Department of Biological Sciences

Extracellular matrix adhesion independent roles of integrins and FA proteins.

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Περίληψη

Η ικανότητα των κυττάρων και των ιστών ενός οργανισμού να μπορεί να αντιληφθεί και να αποκριθεί σε μηχανικές δυνάμεις μετατρέποντας τις σε βιοχημικά σήματα είναι γνωστή ως μηχανοαίσθηση και μηχανομεταγωγή. Η αντίληψη ότι οι μηχανικές αυτές δυνάμεις που ασκούνται στα κύτταρα, επηρεάζουν και ρυθμίζουν ποικίλες κυτταρικές αποκρίσεις και διεργασίες, έχει τροποποιήσει την αντίληψη μας στην κυτταρική και μοριακή βιολογία. Οι κυτταρικές προσδέσεις οι οποίες βασίζονται σε ιντεγκρίνες και καντερίνες αποτελούν τα κύρια συστήματα μέσω των οποίων τα κύτταρα προσδένονται με το εξωκυττάριο τους χώρο ή με τα γειτονικά τους κύτταρα αντίστοιχα. Και τα δύο αυτά συστήματα έχουν δειχθεί να είναι απαραίτητα για την σωστή εμβρυική ανάπτυξη ενώ μεταλλάξεις ή και απώλεια των πρωτεϊνών, μελών των συμπλόκων αυτών, έχουν συνδεθεί με θάνατο κατά την εμβρυική περίοδο και σημαντικές ασθένειες όπως ο καρκίνος. Τα δύο αυτά συστήματα είναι γνωστό ότι τόσο σε κύτταρα όσο και σε ιστούς είναι χωρικά διαχωρισμένα, παρόλα αυτά πολλές μελέτες έχουν δείξει ότι υπάρχει διασταυρωμένη αλληλεπίδραση και επικοινωνία των συστημάτων χωρίς να προσδίδουν την μηχανιστική προσέγγιση πίσω από αυτή την αλληλεπίδραση. Προηγούμενη δουλειά από το εργαστήριο μας, ανακάλυψε ότι μια πρωτεΐνη μέλος των εστιακών προσκολλήσεων, η ΦΑΚ, εμπλέκεται στην μορφογένεση ιστών στην ανάπτυξη του βατράχου μέσω της ρύθμισης της οριοθέτησης της μιτωτικής ατράκτου. Η συνέχεια αυτής της δουλείας έδειξε ότι η ιντεγκρίνη β1 έχει την ικανότητα να ενεργοποιείται στον φλοιό των κυττάρων κατά την μιτωτική διαίρεση, μέσω εφαρμογής μηχανικών δυνάμεων χωρίς την παρουσία προσδέτη. Αυτή η ενεργοποίηση οδηγεί στην δημιουργία ενός μηχανοαισθητήριου συμπλόκου αποτελούμενο από άλλες πρωτεΐνες των εστιακών προσκολλήσεων και ονομάστηκε CMC. Σε αυτή τη μελέτη δείχνουμε ότι τόσο η πρόσδεση κυττάρων σε υποστρώματα ιντεγκρινών όσο και καντερινών οδηγούν στον ορθό προσανατολισμό της μιτωτικής ατράκτου στο επίπεδο της πρόσδεσης και οδηγούν σε πανομοιότυπες αποκρίσεις στα χωρικά σηματοδοτικά μηνύματα που μεταφέρονται από το υπόστρωμα στα κύτταρα. Δείχνουμε ότι ο τύπος του υποστρώματος παρέχει μόνο μηχανικά σήματα στα διαιρούμενα κύτταρα τα οποία δεν εξαρτώνται από τη μοριακή φύση των υποστρωμάτων. Αποδείξαμε επίσης ότι η ενεργοποίηση της ιντεγκρίνης και η δημιουργία του μηχανοαισθητήριου αυτού συμπλόκου είναι απαραίτητα για την ορθή οριοθέτηση της μιτωτικής ατράκτου τόσο σε υποστρώματα καντερινών όσο και σε υποστρώματα ιντεγκρινών αποδεικνύοντας έτσι ότι ο ρόλος των πρωτεϊνών αυτών είναι διαφορετικός από τον ρόλο που έχουν στις εστιακές προσκολλήσεις. Δείξαμε επίσης ότι η ιντεγκρίνη β1 ενεργοποιείται στις περιοχές που παρατηρούνται οι σύνδεσμοι πρόσδεσης των κυττάρων. Αυτή η ενεργοποίηση λαμβάνει χώρα κατόπιν συγκεκριμένου χρονικού διαστήματος και εξαρτάται από την συνένωση των πρωτεϊνών μελών των συνδέσμων πρόσδεσης με τον κυτταροσκελετό της ακτίνης. Αποδείξαμε επίσης ότι αυτή η ενεργοποίηση είναι αποτέλεσμα της άσκησης υψηλής μηχανικής δύναμης στους συνδέσμους αυτούς

η οποία μεταφέρεται από τον κυτταροσκελετό της ακτίνης. Η ενεργοποίηση της ιντεγκρίνης φάνηκε να ρυθμίζει αρνητικά τους συνδέσμους προσκόλλησης οδηγώντας στην διάσπαση τους μέσω ενδοκυττάρωσης. Επίσης δείξαμε ότι κατόπιν ενεργοποίησης της ιντεγκρίνης σε αυτά τα σημεία, στρατολογούνται πρωτεΐνες των εστιακών προσκολλήσεων και δημιουργούν το σύμπλοκο που ονομάσαμε ως υβριδικές προσδέσεις (hybrid adhesions, HA). Η στοιχειομετρία των HA και η διαμόρφωση της δομής της ενεργοποιημένης ιντεγκρίνης έδειξε να έχει διαφορές με αυτή που παρατηρείται στις εστιακές προσκολλήσεις ενώ φάνηκε να παρουσιάζει ομοιότητες με το μηχανοαισθητήριο σύμπλοκο που παρατηρήθηκε στον πλευρικό φλοιό των μιτωτικών κυττάρων, CMC (Cortical Mechanosensory Complex). Ο μηχανισμός μέσω του οποίου πραγματοποιείται η ενεργοποίηση της ιντεγκρίνης σε αυτά τα σύμπλοκα έχει επίσης δειχθεί και φαίνεται να γίνεται μέσω παγίδευσης μορίων ιντεγκρίνης σε ένα δίκτυο κυτταροσκελετού ακτίνης και δεσμίδες ακτίνης και μυοσίνης σε αυτά τα σημεία χωρίς την εναπόθεση προσδέτη. Αυτή η διαδικασία φάνηκε να καθορίζει την τοπολογία της εναπόθεσης και δημιουργίας του εξωκυττάριου στρώματος τόσο σε κύτταρα όσο και σε έμβρυα βατράχου.

Συνοπτικά, δείξαμε ότι το μηχανοαισθητήριο σύμπλοκο το οποίο στρατολογείται στον πλευρικό φλοιό των μιτωτικών κυττάρων εμπλέκεται στην μιτωτική διαίρεση και ο ρόλος των πρωτεϊνών μελών του είναι ανεξάρτητος από τον ρόλο τους στις εστιακές προσκολλήσεις. Αποδείξαμε επίσης ότι στις περιοχές συνδέσμων προσκόλλησης, κάτω από συνθήκες όπου ασκείται έντονη μηχανική δύναμη, ενεργοποιείται η ιντεγκρίνη β1 μέσω ενός μηχανισμού που είναι βασισμένος στην ακτίνη του κυτταροσκελετού και στην δύναμη που ασκείται στο κύτταρο από τις δεσμίδες ακτίνης και μυοσίνης. Αυτή η ενεργοποίηση οδηγεί στην στρατολόγηση πρωτεϊνών μελών των εστιακών προσκολλήσεων και στην δημιουργία των HA τα οποία οδηγούν στην διάσπαση των συνδέσμων πρόσδεσης μέσω ενδοκυττάρωσης και η διαδικασία αυτή φάνηκε να καθορίζει την τοπολογία της εναπόθεσης και δημιουργίας του εξωκυττάριου στρώματος τόσο σε κύτταρα όσο και σε έμβρυα βατράχου.

Abstract:

Mechanosensation and Mechanotransduction are the abilities of a cell to sense and respond to mechanical signals by translating them into biochemical pathways. The realization that mechanical forces influence and regulate numerous cell processes has changed our perspective in cell and molecular biology. Integrin-based adhesions and Cadherin-based adhesions are the two major metazoan adhesion systems that facilitate the cell-ECM and cell-cell adhesion respectively. Both systems have been found indispensable for proper embryonic development and loss of protein members of these complexes leads to embryonic lethality and are implicated in disease. The two systems are known to be spatially segregated in both cells and tissues. However numerous studies underline their crosstalk without providing any mechanistic insight of this interaction. Earlier work from our group revealed that a well characterized member of focal adhesions (FAs), FAK is implicated in tissue morphogenesis in *Xenopus* through the regulation of spindle orientation. Later work suggested that integrin β1 becomes activated through mechanical stimuli in the absence of a ligand at the lateral cortex of mitotic cells. Upon this activation, known FA proteins were shown to be recruited at the mitotic cortex forming the cortical mechanosensory complex (composed of FAK, p130Cas and Src). Here we show that both integrin-based and cadherin-based adhesion drive proper mitotic spindle orientation parallel to the plane of the attachment and promote identical responses to spatial cues provided by adhesion geometry showing that cell subtsrate interactions simply provide mechanistically cues to the dividing cells which are independent from the molecular nature of adhesion. We also show that integrin activation and the CMC are crucial for spindle orientation both on cadherin and fibronectin substrates. This shows that spindle responses to adhesion topology are a result of force anisotropy on the cell cortex and the role of cortical mechanosensory complex in this process is distinct from its role in cell-ECM adhesion. We move on to show that integrin β1 also becomes activated and clustered at adherens junctions. This activation relies on PM tension followed by stabilization through actin trapping within the actomyosin bundles terminating at mature AJs. We go on to show that that integrin β1 activation modulates adherens junction dynamics leading to their disassembly through caveolin based endocytosis. The activation of integrin β1 at AJs leads to the recruitment of FA proteins and to the formation of hybrid adhesion complexes in which the stoichiometry of FA proteins and the conformation of integrin β1 is distinct from that at FAs. At the same time this activation displays similarities to the cortical mechanosensory complex. Finally, we go on to show that integrin activation at AJs under increased tension not only leads to AJ disassembly but also spatially guides ECM deposition in vitro and in the embryo.

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Dedication:

In memory of my grandmother and grandfather, I will always love you. Always and forever.

To my parents, with love and eternal appreciation.

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1. General Introduction

1.1 Cell adhesive interactions

The connection of cells with neighboring cells or to their extracellular environment, known as extracellular matrix (ECM), are well-characterized processes known as cell-cell and cell-ECM adhesions respectively. These interactions are maintained through the adhesive molecules which are mainly found either at the surface of the cells or cell-ECM interactions. The first type of interaction, mediates cell-cell interactions which link neighboring cells into functional tissues and organs. While the second, provides mechanical anchoring of the cells and provide precisely cell positioning within a multicellular organism (Heisenberg and Fässler, 2012). The major molecules facilitating these processes are cadherins and integrins and defects in any of these molecules and in the adhesive process respectively, has been associated with abnormal embryonic development and wound repair. These molecules are linked with a wide variety of disorders such as immune, hematological dermatological and fibrotic, neurodegenerative and cardiovascular diseases. They have also been associated with a noticeable number of muscular dystrophies, tumor malignancies and metastasis. All of these together, underlie the importance of studying their precise role during the adhesion processes (Morgan, Humphries and Bass, 2007; Goldstein, 2007; Geiger and Yamada, 2011; Sabina E Winograd-Katz *et al.*, 2014).

The interactions between cells and the ECM are known to promote a variety of mechanical and biochemical signals which are crucial for cellular functions including cell migration, differentiation, tissue remodeling and morphogenesis, cell proliferation and wound healing (Beauvais-Jouneau and Thiery, 1997; Galbraith, Davidson and Galbraith, 2018). The cell-ECM interaction is maintained through the engagement of cell surface receptors with components of the ECM. This results to the transduction of a cell response through the formation of large multiprotein cell-matrix complexes. These complexes are responsible for the linkage of ECM with the actin cytoskeleton system of the cell (Wehrle-Haller, 2012; Sabina E Winograd-Katz *et al.*, 2014; Humphries *et al.*, 2019). Different types of matrix exist such as the basement membrane, connective tissue, tissue culture surfaces, and tendons. These different matrix types are composed of a variety of ECM components with the most well-characterized being fibronectin (FN), vitronectin (VN), collagen, laminin, perlecan, and glycosaminoglycans (**Figure 1**).

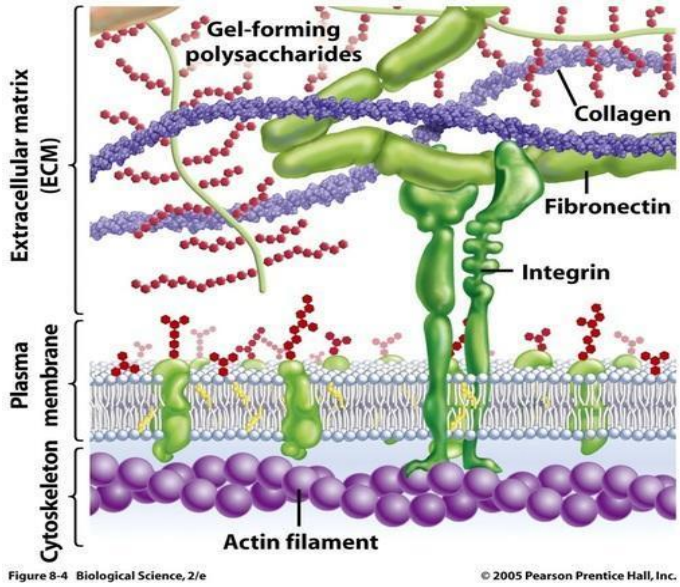


Figure 1: Schematic representation of ECM components and their interaction with the cell.

Adapted from: (Wiesner, Legate and Fässler, 2005)

The ECM provides physical support to cells and tissues and enables microenvironmental sensing through its chemical composition and its mechanical properties. This sensing guides the activation of intracellular signals that are responsible for the migration, proliferation, morphology, pattern of gene expression, stem cell fate choice, and tumor progression (Frantz, Stewart and Weaver, 2010; Goodwin *et al.*, 2017; Geiger and Yamada, 2011). The cell is characterized by diverse surface receptors able to recognize ECM components; integrin and non-integrin receptors. The non-integrin receptors include the syndecans, the proteoglycans, the selectins, CD44, RHAMM, uPAR, and the tyrosine kinase receptors discoidin domain receptor 1 and 2 (Geiger and Yamada, 2011; Smith and Marshall, 2010; Beauvais and Rapraeger, 2004; Frantz, Stewart and Weaver, 2010). Integrin receptors are the major receptors for ECM proteins. They can form simultaneously, distinct adhesions that are characterized by a morphological, dynamical, and structural variability, within the same cell. These adhesions are known as "integrin adhesions" (IAC) and they exhibit high molecular complexity concerning their morphology, composition, and regulation since they provide numerous structural capacities and signaling-sensing activities to the cells (Geiger and Yamada, 2011).

The interactions between cells are mainly responsible for the communication of neighboring cells through the transduction of chemical, electrical and mechanical signals. Three different types of cell-cell adhesion junctions are known in mammals and each is composed of diverse protein components known as cell adhesion molecules (CAMs) (Tight junctions, Adherens junctions (AJs) and Desmosomes) (Gayrard *et al.*, 2018; McEver and Luscinskas, 2018). Some of these CAMs are known to be expressed only by specific tissues like blood or endothelial cells; however, they can be synthesized by other cell types as well. Tight junctions are known to serve as diffusion barriers and

are mainly found in endothelial and epithelial tissues. Proteins associated with the formation of this type of cell-cell adhesion are junctional adhesion molecules (JAMS), claudins and occludin (Steed, Balda and Matter, 2010). Desmosomes are known to connect the intermediate actin filaments of neighboring cells through the formation of proteinic complexes composed of desmosomal cadherins, plakoglobin, plakophilin and desmoplakin (Delva, Tucker and Kowalczyk, 2009). AJs are a type of stable cell contact which serves in the maintenance of tissue integrity and in the translation of actomyosin generated forces in tissue through the formation of an interconnected lateral bridge. This connection links the actin cytoskeleton of two neighboring cells (Meng and Takeichi, 2009; Harris and Tepass, 2010). A wide variety of signaling and scaffolding proteins, actin regulators and adhesion receptors are involved in this complex known as the "cadhesome" (Figure 2) (Zaidel-Bar, 2013; Guo *et al.*, 2014).

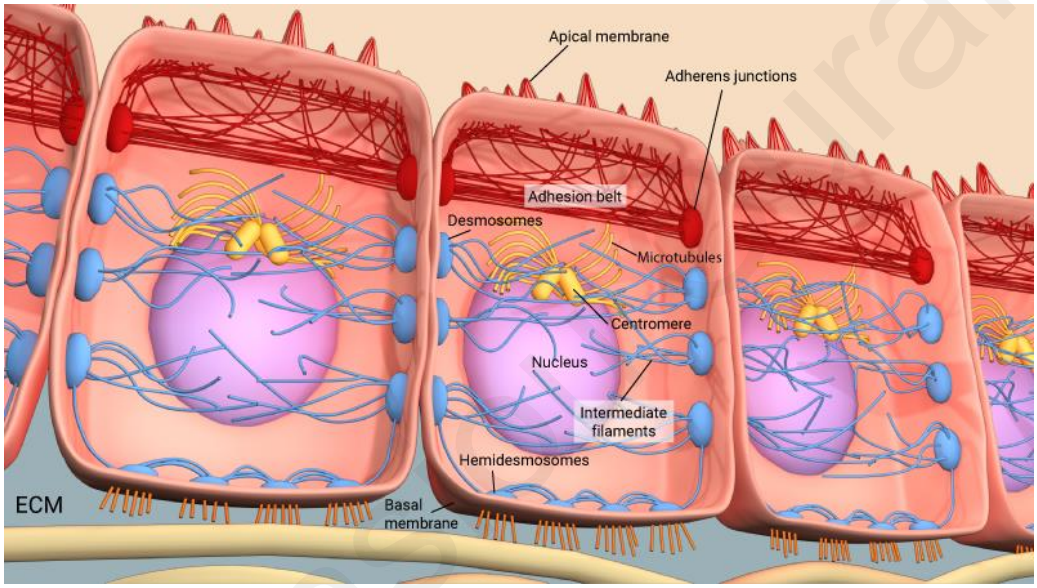


Figure 2: The cell-cell adhesion junctions

The cell-cell junctions complexes and their linkage to actin networks of the cells. Adapted from: MechanoBiology Institute MB info.

1.2 The integrin adhesome; composition, structure and dynamic plasticity.

The integrin adhesome is characterized by a molecular composition complexity which is reflected through the scaffolding and the regulatory interactions that take place to allow the signal transduction between the cells and their ECM. Its exact composition has been under investigation for many years

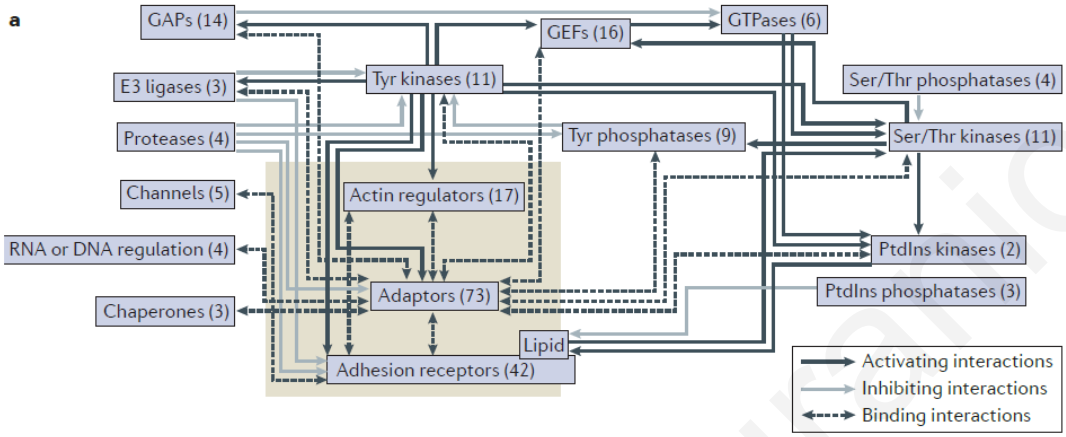


Figure 3: Representative diagram of the integrin adhesion.

The different categories of integrin adhesome components are displayed in combination with their major effect on the adhesome. Adapted from: (Sabina E Winograd-Katz *et al.*, 2014)

since it is a very dynamic structure changing its morphology according to the ECM environment. The regulatory interactions that take place in the adhesome include major signaling molecules like Rho-family GTPases and their regulators and scaffolding interactions. The first group of molecules mainly provides the dynamic plasticity of the adhesion sites. While the second, includes actin-associated and adaptor proteins and are responsible for the mechanical anchoring to the actin cytoskeleton (**Figure 3**). The molecular architecture of the integrin adhesome has been studied for many years and to date, the literature-curated adhesome is composed of 232 components of which 84 are transiently associated with the adhesion site while 148 are intrinsic molecules of the complex (Sabina E Winograd-Katz *et al.*, 2014; Bidone *et al.*, 2019; Green and Brown, 2019a). However, numerous proteomic studies have identified in recent years 2412 proteins that are associated with integrins providing further evidence regarding the complexity of these adhesion complexes (Green and Brown, 2019a; Cavenett, 2013). The integrin adhesome components at the basic level have been extensively studied using 2D cultures. The major components are the ECM, the integrin-associated proteins (IAPs), the cytoskeleton (actin cytoskeleton primarily) and the transmembrane receptors known as integrins (Green and Brown, 2019a; Cukierman *et al.*, 2001, Bidone *et al.*, 2019).

Integrins are transmembrane heteromeric receptors composed of non-covalently linked α and β subunits. They are composed of an extracellular domain, a transmembrane region and a cytoplasmic tail. The extracellular domain binds to ECM proteins. The transmembrane domain provides interaction between integrin subunits and the cytoplasmic tail is indirectly linked to actin through the

formation of large multiprotein complexes (Parsons *et al.*, 2012; Green and Brown, 2019a; Horton *et al.*, 2016) (**Figure 4**).

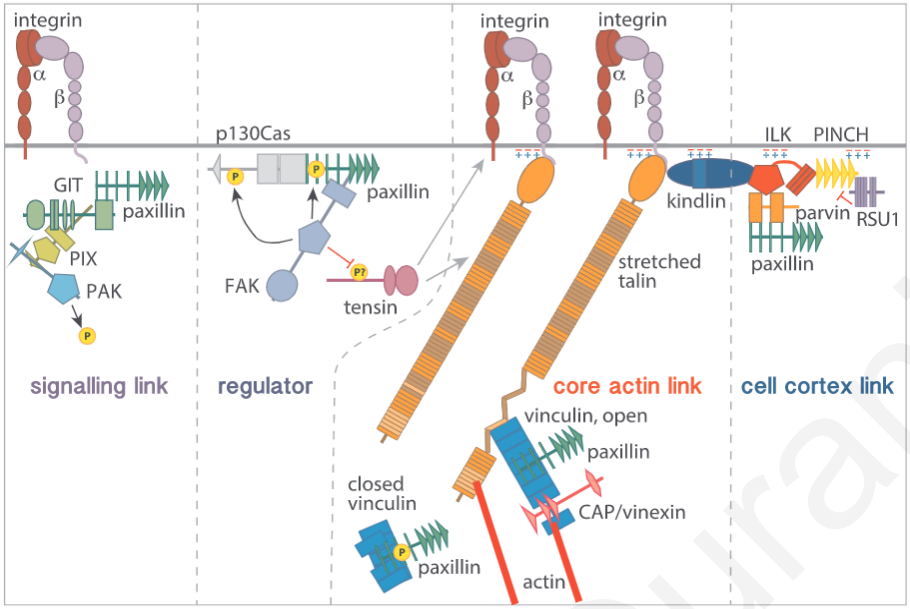


Figure 4: Integrin adhesions structure and composition

Representation of integrin adhesions connection to the actin cytoskeleton through the formation of the multiprotein complex formed on integrin's cytoplasmic tails. The members of the adhesion shown are integrins, talin, paxillin, kindlin, vinculin, p130Cas, FAK, ILK. And PINCH. Adapted from: (Green and Brown, 2019b).

Characteristic examples of proteins that are directly associated with the cytoplasmic tails of integrins, are talin, filamin and α-actinin (Green and Brown, 2019a, Sharma, Ezzell and Arnaout, 1995, Otey, 1990). Proteins that interact indirectly with integrins are vinculin, zyxin, p130Cas, VASP (vasodilator-stimulated phosphoprotein) and tensin (Ramage, 2012; Green and Brown, 2019a, Isenberg, Leonard and Brigitte M Jockusch, 1982, Beckerle, 1997). The integrin adhesions, as mentioned above, are also composed of proteins that serve as regulators like FAK (Focal Adhesion Kinase), paxillin and ILK (Integrin-Linked Kinase) (Otey, 1996)(Green and Brown, 2019a)(Hannigan *et al.*, 1996) and of proteins which regulate the dynamic nature of the adhesion sites like Calpains (calcium-dependent proteases) (Beckerle *et al.*, 1987) P13K kinases (phosphoinositide 3-kinases) and PKC (Protein Kinase C) (Morgan, Humphries and Bass, 2007). Proteins that act as actin regulators are also part of the cell-ECM adhesions. A characteristic example is the Arp2/3 complex (Pollard, 2007). Lastly, different endocytosis regulatory molecules such as caveolin-1 and dynamin-2 have also been identified as members of integrin adhesome (Nethe and Hordijk, 2011; Ezratty, Partridge and Gundersen, 2005).

During cell migration, four different types of cell-ECM adhesions can be present in a cell (separately or simultaneously) and this depends on the cell type, cell environment, morphology and size of the cell (Geiger and Yamada, 2011). The initial step during migration, is the actin-driven formation and

extension of protrusions from the cell membrane and the formation of sensing structures known as filopodia (**Figure 5**). These filopodia mature, become stable and form the different types of adhesion at the front of the cell. During this phase, actin acquires a branched arrangement at the lamellipodium through polymerization. This is achieved by the regulation of the Arp2/3 complex from Rac, Cdc42 and Rho GTPases (Parsons, Horwitz and Schwartz, 2010; Huttenlocher and Horwitz, 2011; Vicente-manzanares and Horwitz, 2011). This initial protrusive activity guides the integrin-expressing cell membranes in close proximity with the ECM components (or ligands). This guides integrin activation and the consequent recruitment of several proteins to nascent adhesions (NAs) (**Figure 5**) (Wehrle-Haller, 2012; Parsons, Horwitz and Schwartz, 2010; Vicente-manzanares and Horwitz, 2011). The first adhesion type during cell migration is the NAs. NAs are small and short-lived adhesions. These adhesions are known to be formed right behind the leading edge of the cell and are composed of a small number of proteins at this point, such as VASP, talin, paxillin, and α-actinin. NAs can either undergo turnover in a time interval of 60 seconds or become mature to a larger protein complex known as Focal Complexes (FCs) which have a longer lifetime (**Figure 5**) (Parsons, Horwitz and Schwartz, 2010; Geiger and Yamada, 2011). A characteristic event driving this maturation, is the binding of vinculin tail to talin which consequently drives the active integrin clustering. This results in a strengthened link between integrins and actin. At this point, the actin at the lamellipodium of the cell reorganizes and polymerizes into bundles. These bundles, through the regulation of actomyosin contraction from Rho and ROCK (Rho-associated protein Kinase), create strong traction forces responsible for the forward movement of the cell. This leads to the maturation of the adhesion complex to Focal Adhesions (FAs) (Askari *et al.*, 2010; Parsons, Horwitz and Schwartz, 2010). FAs are formed also at the center and the periphery of the cell and are not restricted to its leading edge. Characteristic of maturation into FAs, is the alteration in protein composition of the complex. Proteins like zyxin (Beckerle, 1997) are recruited while proteins like paxillin become phosphorylated. For the cell to achieve a polarized forward movement, these complexes have to disassemble from the rear of the cell. This process requires polar myosin activation at the cell rear. The last type of adhesion types is the Fibrillar Adhesions (FBs) and are characterized by an elongated morphology and long-lifetime. These complexes are found in the center of the cell and are formed along matrix fibrils (**Figure 5**) (Parsons, Horwitz and Schwartz, 2010; Vicente-manzanares and Horwitz, 2011). FBs are enriched in a variety of proteins like tensin, parvins and FN (Danen *et al.*, 2002). These complexes lack of any enzymatic activity; thus, no phosphorylation events are detected in this complex. Their formation is initiated by the translocation of integrin α5β1 at the center of the cell by transforming the FA generated actomyosin tension into movement along the actin filaments. The formation of FBs is crucial for the assembly and reorganization of the FN (a well-known ECM component) (Cukierman *et al.*, 2001; Parsons, Horwitz and Schwartz, 2010; Vicente-manzanares and Horwitz, 2011).

The disassembly of FAs happens simultaneously at the rear and front end of the cell where two different populations of adhesions are present. At the rear end of the cell, the disassembly of FAs is a result of a synergistic action of Rho-kinase and myosin II. These two, drive retraction and sliding of the FAs while the cell moves inward. It has been previously shown that this process is based on Rac1 activity through caveolin-1 dependent endocytosis (Parsons, Horwitz and Schwartz, 2010; Nethe and Hordijk, 2011). At the front-end of the cell, actin depolymerizes and reorganizes leading to the disassembly of the FAs. Other member proteins of the integrin adhesions are found to be involved in disassembling mechanisms such as FAK and paxillin and talin through their proteolytic cleavage by Calpain (Cukierman *et al.*, 2001; Parsons, Horwitz and Schwartz, 2010). Work from Patridge et al. has shown that microtubule targeting of FAs drives a dynamin 2-dependent integrin endocytosis which leads to FA disassembly (Ezratty, Partridge and Gundersen, 2005).

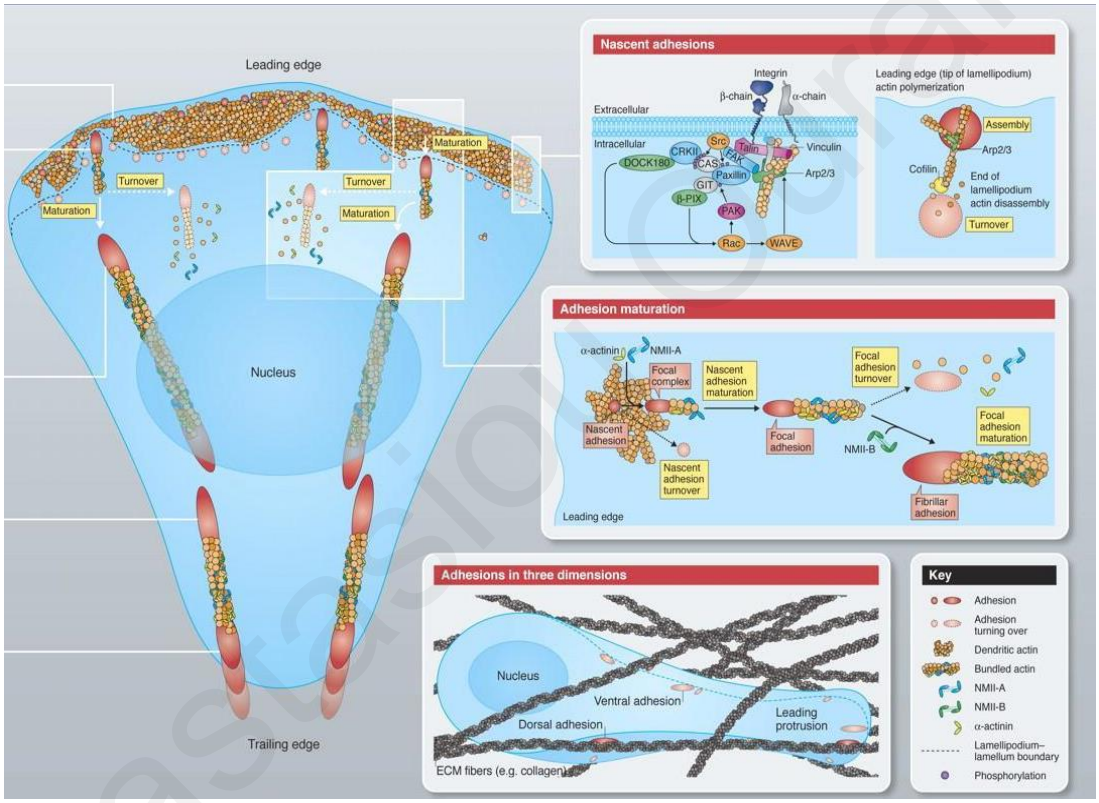


Figure 5: Types of cell adhesions

The different types of cell adhesions. NAs form first at the leading edge of the cell and they are enriched in paxillin and Talin. They have a short life span and they turnover into FCs which are larger protein complexes and they have a different protein composition. FCs mature further and create FAs which leads to actin reorganization of the actin cytoskeleton into stress fibers. At this point the cell movement is promoted through the actomyosin contraction that occurred to the cell. FAs mature into FBs which are located into the center of the cell and are implicated in the formation of FN matrix. Adapted from: (Vicente-manzanares and Horwitz, 2011)

Fundamental processes during embryonic development such as cell proliferation, migration differentiation and cell survival, have been found associated with the structure, molecular

composition and mechanisms of the integrin adhesions. The disruption or malfunction of genes coding for the core proteins of integrin adhesions has been previously shown to result in embryonic lethality (Wiesner, Legate and Fässler, 2005). All these together demonstrate the crucial contribution of cell-ECM adhesion during embryonic development. In this section I present an overview of the major proteins that compose the integrin adhesions and play an indispensable role in cell-ECM adhesion maintenance.

1.2.1 Integrins

1.2.1.1 Structure and Classification

Integrins are a large family of glycoproteins class I transmembrane, heterodimeric receptors that are known to accomplish the connection of cells to the ECM. They are composed of two non-covalently bound α and β subunits which form heterodimers. There are 18 α and 8 β integrin subunits that can associate to each other and form a wide range of distinct heterodimers (24) (Green and Brown, 2019a; Margadant *et al.*, 2011; Jones and Walker, 1999; Elizabeth M. Morse, Brahme and Calderwood, 2014; Campbell and Humphries, 2011; Manakan.B Srichai, 2017; Kechagia, Ivaska and Roca-Cusachs, 2019). Each of these heterodimers is characterized by a unique ECM binding specificity and expression pattern. This specificity guides a differential signal transduction from and to the cell. Integrins heterodimerize in the endoplasmic reticulum and are expressed on cell membranes as heterodimers (**Figure 6**). As members of the glycoprotein family, integrins are composed of a large extracellular domain (globular head), a small transmembrane domain and the tail (except β4 which has a large intracellular domain) (Manakan.B Srichai, 2017; Ffrench-Constant, 2003). These are characteristics for both units of the heterodimer. The integrin extracellular domain is approximately 80-150 kDa and is composed of several subdomains that form a ligand-binding globular N-terminal head. This head is connected to a C-terminal leg which is connected to the transmembrane domain and the intracellular tail of the integrins. Integrin subunits are characterized by major differences regarding their extracellular domains since α-subunits are composed of a β propeller head domain two calf domains and a thigh domain (Ffrench-Constant, 2003; Campbell and Humphries, 2011; Manakan.B Srichai, 2017; Green and Brown, 2019a; Kechagia, Ivaska and Roca-Cusachs, 2019). A vast majority of α subunits contains an I (inserted) domain which is approximately 200aa and this, when present, is responsible for the ligand binding; through its ability to bind divalent cations (Mg^{2+}) via its conserve metal ion-dependent adhesive site (MIDAS) (Ffrench-Constant, 2003; Manakan.B Srichai, 2017). The β subunits are composed of a PSI (plexin/semaphorin/integrin) domain, a hybrid domain, four EGF repeats, a proximal to membrane B-tail domain and an I-like domain which is homologous to the I-domain in α-subunits (Green, Mould and Humphries, 1998); Manakan.B Srichai, 2017) (**Figure 6**). This last domain is responsible for ligand binding in integrins whose α-subunits lack the I-domain. Under these circumstances the ligand is bound at a crevice and is located

in the head between the interfaces of the two subunits. In general, it has been established that the ligand interacts with the MIDAS domain which is ion occupied and the propeller domain of α-subunits. The integrin transmembrane domains (TM) are small, approximately 25-29aa and they form α-helical coiled-coil which heterodimerizes. The TM of α-integrin subunits is characterized by a conserved GFFKR motif and has been shown to have a major role in the transition from inactive to an active state of integrins. The TM of β-subunits, on the other hand, is composed of a homologous HDR(R/K) E motif (Ffrench-Constant, 2003; Manakan.B Srichai, 2017).

The integrin cytoplasmic tail is largely unstructured and short, composed of 10-70aa except for the tail of the β4 subunit which is composed of more than 1000aa. The β-subunit tails are largely conserved while the α-subunit tails display high diversity (beside their conserved GFFKR motifs) and as a result the interactions of β-subunit are better understood. The β-tails are composed of two well-defined Npx motifs.; the NPxY proximal and the NxxY distal motifs. These have been found to represent recognition sequences for phosphotyrosine-binding domains (PTBs), where major integrin-binding proteins like talin and kindlin bind (Ffrench-Constant, 2003; Nevo, 2010; Anthis and Campbell, 2011; Campbell and Humphries, 2011; Manakan.B Srichai, 2017, Kechagia, Ivaska and Roca-Cusachs, 2019). The two homologous domains between α and β subunits are GFFKR and HDR(R/K) E and they form a salt bridge between arginine (R) from α subunit and aspartic acid (D) from β. Several studies showed that this salt bridge formation is responsible for the preservation of the inactive state of integrin heterodimers and its disruption is connected with key events in the regulation of integrin activation. These studies had conflicting results since some suggest the minor contribution of the salt bridge in integrin activation while others suggest that it plays a major role in this process (Czuchra *et al.*, 2006; Müller *et al.*, 2014; Weljie, Hwang and Vogel, 2002; Campbell and Humphries, 2011; Ffrench-Constant, 2003).

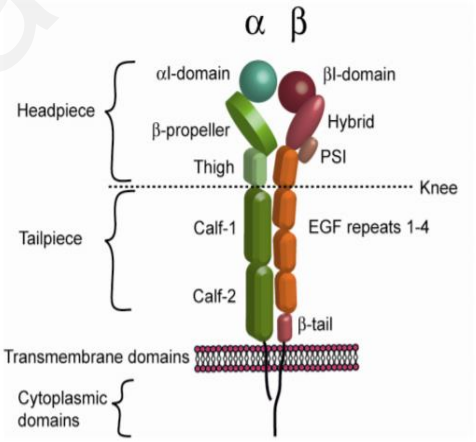


Figure 6: Illustration of integrin α and β subunits structure

Integrin α and β subunits composed of the headpiece (extracellular domain), the transmembrane domain and the cytoplasmic tails. The extracellular domain is subdivided into smaller domains. Adapted from: (Nevo, 2010)

Integrins are classified into several groups according to their ligand selection and the main categories are briefly described below. For example, the integrins that bind to FN are α5β1, α4β1, αvβ3, the integrins that bind to Vitronectin are αvβ1, αvβ5, αvβ3, the integrins that bind to laminin (Huttenlocher and Horwitz, 2011) are α2β1, α3β1, α6β1 and the integrins that are known to recognize collagen are α1β1 and α6β1. Three regions of the heterodimer have been found to recognize the ECM protein motifs, the EGF, a region near the N-terminus of β-subunit and the A-domain (**Figure 7**) (Ffrench-Constant, 2003; Manakan.B Srichai, 2017; Campbell and Humphries, 2011).

- RGD-binding integrins:

This class of integrins is characterized by its ability to bind to ligands that contain the RGD domain (Arg-Gly-Asp) among which are numerous vascular ligands, FN, VN, fibrinogen and thrombospondin (Plow *et al.*, 2000; Campbell and Humphries, 2011). The RGD binding domain is identical between the different integrins and is located between the interface of the two subunits (Campbell and Humphries, 2011; Xiao *et al.*, 2004; Takagi and Springer, 2002). The Arg residue fits in the α-subunit at the β-propeller and the Asp residue interacts with the β-I-domain (cations at this domain). In this group, integrin β1, α3 and α5 integrins, aV integrins and integrin αIIIβ3 are categorized.

- A-domain integrins:

This category is composed of integrins that contain the α-I-domain like α1, α2, α10, α11 and β1. (Humphries, Byron and Humphries, 2006; Campbell and Humphries, 2011). These integrins have the ability to bind to a CFOGER motif that belongs to ECM components like collagen and laminin and this interaction is indispensable for tissue maintenance, repair and normal embryonic development (Harburger and Calderwood, 2009; Takagi and Springer, 2002).

- Non-α-domain integrins:

In this category, integrins that bind laminin receptor are included like β1 integrins, α3, α6, α7 and α6β 4(Humphries, Byron and Humphries, 2006; Campbell and Humphries, 2011)(Takagi and Springer, 2002).

- LDV-binding integrins:

This group of integrins recognizes an LDV motif; which is believed to be similar to the RGD motif, and are present in FN, VCAM-1 (Vascular Cell Adhesion Molecule 1) and MAdCAM-1 (Mucosal Addressin Cell Adhesion Molecule-1). This group consists of integrins like α9β1, α4β7, αEβ7 and β2 families (Humphries, Byron and Humphries, 2006; Campbell and Humphries, 2011).

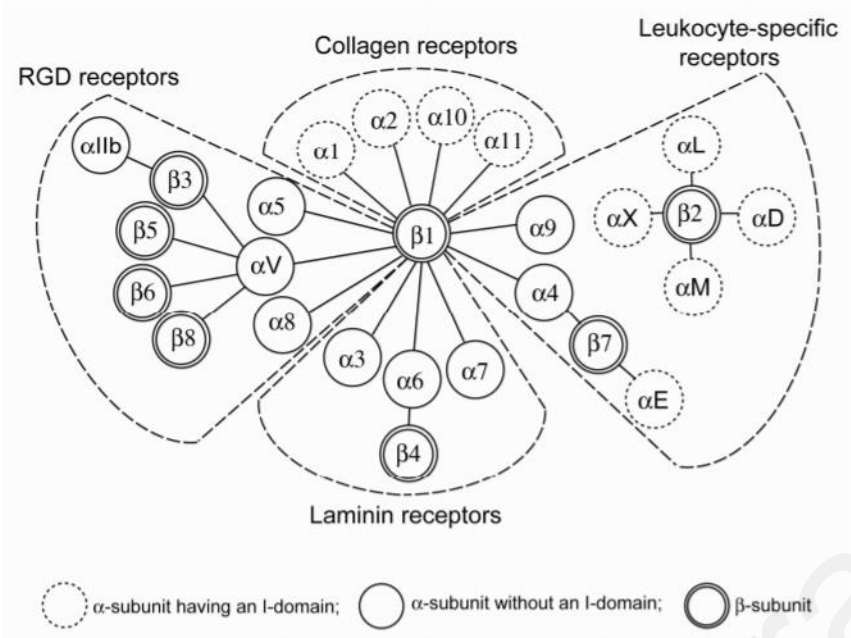


Figure 7: Schematic representation of integrin subunits and their ligands.

Classification of integrins based on their ligand specificity. Adapted from: (Nevo, 2010)

1.2.1.2 Integrin activation and role during development.

As mentioned above, integrins exist in different conformations. These alterations in conformation that can be induced either by cytoplasmic events (inside-out signaling) or binding of integrins to their ligands (outside-in-signaling) and result in the activation of integrins. This activation is the main event for the initial formation of the cell-ECM adhesion (Campbell and Humphries, 2011; Bidone *et al.*, 2019; Green and Brown, 2019a). The conformational changes that take place during these events are based on interactions of the extracellular domain of integrins and more precisely between the head and the legs. Integrin activation reflects ligand affinity (low, intermediate, high) of the extracellular domain and these different ligand affinity conformations exist in a dynamic equilibrium. The topic of integrin activation has been thoroughly studied using monoclonal antibodies (mAbs), ligand affinity chromatography and cryo-electron microscopy (EM) studies (Humphries, Byron and Humphries, 2006; Campbell and Humphries, 2011; Margadant *et al.*, 2011). These studies revealed insights of the integrin conformation alterations and their ligand binding. Integrins exist in a bend head-piece close state at which their affinity for their ligands is low. At this state, the extracellular globular head of the integrin faces the plasma membrane of the cell forming a reversed V shape topology (**Figure 8**). This conformation is held together through bonds between the tail, the legs of the extracellular domain that prevent any intracellular interactions, making the integrins unable to bind ligands extracellularly. Detailed analysis of integrins under the electron microscope using cryo EM, revealed an intermediate state of activation during which the heterodimer is extended with the

head of the extracellular domain unfolded from the V-shaped topology away from the membrane. This stage is also known as primed conformation (extended integrins with head-piece close) and it can bind external ligands with moderate affinity (**Figure 8**). The stage at which integrins are characterized by a high affinity for ligands is described as the extended head-piece open state integrin conformation. In this conformation, the cytoplasmic tails of the heterodimer separate and the β-I-domain and the propeller detach from the hybrid domain. These changes lead to the acquisition of a high affinity binding for their ligands and both the head and the tail become fully activated (**Figure 8**) (Askari *et al.*, 2009; Anthis and Campbell, 2011; Campbell and Humphries, 2011; Nevo, 2010; Luo, Carman and Springer, 2007, (Su *et al.*, 2016; Campbell *et al.*, 2020). The above model has been proposed using fluorescence resonance energy transfer (FRET) experiments and is the predominant model for integrin activation. Experiments by Askari et al showed that the dissociation of the two cytoplasmic domains of the heterodimer was crucial for the acquisition of the open-active conformation (Askari *et al.*, 2010; Horton *et al.*, 2016; Humphries *et al.*, 2019). More recently, experiments using cryo-EM came to similar conclusions (Campbell *et al.*, 2020). As stated, cytoplasmic β-tails of integrins are composed of two NPxY and NxxY motifs. These motifs become exposed upon integrin activation and compose of the binding sites for Talin and kindlin respectively. Talin binding on the NPxY motif drives the disruption of the salt bridge which is formed at the membrane-proximal regions of the two subunits (Tadokoro *et al.*, 2003; Anthis and Campbell, 2011). The following binding of kindlin at the second motif has been found to play a major role during integrin activation since its inhibition leads to blockage of this process (Baade *et al.*, 2019; Nordenfelt, Elliott and Springer, 2016). The clustering of integrins coexists with the binding of these proteins and the downstream multiprotein complex formed at those sites. This protein complex is responsible for downstream transduction of signals (Campbell and Humphries, 2011).

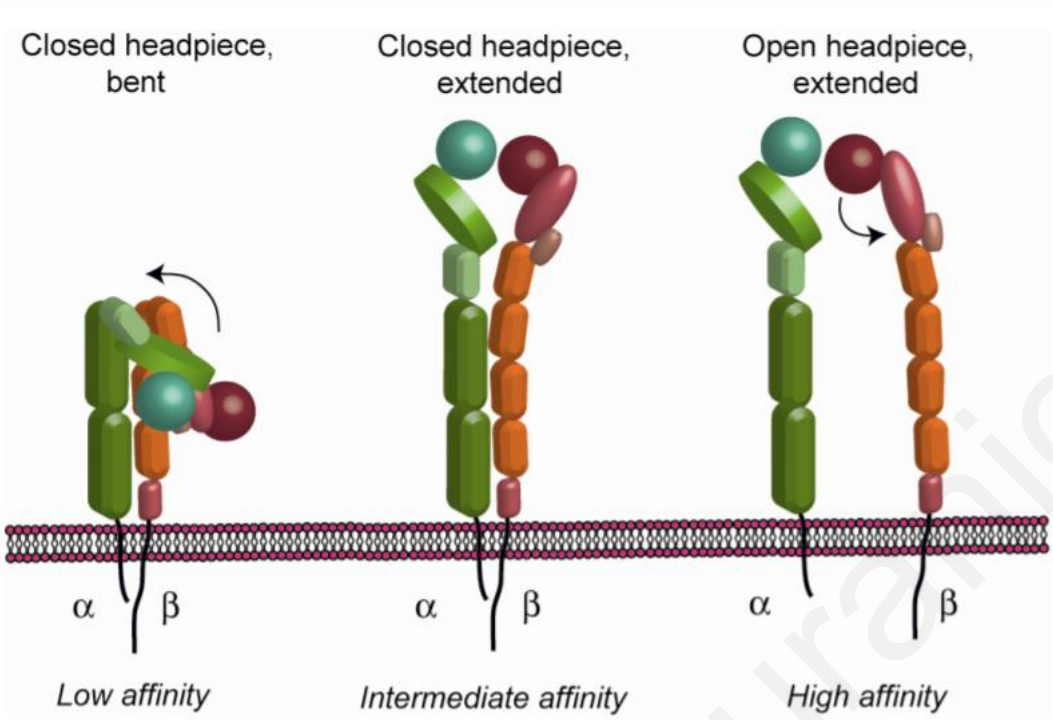


Figure 8: Integrin conformational stages and activation.

Representation of the integrin conformations from the low-affinity stage to the high affinity and the changes observed in the heterodimer. Adapted from: (Gahmberg *et al.*, 2009).

As already mentioned, activation of integrins has been shown to occur either in an outside-in manner or in an inside-out manner. More recently another mode of activation had been proposed and this activation is maintained via plasma membrane tension (Maria and Ferraris, 2010; Ferraris *et al.*, 2014; Petridou and Skourides, 2016; Kim *et al.*, 2020). The outside-in signaling is fundamental for the maintenance of the adhesion since integrins lack intrinsic catalytic activity. Through their binding to their ligand, they can drive signal transduction inside the cell. This signaling is responsible for the formation of FAs and the assembly of actin stress fibers. This eventually leads to integrin clustering and matrix binding (Ffrench-Constant, 2003; Manakan.B Srichai, 2017). During this process, the integrin α-subunit plays an important role since it controls the ligand specificity. Initially, the immediate events that take place at the first 60 seconds include the accumulation of lipid second messenger phosphatidylinositol (4,5) bisphosphate (PIP_2) or phosphatidylinositol (3,4,5) triphosphate (PIP_3) and phosphorylation of numerous protein substrates, at this point actin polymerization guides alterations in cell shape and all these lead to effects on cell survival, proliferation and differentiation. As mentioned above, the integrin cytoplasmic tail lacks enzymatic activity. This means that integrins in the bend head-piece close conformation require the binding of adaptor or structural proteins on specific domains. This binding serves as a connector between integrins and other proteins of cell-ECM adhesions (Ffrench-Constant, 2003; Manakan.B Srichai, 2017). The inside-out signaling is a crucial process in situations where ligands are close to the cells (like blood), in developmental processes and in morphogenetic processes where cells migrate for a

specific amount of time. Under the resting, close conformation, integrins have low affinity for their ligands and through activating signals that emerge from the inside of the cell, their conformation alters, the headpiece extends and their affinity for their ligand increases. The signals emerging from the inside part of the cell are mainly through the binding of other proteins to the cytoplasmic tails of integrins like talin, kindlin, filamin and a-actinin. To date, two different models of how integrins become activated have been proposed. Both theories highlight the necessity of conformational changes, occurred at the head of the extracellular domain of integrins, in ligand binding (Ffrench-Constant, 2003; Manakan.B Srichai, 2017, Arnaout, Goodman and Xiong, 2007, Luo, Carman and Springer, 2007). The integrin TM has been shown to be necessary for the activation. It is widely believed that the separation of TMs of integrin subunits is required for this process. Different models exist in terms of how the separation occurs and the most abundant are the "piston model" and the "scissors model". In the first case, it is suggested that vertical movements between the TM of the subunits lead to a separation while the second model proposes that there is only an increase of the angle of the two subunits TM (Ffrench-Constant, 2003; Wegener *et al.*, 2007; Anthis and Campbell, 2011; Manakan.B Srichai, 2017). The role of the cytoplasmic domain is crucial during this process since, as mentioned above, the adaptor and structural proteins of the adhesion sites bind at this domain to drive increased ligand affinity (Theodosiou *et al.*, 2016, Margadant *et al.*, 2011).

The role of integrins during development has been found to be crucial through a plethora of studies. Work from Hynes et al. elegantly showed that knockout mice displayed severe developmental defects with some displaying embryonic lethality. Mice that were homozygous null for integrin β1 displayed lethality at pre-implantation stages, during day E6.5. Mouse embryos derived from integrin β1-deficient stem cells led to major morphogenetic anomalies including gastrulation and neurulation failure (Taverna *et al.*, 1998, Daniel *et al.*, 2001). Experiments using conditional knockout mice for integrin β1 at neural crest precursor cells resulted in aberrant neurulation and severe defects which led to lethality one month after their birth. This phenotype was accompanied by defective migration of neural crest cells and abnormal maturation of Schwan cells (Taverna *et al.*, 1998) (Daniel *et al.*, 2001). Furthermore, experiments in the inner cell mass of the blastocysts using integrin β1 null blastocysts displayed abnormal migration of the extraembryonic mesoderm and morphogenesis (Zhu *et al.*, 2002). Lastly, experiments using other integrin subunits showed major defects in embryonic development. As Zhu, Motejlek et al. elegantly showed, embryos that lack the gene coding for integrin β8, display embryonic lethality at day 11.5 due to malfunctions in vascular development of the placenta, yolk sac and the nervous system (Hodivala-Dilke *et al.*, 1999; Zhu *et al.*, 2002). The α-integrin subunits have also been studying in terms of embryonic development and it has been shown that integrin α5-null mice show mesodermal defects and extraembryonic defects which lead to embryonic lethality at day E10-11 (Mercurio, 2002).

