin (#MABT26, Merck), MENA (gift from Matthias Krause), phospho-myosin light chain 2 (Thr18/Ser19) (#3674, Cell Signalling) and laminin (#MAB19562, Sigma) were used followed by Acti-Stain 555 (Cytoskeleton) for actin, along with secondary antibodies, anti-mouse-647 (#A21236, Life technologies) and anti-rabbit-405 (#A34556, Life technologies) and anti-rat-647 (#A11007, Life technologies). Coverslips were mounted in Dako mounting medium (S3023, Agilent) and imaged the next day.

For acini growth, cells were seeded on top of X% polymerized matrigel (CB-40230C, Corning) in Millicell EZ SLIDE 8-well glass chamber slide (PEZGS0816, Millipore) in a medium containing 4ng/mL EGF (4ng/mL) and 1% serum and supplemented with 2% of matrigel. The media was changed every 3 days and acini were allowed to grow for 3 weeks. Acini were then fixed in 2% PFA in PBS and permeabilized with 0.5% Triton X-100. Acini were then rinsed in PBS with 100mM glycine and blocked successively with IF Buffer (PBS with 10% FBS, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween-20) and IF Buffer + 20 µg/ml goat anti-Rabbit Fc fragment (111-005-046, Jackson ImmunoResearch). Acini were incubated with the primary antibodies followed by IF buffer washes and then incubation with secondary antibodies and phalloidin. Slides were mounted with Abberior Mount Liquid Antifade

(Abberior) and sealed with valap. Images were acquired with a 40x (NA 0.65) water immersion objective on a Leica SP8 laser-scanning confocal microscope.

Imaging and Analysis

For analysing migration of the leading edge, 1.5×10^6 cells cm^{-2} were plated on ibidi dishes (#80466) with inserts. Inserts were removed 1 day after plating and phase contrast images were acquired on an Olympus IX83 using a 20x objective (NA 0.5) equipped with an Orca-Flash4.0 V3 camera (Hamamatsu). Coordinates of the leading of migration were extracted using the FIJI plugin JFilament. Analysis of autocorrelation of angle of the leading edge and lamellipodia over distance was done using algorithms previously described for analysing autocorrelation of angle of single cell migration (i.e., persistence) over time (Gorelik et al 2014). For hypotonic unjamming, 1×10^6 cells cm^{-2} were plated on ibidi Ph+ microslides (#80446) and treated with hypotonic media (9 parts cell culture medium and 1 part distilled water) after 3 days. Unjamming cells were imaged at 10 min intervals on an Olympus IX83 using a 20x objective (NA 0.5) equipped with an Orca-Flash4.0 V3 camera (Hamamatsu). PIV analysis was carried out as described in Garcia et al. 2015.

To image the transcellular actin network, 1.5×10^6 cells cm^{-2} were plated on fibronectin (10 $\mu\text{g}/\text{mL}$, F1141, Sigma) coated coverslips and stained 3 days later. Z-stacks were taken at 0.2 μm intervals using an SP8 laser-scanning confocal microscope (Leica) and were deconvolved using an adaptive blind algorithm based on theoretical PSFs in Autoquant X2 (Mediacybernetics). All immunofluorescent staining images are maximum z-projections. Colormap views were made using the Fiji plugin ZstackDepthColorCode. Length of FAJs and number of cells crossed by transcellular fibres were measured manually in FIJI.