1.2.2 Talin

Talin is the direct link of integrins with the actin cytoskeleton thus it can be characterized as one of the most important adaptor proteins during this process. In vertebrates, Talin exists in two different isoforms; Talin 1 and Talin 2. Even though the isoforms share approximately 88% similarities in their sequence, they are not functionally redundant since Talin 2 cannot replace the function of Talin 1. Their precise differences are still under investigation. In mammals, Talin 1 is expressed in an ubiquitous manner while Talin 2 displays distribution is specific tissues such as heart, brain and skeletal muscle (Manso *et al.*, 2013; Gough and Goult, 2018). Talin 1 is a 270kDa homodimeric protein and is one of the proteins that is recruited to the cell-ECM adhesion sites. Talin acts as a cytoplasmic ligand of integrin and it consists of an N-terminal globular head which is approximately 47kDa and a large rod-like C-terminal domain which is approximately 220kDa (Cohen *et al.*, 2005; Petrich, 2009; Wehrle-Haller, 2012). The head region is composed of a four-point one, Ezrin, Radixin, Moesin (FERM) domain which is subdivided into F1, F2 and F3 domains and the F0 domain. The F3 domain binds to the membrane-proximal NPxY motif of integrin cytoplasmic β tail while the F1 and F2 domains are known to anchor to the cell membrane. The Talin FERM domain was also shown to interact with negatively charged PIP_2 and FAK. The Talin rod-shaped C-terminus is composed of 62 amphipathic a-helices which form into a series of 13 helical bundles (R1-R13). This domain interacts with vinculin and actin. The C-terminal rod shape domain is followed by a helical dimerization domain (DD) (Cohen *et al.*, 2005; Petrich, 2009; Wehrle-Haller, 2012). At its physiological state, Talin acquires an autoinhibited conformation through the binding of the head domain to the rod intracellularly. For its activation, Src signaling interactions with negatively charged PIP2, or proteolysis are required (Cohen *et al.*, 2005; Petrich, 2009; Wehrle-Haller, 2012). Experiments using magnetic tweezers and molecular dynamics, identified the ability of Talin to become active through force application suggesting that Talin acts as a mechanosensor (Calderwood, Campbell and Critchley, 2013; Kumar *et al.*, 2016; Li, Lee and Zhu, 2016). Talin, as mentioned above, is one of the first proteins recruited at the integrin adhesions and induces integrin conformational changes through its binding to the cytoplasmic integrin tail (**Figure9**) (Wegener *et al.*, 2007; Zhang *et al.*, 2008; Petrich, 2009; Kumar *et al.*, 2016). It was shown that Talin binding to cytoplasmic integrin tail is responsible for the disruption of the salt bridge formed between the two subunits resulting in conformational changes and activation of integrins. The critical role of Talin during embryonic development has been shown *in vivo*. Cells lacking Talin display FA assembly defects and aberrant FAK signaling (Wegener *et al.*, 2007; Zhang *et al.*, 2008; Petrich, 2009; Kumar *et al.*, 2016). Embryos knocked out for Talin display defective cell migration during gastrulation leading to embryonic lethality by E8.5 (Czuchra *et al.*, 2006; Petrich, 2009; Meves *et al.*, 2013).

1.2.3 Vinculin

Vinculin is a key-player in the cell-ECM link. It is a 117kDa protein composed of a C-terminus tail domain that is rod-shaped, an N-terminus globular head domain and a short proline-rich sequence which is found between the head and the tail. Vinculin has been shown to interact with numerous structural adaptor proteins both at the rod-shaped tail and at the globular head domain. Vinculin head domain interacts with talin, a-catenin and a-actinin while the tail domain interacts with actin paxillin, Arp2/3 complex and PIP_2 (**Figure 9**). Normally vinculin adopts an autoinhibitory conformation which prevents talin and F-actin binding since these binding sites are covered. Vinculin activation occurs only at the adhesion sites via talin and actin-binding. This activation is responsible for the maturation of NAs into FCs (Isenberg, Leonard and Brigitte M. Jockusch, 1982; Riveline *et al.*, 2001; Li, Lee and Zhu, 2016; Baade *et al.*, 2019). Experiments using FRET sensors have also demonstrated that vinculin is crucial for the stabilization of FAs (Grashoff *et al.*, 2010). The regulation of integrins through vinculin occurs via the recruitment and increased localization of PIP_2. PIP_2 bind talin resulting in an increased activity of the molecule, for integrins. During embryonic development vinculin has been found to play a major role since vinculin null mice display neural tube defects and defective heart development which lead to embryo lethality at E8-10 (Xu, Baribault and Adamson, 1998; Atherton *et al.*, 2016).

1.2.4 Tensin

Tensin is a cytoskeleton scaffolding protein of approximately 170kDa and it is composed of an N-terminal domain of three actin-binding domains (ABDs), a C-terminal domain which contains a phospho-tyrosine binding domain (PTB) and a Src homology domain (SH2) domain. Tensin appears later at the cell-matrix adhesions and is not present at the NAs.The N-terminal domain of tensin and more precisely the PTB has been shown to directly interact with the integrin cytoplasmic tails (**Figure 9**). This interaction allows tensin to link integrins with actin filaments through its ABD domains. Through its SH2 domain tensin provides tyrosine phosphorylation signaling and is implicated in the assembly and disassembly of the FAs (Lo, 2004).

1.2.5 Integrin-linked protein kinase (ILK)

ILK is a protein composed of three domains. The N-terminus domain consists of four ANK repeats and a pleckstrin homology (PH) like motif. The C-terminus is found in close proximity with the PH motif and is similar to other protein kinase catalytic domains. The role of ILK is mainly to promote protein-protein interactions and up to date several partners of ILK have been identified. The ANK domain of ILK interacts with the 5 Lim domains of PINCH while the C-terminal domain has been found to interact with the integrin cytoplasmic tails, affixin and can be recognized by paxillin (**Figure 9**). Localization of ILK at the cell-ECM adhesion sites occurs after the FA formation and there is

evidence suggesting its presence at the FBs too. Its localization requires two different interactions; first the ILK-PINCH interaction through the ANK domain of ILK. Mutations that disrupt this interaction have shown the inability of ILK to localize at the FA sites. The second interaction that is responsible for ILK localization at those sites is the interaction with its C-terminal domain. Previous studies showed that mutations of the C-terminus prohibited ILK localization at the adhesion sites. The role of ILK in embryonic development is highly underlined by numerous studies. Null mutations of this protein display similar defects to the ones caused by null integrin mutations. Experiments in mouse embryos knocked out for ILK, display embryonic lethality around E8.5-10 while experiments on fibroblasts that were ILK null display F-actin deficient accumulation at the integrin adhesion sites suggesting that ILK is involved in actin regulation while those cells displaying severe differentiation defects (Hannigan *et al.*, 1996; Dedhar, Williams and Hannigan, 1999; Brakebusch, 2003; Hehlgans, Haase and Cordes, 2007).

1.2.6 Paxillin

Paxillin is a 68kDa adaptor protein composed of five leucine and aspartate rich sequences LDXLLXXL (LD) domains at its N-terminal and four Lin11, Isl-1, Mec-3 (LIM) domains at its C-terminal. Both N and C terminuses are known to mediate interactions with numerous proteins of the cell-ECM adhesions. Its N-terminal domain is responsible for paxillin-vinculin interactions as well as for paxillin-FAK interactions. The C-terminal domain of paxillin mediates interaction with tubulin and PTP-PEST (**Figure 9**). It has been previously shown that paxillin interacts directly with some integrin subunit cytoplasmic tails like α4 and β1. Paxillin is characterized by a plethora of sites that become phosphorylated by different proteins like FAK, Src, PAK, and Cdk5. Paxillin controls the spatiotemporal activation of Rho GTPases at the adhesion sites thus regulating the plasticity of the complex. During embryonic development paxillin, null mice die at E9.5 with severe defective phenotypes such as anterior-posterior axis shortening and somite abnormalities (Hashimoto *et al.*, 2001; Riveline *et al.*, 2001; Webb *et al.*, 2004; Stutchbury *et al.*, 2017).

1.2.7 Focal Adhesion Kinase (FAK)

FAK is a non-receptor tyrosine kinase and has a size of 125kDa. FAK is composed of a Focal Adhesion Targeting (FAT) domain at its C-terminus, a central catalytic kinase domain, a (FERM) domain, and proline-rich regions, which act as binding sites for different proteins at its N-terminus domain. Activation of FAK is mediated upon integrin adhesion and after tyrosine phosphorylation. The FERM domain of FAK has been shown to bind peptides of the cytoplasmic tail of β1 integrin, PIP_2 and distinct growth factor receptors (GFRs). The FAT domain guides the targeting of FAK at the FA complexes and contains binding sites for talin and paxillin (**Figure9**). Under physiological

conditions, FAK is autoinhibited and when integrin-mediated adhesion is achieved, FAK becomes auto phosphorylated at a tyrosine known as Y397 creating a high-affinity binding capacity for Src. Binding of Src initiates a cascade of downstream phosphorylation events on FAK molecule, at different tyrosine sites. This, creates more binding sites for proteins such as RhoGAP GRAF and p130Cas. Mice that lack FAK die during early development at day E8.5 due to mesodermal defects and cardiovascular defects (Otey, 1996; Wang *et al.*, 2001; Plotnikov *et al.*, 2012; Gayrard *et al.*, 2018).

1.2.8 p130Cas

p130Cas is known as Crk-associated protein or as breast cancer antiestrogen resistance protein (BRCA1) (Zhang *et al.*, 2013). P130Cas is a non-enzymatic docking protein that localizes and functions at FAs. P130Cas has 4 major domains. The SH3 domain mediates the interactions with FAK and is necessary for the localization of p130Cas at FAs (Meenderink *et al.*, 2010), the SD composed of 15 scattered YXXP motifs, and is located in the central region of the protein, the SBD at which c-Src protein binds and the CHH domain composed of 140 residues and it is unique for this protein (Meenderink *et al.*, 2010; Bae *et al.*, 2014, Camacho Leal *et al.*, 2015). It was previously shown that the SH3 and CCH domains of p130Cas are necessary for the correct localization os p130Cas to FAs (Meenderink *et al.*, 2010) while the SD fails to be phosphorylated in the absence of the SH3 domain. It is believed that p130Cas targeting to FAs is achieved through interactions of SH3 domain with FAK. This provides evidence that FAK is responsible not only for the recruitment of p130Cas at FAs but also for the recruitment of Src. The Src-FAK interaction further phosphorylates p130Cas SD and promotes downstream signaling pathways. Besides, the N-terminal domain of p130Cas has been shown to interact with other proteins like PTP-PEST and protein tyrosine kinase 2 (Pyk2) while its C-terminal domain has been shown to contain binding sites for P13K (**Figure9**). Cells null for p130Cas have a disorganized actin network while the actin-bundling is defective. Aside from these, cells lacking p130Cas have been shown to display problematic cell spreading and reduced FA disassembly rates. *In vivo* experiments using mice suggested that p130Cas knockout led to embryonic lethality during E12.5 with distinct phenotypic characteristics like defects in blood vessels and heart (Nojima, 1999; Garton, Flint and Tonks, 1996; Nakamoto *et al.*, 2000).

1.2.9 c-Src

Src is a member of the Src family kinases (SYFs) and is a non-receptor tyrosine kinase. Its N-terminal domain is a unique, myristoylated domain followed by SH3, and SH2 domains, and a linker compartment. Its C-terminal domain is composed of a tyrosine kinase domain (SH1) (Boggon and Eck, 2004). The SH2 domain of Src is responsible for FAK binding at Y397 resulting in the phosphorylation of downstream targets such as paxillin and p130Cas (**Figure 9**) (Boggon and Eck, 2004; Gayrard *et al.*, 2018). Fibroblast cells, null for Src, display abnormalities at the cell-ECM

adhesions. The size of FBs is notably increased, their spreading and adhesion ability on FN is reduced and their phosphotyrosine levels are dramatically decreased (Kaplan *et al.*, 1995; Strohmeyer *et al.*, 2017; Webb *et al.*, 2004). The family of Src kinases is composed of other proteins that display similarities and homology with Src and as a result *in vivo* experiments in mice display minor phenotypes like osteoporosis and this might be a result of the compensatory role of other family members proteins (Soriano *et al.*, 1991).

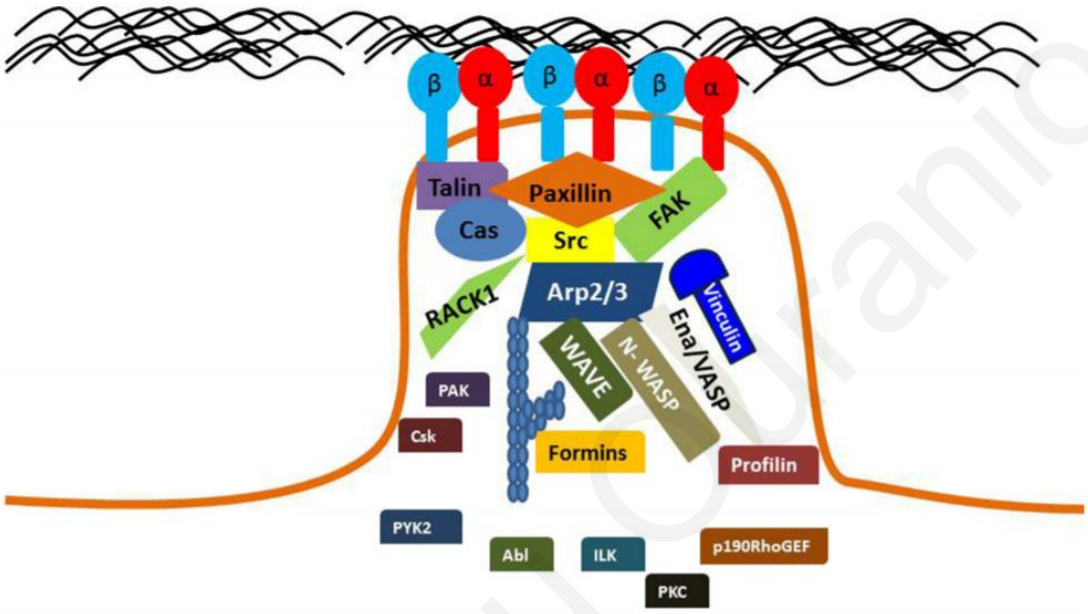


Figure 9: Integrin adhesions: protein interactions

Schematic representation of the protein interactions that take place within the adhesions for the cell to achieve a linkage of ECM with the actin cytoskeleton. Adapted from: (Maziveyi and Alahari, 2017).

1.2.10 Fibronectin

Fibronectin is a major player in integrin adhesions (even though it is an ECM component) since it is the most dominant ligand of cells in culture. Fibronectin (FN) is a protein with size approximately 250kDa and even though a single gene produces the FN protein, alternative mRNA splicing can generate different variants (approximately 20) of FN (Pankov and Yamada, 2002; Schwarzbauer and DeSimone, 2011). FN is composed of two almost identical subunits that are linked together through their C-terminal domains by disulfide bonds. Each monomer comprises a different number of three distinct types of repeats; twelve of type I, two type II and fifteen to seventeen type III. Each one of these repeat types is necessary for the binding with different proteins of the ECM and a plethora of integrin receptors such as α5β1, αvβ1, αvβ3, α4β1 and α3β1. Recent studies have identified the precise sequences where integrins interact with FN with the most known to be the RGD sequence. This RGD is located in FN $III_{9\text{-}10}$ and is located in the central cell-binding domain (**Figure 10**) (Pankov and Yamada, 2002; Schwarzbauer and DeSimone, 2011). FN exists as a soluble molecule

in the plasma and other body fluids and as an insoluble molecule at the cell. The soluble form of the protein is synthesized predominantly in the liver by hepatocytes and displays a simple splicing pattern. In contrast, the insoluble form of the protein is larger and composed of a group of variant FN isoforms. The soluble form of FN is in an inactive form and the formation into the insoluble form of FN requires integrin binding (**Figure 10**). During this tightly regulated process, known as FN fibrillogenesis (or FN matrix assembly), soluble FN is self-associated into fibrils at multiple binding sites forming the FN matrix (Baneyx, Baugh and Vogel, 2002; Pankov and Yamada, 2002; Schwarzbauer and DeSimone, 2011; Mosher, 1993). Experiments *in vivo* using amphibian and avian models showed that blockage of RGD binding sites of FN leads to defects in mesoderm migration during gastrulation suggesting that FN is expressed and assemble into the fibrillar matrix prior the initiation of gastrulation (Winklbauer and Stoltz, 1995; Winklbauer, 1998; Schwarzbauer and DeSimone, 2011). Mice with disrupted FN gene display embryonic lethality with intense phenotypes of defective mesodermal development, defective notochord, and somite development and heart defects (Zhang and Labouesse, 2012; Schwarzbauer and DeSimone, 2011). Studies also demonstrated that FN null embryos are characterized by a shortening of the A-P axis and defective trunk mesoderm (Pulina *et al.*, 2011; Schwarzbauer and DeSimone, 2011; Mosher, 1993; Georges-Labouesse *et al.*, 1996; George *et al.*, 1993).

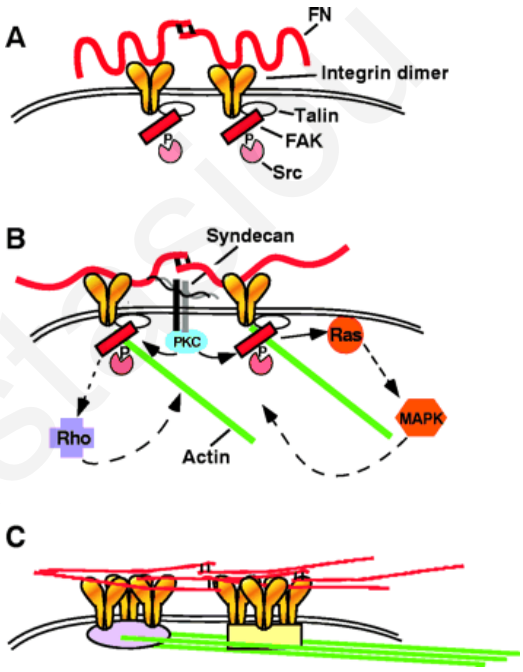


Figure 10: Fibronectin fibril formation and matrix assembly

(A) Binding of soluble FN molecules at the integrin receptors initiates conformational changes that drive to increased clustering of integrins and binding of talin. (B) The initial formation of FN fibrils and subsequent organization of actin cytoskeleton from integrins and Syndecan 4. (C) The concentration of active FN at the sites of integrins promoting FN matrix assembly. Adapted from: (Wierzbicka-Patynowski and Schwarzbauer, 2003).

1.3 Integrin adhesions and mechanotransduction

Mechanotransduction is defined as the ability of cells to sense and respond to mechanical signals applied to them either by their extracellular environment or by their internal environment and tension generation. Tension is defined as the situation under which bidirectional forces are applied to the opposing site of an object (Jansen, Atherton and Ballestrem, 2017). A variety of sensory organelles and structures inside the cell are known to have the ability to convert the mechanical forces into biochemical signals. The most well-described examples are the cilia, the cell-cell contacts and the adhesion of cells to their ECM. The evidence that the activation of integrins can occur through application of force generation by the actin cytoskeleton and contraction resistance, make each component of the cell-ECM link prime candidates of mechanotransduction. Integrin dimer formation is characterized by a variation in force sensing and transducing ability, however, the different dimers can bring a mechanical equilibrium to the cell (Martino *et al.*, 2018; Leiphart *et al.*, 2019). The role of integrins in mechanotransduction and mechanosensation was suggested through experiments using force application both externally and internally of the cell. The internal force application was achieved through an FN-variant coating of beads which did not support FA formation. The external force was applied to the cells with the use of laser tweezers. This force application showed that the adhesion site becomes mature through its strengthening upon strength application. Data also suggested that integrins have the ability to acquire their open-active conformation through mechanical force application (Galbraith, Yamada and Sheetz, 2002). The first experiments were performed using NIH3T3 cells which were allowed to attach and spread on elastic membranes which were coated with FN. Integrins αvβ3 became active and this activation was found to drive increased affinity for integrin ligands which was shown to be P13K dependent. Further experiments using β3 integrins showed that the deletion of this integrin is driving a dramatic increase of traction forces in the cells (Puklin-Faucher *et al.*, 2006; Martino *et al.*, 2018). Initial experiments using integrin β1 suggested its role in mechanotransduction since the deletion of this integrin was shown to cause a decrease in the contractile forces of the cell (Schwartz and DeSimone, 2008; Shiu *et al.*, 2018). Work by Friedland, Lee et al. showed that upon force application on the α5β1 integrin heterodimer, its affinity for FN increased. This resulted to alterations of integrin conformation from its bend head-piece close inactive state to its active open head-piece extended state. In agreement with these results, experiments using atomic force microscopy revealed that forces were strengthening the catch bonds on integrins; known to stabilize the heterodimer in the open-active conformation (Julie C. Friedland, Lee and Boettiger, 2009). A recent study by Ferraris et al. revealed the ability of integrin β1 to activate in response to mechanical tension applied to the cell membrane. They also suggested that this activation was ligand independent since the experiments were performed using an artificial system where cells were able to spread and attach on VN substrates. This cell adhesion was mediated through uPAR and was independent of integrin β1 and their results demonstrated that downstream signaling of integrin β1 was present under these conditions. This suggested that ligands are

dispensable for integrin activation under these conditions (Ferraris *et al.*, 2014; Maria and Ferraris, 2010). Recent work from our laboratory revealed an association of this ligand independent but force-dependent integrin activation with the mitotic division. As Petridou and Skourides elegantly showed, during mitotic divisions integrin β1 becomes activated at the lateral cortex of the mitotic cell in a ligand independent but force dependent manner. This activation guides the formation of a newly discovered complex of proteins known as Cortical Mechanosensory Complex (CMC). CMC is composed of FAK, p130Cas and Src. (Petridou and Skourides, 2014; Petridou and Skourides, 2016). This integrin activation found indispensable for spindle orientation since its blockage using monoclonal antibodies led to the missorientation of the mitotic spindle (Petridou and Skourides, 2016). More recently, work by Jiyoon Kim et al. showed that aIIbb3 integrin becomes activated through shear stress, osmotic pressure and stress. This activation was independent from its intracellular signaling and was shown to be depended on applied mechanical stimuli. The authors also showed that upon mechanical stimuli the lipidic embedding of β3 integrins TMD was altered, the interactions of aIIb with β3 TMD become weak and integrins become activated (**Figure 11**) (Kim *et al.*, 2020).

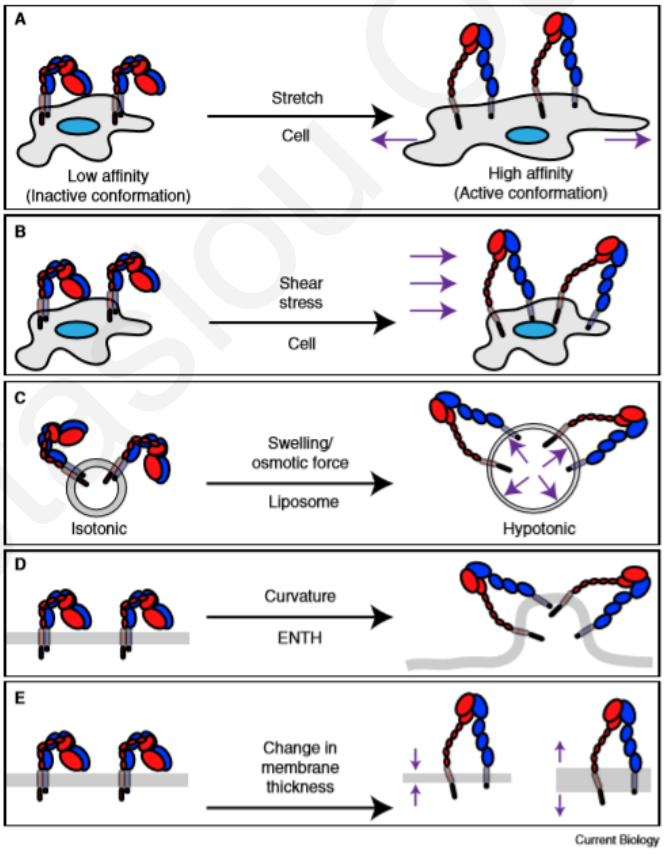


Figure 11: Integrin activation is induced by mechanical forces

Integrin aIIbb3, one major adhesion receptor, can be activated by various mechanical stimuli such as: (**A**) stretch applied on the cell; (**B**) shear stress induced by flow on a cell; (**C**) osmotic force to inflate and stretch the liposome membrane bilayer; (**D**) membrane curvature; and (**E**) change in membrane thickness by lipid composition of different chain length. Adapted from: Kim et al. 2020

During the past decades the mechanotransduction at cell-ECM adhesion sites has been extensively studied. The first evidence suggesting that the FA protein members act as mechanotransducers emerged from experiments between actomyosin stress fibers and variable stiffness cell-cultured substrates composed of ECM-polymers. The cells on rigid substrates have the ability to form stronger adhesions than the ones on softer substrates (Pelham and Wang, 1997; Engler *et al.*, 2006; Schwartz and DeSimone, 2008; Plotnikov *et al.*, 2012). Experiments using variations in ECM-ligand concentration led to variations in cell spreading and FA formation. Increased ligand concentration has been shown to increase cell spreading which was found to regulate important cellular functions through cytoskeletal tension alterations, RhoA mediation and cell shape (Chen *et al.*, 2003; Schwartz and DeSimone, 2008; Hur *et al.*, 2020). Work using cell substrates with variable mechanical properties such as hydrogels, showed an association of the substrate stiffness and human Mesenchymal Stem Cells (hMSC) since the stiffness of these polymer substrates was guiding the fate of hMSCs through the remodeling of FAs and cytoskeleton (Engler *et al.*, 2004; Discher, Janmey and Wang, 2005; Hur *et al.*, 2020). Earlier experiments using contractility inhibitors found an association between the reduction of force application to the adhesion sites and the disassembly of FAs (Chen *et al.*, 2003). Lastly, the application of mechanical force through a micropipette at small dot-like adhesions resulted in local assembly and elongation of the adhesions (Riveline *et al.*, 2001).

The role of some core proteins of the cell-ECM adhesion sites, regarding their mechanical properties, has been studied in-depth and mechanotransducing roles have been identified for almost all the proteins. The ability of cells to sense the initial mechanical forces that are applied to them is crucial since it will determine its downstream responses. Talin and vinculin are the first protein players at the adhesome which bind either directly to integrins or actin cytoskeleton and connect them. It has been shown that both of these proteins have the ability to sense and further transduce mechanical signals (Jansen, Atherton and Ballestrem, 2017, Leiphart *et al.*, 2019). Talin has also been shown to act as a mechanosensory protein. Experiments showed that talin requires tension applied through the actomyosin machinery in order to stretch and acquire an open, active conformation. This was examined using mutants for the actomyosin binding site of talin (located at the C-terminus of the protein) which led to defective FA formation and reduced actin-binding abilities of the protein (Jansen, Atherton and Ballestrem, 2017, Atherton *et al.*, 2015). This ability of talin to stretch and reveal protein binding sites is important for its implication in mechanotransduction and is highly associated with vinculin recruitment at those sites (Ateshian, 2017; Jansen, Atherton and Ballestrem, 2017). Experiments using magnetic tweezers suggested that force can stretch talin and expose the sites for vinculin binding indicating that talin can respond to mechanical tension (Little *et al.*, 2008). Reports have associated vinculin with mechanostransducing and mechanosensing abilities since experiments in cells lacking vinculin display reduced adhesion forces after contractility inhibition. The usage of Förster resonance energy transfer (FRET) sensor has also shown that both vinculin and talin experience force application in living cells (Austen *et al.*, 2015; Kumar *et al.*, 2016) while photokinetic experiments showed a reduced turnover of both proteins when they are active

suggesting that their activation through forces after actin binding is a mechanism to reduce the retrograde actin flow which takes place during cell migration (Leerberg *et al.*, 2014; Atherton *et al.*, 2015; Stutchbury *et al.*, 2017). The recent described protein KANK appears later at the adhesion sites and it has been described as a talin activator at the R7 domain of talin (Sun *et al.*, 2016). This binding occurs in close proximity with the binding domain of F-actin on talin (ABS2) and has been shown to lead to reduced force transmission on talin, reduction of migration, and transition of FAs to FBs (Sun, Guo and Fässler, 2016). Within the adhesion sites, numerous downstream signaling events are taking place and the activation of talin and vinculin drive the recruitment of other molecules such as FAK and Paxillin. These two proteins are characterized by high tyrosine phosphorylation activity and it has been proposed that these tyrosine phosphorylation events are the first responses of the adhesion sites to the applied mechanical stimuli (Jansen, Atherton and Ballestrem, 2017; Mitra, Hanson and Schlaepfer, 2005). Myosin activation subsequent paxillin phosphorylation was found important for the recruitment of vinculin at the FAs (Arold, Hoellerer and Noble, 2002; Ateshian, 2017). FAK's role in mechanotransduction has been extensively studied and it was shown that the phosphorylation of FAK at Y397 was higher on stiff substrates than on soft. FAK null cells are known to display defects in FA disassembly and as a result they have been shown to have a defective response to contractility changes (Bae *et al.*, 2014; Martino *et al.*, 2018; Julie C Friedland, Lee and Boettiger, 2009). These cells also display defective durotaxis; a process during which cells can sense the stiffness of the substrate and orient their movement, upon local application of force, from the soft to rigid substrate (Wang *et al.*, 2001). FAK-null cells were found to have a preferential migration towards softer substrates. As a result, these cells display inability to transduce the mechanical signals applied to them and display lower traction forces. Experiments using cell stretching showed that FAK displayed increased phosphorylation upon lysophosphoric acid (LPA)-induced contractility, another piece of evidence suggesting its role as a mechanotransducing molecule (Nojima, 1999). Lastly, experiments using systems in which cells lack a surrounding membrane and their cytoplasmic part can react with the remaining cytoskeleton, showed that proteins like FAK, paxillin, and p130Cas displayed a preference in binding to these triton-X cytoskeletons (Sawada and Sheetz, 2002). These data suggested that these proteins are able to transduce, via their force-dependent conformational changes, forces that are applied by the matrix. FAK interacts with Src and this interaction has been found to promote downstream signaling within the adhesion sites. However, inhibition of FAK and Src signaling has been shown to block the cell responses under conditions where cyclic stretch is applied (Gauthier and Roca-Cusachs, 2018; Martino *et al.*, 2018). Moreover, experiments using uniaxial stretch on fibroblasts showed increased Src kinase activity which led to a dramatic increase of p130Cas, paxillin, and FAK phosphorylation. Another downstream protein that has been found to respond to increased contractility is p130Cas. This protein was found to have increased phosphorylation upon increased contractility and its implication in mechanotransduction began to unravel. Protein assay experiments *in vitro*, where mechanical stretch was applied to the SD of p130Cas led to the exposure of the 15 tyrosine residues

of this domain and subsequently drove an increased phosphorylation by Src. This resulted in Rap1 activation (Geiger, 2006). Other proteins, at the cell-ECM adhesion sites, that have been suggested to respond to mechanical stimuli and transduce the signal to downstream protein members are zyxin and Hic-5. More precisely, cells null for zyxin displayed decreased actin polymerization (Martino *et al.*, 2018). Beside from the core members of integrin adhesions, numerous structural and regulatory proteins have been found to have mechanotransductory or mechanosensory roles at those sites. For example, actin regulating protein a-actinin binds integrins, vinculin and actin and it is believed to play a role in the transmission of force and FA maturation. YAP and TAZ are two transcriptional activators of the Hippo pathway. Even though the precise mechanism is still unknown, YAP and TAZ have been found activated downstream of integrin activation and cellular tension. Besides the activation of YAP and TAZ upon actin polymerization, events associated with myocardin-related transcription factors (MRTFs) have been reported during this process. MRTFs are known to share transcription targets with TAZ and YAP activation pathways and experiments with application of unicyclic stress MRTFS become active leading to the YAP/TAZ activation. These results suggest that MRTFs are able to translate immediate mechanical stress into transcriptional responses (Sabina E. Winograd-Katz *et al.*, 2014; Sun, Guo and Fässler, 2016). Lastly, experiments in mouse embryonic fibroblasts showed a role of vinexin in rigidity sensing of the cells. Force application had been also associated with FN fibrillogenesis and matrix assembly through *in vitro* experiments using single molecule force spectroscopy. These experiments showed the ability of cryptic type III domains on FN molecule to unfold in response to force applied on the molecule (Martin *et al.*, 2010; Oberhauser *et al.*, 2002). Similar experiments using molecular dynamic simulations revealed the unfolding of RGD cryptic sites after application of specific force magnitude (Gee, Ingber and Stultz, 2008; Martino *et al.*, 2018).

1.4 Cell-cell interactions – Adherens Junctions composition and structure

During development in multicellular organisms, tissues undergo a variety of movements that require intact cell-cell connection. Without the cell-cell adhesion system, processes like gastrulation, neurulation, tissue compartmentalization and establishment of cell polarity are disrupted. As mentioned above, different types of cell-cell adhesion exist and each meets different tissue organizational requirements. These include, cell proliferation, epithelial to mesenchymal transition, and migration. Cell-cell adhesion is mainly achieved through complexes known as adherens junctions (AJs) which are initially believed to be static and stable complexes. Extensive research however provided new insight into these complexes and proved that these complexes are really dynamic (Niessen and Gottardi, 2008; Troyanovsky, 2012; Collinet and Lecuit, 2013). Briefly, AJs are composed of the cadherin transmembrane receptors, which are Ca^{2+} dependent, and their binding partners β, α and p120 catenins. Apart from these, a plethora of other proteins have been found to be associated either directly or indirectly with the cell-cell adhesions. Up to date, more than 170 proteins have been associated with the cadherin adhesion sites and most of them are separated into structural and regulatory proteins. The category of structural proteins includes more than 70 different proteins and consists of other transmembrane receptors such as nectins and immunoglobulin-like receptors (Troyanovsky, 2012; Collinet and Lecuit, 2013). The precise role of all those protein members is not clear and they might act as scaffolding proteins, signaling molecules or both. The larger group of adhesion regulators are phosphatases, tyrosine kinases and GTPases. Protein members like Arp 2/3 complex, VASP and mDial are proteins observed within the cadherin adhesions along with their activators and suppressors. The activators of these proteins are characterized by a Rho-GTPase dependent activation. It has been also suggested that these proteins are implicated in processes such as pushing membranes, protrusion formation and endocytosis. In general, the presence of cadherin adhesion complexes at different cell types is underlined by the fact that some proteins of the complex are observed only in specific cell types like neurojungin in neurons and KRITI in endothelial cells. Evidence suggests that other proteins from the integrin adhesome are present in these complexes like zyxin and tes. It has been also proposed that ZO-1 and cortactin also act as actin adaptors at those sites (**Figure 12**) (Niessen and Gottardi, 2008; Troyanovsky, 2012; Oldenburg *et al.*, 2015).

Cadherins from neighboring cells are connected between them through their ectodomains via homophilic interactions. Their α catenin binding connects them to the actomyosin cytoskeleton. Ca^{2+} ions are known to keep the cadherin molecules in a stable form of open conformation. The precise mechanism with which AJs are formed and undergo maturation is up to this day debatable. However, it has been suggested that the formation of the AJs is a result of two independent activities. The first activity initiates at specific sites of the cell-cell contact following a nucleation process. During this process, trans and cis interactions of the ectodomains of cadherins take place and create clusters in which, cadherins are arranged in specific arrays (Troyanovsky, 2012). These clusters have been identified in epithelial and cultured cells and it is suggested that they are composed of high density

trans-homophilic interactions of cadherins. Upon these interactions, cadherins acquire their open conformation. The second activity is achieved through the actin cytoskeleton which has been shown to control the retraction of membrane protrusions of neighboring cells. Evidence from experiments using Madin-Darby Canine Kidney (MDCK) cells, showed that the initiation of cell contact is achieved through the lamellipodia of the neighboring cells. Experiments on keratinocytes found that the initial cell-cell adhesion is achieved through contact via filopodia (Adams and Nelson, 1998). Actin cytoskeleton reorganization is the common event that underlines these two examples. Further supporting pieces of evidence on this initial cell lamellipodium contact were acquired from experiments performed in *C. elegans* and *Drosophila melanogaster*. During *Drosophila* dorsal closure of the epidermis, the cells of the lamellipodium project. This is observed at the cells of the leading edge of the epithelial sheets. This observation is identical to the *in vitro* setups in cell culture experiments. In *C. elegans*, experiments during ventral enclosure have shown the formation of filopodia. During this process, Cadherins and α-catenin were rapidly recruited at the filopodium contact regions, promoting the formation of cell-cell junctions. Loss of function of cadherins showed to promote defects in junction formation during this process (Tepass, 1999; Gumbiner, 2000; Niessen and Gottardi, 2008; Troyanovsky, 2012; Zaidel-Bar, 2013). Supporting evidence regarding the formation of clusters and the implication of the actin cytoskeleton in this process have recently emerged by a study from Gonschior et al. The group, using super-resolution microscopy, identified nanoscale clusters of cadherins and a stratified organization intracellularly at the regions where the AJs are formed. These nanoclusters were found associated with actin and this association seems to be dependent both on homophilic interactions and actin anchoring via the cadherin tail (Gonschior, Haucke and Lehmann, 2020). Recent studies have shown that other proteins are implicated in the formation of the nascent cadherin adhesions but their importance and precise role in these complexes is still under investigation. Proteins like nectin and afadin have been shown to initiate the formation of nascent cadherin adhesions prior to cadherin clustering. Experiments performed in mammalian cells showed that nectins can interact with the neighboring cell molecules in a similar manner. The interactions between two molecules of nectin lead to their clustering with afadin. Afadin has been shown to have a direct interaction with actin cytoskeleton, hence it is believed that this clustering of nectin and afadin is a crucial point for the recruitment of cadherins and the formation of the cadherin adhesions (Takahashi *et al.*, 1999; Takai and Nakanishi, 2003; Kiss, Troyanovsky and Troyanovsky, 2008). Irie et al. recently proposed a "fork" initiation and "zipper" model for the AJ formation. Experiments performed by another group suggested that nectin trans interactions are "uncooperative", i.e. the molecules of the opposing cells do not unbind in parallel, while the ones observed on cadherins are "cooperative" and display a parallel unbinding between them (Irie *et al.*, 2004; Inagaki *et al.*, 2005). These observations were in favor of the fork initiation and zipper model. They further suggested that the initial contact between cells is achieved through the uncooperative nectin molecules. This should promote the so-called fork initiation during which the nectin-nectin interactions lead to the stimulation of downstream signals. This drives the increase at cell-cell

contacts, and this is the point during which cadherins are recruited. As mentioned above, the importance of nectins during this process has not yet been clarified. The fact that these proteins are found at the cadherin adhesion sites may be tissue or cell type-specific. After the initial interaction, through cell lamellipodium, and the formation of the nascent junction, the complex becomes stable and it extends in a zippering fashion through the cell protrusions (Irie *et al.*, 2004; Tsukasaki *et al.*, 2007). The ectodomains of cadherins undergo trans homophilic interactions and form small clusters. The clusters grow and become more stable upon the recruitment of other molecules at these cell-cell adhesion sites, such as β-catenin and connection with actin. Previous work on E-cadherin has shown that the interaction with β-catenin occurs immediately after these proteins are exported from the endoplasmic reticulum. This suggests that these proteins are recruited at the sites of the adhesion simultaneously while α-catenin interacts with cadherin at a later stage. It is also known that β-catenin interacts with α-catenin and both of them form a stoichiometric complex which interacts with cadherins and actin cytoskeleton (Niessen and Gottardi, 2008; Meng and Takeichi, 2009; Troyanovsky, 2012). This opinion has been overcome in the past years where *in vitro* experiments showed that α-catenin is an allosteric molecule and it is unable to bind actin and β-catenin simultaneously. The binding of α-catenin to actin drives the recruitment of previously described FA protein vinculin. New studies suggest that at the sites of the cadherin adhesions other FA proteins are recruited as well. Proteins such as VASP, zyxin and TES have been identified at the AJs and their recruitment has been shown to be driven by the vinculin/α-catenin interaction (Oldenburg *et al.*, 2015).

Cadherin adhesion's assembly and disassembly are dynamic processes which undergo strict regulation. Evidence emerged connecting the post-translational modifications in the loss of cell-cell adhesion with processes like cadherin endocytosis. It is well established that under normal conditions cadherins undergo constitutive, cyclic endocytosis which eventually will recycle them back to the membrane (Zaidel-Bar, 2013). However, initial experiments using EM during AJ disassembly, showed an accumulation of endocytosis vesicles in close proximity to the plasma membrane (Bryant and Stow, 2004; Warren and Nelson, 1987). These vesicles were clathrin- and dynamin-rich and provided the first evidence on how the disassembly of AJs is promoted. During AJ disassembly, dynamin is recruited at the sites of the adhesions and drives clathrin-dependent endocytosis which subsequently blocks the return of cadherins to the membrane (Kamei *et al.*, 1999; Le, Yap and Stow, 1999; Ivanov, Nusrat and Parkos, 2004). Further evidence suggested that other endocytic pathways are involved in this process since caveolin rich endocytic vesicles have been reported in specific cell types (Lu *et al.*, 2003; Paterson *et al.*, 2003). Recent studies identified other endocytic adaptor molecules such as AP-1B, Dab2 and Numb as factors implicated in cadherin endocytosis clathrin or caveolin based. Proteins like RF6-GTPase are known to modulate the cadherin (E) movement along the endosomal pathway. This GTPase has been found to recruit a kinase responsible for the dynamin-dependent vesicle fission which eventually facilitates the internalization of cadherins (Krishnan *et al.*, 2001; Palacios *et al.*, 2002). Extra regulation of cadherin internalization may be a result of

interactions via proteins known to promote AJ disassembly like Src. Indeed, it was proven recently, that Src phosphorylates β-catenin and as a consequence the interaction between cadherin and α-catenin is lost. This leads to the weakening of the AJs their possible disassembly (Zaidel-Bar, 2013). This evidence is further supported by the current knowledge that cadherins have a PEST sequence. This PEST sequence is masked during the interactions with β-catenin, and acts as a degradation signal. This degradation signal will eventually drive the internalization of cadherins. Other evidence from mammalian systems, suggests that a precise p120-catenin binding domain at cadherins is implicated in endocytosis through its binding to the IL2 receptor on the plasma membrane (Anastasiadis and Reynolds, 2001; Morali *et al.*, 2001). Apart from these, experiments using MDCK cells showed that the posttranslational modification ubiquitination, which acts as an internalization signal, is involved in cadherin adhesion disassembly. Lastly, a number of ligases have also found to target cadherins tails and promote their internalization through the p120-catenin binding domain (Anastasiadis and Reynolds, 2001; Huber *et al.*, 2001; Morali *et al.*, 2001; Marambaud *et al.*, 2002). Taken altogether, the disassembly of the cadherin adhesions is a complex procedure depending on a variety of regulators, signals and modifications (**Figure 12**) (D'Souza-Schorey, 2005).

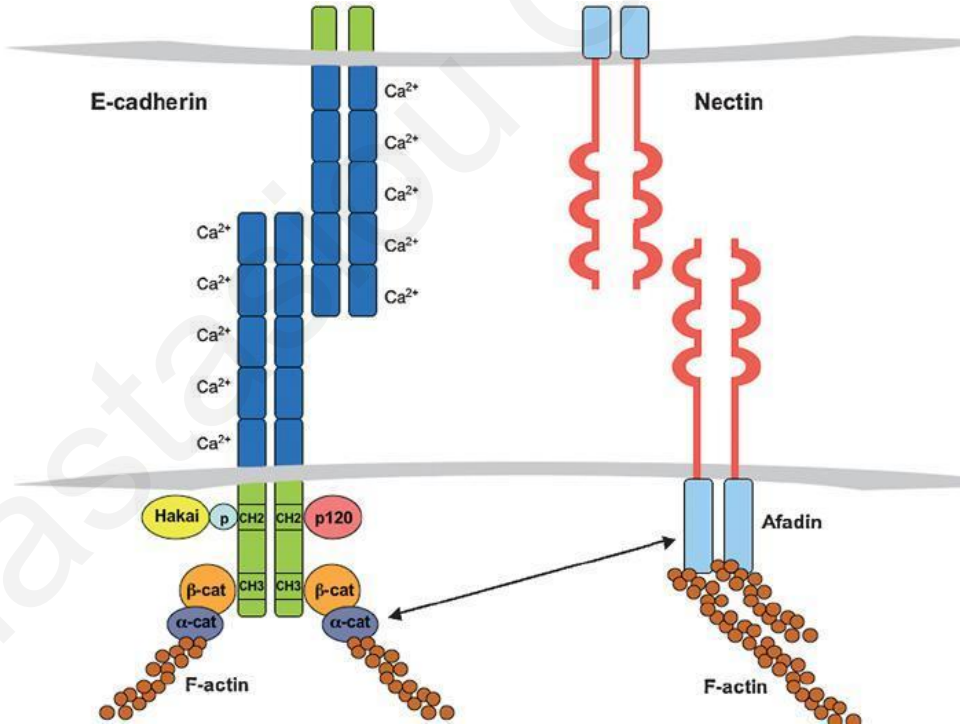


Figure 12*: Cell-cell interactions; AJs and member molecules involved*

Schematic representation of AJ molecular architecture and structure. Adapted from: (Miyoshi and Takai, 2011)

1.5 Cadherin adhesome; member proteins, mechanisms of activation and structure.

This section includes a more detailed overview of the core proteins composing the cadhesome. It describes their mechanisms of activation and their general roles during embryonic development. The importance of this complex is shown by numerous studies that support the notion that the cadherin adhesions are indispensable for the maintenance and repair of tissues, the epithelial to mesenchymal cell transition and the formation of a multicellular organism.

1.5.1 Cadherins-Structure and classification and roles during development

Cadherins are the major component of the AJ complexes. Back in 1982 they were identified, by Yoshida and Takeichi, as membrane glycoproteins that are Ca^{2+} dependent (Yoshida and Takeichi, 1982). Cadherins are a family of proteins whose members are that is characterized by a structural diversity. The diversity and categorization of cadherins are based on variations observed at their extracellular domains. The three major types of cadherins are classical cadherins, protocadherins and atypical cadherins while other categories like desmosomal cadherins are known to exist as well. (**Figure 14**) (Patel *et al.*, 2003; Halbleib and Nelson, 2006; Panorchan *et al.*, 2006; Zaidel-Bar, 2013).

- Classical or vertebrate Cadherins

They are the most well-studied type of the cadherin family receptors and they are expressed in almost all vertebrate tissues (**Figure 14**). They are known to form homophilic cell-cell interactions between adjacent neighboring cells and they are known to be the major players of the AJs. Two different subtypes of this family have been identified; classical cadherins type I and type II. Both types are characterized by an extracellular domain composed of five domains (EC), a small transmembrane domain and a highly conserved cytoplasmic tail. Their major difference is based on the fact that type II cadherins lack a specific tripeptide motif which is located at the most distal EC (EC1) at their extracellular domains. The first category contains neuronal (N) and epithelial (E) cadherins while the second, the vascular epithelium (VE) cadherin (Patel *et al.*, 2003; Halbleib and Nelson, 2006; Harris and Tepass, 2010). The cytoplasmic domain is mainly responsible for the interactions of cadherins with other proteins intracellularly like β-catenin, p120-catenin, and for the connection to actin cytoskeleton. The precise positions for catenin interactions at the cytoplasmic tail of cadherins are known as catenin binding domain (CBD) for the β-catenin and juxtamembrane domain (JMD) for p120-catenin. The interactions with α-catenin are not direct and are achieved through interactions of cadherins with β-catenin (Halbleib and Nelson, 2006; Panorchan *et al.*, 2006).

The first step for the connection of two adjacent cells, as previously discussed, is the close contact of the lamellipodium of neighboring cells. This event is associated with the actin cytoskeleton activity and the adhesiveness of cadherins (Halbleib and Nelson, 2006; Troyanovsky, 2012; Zaidel-Bar, 2013). As mentioned above, cadherins require cis and trans interaction between their molecules for the clustering to initiate. These different interaction forms are crucial for the enchancement of the cadherin adhesions and the reinforcement of the cadherin clusters. The cis interactions occur when cadherins interact with cadherins from the same cell while trans interactions exist when cadherins from the neighboring cells interact between them. It has been clearly shown through FRET studies, co-immunoprecipitation studies and cryo-electron-tomography experiments that the adhesive site of cadherins is located at the EC1 domain of their extracellular domain (Tomschy *et al.*, 1996; Klingelhofer *et al.*, 2000). This domain is composed of seven β-strands and the first one is divided into 2 parts, the A, located C-terminally and the A* which is located at the N-terminal of the domain. It has been also proven that the A* interacts with the B strand. The cadherin dimerization is based on a strand swap model between the cadherins of the adjacent cells. The A strand is composed of 3 residues, one of which is Trp2. In conditions where calcium ions are not present this strand is tightly fixed to the EC1 domain through the formation of a salt bridge (**Figure 13**). This salt bridge formation occurs between a Glu89 of the rest of the molecule and a Trp2 which is located inside the EC1 domain and forms hydrophobic bonds. When calcium ions are present, the close conformation is relatively unstable and in equilibrium with the open conformation. At this point the Trp2 residue is exposed to the external environment. This will eventually drive the connection of two neighboring cadherins through the so-called strand swap and initiates the increase in cadherin adhesives (**Figure 13**)(Miyoshi and Takai, 2011; Troyanovsky, 2012; Troyanovsky, 2005; Haussinger *et al.*, 2004; Vunnam and Pedigo, 2011).

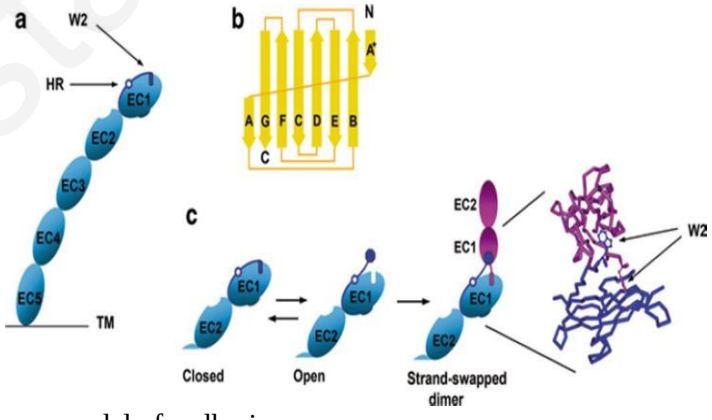


Figure 13: Strand swap model of cadherins

(A) Strand swap model of initial cadherin connection through their A* strand in the presence of calcium ions. The extracellular domain of classical cadherins is composed of 5 EC subdomains. (B) The structure of the EC1 domain including the 7 β-strands and the catalytic A and A* strands. (C) The release of Trp2 residue from the A* strand and the strand swap model with cadherin molecules from the neighboring cell (only EC1 and EC2 domains are shown). Adapted from:(Troyanovsky, 2012).

The two most well-studied members of the classical cadherin group are N- and E- cadherins. N-cadherin is a 130kDa protein whose role is crucial in the nervous system and more precisely in axonal growth and synapse formation (Zaidel-Bar, 2013). E-cadherin is a 120kDa protein whose role is crucial in epithelial cells. The two proteins are characterized by a high similarity regarding their aa sequence. Their extracellular domains display 64% similarity, their cytoplasmic tails display 70% similarity and their catenin binding domain 84% similarity (Stemmler, 2008; van Roy and Berx, 2008). Even though their similarities are major, the variation in cell-type expression has been a major research field for a long time. Initial sorting experiments using sorting of fibroblasts and cadherin expression suggested that cadherins are major players in tissue segregation and cell-sorting during embryonic development (Nose, Nagafuchi and Takeichi, 1988). The precise mechanism through which cadherins facilitate cell sorting remains highly unknown. However, it is suggested that this is achieved through the ability of cadherins to form exclusively homophilic interactions. It has also been suggested that this ability is not only due to the differential cadherin expression but also due to the variation in levels of expression (Foty and Steinberg, 2005). Later experiments using *Drosophila* embryos identified the importance of cell sorting during embryonic development. Studies in gonad development found that the role of DE-cadherin is crucial for the promotion of gonad precursor cells sorting from the other surrounding mesodermal cells (Godt and Tepass, 2003). Besides these, experiments using dominant-negative (DN) mutants of cadherins in endothelial cells and cell epithelium *in vivo* showed a promotion of apoptosis. These results suggest that cadherin expression prevents apoptosis (M L Hermiston and Gordon, 1995). The establishment of apical-basal cell polarity is another major process during which cadherins are known to be major players in. Under normal conditions, the polarity is established through the gradient distribution of several proteins along the cell. Experiments of ectopic transfections of E-cadherin in fibroblasts proved the involvement of cadherins in this process. Cells upon transfection with E-cadherin drove a redistribution of molecules in cells resulting in phenotypes similar to those observed in epithelial cells. Later, it was shown that this redistribution is a result of molecular cues transmitted through cadherins. These cues result from cadherins' connection with the cytoskeleton (McNeill *et al.*, 1990; Perez-Moreno, Jamora and Fuchs, 2003; Wheelock and Johnson, 2003). A lot of experiments have been performed in an attempt to identify the precise role of cadherins in embryonic development. The most well-studied examples are discussed below. Mice, null for E-Cadherin have faulty trophectoderm formation while they display a defective blastocyst. Knockdown experiments for E-cadherin in mice oocytes also result in a lethal phenotype since the embryos formed, are unable to undergo compaction. The downregulation of both maternal E-cadherin expression and the zygotic, using morpholinos, results in embryonic lethality at cell 2 stage embryos (Larue *et al.*, 1994; Kanzler *et al.*, 2003; de Vries *et al.*, 2004). Lastly, the genetic ablation of E-cadherin on skin displays a variety of phenotypes with the most prominent to be embryonic lethality during E15.5 due to skin defects (Young *et al.*, 2003; Tinkle *et al.*, 2004). Embryos knocked out for N-cadherin

display a wide range of defects such as abnormal somites, defective neural tube and impaired heart formation, and die during E10 (Michelle L Hermiston and Gordon, 1995; Radice *et al.*, 1997). Experiments in *Xenopus* during neurulation showed also that premature expression of N-cadherin leads to abnormal histogenesis. This is a strong indication in the implication of N-cadherin in morphogenetic changes that are associated with neurogenesis (Bozdagi *et al.*, 2000; Jüngling *et al.*, 2006). Lastly, phenotypes associated with impaired brain development have been reported in embryos and embryonic cells (ES) with mutant or knock out N-cadherin. Examples are: defective cell fate decisions, axonal guidance, cone growth and defects associated with the glutamatergic neurons (Bozdagi *et al.*, 2000; Jüngling *et al.*, 2006). Even though the two cadherins share a lot of similarities, their replacement with each other is not associated with physiological development, indicating another important role for each cadherin at specific cell types. Replacing E-cadherin with N-cadherin in mice, did not rescue the E-cadherin null phenotype (Tinkle *et al.*, 2004; Calì *et al.*, 2007). In addition to their role in development, both cadherins have been also associated with carcinogenesis. It is well established that the most common cancers are observed in epithelial cells. N-cadherin ectopic expression in epithelial cells leads to defective cell signaling and as a result the cells display loss of polarity and increase in their migratory ability. Besides, N-cadherin expression has been associated with increased motility and migration of cells in *in vitro* experiments. Lastly, a role of E-cadherin as a tumor or invasion suppressor has been identified in gastrula embryos and more precisely during EMT; a process which during tumorigenesis results in degradation of cells (Vleminckx *et al.*, 1991; Birchmeier, 1995; Cavallaro *et al.*, 2001; Derycke and Bracke, 2004; Stemmler, 2008; van Roy and Berx, 2008).

- Atypical cadherins and planar cell polarity (PCP)

This category of cadherins has been identified as a major key player of the PCP in *Drosophila melanogaster* embryos (**Figure 14**). They were shown to regulate a known receptor of the PCP signaling pathway in *Drosophila*, called frizzled receptor (Fz). Members of this family are Fat, Dachsous (Ds) and Flamingo (Fmi) cadherins, and their extracellular domains were shown to have 27 and 34 EC subdomains. Their cytoplasmic domains (Fat and Ds) have been shown to share high homology with domains that are known to interact with β-catenin in classical cadherins. Evidence regarding their ability to bind β-catenin is non-existent however it has been shown that Fat binds to VASP (Halbleib and Nelson, 2006; Panorchan *et al.*, 2006).

- Desmosomal Cadherins

This subfamily of cadherins has two known members; desmocollin and desmoglein and as stated by their names are localized at the desmosomes (**Figure 14**). Three different types of each of these proteins exist and they are known to localize in tissues where high mechanical stress is applied. The general structure of these proteins shares similarities with the structure of the

classical cadherins since they have an extracellular domain of 5 EC subdomains, a transmembrane domain and a cytoplasmic tail known to interact with intracellular proteins. The desmosomal cadherins are known to form heterophilic interactions with neighboring cells, in contrast to classical cadherins, known to form homophilic interactions. There is evidence suggesting that even though they can initiate and maintain cell-cell adhesion in the absence of classical cadherins, their assembly occurs after the formation of AJs during development (Halbleib and Nelson, 2006).

- Protocadherins

This is the biggest family of cadherins as it is composed of more than 60 members, however, their functions during development are still unknown (**Figure 14**). This type of cadherins is expressed primarily in the nervous system and in numerous neuronal tissues. It has been shown that these cadherins are present not only in vertebrates but in different families of sponges too (Patel *et al.*, 2003; Chen and Gumbiner, 2006; Halbleib and Nelson, 2006). They share similar characteristics with classical cadherins since they are also type I transmembrane proteins, however, their extracellular domain has been suggested to consist of six to seven EC subdomains. Their cytoplasmic domain is characterized by a notable diversity and very little evidence exists regarding their binding partners. Apart from these, their ability to display adhesive properties is debatable, while whether they form homophilic or heterophilic interactions with their adjacent cells is still unknown (Halbleib and Nelson, 2006).

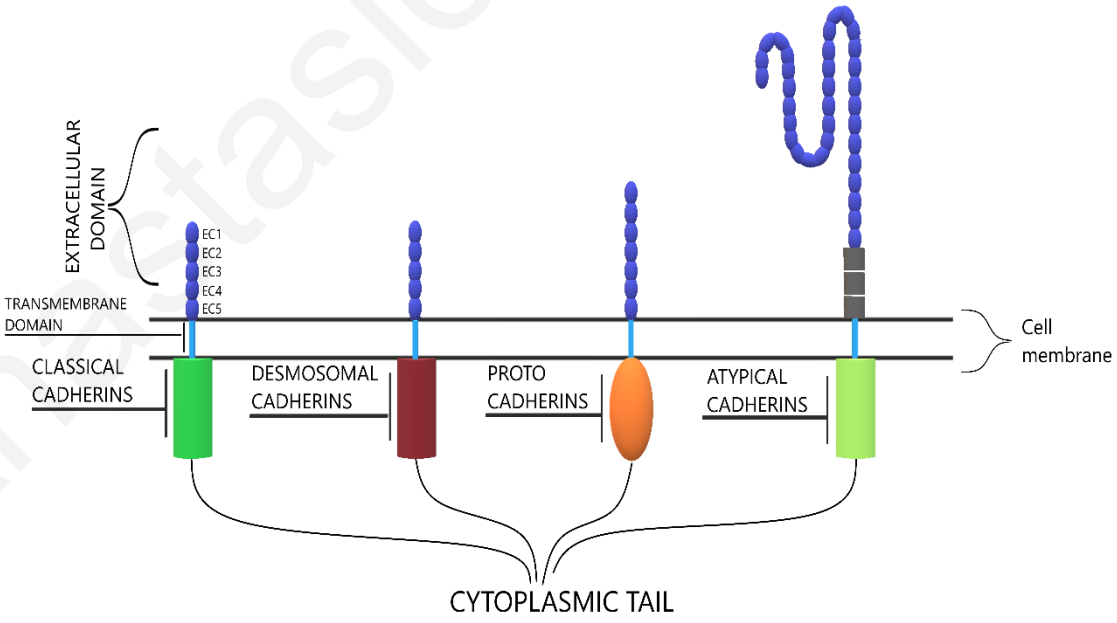


Figure 14: Categorization and structural differences of different cadherin family members

Different types of cadherins composing the cadherin family. Classical cadherins and desmosomal cadherins with 5 subdomains at their extracellular domain, protocadherins with 6-7 EC subdomains and the Atypical

cadherin family which shares similarities with the classical cadherins regarding their cytoplasmic domain, however, are composed of 27-34 EC subdomains extracellularly.

1.5.2 Catenins

These proteins are known to provide an indirect linkage between the Cadherin proteins and the actin cytoskeleton in AJ complexes. They were named after the Latin word catena which translates into the chain (McCrea and Gu, 2010; Miller *et al.*, 2013). All catenin family members contain a domain known as Armadillo which is composed of approximately 42 amino acids in ten or twelve repeats. This domain folds and forms a so-called super-helix of helices composed of a positively charged groove and numerous binding regions (McCrea and Gu, 2010; Miller *et al.*, 2013). These Armadillo repeats to promote the interactions with cadherins while they engage other proteinic interactions too. The catenin family composes of different subcategories and the most noteworthy representatives of these categories are β-catenin, α-catenin, p120-catenin and δ-catenin. This categorization is mainly supported by the different functions of the protein members (McCrea and Gu, 2010; Miller *et al.*, 2013; Yonemura, 2017). Some members of the catenin family can be found at desmosomes where they are involved in the connection with the intermediate filaments and are implicated in cadherin endocytosis, regulation of small GTPases, and in nuclear pathways (McCrea and Gu, 2010). In the next section the major members of the catenin family, that have been identified at AJs, are going to be described in detail (**Figure 15**).

1.5.2.1 β-catenin

The initial characterization of β-catenin was as a segment polarity protein in *Drosophila*. It has been shown to contain an Armadillo motif composed of a triple α-helix and 13 repeats of 42 amino acidic residues (**Figure 15**) (Riggleman, Wieschaus and Schedl, 1989). β-catenin interacts both with α-catenin and cadherins at two distinct regions. With cadherins, it interacts at the most distal region of the intracellular, C-terminal cadherin tail and this interaction has been found to occur directly after the protein exports from the endoplasmic reticulum. The interaction of β-catenin with cadherin at this point is crucial for the stabilization of the protein and the transportation of the cadherin-catenin complex to the membrane. The interaction of β-catenin with cadherins and more precisely with E-cadherin has been extensively studied and it requires a plethora of phosphorylation events at specific residues on both molecules. Initially, the binding affinity of β-catenin for E-cadherin is low. The affinity is subsequently enhanced via phosphorylation events, guided by CKII and GSK-3β kinases at three distinct Serine residues (S684, S686 and S692). These phosphorylation events fascilitate the interaction between the two molecules (Lickert *et al.*, 2000; Piedra *et al.*, 2001; Gayrard *et al.*, 2018).

As mentioned earlier, it has been shown that β-catenin is implicated in AJs' strength reduction, cadherin endocytosis and destabilization of AJs through its interaction with proteins like Src and FAK (Gayrard *et al.*, 2018). Numerous studies have shown that the phosphorylation of β-catenin at specific tyrosine residues facilitates the disassembly of the cadherin-catenin complex. More

precisely, phosphorylation of β-catenin at residues Y489, Y654 decreases its affinity for cadherins and disrupts the complex. Notably, Src protein phosphorylates β-catenin at Y654 (Gayrard *et al.*, 2018). Besides, it is already known that a β-catenin also interacts with the Axin/APC degradation complex and LEF/TCF transcription factors through its Armadillo domain. The N and C terminal regions of the β-catenin molecule are not very well characterized; however, it has been shown that these regions are implicated in the recruitment of cofactors that are related to cell adhesion and signaling (Daugherty and Gottardi, 2007). Aside from these, it is nowadays well established that β-catenin is a core member of the Wnt signaling pathway. Following the initiation and activation of this signaling pathway, cytoplasmic β-catenin has been shown to acquire access to the nucleus. Up to date, the precise mechanism through which the cytoplasmic and nuclear functions of the protein are discriminated against, however, studies suggest that a transcription factor known as BCL-9 is the main regulator of this switch. Evidence shows that the β-catenin Armadillo domain mimics the HEAT repeats that exist on nuclear import factors. This mimic activity fascilitates the transport of the molecule into the nucleus through direct interaction with nucleoporins. β-catenin interacts with specific transcription factors in the nucleus like Tcf/Lef and promotes proliferation. It is also believed that β-catenin moves in and out from the nucleus and through its interactions with distinct proteins its distribution is affected (Suh and Gumbiner, 2003). Recent works suggest that co-activators interact with β-catenin through phosphorylation events. These interactions are responsible for the preferential activation of target genes (Miyabayashi *et al.*, 2007). Lastly, it was recently shown that factors that are associated with transcription initiation by RNA polymerase such as parafibromin/hyrax and chromatin remodeling molecules such as CBP/p300 histone acetylases, Brg-1, TTRAP/TIP60 and mixed lineage leukemia SETI type complexes are modulated via the phosphorylation of β-catenin (Hecht *et al.*, 1999; Urnov and Wolffe, 2001; Brembeck *et al.*, 2004).

1.5.2.2 α-catenin

α-catenin is another protein of the AJs. It has an Armadillo domain and its function and role have been extensively studied both *in vivo* and *in vitro* (**Figure 15**). Experiments in cells lacking α-catenin displayed inability to localize the AJ-associated proteins at the cell membrane. *In vivo* experiments with α-catenin downregulation, resulted in numerous morphogenetic abnormalities (Watabe-Uchida *et al.*, 1998; Yang *et al.*, 2001; Tinkle *et al.*, 2004). As previously described, the initiation of AJs' formation requires the contact between the lamellipodia of neighboring cells and direct interactions with actin cytoskeleton. It is also generally known that actin regulators affect the cadherin adhesion. Taken together, these pieces of evidence suggest that there is an indispensable indirect interaction between cadherins and actin cytoskeleton. It has been later shown, that this linkage was through α-catenin binding. A-catenin in cytosol exists in two different forms, as a monomer and as a dimer. Experiments by Yamada et al. using *in vitro* binding assays showed that α-catenin was able to bind actin, however there was no evidence for interactions with the cadherin-β-catenin complex. Evidence

also suggest that these two interactions are mutually exclusive. It was initially believed that binding of actin with α-catenin was achieved through the dimer form of actin. In contrast, it was believed that the binding with cadherin-β-catenin complex was achieved only when actin was at a monomer form. The binding sites of each actin and cadherin-β-catenin complex are in two distinct regions. The binding to the cadherin-β-catenin complex overlaps with the residues that are responsible for the dimerization of the molecule while the actin-binding domain is located distinct from this point. The direct binding of α-catenin to actin and the cadherin-β-catenin complex is still under investigation and a lot of theories are currently examined. It is currently believed that dynamic crosstalk between α-catenin monomers and dimers is what facilitates the connection with actin in dimer and cadherin-catenin complex in monomer form. It is also well established that besides actin binding, α-catenin mediates the recruitment of other proteins at the AJs sites such as vinculin, and zyxin (Drees *et al.*, 2005; Yamada *et al.*, 2005; Scott and Yap, 2006).

1.5.2.3 p120-catenin

Initially p120-catenin was identified as a substrate of Src and became the most well-studied catenin of the cadherin associated catenins. It is a 120kDa protein and contains an Armadillo domain. It shares structural similarities with β-catenin since they both bind through their Armadillo domain to the JMD domain of cadherins (**Figure 15**). Regardless these similarities, the two proteins are characterized by distinct functions. The binding of p120-catenin to cadherins (through the JMD domain) facilitates the stability of cadherin expression at the cell membrane. It also leads to the strengthening of the cadherin clusters. Experiments using p120 catenin knockout cells demonstrated an inability in cadherins' plasma membrane localization. Additionally, it has been recently proposed that p120 catenin is what inhibits cadherin endocytosis initiation through the interaction of CBD of p120 to the JMD of the cadherin. These sites contain residues that are implicated in clathrin-mediated endocytosis and ubiquitination of cadherins. The binding to cadherins is a dynamic process regulated by phosphorylation events. Phosphorylation on specific residues on cadherin such as Y755/Y756 has been shown to disrupt p120 binding (Ishiyama *et al.*, 2010; Kourtidis, Ngok and Anastasiadis, 2013). The involvement of p120-catenin in the stability of cadherin expression at the membrane is highlighted by studies showing that p120-catenin interacts with regulators of cell migration such as Rho-GTPases. Cadherins act as modulators in Rho-GTPases action and this regulation is facilitated by p120-catenin (Braga *et al.*, 1997; Kodama *et al.*, 1999; Quiros and Nusrat, 2014). Besides, p120 catenin has been proposed to have a signaling function in the regulation of gene transcription and more precisely in the regulation of Cyclin D11 and Wnt-11. These two are well-characterized target genes of the canonical Wnt/β-catenin pathway (Kourtidis, Ngok and Anastasiadis, 2013). Lastly, numerous studies have associated p120 with the microtubule network. P120 catenin interacts with microtubules through the Armadillo domain and this interaction is blocking the E-cadherin interaction with p120-catenin. Evidence for interaction of the N-terminal domain of p120-catenin

with microtubules also exists and is facilitated through protein Dynein (Yanagisawa *et al.*, 2004; Ligon and Holzbaur, 2007; Kourtidis, Ngok and Anastasiadis, 2013).

The role catenins in development has been under investigation for many years. For example, it is well established that the role of β-catenin in development includes the maintenance of the cadherin function and the transduction of Wnt-signals (Zhurinsky, Shtutman and Ben-Ze'ev, 2000; Cadigan and Peifer, 2009; Chien, Conrad and Moon, 2009; McCrea and Gu, 2010). Knock out embryos for β-catenin display embryonic lethality early during development. Experiments have also identified an important contribution of β-catenin in cancer since mutant forms of β-catenin have been shown to increase the transcription of target genes from the Wnt pathway that responds to the nuclear β-catenin. *In vivo* experiments using knockout embryos for p120 catenin lead to embryonic lethality. How exactly the downregulation of p120-catenin affects precise molecular pathways and results to these phenotypes remains unclear (Fang *et al.*, 2004). Further experiments in mice with mutant forms and/or downregulation for p120 catenin led to hyperproliferation of cells and hyperplasia. These experiments also identified an interaction of p120-catenin with the nuclear factor NFκB which was associated with the observed phenotypes (Lynch and Hardin, 2009; Bulgakova and Brown, 2016). *Xenopus* studies for all catenins have identified the crucial role of these proteins during embryogenesis and their downregulation was associated with defective gastrulation and neural crest cell migration (Gu *et al.*, 2009; Dzamba *et al.*, 2009; Ninomiya *et al.*, 2012). Loss of other catenin family members like plakophilin in mice, promotes skin and heart phenotypes. Nevertheless, the fact that these phenotypes are a result of desmosomal plakophilins' association with Rho-GTPases is still under investigation (Hatzfeld, 2007).

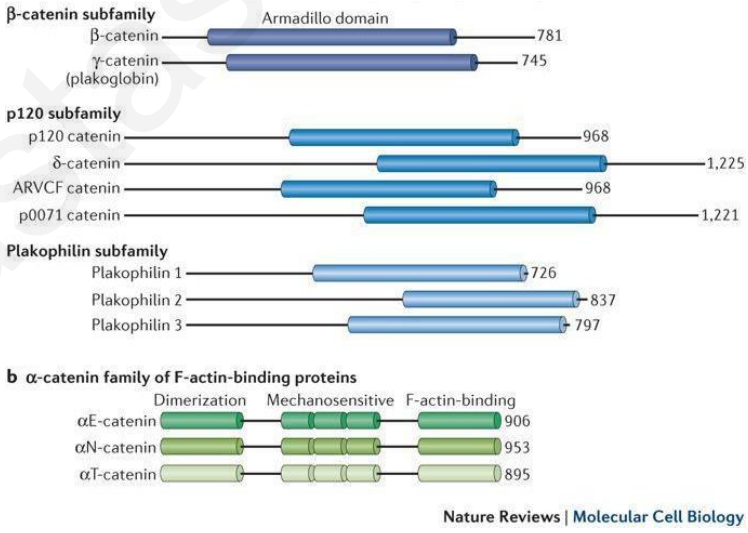


Figure 15: Subcategories of the catenin protein family

Schematic representation of the categorization of catenins and their core protein members. Adapted from: (McCrea and Gottardi, 2016)

1.6 Cadherin adhesion, AJs and mechanotransduction

The formation of a multicellular organism and its maintenance involves perfect communication between cells. The force transitions from cell to a cell are known to drive the induction of morphogenetic movements like polarization of the cells, migration, apical constriction, cell intercalation and cell remodeling (Montell, 2008; Leckband *et al.*, 2011; Takeichi, 2014; Ladoux *et al.*, 2015). It is therefore crucial to unravel the mechanisms through which cells sense and transmit the mechanical signals that are applied to them. It is also important to understand how these signals are further translated into biochemical signals inside the cells and promote different procedures. As mentioned before, the cell has the ability to respond to mechanical forces applied to it through changes in its cytoplasmic environment. These changes occur through myosin II which is bound to actin. The binding of this complex at cell-cell contacts is achieved through adaptor proteins. This eventually results in an equilibrium of the forces applied externally and internally to the cell, which most of the times, is translated into downstream biochemical signals.

The first evidence for the mechanical coupling at the AJs emerged from experiments using PDMS surfaces and beads coated with N-cadherin. The beads on the cell surfaces were dragged laterally and the force application on the PDMS was measured. The tension applied found to be similar to the one observed on integrins on ECM components suggesting that cadherins are mechanically coupled to actin. Same evidence arises from experiments on stiff and soft tissues *in vitro* where stiffer tissues display higher tension suggesting the ability of cadherins to adapt and transmit mechanical stimuli (Lambert, Choquet and Mège, 2002; Ladoux *et al.*, 2010; Tabdili *et al.*, 2012; Barry *et al.*, 2014). Further experiments using an E-cadherin FRET sensor found that (in epithelial cells) the cytoplasmic domain of E-cadherin is under high tension. This observation was found related to the binding of E-cadherin to both actin and β-catenin (Borghi *et al.*, 2012). In *Drosophila*, during border cell migration, it was observed that E-cadherin tension was asymmetrically distributed. More tension was found at the migrating site of cells thus, proving the importance of tension application and sensing through E-cadherin (Cai *et al.*, 2014). The molecular mechanisms of mechanotransduction and mechanosensation at the AJs remains highly unexplored and evidence for these mechanisms emerges mainly through *in vitro* models like single-molecule force clamp spectroscopy (Ladoux *et al.*, 2015). Experiments using this model were performed on a moving platform using actin optical traps. This moving platform was composed of a complex of catenin α and β, actin, and the tail of E-cadherin. The results showed that actin-binding is achieved upon tension application provided by the movement of the platform. This concluded the importance of tension in the stabilization of the connection of this complex with actin. It also made clear that α-catenin had the ability to undergo conformational changes that are force-dependent (Yao *et al.*, 2014). Using magnetic tweezers Yao et al. elegantly showed that upon force application, α-catenin alters its conformation in a way that the binding of the molecule to actin and vinculin is facilitated. Lastly, evidence emerged that the

recruitment of vinculin at the AJs during cell-cell remodeling is also a result of a force-induced signal. In agreement, vinculin recruitment was shown to be dependent on Myosin II and α-catenin (Cohen *et al.*, 2005; Bois *et al.*, 2006; Yao *et al.*, 2014). Further stretch application on cells showed direct recruitment of vinculin through interactions with both Myosin II and α-catenin in a force-dependent manner (Yao *et al.*, 2014). Later work using an α-catenin conformational sensor showed that α-catenin is a reversible, tension-activated molecule that links cadherins and actin and has a crucial role in mechanotransduction at AJs (Kim *et al.*, 2015). All these might result in the transfer of forces during numerous morphogenetic movements where cadherins and other proteins of AJs have been shown to be major key players. One good example is experiments in *Drosophila* larvae during embryonic germ band extension. During this process the epithelium undergoes an elongation via morphogenetic movement known as intercalation and has been shown to be dependent on remodeling of the AJs. This remodeling occurs via Myosin-ii and α-catenin and more recent experiments using optical tweezers managed to reproduce the main deformation of cells during this procedure. Their results suggested the existence of tension showing the co-dependence of tension with α-catenin and myosin-II (Cavey *et al.*, 2008; Rauzi, Lenne and Lecuit, 2010; Bambardekar *et al.*, 2015). To conclude, the mechanical molecular mechanisms that take place at AJs have not been extensively studied to date and many aspects of them remain currently unknown.

2. Scientific Hypothesis and Aims

2.1 Scientific Hypothesis:

The ability of cells to sense extracellular forces and translate them into biochemical signals through which cells can activate different molecular pathways has been extensively studied for many years. This ability of cells has been associated with numerous developmental processes like spindle orientation, tissue morphogenesis, cell adhesion and motility. However, the precise mechanisms through which cells sense and transduce forces remain largely unknown. Previous work from our laboratory showed that upon force application, integrin β1 becomes activated at the lateral cortex of the mitotic cell in a ligand independed but force depended manner and as a result a number of FA proteins are recruited there forming the CMC complex. We hypothesize that the role of this complex and the activation of integrin β1 in spindle orientation derives primarily from the ability of cells to sense cortical forces and is independent from their role in cell-ECM adhesion. Finally, preliminary data suggested that activation of integrin β1 and recruitment of FA proteins takes place at interphase cells and more precisely during the formation of radial AJs. This suggests that integrin activation is observed during other cellular processes where high mechanical tension is observed. We wanted to provide insight into the molecular mechanism underlying this integrin activation and explore the possibility that this activation is not limited to the control of spindle orientation but can be involved in multiple processes.

2.2 Aims:

Aim 1: We aim to explore the possibility that Cell-ECM interactions are dispensable for the proper orientation of mitotic spindle parallel to the substrate. The first aim of the project focuses on carrying out experiments aimed to examine if cell-ECM interactions are dispensable for the proper spindle orientation parallel to the substrate plane. We will try to prove that chimeric N-Cadherin Fc micropatterned surfaces have the ability to act in a similar way as FN patterns and display spindle orientation in response to mechanical external cues. We propose to examine how the inhibition of integrin β1 alters the spreading of cells attached in the absence of cell -ECM interactions and how spindle orientation is affected under these conditions. In addition, we will determine if the implication of CMC proteins in spindle orientation have a distinct role from their role in cell adhesion. Overall this aim (in combination with previous work from our group), will attempt to explore the possibility that spatial cues guiding spindle orientation do not depend on integrin ligand engagement from the ECM and that the involvement of FA proteins, like p130Cas and c-Src, in this process is independent of their role in the cell-ECM adhesion process.

Aim 2: Adherens Junctions topological clustering and formation drives to the activation of integrin β1 and recruitment of FA proteins. Previous studies suggest that cells attached on cadherin substrates generate linear AJs. Here for the first time we show that these linear AJs display over time localization and activation of integrin β1 and other FA proteins. This aim focuses on exploring the activation of integrin β1 at the sites of the AJ formation and clustering. We plan to explore the spatial and temporal aspect of integrin β1 activation as well as to determine the precise status of integrin beta 1 associated with AJs and compare it to that at FAs. Finally, we will explore the composition of this AJ elicited integrin beta 1 based complex.

Aim 3: We aim to unravel the mechanism underlying integrin β1 activation at AJs and examine the possible physiological relevant roles and downstream signaling events of this activation. The mechanism through which integrin activation is spatially governed by AJ topology remains unclear. Considering the fact that these structures are a result of a high tension applied to the cell, and considering previous knowledge showing that they are connected to actomyosin bundles, we propose that the activation and clustering of integrin β1 is force depended and ligand independent. In this aim we will examine how integrin β1 activation depends on the clustering and formation of AJs, explore if the activation of integrin β1 is driven by force and not by the presence of a ligand and determine a precise role of this activation through AJ spatial distribution on ECM deposition and other processes governed by integrin signaling.

3. Materials and Methods

3.1 DNA Constructs

All constructs were generated using standard molecular biology techniques and were verified by DNA sequencing.

3.1.1 Provided plasmids

The plasmid PLZRS-MS-GFP Cas wt construct was provided by S. Hanks (P130Cas Src-binding and substrate domains, Dynamics and mechanism of p130Cas localization), the pCS2++ N—cadherin ΔCP GFP and the pCS2++ N—cadherin wt plasmids were kindly provided by Carl Philipp Heisenberg, the pCDNA3.1 E-cadherin ΔCP YFP and m-cherry were kindly provided by Kalina Hristova (Singh *et al.*, 2017), the mEmerald-Integrin-Beta1-N18 was a gift from Michael Davidson (Addgene no. 54129), the alpha-catenin conformational sensor was a gift from Deborah Leckband (Addgene no. 71709), the Sharpin-GFP construct was kindly provided by Maddison Parsons, the PAIpFN-GFP construct was kindly provided by Harold Erickson and the FAK chicken variant used for the creation of pCS108 plasmid was purchased from GenBank AAA48765.1. The pCDNA3.1 E-cadherin–GFP wild-type construct, the B-catenin-GFP, and the LGN-C′ membrane cherry construct (pTK38_mCherry- LGN-C) were purchased from Addgene (no. 28009, no.16838 and 46346). The Fusion Red Talin construct was purchased from evrogene (no. FP432).

3.1.2 Plasmid Generation

All plasmids generated in this project by PCR are listed in Table 1. The amplification reactions of the DNA were performed using the Invitrogen (1234-040) Accuprime TM Pfx Supermix which is composed of 22u/ml Thermococcus species KOD thermostable polymerase complexed with anti-KOD antibodies, 330μM dNTPs, 66mM Tris-SO4(pH 8.4), 30,8 mM (NH4)2SO4, 11mM KCL, 1.1mM MgSO4, stabilizers and Accuprime proteins.

Generated Constructs	**Primers**	**Template DNA**
pCS108 E-cadherin ΔCP-YFP	F_Ecadh: R_YFP:	pCDNA3.1 E-cadherin ΔCP YFP
pCS108 palm site-mEmerald Integrin-β1 tail	F1: ATGATGACGACCAAAAGATTAAGCTTTTAATGATAATTCATGACAG F2: AACCAAACAGGTTGAAAAAAATGATGACGACCAAAAGATT F3: AAATCGATATGCTGTGCTGTATGAGAAGAACCAAACAGGTTGAAAAAA R: AAGCGGCCGCTTACTTGTACAGCTCGTCCA	the mEmerald-Integrin-Beta1-N18
pCS108	F/HA R/ΔFAT	pCS108 HA-FAK
Pcs108 alpha catenin conformational sensor	F: AAAGTCGACATGACTGCTGTCCATGCAGGC R: TTTGCGGCCGCTTAGATGCTGTCCATAGC	pCS108

Table 1: List of constructs generated by PCR and the primers used for each cloning.

3.2 mRNA synthesis

3.2.1 mRNA synthesis

All constructs were transcribed *in vitro* using the commercially available kits from Ambion for mRNA synthesis mMessage mMachine SP6 or T7.

3.3 Cell culture

3.3.1 Cell lines

HeLa and U2OS cell lines were purchased from ATTC (American Type Culture Collection) and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). p130Cas−/− and p130Cas-reconstituted cells were provided by S. Cabodi. They were cultured in DMEM with 10% FBS and 1% nonessential amino acids. The HeLa N-Cadherin selected cells were manufactured in the laboratory and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).

3.3.2 Cell transfections

All cell lines HeLa, U20S, p130Cas-/- and p130Cas-reconstituted cells were transfected with the indicated plasmids using Lipofectamine 2000 from Invitrogen, electroporation (Invitrogen) or with Calcium Phosphate protocol as previously described (Guo *et al.*, 2017), according to the manufacturer's protocol.

3.3.3 Drug and antibody treatments in cultured cells

In order to examine whether cell spreading and spindle orientation on N-cadherin substrates depended on AJs, we treated HeLa cells with EGTA, which selectively chelates Ca2+ ions. HeLa cells were treated with 1.5 mM EGTA for 20 minutes at 37°C before fixation. For examining the possibility that ECM ligands secreted by the cells within the 30 minutes of attachment and the possibility that this secretion played a role in this context, cells were treated prior seeding on N-cadherin substrates for 2 hours with 20mM Brefeldin-A, secretory pathway inhibitor (Helms and Rothman, 1992) at 37°C, mechanically disrupted with MACS buffer containing 20 mM brefeldin-A and seeded in the presence of brefeldin-A for 30 minutes prior fixation. For Src inhibition, cells were allowed to attach and spread on substrates for 20 minutes and then treated with 4 μM PP2 inhibitor

(Sigma-Aldrich) for 30 minutes before fixation. For the disruption of the actin cytoskeleton HeLa cells were treated with 0.3μg/ml of Cytochalasin D (citation) for 20 minutes prior live imaging. For the disruption of cell contractility cells were treated with 0.3μg/ml of the well-characterized ROCK inhibitor for 20 minutes prior live imaging. For the inhibition of the protein synthesis, Hela cells were treated with 2.5mM of the commercially available inhibitor Cycloheximide (CHX) (Poehlsgaard and Douthwaite, 2005) for 12h prior MACs buffer mechanical disruption. The MACs buffer contained 2.5 mM CHX, and cells were allowed to attach and spread in the presence of CHX for different time intervals prior to fixation (30, 60, 90 minutes). For caveolin-1 endocytosis inhibition we used 2.5mM of the well-characterized inhibitor Methyl-β-Cyclodextrin inhibitor (Santa Cruz Biotechnology). Cells were treated with the inhibitor for 1hour prior to MACS buffer mechanical disruption at 37°C. Cells were mechanically disrupted and allowed to spread for 60 minutes in the presence of the inhibitor. For integrin inhibition experiments, cells were incubated with the AIIB2, P4C10, and P5D2 integrin β1 inhibitory antibodies (1:100; 0.5μg/ml; Hybridoma Bank) for 30 minutes at 37°C before fixation on substrates and for integrin overactivation experiments, Hela cells were treated with 0.625μg/ml 9EG7 for 30 minutes prior fixation at 37°C. For experiments requiring MT network disruption we used 5μM of the well characterized inhibitor of MT network Nocodazole (Invitrogen) for 1 hour prior to cell mechanical disruption with MACS buffer and seeding at 37°C. For all experiments, cells were mechanically detached in MACS buffer [containing 1× phosphate-buffered saline (PBS) (pH 7.4), 2 mM EDTA, and 0.5% bovine serum albumin] and seeded in DMEM for 30 min before fixation.

3.3.4 Selection of Hela cells that attach on N-cadherin Fc

Hela cells display a variation regarding their β-catenin and N-Cadherin levels of expression. In order to minimize this variation and to create a more uniform population of cells, plastic 12 well plates (Santa Cruz Biotechnology) were charged using piranha solution, silanized treated as mentioned above and coated with IgG-goat anti-human and N-cadherin Fc using the same protocol used in the glass-coverslip coating. Hela cells were then mechanically detached in MACS buffer and allowed to spread for 10-15 minutes in DMEM serum-free medium at 37°C. Following, cells were mechanically disrupted, and the medium was replaced with fresh Hela cells culture medium as described above. Cells were then cultured and immunofluorescence experiments in combination with β-catenin and N-cadherin levels statistical analysis were performed in order to ensure that the population of N-cadherin selected Hela were uniform and represented the population we aimed to isolate.

3.3.5 Cell adhesion on substrates

All cell adhesion experiments were performed using previously described protocols with modifications (30, 57 from paper). Briefly, glass coverslips were charged using piranha solution (Sulphuric acid and Hydrogen Peroxide 3:1) for 1 hour at 25°C. The coverslips were then thoroughly washed with distilled water and dried at 50°C for about 15 minutes. Then, coverslips were exposed to UV ozone cleaner for charging and were exposed to UV light of 185nm and 254nm for 10 minutes. Several different silanes were optimized in order to determine the optimal conditions regarding cell seeding and spreading: (3-Aminopropyl) triethoxy-silane (Alfa Aesar), N-3(Trimethoxysilypropyl)diethylenetriamine (Sigma Aldrich), (3-Aminopropyl) trimethoxy-silane (Sigma Aldrich) and (3-Aminopropyl) triethoxy-silane (Sigma Aldrich). The first three silanes were diluted in isopropanol solution in a 30% concentration. Glass coverslips were incubated in the solution at room temperature for 1 hour and 30 minutes followed by three washes with clean isopropanol and baking at 100°C for about 30 minutes. Coating with the last silane from Sigma Aldrich required slightly different conditions, coverslips were incubated in 100% silane for 5 minutes and then clean isopropanol was added. Coverslips were incubated with silane and isopropanol for 20 minutes and washed three times with isopropanol. They were then dried at 100°C for about 30 minutes. Coverslips were either stored in sealed containers for use for up to one week or used immediately. For FN coating, silanized coverslips were incubated with bovine plasma FN (10 μg/ml; Invitrogen) in 1× PBS for 60 minutes at 37°C, followed by thoroughly washed with 1xPBS solution. For N-cadherin and E-cadherin substrate generation, silanized coverslips were initially incubated with goat anti-human IgG (10 μg/ml; Sino Biological) in 1× PBS for 1 hour and 30 minutes at 37°C. Coverslips were then incubated in human N-cadherin Fc (Sino Biological) or E-cadherin Fc (Sino Biological) in 1× PBS at a concentration of 10 μg/ml for 60 minutes at 37°C and then washed to remove the excess protein with 1xPBS (Lambert, Padilla and Mege, 2000; Vega L *et al.*, 2014).

3.3.6 Micropatterned substrate generation

For the generation of N-cadherin Fc and FN micropatterns we used a wide variety of approaches to achieve the optimal condition under which cells were able to seed and adhere to non-ECM and ECM proteins. Briefly, circular glass coverslips were sonicated for 15 minutes with heat, washed with distilled water and isopropanol, and dried at room temperature. Coverslips were then charged using piranha solution for 30 minutes at 25°C, washed three times with distilled water, and dried at 50°C for about 10 minutes. Then, coverslips were exposed to ultraviolet (UV)/ozone (185 and 254 nm) for 10 minutes in order to be sterilized. The glass coverslips were then incubated in PLL(20)g[3.5]-PEG(2) (100 μg/ml; SuSoS Surface Technology) for 30 minutes at 37°C. L- and linear-shaped patterns were generated by exposure of coverslips to UV/ozone (E511, Ossila) (185 and 254 nm) for

10 minutes, using a custom photolithography mask purchased from JD Photo Data. Coverslips were then thoroughly washed with 1× PBS, dried at room temperature, and silanized using vapor (3-aminopropyl) triethoxysilane (Sigma- Aldrich) for 5 seconds. The patterned coverslips were then washed with distilled water for three times. Coverslips were then incubated with goat anti-human IgG-Fc antibody (10 μg/ml; Sino Biological) for 1 hour and 30 minutes and subsequently incubated with human N-Cadherin Fc (10 μg/ml; Sino Biological) in 1× PBS for 60 minutes at 37°C. For FN stripes coverslips were incubated for 60 minutes at 37°C in FN (10 μg/ml; Invitrogen) diluted in 1xPBS (Théry *et al.*, 2007; Tseng *et al.*, 2012).

3.3.7 Micropatterned substrate generation using scratches

For the generation of FN and N-cadherin Fc substrates with scratches we firstly charged glass coverslips using piranha solution (Sulphuric acid and Hydrogen Peroxide 3:1) for 1 hour at 25°C, washed three times with water, and dried at 100°C for about 10 minutes. Then, coverslips were treated with 20% (3-aminopropyl) triethoxysilane (Sigma- Aldrich) in isopropanol 1 hour and 30 minutes, washed three times with isopropanol, and dried at 100°C for about 30 minutes. Coverslips were subsequently coated with goat anti-human IgG-Fc antibody (10 μg/ml; Sino Biological) for 1 hour and 30 minutes at 37°C and then incubated with human N-Cadherin Fc (10 μg/ml; Sino Biological) in 1× PBS for 60 minutes at 37°C. The coated coverslips were then scratched and either washed with 1xPBS directly prior cell seeding, blocked with 3% heat-inactivated BSA solution (Sigma Aldrich) diluted in 1xPBS for 20 minutes at 25°C or blocked with 3% heat-inactivated BSA solution (Sigma Aldrich) diluted in 1xPBS for 20 minutes at 25°C and coated with FN (10 μg/ml; Invitrogen) diluted in 1xPBS for 60 minutes at 37°C in order to coat the scratched surfaces with FN.

3.4 *Xenopus* Frogs, embryos and embryo manipulation

3.4.1 Frogs

Adult frogs obtained from the international suppliers NASCO (United States) and *Xenopus* Express (France/UK). Newly obtained frogs were kept in different aquarium tanks from the older frogs and we provided them with a recovery period of one-month prior experimentation (Sive, Grainger and Harland, 2000, 2010).

3.4.2 Egg collection and *in vitro* fertilization

We performed *in vitro* fertilization in adult frogs which was initiated with the induction of ovulation through the injection of 600-750 units of Chorionic Gonadotropin (hCG; Chorulon/Sigma) into the dorsal lymph sac of the female frog. More precisely the injection was performed posteriorly, at the level of the hindlimb near the lateral line stitch. This was achieved with the usage of a fine needle (26-gauge, Fisher) attached to a 1ml syringe. Primed frogs were kept at 18-20°C and ovulation was initiated approximately 12 hours after the injection. The injected frogs that were ready to ovulate were characterized by a red and swollen cloaca (Sive, Grainger and Harland, 2000, 2010). For the egg collection, female frogs were physically restrained by firmly holding them in place, while lateral and vertical pressure was applied by massaging the belly (a procedure which lasts approximately 2-3 minutes). Eggs were then collected in a clean glass petri dish which contained 0.3x Marc's Modified Ringer's Solution) MMR. This procedure was repeated up to 6-8 times per day and each batch of obtained oocytes was kept in a separate glass petri dish (Sive, Grainger and Harland, 2000, 2010). *In vitro* fertilization was performed immediately after laying off the eggs and time of fertilization was noted. Testes isolation was performed after male frog sacrifice by submerging it in 0.05% Benzocaine for 30 minutes at room temperature. Testes are found at the base of the fat bodies and they were removed from the body by the use of scissors and forceps (Sive, Grainger and Harland, 2000, 2010). The isolated testes are stored in 10% newborn calf serum, 90% Leibovitz (L15-Medium Leibovitz), and antibiotic (0.05mg/ml gentamicin) at 4°C for 5-7 days. Prior fertilization, MMR buffer was removed from the petri dish containing the *Xenopus* embryos using a plastic pipette and a small piece of testes was cut and macerated using forceps. Then, the small piece of testes was mixed with the eggs in order to distribute the sperm evenly throughout the eggs, and eggs were left for 20 minutes for fertilization to occur. Successfully fertilized embryos were identified as those displaying rotation in a way that their animal hemisphere l faced upwards (their animal dark pole half). Embryos were then stored in 0.3x MMR.

3.4.3 Microinjections and embryo maintenance

Embryos are externally protected by a thick membrane known as "jelly membrane" which has to be removed in order to make micromanipulation procedures possible. Initially, *Xenopus* embryos were treated in order to remove the jelly membrane. To achieve this, embryos were bathed and swirled in 1.8% Cysteine buffer (Sive, Grainger and Harland, 2000, 2010; Wlizla, McNamara and Horb, 2018) diluted in 1/3 MMR solution for 2-4 minutes at room temperature, until embryos became closely packed to each other. Embryos were then washed using 1/3MMR solution for approximately 10 minutes. The embryos used for microinjection experiments were bathed in 4% Ficoll solution diluted in 1/3 MMR while the ones used for other experiments without any further manipulation were maintained in 0.1 MMR solution (Sive, Grainger and Harland, 2000, 2010). Microinjections were performed using a small glass capillary pulled needle, forceps, a Singer Instruments MK1 micromanipulator and a Harvard Apparatus pressure injector. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) injected using mRNA encoding proteins of interest and staged according to *Xenopus* fate map (Dale and Slack, 1987). Following the microinjection procedures, embryos were kept in Ficol for approximately 1 hour at room temperature and then maintained in 0.1xMMR until they reached desired stages. The embryos were then processed for dissection, fixation, immunofluorescence experiments and imaging. The amount of mRNA injected at each experiment varied according to the mRNA injected and is clearly stated in the Results sections.

3.4.4 ACs Explants and Sagittal Sections

All explants were performed with the use of hair knives and forceps.

For the experiments using ACs or sections from *Xenopus* Embryos, the embryos were fixed at desired stages (8-12) with MEMFA for 2h at room temperature, ACs or sections were dissected, postfixed with MEMFA for 30 minutes at room temperature and immunofluorescence experiments were carried out.

3.5 Immunofluorescence

3.5.1 Immunofluorescence in cultured cells

Immunofluorescence on Hela, Hela N-Cadherin selected cells U20S, Cas wt-ires-GFP reconstituted cells and Cas -/- cells were carried out using previously described immunofluorescence protocols as follows: the cells were washed 3 times with 1xPBS and fixed using 4% Paraformaldehyde (PFA; Sigma Aldrich) solution diluted in 1xPBS for 10 minutes at room temperature. Fixation was followed by incubation of cells in 50mM Glycine diluted in 1xPBS (Sigma Aldrich) with adjusted pH=8. Cells were then permeabilized using different Triton-X (Biorad) solution concentrations (diluted in 1xPBS) depending on the primary antibodies and cell lines used. The Triton-X concentration varied from 0.03% to 0.2%- and 6-15-minute treatment. Following permeabilization cells were washed 3 times with 1xPBS and blocked using 10% normal donkey or goat serum (Jackson Immunoresearch) diluted in 1xPBS for 30 minutes at room temperature. Cells were then incubated with primary antibodies listed in Table 2 either for 1 hour and 30 minutes at room temperature or overnight at 4°C, followed by consecutive washes with 1xPBS and incubation with secondary antibodies mentioned in Table 3. For actin staining, Phalloidin was used (Phalloidin 488 A12379, Phalloidin 555 A34055, Phalloidin 633 A22284; Invitrogen and Phalloidin 405 ab176765; Abcam) and for DNA staining TOPRO-3 or Hoechst (Invitrogen) were used. Mounting of the cells was performed using diamond prolong antifade media (Invitrogen). For the staining of active or total integrin β1, cells were fixed with normal donkey or goat serum (Jackson Immunoresearch) right after glycine incubation, prior to the incubation with Triton-X. This allowed the preservation of specificity of the antibodies against their epitopes.

3.5.2 Live immunofluorescence

Live imaging of active integrin β1 was performed using 0.15μg/ml of 9EG7 antibody in cultured cells for 30 minutes. Cell media was then washed and cells were incubated with Cy3 anti-rat secondary antibody for 30 minutes. Cells were then washed with media and imaged.

3.5.3 Immunofluorescence in *Xenopus* embryos

For whole-mount immunofluorescence of *Xenopus* embryos or *Xenopus* Embryo Animal Caps (ACs) the immunofluorescence experiments were performed as follows: whole-mount embryos were fixed in 1 x MEMFA (Aldehyde Fixative) for 2 hours at room temperature followed by PBST permeabilization for 2-3 hours at room temperatures or overnight at 4°C. Embryos were then blocked for 30 minutes in 10% normal Donkey Serum and then incubated with primary antibodies (Table 2)

overnight at 4°C or 5-6 hours at room temperature. Washes with PBST were then performed for 1 hour at room temperature followed by incubation of secondary antibodies (Table 3) for 2 hours at room temperature. The embryos were then washed again with PBST several times for approximately 1 hour at room temperature, postfixed using MEMFA solution and imaged. Clearing of the embryos was performed with methanol dehydration followed by immersion of the embryos in Murray's Clearing Medium. Immunofluorescence experiments using *Xenopus* ACs were performed similarly to the experiments on whole-mount *Xenopus* embryos but the permeabilization was performed for 2 hours at room temperature.

Primary Antibodies (IF)	**Dilution in culture cells**	**Dilution in *Xenopus* Embryos**
Integrin β1 active 9EG7 rat (550531 BD Pharmigen)	Live staining 1:250, on fixed cells 1:1000	-
Integrin β1 active HUTS21 mouse (556048 BD Pharmigen)	1:1000	-
β-Catenin rabbit (11279H20B, Sino Biological)	1:1000	1:500
β-Catenin mousesc-7199, Santa Cruz Biotechnolog	1:700	-
β-Tubulin mouse(E7, Developmental Studies Hybridoma Bank)	1:200	-
a-Tubulin rat (sc-53030, Santa Cruz Biotechnology)	1:500	-
NuMA rabbit (ab36999, Abcam)	1:1000	-
LGN (ABT174, Millipore)	1:1000	-
paxillin mouse (10029-1-Ig, Proteintech)	1:1000	-
FAK mouse (66254-IIg, Proteintech),	1:1000	-
p130Cas mouse (sc365200, Santa Cruz Biotechnology)	1:500	-
Src mouse (sc8056, Santa Cruz Biotechnology)	1:500	-

Fibronectin mouse (4H2 Developmental Studies Hybridoma Bank)	-	1:500
Integrin β1 mouse (8c8 Developmental Studies Hybridoma Bank)	-	1:100
Integrin β1 AIIB2 rat (Developmental Studies Hybridoma Bank)	1:100	-
Integrin β1 TS2/16 (sc53711 Santa Cruz Biotechnology)	1:100	-
Mab13 Integrin β1 inhibitory rat (MABT821) Millipore	1:100	-
Integrin β1 P5D2 mouse (Developmental Studies Hybridoma Bank)	1:100	-
Integrin β1 P4C10 mouse (Developmental Studies Hybridoma Bank)	1:100	-
Fibronectin rabbit HFN-11 mouse (C	1:500	-
Fibronectin h-300 rabbit (sc9068 Santa Cruz Biotechnology)	1:500	-
Laminin mouse (sc74418 Santa Cruz Biotechnology)	1:200	-
Collagen mouse (NB600-408 Novus Biological)	1:500	-
p-Myosin rabbit (PA5-17726 Thermo Scientific)	1:500	-
p120Catenin mouse (66208 Proteintech)	1:1000	-
N-Cadherin mouse (MA1-159 Thermo Scientific)	1:1000	-
Talin 1 mouse (ab71333 Abcam)	1:1000	-

Caveolin 1 mouse (sc70516 Santa Cruz Biotechnology)	1:500	-
Tensin-1 rabbit (SAB4200283 Sigma)	1:800	-
Vinculin rabbit (26520-I-AP Proteintech)	1:1000	-
ILK (65.1) mouse (sc20019 Santa Cruz Biotechnology)	1:500	-
Zyxin mouse (610521 Transduction)	1:500	-
Integrin a5 inhibitory P1D6 mouse (Developmental Studies Hybridoma Bank)	1:100	-
Integrin a5 (A-11) mouse (sc166665 Santa Cruz Biotechnology)	1:100	-
Integrin av (C-9) mouse (sc376199 Santa Cruz Biotechnology)	1:100	-
A-Catenin (Pa5-18512 Thermo Scientific)	1:1000	-
A3 integrin alpha-3 CD49c-P1B5 (Developmental Studies Hybridoma Bank)	1:50	-
A6 integrin P5G10(Developmental Studies Hybridoma Bank)	1:50	-

Table 2: List of primary antibodies used in immunofluorescence experiments in cultured cells and *Xenopus* whole-mount embryos or sections. Abbreviations: IF; Immunofluorescence

Secondary Antibodies (IF)	Dilution
Alexa Fluor 488 anti-mouse (A11029, Invitrogen)	1:500
Alexa Fluor 488 anti-rabbit (A11034, Invitrogen)	1:500
Alexa Fluor 488 anti-rat (A21208 Invitrogen)	1:500
Alexa Fluor 633 anti-mouse (A21052, Invitrogen)	1:250
Alexa Fluor 633 anti-rabbit (A21070, Invitrogen)	1:250
Cy3 anti-mouse (711-165-150 Jackson Immunoresearch)	1:500
Cy3 anti-rabbit (711-165-150 Jackson Immunoresearch)	1:500
Cy3 anti Rat (712-165-153 Jackson Immunoresearch)	1:500
Cy3 anti-Goat (705-165-147 Jackson Immunoresearch)	1:500
647 anti-mouse (715-165-151 Jackson Immunoresearch)	1:250
647 anti-rabbit (711-605-152 Jackson Immunoresearch)	1:250
647 anti-Goat (705-605-147 Jackson Immunoresearch)	1:250

Table 3: List of secondary antibodies used in immunofluorescence experiments in cultured cells and *Xenopus* whole-mount embryos or sections. Abbreviations: IF; Immunofluorescence

3.6 Imaging

Embryos, explants and cells in cultures were imaged using: Zeiss Axio Imager Z1 with Zeiss Axiocam MR3, Zeiss Lumar V12 stereomicroscope and LSM710 Confocal microscope from Zeiss.

3.6.1 Fluorescent Recovery After Photobleaching (FRAP)

The experiments using FRAP were performed using a Plan Apochromat 63x oil lens at the confocal microscope (LSM 710). The 488nm and 543 nm laser were used for GFP and Fusion Red excitations. Regions enclosed in a rectangle from Zen Blue software, composed of the photobleached regions and the fluorescence in the rectangular regions were measured before bleaching at low power amounts. Low levels of energy power were used for the recovery until the intensity reached the initial or close to initial intensity. The recovery curve and the analysis of the fluorescence were automatically acquired using Zen Blue software and the recovery rate was calculated using the formula $R = 1/t$.

3.6.2 Time-lapse imaging of cell doublets

Live imaging of cell doublets described was performed by placing the cells on glass coverslips coated with 10% PLL poly-L-Lysine diluted in 1xPBS solution. The coverslips were placed on small pieces of double face tape and surrounded by a PDMS gasket approximately 2mm thick filled with media. Optical sections of the doublets were acquired every 1 minute for a 30-minute period.

3.7 Images and Statistical Analysis.

All intensity profiles and color-coded images and intensity profiles were acquired using the ZEN2010, ZENlite, and AxioVision4.8 softwares. All graphical representations, scatter plots and statistical analysis of the data were performed using ImageJ, Adobe Photoshop, GraphPad Prism, Office Microsoft Excel and MATLAB.

3.7.1 Spindle orientation

For the quantification of spindle orientation in culture cells at the XZ plane, the angle between the line connecting the two spindle poles and the plane of the substrate of cultured cells was measured. Statistical analysis included unpaired t-test for parametric distributions and Mann-Whitney tests for

non-parametric distributions. For the quantification of spindle orientation in cultured cells at the XY plane, the angle between the line connecting the two spindle poles and the long axis of the micropattern in cultured cells was measured. Statistical analysis included unpaired t-test for parametric distributions and Mann-Whitney tests for non-parametric distributions.

3.7.2 Deconvolution

Images were acquired using a confocal microscope with a 63X oil immersion lens and a 1au pinhole using the Deconvolution algorithm from AxioVision software 4.8. The software automatically defined the excitation and emission wavelengths of the fluorophores used in each experiment and automatically calculated the theoretical PSF, as well as the immersion liquid the lens required for imaging.

3.7.3 Intensity quantifications

Cell spreading measurements were done using the AxioVision 4.8 software. Intensities of the proteins of interest were measured and analyzed using the Zen (Blue edition) software and the Imaris software by BITPLANE, GraphPad Prism and Microsoft Excel.

3.7.4 Correlation coefficient quantifications

Profiles for co-localization of proteins of interest were generated using the Zen (Blue edition) software. All measurements were analyzed with GraphPad Prism and Microsoft Excel. The colocalization coefficient quantification was performed using intensities measured using Zen (Blue edition) software and the Imaris software by BITPLANE and analyzed with ImageJ using algorithms from already described methods of analysis (MANDERS, VERBEEK and ATEN, 1993; Costes *et al.*, 2004).

4. Chapter I

4.1 Introduction Chapter I

4.1.1 Mitotic spindle orientation

4.1.1.1 Mitotic spindle orientation in development and disease

From development to adult life, all cells derive from pre-existing cells. This process is known as cell division and results in the division of cell genetic material and components to the two derived daughter cells. Division can be either asymmetrical, where the daughter cells acquire different characteristics and hence a distinct cell fate; or symmetrical, where the daughter cells acquire the same characteristics with the mother cell and promote tissue growth and epithelial maintenance. The orientation of cell division is defined as the process during which the division axis of the cell is decided so that the daughter cells will obtain a correct position with respect either to a substrate or to their adjacent cells (**Figure 16**). Defects during this fundamental process have been associated with aberrant embryogenesis and numerous diseases such as tumorigenesis, neurological diseases and polycystic kidney disease (D, 2011; Pease and Tirnauer, 2011; Noatynska and Gotta, 2012).

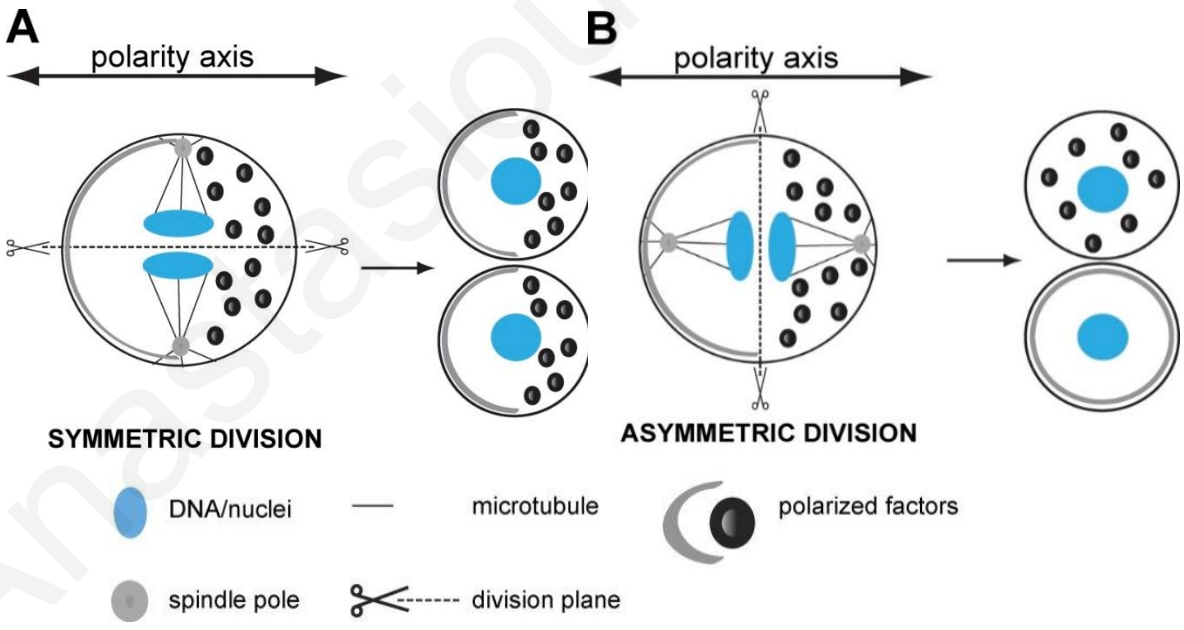


Figure 16: Symmetric and asymmetric cell division

Schematic representation of the differences between symmetric and asymmetric cell division. (A) Divisions perpendicular to the division axis drive symmetric divisions and in this case daughter cells can acquire a distinct cell fate from the maternal cell. (B) Divisions parallel to the division axis result in asymmetric divisions where daughter cells promote tissue growth and maintenance of the epithelium. Adapted from: (Noatynska, Gotta and Meraldi, 2012)

Studies using a variety of models have contributed to the characterization of the importance of cell divisions in development and human diseases. Experiments in MDCK cells cultured in cysts, a model known to represent the epithelia *in vitro*, showed the importance of spindle orientation. The downregulation of elements associated with the proper spindle orientation were shown to lead to defects in single lumen formation *in vitro* (Bañón-Rodríguez *et al.*, 2014). Studies in *C. elegans* suggested the importance of spindle orientation in cell fate determination during development through the downregulation of genes associated with this process. During *C. elegans'* development first division results in 2 daughter cells that are district regarding their size; a large anterior and a small posterior. The smaller daughter cell undergoes asymmetric divisions in order to produce founder cells, each of which is a progenitor of distinct cell types. These asymmetric divisions are responsible for the formation of the principal axes of the body through five asymmetric divisions. Disruption of genes associated with this process leads to defective spindle orientation, loss in cell asymmetry, and to animal lethality (Werts, Roh-Johnson and Goldstein, 2011; Rose and Gonczy, 2014). In *Drosophila*, asymmetric spindle orientation maintenance is responsible for the development of the fly nervous system. Three different protein complexes have been associated with this process. Disruption in the function of those protein complex members is associated with abnormally increased proliferation of progenitor neural cells and the formation of tumors and lethality in flies (Lee *et al.*, 2006; Cabernard and Doe, 2009). Another important role of spindle orientation in *Drosophila* development is during the formation of the mechanosensory organs of the nervous system. This process is maintained through asymmetric divisions in a process known as planar cell polarity (PCP). Downregulation or mutations in genes associated with asymmetric divisions during PCP leads to defective bristle formation (Bellaiche *et al.*, 2001; Gho and Schweisguth, 1998). Asymmetric cell divisions are also known to exist in the development and formation of the *Drosophila* epithelium at a late stage. This epithelium is responsible for the proper transfer of electrolytes and ions in and out of the fly skin. Defects in genes associated with the cell division during this process have been associated with defective epithelium formation and defects in ion-exchange leading to dehydration and lethality to the flies (Lechler and Fuchs, 2005; Poulson and Lechler, 2010; Williams *et al.*, 2011). The role of spindle orientation in *Drosophila* has been also associated with intact organogenesis. Defects in the orientation of spindle during organogenesis lead to lethality as a result from different organ defects (Baena-Lopez, Baonza and Garcia-Bellido, 2005). The role of cell divisions in vertebrate development has been extensively studied both in *Xenopus* and *Zebrafish.* Disruption of the symmetric cell divisions promotes defects in gastrulation, neurulation and axial extension (M. Marsden and DeSimone, 2001; Wallingford and Harland, 2002; Campinho *et al.*, 2013). In mammals, spindle orientation is crucial for the organ morphogenesis and epidermal development of skin (Lechler and Fuchs, 2005; Fischer *et al.*, 2006; Zhang *et al.*, 2012).