FIGURES

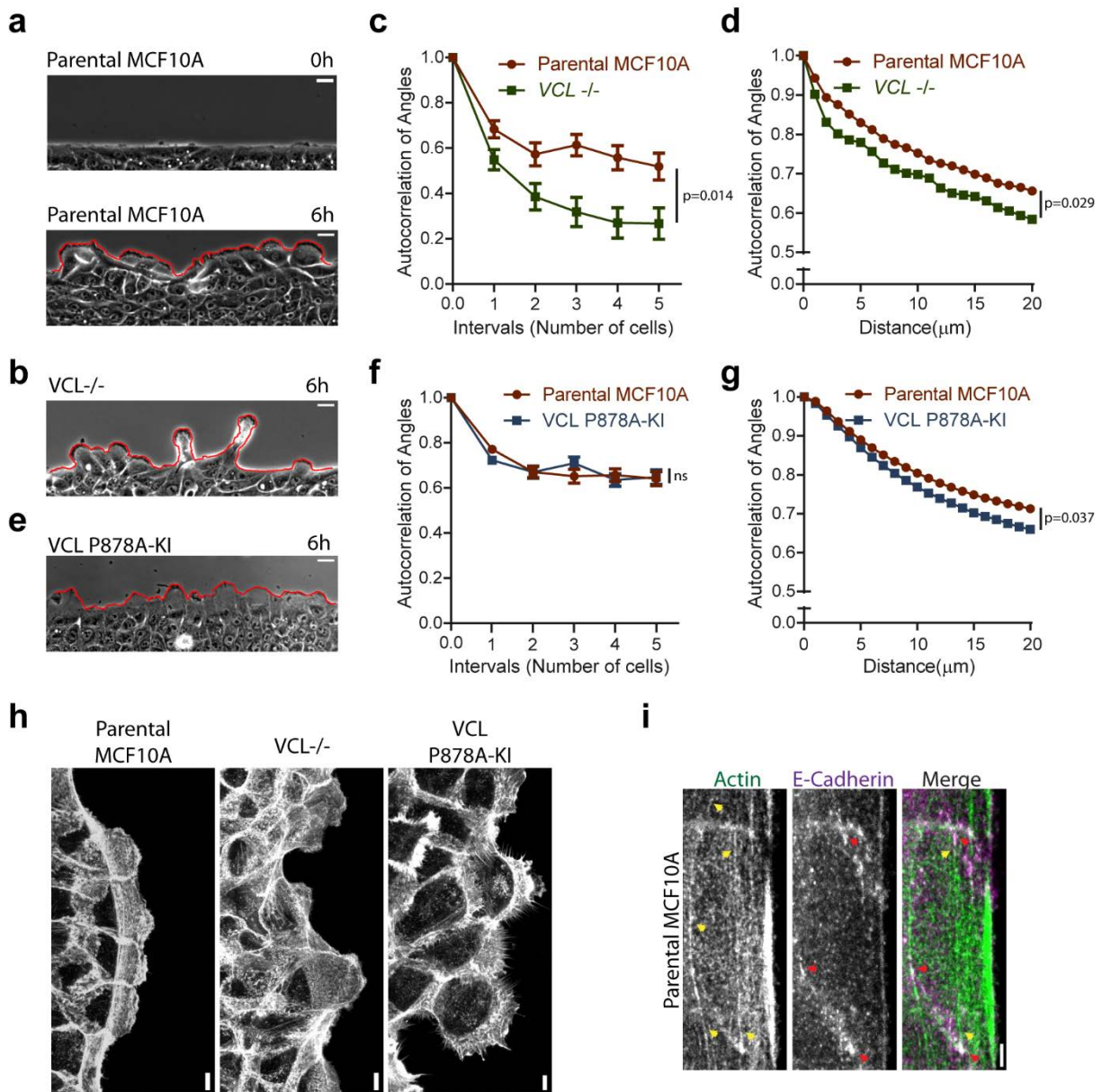


Figure 1. Vinculin coordinates lamellipodia during collective migration. **a,b,e** Migration of a leading edge of Parental MCF10A, VCL^{-/-} and VCL P878A-KI cells imaged in phase contrast. **c,f** Autocorrelation of angle of leading edge over multiple cell lengths. 1 Cell length corresponds to 20 μm **d,g** Autocorrelation of angle of lamellipodia along the leading edge over short distances. (Mean \pm SD, One-way ANOVA of coefficient of exponential and plateau fits $n=10$, $N=3$ for b,c and $N=2$ for e,f. 1 representative experiment shown.) **h** Staining of actin in Parental MCF10A, VCL^{-/-} and VCL P878A cells 6h after migration of the leading edge. Scale bar = 5 μm . **i** Staining for actin and e-cadherin in Parental MCF10A cells grown constrained by a plastic insert 0h after removal of the insert. Scale bar = 3 μm . Yellow arrows show actin

fibres that cross multiple cells. Red arrows show E-cadherin puncta at points where actin fibres cross cell boundaries.

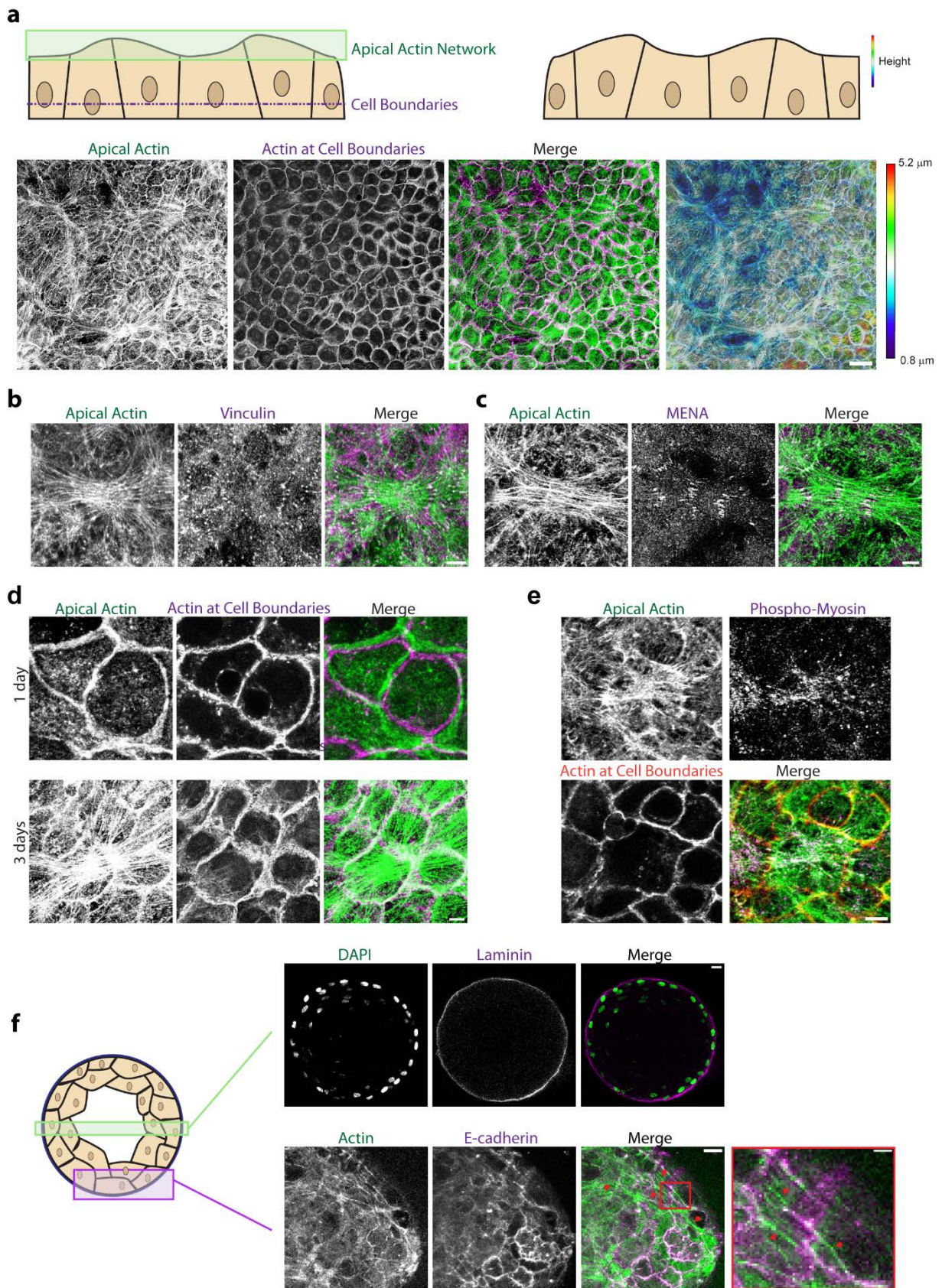


Figure 2. Parental MCF10A cells organize apical stress fibers to form a network of transcellular actin fibres. a Actin staining in Parental MCF10A monolayers 3 days post

seeding with apical planes showing the transcellular actin network and middle plane showing cell boundaries, along with a topographical color map of the monolayer (right panel). Scale bar = 20 μm **b,c** Staining of actin, vinculin and MENA in Parental MCF10A cells. Scale bar = 5 μm **d** Actin staining showing apical actin and actin at cell boundaries in Parental MCF10A monolayers 1- and 3-days post seeding. Scale bar = 5 μm **e** Staining of phospho-myosin along with apical actin and actin at cell boundaries in Parental MCF10A monolayers 3 days post seeding. **f** Staining of nuclei (DAPI), basal membrane (laminin), actin and E-cadherin in MCF10A acini Scale bar = 15 μm , 5 μm for zoomed in image (red rectangle).

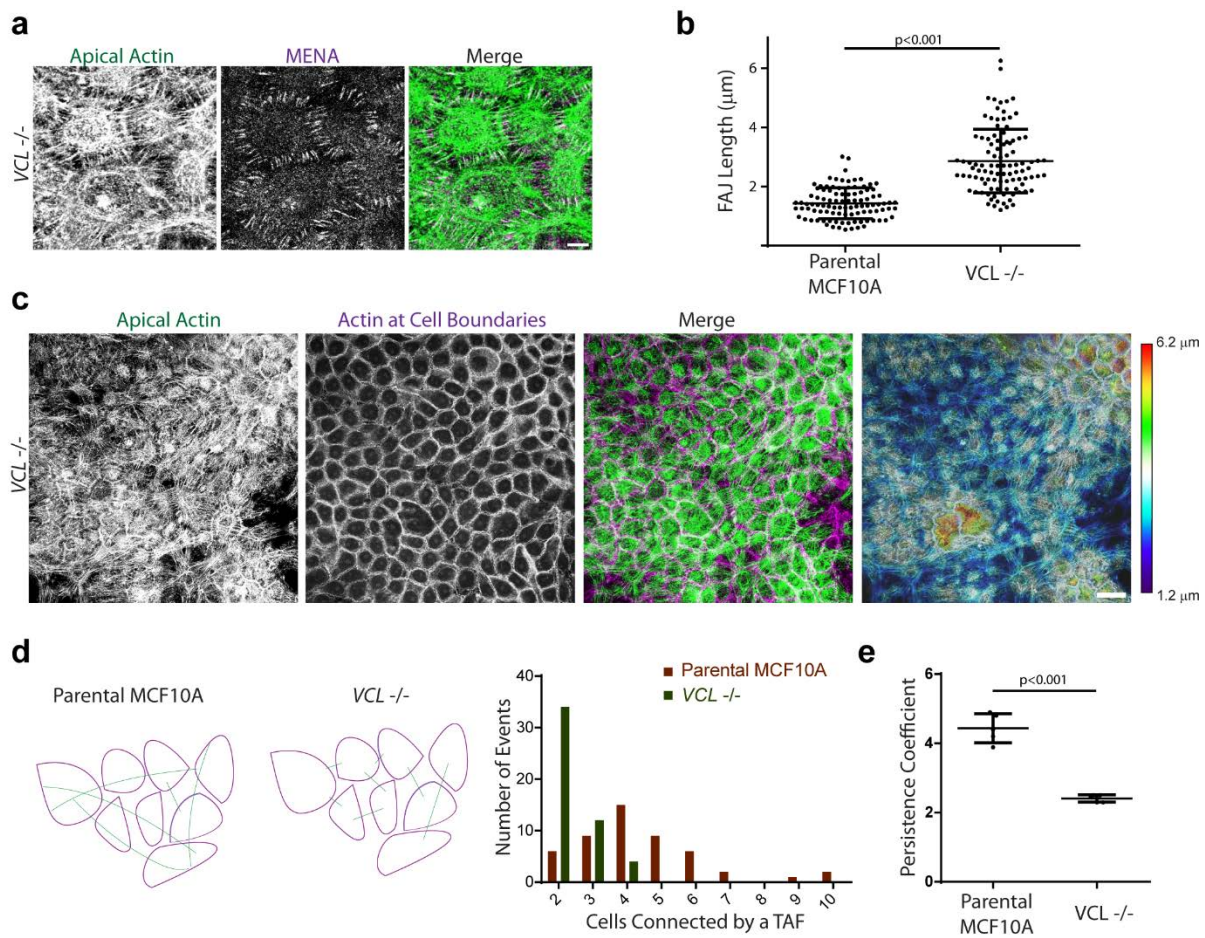


Figure 3. Vinculin coordinates the network of transcellular actin fibres across multiple cells. **a** Staining of actin and MENA in VCL^{-/-} cells Scale bar = 5 µm **c** Quantification of length of focal FAJs (Mean ± SD, t-test n=100, N=2, 1 representative experiment shown.) **c** Actin staining showing apical actin and cell boundaries in VCL^{-/-} monolayers, along with a topographical color map of the monolayer (right panel). Scale bar = 20 µm **d,e** Quantification of coordination of transcellular network. Histogram of number of cells connected by transcellular actin fibres and coefficient of persistence of fibres in Parental MCF10A and VCL^{-/-} monolayers (Mean ± SD, t-test n=5, N=3, 1 representative experiment shown.)