3.1.1.2 Intrinsic and extrinsic signaling during spindle orientation

The establishment of the division axis and the orientation of the spindle of the cell along this axis is a process known as spindle orientation (Michelle S. Lu and Johnston, 2013). The symmetric division is achieved both in single cells in culture that are in contact with their substrate and within the tissues of the epithelium. In the first case the spindle is oriented parallel to the substrate of the cell which is normally composed of ECM-components, while in the second case, cells orient their spindle parallel to the plane of the epithelium known as orientation. This process is influenced by a wide range of different factors such as; internal cues and molecules that localize at the cell cortex, known as intrinsic signals and cell shape, cell adhesion geometry, and external forces, known as extrinsic signals (Nestor-Bergmann, Goddard and Woolner, 2014). Spindle orientation both in culture cells and cells of epithelium can be either in respect to the z-axis or the xy-axis. In cell cultures, the orientation of z-axis is achieved mainly through the retraction fibers (RFs) which are actin-rich protrusions (Toyoshima and Nishida, 2007). RFs connect the cells with their substrate while on tissue this orientation refers to the orientation of cells along the plane of the epithelium which occurs in a parallel manner. The xy-axis orientation is controlled mostly by the extrinsic signals applied to the cells (Théry *et al.*, 2007; Thery *et al.*, 2005). The signaling that takes place inside of the cell during mitosis has been extensively studied both *in vitro* and *in vivo*. The predominant model of how the spindle is oriented suggests that the astral microtubules (AT) anchor at the cortex of the cell on predetermined regions. These regions are known as spindle capture sites. At those sites, the minus end of microtubules is attached through motor protein dynein, pulls the other side of the microtubules and drives the correct spindle positioning (McNally, 2013). Numerous studies have identified that the localization of dynein and effectively dynactin at the cortex of cells during mitosis was achieved through a conserved molecular complex known as cortical machinery or LGN complex. The members of this complex are the heterotrimeric protein Gαι, LGN and NuMA in mammals, Gαι, Pins and Mud in *Drosophila* and GOA1/GPA16, GPR1,2 and LIN5 in *C. elegans* (**Figure 17**). This complex localizes asymmetrically at the cortex of the cell and displays enrichment at the spindle capture sites. Gαι subunits are localized, through a myristoylation site, at the membrane of the cell. This is the point where the whole complex anchors during mitosis. LGN during interphase exists in a closed inactive conformation and has a low affinity for Gαι. This inactive state is achieved through the interaction of N and C termini of LGN between them and prohibits its localization at the membrane. NuMA during interphase is localized in the nucleus and when the cell enters mitosis, it localizes both at the cell membrane and the spindle poles. It is well established that LGN interacts through its N-terminal domain with NuMA and through its C-terminal with Gαι (di Pietro, Echard and Morin, 2016; Morin, Jaouen and Durbec, 2007; Peyre *et al.*, 2011) (**Figure 17**). However, the precise mechanism on how this complex is directed at those sites is still not clear. Other protein players have been found associated with the localization and selective distribution of these polarity cues such as; Afadin, Huntingtin and aPKC. Afadin binds simultaneously actin and LGN through its

TPR domain, preventing the binding of NuMA at LGN. This interaction has been shown to have a lower affinity than the NuMA-LGN interaction has. During mitosis, NuMA is released from the nuclear envelope, dissociates the Afadin/LGN complex and promotes the binding of LGN to the TPR domain of the protein. It has been also suggested that this Afadin/LGN complex serves for the initial localization of LGN at the cell membrane via actin-binding and is important for the localization of LGN at the cell cortex and interaction with Gαι (Speicher *et al.*, 2008; Segalen *et al.*, 2010; Carminati *et al.*, 2016). These results are in agreement with studies showing that the actin cytoskeleton is implicated in the localization of LGN at the cell membrane. These experiments were performed using Latrunculin B and they clearly showed that upon actin cytoskeleton disruption, LGN was unable to localized at the cell cortex (Matsumura *et al.*, 2012). The precise mechanism on how the Afadin interacts with Gαι is still unknown. Another protein found to associate with the cortical machinery is Huntingtin (HTT). This protein has been shown to interact with the Gαι, LGN and NuMA in studies of cells in culture *in vitro* and in mice and *Drosophila in vivo.* HTT found localized at the spindle poles during mitosis and its absence led to the decrease of cortical LGN and NuMA. It has been also suggested that HTT facilitates the transport of LGN and dynein complex to the cortex via AMTs (Godin *et al.*, 2010; Elias *et al.*, 2014). Recently, a protein known as Wart (Hippo pathway protein member) has been associated with spindle orientation. This protein was shown to facilitate the phosphorylation of NuMA, an indispensable process for its cortical localization, both in *Drosophila* embryos and *in vitro* using cell cultures (VanHook, 2015). It is well established that the localization of NuMA at the cell cortex during mitosis is facilitated by a kinase known as Aurora A. The mechanisms through which Aurora and Wart act are distinct from each other however both have been shown to localize on the spindle poles during mitosis. This suggests that both of these proteins facilitate the phosphorylation of NuMA, its release from spindle poles and its interaction with LGN (Dewey, Sanchez and Johnston, 2015; Gallini *et al.*, 2016). Another factor known to phosphorylate NuMA and found crucial for spindle orientation is the Abelson murine leukemia viral oncogene homolog 1 (ABL1) (Matsumura *et al.*, 2012). Besides these, the importance of the asymmetric localization of the cortical machinery during mitosis has been studied extensively. It was shown that upon LGN polarity disruption the spindle acquired a randomized orientation. Also, other proteins like Polo-like kinase (Plk1) have been associated with the cortical machinery. Specifically, it was shown that Plk1 localizes at spindle poles only when the pole is in close proximity to the cortex of the cell. This protein led to the dissociation of dynein from the LGN/NuMA complex and leads to the movement of the spindle in the opposite direction at the cortex. A fact that suggests that this protein drives the centering of the mitotic spindle (Kiyomitsu and Cheeseman, 2013). Lastly, a gradient that derives from Ran-GTP was found to cause disruption of the LGN/NuMA complex from the cortex of the mitotic cell when the chromosomes are close to the cortex (Kiyomitsu and Cheeseman, 2013). The fact that experiments in cells, using downregulation of LGN, displayed proper cell division orientation suggests that alternative mechanisms are implicated in this process. Studies by Toyoshima, Matsumura et al. and Matsumura, Hamasaki et al. found an association of

cortical machinery and phosphoinositides (PIP). The authors elegantly showed that NuMA interacts with PIP and PIP2 and that interaction facilitates the localization of NuMA at the cortex of the mitotic cell (Toyoshima and Nishida, 2007; Matsumura *et al.*, 2016; Matsumura *et al.*, 2012). More recent studies have suggested the involvement of proteins such as Disc large (Dlg) in the orientation of mitotic spindle. Numerous studies both *in vitro* and in *Drosophila* embryos *in vivo* have shown that this protein acts as an adaptor of cortical cell machinery at the cell cortex while it provides positioning information for LGN. Its precise role remains unknown but its association with spindle orientation and the cortical machinery is clear (Bergstralh, Dawney and St Johnston, 2017).

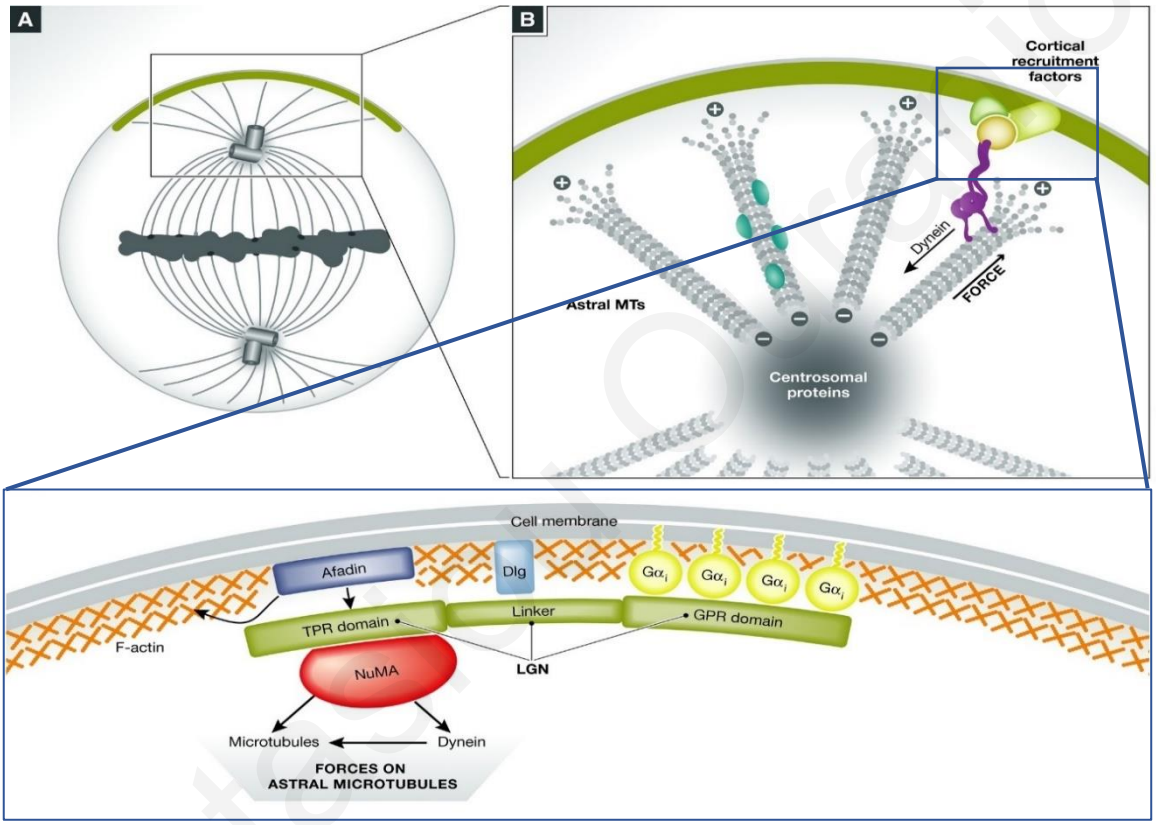


Figure 17: Regulation of spindle orientation; The cortical machinery and the astral microtubules

(A) Illustration of the mitotic cell including the chromosomes, spindle (astral microtubules, the centrosomes and the cortex of the cell. (B) Schematic representation of the cortical machinery, the dynein complex and the structure of astral microtubules during cell division. (C) A closeup illustration of the previously described protein members of the cortical machinery and the localization at those sites of other proteins known to interact with the LGN-complex. Adapted and modified from: (di Pietro, Echard and Morin, 2016)

The application of external signals to the mitotic spindle has been under investigation for more than 100 years when first experiments with frog embryos displayed a characteristic shape anisotropy and suggested an association of division plane and cell shape. This observation became known as "Hertwig rule" or long axis rule and is up to this day widely accepted for the prediction of the cell division plane (Hertwig, 1884). Computational models and experiments using urchin eggs in

chambers of pre-designed geometry showed that the length of microtubules emerging from spindle poles and hence the cell geometry could predict the division axis (Minc, Burgess and Chang, 2011). However, it has been shown that cells in cell culture acquire a spherical shape while they undergo mitosis and hence the geometry and shape role in spindle orientation was debatable. The first experiments providing evidence for the implication of external cues in the mitotic division were experiments performed by Thery et al. who used micropatterned adhesive substrates containing ECM components, for cell culture. They used micro-contact printing techniques for the development of different adhesive shapes on glass coverslips and cells were allowed to attach and spread at those shapes. HeLa cells found to adapt these shapes when plated on them and when cells were undergoing mitotic division, the spindle found to align with the longer axis of the cell, which was determined during interphase. This was the first proof that the external signals of the cell are essential for the proper orientation of the mitotic spindle (Thery *et al.*, 2005). Later, they proved that the adhesion environment was applying mechanical forces to the cell. This suggests that the mechanical force application from the extracellular environment is involved in spindle orientation. As Fink et al. elegantly showed, when uniaxial stretch was applying on dividing Hela cells, the spindles were rotated towards the side of the stretch application, suggesting that spindles align with the forces applied from the external environment of the cells (Fink *et al.*, 2011). Studies by Seldin et al. confirmed the same observation in cultured keratinocytes. The showed that the application of unidirectional stretch on keratinocytes leads to spindle alignment with the direction of the stretch application (Seldin *et al.*, 2013). The interesting fact about cells in culture is that they normally round up during mitosis. Their divisions are aligned with their adhesive substrate and the cell shape that they acquired during interphase. This suggests that the orientation of the spindle during mitosis in cultured cells depends on the adhesion geometry, the cell had during interphase and it is regulated by the RFs. RFs connect the mitotic cell with the substrate during cell division. The first experiments performed in order to prove this theory were performed in Hela cultured cells. These cells were grown on adhesive substrates of different shapes where the distribution of RFs was predetermined (Théry *et al.*, 2007). This suggested that RFs serve as a memory mechanism of the cell for it to divide in this direction. However, given the fact that the information provided by the external environment is transmitted to the cell in real-time, whether RFs serve simply as anchoring cables for the mitotic cell, remains to be explored. In an attempt to show that RFs provide external forces to the cell during mitosis, Fink et al. used micropatterns of different shapes such as bar-shaped or cross-shaped that were FN coated. Under normal conditions the spindle of cells on these micropatterns aligned along the long axis of the shape. Laser ablation experiments on these cells revealed that the cells on cross-bar patterns display rotation of their spindle with preference to the axis where the RFs are longer after the ablation. The case however, was different for cells on bar-shaped micropatterns where no available RFs, longer in size, were present. The cells under these conditions display no spindle rotation. These pieces of evidence agree with other experiments that showed that RFs face high tension during this process and prove that spindles can sense and respond to external forces applied

to the cell externally. They also suggest that these results are not due to a memory mechanism that the cell acquires during interphase (Fink *et al.*, 2011). These studies have provided evidence for the force-dependent mechanisms of spindle orientation that are independent of the cell shape. Evidence supporting these observations also derived from works in *Zebrafish* and *Drosophila* where the alignment of spindle with the externally applied forces is necessary for the embryogenesis (Campinho *et al.*, 2013; Michelle S Lu and Johnston, 2013; Mao *et al.*, 2011; LeGoff, Rouault and Lecuit, 2013). In *Zebrafish* development, a procedure known as epiboly has been extensively studied in spindle orientation, since during this procedure the rate of cell proliferation in the epithelium is high. The mitotic spindles of the cells during this procedure align with the plane of the epithelium and the axis of tissue spreading at the animal-vegetal axis. Using laser ablation experiments, Campinho et al. elegantly showed that the tension applied to these cells is along the animal-vegetal pole of the embryo. Upon real-time tension application at a distinct direction, the authors observed the same phenotype observed in the *in vitro* experiments, where the cell was re-orienting its spindle along the major force vector (Campinho *et al.*, 2013). In *Drosophila*, and precisely on wing disc epithelium, cell divisions are taking place and the spindle orientation of cells is located in the center. Experiments showed that spindle orientation aligns with the proximal-distal axis in the center while the spindle orientation of cells at the periphery is perpendicular to this axis. This observation suggested that due to different proliferating rates at those cells, an anisotropic force is created, and this force determines the final orientation of the mitotic spindles (Mao *et al.*, 2011; LeGoff, Rouault and Lecuit, 2013). Laser ablation experiments by Mao et al. showed that cells at the periphery displayed tension application perpendicular to the proximal-axis and drove cell elongation, increased proliferation rates in the center of the disc and different orientation of spindles within the disc (Mao *et al.*, 2011). Lastly, experiments in *Xenopus* embryonic epithelia suggested that the force distribution is what determines the spindle orientation rather than the cell shape (Petridou and Skourides, 2014). In conclusion, the fact that the cell shape is altered upon the application of force creates a big question regarding the possibility of separation of these parameters especially in the tissue context (Nestor-Bergmann, Goddard and Woolner, 2014).

4.2 Intracellular transduction and translation of the extrinsic signals that guide spindle orientation.

Even though the importance of the extrinsic signals during spindle orientation has been established, the precise mechanisms through which cells are able to sense, transduce and translate these forces into biochemical responses are still under investigation and began to unravel recently. Two different aspects of force application on mitotic cells are under investigation at the moment; how the force is sensed via RFs and transduced to the cortex of the cell and how these signals are further translated into biochemical signals inside the cell.

4.2.1 Intracellular transduction of extrinsic signals:

Cells undergoing mitosis maintain the connection with their substrate via RFs and it has been shown that this connection is required for both Z-axis and XY axis orientation (Petridou and Skourides, 2014). Initially it was suggested that the transduction of mechanical forces applied externally to the cell during mitosis are transferred through the RFs, however, work from Petrdiou et al. has recently shown that the FA member FAK, is crucial for this process. Experiments *in vivo* and *in vitro* identified FAK as a regulator of mitotic spindle orientation since the absence of FAK led to spindle misorientation in culture cells and *Xenopus* embryos. The authors clearly showed that even though spindle integrity, centering and cortical microtubule capturing were not affected in the absence of FAK, the cells failed to respond to external forces. Similar results were observed at the *Xenopus* epithelial cells, where FAK function in spindle orientation was found crucial for the epithelial morphogenesis. They also suggested that even though cells lacking FAK were able to form RFs during mitosis, they failed to orient their spindle. Evidence proposes that RFs are not sufficient to transmit the externally applied forces to the cell cortex, but these forces are transmitted via a link of RFs to a mechanosensing protein complex or the substrate interphase. The authors also showed that even though FAs disassemble during mitosis, a pool of FA-positive complex is present at the RFs-ECM interface and this might have been the link for signal sensing and translation to the mitotic cell (Petridou and Skourides, 2014). FA proteins have been found to be indispensable for spindle orientation before. Other members of integrin adhesome, the integrins have been also found indispensable for spindle orientation. The first evidence regarding integrin importance in spindle orientation emerged from Toyoshima et al. who elegantly showed that during symmetric cell divisions in adherent cells, integrin β1 was found indispensable for the determination of the division axis parallel to the substrate. Experiments were performed using a variety of methods for the downregulation of integrin β1 such as inhibition of integrin-FN interaction by RGD treatment and loss of integrin function using inhibitory antibodies. The results suggested that the integrin mediated adhesion signaling was promoting the accumulation of PIP3 at the cell cortex. PIP3 accumulation

suggested to act as a molecular cue which resulted to the orientation of the spindle parallel to the Z-axis (Toyoshima and Nishida, 2007). Another study using mammalian mice skin showed that integrin β1 knockout mice display randomized orientation of their spindles (Lechler and Fuchs, 2005), however, a direct role of integrin in spindle orientation has not been addressed yet. This is due to the fact that integrins in epithelial tissues control basement membrane deposition. This deposition guides the establishment of epithelial polarity, a process fundamental for correct spindle orientation. This suggests that the integrin effects in spindle orientation might simply be a result of secondary effects due to defects in epithelial polarity. Later studies involved integrin β1 in the follicle cells of ovarian epithelium monolayer in *Drosophila*. The authors of this study, elegantly showed that the integrin signaling but not the integrin-based adhesion was required for the spindle orientation. There results suggest that the role of integrin during spindle orientation is independent from its role in cell-ECM adhesion (Fernandez-Minan, Martin-Bermudo and Gonzalez-Reyes, 2007). Surprisingly, later work by Ferraris et al. elegantly showed that integrin β1 can become activated through membrane tension independently of its ability to bind ligand. This activation was shown to elicit an integrin signaling similar to the one observed to ligand based active integrin β1 (Maria and Ferraris, 2010; Ferraris *et al.*, 2014). Even though the evidence was convincing, the physiological relevance of this mode was not clear. Data emerged from Petridou et al. on *Xenopus* outermost epithelium, where no cell-ECM interactions exist, showed that FAK is associated with correct spindle orientation (Petridou and Skourides, 2014). This led to the impressive correlation of these proteins in spindle orientation. Another study performed by Petridou and Skourides identified the presence of two distinct pools of active integrin β1 at cells in culture during mitosis. These 2 pools were distinct from each other, with the first identified at the RFs-ECM contacts, while the other identified at the lateral cortex of the mitotic cells. This observation in combination with previous results implicating FAK in spindle orientation led to the identification of a Cortical Mechanosensory Complex (CMC) at the lateral cortex of the mitotic cells composed of integrin β1, FAK, p130Cas, and Src. This complex was shown to become asymmetrically distributed upon the activation of integrin at those sites. The activation of integrin at those sites was shown to be ligand-independent and force-dependent (**Figure 18**). (Petridou and Skourides, 2016). All these together, lead to the conclusion that the mechanism through which the cell can sense and transduce the mechanical forces applied to it externally during mitosis is through this CMC complex. These data also suggest that this complex might be implicated in the consequent transduction of signal intracellularly to the spindle. To conclude, the precise mechanism through which these forces are sensed is not clearly understood and if the implication of these proteins is due to their implication in cell-ECM adhesion or not is left to be explored.

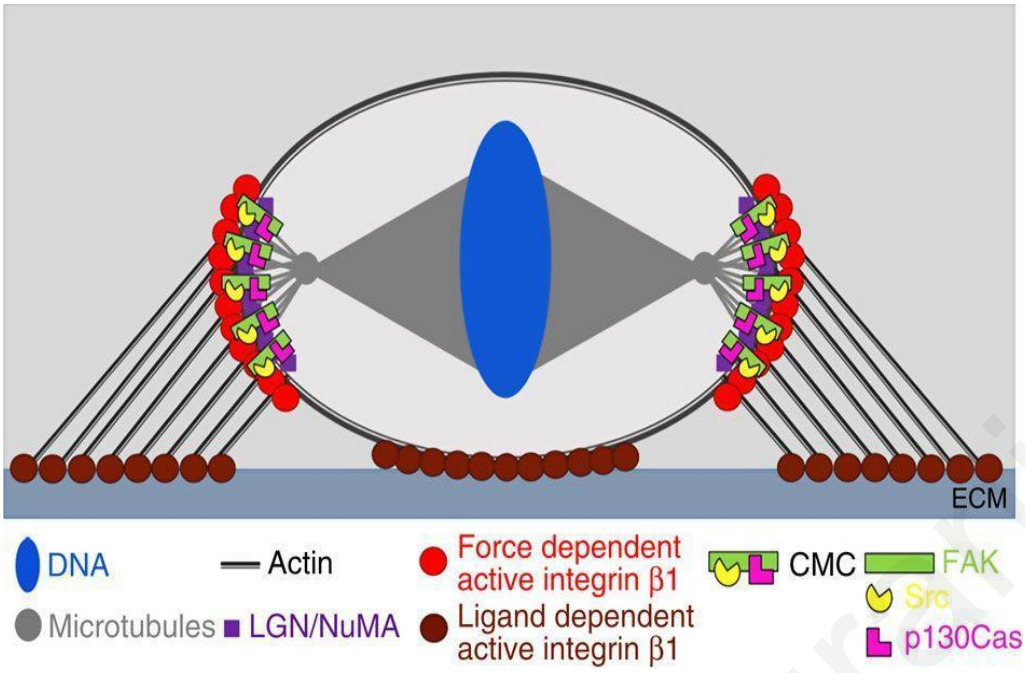


Figure 18: Illustrated representation of the mitotic cell.

The illustration shows the two distinct pools of active integrin β1 during mitosis. At the lateral cortex of the mitotic cell a pool of ligand-independent integrin β1 is shown which results in the subsequent recruitment of FAK, p130Cas and Src, while a poll of ligand-dependent integrin is found at the cell-ECM contacts connecting the mitotic cell through RFs with the ECM. Adapted from: (Petridou and Skourides, 2016)

4.2.2 Intracellular translation of extrinsic signals:

A wide variety of proteins has been associated with spindle orientation, like microtubule and actin-associated proteins such as EB1, myosin X, contractin and ezrin, kinases such as Src, Aurora, P13K and ABL1, members of the PCP, and transmembrane receptors. But only a few pieces of evidence show that the force transduced to the cell externally during mitosis, has any effects on their function. Work by Fink, Carpi et al. identified the presence of subcortical actin clouds which were formed during mitosis, and these clouds were found associated with spindle movement. Experiments using laser ablation on cells attached on micropatterned coated surfaces revealed that these actin clouds were polarized along the non-ablated axis showing a response from the cell spindle to the applied forces (Fink *et al.*, 2011). This communication with the spindle was lost upon Nocodazole treatment. This treatment led to microtubule network disruption through nocodazole. These results showed the the necessity of the communication between actin clouds and the spindle of the mitotic cells (Fink *et al.*, 2011). A recent study identified an implication of myosin 10 in these actin clouds. As Kwon et al. elegantly showed, myosin 10 pulls the centrosomes towards the direction of these actin clouds (Kwon *et al.*, 2015). This might also be a link with the pre-mentioned CMC complex. This complex is composed of proteins from FAs and as known, these proteins interact directly with actin cytoskeleton. This could presumably means that the CMC during mitosis interact with these subcortical actin clouds and this is what guides the correct orientation of the mitotic spindle. Another involvement of the CMC might be through the stabilization of the MTs at the spindle capture sites since previous studies have identified the targeting of MTs at active integrin sites (Seldin *et al.*, 2013, Byron *et al.*, 2015). Other possible mechanism of translation of signals applied externally to the mitotic cells, includes the involvement of the cortical machinery complex. Experiments on micropatterns showed that both LGN and NuMA have been found to localize at the cell cortex upon force application and further evidence suggests that these two proteins have the capacity to respond to the forces applied by RFs. Lastly, experiments using uniaxial stretch on cells showed that NuMA becomes enriched at the cortex, whereas rotation of the spindle is induced towards the stretch application site and this observation is lost upon NuMA knockdown. These results, in combination with existing knowledge that NuMA interacts with a family of actin proteins and therefore is linked to the actin cytoskeleton to the cortex, provides further evidence for its association with this process (Seldin *et al.*, 2013).

4.3 Results Chapter I: The responses of the mitotic cells to the substrate topological clues are independent of the molecular nature of adhesion

4.3.1 The orientation of the mitotic spindle on planar substrates is parallel to the plane of attachment irrespective of the molecular nature of the cell adhesion.

As mentioned before, cells in culture on ECM-substrates have been shown to orient their spindle parallel to the substrate of attachment. Experiments using Hela and NRK cells have identified that spindle orientation under these conditions, dependents on cell-substrate adhesion (Toyoshima and Nishida, 2007). It is also known that cells on glass surfaces coated with PLL fail to orient their spindles since they are not able to properly spread on them. Experiments using FN-coated glass surfaces showed that cells were able to orient their spindles parallel to the substrate; however, upon treatments with RGD peptides, known to block attachment through integrins, spindles of cells were shown to be misoriented. Further experiments performed using inhibitory antibodies against integrin β1 have shown that upon inhibition cells displayed again spindle misorientation with respect to the substrate (Toyoshima and Nishida, 2007; Petridou and Skourides, 2016). Recent experiments, using VN coated coverslips, showed that two different pools of integrins exist; one at the lateral cortex of the mitotic cell and one at the points where the cell is connected with the ECM through RFs (Petridou and Skourides, 2016). The importance of this later interaction was not clarified and in combination with all the earliest evidence, left a major question unanswered. Is the integrin-substrate (ECM) adhesion what guides the spindle orientation and is this interaction indispensable for this process? To examine the precise role of integrin-dependent cell adhesion in spindle orientation parallel to the plane of attachment we initially utilized a modified, pre-described system. Briefly, we allowed cells to attach on glass coverslips coated with a chimeric protein composed of the extracellular domain of N-cadherin which was fused to its C-terminal domain with the Fc domain of human immunoglobulin G1 (IgG1) (Lambert, Padilla and Mege, 2000; Vega L *et al.*, 2014). These cells were compared to cells that were seeded on FN substrates (known to attach and spread through the formation of integrin adhesions). In order to preclude any possibility that integrin-based adhesion was contributing to the cell attachment on these substrates and that the cells were devoid of FA formation, we allowed cells to attach and spread for a 30-minute interval under serum-free conditions. Most of the cells attached on N-cadherin Fc were able to spread fully in a time interval of 30 minutes and formed the pre-described structures known as linear AJs (Gavard, Lambert, Grosheva, Marthiens, Irinopoulou, J. F. Riou, *et al.*, 2004; Craig T. Lefort, Wojciechowski and Hocking, 2011).

Cells that were attached and spread on N-cadherin Fc displayed linear AJs that were β-catenin rich and displayed no detectable formation of FAs or integrin activation (**Figure 19 A** and **B**). This allowed us to ensure that the cells on N-cadherin Fc were devoid of any visible FAs and suggested that cells were able to seed and spread only through the formation of AJs. When the cells entered mitosis, we observed that the adhesion complexes were disassembled both on FN and N-cadherin Fc and the enrichment of the receptors (β1-integrin and N-cadherin respectively) was at minimum levels at the ventral surface of the cells. The receptors however, were found to be redistributed at the cortex of the mitotic cells (**Figure 19 C**). The ability of Hela cells to orient, their spindle parallel to the plane of the substrate was evaluated both on N-cadherin Fc and FN substrates. We observed that cells on N-cadherin Fc were able to orient their spindle parallel to the plane of the substrate in an indistinguishable manner from that of cells dividing on FN (**Figure 19 C, D).** These data together suggest that integrin-dependent adhesion is not driving spindle orientation of cultured non-polarized cells at the plane of the substrate.

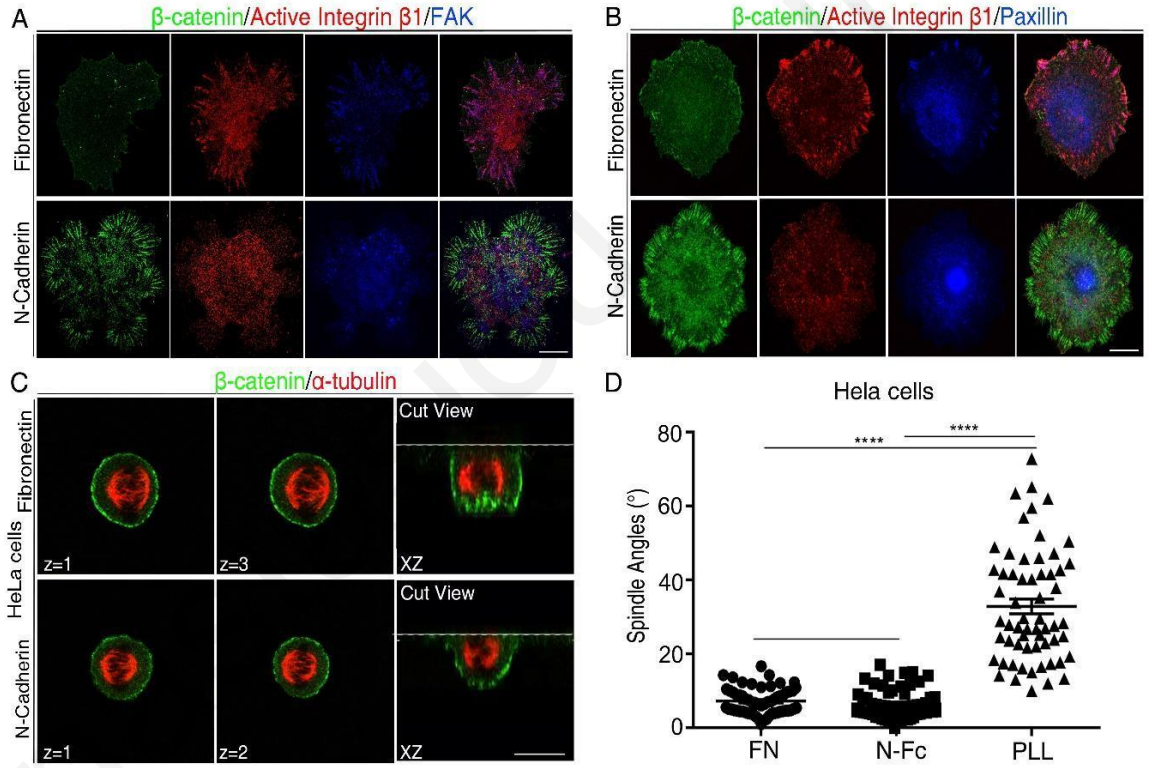


Figure 19: Spindle orientation parallel to the substrate on cadherin- and integrin-based adhesions.

(**A–B**) Confocal images of interphase HeLa cells on coverslips coated with Fibronectin (FN) or N-cadherin Fc (N-Fc). Cells were stained for β-catenin, active integrin β1, and either FAK, N=220 (A), or Paxillin, N=256 (B). N, number of interphase cells across all conditions from three independent experiments. (**C**) Representative Z-stacks at the plane of the spindle poles and side projections (xz) of metaphase HeLa cells on FN and N-Fc substrates. Cells were stained for α-tubulin and β-catenin. N = 180 total number of metaphase cells across all conditions from four independent experiments. (**D**) Distribution of spindle-to-substrate angles in (**C**). Mean±s.e.m: HeLa cells on FN 7.235 ± 0.4565°, N=60; HeLa cells on N-Fc, 6.428 ± 0.5170° N=60; HeLa on PLL, 32.87 ± 1.961° N=60; *P* values calculated using Mann–Whitney test; N, number of metaphase cell, for each condition ls from three independent experiments.

In order to avoid the possibility that this observation was cell-type specific we performed the same experiments on U2OS cells, which are bone osteosarcoma cells known to express N-cadherin. These cells on N-cadherin Fc displayed the same characteristics as Hela cells since at the 30-minute interval were able to spread and adhere in the absence of any detectable FAs or integrin activation. Spindle orientation evaluation showed that these cells orient their spindles along the plane of the substrate both on N-cadherin Fc and FN substrates. (**Figure 20 A, B, C**).

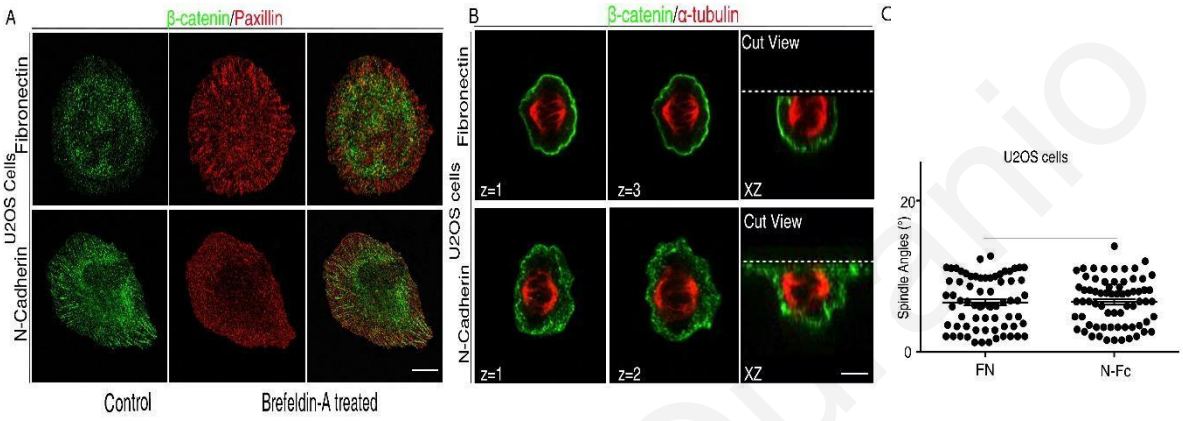


Figure 20: Spindle orientation and cell spreading of U2OS cells on cadherin planar substrates

(**A**) Representative images of interphase U2OS cells on Fibronectin (FN) or N-cadherin Fc at the plane of cell-substrate attachment. Cells were stained for β-catenin and paxillin. N=355 total number of interphase cells, across all conditions, from three independent experiments. (**B**) Z-stacks at the plane of the spindle poles and side projections of metaphase U2OS cells on FN and N-Fc substrates. Cells were stained for α-tubulin and β-catenin; N = 100 total number of metaphase cells across all conditions from four independent experiments. (**C**) Distribution of spindle-to-substrate angles in (E). Mean ± s.e.m. U2OS cells on FN 6.566 ± 0.4883°, N=50; U2OS cells on N-Fc 6.702 ± 0.4522°, N=50. *P* values calculated using Mann–Whitney test; N, number of metaphase cells, for each condition from three independent experiments. Scale bars, 10μm.

4.3.2 Cell spreading and mitotic spindle orientation on N-cadherin Fc is AJ driven

AJ formation and stabilization rely on Ca^{2+} ions while FAs form upon integrin binding to the ECM, a binding facilitated by Mg and Mn ions (Rothen-Rutishauser *et al.*, 2002; Xia and Springer, 2014). To ensure that the cell spreading and the orientation of the mitotic spindle parallel to the plane of the substrate on N-cadherin Fc substrates was purely through AJs adhesion, we used the widely known chemical thylene glycol-bis (β-aminoethyl ether)-*N*, *N*, *N′*, *N′*-tetraacetic acid (EGTA). EGTA selectively chelates Ca^{2+} ions, thus we expected AJ disassembly and rounding up of cells on N-cadherin Fc while no effects were expected on the cells attached on FN through integrin-based adhesion (Rothen-Rutishauser *et al.*, 2002; Xia and Springer, 2014). Hela cells were treated with EGTA for a 20-minute interval and we observed that cells attached on N-cadherin Fc became round while cells on FN displayed no morphological alterations. This suggested that EGTA is not affect them (**Figure 21 A**) and confirmed that the cells attached to N-cadherin Fc substrates are attached and spread on the substrate specifically through AJ formation. To further explore the effect of AJs disassembly on these cells, we examined the ability of cells to orient their spindle under EGTA treatment on both N-cadherin Fc and FN substrates. Hela cells were monitored after the addition of EGTA for 20-minutes and were fixed at the point where rounding of cells but not detachment was observed. This treatment showed that mitotic Hela cells on N-cadherin Fc were not able to orient their spindle while spindle orientation of mitotic cells on FN was not affected (**Figure 21 B, C**). This shows that the formation of AJs on cells on N-cadherin Fc was responsible for the guidance of spindle orientation parallel to the substrate at those cells. In this context, another possibility was that the cells are able to secrete ECM ligands during their spreading time interval, suggesting that secreted ligands are responsible for the orientation of the spindle parallel to the substrate plane on these N-cadherin substrates. Even though the cells were attached in the complete absence of any soluble ligand provided by the cell culture-medium (serum-free conditions) and even though the time interval provided for them to spread was not sufficient for ligand deposition, we wanted to eliminate this possibility. In order to do that we used a well characterized inhibitor of the secretory pathway known as Brefeldin A (Helms and Rothman, 1992; Nebenfuhr, Ritzenthaler and Robinson, 2002). This inhibitor has been shown to disrupt the Golgi complex. Hela cells were treated before spreading for 30 minutes with the inhibitor, however, this inhibition failed to induce any spindle misorientation of cells on N-cadherin Fc (**Figure 21 D, E**). Together these results suggest that integrin-based adhesion is dispensable for the orientation of the spindle at the plane of the substrate on N-cadherin substrates.

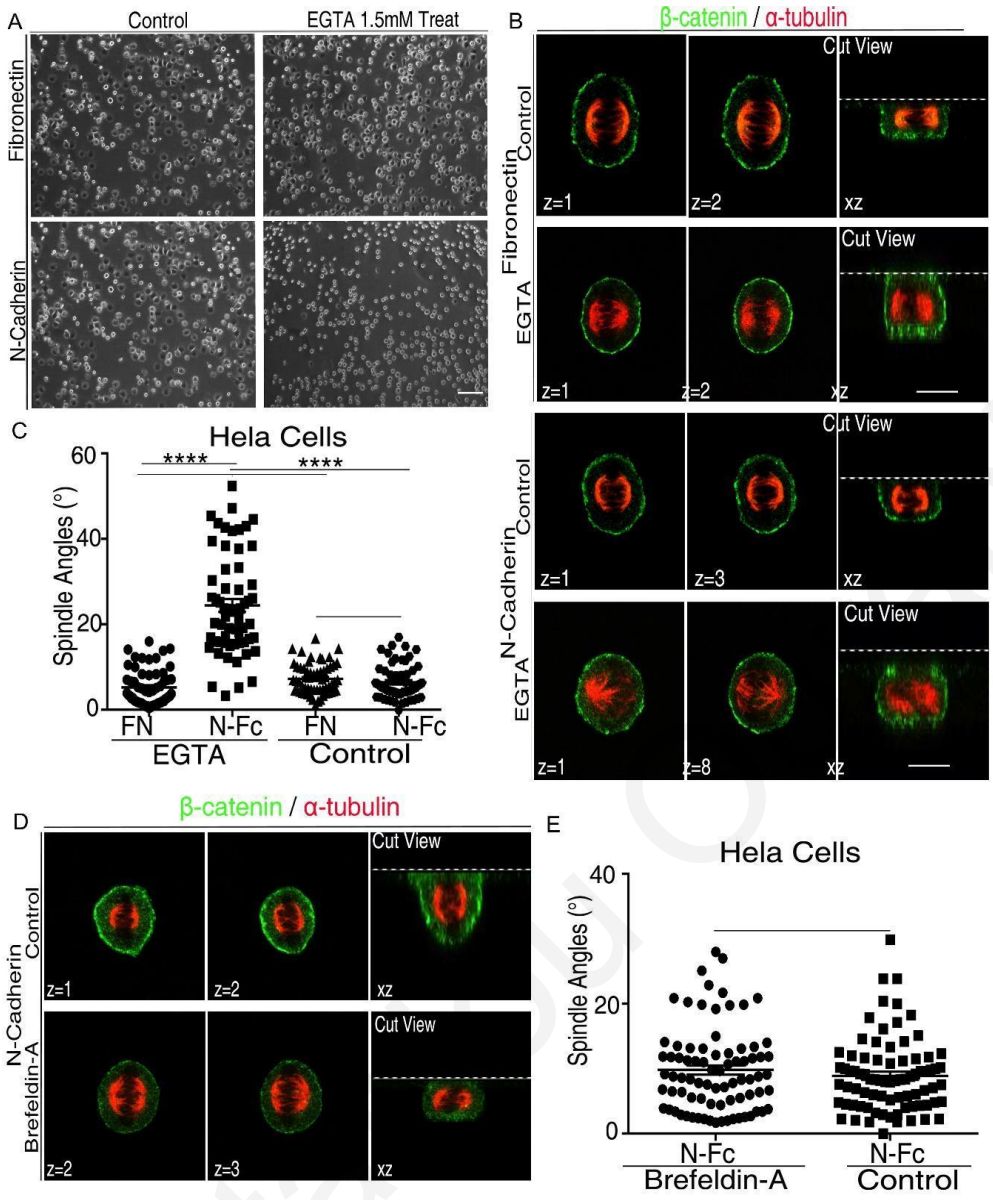


Figure 21: Cadherin engagement is necessary for correct spindle orientation on cadherin substrates.

(**A**) Phase-contrast images of control and EGTA-treated HeLa cells on FN and N-cadherin Fc. N=363 total number of cells, across all conditions, from three independent experiments. (**B**) Optical sections and side projections (xz) of control and EGTA-treated metaphase HeLa cells on Fibronectin (FN) or N-cadherin Fc (N-Fc) substrates. Cells were stained for β-catenin and α-tubulin, and images were acquired at the plane of the spindle poles. N=240 total number of metaphase cells across all conditions from three independent experiments. (**C**) Distribution of spindle-to-substrate angles in (**B**). Mean±s.e.m: control on FN 7.235 ± 0.4565°, N=60; EGTA-treated on FN 5.210 ± 0.5494°, N=60; control on N-Fc 6.428 ± 0.5170°, N=60; EGTA-treated on N-Fc 24.42 ± 1.514°, N=60. *P* values calculated using Mann–Whitney test; N, number of metaphase cells, for each condition, from three independent experiments. (**D**) Representative optical sections and side projections of representative control and Brefeldin-A–treated metaphase HeLa cells on N-Fc. Cells were stained for β-catenin and α-tubulin. N=160 total number of metaphase cells across all conditions from three independent experiments. (**E**) Distribution of spindle-to-substrate angles in metaphase control and Brefeldin-A–treated HeLa cells on N-Fc. Control on N-Fc 8.910 ± 0.6321°, N=80; Brefeldin-A–treated on N-Fc 9.809 ± 0.7211°, N=80. *P* values calculated using Mann–Whitney test; N, number of metaphase cells, from each condition, from three independent experiments. Scale bars, 10μm

4.3.3 The localization and the recruitment of LGN and NuMA to the cortex of the mitotic cells on N-cadherin Fc are polarized even in the absence of cell-ECM interactions.

The role of the cortical machinery complex composed of LGN, NuMA and Gαι has been extensively discussed in the introduction. This complex has been found to localize asymmetrically at the cortex of mitotic cells and displays enrichment at the spindle capture sites. It has been also shown that its importance in mitosis is underlined by its indirect connection with AMTs and through the direct binding with motor protein dynamin, known to be connected with the ATMs (Morin, Jaouen and Durbec, 2007; Peyre *et al.*, 2011; Noatynska and Gotta, 2012; McNally, 2013; di Pietro, Echard and Morin, 2016). The correct positioning and anchoring of the spindle at the sites of the cortex is a result of the spatially restricted localization of LGN and NuMA on the cortex. This polarized localization, leads to the anchorage of ATMs at those points of the cortex and generates pulling forces applied to the spindle poles (Peyre *et al.*, 2011; McNally, 2013). This results in the correct positioning of the mitotic spindle of the cell. Both LGN and NuMa become polarized when cells attached on FN substrates enter mitosis and they display clear polarization and enrichment at the lateral cortex of the mitotic cells at points where the spindle is captured (Peyre *et al.*, 2011; Kiyomitsu and Cheeseman, 2013; Petridou and Skourides, 2016). To examine what happens under the conditions where cells are attached on an ECM-free substrate, we allowed Hela cells to adhere and spread on FN and N-cadherin Fc and stained them with antibodies against NuMA and LGN. On both substrates, we observed that cells undergoing mitosis, displayed enrichment of both LGN and NuMA at the sites where spindle was captured to the lateral cortex of the mitotic cells while they displayed no enrichment at the apical (dorsal) and basal (ventral, adherent) site of the cell (**Figure 22 A**). This shows that both proteins display correct polarity regardless of the substrate of cell attachment and suggests that integrin-based adhesion has no implication in the recruitment or polarization of this cortical machinery at the mitotic cell cortex. It was recently suggested that E-cadherin serves as a binding partner of LGN and that this interaction is responsible for the recruitment of LGN at the lateral cortex of the mitotic cells (Gloerich *et al.*, 2017; Hart *et al.*, 2017). This is not the case for cells attached on N-cadherin Fc since no ventral localization of the LGN protein is observed (**Figure 22 A**). This also shows that the binding of LGN to N-cadherin is not driving its localization at the cell cortex. If this was the case it was expected to see LGN enrichment at the site where cells were connected with the ECM at the ventral side, and also observe cell divisions perpendicular to the plane of attachment, something that was clearly not taking place. To examine this possibility in more detail, we generated micropatterned N-cadherin substrates with stripes of N-cadherin Fc. Hela cells were allowed to spread on these micropatterns for 30 minutes and stained for LGN and β-catenin. Confocal imaging of these cells was performed at their basal sites (where cells were in contact with the N-cadherin Fc coated areas) and did not reveal any polarized enrichment of LGN on the N-cadherin Fc stripes (**Figure 22 B**). No LGN enrichment was observed on the linear AJs that formed at the N-cadherin Fc stripes also, confirming that LGN is not recruited to the cell cortex by N-cadherin based AJs. To conclude, these

experiments show that the cortical machinery complex composed of Gαι, LGN, and NuMA, is recruited and becomes polarized at the lateral cortex of the mitotic cells in the absence of any cell-ECM interactions. These results agree with the data showing that cells can orient their spindle parallel to the plane of the substrate on N-cadherin Fc without any cell-ECM interactions. In order to explore the differences between previously published work on E-cadherin (Gloerich *et al.*, 2017; Hart *et al.*, 2017) and our results using N-cadherin, we transfected Hela cells with E-cadherin fused to GFP and allowed them to attach on E-cadherin Fc. These cells were able to seed and spread on E-cadherin Fc as expected while control Hela cells failed to spread on this substrate. Evaluation of spindle orientation of these cells showed that even though both control and E-cadherin-GFP expressing cells attached on FN substrates, were able to orient their spindle parallel to the plane of attachment, only cells expressing E-cadherin-GFP were able to orient their spindle parallel to the substrate on E-cadherin Fc (**Figure 22 C, E**). These data show that Hela cells upon exogenous expression of E-cadherin can spread and attach on E-cadherin Fc and they can orient their spindles parallel to the plane of the substrate. Thus, mitotic cells on E-cadherin Fc planar substrates display spindle orientation parallel to the plane of the substrate as effectively as cells attached on N-cadherin Fc and FN. This is another confirmation of the ability of cells to orient their spindle parallel to the plane of attachment in the absence of cell-ECM interactions. These experiments also suggest that the E-cadherin Fc based AJs fail to recruit LGN to the basal side of the cell similarly to N-cadherin based AJs since such localization would presumably lead to the perpendicular orientation of the cell spindles with respect to the plane of attachment. We then wanted to examine the precise localization of cortical machinery complex proteins (LGN and NuMA) on E-cadherin Fc. Hela transiently expressing E-cadherin GFP were plated on E-cadherin substrates and stained against these proteins. No enrichment of LGN was observed at the sites of cell-ECM attachment and both LGN and NuMA displayed identical polarization and enrichment as previously observed on N-cadherin Fc and FN (**Figure 22 D**). In order to further evaluate the contribution of the cortical machinery complex to spindle orientation of cells attached on non-ECM based substrates we used a construct composed of the C-terminal of LGN, known to act as dominant-negative and by disrupting the interaction of LGN with NuMA. This construct competes with endogenous LGN for the binding to NuMA and leads to the disruption of cortical MTs anchoring (Du and Macara, 2004; Morin, Jaouen and Durbec, 2007; Pirovano *et al.*, 2019). The spindle orientation was evaluated on Hela cells transiently expressing the LGN-C terminus both on N-cadherin Fc and FN. Spindle orientation quantification showed that cells expressing this construct displays spindle misorientation on both N-cadherin Fc and FN (**Figure 22 F**). This shows the importance of the cortical machinery complex in spindle orientation regardless of the nature of the substrate. Conclusively, the above data show that the cortical machinery complex composed of Gαi, LGN and NuMA, is localized and recruited at the lateral cortex of the mitotic cells and display correct polarity both on N and E-cadherin substrates in the absence of any cell-ECM interactions. These data also show that integrin signaling is dispensable for the exclusion of this complex from the cell-substrate contact sites. Additionally, the data suggest that the interactions

between E-cadherin and LGN do not play a central role in the distribution of the protein at the lateral cortex during mitosis and it is rather a secondary cue, possibly serving as a factor for the refinement of LGN localization at those sites.