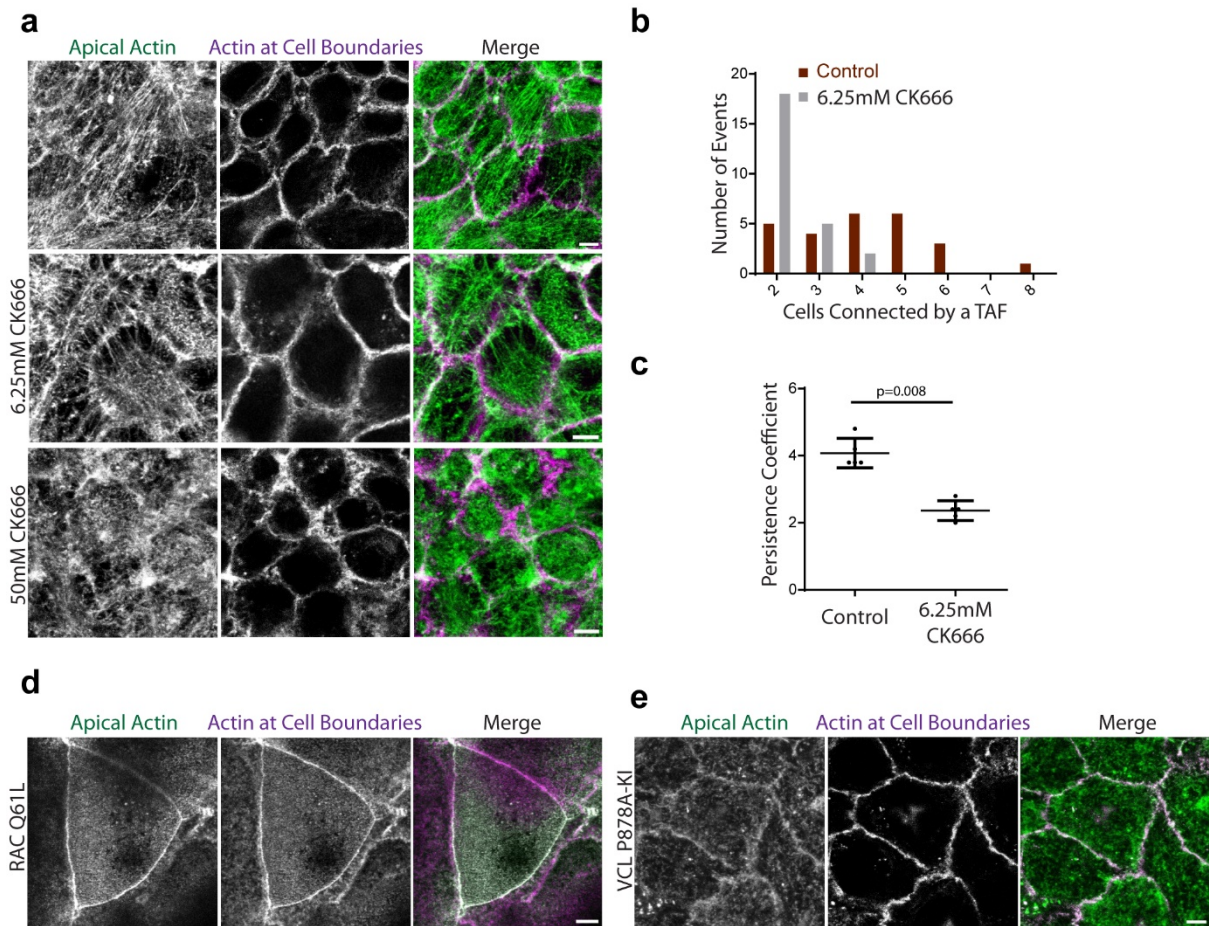


Figure 4. Arp2/3 activity is finely tuned to form transcellular actin fibres. **a** Actin staining showing apical actin and cell boundaries in Parental MCF10A monolayers grown with and without CK666. Scale bar = 5 μ m **b,c** Histogram of number of cells connected by transcellular fibres and coefficient of persistence of fibres. (Mean \pm SD, t-test $n=5$, $N=2$, 1 representative experiment shown.) **d,e** Actin staining showing apical actin and actin at cell boundaries in RACQ61L(**d**) and VCL P878A-KI cells(**e**). Scale bar = 5 μ m

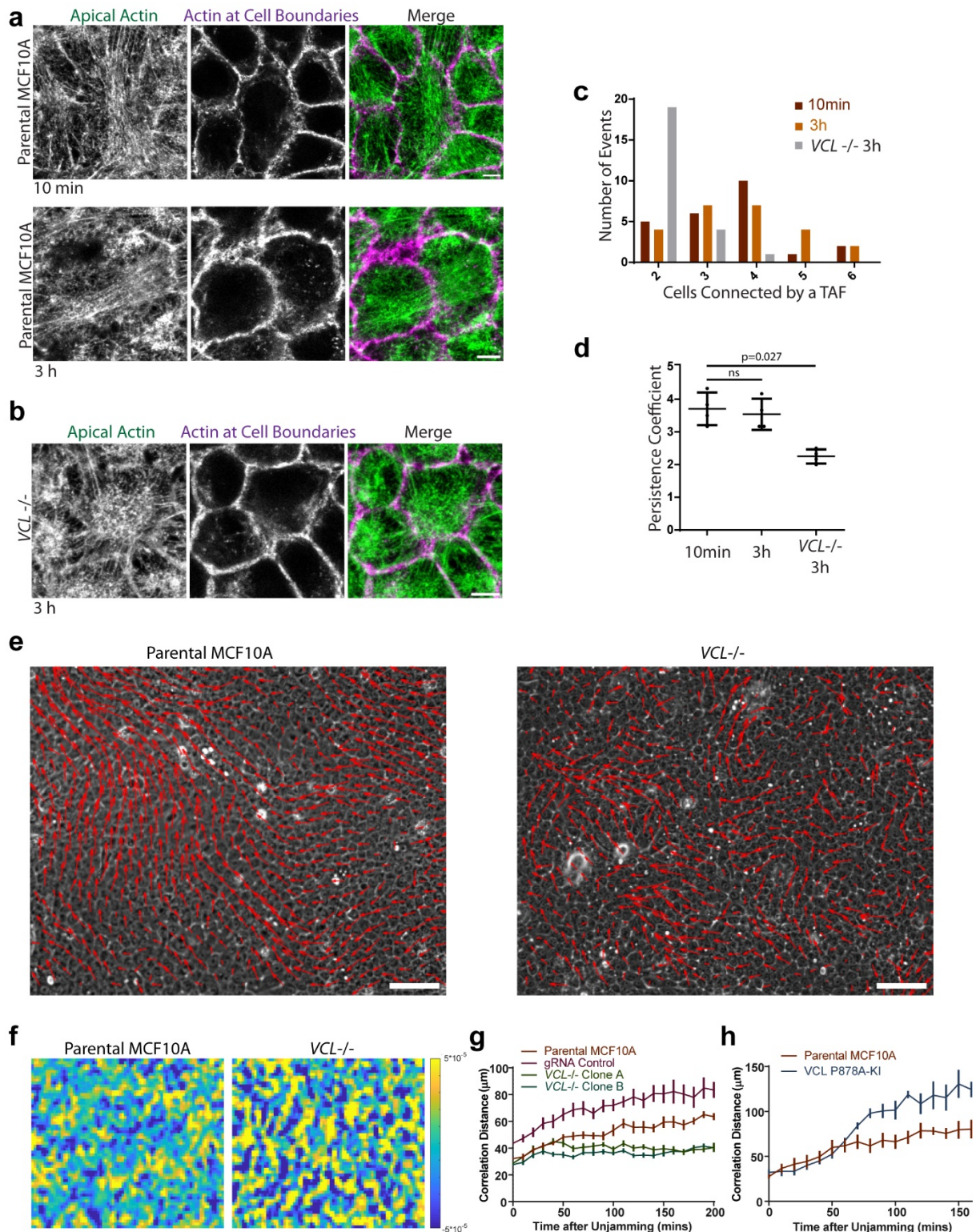


Figure 5. Vinculin and the vinculin-Arp2/3 interaction coordinate collective migration during hypotonic unjamming. a,b Actin staining showing apical actin and actin at cell boundaries in Parental MCF10A monolayers 10min and 3h(**a**) after hypotonic unjamming and VCL^{-/-} 3h after hypotonic unjamming(**b**). Scale bar = 5 μm **c,d** Histogram of number of cells connected by transcellular actin fibres and coefficient of persistence of fibres. (Mean \pm SD, t-

test n=4, N=2, 1 representative experiment shown.) **e,f** Displacement vectors from PIV analysis overlaid on phase contrast images(**e**) and vorticity maps of corresponding fields (**f**) of Parental MCF10A and VCL^{-/-} monolayers after hypotonic unjamming. Scale bar = 100 μm
g,h Correlation distance of migration for Parental MCF10A, VCL^{-/-} and VCL P878A-KI cells after hypotonic unjamming. (Mean \pm SD n=5, N=3, 1 representative experiment shown.)

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5 Perspectives and Discussion

Over the course of this project, we have shown that the vinculin-Arp2/3 interaction antagonises branched actin activity in several cell behaviours - single cell migration, proliferation, cell junction formation, cell junction maturation and collective cell migration. As with any research project, this work raises several open questions which can be the basis for future projects. In this section we will explore some of these questions along with possible experiments to unravel the relevance of this interaction further.

5.1 Molecular Basis of Interaction

Perhaps the most pressing question raised by this novel function is how the vinculin antagonises Arp2/3 and how this interaction occurs at the molecular level. We propose 2 possible mechanisms for this function - destabilisation/unbranching of branched actin networks or direct inhibition of the soluble Arp2/3 complex. These 2 possibilities are fundamentally different as the former would occur after creation of the branch while the latter would occur before creation of the branch. This difference could potentially be used to further understand the molecular basis of the interaction.

Vinculin has been shown to unbranch Arp2/3-generated networks through imaging of labelled actin filament immobilised onto beads[160]. However, it is only the bundling activity of the dimerising vinculin tail that has been implicated in this process. It is possible that the vinculin-Arp2/3 interaction contributes to the bundling of branched networks. Adding P878A mutant vinculin to such an assay and measuring the kinetics of bundling would show us whether the P878A interaction is involved in unbranching of networks. Any effect on bundling could either occur by simply increasing recruitment of vinculin and its bundling tail to sites of branching actin or it could be that the vinculin-Arp2/3 interaction destabilises the branch. Any lack of change in bundling with WT and mutant vinculin could also point to another possibility, that vinculin

contributes to unbranching by competitively reducing binding of branched actin stabilisers like cortactin. This possibility could also be tested for by adding cortactin to this assay.

The second possibility is that vinculin binding to Arp2/3 directly inhibits Arp2/3 activity in a manner similar to Arpin. Measuring the rate of actin polymerisation in in-vitro branched actin polymerisation assays, with and without the interaction would allow us to understand if vinculin binding results in a direct inhibition. Adding the vinculin linker directly to polymerisation assays does not have an effect on actin polymerisation. These results remain inconclusive since we were unable to reconstitute the interaction by simply having full length vinculin in solution with actin and Arp2/3.

This lack of interaction in-vitro points toward post-translation modifications of vinculin or Arp2/3 that regulate their interaction. It has been shown that phosphorylation of vinculin amino acids Y100 and Y1065 in the head and tail domains respectively leads to increased interaction with Arp2/3[217]. We also found in preliminary experiments that using a lysis buffer containing phosphate inhibitors also leads to an increase in interaction between the Arp2/3 complex and the vinculin linker. This suggests that there is more to the interaction than just phosphorylation of the vinculin head and tail. A potential site of phosphorylation on the vinculin linker, Y822, does not seem to have an effect on the interaction[217]. This points towards phosphorylation of Arp2/3 as a potential regulation of the interaction.