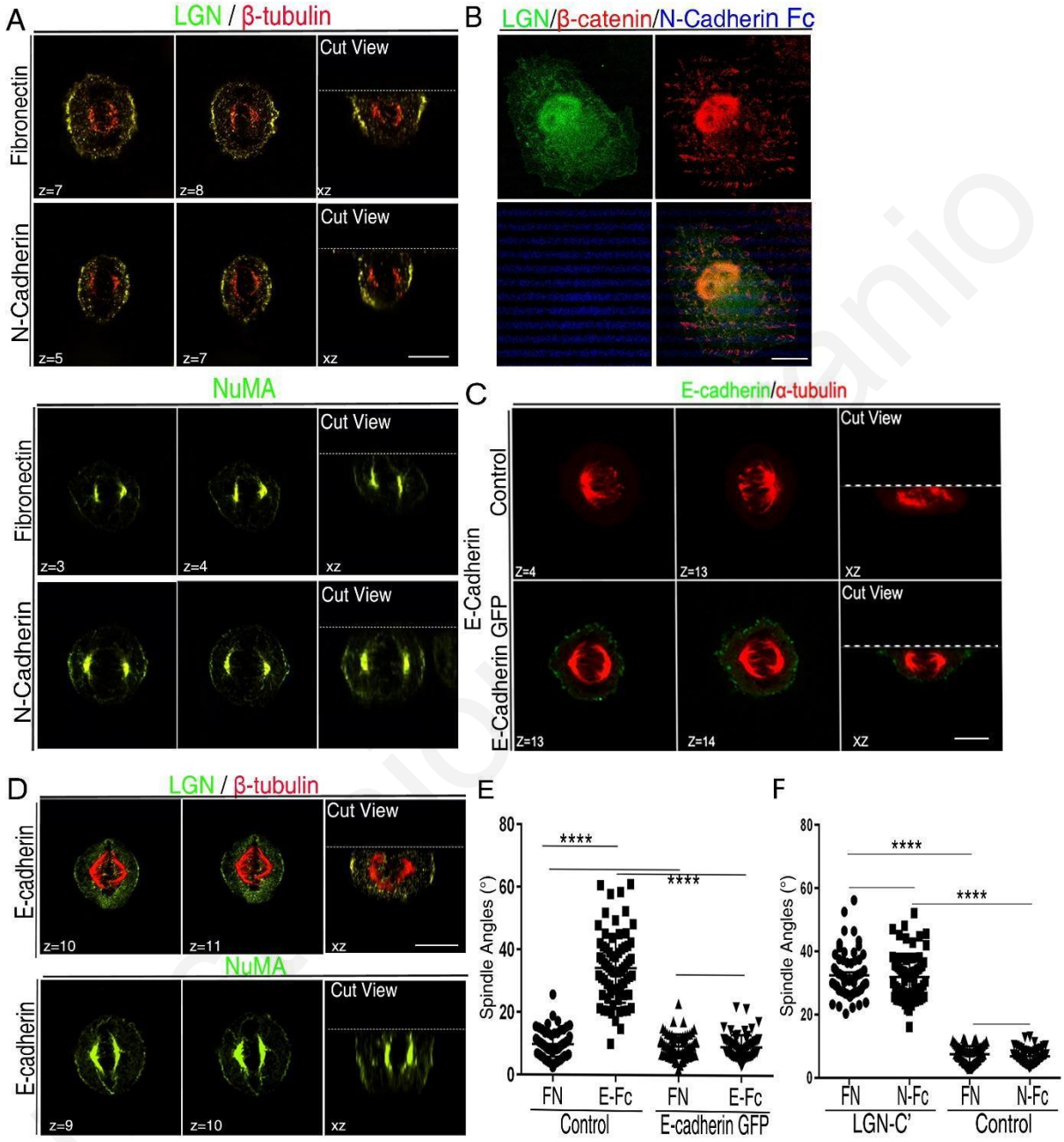


Figure 22: The spatial distribution of LGN and NuMA does not depend on the molecular nature of the adhesion.

(**A**) Representative optical sections and side projections (xz) of HeLa cells seeded on Fibronectin (FN) and N-cadherin Fc (N-Fc). Images were acquired at the plane of the spindle poles. Cells were stained for α-tubulin, LGN, and NuMA as indicated. N=215 total number of metaphase cells across all conditions from three independent experiments. (**B**) Representative confocal image of interphase HeLa cells expressing LGN-GFP on N-Fc linear micropatterns and labeled for LGN, β-catenin, and N-Fc. Images were acquired at the plane of attachment. N=112 total number across all conditions of interphase cells from three independent experiments. (**C**) Representative optical sections and side

. (**C**) Representative optical sections and side projections (xz) of control and E-cadherin–GFP–expressing metaphase HeLa cells stained for β-tubulin. N=277 total number of metaphase cells, across all conditions, from three independent experiments. Scale bars, 10μm. (**D**) Representative optical sections and side projections of HeLa cells expressing E-cadherin–GFP on E-cadherin Fc substrate. Cells were stained for α-tubulin, LGN, and NuMA as indicated. Images were acquired at the plane of the spindle poles. N=277 total number of metaphase cells across all conditions from three independent experiments. (**E**) Distribution of spindle-to-substrate angles in metaphase control and E-cadherin–GFP–expressing HeLa cells on FN or E-cadherin Fc (E- Fc). Mean±s.e.m: control on FN 9.769 ± 0.4638°, N=74; control on E-cadherin 34.17 ± 1.305°, N=71; E-cadherin GFP on FN 9.315 ± 0.4771°, N=65; E-cadherin GFP on E-cadherin Fc 9.184 ± 0.4588°, N=67. *P* values calculated using Mann–Whitney test; N, number of metaphase cells from three independent experiments. (**F**) Distribution of spindle-to-substrate angles in control and LGN-C'mCherry–expressing metaphase HeLa cells on FN or N-Fc. Mean±s.e.m: LGN-C' cherry on FN 3.248 ± 0.8769°, N=74; LGN-C' cherry on N-Fc 7.509 ± 0.3213°, N=71; control on FN 3.189 ± 0.8956°, N=65; control on N-Fc 6.878 ± 0.2764°, N=67. . *P* values calculated using Mann–Whitney test; N, number of metaphase cells, from each condition, from three independent experiments. Scale bars, 10μm.

4.3.4 Spindle orientation on planar cadherin substrates is determined through the formation of forces derived from asymmetrically distributed RFs on the cell cortex.

Previous studies have shown that spindle orientation in cultured cells depends on the distribution and force application of the RFs at the cell cortex. RFs connect the cells with the substrate during mitotic division. The force application through RFs to the mitotic cell cortex has been previously studied. Studies using micropatterned surfaces of defined geometry, where cells can adhere and divide, showed that cells acquire the specific shape and adhesion geometry of these surfaces and orient their spindles with respect to the adhesion geometry. The spindle orientation under these conditions was found to be associated with the forces applied to the cortex of the cell through the anisotropic distribution of RFs (Thery *et al.*, 2005; Théry *et al.*, 2007; Fink *et al.*, 2011; Nestor-Bergmann, Goddard and Woolner, 2014; Petridou and Skourides, 2016). These micropatterned surfaces were found to direct spindle orientation predictably through the specific distribution of RFs in a way that spindles were oriented along the greatest force vector. These previously performed studies suggested that the anisotropic force distribution provided to the cell cortex by the RFs, provide a memory of the adhesion geometry of the cell during interphase (Thery *et al.*, 2005; Théry *et al.*, 2007; Fink *et al.*, 2011). The orientation of mitotic spindle parallel to the plane of attachment, has been suggested to be a consequence of the lack of RFs and therefore the absence of forces at the apical and basal sites of the adherent cells undergoing mitosis (Petridou and Skourides, 2016). Taking this evidence into consideration and provided that N- and E-cadherin Fc substrates orient the spindle parallel to the substrate, we wanted to examine the possibility that this type of adhesion will also guide the orientation of the spindle in response to defined adhesion geometry within the XY plane. Initially we wanted to explore the possibility that Hela cells on N-cadherin Fc substrates can have correct spatial memory through the formation of RFs. To do that, we compared RF formation of Hela cells on FN and N-cadherin Fc substrates. As shown in Figure 22 A, on both substrates, cells undergoing mitosis display RFs formation. The cells undergoing mitosis on N-cadherin Fc substrates display RFs that are positive for β-catenin but negative for active integrin β1, while cells on FN displayRFs positive for active integrin β1 with almost non-existent staining of β-catenin (**Figure 23 A**). In an attempt to explore these differences further we generated micropatterned surfaces both on FN coated and N-cadherin Fc coated glass coverslips. For the generation of the FN micropatterned surfaces we used a previously described protocol (Thery and Piel, 2009) while for the N-cadherin micropatterned surfaces, we developed a protocol that would allow patterning on silanized glass surfaces. Briefly, glass coverslips were coated with PLL-PEG and were then irradiated with deep UV, through a custom photomask with L and bar shaped patterns, to remove PEG from the micropattern as previously described (Thery and Piel, 2009). The coverslips were subsequently subjected to vapor APTES deposition, which resulted in the silanization of the patterned area, leaving the remaining PLL-PEG coated area intact. This allowed the specific and high-density immobilization of proteins on the micropatterns and the generation of high-quality N-cadherin Fc patterned surfaces. We generated both L-shaped and linear micropatterns for both N-cadherin Fc and FN substrates in order to explore

our hypothesis. Initially, we allowed Hela cells to attach on N-cadherin and FN coated L-shaped micropatterns to study the ability of cells to attach and spread on these surfaces. Hela cells were able to spread on both substrates however on FN micropatterns, cells displayed clear FA formation and activation of integrin β1 at the periphery while cells on N-cadherin Fc micropatterns, displayed linear AJ formation (**Figure 23 B**). We further attempted to compare and assess the ability of cells to orient their mitotic spindle on these micropatterned surfaces. In order to do that we used both L-shaped and linear micropatterns and examined the orientation of the spindle of mitotic Hela cells on both N-cadherin and FN coated micropatterns (**Figure 23 C**). It was previously shown that the cells on L-shaped micropatterns coated with FN, orient their spindle parallel to the hypotenuse of the L-shaped patterns and along the long axis on linear patterns. Hela cells on both N-cadherin Fc and FN display equal ability in orienting their spindles within the plane of attachment suggesting that N-cadherin adhesion geometry guides spindle orientation similarly to FN (**Figure 23 D**). These results together indicate that AJs have the ability to guide spindle orientation based on the geometry of adhesion and that this is a result of the formation, asymmetric distribution and the force generation on the cell cortex through RFs. These results are identical to what it has previously shown for cells on FN micropatterned substrates and in combination with our previous results, they show that spindle orientation parallel to the plane of attachment, can also be guiuded by cadherin-based adhesion. Overall, this new evidence shows that the nature of adhesion provides purely mechanical cues which are independent of the molecular nature of the adhesion.

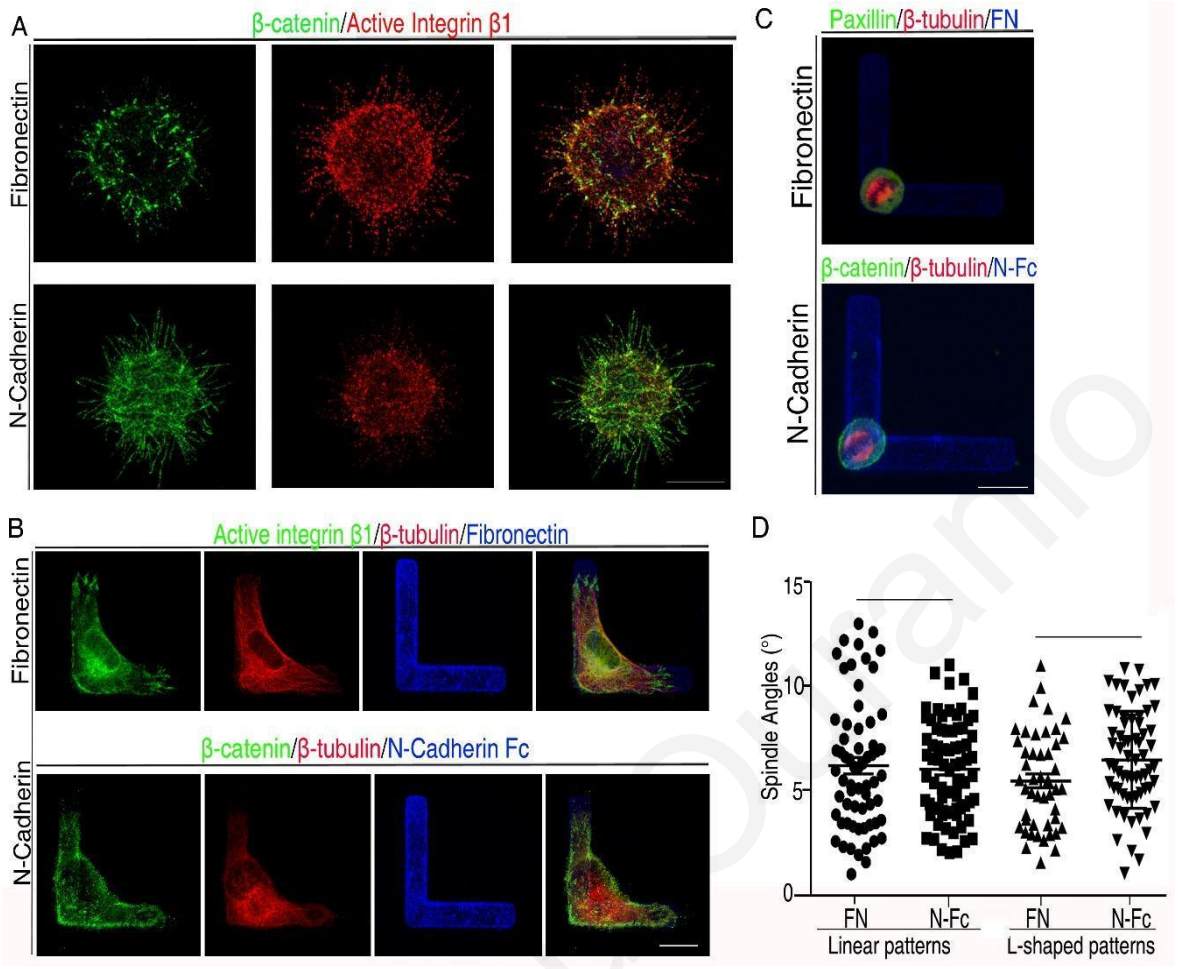


Figure 23: Spindle responses to adhesion geometry are independent of the molecular nature of the adhesion.

(**A**) Optical sections and side projections (xz) of mitotic HeLa cells on Fibronectin (FN) or N-cadherin Fc (N-Fc). Cells were stained for active integrin β1 and β-catenin. Images were acquired at the plane of spindle poles. N=197 total number of metaphase cells across all conditions from three independent experiments. (**B**) Representative images of interphase HeLa cells on Fibronectin (FN) or N-Fc L-shaped micropatterned substrates. Cells were stained for active integrin β1, β-catenin, β-tubulin, Fibronectin (FN), and N-Fc as indicated. Images were acquired at the plane of attachment. N=152 total number of interphase cells across all conditions from three independent experiments. (**C**) Metaphase HeLa cells on FN or N-Fc L-shaped micropatterned surfaces. Cells were stained for Paxillin, β-catenin, β-tubulin, FN, and N-FC as indicated. The images were acquired at the plane of the mitotic spindle. N=82 total number of metaphase cells across all conditions from three independent experiments. (**D**) Distribution of XY spindle angles in metaphase HeLa cells on FN or N-Fc L-shaped and linear micropatterns. Mean±s.e.m: Fibronectin bar patterns 6.399 ± 0.4586°, N=44; Fibronectin L-shaped patterns, 5.829 ± 0.4129° N=31; N-Fc linear patterns 6.235 ± 0.2674°, N=85; N-Fc L-shaped patterns 6.402 ± 0.2679°, N=82. *P* values calculated using Mann–Whitney test; N, number of metaphase cells, from each condition, from three independent experiments. Scale bars, 10μm.

4.3.5 Integrin β1 activation is necessary for spindle responses to planar substrates independently of the molecular nature of the adhesion.

It has recently been suggested that during mitosis two different pools of integrin β1 are observed on cultured cells. The one at the points where RFs connect the cell to the substrate and the second at the lateral cortex of the mitotic cell at the points where the RFs terminate. This study also suggested that the implication of integrin β1 in mitosis has a distinct role from its role in cell adhesion. It was shown that integrin β1 becomes activated on the lateral cortex of the cells during mitosis and effectively guides the recruitment of other FA proteins like (FAK, p130Cas, and Src) which form a complex called CMC. The protein members of this complex were found indispensable for spindle orientation since they were shown to govern spindle responses to the mechanical force applied to the cell during mitosis (Petridou and Skourides, 2016). As our results confirm, cells on N-cadherin Fc substrates do not display activation of integrin β1 at the cell-substrate plane but orient their spindle parallel to the plane of attachment in an identical manner as cells on FN substrates. This suggests that integrin β1 on these substrates is not required for cell spreading. However, we wanted to examine the possibility that integrin β1 has a role in spindle orientation under integrin-independent-adhesion conditions. Initially, we examined the state of integrin β1 on both N-cadherin Fc and FN during interphase and mitosis. As shown in **Figure 24 A**, interphase cells attached on FN exhibit activation of integrin β1 at the sites of FAs while interphase cells on N-cadherin Fc do not display any integrin activation at the sites of cell-substrate adhesion. During mitosis, cells on both N-cadherin Fc and FN display identical activation of integrin β1 at the lateral cortex (**Figure 24 B**). This suggests that activation of integrin β1 at the lateral cortex of the mitotic cells is independent of the molecular nature of adhesion. We then moved on to address a potential implication of cortical activation of integrin β1 in spindle orientation under conditions where cell-ECM interactions were absent. In order to do that, we took advantage of well-characterized commercially available antibodies known to inhibit integrin β1 (AIIB2, P4C10 and P5D2) (Byron *et al.*, 2009). AIIB2 is a well-characterized allosteric inhibitory antibody for integrin β1, which binds to the α2 helix region of the βI domain (Hall *et al.*, 1990). P4C10 is an inhibitory antibody reported to act in the same region as AIIB2 with a similar way for the inhibition of integrin β1 activation (Kovach *et al.*, 1992; Takada and Puzon, 1993). Lastly, the P5D2 antibody, which has been shown to bind at a region close to the ligand-binding pocket on integrin β1 and to facilitate conformational changes that reduce the affinity for integrin ligand, keeping the integrin in a close-inactive state (Xanthis *et al.*, 2019). Initially we tested AIIB2 treatment on Hela cells. Cells were allowed to attach and spread on N-cadherin Fc and FN substrates for 20 minutes and then treated with AIIB2. We observed that treatment with AIIB2 had no effect on cell spreading on N-cadherin Fc substrates; however, the cells treated with this antibody displayed major spindle misorientation with respect to the plane of the substrate. On FN, cells displayed defective spreading, however the minority of cells able to spread and divide displayed spindle misorientation similarly to the ones on N-cadherin Fc (**Figure 24 C, D, E**). To verify our results, we used P4C10 and P5D2 and we observed that upon treatment with both antibodies, spindle misorientation was

elicited both on N-cadherin Fc and FN (**Figure 24 F, G**). Collectively, these data confirm the role of integrin β1 in the orientation of mitotic spindles independent from the molecular nature of the adhesion.

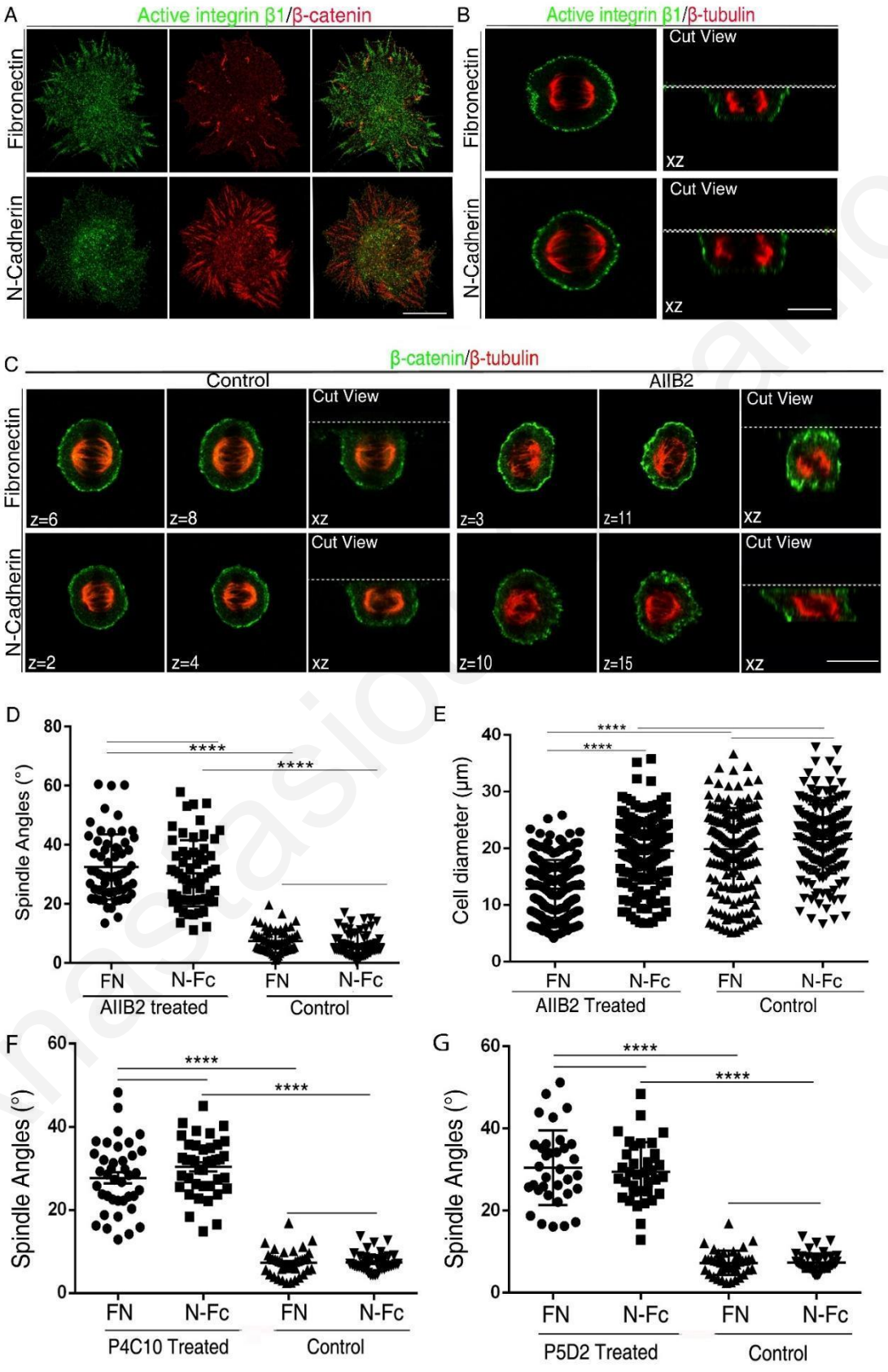


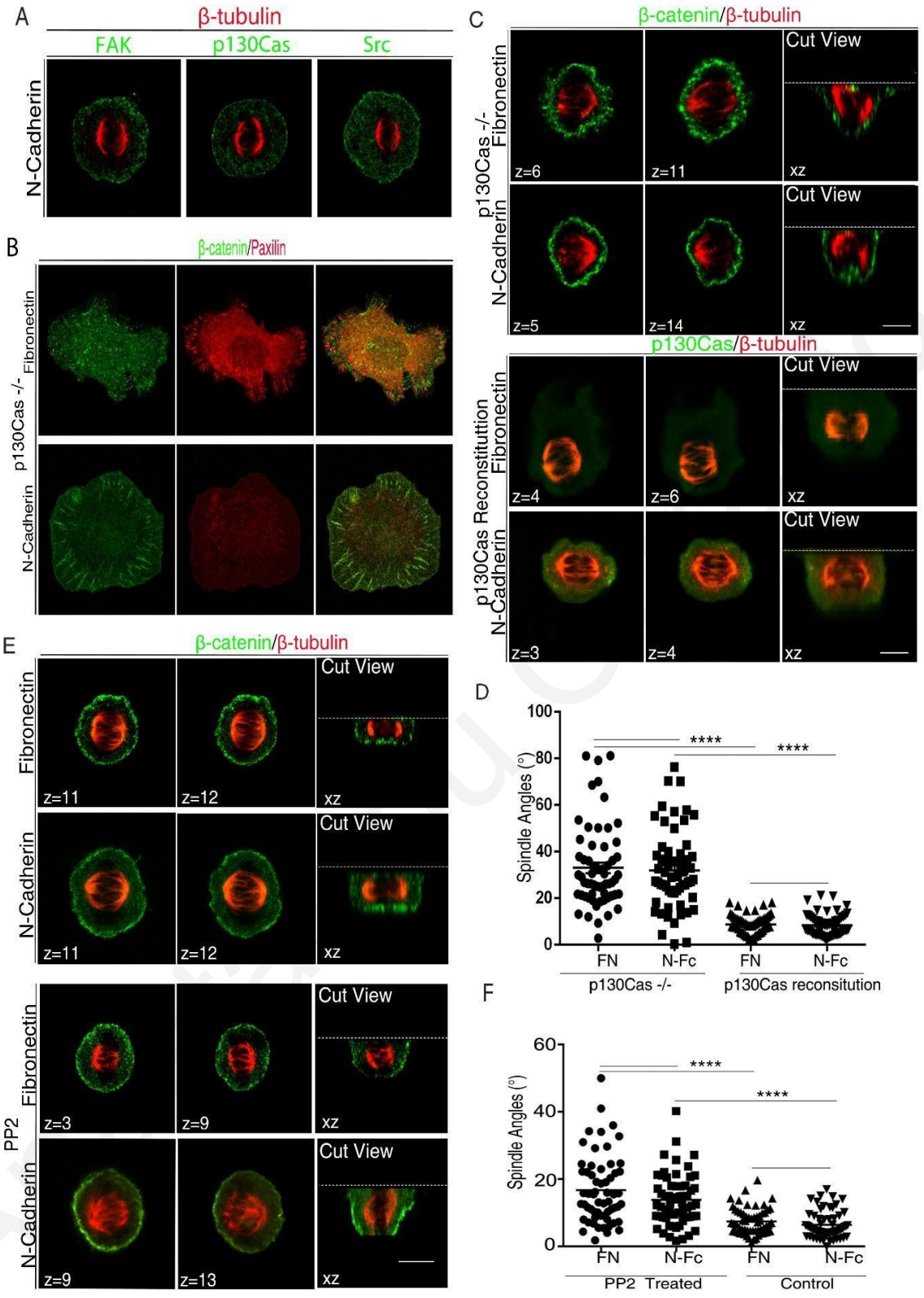
Figure 24: Integrin activation is indispensable for spindle orientation on planar substrates

(**A**) Optical sections of interphase HeLa cells on Fibronectin (FN) or N-cadherin Fc (N-Fc). Cells were stained for β-catenin and active integrin β1, and images were acquired at the cell-substrate plane. N=357 total number of interphase cells across all conditions from three independent experiments. (**B**) Optical sections and side projections (xz) of control HeLa cells on FN or N-Fc at the plane of the spindle pole. Cells were stained for β-tubulin and active integrin β1. N=205 total number of metaphase cells across all conditions from three independent experiments. (**C**) Optical sections and side projections of control and AIIB2-treated metaphase HeLa cells on FN or N-Fc. Cells were stained for β-tubulin and β-catenin. Images were acquired at the plane of each spindle pole. N=342 total number of metaphase cells across all conditions from three independent experiments. (**D**) Distribution of spindle-to-substrate angles in control and AIIB2-treated metaphase HeLa cells on FN or N-Fc. Mean±s.e.m: Control on FN, 7.358 ± 0.3919°,N=85; AIIB2 treated on FN 31.63 ± 1.110°, N=85; control on N-Fc 6.665 ± 0.3849°, N=86; AIIB2 treated on N-Fc 30.82 ± 1.104°, N=86. *P* values calculated using Mann–Whitney test; N, number of metaphase cells, from each condition, from three independent experiments. (**E**) Distribution of the cell spread of control and AIIB2-treated interphase HeLa cells on FN or N-Fc. Mean±s.e.m: Control on FN, 19.89± 0.5394μm, N=180; AIIB2 treated on FN 12.88 ± 0.3790μm, N=181; control on N-Fc 21.67 ± 0.4622μm, N=176; AIIB2 treated on N-Fc 19.56 ± 0.5394μm, N=181. *P* values calculated using Mann–Whitney test; N, number of interphase cell diameter, from each condition, from four independent experiments. (**F**) Distribution of substrate-to-spindle angles of metaphase control and P4C10-treated HeLa cells on Fibronectin (FN) and N-cadherin Fc (N-Fc) substrates. Data represent the mean±s.e.m. Control on FN, 7,272 ± 3,019°, N=40; P4C10-treated on FN 27,757± 8,282°, N=39; control on N-Fc 7.391 ± 2,212°, N=39; P4C10-treated on N-Fc 30,373±6,761°, N=39. *P* values calculated using Mann–Whitney test. N = number of metaphase cells, from each condition, from three independent experiments. (**G**) Distribution of substrate-to-spindle angles of metaphase control and P5D2-treated HeLa cells in FN and N-Fc substrates. Data represent the mean±s.e.m. Control on FN, 7,295 ± 0.4646° N=41; P5D2-treated on FN 30.481 ±1,561°, N=34; control on N-Fc 7.355 ± 0.3525°, N=40; P5D2-treated on N-Fc 29,96 ± 1,201°, N=36. *P* values calculated using Mann–Whitney test. *N* = number of metaphase cells from two independent experiments. Scale bars, 10μm.

4.3.6 The CMC member proteins p130Cas and Src are necessary for spindle orientation in the absence of cell-ECM interactions.

We have provided evidence that integrin β1 activation is necessary for spindle orientation with a role distinct from its role in cell-ECM adhesion. These results agree with previous studies suggesting that force-dependent activation of integrin β1 at the lateral cortex of the mitotic cell is what facilitates spindle responses to mechanical cues. It was also suggested that this integrin β1 activation drives the recruitment of other FA proteins such as FAK, p130Cas and Src and the establishment of the CMC through a yet unknown mechanism. This complex appears to spatially bias AMTs, guiding spindle responses to the externally applied RF driven forces (Petridou and Skourides, 2016). The above observations, along with experiments showing that CMC protein members mediate spindle orientation (Petridou and Skourides, 2016), and our results showing that integrin β1 activation is indispensable in spindle orientation in a cell-ECM adhesion independent manner, suggest that, the proteins of the CMC are necessary for the correct spindle orientation irrespectively of the molecular nature of the substratum. To explore this possibility, we initially examined the precise localization of each protein member of the CMC; FAK, p130Cas and Src, at the lateral cortex of the mitotic cells on N-cadherin Fc and FN substrates. Hela cells were allowed to spread on both substrates for a 30-minute interval and stained against CMC protein members. All the protein members of the CMC, similarly to active integrin β1, localize at the sites of lateral cortex of the mitotic cells both on N-cadherin and FN substrates (**Figure 25 A**) suggesting a role in spindle orientation on cadherin-based substrates. We then wanted to address the role of p130Cas in spindle orientation using mouse embryonic fibroblasts (MEFs) knocked down for 130Cas. These cells have been described elsewhere regarding their spindle orientation defects but in brief, they have been found to adhere and spread on FN (Petridou and Skourides, 2016) however, they display major misorientation defects at the plane of the substrate. This misorientation is rescued upon the exogenous re-introduction of wild type p130Cas-ires GFP on FN. First, we allowed these cells to attach and spread on N-cadherin Fc in an attempt to characterize their ability to form linear AJs. At a 30-minutes interval, p130Cas null MEFs display linear AJs formation in the absence of any detectable FAs (**Figure 25 B**). Then we assessed the spindle orientation of these cells both on N-cadherin Fc and FN showing that the spindle was misoriented on both substrates in respect to the plane of the substrate. Using a construct of p130cas-wt in a vector that expresses GFP from an internal ribosome entry sequence (IRES) for tracking the transfected cells (Meenderink *et al.*, 2010), we transiently transfected those cells and observed the effects of the re-introduction of the protein on spindle orientation. On both FN and N-cadherin Fc substrates, the spindle orientation defects were rescued (**Figure 25 C, D**) suggesting that p130Cas is necessary for correct spindle orientation in an adhesion-independent manner, thus uncoupling the role of p130Cas during mitosis from its role in cell adhesion. We moved on to address the role of another CMC protein member; Src, which has previously described to be crucial for the correct spindle orientation on planar substrates and micropatterns. To do that, we took advantage of the well-

characterized PP2 inhibitor (Thery *et al.*, 2005; Petridou and Skourides, 2016). Hela cells attached on N-cadherin Fc and FN were treated with the inhibitor after seeding for 30 minutes and their spindle orientation was evaluated (**Figure 25 E, F**). We observed that treatment with the inhibitor led to defects in spindle orientation of cells on both substrates suggesting that the role of Src in spindle orientation is distinct from its role in cell-ECM mediated adhesion. Collectively these experiments show that the role of integrin β1 and the role of CMC in spindle orientation responses to planar substrates is distinct from the role of the member proteins in cell-ECM mediated adhesion.


A
β-tubulin
FAK
p130Cas
Src
N-Cadherin
B
β-catenin/Paxilin
p130Cas -/-
Fibronectin
N-Cadherin
C
β-catenin/β-tubulin
p130Cas -/-
Fibronectin
N-Cadherin
Cut View
z=6
z=11
z=5
z=14
xz
p130Cas/β-tubulin
p130Cas Reconstitution
Fibronectin
N-Cadherin
z=4
z=6
z=3
z=4
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Spindle Angles (°)
100
80
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FN
N-Fc
FN
N-Fc
p130Cas -/-
p130Cas reconsitution
E
β-catenin/β-tubulin
Fibronectin
N-Cadherin
PP2
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z=11
z=12
z=3
z=9
z=13
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F
Spindle Angles (°)
60
40
20
0
****
****
FN
N-Fc
FN
N-Fc
PP2 Treated
Control

Figure 25: Integrin activation is indispensable for spindle orientation on planar substrates

(**A**) Optical sections of interphase HeLa cells on Fibronectin (FN) or N-cadherin Fc (N-Fc). Cells were stained for β-catenin and active integrin β1, and images were acquired at the cell-substrate plane. N=357 total number of interphase cells across all conditions from three independent experiments. (**B**) Optical sections and side projections (xz) of control HeLa cells on FN or N-Fc at the plane of the spindle pole. Cells were stained for β-tubulin and active integrin β1. N=205 total number of metaphase cells across all conditions from three independent experiments. (**C**) Optical sections and side projections of control and AIIB2-treated metaphase HeLa cells on FN or N-Fc. Cells were stained for β-tubulin and β-catenin. Images were acquired at the plane of each spindle pole. N=342 total number of metaphase cells across all conditions from three independent experiments. (**D**) Distribution of spindle-to-substrate angles in control and AIIB2-treated metaphase HeLa cells on FN or N-Fc. Mean±s.e.m: Control on FN, 7.358 ± 0.3919°,N=85; AIIB2 treated on FN 31.63 ± 1.110°, N=85; control on N-Fc 6.665 ± 0.3849°, N=86; AIIB2 treated on N-Fc 30.82 ± 1.104°, N=86. *P* values calculated using Mann–Whitney test; N, number of metaphase cells, from each condition, from three independent experiments. (**E**) Distribution of the cell spread of control and AIIB2-treated interphase HeLa cells on FN or N-Fc. Mean±s.e.m: Control on FN, 19.89± 0.5394μm, N=180; AIIB2 treated on FN 12.88 ± 0.3790μm, N=181; control on N-Fc 21.67 ± 0.4622μm, N=176; AIIB2 treated on N-Fc 19.56 ± 0.5394μm, N=181. *P* values calculated using Mann–Whitney test; N, number of interphase cell diameter, from each condition, from four independent experiments. (**F**) Distribution of substrate-to-spindle angles of metaphase control and P4C10-treated HeLa cells on Fibronectin (FN) and N-cadherin Fc (N-Fc) substrates. Data represent the mean±s.e.m. Control on FN, 7,272 ± 3,019°, N=40; P4C10-treated on FN 27,757± 8,282°, N=39; control on N-Fc 7.391 ± 2,212°, N=39; P4C10-treated on N-Fc 30,373±6,761°, N=39. *P* values calculated using Mann–Whitney test. N = number of metaphase cells, from each condition, from three independent experiments. (**G**) Distribution of substrate-to-spindle angles of metaphase control and P5D2-treated HeLa cells in FN and N-Fc substrates. Data represent the mean±s.e.m. Control on FN, 7,295 ± 0.4646° N=41; P5D2-treated on FN 30.481 ±1,561°, N=34; control on N-Fc 7.355 ± 0.3525°, N=40; P5D2-treated on N-Fc 29,96 ± 1,201°, N=36. *P* values calculated using Mann–Whitney test. *N* = number of metaphase cells from two independent experiments. Scale bars, 10μm.

4.4 Discussion Chapter I

The proper spindle orientation is fundamental for a wide range of processes within a multicellular organism such as tissue morphogenesis and homeostasis, epithelial integrity, cell proliferation and cell fate decision (Gillies and Cabernard, 2011; Michelle S Lu and Johnston, 2013; VanHook, 2015). The role of spindle orientation has been extensively studied throughout the years and defects in this process have implicated spindle orientation with different developmental diseases such as numerous neurological diseases, polycystic kidney disease and cancer (Fischer *et al.*, 2006; Godin *et al.*, 2010; Noatynska, Gotta and Meraldi, 2012; Petridou and Skourides, 2016). The correct positioning of the mitotic spindle of the cell is a crucial procedure for the proper cell division and is has been shown to be guided by the evolutionary conserved cortical machinery complex composed of Gαi, LGN and NuMA (Du and Macara, 2004; Peyre *et al.*, 2011; McNally, 2013; Matsumura *et al.*, 2016; Bergstralh, Dawney and St Johnston, 2017; Pirovano *et al.*, 2019). This complex is established at the lateral cortex of the mitotic cell and becomes polarized. This polarization is what establishes the anchoring points of ATMs at the lateral mitotic cortex of the cell. This is achieved through the interactions of NuMA with the motor protein dynein. The dynein-dynamin protein complex is what eventually exerts pulling forces on AMTs in order to correctly position the spindle. Evidence on Hela cells suggested that interphase Hela cells on FN substrates display an equal distribution of LGN on the cortex of the cell and this distribution changes during mitosis in a way that it corresponds to the final orientation of the spindle (Peyre *et al.*, 2011; McNally, 2013; di Pietro, Echard and Morin, 2016; Petridou and Skourides, 2016). The involvement of integrins in spindle orientation has also been under investigation for many years providing solid evidence for their role in spindle axis determination through their role in cell-ECM adhesion and signaling (Lechler and Fuchs, 2005; Toyoshima and Nishida, 2007; Kuo *et al.*, 2012; Xanthis *et al.*, 2019). Evidence supporting such a role has been generated both *in vivo* and *in vitro*. For example, experiments on integrin β1 knockout mice and non-polar cells in culture showed that disruption of integrin β1 cell adhesion led to spindle misorientation at the plane of the substrate. Further experiments in epithelial cells have been shown that integrin β1 regulates the divisions in epithelial cells (Lechler and Fuchs, 2005; Toyoshima and Nishida, 2007; Kuo *et al.*, 2012; Xanthis *et al.*, 2019). However, if the involvement of integrin in spindle orientation was associated with its role in cell adhesion was not clarified. A recent study performed by Petridou and Skourides. showed that during mitosis at *Xenopus* epithelial, FAK (another protein member of FAs) is indispensable for spindle orientation. The cells at the outermost superficial cell layer of *Xenopus* are known to not have contact with the ECM and hence they do not form FAs (Petridou and Skourides, 2014). This hypothesis was further supported by experiments that showed that these cells are not in contact with the FN matrix (which is formed during development) and the inhibition of the FN matrix assembly did not affect the orientation of cell spindles during mitosis (Ramos and DeSimone, 1996; DeSimone, Dzamba and Davidson, 2007; Rozario *et al.*, 2009). This raised the possibility that during mitosis, integrin and other FA proteins might have a

role in spindle orientation and this role is distinct from their role in cell adhesion. First evidence arising regarding a differential model of activation for integrin β1 during cell division was firstly provided by Ferraris et al. who used cells on VN and the ability of uPAR to bind VN showed that integrin activation can be activated through tension applied to the cells mechanically, they also showed that the signaling from integrin β1 led to the phosphorylation of downstream known FA targets such as p130Cas (Maria and Ferraris, 2010; Ferraris *et al.*, 2014). This unravels an ability of integrins to respond and become activated directly through tension in the absence of any ligand. Later, Petridou and Skourides showed that during mitosis a pool of active integrin β1 observed not only at the cell-ECM plane but at the lateral cortex of the mitotic cells and this activation found to lead to the formation of a complex known as CMC composed of FAK, p130Cas and Src. The proteins of this complex were found indispensable for the orientation of the mitotic spindle. Additionally, they showed that integrin β1 activation at the cortex was distributed asymmetrically at the points where the most forces were applied through RFs and these sites corresponded to the spindle capture sites where the cortical machinery complex is localized. They also suggested that integrin activation at those sites was tension dependent and ligand independent. This finding was in agreement with more recent data showing that integrin activation can become active through membrane curvature and tension (Kim *et al.*, 2020). These data suggest that both integrin β1 and CMC proteins (crucial in FA formation) are major players in spindle orientation both *in vivo* and *in vitro*. All these data together provide evidence regarding the role of integrins during mitosis which is associated with the activation of integrins through plasma membrane tension application. However, they do not clarify the precise role of cell-ECM interactions during this process nor do they constitute proof that FA proteins and integrins play roles in spindle orientation that are distinct from their role in cell adhesion (Petridou and Skourides, 2016).

The major finding in this study is the evidence that integrin-based cell-ECM adhesion is dispensable for the generation of spindle orientation cues provided by cell adhesion. We generated substrates where cells attached and spread through the formation of linear AJs (N-cadherin Fc substrates) as it has been previously shown (Gavard, Lambert, Grosheva, Marthiens, Irinopoulou, J.-F. Riou, *et al.*, 2004; Craig T Lefort, Wojciechowski and Hocking, 2011; Vega L *et al.*, 2014). Cells under serum-free conditions, form linear AJs and display no detectable activation of integrin β1 or localization of FA proteins at their basal surface (FAK and paxillin). These cells can orient their spindles parallel to the plane of the substrate, in a similar manner to cells seeded on FN substrates. This clearly shows that cells on planar cadherin substrates are able to orient their spindles parallel to the plane of attachment without any interactions of the cells with the ECM. To ensure that this was not a cell type-specific observation, the experiments were performed on different cell types, known to express N-cadherin Fc, U2OS and the results were identical. Additionally, using different approaches (Brefeldin-A and EGTA), we ensured that the cell spreading on these substrates is achieved through the formation of AJs and is independent of any cell-ECM interactions. Analysis of their ability to orient their spindles parallel to the plane of the substrate revealed that the inhibition of the secretory

pathway and hence the presence of ligands did not affect the ability of cells to orient their spindle on N-cadherin Fc. This shows that the ability of cells to orient their spindle on N-cadherin Fc is independent of ECM molecules. Previous studies showed that, the orientation of spindle on cells in culture was achieved through the formation of RFs and that distribution of RFs was guiding spindle orientation on ECM based substrates (Thery *et al.*, 2005; Théry *et al.*, 2007; Fink *et al.*, 2011). We show that cells on N-cadherin are also able to form RFs during mitosis and that those RFs are integrin β1 negative but cadherin and catenin rich. We also show that cells on micropatterned surfaces of defined adhesion geometry coated with non-ECM substrates are able to guide the spindle orientation of Hela cells. These pieces of evidence together show the mechanical nature of RF contribution during mitosis. More precisely, they show that the adhesion provides mechanical cues during mitosis and that these cues are independent of the molecular nature of adhesion. Since the cortical machinery complex is responsible for the anchoring of AMTs and guides the positioning of mitotic spindles we examined the localization of core members of the cortical machinery complex under conditions where cell-ECM interactions were not taking place. We found that both LGN and NuMA become polarized at the lateral cortex of mitotic cells on N-cadherin Fc in an identical manner to the one observed on cells on FN. This suggests that the cortical machinery complex becomes distributed and guides the spindle position independent of the molecular nature of adhesion. Evidence also suggested that LGN has a direct interaction with E-cadherin and that E-cadherin is implicated in spindle orientation at the attachment plane of epithelial cells (Gloerich *et al.*, 2017; Hart *et al.*, 2017). We used Hela cells attached on N-cadherin substrates and compared to FN with respect to the cortical machinery components localization and E-cadherin GFP transiently transfected Hela compared to Hela on FN. If any interaction between E-cadherin or/and N-cadherin with LGN was taking place, we expected to observe basal enrichment of LGN at those substrates. Besides, we expected the spindle orientation to be perpendicular to the plane of the substrate if E-cadherin was responsible for the recruitment of LGN but these notions are clearly do not take place. In order to ensure our results, we created micropatterned substrates at which specific linear regions were coated with N-cadherin Fc and the rest of the surface was uncoated. We observed that the localization of LGN under these circumstances was absent from the regions where cadherin was enriched (the cadherin coated regions) and the linear AJs were formed. Overall, this set of experiments suggest that no direct interaction of LGN with either N-cadherin or E-cadherin on planar and micropatterned surfaces is taking place during mitosis. Even though the possibility that such interaction takes place cannot be precluded, we observed that both LGN and NuMA displayed exclusive cortical localization with no basal localization on N-cadherin and E-cadherin substrates during mitosis. These suggest that even if such interaction is taking place it is clearly not the major determinant for the localization of LGN at the mitotic cell cortex. These results are in agreement with previous studies showing that LGN localization at the cortex of the cell during interphase is minimal and it increases only when the cell undergoes mitosis (Kaushik *et al.*, 2003). This increased localization has been found to be associated with LGN binding to NuMA and this interaction is mutually exclusive with interactions of LGN to

E-cadherin since these are suggested to occur in the same region. As previously mentioned, the cues provided by the adhesion substrate to the cell determine the distribution of forces on the mitotic cell cortex during mitotic divisions. However, how these cues are transmitted to the spindle is yet unknown. The protein members of the CMC complex have been found crucial for this process since upon integrin activation at the cell cortex during mitosis these proteins (FAK, p130Cas and Src) are recruited to this site (Petridou and Skourides, 2016). We wanted to examine if this complex has a role in spindle orientation in a cell-ECM independent context. We used Hela cells on N-cadherin Fc and FN and compared the localization of core members of this complex integrin β1, FAK, p130Cas and Src. The localization of these proteins found to be identical on both substrates. We then attempted to disrupt the function of these proteins in order to identify any implication in spindle orientation under these conditions. We used p130Cas-/- MEFs and compared their spindle orientation ability on N-cadherin and FN. Cells were not able to orient their spindle on both substrates, a phenotype rescued by the re-introduction of p130Cas-ires-GFP construct into the cells. These results suggest that p130Cas is crucial for correct spindle orientation even in cell-ECM independent conditions. Then using an inhibitor for Src we compared treated cells on N-cadherin Fc and FN and again we show that Src is necessary for the proper orientation of the mitotic spindle irrespectively of the molecular nature of the adhesion. These data together clearly show that the CMC members have a central role in spindle orientation irrespectively of the molecular nature of adhesion. It was also suggested by a previous study that this complex is recruited upon the activation of integrins. This activation was characterized by a unique conformation and was found to be ligand independent but force dependent. However, even though the differentiation of the mode of integrin activation is really hard to be claimed, our results support the notion that CMC is required for correct spindle orientation responses in a cell-ECM adhesion independent context. These results also suggest that the role of these proteins in cell adhesion is distinct from their role in spindle orientation. Overall this work shows that the role of integrin β1 is adhesion independent and possibly ligand independent and determines its role in sensing mechanical cues that guide the spindle orientation in an adhesion independent manner. Besides, this study shows that the CMC members p130Cas and Src have a role in the responses of the spindle and we identified that this role is distinct from their role in cell adhesion. However, the mechanism through which the CMC influences spindle capture on the cortex is still unclear.

It has been established that neither of the cortical machinery complex components localization is affected upon integrin β1 inhibition or in the absence of CMC proteins. Intrinsic signals similar to the Ras-related nuclear protein GTP gradient have been shown to be responsible for the cortical asymmetric distribution of the complex and that this distribution is not associated with the extrinsic cues applied to the cell cortex through mechanical stimuli (Kiyomitsu and Cheeseman, 2013, Cavazza and Vernos, 2015). This is supported by the fact that LGN and NuMA under CMC member inhibition are found correctly localized and polarized at the cortex of mitotic cells (Petridou and Skourides, 2016). It is also known that PI3K has been implicated in spindle orientation (Toyoshima and Nishida, 2007) of adherent cells. PI3K found localized at the cortex of mitotic cells and this

localization was not associated with integrins. It is also known that FAK interacts with PI3K at the cell membrane. Their in-between interaction and binding with Rac1 have been found to reorganize the actin cytoskeleton (Kallergi *et al.*, 2007). It was shown by recent studies that an actin pool is observed during mitosis at the subcortical regions of the cell and that this actin pool or clouds have been found to influence the AMTs capture sites by generating forces on the centrosomes (Fink *et al.*, 2011; Kwon *et al.*, 2015). It was also shown that the binding of ATMs by myosin10 was indispensable for the centrosome positioning according to these actin clouds. This may suggest that proteins known to bind actin and/or actin regulators may have a role in spindle orientation through regulation of these subcortical actin clouds (Kwon *et al.*, 2015). It is a possibility that the CMC and the PI3K dependent pathways act together in spindle orientation if we consider the previously described evidence showing that PI3K in adherent cells accumulates PIP3 at the mid-cortex of cells in a manner that is integrin dependent. However, data on MDCKs show that the mechanism through which PI3K is implicated in spindle orientation is not conserved in polarized cells, something that is not the case for CMC, suggesting that these two pathways may not be associated (Toyoshima and Nishida, 2007). In addition, p130Cas (another core member of the CMC complex) acts as actin regulator. It has been well established that the substrate domain of p130Cas acts as the binding site for adaptor proteins Nck which are crucial for the regulation of actin dynamics (Hehlgans, Haase and Cordes, 2007; Rivera *et al.*, 2006). Apart from these, p130Cas activates Rac as a downstream target through phosphorylation and it is also established, from experiments performed by Sharma and Mayer, that Rac activity is implicated in spindle orientation in mammalian oocytes (Sharma and Mayer, 2008; Halet and Carroll, 2007). The interaction of Rac with p130Cas promotes p21-activated Kinase (PAK) and drives further actin reorganization (Halet and Carroll, 2007). Other evidence suggests that proteins of the PAK family interact with another member of the CMC, paxillin, and are also involved in spindle orientation (Hashimoto *et al.*, 2001). All these together suggest that perhaps the members of the CMC act as the FA proteins in cell adhesion and connect the cell with the actin cytoskeleton. This connection could presumably drive the spindle orientation and could possibly explain how the signals transduced from the mechanical stimuli on the cell cortex are translated into biochemical signals in the cell.

The possibility that the CMC complex has an actin regulatory factor as the FA complex may be of high importance for the role of these proteins in the regulation of several processes with distinct contexts. It is clear that ligand independent activation of integrins can take place both as a result of tension as well as bending of the PM. This would suggest a possible role for integrin in developmental processes where high tension is applied to the cells. Unpublished data from our laboratory suggest that CMC proteins and integrin β1 are involved in a process known as apical constriction during neural tube closure in *Xenopus*. The cells during this procedure are not associated with the ECM while they subject to constant mechanical stimulation through morphogenetic movements and tissue rearrangements. This might suggest an implication of CMC in other tension-dependent developmental processes. It would be extremely interesting to identify the core protein members of

both FAs and CMC and analyze them for differences in their members' interactions, their stoichiometry, composition and characterization of the members. This will also provide a deeper understanding of the evolution of these proteins and their precise role in important developmental processes through their implication in possible distinct complexes.

4.5 Conclusions Chapter I

To conclude, we showed that the orientation of the mitotic spindle in non-polar adherent cells is dispensable from the molecular nature of adhesion by creating N-cadherin Fc substrates where cells attached and allowed to spread in the absence of cell-ECM interactions. These cells compared with cells on FN substrates with respect to their ability to orient their spindles parallel to the plane of the substrate. Additionally, through experiments using inhibitors for the protein secretion through Golgi and dissociation of AJs, we suggested that the cell spreading under these conditions (on N-cadherin Fc) was purely based on AJs formation and had no detectable interactions with the ECM or any detectable integrin ligands. These together show that the molecular nature of the adhesion is dispensable for planar spindle orientation on N-cadherin Fc substrates. We moved on and showed that the proteins of the cortical machinery LGN and NuMA are properly localized and polarized at the mitotic cell cortex on the sites where the spindle is captured on cells plated on N-cadherin showing that the localization of these proteins is cell-ECM independent. Through exogenous E-cadherin GFP expression, we showed that the recruitment and polarization of LGN at the cell cortex is not a result of interactions of the protein with E-cadherin during mitosis. Further experiments using micropatterned substrates of different adhesion geometry and spindle orientation assessment allowed us to show that the molecular nature of the adhesion provides only mechanical cues through the formation of the RFs and that these mechanical cues are independent of the molecular nature of the cell adhesion. Besides these, we showed that the role of integrin β1 in mitotic spindle orientation is independent of its role in cell adhesion. This was achieved with experiments using inhibitory antibodies against integrin β1 on cells on N-cadherin compared to cells on FN. Integrin is required for the proper spindle orientation, however its role in this process is found to be distinct from its role in cell adhesion. We moved on to characterize the localization of CMC proteins members FAK, p130Cas and Src at mitotic cells on N-cadherin Fc where cell-ECM interactions were non-existent. We further utilized cells lacking core member protein of the CMC p130Cas and assessed the ability of these cells to orient their spindle under cell-ECM interaction-free conditions. These suggest that even though cell-ECM interactions do not exist, p130Cas is indispensable for proper spindle orientation in agreement with a previous study suggesting a role of this protein in spindle orientation. We used an inhibitor for another core protein member of the CMC, Src, and we evaluated the ability of cells to orient their spindle on N-cadherin and FN showing that again Src is indispensable for proper spindle orientation even in conditions where the adhesion was not based on cell-ECM interactions. These findings together propose that the spindle orientation on planar substrates does not depend on cell-ECM interactions and the involvement of the integrin pool observed at the cell-ECM interface in previous studies serves purely as mechanical anchoring of cells. Cells on N-cadherin Fc are able to attach, spread and divide parallel to the plane of attachment and display identical force distribution at the cell cortex to the cells under cell-ECM interaction dependent substrates. Lastly, the role of CMC proteins and integrin activation during this process is found

crucial even under conditions where ECM interactions with cells are nonexistent. This strengthens the notion that the activation of integrins on the cell cortex derives probably from mechanical stimuli. During development, cells experience mechanical stimuli from different sources due to morphogenetic movements and tissue rearrangements. Our findings in combination with the existing knowledge, suggest that this central force sensing protein complex known as CMC is likely involved in other important morphogenetic processes too. These data also suggest a significance in the evolution of these proteins. The notion that CMC forms under adhesion independent fashion may be a supporting statement that these proteins may be important for other adhesion independent processes. For example, previous work from our laboratory has already identified some of these protein members in motile ciliogenesis forming the so-called ciliary adhesion complexes (Antoniades, Stylianou and Skourides, 2014). These complexes are also associated with actin suggesting that these proteins may have retained conserved features that allow them to be required in different functions.

5. Chapter II

5.1 Introduction

5.1.1 *Xenopus* Laevis; an experimental model in cell and developmental biology

Xenopus is a genus of aquatic frogs native to sub-Saharan Africa. Twenty species have been described however the two best-known species of this genus are *Xenopus laevis* and *Xenopus tropicalis*. These two are commonly used as model systems in a broad range of diciplines from toxicology to embryology and neurosicnece. *Xenopus* in general is a well-studied model that has many advantages in comparison to other animal models, making it an excellent experimental model. The main advantages of *Xenopus* are the easy animal maintenance, the ability to easily perform *in vitro* fertilization (IVT), the large number of eggs provided and their large size. *Xenopus* embryos grow fast and develop into a whole organism with fully functional organs within a couple of days after the fertilization (**Figure 25**), allowing us to explore effects deriving from gain or loss of function experiments in real-time and in a small amount of time. The major advantage of *Xenopus* is however the well-characterized fate maps of the embryo. As a result of that, we know the precise fate of each cell allowing us to perform targeted loss or gain of function experiments in the tissues of interest. Gain or loss of function approaches are performed mainly using mRNAs encoding the proteins of interest, morpholino oligonucleotides (MOs), which are DNA analogs that block either mRNA splicing or translation, as well as more recently developed approaches such as Transcription Activator-Like effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs). The major disadvantage of *Xenopus laevis* is its pseudo tetraploid genome, which makes genetic manipulation and genetic experiments really challenging.

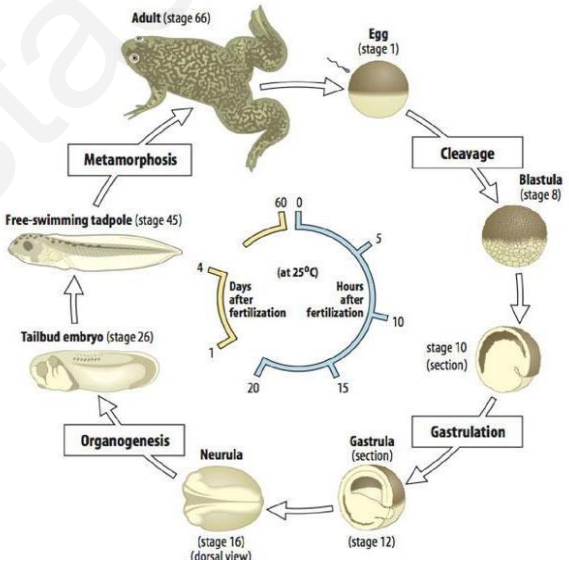


Figure 26: *Xenopus* embryonic development stages and metamorphosis

Adapted from Xenbase: (Karimi *et al.*, 2018)

5.1.2 *Xenopus* embryonic development

Xenopus embryonic development is divided into 3 major stages, the first is the development of the oocyte into a zygote and the blastula formation through the consecutive cleavages. The second is the gastrulation during which the three major germ layers of the body are formed and the third is neurulation. During neurulation, the progenitors of the neural system, neural tube and neural crest cells are formed. This stage is followed by the organ formation (**Figure 26**). These stages together result in the formation of the initial basic body plan and then to the fully developed *Xenopus* embryo, known as tailbud, which is a functional organism (Sive, Grainger and Harland, 2000, 2010; Ma and Liu, 2015; Wang *et al.*, 2015).

- Blastula formation

  Xenopus oocytes are composed of two different types of ECM. The outer jelly membrane which protects the embryo from the external environment and the inner vitelline membrane which is in contact with the embryo. The *Xenopus* embryos display characteristic morphology since they are composed of two different poles; the animal pole (AP) and the vegetal pole (VP). The animal pole is characterized by a pigmented dark color, while the vegetal pole is denser and is mainly composed of yolk cells (Sive, Grainger and Harland, 2000; Sive, Grainger and Harland, 2010). Fertilization occurs when a single sperm enters the oocyte and during this procedure an increase of calcium is observed. This leads to the lifting of the vitelline membrane and formation of the zygote. At this point, rotation of the embryo takes place, resulting in the rearrangement of the two poles in a way that the darker AP is facing up (Sive, Grainger and Harland, 2000; Whitaker, 2006). After fertilization (approximately 2 hours), the cleavages initiate. The first cleavage takes place is at the AP and drives the left-right axis (L-R) formation, whereas the second one is perpendicular to the first and drives the dorsal-ventral (D-V) axis formation. The third division is what separates the AP from the VP and then consecutive cleavages take place, resulting in the creation of inner and outer cells with distinct fates (Elinson, 2011). The blastula consists of a multilayer wall with varying thickness. The blastocoel roof (BCR) for example, is an epithelium that contains a cell layer located at the outer part which is connected without basal lamina to two inner layers where cells, with the help of tight junctions, display apicobasal polarity. BCR is where the FN matrix is firstly observed and it covers it (Winklbauer, 1998; Keller, 2002; Seifert *et al.*, 2009). The top part of the AP, known as the animal cap (AC), will give the epidermis while the VP will develop into endodermal tissues. At the connection of the two poles a marginal zone (MZ) exists, which will give the mesodermal tissues (Winklbauer, 1998; Sive, Grainger and Harland, 2000).

- Gastrulation

  Gastrulation can be characterized as the most important period during embryonic development. This is the stage during which the three germ layers of the embryo will develop and acquire their final positioning in the developing embryo. This will be achieved through massive cell morphogenetic movements and cell rearrangements. The three germ layers are known as the ectoderm, mesoderm and endoderm and each one of them will give rise to different organs and tissues. The external germ layer known as the ectoderm, gives rise to skin, nervous system, ears, lens and cement glands. The middle germ layer known as mesoderm, gives rise to the notochord, the gastrointestinal tract, the somites, kidneys, heart, gonads and blood, and blood vessels. The inner layer is the endoderm which forms the epithelium of the gastrointestinal tract, liver bladder and lungs. Morphogenetic movements such as invagination, involution, convergent extension, mesodermal migration and epiboly, take place and eventually place the cells of these three germ layers at the proper positioning in the developing embryo, in order to give rise to the various organs and tissues. Along the BCR of *Xenopus,* mesodermal cells undergo migration, and for this cell movement it has been shown that FN matrix is required. Epiboly is also known to initiate at the BCR, and it has been shown that the cue for the initiation of epiboly is the formation of FN matrix which is located beneath the inner cell layer of the BCR (Winklbauer and Stoltz, 1995; Winklbauer, 1998; Sive, Grainger and Harland, 2000, 2010; Keller, 2002).

- Neurulation and organogenesis

  Following gastrulation, neurulation initiates which will lead to the formation of the neural tube and the neural crest cells. The dorsal ectoderm of the embryo thickens and undergoes cell shape rearrangements which eventually lead to the neural folds' formation. The neural folds elevate, move toward the midline of the embryo and fuse to form a tubular structure, the neural tube. During this process both the cells of the ectoderm and the cells from the underlying mesoderm undergo alterations in order to achieve the formation of the neural tube. After this stage, the already elongated tailbud that has been formed through elongation of the previously mentioned group of cells at the AP axis, undergoes organogenesis (Keller, Shih and Sater, 1992; Sive, Grainger and Harland, 2000, 2010) .

5.1.3 FN matrix assembly and its role in development.

As mentioned above, the formation of the FN matrix has been associated with morphogenetic movements during gastrulation, thus making its formation essential for many critical processes during the development of vertebrates (Winklbauer and Keller, 1996; Dzamba *et al.*, 2009; M. Marsden and DeSimone, 2001).

The role of FN matrix assembly in both cell intercalation, convergent extension and mesendodermal migration has been shown to be crucial. When this process is inhibited during BCR cells radical intercalation, it leads to a failure of epiboly spreading and abnormal thinning of the BCR (Davidson *et al.*, 2002; Davidson, Keller and DeSimone, 2004; Rozario *et al.*, 2009). Furthermore, FN fibril formation has been shown to contribute to the maintenance of spindle orientation of cells at the BCR and this has been attributed to the FN matrix dependence of cells during intercalation (Wei and Mikawa, 2000; M. Marsden and DeSimone, 2001; Gong, Mo and Fraser, 2004). The cleft formation during epithelial branching is another process during which FN matrix assembly has been found important. Experiments using knockdowns of FN, led to the blockage of cleft formation, while the exogenous addition of FN led to their promotion (Sakai, Larsen and Yamada, 2003). Additionally, various studies associated FN with normal mesendodermal protrusive processes (Winklbauer and Keller, 1996; Winklbauer, 1998; Winklbauer *et al.*, 1992) and tissue maintenance (Nagel *et al.*, 2004). Experiments in *Zebrafish* identified the importance of the FN matrix, using MOs in mesoderm migration and somite boundary formation (Pulina *et al.*, 2011). Finally, the lack of FN fibrils during mesendodermal cell migration across BCR has been shown to lead in an increased cell velocity. This increase has been linked to defects in gastrulation (Rozario *et al.*, 2009). Evidence also suggests that the FN fibrillar matrix is involved in primitive streak formation in the chicken (Duband and Thiery, 1982) and in convergence extension in *Xenopus* embryos (M. Marsden and DeSimone, 2001; Marsden and Douglas W DeSimone, 2003; Rozario *et al.*, 2009). Overall, the formation of FN matrix has been associated with normal development and morphogenesis, while cell signaling through FN matrix has been shown to be crucial for a variety of cellular processes like cell fate determination, differentiation, proliferation and survival. Achieving the understanding of how this matrix is formed, is extremely important for uncovering the processes driving embryogenesis and development.

The formation of FN fibrils at the BCR of *Xenopus* is similar to the one observed in cultured cells *in vitro*. As mentioned above, the formation and assemble of FN matrix is mediated by the FBs. During the formation of FBs, the α5β1 integrin translocates from these sites to the center of the cell through transformation of the actomyosin generated tension into movement along actin filaments. FBs formation is also linked with cell shape alterations which eventually lead to conformational changes in the FN molecule. These changes, promote the elongation of the FN fibrils and their assembly to a matrix. It has also been suggested that the FN fibrillogenesis requires integrin activation and is promoted by cytoskeletal tension (LaFlamme, Akiyama and Yamada, 1992). *In vivo*, at the *Xenopus* BCR, the FN matrix assembly initiates during early gastrulation as a cell-autonomous process

(Winklbauer and Stoltz, 1995). This process has been shown to require free cell surfaces but at the same time a cohesion between cells. This precise location allows the application of mechanical forces in order to generate the FN matrix (Winklbauer, 1998). In *Xenopus*, a non-fibrillar matrix begins to form at the free surfaces of the cells in the BCR, a region rich in the so-called soluble FN (Lee, Hynes and Kirschner, 1984). Initially, puncta of FN assemble at the surfaces of the cells and this gives rise to the matrix which eventually will cross the cell boundaries and thicken over time. It has been shown that tension, integrins, FA proteins and cadherins have a role in the formation of this matrix, however their precise roles and contribution are still unclear (Kragtorp and Miller, 2006, Winklbauer and Stoltz, 1995; Cousin and Alfandari, 2004; DeSimone, Dzamba and Davidson, 2007; Dzamba *et al.*, 2009; Hunt and Schwarzbauer, 2009; Rozario *et al.*, 2009). Julich et al. showed that integrin α5β1 heterodimers are associated with each other on neighboring cells when integrins are in the closed, inactive conformation. N-cadherin was shown to be a major factor for the stabilization of this inactive form and the inhibition of FN fibril formation. Downregulation of N-cadherin resulted in activation of integrins and subsequent formation of FN matrix (Marsden and Douglas W DeSimone, 2003; Davidson *et al.*, 2006; Jülich *et al.*, 2015). These data suggest that the differential molecular interactions and differential strength of adhesion are what gives cadherins these different roles during FN matrix assembly. The first clues suggesting that the implication of cadherins and tension from tissues are necessary for this matrix assembly, derived from experiments perfomed by Dzamba et al. (Dzamba *et al.*, 2009). The role of cell tension, is characterized as a key point for the formation of FN matrix on stiff cell-substrate experiments, but the formation of the matrix *in vivo* was less understandable up until recently (Zhang *et al.*, 1993; Zhong, Kinch and Burridge, 1997; Cukierman *et al.*, 2001; Pankov and Yamada, 2002). Experiments by Dzamba et al. suggested that the non-canonical Wnt/PCP pathway has a role in this process (Dzamba *et al.*, 2009). This work, together with experiments performed in *Zebrafish* suggested a model for FN fibril formation, in which changes in cell-cell adhesion from cadherins, result in the reorganization of the actin cytoskeleton. This event, was shown to be dependent on Rac and Pak and found indispensable for the translocation of integrins to bound FN at cell-cell contact sites where the matrix formation is initiated. It was also proposed that AJs have a role similar to the role of FAs in this procedure and they generate tension on integrins, necessary to expose binding sites within FN (LaFlamme, Akiyama and Yamada, 1992; Dzamba *et al.*, 2009). However, further evidence for the precise mechanism in this process has not been revealed yet. Earlier experiments demonstrated the implication of other FA proteins during this process such as FAK and PTP-PEST phosphatase. FAK downregulation led to FN matrix assembly defects while overexpression of PTP-PEST phosphatase also affected FN matrix formation (Cousin and Alfandari, 2004; Kragtorp and Miller, 2006).

In conclusion, the FN matrix assembly is a complex process during development that remains under investigation and raises a range of questions regarding its mechanistic impact during this process. More recent evidence showed that FAK downregulation drives spindle misorientation at the outer-most epithelium of the BCR where integrin-FN interactions do not take place (Petridou and

Skourides, 2014). This, in combination with other evidence suggesting the formation of a complex composed of FA proteins and integrin β1 during spindle orientation that gets activated in a tension-dependent manner, indicate the potential involvement of such proteins in this process. Their role is most probably different than their traditional cell-adhesion role (Petridou and Skourides, 2016; Anastasiou, Hadjisavva and Skourides, 2020). Recent evidence, arising the last years, suggests a strong interplay between integrins and cadherins in different aspects of cellular functions and their mechanosensing and mechanotransduction abilities. This interplay, their mechanotransducing abilities, their association with similar downstream targets and their implication in this process by previous evidence may be the missing link on how the FN matrix is guided and provide a strong insight in the understanding of this crucial developmental process.

5.1.4 Integrins and cadherin crosstalk

Molecular crosstalk can be defined as the signaling communication between different pathways. Such communication allows cells to interact with either neighboring cells and/or distant components. These interactions result in the regulation of crucial events of synergistic or antagonistic nature. These events eventually regulate different biological outcomes. The ability of numerous molecular signaling pathways to interact between them has been studied extensively since the molecular crosstalk events take place in a wide variety of developmental and cellular processes. One of the most attractive examples of signaling crosstalk, that has been under investigation for decades, is the one between integrins and cadherins. Both of these pathways have crucial roles during development and tissue homeostasis. Defects in any of them during development result in embryonic lethality, diseases and cancer. As previously mentioned, integrins are the major receptors for cell-ECM interactions and cadherins are the major cell-cell interaction receptors. Both of these protein families are characterized by the ability to connect the cell to the actin cytoskeleton through numerous protein interactions and have been characterized as major players in the function of tissues and architectural development in both adult and embryonic organisms (Hynes, 2002; Wheelock and Johnson, 2003; Thiery *et al.*, 2009). A large amount of evidence suggests that there is a precise crosstalk between these two different adhesion systems. This crosstalk is believed to influence the positioning, turnover, expression and functions of the adhesion receptors of the two systems. However, the molecular mechanisms and the exact molecules involved in the integrin-cadherin crosstalk are not clear. This section discusses the most recent evidence regarding integrin-cadherin crosstalk, as well as the importance of understanding and unraveling the relationship between the two adhesion systems (**Figure 27**).

Integrins and cadherins form adhesion complexes either with the ECM or with adjacent cells, as components of focal adhesions and adherens junctions respectively. These complexes are known to facilitate the connection and remodeling of the actin cytoskeleton through the direct or indirect interaction of both adhesion molecules with the Rho family of GTPases. This engagement leads to the stimulation of these protein family members and leads to the actin cytoskeleton remodeling. (Parsons, 1996; Etienne-Manneville and Hall, 2002; Parsons, Horwitz and Schwartz, 2010; Watanabe *et al.*, 2010). The functions of the actin cytoskeleton are not limited to its structural abilities, but in combination with the myosin network forms an internal cell component responsible for generating tension in response to externally applied mechanical stimuli. These responses induce differential biochemical signals which eventually regulate fundamental biological processes such as cell proliferation, differentiation and migration, as well as tissue morphogenesis.

In addition to interactions with actin, two more types of interactions have been proposed in the integrin cadherin crosstalk. The first refers to a so-called long-range input-output signaling, where signals from the one type of adhesion system drives changes (either activation or deactivation) in the function of the other type of adhesion. For example, one type of this adhesion might drive changes

in membrane trafficking, cytoskeletal association and binding affinity of members of the other adhesion system (Avizienyte *et al.*, 2002). The second type of interaction refers to the association of the two adhesion systems at the membrane plane. In this case, different types of proteins, such as growth factors receptors or tetraspanins, facilitate interactions of both adhesion receptors; integrins and cadherins (Chattopadhyay *et al.*, 2003). These interactions may converge in a pathway and result in complex interactions (**Figure 27**).

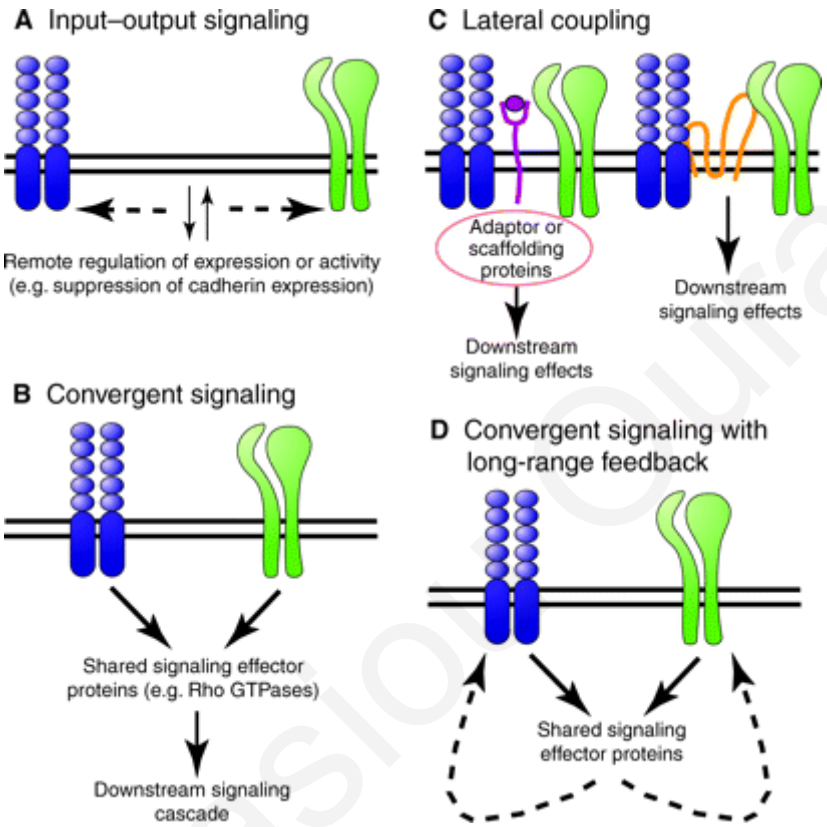


Figure 27: Types of adhesive interactions

(A) The mode of input-output signaling describes the interactions that emerge from one adhesion system and simultaneously affect the expression or activity of another adhesion system. (B) Signaling of both adhesion systems results in common downstream effector molecules like cytoskeletal components, kinases, and various adaptor proteins and it is known as convergent signaling. (C) Interaction of the two adhesion systems laterally on the cell membrane for signaling that does not include adhesion. (D) The combination of more than one of the previously mentioned interactions might result in a cross-talk between the two adhesion systems. Adapted from: (Weber, Bjerke and DeSimone, 2011)

5.1.4.1 Crosstalk through Rho GTPases

The interaction of both cadherins and integrins with Rho GTPases has been extensively studied in a crosstalk-independent context (**Figure 28**). It is well established that this protein family is essential for the assembly of FAs and AJs. For the assembly of AJs both Rac and Cdc42 are important and their increased activity has been associated with the disruption of AJs. Thus, indicating the requirement of tight regulation of these molecules (Zhong, Kinch and Burridge, 1997; Hall, 1998; Irie *et al.*, 2004; Takeichi, 2014). It is well established that the formation of AJs leads to increased Rac1 through its engagement, and simultaneously drives RhoA activity inhibition. It has been also described that the Rho family activation occurs through the formation of integrin adhesions. Moreover, Rho activation through integrin adhesion, above a certain threshold, has been proposed to have a downstream activity responsible for the regulation of AJs formation in epithelial cells (Zhong, Kinch and Burridge, 1997; Hall, 1998; Van Aelst and Symons, 2002). For example, in colon cancer cells it was suggested that activation of Rac1B through integrin signaling leads to the AJ formation (Chartier *et al.*, 2006). Additional pieces of evidence show that p190 Rho known as RhoGAP has an important role in FA-AJ crosstalk through different input-output pathways. RhoGAP has been shown to interact with p120 catenin which binds to cadherins and its overexpression has been associated with the inhibition of Rho subfamily locally, through the activation of Rac and Cdc42 expression (Wildenberg *et al.*, 2006; Bass *et al.*, 2008). RhoGAP is also activated through integrin adhesion and has been shown to regulate cadherin cell-cell adhesion in epithelial cells (Playford *et al.*, 2008). Additional evidence indicates the implication of this family of proteins in convergent signaling of integrins and cadherins. An example is, the implication of both adhesion systems in increased cell proliferation through increase in Rac1 expression, which consequently drives an increase in cyclin D1 expression (Fournier *et al.*, 2008). Apart from these, Rho-kinase (ROCK) and consequently Rho subfamily member proteins, have been shown to have different effects on both types of adhesions. ROCK is controlled by cell-ECM adhesion through the cell shape, and the tension applied on the cytoskeleton at those adhesion sites. This is achieved through a positive feedback loop, where tension applied to the cells from cell-ECM interplay drives ROCK activation and leads to the formation and/or maturation of FAs (Bhadriraju *et al.*, 2007) (**Figure 28**). At the same time, RhoA activation drives the disruption of AJs (Sahai and Marshall, 2002) through an increase in the actomyosin contractility. Other members of the Rho family have also been associated with the stabilization of AJs through the reorganization of the actin cytoskeleton (Van Aelst and Symons, 2002). Lastly, Cdc42 has been proposed to limit RhoA signaling as a response to the excessive application of tension on the cell. Thus, facilitating the AJs maintenance (Wildenberg *et al.*, 2006). Overall, a

plethora of studies show the implication of Rho family proteins in the integrin-cadherin crosstalk, effectively showing their communication.

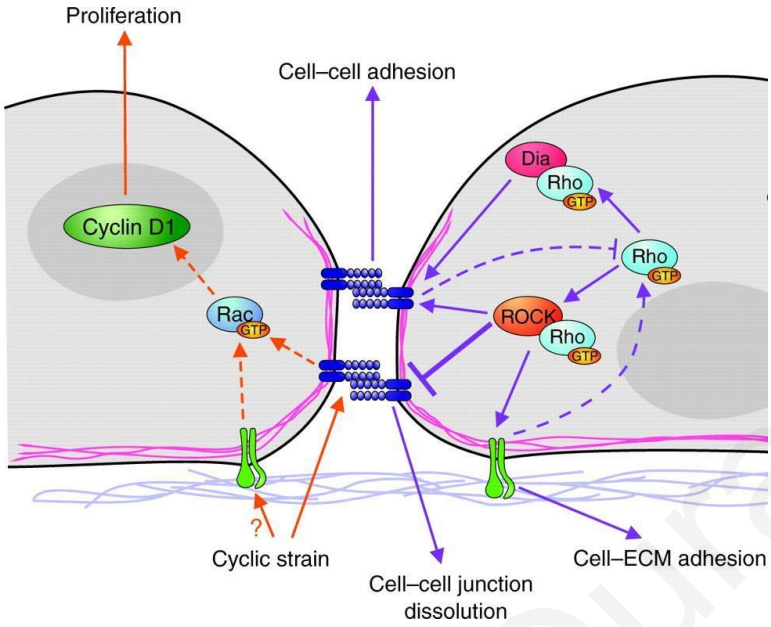


Figure 28: Evidence of crosstalk between cell-cell and cell-ECM adhesion systems with RhoGTPases.

Finally, Rac is activated by both cadherins and integrins and positively regulates proliferation, through an increase in cyclin D1 expression. The two receptors have an antagonistic influence in Rho GTPase activity and Rho. Cell-ECM adhesions are disrupted at high levels of Rho and ROCK actin contractility, while Rho signaling up to specific thresholds, drives actin cytoskeleton reorganization at cell-cell sites leading to their enhancement. The effects on the cell-ECM adhesion on Rho and ROCK are always towards the enhancement of the complexes. Adapted from: (Weber, Bjerke and DeSimone, 2011)

5.1.4.2 Crosstalk through tyrosine kinases, phosphatases and other adaptor proteins.

Several FA proteins or FA-linked proteins have been associated with the AJs and and vice versa (Gomez, McLachlan and Yap, 2011; Weber, Bjerke and DeSimone, 2011; Mui, Chen and Assoian, 2016; Giannone and Sheetz, 2006; McLachlan *et al.*, 2007; Langhe *et al.*, 2016). These pieces of evidence suggest that these two spatially discrete signaling pathways are probably not as distinct as initially believed.

Src is an important member of cell-ECM adhesion complexes, FAs. However, Src has been shown to localize at AJs in a number of different studies (Avizienyte *et al.*, 2002; McLachlan *et al.*, 2007; Gayrard *et al.*, 2018). Upon integrin activation, Src activation leads to a cascade of events including events that are associated with RhoGTPases (Huveneers and Danen, 2009). For example, in endothelial cells it was shown that the increased activation of Src at cell-ECM sites leads to the accumulation of phosphorylated myosin at those sites and the disruption of AJs. However, the moderate activation of Src and the contractility induced by ROCK, are necessary for the

strengthening of the AJs. These together highlight the significance of tight regulation of Src at both complexes on cell-cell and cell-ECM sites (Avizienyte *et al.*, 2004; Martinez-Rico *et al.*, 2010). At AJs, Src has also been found to be a downstream target of E-cadherin. The Src, cadherin driven, activation leads to a positive-feedback loop through PI3K signaling, which promotes cell-cell contacts. Aside these, this activation also increases the activity of Src and has negative results on AJs since it promotes their disruption. AJ complexes' disassembly is associated with the increased phosphorylation of myosin at the sites of cell-ECM contacts (McLachlan *et al.*, 2007). Several studies *in vitro* have shown that the interaction between Src and E-cadherin leads to the recruitment of ligases that in turn drive the degradation of E-cadherin and the weakening of adhesions. Other *in vitro* studies showed that the initial relaxation of E-cadherin by Src is associated with increased β-catenin activity (Riveline *et al.*, 2001; Fujita *et al.*, 2002). Later studies have also identified the ability of Src to phosphorylate AJ-localized β-catenin on Tyr 654 (Y654) and this phosphorylation was found to be associated with the unbinding of catenin from E-cadherin. This suggests that the only association between Src and AJs is through β-catenin at AJs (Gayrard *et al.*, 2018). Even though the precise function of Src at AJs is not clear at this point, it is well established that it has a role which is connected to its role in the FAs. All these together show that an interplay of proteins between these two complexes is taking place.

Another FA protein found to be implicated in both complexes is FAK which is a downstream effector of integrin activation and is involved in Src-mediated signaling (Playford *et al.*, 2008). In various experiments using colon cancer cells, it was observed inhibition of epithelial to mesenchymal transition of cells caused by TGF(β). This inhibition is associated with increased ECM components expression, increased engagement of integrins and increased FAK activation (Wang *et al.*, 2004) which promotes the expression of E-cadherin and cell-cell adhesion (Wang *et al.*, 2004; Yano *et al.*, 2004). This observation agrees with other experiments where FAK knockouts were used and showed a negative, FAK-dependent promotion of epithelial to mesenchymal transition. This inhibition is associated with problematic AJ formation and assembly (Yano *et al.*, 2004). More recent studies showed that FAK binds to VE-cadherin and phosphorylates β-catenin on Tyr 142 (Y142) in vascular endothelial growth factor-stimulated human umbilical vein endothelial cells (HUVECs) (Chen *et al.*, 2012). This interaction found associated with the disruption of the β-catenin/VE-cadherin complex and shown to led to decreased AJ stability and increased permeability of the cells. Further experiments using Blebbistatin, a cell contractility inhibitor, showed that the interaction between FAK and VE-cadherin was not affected, suggesting that factors other than tension are driving this interaction (Chen *et al.*, 2012). The connection between integrin-cadherin crosstalk and FAK has also been studied in other contexts, where force was implicated. Experiments using MEFs and Vascular Smooth Muscle Cells (VSMCs) cells on substrates of different stiffness showed that FAK and p130Cas are required for the entry of cells at the S-phase of the cell cycle. In addition, FAK and p130Cas found involved in driving Rac activation, which in turn triggered cell-cell adhesion pathways through increased N-cadherin expression (Bae *et al.*, 2014; Mui *et al.*, 2015). The authors

used micropatterned surfaces of different sizes where cells were able to spread up to a certain point and showed that FAK/p130Cas and Rac lead to the stimulation of N-cadherin. The above results suggest that this interaction determines the ECM-dependent spreading of the cells. These lead to the conclusion that all of the above-mentioned observations are required for the cell entry in S-phase and the cell proliferation (Chen *et al.*, 2012; Bae *et al.*, 2014).

Paxillin in combination with FAK has also been associated with the assembly of N-cadherin dependent junctions. Paxillin at those sites was shown to inhibit cell migration (Yano *et al.*, 2004). Experiments using paxillin siRNAs showed reduced recruitment of FAK at FAs of Hela cells, leading to decreased Rac activity and the formation of N-cadherin cell-cell adhesions (Bae *et al.*, 2014). Collectively these findings suggest that different FA proteins including paxillin, FAK and p130Cas can differentially regulate N-cadherin function in different contexts.

Vinculin is the most well-characterized protein, crucial for both cell-cell and cell-ECM adhesions. Vinculin is found in an autoinhibited form as discussed in general introduction section, with its head domain bound to its tail domain. At FAs, the vinculin head domain is interacting with talin while the tail domain with actin (Isenberg, Leonard and Brigitte M Jockusch, 1982; Riveline *et al.*, 2001; Li, Lee and Zhu, 2016; Verdanova *et al.*, 2017). These interactions control the ability of vinculin to bear forces applied at FAs and to regulate their assembly and disassembly (Grashoff *et al.*, 2010; Weber, Bjerke and DeSimone, 2011; Leerberg *et al.*, 2014; Mui, Chen and Assoian, 2016). On the other hand, at AJs, vinculin is bound to a cryptic site of α-catenin under conditions where the tension is high, leading to the reinforcement of AJs (Weber, Bjerke and DeSimone, 2011; Mui, Chen and Assoian, 2016; Yonemura, 2017). The role of vinculin at the different complexes has been shown to be controlled by phosphorylations on different Tyrosine residues. For example, at AJs upon force exertion on E-cadherin, the phosphorylation of vinculin on Y822 is increased and this leads to the ability of vinculin to associate with a number of proteins at those sites (Bays *et al.*, 2014). In contrast, it has been shown that at FAs Src phosphorylates vinculin on Y100 and Y1065 and this, in combination with other vinculin interactions, regulates the transition of force from ECM to FAs and the actin cytoskeleton (Auernheimer and Goldmann, 2014; Bays *et al.*, 2014). In combination, these findings suggest that the spatial regulation of vinculin phosphorylation is an important parameter that separates the function of vinculin at AJs and FAs.

Fer is another protein implicated in both FAs and AJs. Fer is an actin-organizing protein which interacts with contractin (Arregui *et al.*, 2000). This protein has been proposed to be required for cell spreading on FN through the activation of contractin. It has also been shown to drive cell motility. Activated contractin localizes at AJs upon E-cadherin engagement and this activation is a result of Src and/or Fer interactions with Fer (El Sayegh *et al.*, 2005; Sangrar *et al.*, 2007). These studies suggest that the actin reorganization, promoted by contraction through Fer, is implicated both in cell-cell and cell-ECM adhesions.

Other proteins such as Elmo and DOCK have also been associated with both complexes through experiments performed in MDCK cells (Toret, Collins and Nelson, 2014). These proteins are known FA proteins that colocalize at those sites upon Rac activation. Both Emo and DOCK drive actin reorganization. This reorganization is responsible for the recruitment of E-cadherin at cell-cell adhesions, suggesting a role for these proteins in the maturation of AJs.

Finally, studies by Oldenburg et al. using HUVEC and MDCK cells have unraveled the implication of additional FA proteins like zyxin, Tes and VASP at AJs (Oldenburg *et al.*, 2015). These proteins act as actin regulators at FAs and the study clearly states that their implication in AJs is independent of the connection of vinculin and α-catenin with the actin cytoskeleton (Oldenburg *et al.*, 2015). The study also proposes that the localization of these proteins at AJs is achieved through an unidentified mechanosensitive module which takes place at those sites.

A number of studies demonstrated that several phosphatases are implicated in the cadherin-integrin crosstalk. PTP1B, a member of the family of protein tyrosine phosphatases (PTP), has been shown to localize at both FAs and AJs. This is accomplished via an interaction of PTP1B with the cytoplasmic tail of cadherins at AJs and through dephosphorylation of Src at FAs. These interactions were found to affect the stability of both complexes (Balsamo *et al.*, 1998; Arregui *et al.*, 2000; Stoker, 2005; Sallee, Wittchen and Burridge, 2006). Another member of this family, PTPμ, has been found associated with a complex between integrin α3β1 and tatraspanin. These complexes have been also been proposed to be associated with E-cadherin (Chattopadhyay *et al.*, 2003). However, even though both of these phosphatases have a crucial role in later interactions taking place at FAs or AJs, their precise implication and role are still unknown. Aside these, numerous other adaptor proteins have been identified at both complexes including RACK1, a scaffolding protein implicated in the α3β1 integrin, tatraspanin and E-cadherin lateral signaling mentioned above.

Pkg is a protein known to connect desmosomes with intermediate filaments and has been shown to have a role in tissue integrity at the AJs (Besson, Wilson and Yong, 2002; Chattopadhyay *et al.*, 2003). Studies in keratinocytes have identified that this protein is a target of Src activity and acts in the FN expression through Src inhibition (Besson, Wilson and Yong, 2002; Chattopadhyay *et al.*, 2003). As described above, several proteins with known functions at FAs have been associated with integrin-cadherin crosstalk through signaling pathways that are not yet fully understood. Some reports identify cadherins as regulators of integrin signaling. Work performed in *Xenopus* embryos showed an interaction between FAs and cadherin-11. Cadherin-11 found co-localize with integrin β1 and paxillin and found to interact directly with syndecan 4, an FN binding protein. This study unraveled a novel role of a cadherin family protein at the FAs and provided further evidence regarding the direct interaction between proteins of FAs and AJs (Langhe *et al.*, 2016).

5.1.5 The connection between integrin-cadherin crosstalk and the actin cytoskeleton and regulation of mechanotransduction.

Different cellular processes result in the production of mechanical forces. These are exerted at the same time on cell-cell and cell-ECM contacts. The variation in force application across the cell results in an alternation of the balance of the tension across these adhesion systems. The balance of tension and the so-called tension homeostasis are important for adhesion organization and maintenance or remodeling of the cell. A number of studies suggested that the integrin-cadherin systems act antagonistically in terms of force application. For example, Wang et al. and Yano et al. demonstrated that FAK activation downstream of integrin engagement leads to loss of VE-cadherin (Yano *et al.*, 2004; Discher, Janmey and Wang, 2005; Wang *et al.*, 2006). On the other hand, experiments on soft substrates, where connection to the ECM is unstable and FAs are small, revealed enhanced cell aggregation and compaction (Guo *et al.*, 2006). However, the suggestion that the two adhesion systems act antagonistically is too simplistic. Especially if we take into consideration the different types of interactions between these systems. The interactions of the two adhesion systems may act synergistically or independently from each other in order to achieve the tensional homeostasis. Using an inducible endothelial-specific integrin β1 knockout mouse model tamoxifen-inducible Cre strain, Yamamoto 2015 showed that endothelial integrin β1 leads to suppressed endothelial proliferation, stabilization of cell junctions and regulation of VE-Cadherin trafficking (Yamamoto *et al.*, 2015). This study also showed that integrin β1 controls phospho-MLC (p-MLC) levels, endothelial actomyosin contractility and thereby VE-cadherin localization at cell-cell contacts through the Rap1/MRCK and Rho/Rho-kinase pathways, which have partially redundant roles in the regulation of vessel wall integrity. Defects in these processes have been shown to drive junctional defects and lead to leaky and unstable blood vessels (Yamamoto *et al.*, 2015). Studies by Ouyand et al. and others examined how cadherins and integrins regulate their mutual distribution in the cells and how they establish polarized signaling pathways by distinct molecular components. They created FN-coated micropatterned strips to investigate the PI3K and Rac signaling at the free tip versus the tip connected to another neighboring cell (Ouyang *et al.*, 2013). They observed that the P13K and Rac activities were stimulated by integrin at the cell-free end, while N-cadherin and p120 catenin excluded α5β1 from junctions. This showed to drive the suppression of P13K and Rac (Ouyang *et al.*, 2013). Under these conditions, it was also shown that the Myosin II light chain and actin filaments are locally associated with the cell-cell junctions. Data from ectopic expression of mutant forms of N-cadherin and p120-catenin showed that the myosin II light chain and actin filaments localize at these areas where they are regulated by catenin and N-cadherin. Nevertheless, key questions, in terms of how cell organization, and eventually the positioning of the adhesion systems, affects the tension between cell-ECM and cell-cell junctions and drives differential signaling, remain to be answered (Ouyang *et al.*, 2013). It has been also shown by Mertz et al. that the interaction between integrins and cadherins

can also direct the localization of forces within cell aggregates. They cultured keratinocytes on 2D FN-coated silicone gels and incubated the cells at low and high Ca^{2+} concentration to preclude or support cadherin adhesion respectively. High Ca^{2+} led to cell aggregates and traction stresses at the periphery of cells while they had an inward alignment. In contrast, Ca^{2+} prevented cadherin adhesion and led to an evenly distributed actin cytoskeleton. Taking advantage of function-blocking antibodies, the authors also observed that the effects of high Ca^{2+} concentration were mediated by E-cadherin which showed to control the localization of F-actin and traction forces (Mertz *et al.*, 2013). However, how cadherin-mediated stress is translated into a biochemical outcome is still unknown. In the same concept, using embryonic stem cells human (hESCs) on micropatterned matrigel islands of different geometries, Toh et al. showed that the adhesion geometry regulates the distribution of cadherins and integrins in this context. Under these conditions, they observed a heterogeneous distribution of myosin light chain and actomyosin tension which resulted in spatial restricted differentiation of colonies into mesodermal tissue (Toh, Xing and Yu, 2015). Overall, the association of activated myosin II with integrins and areas of high tension was found to drive mesendoderm differentiation while its association with cadherins and areas of low tension was found to drive the maintenance of pluripotency. These observations have led to the conclusion that the spatial polarization of integrins and cadherins creates a variation in mechanical force application throughout the cells, which drives heterogeneity during stem cell differentiation (Toh, Xing and Yu, 2015). Work by Danuser et al. using a computational model and *in vitro* approaches (such as force application) to the cells using beads, quantified force transition within multicellular clusters for the first time. They suggested that like in two-cell systems, in cell clusters, the total amount of force application on cells has to be zero. They showed that the force distribution at cell-cell junctions is dynamic and alters, based on variations observed locally at cell-ECM adhesion and actomyosin contractility (Ng *et al.*, 2014). Taking together these studies, we can conclude that a direct communication between integrins and cadherins is taking place as a result of a shift in actomyosin contractility which was shown to determine the organization of biochemical and mechanical signals at the cell and tissue level.

5.1.6 Implication of the integrin-cadherin crosstalk in cellular and developmental processes.

Almost all crucial cellular and developmental processes have been associated with the function of integrins and/or cadherins. These include cell proliferation, migration, differentiation and apoptosis. Most of the studies described in the previous sections of this thesis have been performed *in vitro,* however a number of studies using *in vivo* models provided further insights into the physiological relevance and importance of these interactions during critical cellular processes and during development (Weber, Bjerke and DeSimone, 2011; Mui, Chen and Assoian, 2016, Fouquet *et al.*, 2004; Kang *et al.*, 2007).

- Cell Migration

  This process is a result of a complicated and coordinated complex of mechanical and biochemical signaling events including the spatial and temporal regulation of the AJs and FAs and tension generation intracellularly. Migration is achieved through a mechanism of distributed traction during which the leading-edge of cells and the ones that follow become polar and migrate towards a specific direction (Weber, Bjerke and DeSimone, 2011; Mui, Chen and Assoian, 2016). Initial experiments on ovarian carcinoma cells showed that for cell migration, activation of integrins through binding with collagen is required. This activation was found to be the result of E-cadherin downregulation through metalloproteinases showing the antagonistic effect of the two adhesion systems in migration (Desai *et al.*, 2009; Borghi *et al.*, 2010). However, more recent studies using physiological conditions and not carcinoma cells identified that there was a communication of AJs and FAs during migration through mechanical stress application. Work by Liu et al. has shown that the increased endogenous stress between cells leads to increased AJs size (Liu *et al.*, 2008). Work by Maruthamuthu et al. showed that increased traction from ECM also has effects on AJs, since they observed proportional increase in tension at cell-cell adhesions during this process (Maruthamuthu *et al.*, 2011). Work by another group tried to identify the precise role of cadherin adhesions in cell polarity regulation during directed cell migration (Desai *et al.*, 2009). Desai et al. used micropatterned substrates where they performed scrape wounds and they observed that after 4 hours, cells had their centrosomes oriented close to the nucleus and away from cell-cell contacts which was followed by increased protrusive and migratory activity. Then, using E-cadherin mutants, they showed that this process is directed by E-cadherin through Cdc42 signaling and the actin cytoskeleton. The cell-cell adhesion sites displayed suppressed protrusive activity in contrast to the sites of cell-ECM adhesions.

  The polarization of E-cadherin and actomyosin contractility are also present in collective migration of border cells in *Drosophila* ovary (Desai *et al.*, 2009). The authors here created an E-cadherin FRET sensor in an attempt to directly investigate the close relation between

cadherins and tension. They observed that E-cadherin is under high tension at the front of migrating border cells and this tension directs Rac1 localization at the front of the cells. This was shown to enhance tension at E-cadherin adhesions at cell-cell borders. Rac activity is also a major target of integrin signaling. Interactions of the cells with their substrate are crucial for migration and lead to alterations in Rac activity. This could presumably mean that both cell-cell and cell-ECM receptors help to integrate Rac signals to direct migration (Desai *et al.*, 2009; Cai *et al.*, 2014).

During development, cell migration is a crucial process for morphogenesis in which both cadherins and integrins have been found crucial. Experiments in cranial neural crest cells showed that N-cadherin adhesions are a prerequisite for cell migration since they target the localization of Rho-GTPases. Knock-out experiments of N-cadherin further supported this hypothesis since downregulation of N-cadherin in these cells led to the inhibition of migration (Theveneau *et al.*, 2013). Moreover, experiments in placode cells also showed that an asymmetric force distribution on these cells was resulting in a dramatic reduction of FA distribution in an N-cadherin dependent manner. These experiments showed that N-cadherin contacts inhibit protrusion formation and control the direction of their migration (Breau and Schneider-Maunoury, 2015). Finally, experiments in *Xenopus* embryos during gastrulation showed that cadherin adhesions are formed in response to FA-generated signals. Knock-down experiments using MOs against FAK showed that the actin cytoskeleton was disrupted, the spatial distribution of keratin was altered and the binding of plakoglobin to C-cadherin was dramatically decreased (Weber, Bjerke and DeSimone, 2011). These led to delayed cell migration due to impaired cell spreading and impaired generation of forces which resulted in disrupted mesendoderm tissue polarity. Collectively, these studies underline the importance of integrin-cadherin crosstalk during cell migration. They suggest that cadherin organization is what guides collective cell movement, while FAs control the degree of traction with an overall effect in spatial force distribution control and guidance of migration.

- Endothelial cell biology

Blood vessels are composed of vascular endothelial cells and are known to be exposed to different types of mechanical stress such as cyclic strain, hydrostatic pressure and shear stress (Weber, Bjerke and DeSimone, 2011). Vascular endothelial cells are tightly connected to each other and their external matrix creates a barrier against the bloodstream, which enables or prohibits molecule exchange. Work from different groups showed that the forces applied to vessels are partially transduced by adhesion complexes. Work by Schwartz et al. elegantly showed that VE-cadherin, VEGFR and PECAM1 compose a mechanosensory complex that has been shown to activate integrins in response to fluid shear in endothelial cells. These experiments included the use of tension sensors for VE-cadherin and PECAM1

and VE-chimeric proteins. The results revealed a role of VE-cadherin as an adaptor protein between PECAM and VEGFR, which drives the activation of P13K and mediates integrin activation. This process is partially coordinated by Shc adaptor protein and was shown to lead to the regulation of cell alignment in the direction of shear stress.

Another study by Coon et al, using magnetic twisting cytometry on endothelial cells, showed that force on VE-cadherin drives the recruitment of F-actin and vinculin. This leads to cell stiffening, as a result of ROCK activation, driving alterations in cellular traction forces. These studies suggest that force activation of VE-cadherin triggers the downstream activation of PI3K and this leads to effects on integrin activation (Coon *et al.*, 2015; Moissoglu and Schwartz, 2006). Moreover, they suggest that different downstream effects can emerge in response to fluid shear stress and twisting forces. This proposes that cells can recognize different types of forces. However, how these mechanical stimuli are created, are translated and transduced from cadherin complexes to integrin complexes is still unknown.

- Early embryonic morphogenesis

  An important process during vertebrate development is the formation of the FN matrix at the blastocoel roof plate (BCR). Studies in *Xenopus* and *Zebrafish* embryos showed that cadherins interact with integrins through the organization of integrin ligands. Experiments in *Xenopus* embryos showed that the major cadherins at this stage are E and C-cadherin. These two lead to increased mechanical tension which drives the promotion of the FN matrix assembly, a crucial procedure during *Xenopus* morphogenesis (Weber, Bjerke and DeSimone, 2011; Mui, Chen and Assoian, 2016). Another study in *Zebrafish*, using fluorescence cross-correlation spectroscopy, aimed to identify specific protein-protein interactions between these two complexes. The formation of the FN matrix during development is an important process which is responsible for body elongation and segmentation. Additional pieces of evidence suggest that the PCP pathway is involved in the formation of the FN matrix by regulating the adhesion of cadherins and the tension generated in the tissue. It has been also proposed that PCP is probably the pathway is the link between cadherins and integrins in the embryo (Marsden and Douglas W DeSimone, 2003; Dzamba *et al.*, 2009). As Dzamba et al. elegantly showed, cadherins regulate integrin-dependent deposition and the assembly of ECM through noncanonical Wnt signaling. Wnt/PCP signaling was shown to increase cell-cell adhesion and tension applied to the ectoderm (responsible for the spatial deposition of FN at free cell surfaces) (Dzamba *et al.*, 2009). A recent study has identified the molecular mechanisms through which β-Parvin interacts with both cadherins and integrins during *Xenopus* gastrulation. Parvin is a scaffolding molecule known to localize at both cell-cell and cell-ECM adhesions. Disruption of interactions of two distinct motifs of Parvin, known to interact with FN and cadherins differently, resulted in disruption of cell intercalation taking place during epiboly and convergent extension. These

experiments suggest a role of Parvin in both cadherin and integrin adhesion (Knapp, 2019). Besides, it has been proposed by Marsden and Desimone. and Davidson et al. that FN is required during convergence extension where, the mesodermal cells are radically intercalated, form the notochord and guide the elongation of the embryo (Marsden and DeSimone, 2003; Davidson *et al.*, 2006). This was shown using different approaches for the inhibition of integrin α5β1 and alterations in cadherin adhesion. It was proposed that integrin α5β1 signaling alters cadherin adhesion during cell sorting behaviors and cell intercalation but the precise signaling mechanisms between the two systems is still unclear (Marsden and DeSimone, 2003; Dzamba *et al.*, 2009).

Evidence suggests that a crosstalk between cadherins and integrins takes place in crucial morphogenetic movements and embryonic development. It is also well established that both cell-cell and cell-ECM adhesions are of high importance throughout development. However, the mechanisms through which these two families of receptors communicate between each other and their precise synergistic or antagonistic roles during these processes remain highly unknown.

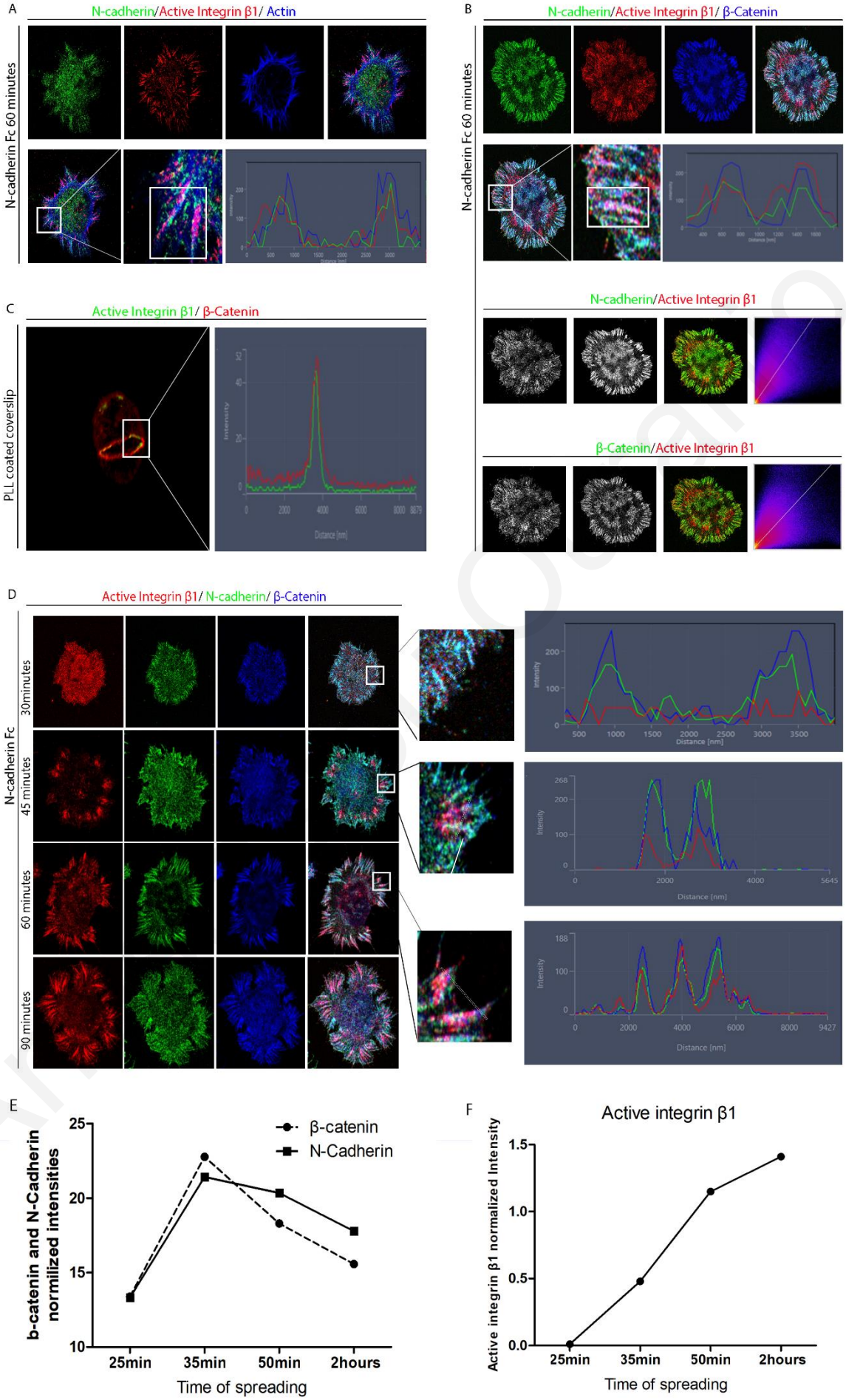
5.2 Results Chapter II: The spatial distribution of AJ formation and clustering leads to tension-driven activation of integrins and guides extracellular matrix deposition topology.

5.2.1 Integrin β1 activation is guided spatially by adherens junction formation.

It has been shown that adherent non-polarized cells attached on N-cadherin Fc substrates generate FA-like linear AJs which are associated with actin stress fibers (Gavard, Lambert, Grosheva, Marthiens, Irinopoulou, J. F. Riou, *et al.*, 2004; Craig T Lefort, Wojciechowski and Hocking, 2011; de Rooij, 2014; Anastasiou, Hadjisavva and Skourides, 2020). As discussed at the previous section after about 30 minutes, these structures display no visible FA formation and no detectable integrin activation (Anastasiou, Hadjisavva and Skourides, 2020). When we allowed cells to remain attached on these substrates for longer than 60 minutes, integrin β1 activation was detected at the ventral cell surface even in the absence of serum. Surprisingly, this integrin activation, observed as clusters, coincided spatially with linear AJs and both of them are associated with actin bundles, suggesting that integrin activation and clustering is spatially guided by the formation of AJs (**Figure 29 A, B**). The majority of cells under these conditions display integrin activation along AJs at the 60-minute interval; however, not all linear AJs of a cell display activation simultaneously. Some AJs display integrin activation earlier while others are doing so at later time points (**Figure 29 B**). The formation of these linear AJs on glass cadherin substrates could be an artifact due to the extremely high stiffness of the substrate or due to the high affinity for protein of the silanized glass. Thereafter, in order to examine the activation of integrin on these structures under more physiological conditions, we used cell doublets where cell-cell contacts are observed. It has been previously shown that cell doublets form in a stepwise fashion which involves the attachment of the two cells and the generation of small scattered cadherin puncta at the cell-cell contact area. These puncta migrate radially and become clustered at the rim of the contact area in an actomyosin driven process (Chu *et al.*, 2004; de Rooij, 2014). The clustering of neighboring cadherins at the rim of the cell doublet has been shown to create a circular AJ with both actin and cadherins absent from the center of the cell-cell contact region. Similar circular AJs were formed using PLL-impregnated polyacrylamide gels where cells are unable to attach, thus reinforcing cell-cell interaction. We mechanically disrupted cells and allowed them to seed on PLL-gels under serum-free conditions. As shown in Figure 28 C, circular AJ formation is observed at the rim of the cell doublets. In an attempt to examine whether activated integrin β1 is observed along AJs under these conditions, we allowed cells to attach for 15 minutes, fixed them and stained using antibodies against activated integrin β1 (**Figure 29 C)**. Activation of integrin β1 is clearly displayed at the centered ring, formed by the fusion of the neighboring cells. This suggests that the formation of AJs in cell doublets elicits integrin activation which spatially coincides with AJs and this observation is identical to what we observed in cells attached on N-cadherin Fc-coated

glass surfaces (**Figure 29 C**). This shows that the guided integrin β1 activation at the sites of AJs is not an artifact of the cells attached on N-cadherin Fc on silanized glass surfaces (**Figure 29 C**).

Integrins and cadherins are the two major adhesion systems in multicellular organisms. The first connects the cell with the ECM while the second, connects neighboring cells between them. These receptors display a clear spatial segregation both in cultured cells as well as tissues and even though the crosstalk between them has been studied for many years. The evidence so far suggest that their interactions are indirect through their common connection with the actin cytoskeleton (Weber, Bjerke and DeSimone, 2011; Tseng *et al.*, 2012; DeMali, Sun and Bui, 2014; Mui, Chen and Assoian, 2016). In contrast to what has already been known, the above data suggest that these two adhesion receptors have a much closer association. In an attempt to unravel a potential direct interaction, we decided to investigate the temporal aspect of integrin activation at the sites of AJs. In order to do that, we seeded Hela cells on N-cadherin Fc substrates and allowed them to spread under serum-free conditions. Cells were fixed at different time points (30 minutes, 45 minutes, 60 minutes and 90 minutes after seeding) and stained against active integrin β1, N-cadherin and β-catenin. As shown in Figure 28 D, E, F, N-cadherin and β-catenin positive AJs are observed at the 30-minute interval. However, no integrin β1 activation is observed at this time point. At the 45-minute interval, small clusters of active integrin β1 are observed at the vicinity of the AJs, which however are not colocalized fully with AJs. At 60 minutes, the majority of cells display colocalization of active integrin β1 with both N-cadherin and β-catenin at the sites of AJs, suggesting that the initial integrin β1 activation occurs at the surrounding areas of AJs and then become clustered along AJs. Finally, at the 90-minute interval, the majority of cells display clear integrin β1 activation along AJs which spatially coincides with N-cadherin and β-catenin (**Figure 29 D, E, F**). Collectively, these results suggest that the topology of integrin β1 activation at those sites is determined by the spatial distribution of AJs.