Interestingly, we also found in preliminary experiments that the amount of Arp2/3 pulled down with the vinculin linker at different time points post-seeding recapitulated the kinetics of enrichment of Arp2/3 at cell junctions. We found more interaction between Arp2/3 and the linker 1 day post seeding cells compared to 6h and 3 days just as there is more Arp2/3 co-localised with endogenous vinculin at cell junctions at 1 day compared to 6h and 3 days. Given that the linker is not subject to the same regulatory mechanisms as full length vinculin, this does suggest that there is something special about the pool of Arp2/3 at cell junctions that interacts with vinculin.

Understanding the effect of Arp2/3 modification on the regulation of this interaction could prove be much more challenging since the binding site on Arp2/3 is not known. Over-expressing the different Arp2/3 subunits in separate cell lines and comparing the amounts of vinculin and Arp2/3 complex pulled down in co-immunoprecipitation assays for the subunits would allow us to see if vinculin can bind any of the subunits individually. Additionally, ARPC1B and ARPC5L have been shown to come together to form Arp2/3 complexes which establish branched actin networks with higher stability

that complexes which contain ARPC1A and ARPC5[78]. We have seen that all 4 of these subunits of Arp2/3 are co-localised with vinculin and preliminary results show that all 4 of these subunits are pulled down with the vinculin linker. This suggests that vinculin does not have any specificity of complexes defined by these subunits. To go a bit further, we could analyse the amounts of Arp2/3 complex pulled down with vinculin from cells in which these different complexes are knocked down using siRNA against their respective constituent proteins.

5.2 Cellular Localisation of Interaction

We have shown that the vinculin interaction antagonises Arp2/3 at lamellipodia and cell junctions. However, it remains unclear whether it is vinculin at the adhesion that interacts with Arp2/3 or vinculin in the cytoplasm. This distinction is especially relevant since vinculin is in its closed conformation in the cytoplasm and in an open conformation at cell adhesions.

It has been shown that vinculin precipitates more Arp2/3 when lysates are incubated with exogenous PIP2[192]. This suggests that it is activated vinculin that interacts with Arp2/3. Thus it is likely vinculin at adhesion sites that interacts with Arp2/3. This hypothesis could be tested further using vinculin mutants where the binding of the head to the tail is impaired. A mutant vinculin referred to as a T12 mutant (D974A, K975A, R976A, R978A) remains in the open conformation[148] and co-immunoprecipitation experiments with such a mutant would allow us to see any changes in binding affinity towards Arp2/3.

A second question this raises is whether the interaction occurs through the same mechanism at lamellipodia and cell-junctions. We have shown here that vinculin antagonises Arp2/3 activity at lamellipodia by changing the dynamics of polymerising networks. On the other hand, we have shown that vinculin antagonises Arp2/3 activity at cell junctions by impacting the recruitment of Arp2/3. The difficulty of imaging at the apical surface of the cell prohibits us from looking directly at actin polymerisation at adherens junctions. High resolution imaging of cadherin based cell adhesions has previously been achieved by plating cells on coverslips coated with E-cadherin[185]. To investigate any differences between the interaction in lamellipodia and at adherens junctions, one could possibly perform TIRF-SIM imaging to look at protrusion dynamics and actin retrograde flow on coverslips coated with E-cadherin, just as we have done on fibronectin coated surfaces.

If it is indeed open vinculin that interacts with Arp2/3, it may be interesting to consider the effect of a Y822 phosphorylation that is seen only at adherens junctions[183]. It has been shown that increasing Y822 phosphorylation does not impact the amount of vinculin-Arp2/3 interaction[217]. This suggests either that the Y822 phosphorylation has no impact on the interaction or that vinculin phosphorylated at Y822 does not interact at all with Arp2/3. It is however uncertain whether these experiments were done in conditions of cell density conducive to formation of adherens junctions. It would be interesting to perform co-immunoprecipitation experiments with Y822E and Y822D mutations of vinculin which are phosphomimetic and non-phosphorylatable respectively.

The mode of recruitment of Arp2/3 to cell junctions is also not entirely clear. It is dependant on vinculin but independent of its direct interaction with Arp2/3 and must be due to signalling downstream of vinculin, at adherens junctions. To understand this further, let us consider a comparison of cell-cell adhesions with cell substrate adhesions. At cell substrate adhesions, Arp2/3 is co-localised with vinculin only at newly forming adhesions and when cells begin spreading on fibronectin, not at focal adhesions[192]. Concomitantly we found that perturbing just the vinculin-Arp2/3 interaction does not have an effect on focal adhesion size. When cells form these early adhesions with the substrate during cell spreading, Arp2/3 activity is initiated by N-Wasp, while it is activated by WAVE during persistent migration[77]. We could expect a similar mechanism with N-Wasp recruiting Arp2/3 dependant on vinculin signalling at early stages of adherens junction formation and the vinculin-Arp2/3 interaction then retaining Arp2/3 at the junction to support maturation of the adhesion. N-Wasp has been reported to be present at adherens junctions colocalising with cortactin[219]. Looking at the kinetics of Arp2/3 recruitment in cell lines where N-Wasp is knocked down would give us further understanding of how Arp2/3 is recruited by vinculin.

5.3 Impact on Cell Behaviour

We have found that the knocking out vinculin leads to decreased collectivity of migration as well as formation of a short range transcellular actin network, while disrupting the vinculin-Arp2/3 interaction leads to an increase in collectivity and lack of a transcellular network. This suggests that cells without the network (VCL P878A-KI) are inherently very collective, and the transcellular networks constrains collective migration. A long range network gives rise to larger domains while a short range network gives rise to smaller domains of the size of 1 or 2 cells. Whether this is causative or

merely correlated is yet to be determined.

We have also found that downregulating Arp2/3 with low concentrations of CK666 leads to formation of a short range network, while upregulating Arp2/3 with a RACQ61L mutant leads to lack of the network. Testing these cells in hypotonic unjamming experiments to see if their collectivity is similar to the VCL^{-/-} and VCL P878A-KI cell lines respectively, would take us a step closer to understanding if this relationship is causative. We could also employ live imaging of the actin network while cells are unjamming to see if the domains of collective migration are constrained by the bundles of the transcellular actin networks.