A
N-cadherin/Active Integrin β1/ Actin
N-cadherin Fc 60 minutes
B
N-cadherin/Active Integrin β1/ β-Catenin
N-cadherin Fc 60 minutes
N-cadherin/Active Integrin β1
β-Catenin/Active Integrin β1
C
Active Integrin β1/ β-Catenin
PLL coated coverslip
Intensity
Distance [nm]
D
Active Integrin β1/ N-cadherin/ β-Catenin
N-cadherin Fc
30minutes
45 minutes
60 minutes
90 minutes
Intensity
Distance [nm]

E
β-catenin
N-Cadherin
b-catenin and N-Cadherin normilized intensities
25
20
15
10
25min
35min
50min
2hours
Time of spreading

F
Active integrin β1
Active integrin β1 normalized Intensity
1.5
1.0
0.5
0.0
25min
35min
50min
2hours
Time of spreading

Figure 29: Activation of integrin β1 is spatially guided by the formation of AJs.

A) Representative image using confocal microscopy of Hela cells attached to N-cadherin Fc for 60 minutes and intensity profile showing the connection of AJs with the actin cytoskeleton. Cells are stained with β-catenin, active integrin β1, and actin antibodies. **B**) Confocal representative image of Hela cells attached to N-cadherin Fc for 60 minutes and stained for β-catenin, active integrin β1 and N-cadherin and intensity profile showing the co-localization of these receptors and β-catenin at the sites where linear AJs are formed. Co-localization images of β-catenin with active integrin β1 and N-cadherin with active integrin β1 showing the spatial relation of these proteins. **C**) Confocal 3D reconstruction of Hela cells forming cell doublets on polyacrylamide gels fused with PLL, stained with β-catenin and active integrin β1 and intensity profile showing the co-localization of these proteins at the sites of the central ring during doublet formation. **D**) Representative images of Hela cells on N-cadherin Fc at different time-points. Cells are stained with antibodies against N-cadherin, β-catenin and active integrin β1 and intensity profiles show the co-localization of these proteins at AJ sites. **E-F**) Graphical representation of normalized intensities of N-cadherin, β-catenin and active integrin β1 over time.

5.2.2 AJ-guided integrin β1 activation which is guided by the AJs leads to the formation of Hybrid Adhesions (HAs).

It has been established that integrin β1 becomes activated along AJs after the clustering of cadherins. These receptors were found colocalized at those sites and have been shown to share topological profiles. This raised questions regarding the precise relationship of the two types of receptors at the individual adhesions in terms of spatial and temporal interactions. Especially if we consider that the activation of integrins is the first step in the establishment of the contacts of cells with the ECM (Horton *et al.*, 2016; Green and Brown, 2019a; Humphries *et al.*, 2019). The interactions of integrins with the ECM drives the recruitment of additional FA proteins at the sites where the cell connects with the ECM. The proteins that are recruited at FAs act either as signaling and scaffolding molecules or act as connections of the integrin-adhesion sites with the actin cytoskeleton (Horton *et al.*, 2016; Baade *et al.*, 2019; Green and Brown, 2019a; Humphries *et al.*, 2019; Kechagia, Ivaska and Roca-Cusachs, 2019). A number of these proteins, have also been shown to associate with AJs and have been found involved in the stabilization, disassembly or/and actin cytoskeleton connections take place at these sites (Martinez-Rico *et al.*, 2010; Weber, Bjerke and DeSimone, 2011; Chen *et al.*, 2012). The fact that the recruitment of these proteins at FAs occurs downstream of integrin activation, raised the question if FA protein recruitment occurs at the AJs too.

In order to investigate this, we initially performed experiments using high-resolution confocal imaging in conjunction with deconvolution which allowed us to get a better understanding of the spatial distribution of integrin and cadherin receptors along the colocalization sites. Hela cells were allowed to attach and spread on N-cadherin Fc for 60 minutes and stained with antibodies for integrin β1 and β-catenin (since N-cadherin staining and β-catenin were shown to be identical). To increase the lateral resolution of the images we used confocal microscopy with sub-1AU pinhole and deconvolution using theoretical PSF. What we observed was that the two receptors are in fact spatially segregated within each AJ and display different topologies. As shown in Figure 29, the two receptors were found either mixed on the same linear AJ, integrin activation was observed either at the end or at the beginning of the AJs (labelled with b catenin), integrin activation was observed parallel to the AJs and finally integrin activation was observed at both sites of the linear AJs. These experiments revealed that different types of adhesions between integrin and cadherin exist. These different types are characterized by a distinct spatial relationship between the two adhesion receptors which however display mutually exclusive topology in each case. This suggests that integrin activation guides the recruitment of other proteins at those sites and there is a possibility that this activation is involved in the turnover of the AJs (**Figure 30**).

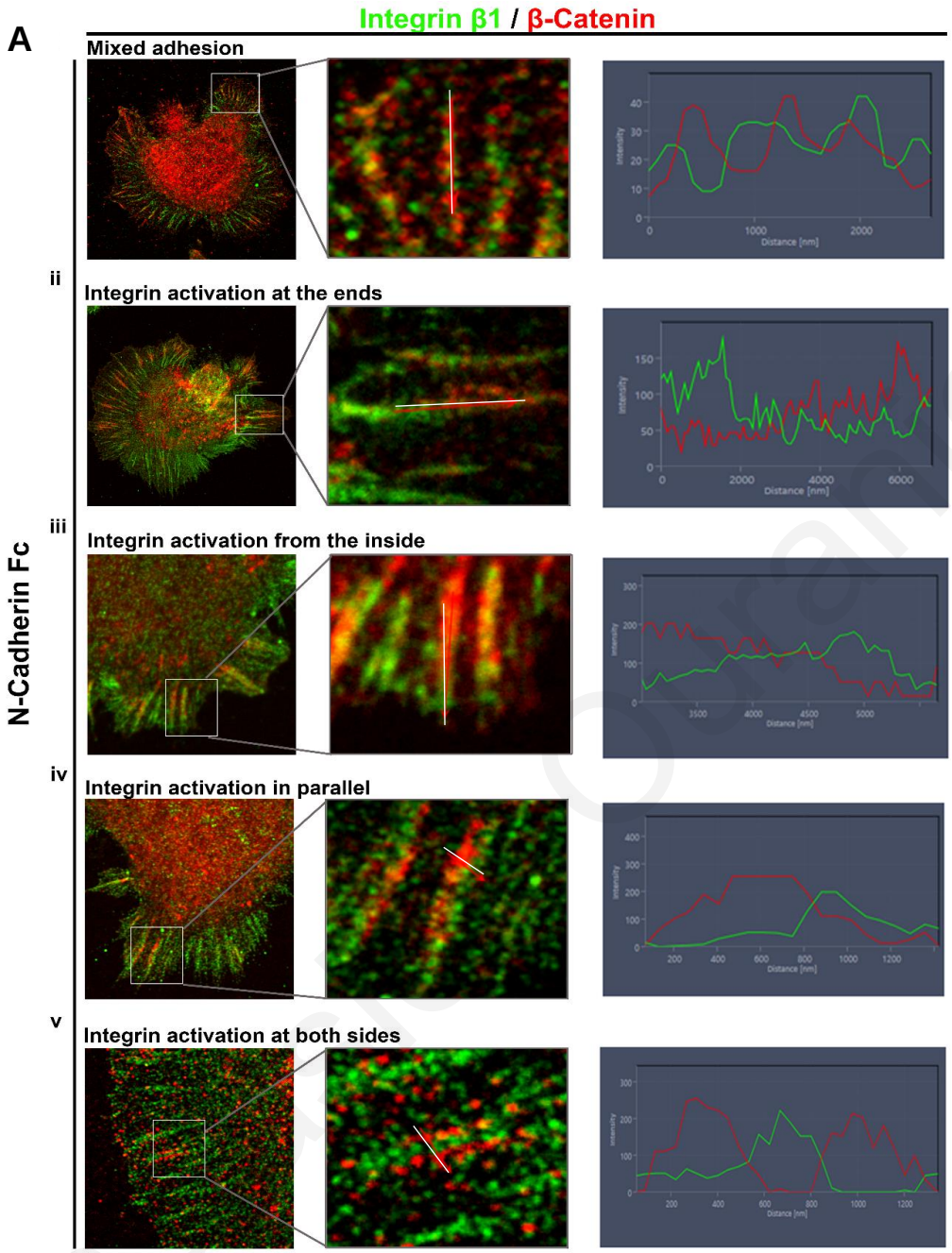


Figure 30: Adherens Junctions' driven activation of integrin β1 is spatially segregated from the AJs components within individual adhesions.

A) (**i-v**) High-resolution confocal images using deconvolution algorithms of Hela cells seeded on N-cadherin Fc for 60 minutes and profiles drawn either parallel or perpendicular to the linear adhesion showing the spatial relationship of two proteins. Cells are stained with antibodies against β-catenin and active integrin β1. The different spatial relationships of β-catenin and active integrin β1 along the same adhesion are as shown **i.** integrin becomes activated and appears mixed within the adhesion with β-catenin, **ii**. Integrin activation is observed at the points where the linear adhesions terminate, **iii.** Integrin activation at the inside of the cell where adhesion begins to form, **iv**. Integrin activation appears side to side with β-catenin and **v**. integrin activation gets activated parallel to the adhesion at both sides. (Figure adapted from Master Thesis, Rania Hadjisavva)

Talin is a major protein member of the integrin adhesome. It is recruited at the cell-ECM adhesion sites directly after integrin activation, interacts with integrin cytoplasmic tails and promotes integrin activation (Zhang *et al.*, 2008; Anthis and Campbell, 2011; Atherton *et al.*, 2015; Li, Lee and Zhu, 2016; Verdanova *et al.*, 2017). Talin recruits vinculin at FAs (Arold, Hoellerer and Noble, 2002; Calderwood, Campbell and Critchley, 2013; Atherton *et al.*, 2015; Kumar *et al.*, 2016; Verdanova *et al.*, 2017) and considering the fact that vinculin is a major member of the AJ complex raises the possibility that talin may also be recruited at AJs. Tensin is another member of the mature FA complexes involved in their connection to the actin cytoskeleton. It is composed of an SH2 domain which has been shown to interact with several FA proteins and has been reported to interact with integrin cytoplasmic tails (Lo, 2004; Stutchbury *et al.*, 2017). Studies have shown that this protein is recruited at the sites of the AJs however its precise role in these complexes remains highly unknown (Craig T Lefort, Wojciechowski and Hocking, 2011; Oldenburg *et al.*, 2015). Additional proteins with important roles at FAs are Paxillin and FAK. Both of them are known to be recruited at the FAs upon integrin activation and their targeting drives the downstream activation of other FA proteins. Both of these proteins have been reported to localize at the AJs. Specifically, FAK has been shown to interact directly with VE-cadherin and to affect the stabilization of AJs, while paxillin, in association with FAK, has been reported to promote N-cadherin based AJs formation and stabilization (Wang *et al.*, 2006, 2019; Chen *et al.*, 2012; Tabdili *et al.*, 2012; Theodosiou *et al.*, 2016). The observation of all these proteins at AJs and their well characterized role at FAs raised the question whether they also localize at AJs under these conditions. Hela cells were allowed to seed and spread on N-cadherin Fc substrates for 30 and 60 minutes, the timepoints where AJs are devoid of integrin activation and the timepoint during which clear integrin activation is observed at the AJs. Cells were stained against integrin β1, β-catenin and different FA proteins (Talin, Vinculin, paxillin, FAK and Tensin). All of these proteins are shown to localize at the AJs, however, careful examination of their topology at those sites, through intensity profiles, revealed differences between them. FAK and paxillin are found on these sites upon integrin activation while proteins like talin, vinculin and tensin behave in a similar manner to active integrin β1 since they were found to be enriched on all integrin β1 positive AJs (**Figure 31**). Colocalization co-efficient quantification of all the proteins also revealed that all the proteins, besides Vinculin, display higher co-localization with integrin β1 than with β-catenin, suggesting that they probably require integrin β1 activation for their recruitment at these sites. Previous evidence showing that proteins like FAK and tensin are recruited at AJs, may suggest that their recruitment is a downstream effect of integrin activation which had not been examined (**Figure 31**) (Oldenburg *et al.*, 2015). Overall, these results suggest that upon integrin activation at AJs, several FA proteins are recruited forming the so-called Hybrid Adhesions (HAs). This complex is characterized by similarities regarding the recruitment of FA proteins, however proteins like tensin, known to be a member of mature FAs, are found simultaneously with integrin activation at those sites suggesting that the formation of this complex is not identical to the one observed at the sites of cell-ECM connection.

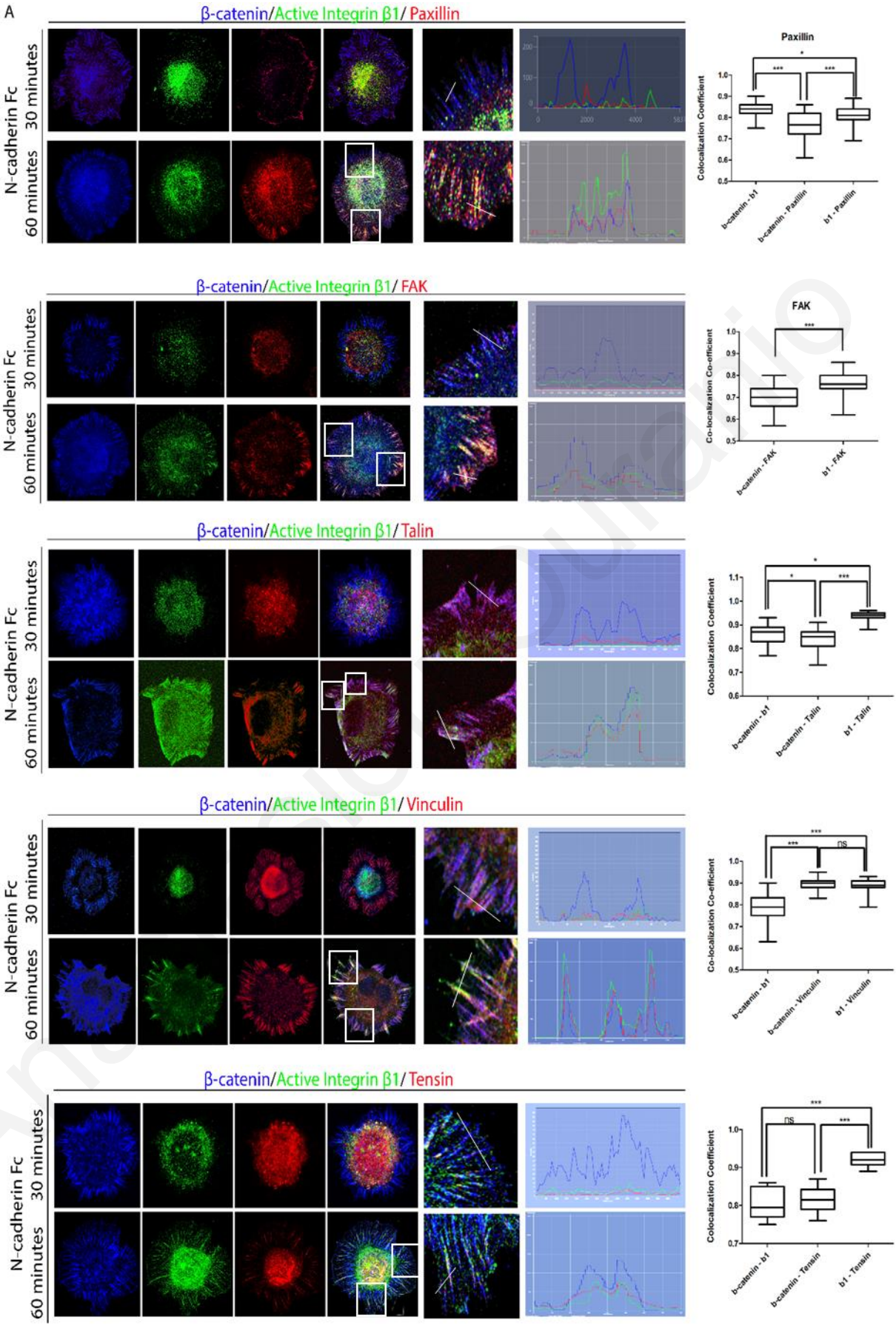

A
β-catenin/Active Integrin β1/ Paxillin
N-cadherin Fc
30 minutes
60 minutes
Paxillin
Colocalization Coefficient
b-catenin - b1
b-catenin - Paxillin
b1 - Paxillin
β-catenin/Active Integrin β1/ FAK
FAK
Co-localization Co-efficient
b-catenin - FAK
b1 - FAK
β-catenin/Active Integrin β1/ Talin
b-catenin - Talin
b1 - Talin
β-catenin/Active Integrin β1/ Vinculin
ns
b-catenin - Vinculin
b1 - Vinculin
β-catenin/Active Integrin β1/ Tensin
b-catenin - Tensin
b1 - Tensin

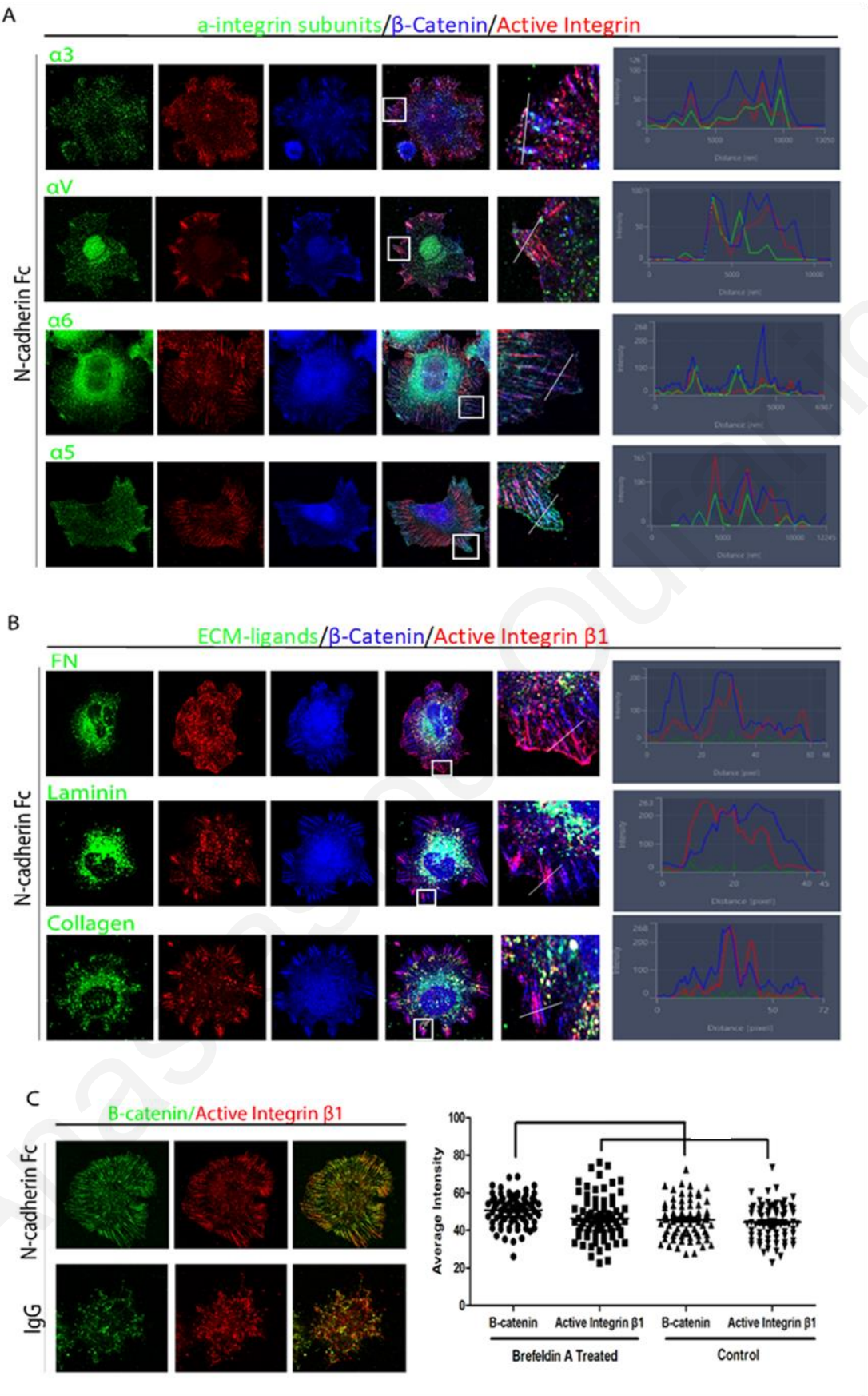
Figure 31: FA proteins are recruited at the sited of AJs and display co-localization with active integrin β1.

A) Representative confocal images of FA proteins localization at AJs of Hela cells on N-cadherin Fc at 30 and 60 minutes. Cells were stained against active integrin β1, β-catenin and each of the FA markers separately (Paxillin, FAK, Talin, Vinculin, Tensin). Profiles showing the co-localization of each of these proteins at AJs sites at 60 minutes and colocalization coefficient of the proteins with active integrin β1 and β-catenin. For paxillin: colocalization coefficients: β1-integrin and β-catenin: 0.863 ± 0.273 (N=57), β1-integrin and paxillin: 0.813 ± 0.263 (N=57), β-catenin and Paxillin: 0.744 ± 0.126 (N=57). For FAK: colocalization coefficients: β1-integrin and FAK: 0.786 ± 0.237 (N=63), β-catenin and FAK: 0.698 ± 0.256 (N=63). For paxillin: colocalization coefficients: β1-integrin and β-catenin: 0.863 ± 0.273 (N=57), β1-integrin and paxillin: 0.813 ± 0.263 (N=57), β-catenin and Paxillin: 0.744 ± 0.126 (N=57). For talin: colocalization coefficients: β1-integrin and β-catenin: 0.872 ± 0.142 (N=54), β1-integrin and talin: 0.922 ± 0.031 (N=54), β-catenin and talin: 0.805 ± 0.203 (N=54). For vinculin: colocalization coefficients: β1-integrin and β-catenin: 0.798 ± 0.302 (N=51), β1-integrin and vinculin: 0.889 ± 0.214 (N=51), β-catenin and vinculin: 0.902 ± 0.107 (N=51). For tensin: colocalization coefficients: β1-integrin and β-catenin: 0.793 ± 0.095 (N=62), β1-integrin and tensin: 0.824 ± 0.073 (N=62), β-catenin and tensin: 0.921 ± 0.012 (N=62).

Since integrin β1 activation is a prerequisite for the localization of these proteins at the AJs and since in nature integrins exist as heterodimers, we moved on to examine the localization of different α integrin subunits at HAs. As shown in Figure 31 A, different α integrin subunits were observed at the AJs distinctly from what has been observed at the FA complexes, where the predominant integrin heterodimers rely on the ligands presented at the ECM (**Figure 32 A**). This suggests that the ligands of different integrin subunits may be present at the sites of AJs. Hence, we went on to examine this possibility. Even though cells were allowed to spread under serum-free conditions, we could not preclude the possibility that rapid integrin β1 activation observed at the sites of HAs occurred through its interactions with ECM ligands. We stained Hela cells on N-cadherin (after 60 minutes of seeding) with different antibodies against the main ligands of integrin subunits observed at the sites of HAs and specifically fibronectin, laminin and collagen. As shown in Figure 31 B, under these conditions' cells display no detectable ECM molecules at the sites of the HAs suggesting that integrin activation at the AJs is independent of the presence of deposited ligands (**Figure 32 B**). In an attempt to further investigate this suggestion, we used the well-characterized Golgi-secretion inhibitor, Brefeldin A. Hela cells were pre-treated with the inhibitor and allowed to attach and spread on N-cadherin Fc substrates in the presence of the inhibitor. As a control, N-cadherin Fc substrates blocked with Igg were used. Cells were then stained against integrin β1 and β-catenin. Cells on blocked substrates were not able to spread suggesting that ligand secretion was a prerequisite for their spreading. Cells on N-cadherin Fc were able to spread normally, formed AJs and displayed active integrin β1 at the 60-minute interval. This suggests that the activation of integrin β1 at AJs was independent of any deposited ECM ligands (**Figure 32 C**). Finally, in order to further eliminate the possibility that ligands are secreted under these conditions, we used the well-characterized protein synthesis inhibitor Cyclohexamide (CHX). Hela cells were treated with CHX for 12 hours and then allowed to spread on N-cadherin substrates under serum-free conditions. FN substrates were used as a control, since it is well known that the spreading of cells on FN requires ligands. Our results show that the cells on FN display weak FA formation, suggesting that the loss of expression of FA components and ligands

led to the reduced ability of cells to form FA complexes. Cells on N-cadherin Fc formed linear AJs displaying robust integrin β1 activation and FA protein recruitment. Quantification of integrin activation showed that the treatment with CHX did not affect the extent of integrin β1 activation, suggesting that this activation is independent of any detectable ligand (**Figure 32 D**).

Overall this set of experiments shows that the cadherin-based adhesion between adjacent cells elicit the activation and clustering of integrins. This results to the formation of an FA-like complex, the HAs. Our results using antibodies against ligands of the ECM, integrin alpha subunits, Brefeldin-A, and CHX suggest that the formation of the HAs occurs in the absence of any detectable, deposited ligand.


A
a-integrin subunits/β-Catenin/Active Integrin
α3
αV
α6
α5
N-cadherin Fc
B
ECM-ligands/β-Catenin/Active Integrin β1
FN
Laminin
Collagen
N-cadherin Fc
C
B-catenin/Active Integrin β1
N-cadherin Fc
IgG
Average Intensity
100
80
60
40
20
0
B-catenin
Active Integrin β1
B-catenin
Active Integrin β1
Brefeldin A Treated
Control

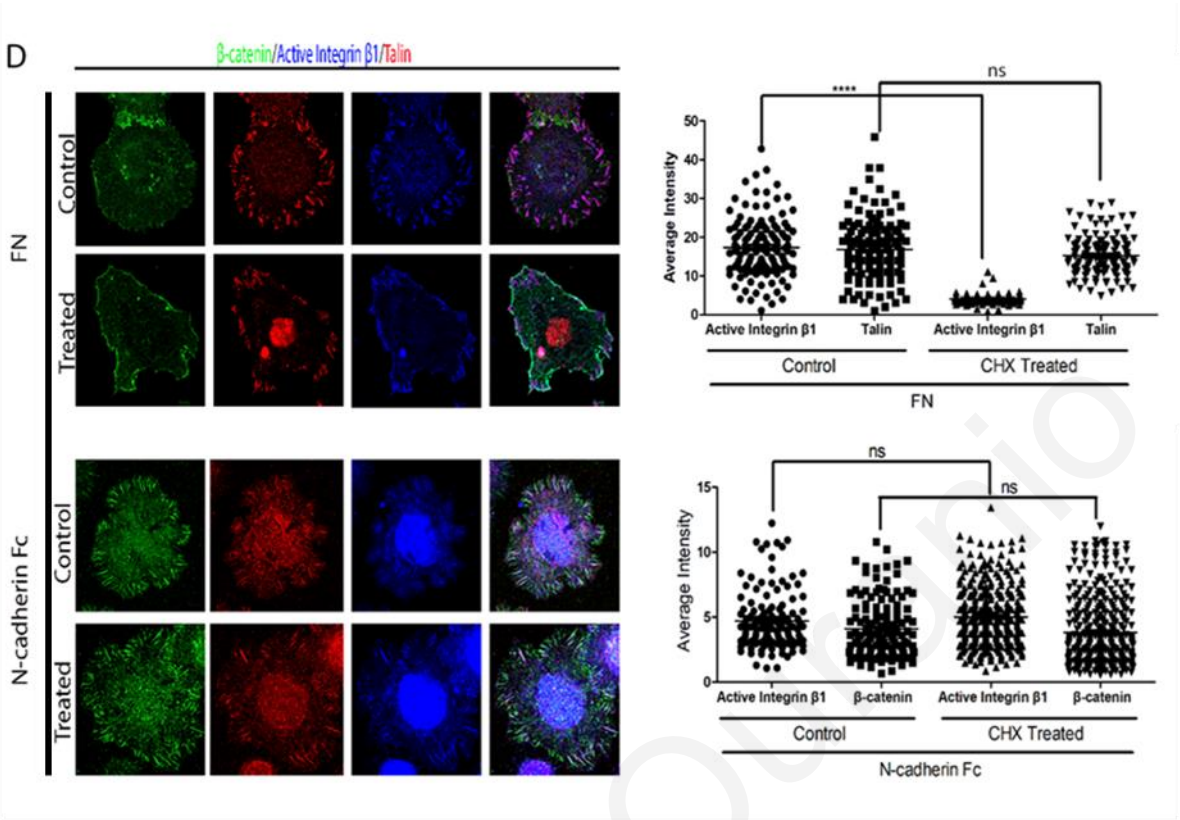


Figure 32: Integrin β1 activation at the sites of AJs takes place in the absence of any detectable, deposited ligand.

A) Representative images using confocal microscopy of Hela cells seeded on N-cadherin Fc for 60minutes and stained against different integrin α-subunits (α5, αV, α6 and α3), active integrin β1 and β-catenin and profiles showing the co-localization of different integrin α-subunits at these sites. **B**) Confocal representative images of Hela cells on N-cadherin Fc stained with antibodies for different ECM ligands (collagen, laminin and FN) active integrin β1 and β-catenin and profiles showing that no co-localization of these ligands is present at those sites. **C**) Optical images using a confocal microscope of Hela cells on N-cadherin Fc and N-cadherin Fc coated with IgG substrates treated with Brefeldin-A. Cells were treated prior to spreading with the inhibitor for 2 hours and spread in the presence of the inhibitor. Cells were stained with antibodies for integrin β1 and β-catenin. Scatter plot showing the intensities of integrin β1 and β-catenin of cells treated with Brefeldin A and control cells integrin β1 Bref-A: 46.34 ± 1.361 (N = 86), β-catenin Bref-A: 45.75 ± 09588 (N = 86), integrin β1 Control: 44.34 ± 1.0861 (N = 76), β-catenin Control: 45.75 ± 1.108 (N = 76). **D**) Representative images using confocal microscopy of Hela cells seeded on N-cadherin Fc and FN for 60minutes after 12-hour treatment with CHX compared to control cells. Cells were stained against active integrin β1, Talin and β-catenin. Scatter plots showing the intensity of active integrin β1, Talin and β-catenin on control and CHX treated cells on N-cadherin Fc and FN. Average intensities: Active Integrin β1 control cells on FN: 17.40 ± 0.698 (N = 123), Active Integrin β1 CHX treated cells on FN: 4.078 ± 0.109 (N = 123), Talin control cells on FN: 16.88 ± 0.734 (N = 123), Talin CHX treated cells on FN: 15.308 ± 0.265 (N = 123). Active Integrin β1 control cells on N-cadherin Fc: 4.740 ± 0.189 (N = 134), Active Integrin β1 CHX treated cells on N-cadherin Fc: 5.020 ± 0.1252 (N = 134), β-catenin control cells on N-cadherin Fc: 4.104 ± 0.197 (N = 134), β-catenin CHX treated cells on N-cadherin Fc: 3.819 ± 0.148 (N = 134).

5.2.3 Active Integrin β1 at AJs displays a unique signature conformation

Activation of integrin β1 is a well-studied process through which integrins alter their affinity for ligands and regulate their function (Czuchra *et al.*, 2006; Anthis and Campbell, 2011; Kechagia, Ivaska and Roca-Cusachs, 2019; Leiphart *et al.*, 2019). It is well documented that integrins exist in different conformations in nature and each conformation represents different affinity state for their ligands. The lowest affinity for their ligands exists when integrins are in the so-called bend, head-piece close conformation. At this close conformation, integrins acquire a bent V-shape where their headpieces are bent next to their extracellular parts, known as legs, and are located close to the cell membrane. The integrin subunits in this conformation, have been shown to be held together through the formation of a salt bridge between the cytoplasmic tails of the two integrin subunits, β and α. The close conformation undergoes reversible conformational changes either upon ligand binding to the extracellular heads or through the association of the cytoplasmic tail of integrins with intracellular proteins, such as talin. This results in the increase in ligand binding affinity through the extension of the extracellular heads of integrin subunits. This initial activation of integrin molecules leads to further conformational changes, resulting in the highest affinity state for their ligands in which the heads of the subunits are completely extended and the cytoplasmic domains are separated. This state of activation is known as extended head-piece open conformation. These three different conformations of integrins molecules are the most well-described, even though it is strongly believed that additional conformations exist in nature. Previous studies have shown that the extended open state is ligand-bound and is observed during cell ECM adhesion while the other two conformations are non-adhesive (Czuchra *et al.*, 2006; Anthis and Campbell, 2011; Kechagia, Ivaska and Roca-Cusachs, 2019; Leiphart *et al.*, 2019). Conformation specific antibodies have been previously characterized and it has been shown that these antibodies recognize epitopes that are exposed only when integrins acquire a specific conformation (Kovach *et al.*, 1992; Green, Mould and Humphries, 1998; Askari *et al.*, 2009; Byron *et al.*, 2009; Campbell and Humphries, 2011). We wanted to examine the possibility that the conformation of integrin β1 at the sites of AJs displays differences to the one observed at FAs since no detectable ECM ligands were observed at AJs. In order to do that we selected specific integrin β1 antibodies that recognize different subunits of the extracellular domain of integrins in an effort to differentiate the states of activation observed in the two complexes. Precisely, we used the 9EG7 antibody, known to bind the EGF repeats of the integrin β subunit, the 12G10 antibody, known to binds the beta A subunit and HUTS21 which binds the hybrid domain. We used Hela cells and allowed them to attach and spread on FN and N-cadherin Fc and stained the cells with 9EG7 and 12G10 or with 9EG7 and HUTS21 (**Figure 33 A**). We observed that the 9EG7 antibody was clearly staining both FAs and AJs and this staining was distinct from what we observed for 12G10. 12G10 displayed almost no staining on AJs while its staining on FAs was quite strong. In contrast to these, the staining of HUTS21 showed a similar pattern to the one observed with 9EG7 at the AJs and stained quite strongly both FAs and AJs. In an attempt to further examine these

differences, we quantified the ratio of 9EG7 to 12G10 and the ratio of 9EG7 to HUTS21 both on AJs and FAs. What we observed was that both ratios varied significantly at the two complexes (**Figure 33 B, C**). These data suggest that the activation of integrin β1 at the AJs displays a distinct conformation from the activation of integrin β1 at FA complexes. Some of the epitopes were shown to be bound with lower affinity at AJs compared to FAs, suggesting that this state of activation may be an intermediate state.

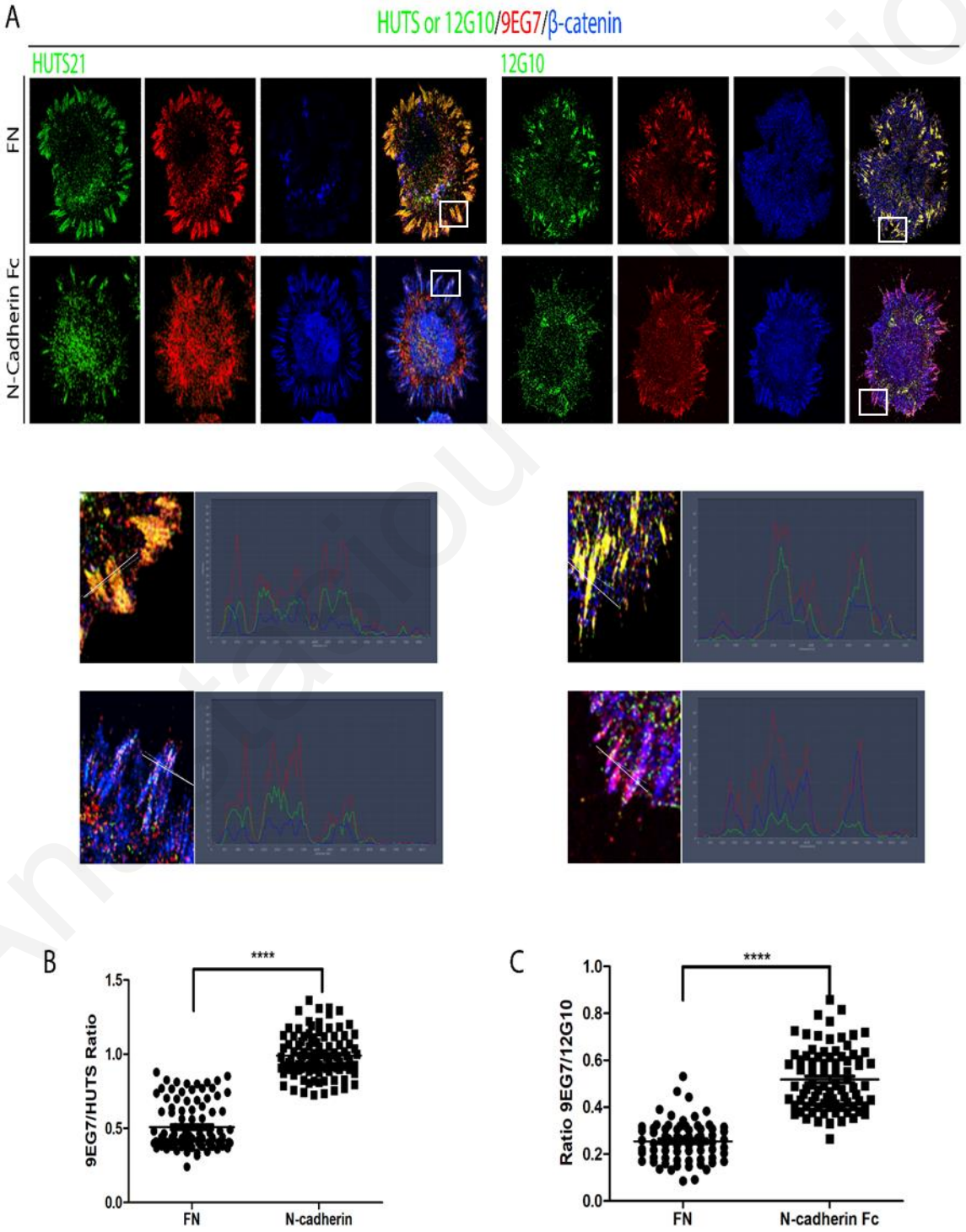


Figure 33: Integrin β1 activation associated with the AJs, displays a unique signature conformation **A**) Representative confocal images and co-localization profiles of Hela cells seeded on N-cadherin Fc and FN and stained against active integrin β1 antibodies that represent different conformational signatures. Cells were stained for active integrin β1 9EG7, β-catenin and either active integrin β1 HUTS21 (Left panel) or 12G10 (Right panel). **B-C**) Scatter plots showing the ratios of different conformation-specific antibodies (9EG7 and HUTS21, 9EG7 and 12G10) of cells seeded on N-cadherin Fc compared to FN. 9EG7/HUTS21 Ratio FN: 0.5082 ± 0.015 (N = 100), 9EG7/HUTS21 Ratio N-Cadherin Fc: 0.9911 ± 0.014 (N = 100). 9EG7/12G10 Ratio FN: 0.2546 ± 0.011 (N = 82), 9EG7/HUTS21 Ratio N-Cadherin Fc: 0.5179 ± 0.014 (N = 82).

Previous studies, including work from our laboratory, elegantly showed that during mitosis, a pool of integrins at the lateral cortex of the mitotic cells becomes activated through tension in the absence of ligands, suggesting the ability of integrins to become active in a ligand-independent manner (Maria and Ferraris, 2010; Ferraris *et al.*, 2014; Petridou and Skourides, 2016). These notions have recently been proven by a work from Kim et al. This study showed that membrane tension can activate integrins in a ligand independent fashion (Kim *et al.*, 2020). This conformation has been characterized as an extended head-piece close conformation and this mode of activation found similar to the one described in spindle orientation (Petridou and Skourides, 2016). These data in combination with our results showing that no detectable ECM ligands are present at the sites of AJs and the fact that the conformation of active integrin at these sites is distinct from the one observed at the classic FA complexes, we moved on to compare the conformational state of integrin β1 activation observed at the AJ sites with the one observed at the lateral cortex of the mitotic cells during cell division. Observation of interphase and metaphase cells on N-cadherin, and their comparison with cells on FN revealed strong 9EG7 staining at the cortex of mitotic cells, while this was not the case for 12G10 staining (**Figure 34 A**). The staining of HUTS21 found to be comparable to the one of 9EG7 but the staining of 9EG7 was the strongest both on FN and N-cadherin cell cortices, suggesting that the state of activation at the lateral cortex of the mitotic cells is the same to the one observed at the AJ sites. We then quantified the ratio of 9EG7 to 12G10 and the ratio of 9EG7 to HUTS21 both on the mitotic cortex and FAs and what we observed was that both ratios varied significantly at the two types of complexes (**Figure 34 B, C**) showing that the conformational state of active integrin during mitosis is comparable to the one observed at AJs and is distinct from the one observed at FAs which is ligand driven. Overall, these data suggest that the activation of integrins at AJs, at least during the early stages, exists in the extended head-piece close conformation, which probably does not require the binding of a ligand. This conformational state cannot promote adhesion and is distinct from the one that is observed at FAs.

To test the possibility that ligand-binding is indispensable for integrin clustering and localization at AJ sites, we generated a construct composed of the cytoplasmic tail of integrin β1 fused to emerald and a palmitoylation site. This construct has the ability to bind talin intracellularly, through its intact

cytoplasmic tail, and in the absence of an α subunit, that masks the talin binding domain., However, it cannot bind ligands extracellularly. Similar constructs have been described previously and have been used as dominant negatives for the disruption of cell-ECM interactions (Arold, Hoellerer and Noble, 2002; Czuchra *et al.*, 2006; Anthis and Campbell, 2011; Meves *et al.*, 2013). Hela cells were transiently transfected with this construct, allowed to attach and spread on N-cadherin Fc for 60 minutes, fixed and stained against active integrin β1 and β-catenin. This construct was shown to cluster at the AJ sites even though it could not bind any extracellular ligand, suggesting that the binding to a ligand is dispensable for the clustering of integrin β1 at these sites. This construct was found on AJs that displayed no activation of endogenous integrin β1, suggesting that the actomyosin bundles that are connected to the AJs and terminate at those sites can trap and cluster these receptors at the N-cadherin based AJs. (**Figure 34 D**). These results do not prove definitively that ligand binding does not take place at these sites, since the construct retains its ability to bind talin intracellularly. However, their combination with the data showing the similarities of conformational state of integrins at AJ sites and the mitotic cell cortex raises the possibility that the initial extension and clustering of integrins at those sites do not depend on deposited ligand binding.

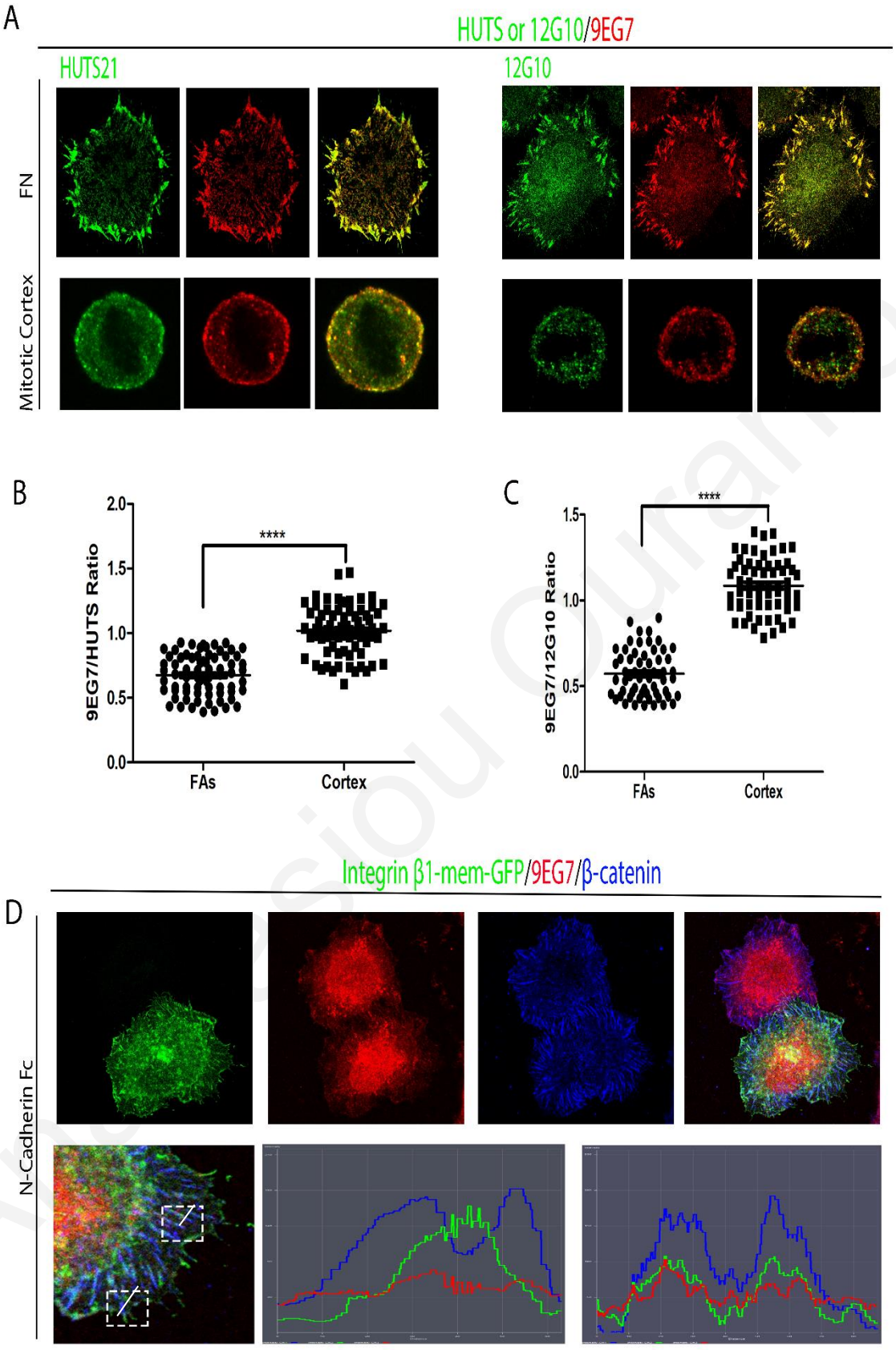

A
HUTS or 12G10/9EG7
HUTS21
12G10
FN
Mitotic Cortex
B
9EG7/HUTS Ratio
2.0
1.5
1.0
0.5
0.0
****
FAs
Cortex
C
9EG7/12G10 Ratio
1.5
1.0
0.5
0.0
****
FAs
Cortex
D
Integrin β1-mem-GFP/9EG7/β-catenin
N-Cadherin Fc

Figure 34: Integrin β1 at the sites of AJs display similaritites in conformation with active integrin β1 at the mitotic cell cortex.

A) Representative confocal images of interphase and metaphase Hela cells seeded on FN and stained against active integrin β1 antibodies that represent different conformational signature. Cells were stained for active integrin β1 9EG7 and either active integrin β1 HUTS 21 (left panel) or 12G10 (right panel). **B-C**) Scatter plots showing the ratios of different conformation-specific antibodies (9EG7 and HUTS21, 9EG7 and 12G10) of interphase cells seeded on FN compared to metaphase cells seeded on FN. 9EG7/HUTS21 Ratio FN-FAs: 0. 676 ± 0.018 (N=72), 9EG7/HUTS21 Ratio FN-cortex: 1.086± 0.021 (N=80). 9EG7/12G10 Ratio FN-FAs: 0.5722±0.018(N=81), 9EG7/12G10 Ratio FN-cortex: 1.086±0.021 (N=80). **D**) Representative confocal images of Hela cells expressing integrin β1tail-mem GFP construct. Cells were stained for active integrin β1 and β-catenin. Profiles show the co-localization of this construct at the sites of AJs in the presence and absence of endogenous active integrin β1.

5.2.4 Clustering and activation of integrins at the AJs modulates the dynamics of AJ complexes.

Since integrin activation and clustering on AJs were found to be spatially segregated from the N-cadherin/b-catenin complexes, we wanted to investigate any potential effects of this activation at these complexes. We initially wanted to explore the temporal relationship between integrin β1 activation and β-catenin at AJs. We performed time-course experiments using Hela cells attached on N-cadherin coated coverslips and allowed to spread for different timepoints. Then the levels of active integrin β1 and β-catenin were assessed using specific antibodies (**Figure 35 A**). As time progressed, we observed that the levels of β-catenin dropped while the levels of active integrin β1 increased (**Figure 35 B**). This inverse relationship over time suggested that active integrin β1 at HAs may be responsible for the disassembly of AJs. To further investigate that, we quantified the intensity of β-catenin, N-cadherin and active integrin β1 on AJs over time. We observed that the alterations in the intensity of catenin and cadherin were identical, suggesting that the effects on AJs are not a result of dissociation of catenin from N-cadherin as previously suggested (D'Souza-Schorey, 2005)(Meng and Takeichi, 2009). Integrin β1 displayed activation and clear polarization along some of the linear AJs while other AJs of the same cell remain negative for active integrin β1 at 45 minutes. This allowed us to quantify the levels of β-catenin on AJs that were both positive and negative at the same cells. This quantification revealed that the levels of β-catenin dropped dramatically at AJs where active integrin β1 was present, in contrast to neighboring AJs that did not display any integrin activation (**Figure 35 D, E**). In addition, we compared the intensity of both active integrin β1 and β-catenin on a scatter plot where each point represents values form an individual AJ, and observed a clear inverse correlation between the intensity levels of integrin β1 and β-catenin (**Figure 35 C**). Collectively, these results provide further evidence for the notion that integrin β1 activation at cadherin/catenin positive adhesions leads to the disassembly of AJs. The data also suggest that the effects of integrin β1 activation are specific to the AJs that display integrin β1 activation making it highly localized.

In order to address the possibility that integrin activation is involved in the regulation of AJ dynamics, we took advantage of the well-characterized integrin β1 inhibitory antibody AIIB2 and examined the effects of integrin inhibition in AJ disassembly. Hela cells were mechanically disrupted and treated with the integrin inhibitory antibody before spreading on N-cadherin Fc substrates. The cells were then allowed to spread in the presence of the antibody and the effects of integrin β1 inhibition were assessed. We observed that treatment with the antibody led to a dramatic reduction in integrin activation, as expected, accompanied by increased β-catenin levels and inhibition of AJ disassembly (**Figure 35 F**). In order to further investigate the effects of integrin β1 activation we followed an alternative approach using overexpression of SHARPIN, a well-documented protein which acts as an integrin β1 inhibitor (Rantala *et al.*, 2011). SHARPIN is a cytosolic protein and has been shown to bind on a conserved region of the α subunit of integrins, sequestering their talin- and kindlin-binding ability. As a result, this blocks their ability to switch from an inactive close conformation to an active open one (Rantala *et al.*, 2011). We transiently transfected Hela cells with SHARPIN-GFP construct and allowed them to spread on N-cadherin Fc substrates for 60 minutes (**Figure 35 G**). We then examined the effects of this integrin inhibitor in both integrin activation, β-catenin intensity and AJ disassembly. Cells expressing SHARPIN-GFP displayed a robust increase in β-catenin intensity while the intensity of integrin β1 activation was dramatically dropped, as expected. This result was the contrary to what we observed in control non-transfected cells. Quantification of β-catenin intensity on both control and SHARPIN expressing cells confirmed that the inhibition of integrin β1 leads to the enhancement of β-catenin (**Figure 35 G**) and hence the AJs, suggesting that integrin β1 activation is responsible for the disassembly of AJs.

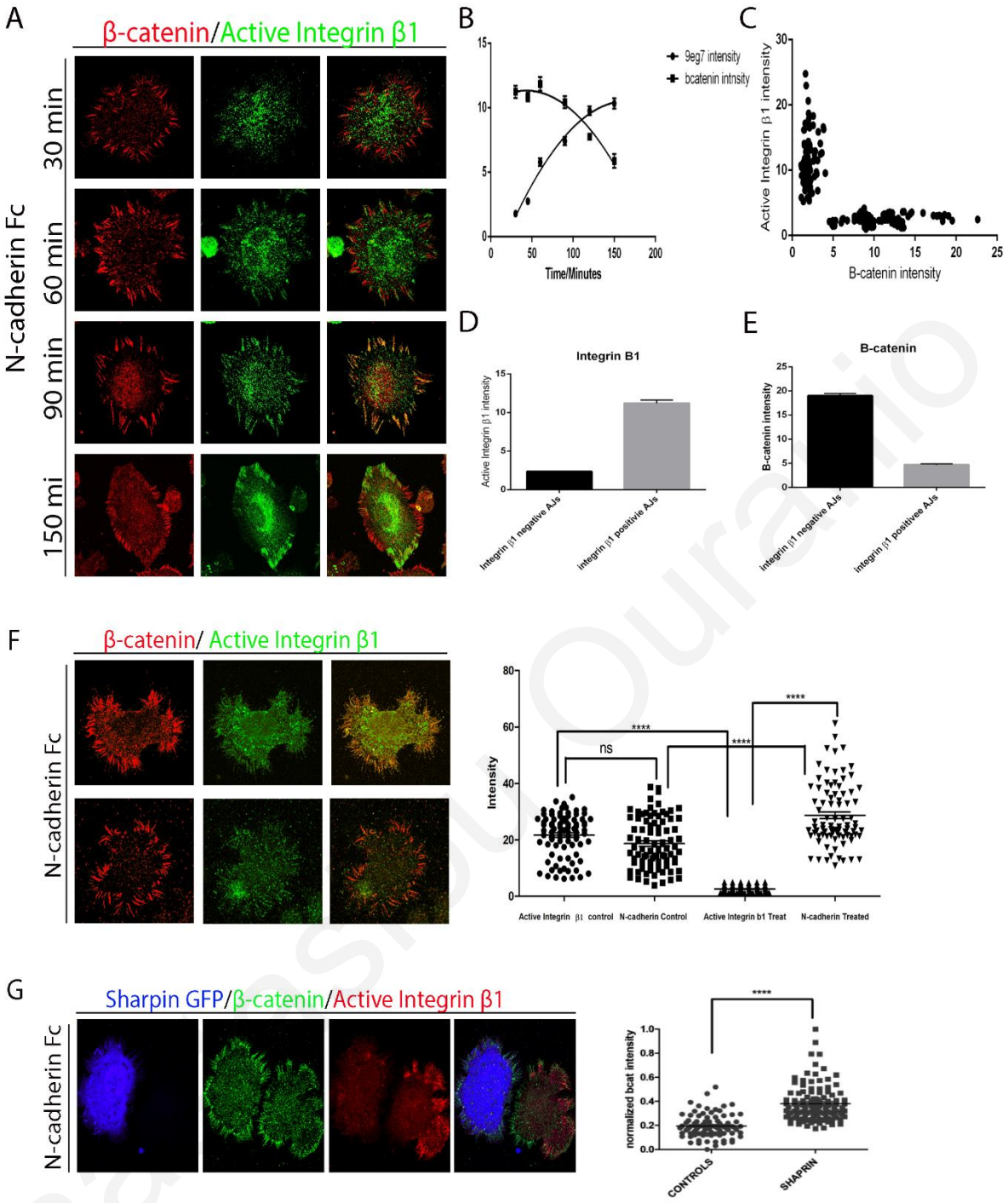


Figure 35: Integrin β1 clustering drives AJs' disassembly.