The loss in collectivity in vinculin knockouts and the gain in VCL P878A-KI could also possibly be caused by the difference in recruitment of Arp2/3 to early cell junctions. We have seen that the recruitment of Arp2/3 influences the strength of early junction. Compared to Parental MCF10A cells, VCL^{-/-} with weaker early junctions are less collective in mature monolayers while VCL P878A-KI with stronger early junctions are more collective. It is possible that early Arp2/3 recruitment provides the actin network that gets reorganised into linear bundles. This possibility can be explored further by studying actin organisation and collectivity of cells treated with CK666 at different times over the course of maturation of the monolayer. If this hypothesis were true, one would expect that adding CK666 at early stages of maturation of the monolayer would have an effect on actin architecture and collectivity, but adding CK666 at later stages would not have such an effect.

We have also seen that the structure of focal adherens junctions is changed in vinculin KO cells and that more MENA is seen at the junction. It has been reported that MENA is recruited to focal adherens junctions independent of vinculin, but dependant on tension at focal adherens junctions[130]. This suggests that VCL^{-/-} monolayers generate more tension at focal adherens junctions and we believe this could have an effect on maintaining tissue architecture. In preliminary experiments, we found that acini made by VCL^{-/-} cells do not have a transcellular actin network and are more prone to local deformations at the outer surface. This indicates that vinculin and the transcellular actin network are essential for maintaining tissue structure. It would be interesting to test the capability of VCL^{-/-} and VCL P878A-KI cells to form more advanced tissue architecture for instance by growing them in 3-D printed tissue scaffolds.

Finally, we have shown that vinculin antagonises Arp2/3, a protein upregulated in many cancer types. Thus we have proposed a novel mechanism for the tumor suppressor role of vinculin which can control several checkpoints of cancer progression

- proliferation, migration and and destabilisation of cell-cell adhesions. It would be interesting to study the possibility of vinculin being a possible drug target for cancer treatment.

Inhibiting Arp2/3 itself, has proved difficult in terms of cancer drug discovery since its known inhibitor CK666 has to be used at very high concentrations to perturb Arp2/3 activity. Potentially increasing vinculin activity could be a way to target Arp2/3 activity in cancer. That fact that vinculin is a very unique and conserved protein could provide credence to using it as a drug target without off-target effects. It is imperative to understand the molecular basis of the interaction further and potentially elucidate a crystal structure which can then be used for molecular dynamics simulations and drug discovery.

Knocking out vinculin or introducing the P878A mutation in pre-transformed cell lines and transplanting them into mice would allow us to look at their efficacy for cancer progression. Interestingly, while attempting to over express vinculin, we found that that these cells lose vinculin expression over time possibly because cells which express vinculin are being selected out of the population over time in culture. This suggests that cells overexpressing vinculin would not be able to proliferate and contribute to cancer progression. Generating cell lines where vinculin expression is increased, using CrispRa[220], and transplanting them into mice could be a potential way to test if increasing vinculin and its Arp2/3 interaction could lead to decreased cancer progression.

5.4 Conclusion

The overarching theme of this project is that the vinculin-Arp2/3 interaction leads to antagonism of branched actin activity and this antagonism leads to changes in cell behaviour. We have seen that vinculin interacts with the canonical Arp2/3 complex. We have seen that perturbing the interaction increases actin polymerisation in lamellipodia leading to increased size of lamellipodia, increased cell spreading and ultimately increased persistence of migration. Consistent with an inherent link between migration and proliferation, perturbing the interaction also leads to increased cell cycling at high cell densities. At adherens junctions, vinculin plays a key role in maintaining junction stability, not only by structurally reinforcing the junction but also by recruiting and retaining Arp2/3 over the course of junction maturation. Vinculin also organises actin fibres at mature adherens junctions and coordinates cells during collective migration. Together, all the results point towards a novel role of

vinculin that can explain its tumor-suppressor activity.

This project has built on previous knowledge to provide an answer to an important question that has been puzzling the field for years - "What is the function of the vinculin-Arp2/3 interaction?" In addition we have made several unexpected new observations like vinculin recruiting Arp2/3 to cell junctions and organising a transcellular actin network. I believe that these findings have not only made a significant contribution to the field and but also open up several new avenues of research for the future.

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Titre : L'interaction Vinculin-Arp2/3 antagonise l'actine branchée pour contrôler les comportements cellulaires individuels et collectifs**Mots clés :** Vinculine, Arp2/3, Actine, Migration Cellulaire Individuelle et Collective, Prolifération Cellulaire, Cancer

Résumé : Le développement, la croissance et le maintien des tissus sont des propriétés découlant du comportement individuel des cellules. Ce comportement est finement contrôlé par de multiples voies de régulation en réponse aux stimuli environnementaux. Les forces sont détectées au niveau des adhésions focales (FAs), et des jonctions adhérentes (AJs), des structures d'adhérence qui relient l'environnement d'une cellule à son cytosquelette d'actine. En réponse à ces forces, le cytosquelette est remodelé et régule des comportements cellulaires complexes comme la prolifération, migration et maintien des jonctions cellulaires sous le contrôle de l'actine branchée. Ces processus sont dérégulés durant la progression des cancers. La vinculine (VCL), décrite comme suppresseur de tumeur, est une protéine structurale et mécanotransductrice aux FAs et les AJs. En plus de renforcer le lien entre le cytosquelette d'actine et les structures d'adhérence, VCL pourrait jouer un rôle dans la régulation du cytosquelette d'actine en interagissant avec Arp2/3, le nucléateur d'actine branchée. Le but de ce projet était de déterminer les effets de VCL sur l'actine branchée et le comportement des cellules. Pour cela, nous avons utilisé des techniques d'édition du génome afin de perturber cette interaction.

Nous avons étudié les effets de l'interaction VCL-Arp2/3 sur le comportement cellulaire individuel en exprimant un peptide VCL 811-881 dans les cellules parentales MCF10A. Nous avons constaté que ce peptide se lie au complexe Arp2/3 canonique, et que les cellules l'exprimant sont capables de migrer de manière plus persistante, de s'étendre sur une plus grande surface, de former des lamellipodes plus grands et de continuer à proliférer à de fortes densités cellulaires. Ces phénotypes indiquent une activité d'Arp2/3 augmentée. On retrouve ces phénotypes avec des lignées cellulaires KO de VCL (VCL^{-/-}) et une lignée cellulaire mutante dans laquelle VCL ne se lie pas à Arp2/3 (VCL P878A-KI). L'imagerie TIRF-SIM a révélé que la vitesse

d'assemblage du réseau d'actine est augmentée dans les cellules VCL^{-/-}, VCL P878A-KI ou exprimant le peptide VCL 811-881 par rapport aux cellules parentales MCF10A. Ensemble, ces résultats démontrent que l'interaction VCL-Arp2/3 antagonise la formation des réseaux d'actine branchée dans le lamellipode, et que VCL 811-881 agit comme un dominant négatif de VCL.

Pour comprendre le rôle de l'interaction VCL-Arp2/3 sur les comportements cellulaires collectifs, nous avons d'abord étudié les AJs qui maintiennent les cellules ensemble et leur permettent de transmettre des signaux mécaniques. Nous avons trouvé que VCL contrôle le recrutement d'Arp2/3 aux AJs et la stabilité des jonctions cellule-cellule. Une fois les AJs matures, les cellules parentales MCF10A peuvent organiser des réseaux d'actine transculturelles sur de longues distances et coordonnés entre plusieurs cellules. En condition d'unjamming hypotonique, ces cellules migrent collectivement au sein de domaines restreints par ce réseau d'actine transculturel. Les cellules VCL P878A ne développent pas ce réseau et migrent de manière plus collective, alors que les cellules VCL^{-/-} migrent moins collectivement et développent un réseau à très courte distance. Nos résultats indiquent donc que l'activité d'Arp2/3 doit être finement régulée aux AJs pour former ce réseau et contrôler les migrations collectives des cellules.

Ainsi, nous montrons un nouveau rôle pour VCL dans la régulation de l'actine branchée par son interaction directe qui antagonise Arp2/3. La perturbation de cette interaction entraîne plusieurs phénotypes caractéristiques des cellules cancéreuses : une prolifération et persistance de migration accrue, une perturbation des jonctions cellulaires et une dérégulation des migrations collectives. Étant donné que l'activité d'Arp2/3 est surexprimée dans plusieurs types de cancer, nos résultats expliquent comment VCL pourrait agir comme suppresseur de tumeur.

Titre : Vinculin-Arp2/3 interaction antagonises branched actin to control single and collective cell behaviours**Keywords :** Vinculin, Arp2/3, Actin, Single and Collective Cell migration, Cell Proliferation, Cancer

Abstract : Development, growth and maintenance of tissues are emergent properties arising from individual cell behaviour. Cell behaviour is finely tuned by a multitude of regulatory pathways in response to stimuli received from their microenvironment. Physical forces are sensed at cell-substrate contacts called focal adhesions (FAs) and cell-cell contacts called adherens junctions (AJs) which connect the exterior of a cell to its actin cytoskeleton. In response to force sensing, the actin cytoskeleton is remodelled to regulate complex cell behaviours such as proliferation, migration and cell-junction maintenance that are under the control of branched actin. During cancer progression, these three processes are deregulated. Vinculin (VCL), described as a tumour suppressor, is a structural and mechanotransductory protein present in both FAs and AJs. In addition to reinforcing the link between the actin cytoskeleton and adhesive structures, VCL is likely to play a second regulatory role on the actin cytoskeleton by interacting with the branched actin nucleator Arp2/3. The goal of this project was to determine the effects of VCL on branched actin, and ultimately cell behaviour. To this end, we used CRISPR-Cas9 genome editing techniques to perturb this interaction.

We began studying the effects of the interaction on individual cell behaviour by expressing a VCL 811-881 peptide in Parental MCF10A cells. We found that the peptide binds the canonical Arp2/3 complex, and cells expressing the peptide are able to migrate more persistently, spread over a larger area, make larger lamellipodia and continue to proliferate at high cell densities. All these phenotypes indicate that Arp2/3 activity is increased in these cells. VCL knockout (VCL^{-/-}) cell lines and a mutant cell line where VCL cannot bind Arp2/3 (VCL P878A-KI) both behave similarly. TIRF-SIM imaging revealed that the actin network assembly rate was increased in VCL^{-/-}, VCL

P878A-KI and VCL 811-881 expressing cells compared to Parental MCF10A. Together, this demonstrates that the function of the VCL-Arp2/3 interaction is to antagonize generation of branched actin networks in the lamellipodium, and that the VCL 811-881 peptide acts as a dominant negative of VCL function.

To understand the role of the VCL-Arp2/3 interaction on collective cell behaviour, we first studied AJs which not only hold cells together but also allow them to pass on mechanical signals. We found that the VCL controls Arp2/3 recruitment to AJs and cell-cell junction stability. Once AJs were mature, Parental MCF10A cells were able to organise long-range transculturel actin networks coordinated across multiple cells. During hypotonic unjamming, these cells migrate collectively as domains constrained by the long-range actin network. VCL P878A cells are not constrained by any long-range transculturel actin network and migrate more collectively while VCL^{-/-} cells develop a very short-range actin network leading to reduced collective migration. Our results indicate that Arp2/3 activity has to be finely regulated at AJs by VCL to form this long range network and regulate collective migration.

Thus, we have established as a novel role for VCL in regulating the actin cytoskeleton through a direct interaction that antagonizes Arp2/3. Perturbation of this interaction leads to several phenotypes characteristic of cancer cells – increased proliferation, increased persistence of migration, perturbation of cell junctions and misregulation of collective migration. Since Arp2/3 activity is known to be upregulated in several cancer types, our results provide a potential mechanism for vinculin's role as a tumour suppressor.