A) Representative confocal images of Hela cells at different timepoints seeded on N-Cadherin Fc and stained against active integrin β1 and β-catenin. **B**) Time-plot showing the fluctuations of intensities for active integrin β1 and β-catenin over time. **C**) Scatter plot comparing the intensities over time of both integrin β1 and β-catenin. **D-E**) Box-plots showing the intensity levels of active integrin β1 and β-catenin and comparing them at AJs displaying or not activation of integrin β1 Intensities: active Integrin β1 at negative AJs: 2.358 ± 0.039 (N = 127), active Integrin β1 at positive AJs: 11.23 ± 0.405 (N = 132), β-catenin at negative AJs: 19.01 ± 0.469 (N = 143), β-catenin at positive AJs: 4.683 ± 0.183 (N = 121). **F**) Representative images of Hela cells on N-cadherin Fc treated with AIIB2 compared to control. Cells were stained for active integrin β1 and β-catenin. Scatter plot showing the fluctuations in intensities of both active integrin β1 and β-catenin on cells treated with AIIB2 or controls. Intensities: active Integrin β1 control: 21.66 ± 0.816 (N = 86), β-catenin control: 18.65 ± 0.9639 (N = 86), Active Integrin β1 AIIB2 treated: 2.594 ± 0.127 (N = 86), β-catenin control: 28.67 ± 1.204 (N = 86). **G**) Representative confocal images of Hela cells transfected with SHARPIN-GFP construct seeded on N-cadherin Fc and stained for active integrin β1 and β-catenin. Scatter plot showing the differences in β-catenin intensity of control cells and cells transfected with SHARPIN-GFP. Intensities: β-catenin control: 0.234 ± 0.622 (N = 94), β-catenin SHARPIN expressing: 0.4563 ± 0.547 (N = 93).

Previous studies have documented that the disassembly of AJs is largely dependent on endocytosis and endocytic vesicles which in turn depends on an intact microtubule network (Kamei *et al.*, 1999; Lu *et al.*, 2003; Bryant and Stow, 2004; Chilov *et al.*, 2011; Byron *et al.*, 2015). It was previously shown that the microtubules target FA complexes but their targeting at cells on N-cadherin Fc found to be limited since it failed to enter the cell periphery. Thus, we asked if integrin activation guides the targeting of microtubules at the sites of HAs, promoting AJ turnover. As previously shown (MSc Thesis Rania Hadjisavva) bundles of microtubules are targeted only at HAs that display active integrin β1, remaining out of the cell lamella and areas with no detectable integrin β1 activation. These results suggested that integrin β1 activation stabilizes the microtubule network at the cell periphery, enabling the targeting of HAs (**Figure 36 A**). We thus moved on to explore the possibility that the microtubule targeting is involved in AJ turnover. To do that, Hela cells were treated with a MT polymerization inhibitor, Nocodazole D, and the intensity of active integrin β1 was examined in control and treated cells (**Figure 36 B**). As shown in Figure 35 B, depolymerization of the microtubule network did not affect integrin activation. However, treated cells displayed higher β-catenin intensity than untreated cells, suggesting that the microtubule network is required for the disassembly of AJs similarly to what has been proposed for FAs (**Figure 36 B**).

We moved on to examine the potential role of endocytosis in this process. There are well-documented evidence indicating that AJ disassembly highly relies on the endocytosis of major AJ protein-members. We thus wanted to address if the disassembly of these complexes which was taking place after integrin activation, was through endocytosis (Bryant and Stow, 2004; Ivanov, Nusrat and Parkos, 2004; Collinet and Lecuit, 2013). Hela cells attached on N-cadherin Fc substrates were stained with caveolin-1, a known endocytic marker. Caveolin-1 is a known scaffolding protein making up the main component of the caveolae plasma membranes in the majority of cell types and

is required in receptor-based endocytosis through the formation of membrane invaginations known as caveolae. It is also known that caveolin interacts with integrin β1 and localizes at FAs (Lu *et al.*, 2003; Ivanov, Nusrat and Parkos, 2004; Nethe and Hordijk, 2011). Staining with the antibody revealed caveolin-1 vesicles at the ventral site of the cells that displayed linear AJs but no integrin β1 activation. In cells that displayed integrin activation, caveolin-1 vesicles were localized near the cell membrane, at the sites of AJs (**Figure 36 C**). This suggests that integrin activation recruits caveolin at AJs/HAs and consequently drives the disassembly of AJs. To examine this further we moved on and used a well-characterized inhibitor of caveolin-mediated endocytosis, Methyl- β -Cyclodextrin. Hela cells were treated with the inhibitor for 2 hours and then mechanically disrupted. They were allowed to attach and spread on N-cadherin substrates (in the presence of the inhibitor) for 1 hour and stained for active integrin β1 and β-catenin. We observed that cells treated with the endocytosis inhibitor displayed increased levels of β-catenin compared to control cells (**Figure 36 D**). The intensity of β-catenin in control cells that did not display integrin β1 activation, was similar to that in treated cells that were also negative for β1 activation. In cells displaying active integrin β1, a dramatic increase in β-catenin intensity was observed upon the inhibitor treatment, unlike non-treated cells in which integrin activation was followed by decrease in b-catenin intensity as previously described (**Figure 36 D**). All these together suggest that caveolin-based endocytosis at the AJs occurs upon integrin β1 activation and is obligatory for the disassembly of AJs.

To further understand the role of localized integrin activation in the regulation of AJ dynamics, we moved to a more physiologically relevant context, the cell doublets as described in previous sections. We examined the AJ formation over time from initial contacts to the formation of mature adhesion and the formation of the clear central ring between the two adjacent cells. Cell doublets were treated with integrin β1 activating and inhibitory antibodies and the effects on AJ maturation process were assessed. Cells expressing membrane-targetted GFP (mem-GFP) were generated as previously described and treated with inhibitory or activating integrin β1 antibodies for 30 minutes prior to their attachment on PLL infused polyacrylamide gels in order to form doublets. The whole process was documented using a fluorescence microscope and time lapse movies were generated for a period of 20 minutes. As shown, treatment with the inhibitory and activating antibodies had opposing effects on AJ formation and dynamics (**Figure 36 E**). Previous work has shown that integrin activation leads to the enhancement of the AJ ring on cell doublets and that may be a result of early downstream interactions of recruited FA proteins at those sites. These interactions were shown to enhance the stability of AJs (D'Souza-Schorey, 2005; Borghi *et al.*, 2010; Mui, Chen and Assoian, 2016; Gayrard *et al.*, 2018; Hur *et al.*, 2020). However, other studies showed that effects of FA proteins at AJs require strict modulation after certain threshold. The increased expression of these proteins upon the certain thresholds was found to have a disassembling effect on these complexes (D'Souza-Schorey, 2005; Borghi *et al.*, 2010; Mui, Chen and Assoian, 2016; Gayrard *et al.*, 2018; Hur *et al.*, 2020). In contrast to these, the inhibition of integrins was shown to drive to the inability of cell doublets to fuse. These pieces of evidence showed the importance of integrin activation and subsequent

recruitment of downstream factors at those sites (D'Souza-Schorey, 2005; Borghi *et al.*, 2010; Mui, Chen and Assoian, 2016; Gayrard *et al.*, 2018; Hur *et al.*, 2020). Collectively, the above data show that the activation of integrin along AJs and the formation of the HAs leads to the disassembly of the AJs in an endocytosis and microtubule network-dependent process.

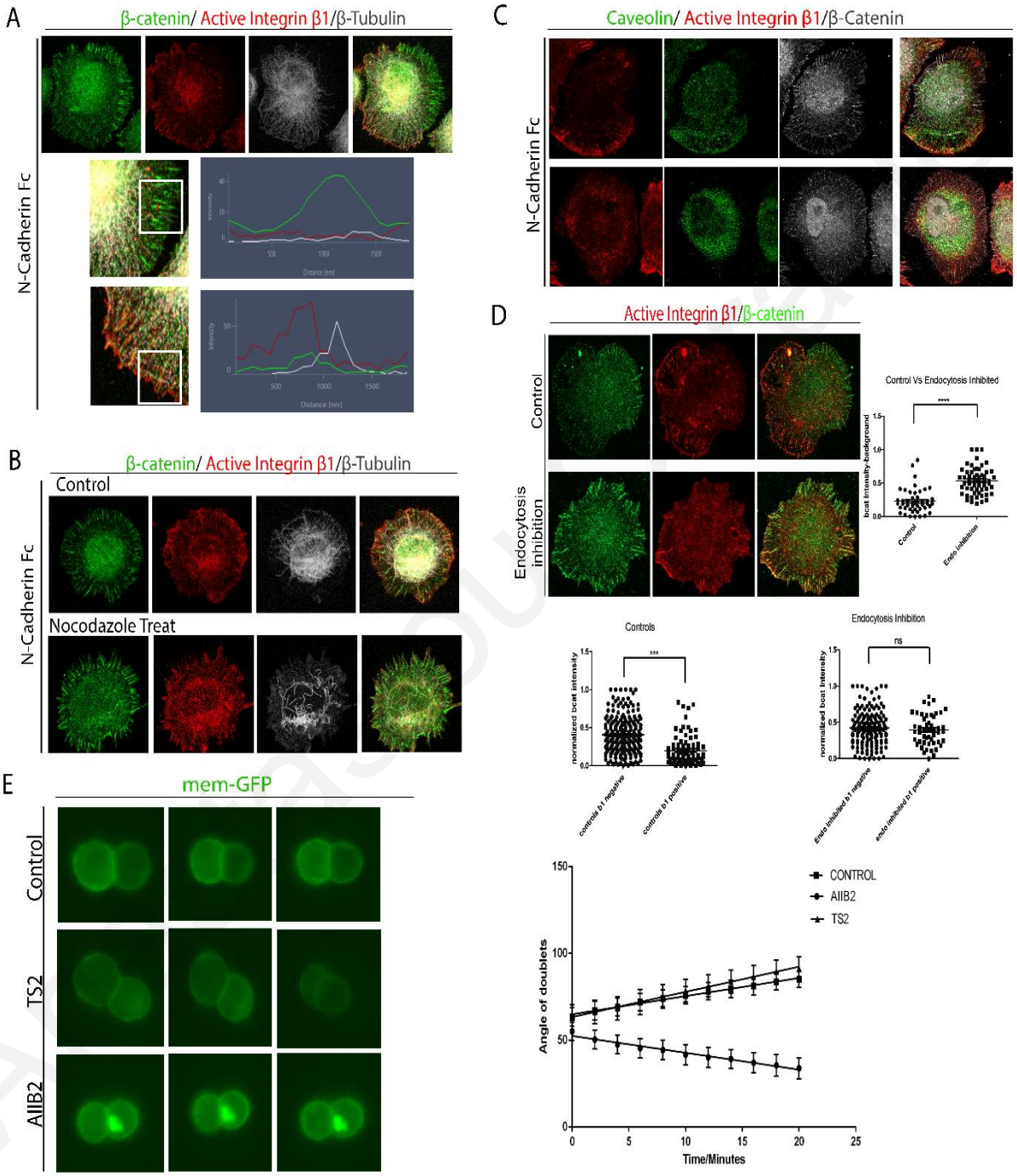


Figure 36: Integrin β1 activation and clustering at the sites of AJs modulates the dynamics of these complexes.

A) Representative confocal images of Hela cells seeded on N-Cadherin Fc, stained against active integrin β1, β-catenin and β-tubulin. **B**) Representative confocal images of Hela cells seeded on N-Cadherin Fc and treated with Nocodazole D compared to control cells. The cells were stained with antibodies for active integrin β1, β-catenin and β-tubulin. **C**) Optical images from a confocal microscope of Hela cells seeded on N-cadherin Fc stained for active integrin β1, β-catenin and caveolin-1. **D**) Confocal images of Hela cells seeded on N-cadherin Fc treated with an endocytosis inhibitor compared to control cells. Scatter plots for the fluctuations of intensities of β-catenin at AJs negative or positive for active integrin β1 for the treated and control cells. Intensities: β-catenin controls: 0.258 ± 0.039 (N = 50), β-catenin treated with endocytosis inhibitor: 0.523 ± 0.045 (N = 80), Intensities: β-catenin controls for active integrin β1 negative AJs: 0.524 ± 0.029 (N = 100), β-catenin controls for active integrin positive β1 AJs: 0.252 ± 0.062 (N = 100), Intensities: β-catenin treated with endocytosis inhibitor for active integrin β1 negative AJs: 0.482 ± 0.015 (N = 100), β-catenin treated with endocytosis inhibitor for active integrin positive β1 AJs: 0.479 ± 0.021 (N = 100). **E**) Representative images of selected time points of Hela cells transfected with membrane-GFP, forming doublets. Hela cells were imaged under normal conditions, upon treatment with activating and inhibitory integrin antibodies. Graphical Representation of the angle formed between the two cells during doublet formation under the previously mentioned conditions.

5.2.5 Activation of integrin β1 at AJs is associated with cadherin clustering.

A lot of evidence up until now suggests crosstalk between the two major adhesion systems through their receptors integrins and cadherins (Avizienyte *et al.*, 2004; Yano *et al.*, 2004; Wang *et al.*, 2006, 2019; Weber, Bjerke and DeSimone, 2011; Chen *et al.*, 2012; Toh, Xing and Yu, 2015). These pieces of evidence are mostly concentrated on how different proteins from both AJs and FAs are involved in the maintenance or modulation of the other adhesion system. The surprising finding that integrin β1 activation is promoted at the AJs has not been reported elsewhere; however, a lot of questions remain to be answered in order to better understand the mechanism that drives integrin activation at these sites. To that end, we proceeded to explore the role of cadherins as spatial cues for the activation of integrin at the AJs. We also wanted to explore the possibility that cadherins are implicated in the activation of integrins at AJs per se. The stepwise process of linear AJ formation on substrates with N-cadherin Fc coated surfaces has already been described. Briefly, the cells on these substrates attach through the formation of AJs with the N cadherin coated surface of glass and display enrichment at the interphase of the cell. The distribution of cadherins however is homogeneous throughout the contact areas of the cells. Over time these linear structures are formed at the periphery of the cell, where cadherin clusters become clearer and well defined, and have been shown to be associated with actomyosin bundles. Reports have shown that cadherins act as ligands for integrin β1 (Whittard *et al.*, 2002; Yano *et al.*, 2004; Wang *et al.*, 2006) thus, suggesting that the activation of integrin at

HAs/AJs might be a result of their interaction with cadherins which serve the major AJ receptors. In an attempt to investigate this hypothesis, we utilized micropatterned surfaces coated with N-cadherin Fc. These surfaces have predefined areas coated with N-cadherin Fc where cadherins are expected to form linear clusters and AJs. Hela cells were allowed to attach and spread on these micropatterned surfaces under serum-free conditions, fixed at two different time points (30 minutes and 60 minutes) and stained against active integrin β1 and α -catenin. The timing of cells on these patterned surfaces display alterations regarding the timing observed in surfaces coated homogeneously with N-cadherin Fc. Thus, we used different time points in order to identify the precise point in time at which cadherins become clustered and integrin β1 activation takes place on. At 30 minutes cadherins display a uniform within the cell and no clustering was observed (hence no binding to actomyosin bundles exists). Consequently, no integrin activation is observed along N-cadherin Fc coated areas. However, the enrichment of α-catenin, at the areas where N-cadherin Fc is located, is clear and this evidence suggests that N-cadherin Fc does not act as a ligand for integrin β1 as previously reported (**Figure 37 A**) (Whittard *et al.*, 2002). At the latter time-point, we observed clear formation of linear AJs along N-cadherin Fc coated stripes and clear clustering of cadherins (and hence binding to actomyosin bundles). At this time point we also observed clear activation of integrin β1 at the sites where the clustering of cadherins (and hence the linear AJs formation) was well defined. The clusters expanded over time and covered the entire cadherin-coated stripes. Integrin activation under these conditions displays an identical pattern to that of catenin since it expands along with the same stripes (**Figure 37 B**) (Figure adapted from MSc Thesis Rania Hadjisavva). These data suggest that the ligation of cadherins to cadherin-coated surfaces, is not sufficient to elicit integrin activation and that this activation requires the connection of cadherins to actomyosin bundles and their clustering.

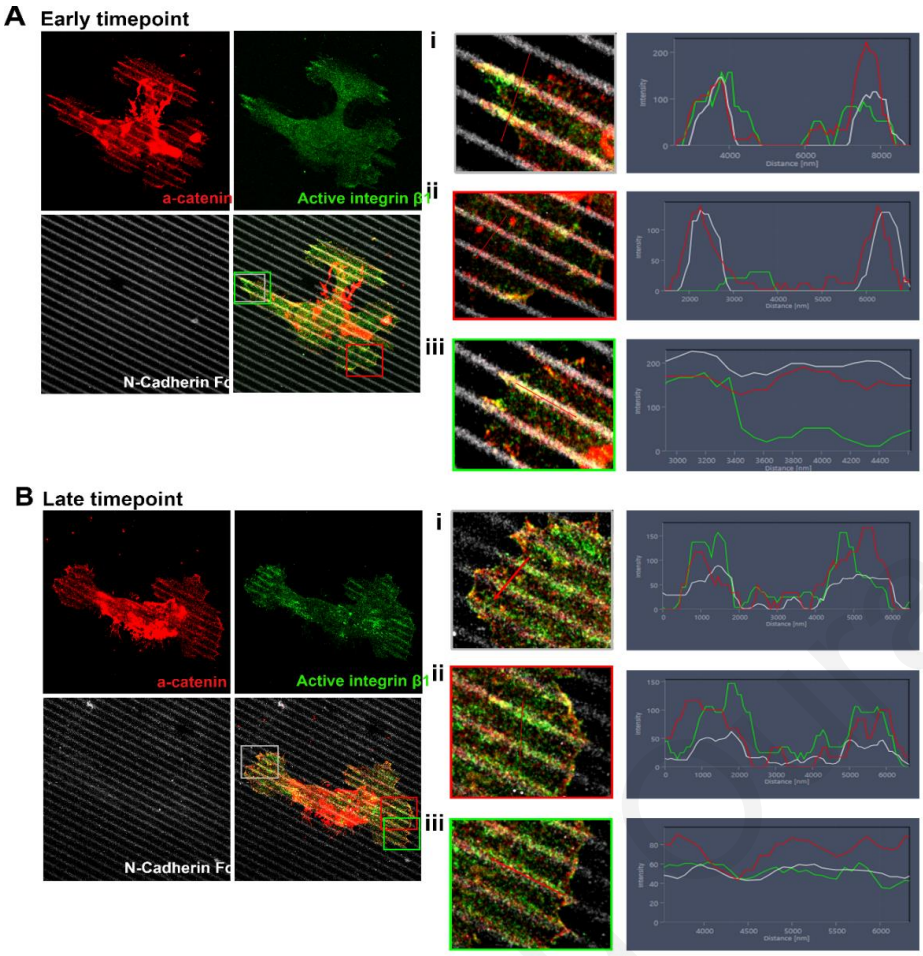


Figure 37: Adherens Junction clustering dictate spatially the activation of integrin β1 at AJs.

A) Confocal images of Hela cells at the plane of cell-micropatterns and co-localization profiles from different points within the same cell. Cells were stained for active integrin β1 and α-catenin. **B**) Representative images of Hela cells on N-cadherin Fc stripped micropatterned glass surfaces and co-localization profiles from different points within the same cell. Cells were stained for active integrin β1 and α-catenin. (Adapted from MSc thesis, Rania Hadjisavva).

Since our data suggested that actomyosin bundle connection to AJs drives the activation of integrin, we attempted to examine the effects of the dissociation of cadherins from actomyosin bundles, following different approaches. Initially, we took advantage of a well-characterized N-cadherin mutant lacking the cytoplasmic domain (N-Cadherin ΔCP) fused to GFP (Thoumine *et al.*, 2006; Ozaki *et al.*, 2009; Garg *et al.*, 2015). This construct retains the ability to promote ligation of cadherins with the cadherins of neighboring cells (or otherwise provided extracellular cadherins) but lacks the ability to bind intracellular binding partners of N-cadherin responsible for the connection of cadherins to actin. As described in the introduction of this thesis, the connection between cadherins and bundles of actin is achieved through the binding of cadherins to β-catenin. B-catenin binds alpha-catenin, which in turn interacts with vinculin which directly binds actin. N-Cadherin ΔCP was expected to act antagonistically to endogenous cadherins for ligand binding (cadherins on the coated substrate) without affecting cell spreading. It was also expected to prevent binding to actin bundles and thus prevent cadherin clustering. Hela cells were transiently transfected with N-Cadherin ΔCP and allowed to attach and spread on N-cadherin Fc coated glass surfaces for 60 minutes. They were

then stained against actin and β-catenin. In previous sections we observed that at the 60 minute-mark cadherin clusters are very well defined and integrin activation is observed along the AJs. For this reasons, this time-point was considered ideal for this set of experiments. We observed that cells were able to spread normally on N-cadherin Fc as expected. Examination of β-catenin staining revealed a dose-dependent inhibition of clustering of the endogenous cadherins. Cells expressing high levels of N-Cadherin ΔCP displayed complete absence of cadherin clusters and hence no formation of linear AJs, suggesting that the spreading achieved in those cells was a result of the ligation of exogenous N-Cadherin ΔCP with the substrate (**Figure 38 A**). Apart from these, we observed that the connection with actin bundles was lost in cells expressing high amounts of N-cadherin ΔCP. This suggests that this construct prevents the connections of actin bundles to AJs and acts antagonistically with endogenous cadherins (**Figure 38 A**). Then we went on to examine the effects of this construct on integrin β1 activation at AJs. Transiently transfected Hela cells were allowed to attach and spread on N-cadherin Fc coated coverslips and stained against β-catenin and active integrin β1. Cells expressing high levels of the construct displayed no cadherin clustering and no integrin activation, while cells with medium expression levels had a significantly reduced integrin activation in comparison to control cells (**Figure 38 B**). Quantification of integrin β1 activation revealed that the expression of N-cadherin ΔCP suppressed integrin activation, suggesting that the clustering of cadherins and the connection with actin bundles is necessary for integrin activation at the sites of AJs (**Figure 38 C**).

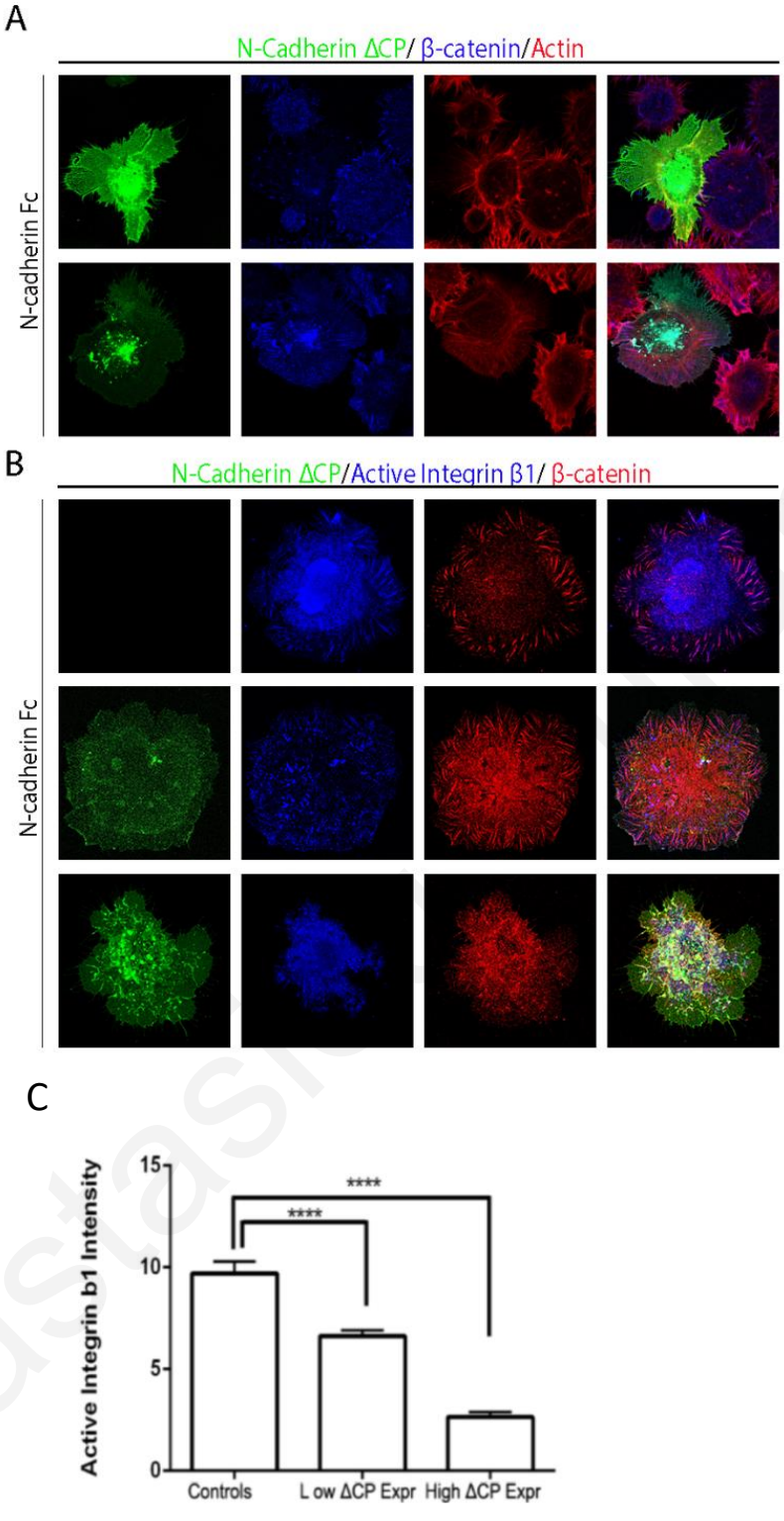


Figure 38: Cadherin clustering is a prerequisite for the AJ associated integrin β1 activation.

A) Representative confocal microscopy images of Hela cells on N-cadherin Fc expressing high (top) and moderate (bottom) levels of N-cadherin ΔCP-GFP construct and stained with antibodies against active integrin β1, β-catenin and actin. **B**) Representative images from a confocal microscope of Hela cells on N-cadherin Fc, expressing high (top) and moderate (middle) levels of N-cadherin ΔCP-GFP construct compared to control cells that do not express the construct. Cells were stained with antibodies against active integrin β1 and β-catenin. **C**) Bar plot showing the fluctuations of active integrin β1 in cells of different expression levels of the construct. Intensities: active integrin β1 control cells: 10.158 ± 1.492 (N = 70), active integrin β1 moderate expressor cells: 6.783 ± 1.285 (N = 70), active integrin β1 high expressor cells: 2.739 ± 0.991 (N = 70).

To further examine the role of actin-mediated cadherin clustering in integrin activation at AJs we moved on to perform live imaging of AJ dynamics is cells, after disrupting the actin cytoskeleton using Cytochalasin D. Hela cells transiently transfected with Talin-Fusion Red and N-cadherin GFP were allowed to attach and spread on N cadherin Fc coated coverslips and visualized over time using confocal live imaging. The cells were plated on silicone chambers, allowed to attach on the substrates and visualization was initiated immediately after the formation of first linear AJs. As described in section 4.2.2 integrin activation at AJs is temporally and spatially correlated with the recruitment of Talin at these sites. Since the visualization of active integrin β1 with the use of activating antibodies could potentially promote activation and/or promote inhibition of inactivation, we considered using the Talin-Fusion Red as an alternative surrogate for active integrin at AJs. Cells on FN coated coverslips were used as a control since FA formation and the recruitment of proteins such as talin, primarily depends on integrin binding on components of the ECM. After the initial visualization of the cells and the formation of either linear AJs or FAs, with clear localization of Talin at those sites, the cells were treated with low concentration amounts of Cytochalasin D. Upon treatment, Talin localization at AJs displayed a rapid decrease, suggesting that actin is required for its localization there and therefore for integrin activation. At the same time localization of N-cadherin was affected at a slower rate (**Figure 39 A-B**). This suggested that localization of Talin and hence integrin activation is not a direct result of cadherin clustering, since loss of Talin localization was observed prior to the disassembly of AJs (**Figure 39 A-B**). Cells on FN displayed constant localization of Talin at the sites of FAs with minimal effects on localization, suggesting that in the presence of an immobilized ligand, Talin and hence integrins do not exclusively depend on actin cytoskeleton for their clustering and consequent activation (**Figure 39 A-B**). This was further confirmed through FRAP experiments where both N-cadherin and Talin were photobleached in cells on N-cadherin Fc. We observed that the recovery time of N-cadherin was slower than the recovery time of Talin (N-cadherin $t^{1/2:}$13.01+/- 0.6 seconds, Talin $t^{1/2}$:1.4 +/- 0.8 seconds), suggesting that Talin localization and consequently integrin activation at AJs do not explicitly depend on immobilized connections via ligands, rather is a result of actin connection to these proteins specifically at AJs (**Figure 39 C**). The FRAP experiments also confirmed our observations on rapid decrease of talin localization in comparison to that of N-cadherin and showed that integrin activation does not depend directly on

cadherin clustering but on actin cytoskeleton connections. These results are in agreement with our previous results that showed Golgi secretion, protein synthesis inhibition, presence of different alpha integrin subunits and absence of deposited ECM ligands at AJs. All these, suggest that no deposited ligand is present at these regions and if any ligand is present this is not immobilized on the glass surface but it is rather in a soluble form. Overall, these experiments show that the spatial distribution of AJs is what guides the activation of integrin β1 and that this activation depends on cadherin clustering at the sites of AJs. They also show the important role of intact actin cytoskeleton for the retention of integrin activation and clustering at AJs.

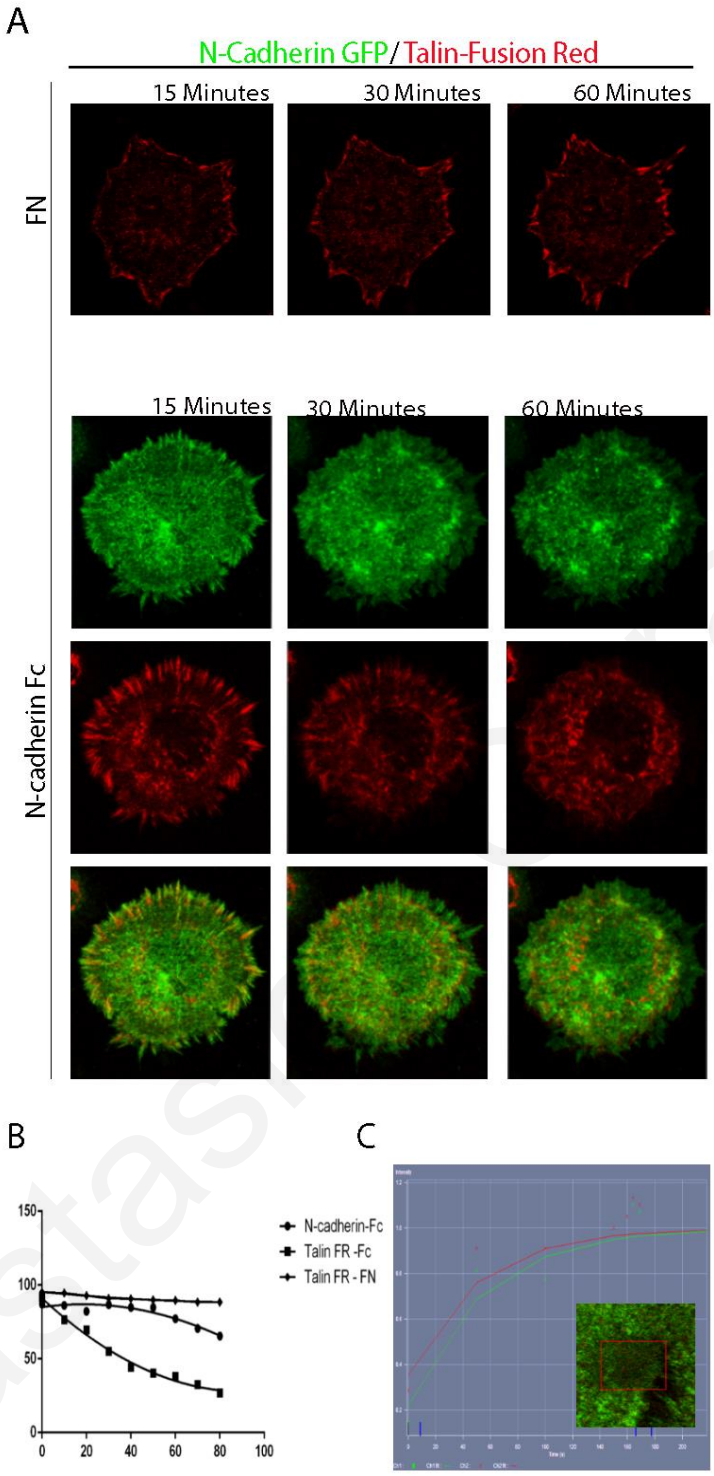


Figure 39: Clustering of cadherins and intact actin cytoskeleton are indispensable for AJ associated integrin β1 activation.

A) Representative confocal microscopy images of Hela cells on N-cadherin Fc and FN during live imaging after Cytochalasin D treatment. Cells are transiently expressing N-cadherin GFP and Talin-Fusion Red constructs. **B**) Time plot showing the fluctuations in intensities of both N-cadherin GFP and Talin-Fusion Red in cells on N-cadherin Fc and FN over time, upon Cytochalasin treatment. **C**) FRAP experiments of cells seeded on N-cadherin Fc showing the recovery time of both proteins: N-cadherin $t^{1/2:}$13.01+/- 0.6 seconds, Talin $t^{1/2}$:1.4 +/- 0.8 seconds.

5.2.6 Actin trapping mechanism is what drives clustering and activation of integrin β1 at AJs.

Up to this point it has been shown that the activation of integrin β1 at AJs requires an intact actin cytoskeleton and the clustering of cadherins through their connection to actomyosin bundles. However, the mechanism through which cadherin clustering elicits the activation of integrin β1 remains unknown. It has been recently proven that plasma membrane tension has the ability to activate integrins in the absence of a ligand (Maria and Ferraris, 2010; Ferraris *et al.*, 2014; Petridou and Skourides, 2016; Kim *et al.*, 2020). AJs are also well-described regions where high tension is applied, since these sites are connected to dense actomyosin bundles. In an attempt to investigate a potential role of AJ-applied tension in integrin activation we took advantage of a commercially available α-catenin FRET sensor. This sensor is composed of α-catenin and fluorescent proteins YFP and CFP. These fluorescent proteins were inserted in central locations of the α-catenin molecule in a way that the molecule was able to respond to force application and tension (Kim *et al.*, 2015). Interactions of cadherins with catenins and hence connection to the actin cytoskeleton and leads to an increase in tension applied on AJs. This tension drives changes in the conformation of the a-catenin sensor and results in the separation between CFP and YFP. This is followed by decrease in FRET/CFP ratio. In the absence of force (inhibition of cadherins or dissociation from the actin cytoskeleton) the sensor acquires a conformation in which CFP and YFP were brought together and this results in increase in the FRET/CFP ratio (Kim *et al.*, 2015). Hela cells were transiently transfected with the sensor and the activation of integrin β1 was compared to AJs that were positive for integrin activation and negative for integrin activation. We initially examined the FRET efficiency in cells on N-cadherin Fc that were positive or negative for integrin activation and observed that the FRET efficiency was decreased at AJs that were integrin β1 positive, suggesting a correlation between integrin β1 activation and tension application at the sites of AJs (**Figure 40 A**). We moved on and quantified the YFP/CFP ratio of AJs that were active integrin β1 positive and active integrin β1 negative. We observed that the ratio of FRET was associated with integrin activation at AJs suggesting that tension is necessary for integrin β1 activation at AJs (**Figure 40 B**). This can also explain the phenomenon observed in some cells where integrin β1 activation and clustering take place only at some of the AJs within the same cell during the early time points. In an attempt to directly address the role of tension on integrin activation guided by the clustering of cadherins at AJs, we used a widely used inhibitor of the cell contractility, ROCK. ROCK phosphorylates the myosin light chain which in return drives the formation of contractile pulling forces at the actin cytoskeleton. This leads to the exertion of mechanical forces within the cells (Sahai and Marshall, 2002; Bhadriraju *et al.*, 2007). Hela cells were co-transfected with Talin-Fusion Red, as a surrogate for active integrin, and N-cadherin GFP. Cells were allowed to seed and spread under serum-free conditions both on N-cadherin Fc and FN substrates. After the initial formation of AJs and subsequent localization of Talin at AJS, cells were treated with low amounts of the ROCK

inhibitor. The effects on localization of both Talin and N-cadherin were monitored using confocal microscopy. We observed that upon treatment, Talin localization at AJs was rapidly decreased in cells on N-cadherin Fc, while its localization at FAs was not affected (at least not drastically) in cells on FN (**Figure 40 C-D**). This shows that integrin activation and clustering at AJs are dependent on tension applied at AJs. This tension is generated by actomyosin bundles connected to the AJs and is applied at AJs. FRAP experiments showed that Talin on N-cadherin Fc displayed faster recovery rate in comparison to N-cadherin (N-cadherin $t^{1/2:}$14.3 +/- 1.63 seconds, Talin $t^{1/2}$: 2.3+/- 0.64 seconds). This provides further evidence that Talin localization and consequently integrin activation at AJs, is a result of actomyosin connection and tension generation at these sites (**Figure 40 E**). In combination with results regarding the absence of immobilized ligands at AJs, the unique integrin conformational state and the fact that on FN, where ligands are immobilized Talin retains its localization, our results suggest that the activation of integrin at AJs is force-dependent and is highly likely to be independent of deposited ligands. These results also suggest that integrin activation at AJs is possibly a result of their trapping through AJ-terminating actomyosin bundles that form upon cadherin clustering. An alternative explanation could be that integrin is bound to non-immobilized ligands, but their activation cannot be maintained in the absence of tension generated through actomyosin bundles.

To further explore the possibility that actomyosin bundles terminating at AJs lead to the trapping of active integrins and guide integrin activation, we moved on to generate i) substrates composed of FN-coated regions adjacent to N-cadherin Fc coated regions and ii) substrates composed of N-cadherin Fc coated regions adjacent to regions blocked with BSA in order to prevent cell adhesion. This allowed us to examine if and how the distribution of AJs at the cadherin coated regions affect integrin activation and consequent FA formation along the FN coated regions. On substrates where AJ-adjacent regions were blocked using BSA, we observed that integrin activation was taking place in an identical way as on regular N-cadherin Fc substrates (**Figure 40 F**). On N-cadherin Fc substrates with adjacent FN coated regions however, we observed that integrin activation was taking place along FN-coated in close proximity with the regions where AJs were formed on the adjacent N-cadherin Fc regions. Precisely, even though the two receptors were segregated at the two types of substrates, we observed that integrin activation and clustering was taking place only at the regions where actomyosin bundles terminated at the linear AJs (**Figure 40 F**). These data clearly show that even when immobilized integrin ligand is provided, the activation and clustering of integrin β1 follows the distribution of AJs, suggesting that AJs dictate the spatial distribution of integrin activation and the subsequent FA formation.

Collectively, these results show that integrin β1 activation and clustering at AJs depend on tension application and intact actin cytoskeleton. They also suggest that integrin activation does not depend on the presence and/or binding to immobilized ligands. All these together propose that the clustering of integrins at AJs is a result of the trapping in an actomyosin bundle network which terminates at these complexes and is directly associated with them.

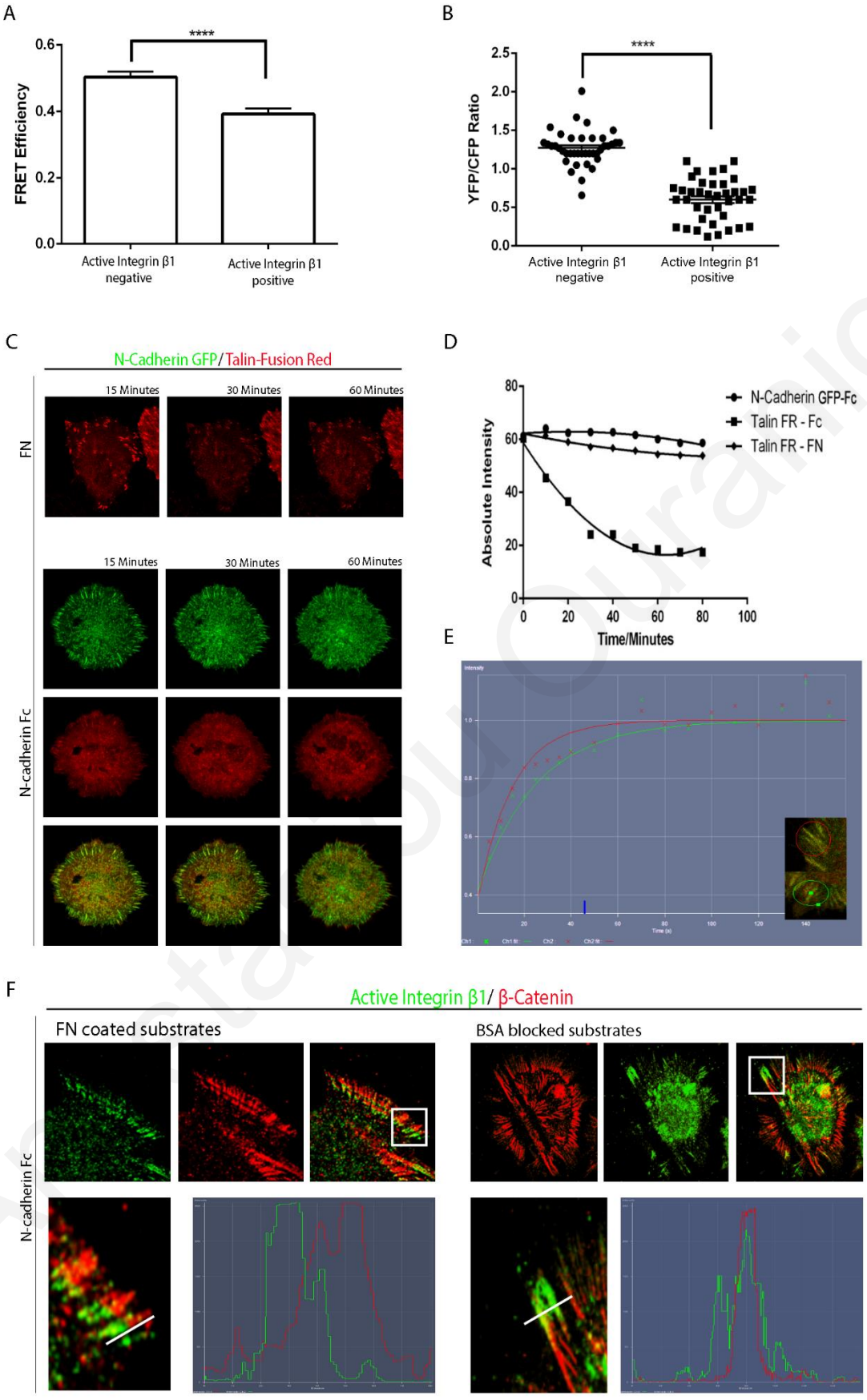


Figure 40: An actin trapping mechanism is modulating the clustering and activation of integrins at AJs.

A) Graphical representation in bar-graph showing the FRET efficiency of Hela cells transfected with the conformational FRET sensor α-catenin seeded on N-cadherin substrates. The FRET efficiency was compared in individual AJs displaying integrin β1 activation with individual AJs displaying no integrin β1 activation. Intensities: active integrin β1 negative cells: 0.5032 ± 0.0168 (N = 65), active integrin β1 positive cells: 0.3917 ± 0.01681 (N = 62). **B**) Scatter plot showing the ratio of YFP/CFP in cells displaying active integrin β1 at individual adhesions compared to cells displaying no active integrin β1. Each point represents an individual adhesion. Intensities: active integrin β1 negative cells: 1.282 ± 0.0252 (N = 45), active integrin β1 positive cells: 0.6204 ± 0.0548 (N = 62). **C**) Representative confocal microscopy images of Hela cells on N-cadherin Fc and FN during live imaging after ROCK inhibitor treatment. Cells are transiently expressing N-cadherin GFP and Talin-Fusion Red constructs. **D**) Time plot showing the fluctuations in intensities of both N-cadherin GFP and Talin-Fusion Red in cells on N-cadherin Fc and FN over time, upon ROCK inhibitor treatment. **E**) FRAP experiments of cells seeded on N-cadherin Fc showing the recovery time of both proteins: N-cadherin $t^{1/2:}$14.3 +/- 1.63 seconds, Talin $t^{1/2}$: 2.3+/- 0.64 seconds. **F**) Representative images from a confocal microscope of Hela cells at substrates composed of N-cadherin Fc coated regions adjacent to FN coated regions (left) and at substrates composed of N-cadherin Fc coated regions adjacent to block regions using BSA. Images were taken at the plane of cell attachment at the substrates and the cells were stained with active integrin β1 and β-catenin. Co-localization profiles also show the localization of the two proteins at the adjacent sites on the substrates.

5.2.7 The spatial distribution of AJs determines the deposition topology of ECM.

Previous works have shown that during development, the formation of the ECM matrix is crucial for a wide variety of developmental processes and morphogenetic movements. Such examples are the cell intercalation, convergent extension and mesendoderm migration (Mosher, 1993; Davidson, Keller and DeSimone, 2004; Rozario *et al.*, 2009). The role of ECM in vertebrate has been found to be indispensable, while its importance in cleft formation during epithelial branching, mesoderm migration and somite boundary formation has also been described (Duband and Thiery, 1982; Ramos and DeSimone, 1996; M. Marsden and DeSimone, 2001; Sakai, Larsen and Yamada, 2003; Gonschior, Haucke and Lehmann, 2020). The formation of ECM matrices is crucial for normal development and mutations in genes coding for proteins of the ECM components have been proven to associate with a wide variety of diseases such as the connective tissue syndromes, Alport and Ehlers-Danlos syndrome, muscular dystrophies and cancer (Bateman, Boot-Handford and Lamandé, 2009; Jansen, Atherton and Ballestrem, 2017). A well-studied ECM matrix is the FN matrix which forms during early *Xenopus* development at the cells of the BCR (Winklbauer *et al.*, 1992; Winklbauer and Stoltz, 1995; Winklbauer and Keller, 1996; Winklbauer, 1998; M. Marsden and DeSimone, 2001; Davidson, Keller and DeSimone, 2004; Nagel *et al.*, 2004; Rozario *et al.*, 2009; Schwarzbauer and DeSimone, 2011). Both integrins and cadherins have been reported to implicate in the formation of this matrix both *in vitro* and *in vivo*. Experiments in *Xenopus* showed that cadherins generate tension during gastrulation and these forces lead to the promotion of FN matrix assembly through Wnt/PCP pathway (Weber, Bjerke and DeSimone, 2011; Mui, Chen and Assoian, 2016). Moreover, studies in *Zebrafish* during FN fibrin formation showed that integrin α5β1 is associated with neighboring cells when integrins are in their close, inactive conformation and this is modulated by cadherins. In addition, experiments in *Xenopus* showed that integrins regulates cadherin adhesion through their binding to FN (Marsden and Douglas W DeSimone, 2003; Davidson *et al.*, 2006; Hunt and Schwarzbauer, 2009; Jülich *et al.*, 2015). This information indicates a crosstalk between integrins and cadherins during development, specifically during FN matrix formation. However, the mechanisms through which the two families of receptors communicate and their precise synergistic or antagonistic roles during this process remain highly unknown. The fact that during development, the formation of cell-cell junctions precedes the formation of cell-ECM adhesions and FAs, together with the fact that integrins become activated at AJs in a tension driven manner (in the absence of deposited ECM ligands), raises the possibility that ECM deposition topology may be influenced by this process. Interestingly. The FN fibril formation has been shown to depend on the presence of AJs and tension (Dzamba *et al.*, 2009; Rozario *et al.*, 2009).

In order to examine the possibility that AJ topology and AJ-driven integrin activation guide the topology of ECM deposition, we transiently transfected Hela cells with GFP-Fibronectin and allowed them to attach and spread on N-cadherin Fc substrates for approximately 2 hours. Within the 2 hours cells would have the ability to secrete FN, however their AJs would have not been disassembled

completely (based on the experiments described in previous sections). As shown in Figure 41 A, the first signs of ECM deposition on the glass coincide with the localization of AJs (**Figure 41 A**). This is in agreement with our suggestion, that the clustering of integrins at AJs guides spatially the deposition and accumulation of ECM. To further investigate this suggestion, we went on and generated i) substrates composed of areas coated with FN and areas coated with N-cadherin Fc and ii) substrates composed of areas coated with N-cadherin Fc and non-coated regions. These regions were adjacent to each other and allowed the examination of the spatial relationship between cadherins and integrins in the presence and absence of integrin ligands. We observed that the activation of integrins and the consequent FA formation was spatially following the clusters of AJs both on substrates where ligands (FN) were present as well as on substrates where cells were allowed to deposit their own ligands. This suggested that AJ spatial distribution guides FA formation. As shown in Figure 41 B, both receptors are linked to the same actomyosin bundle, even though they are spatially separated due to spatial separation of the respective substrates (**Figure 41 B**). Cadherin is distributed at the distal end of the cell lamellum, while active integrin is clustered along the FN-coated regions following however the spatial distribution of cadherins (**Figure 41 B**). These set of experiments suggest that integrin activation at AJs is guided by the spatial distribution of AJs and that the distribution of AJs is what determines the regions where FAs will form in the presence of ECM ligands, thus influencing ECM remodeling.

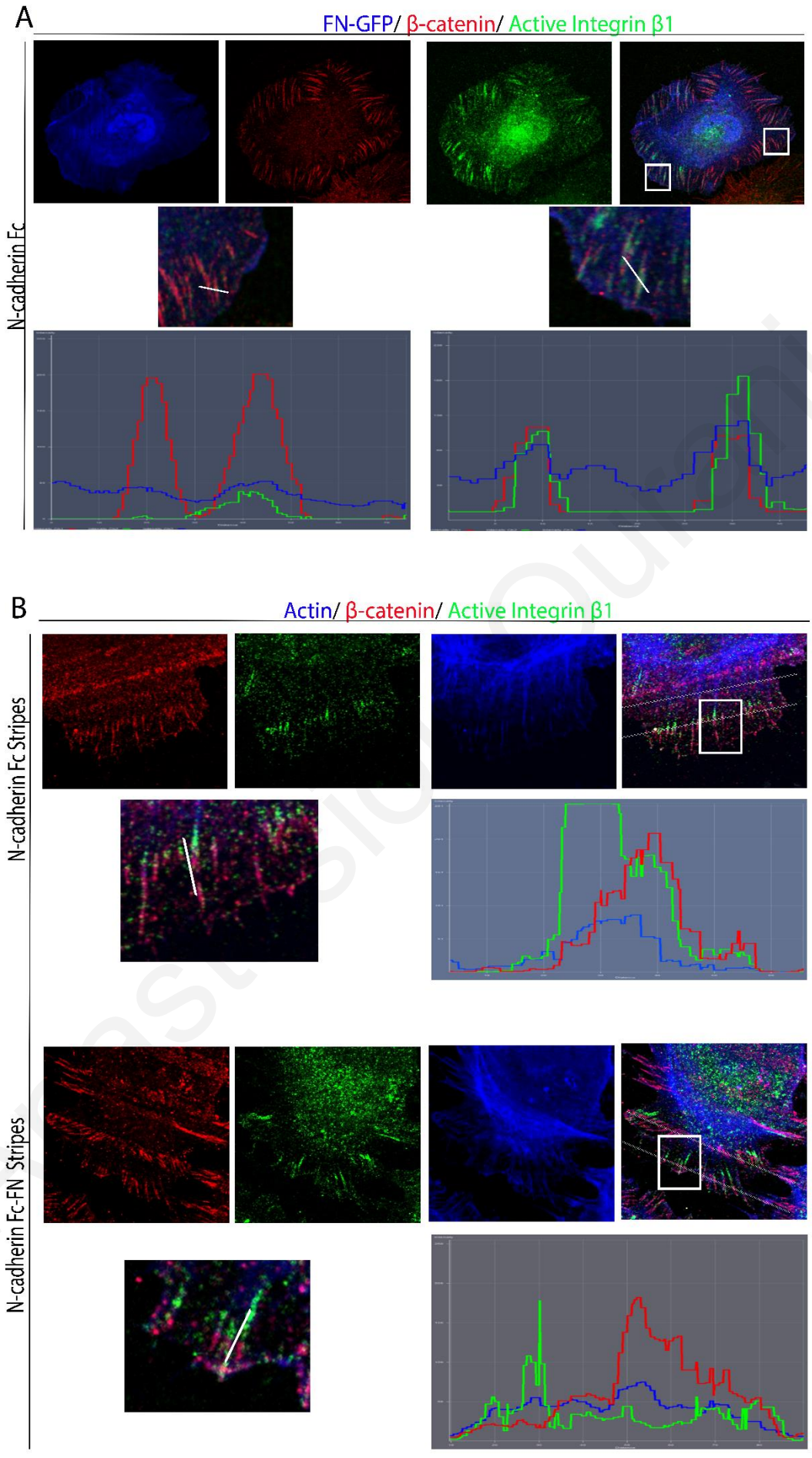


Figure 41: The spatial distribution of cadherins determines the deposition topology of ECM

A) Representative images and co-localization profile from a confocal microscope of Hela cells transfected with FN-GFP and seeded on N-cadherin Fc substrates. Hela cells were stained with active integrin β1 and β -catenin. The profiles show regions before (left) and after (right) integrin activation at AJs and the co-localization of both active integrin β1 and β -catenin with FN. **B**) Optical sections of Hela cells, at the plane of cell attachment on substrates composed of regions of N-cadherin Fc, coated and adjacent plain glass (top) or substrates composed of regions of N-cadherin Fc coated and adjacent FN regions (bottom). Cells were stained with antibodies for active integrin β1 and β -catenin and actin. The profiles show the co-localization of active integrin β1 and β -catenin with actin at different regions of the mixed substrates.

In order to gain a better understanding of this process *in vivo*, we turned into *Xenopus* embryos. As previously described, *Xenopus* is widely used for studies of the ECM formation since the FN matrix assembly in *Xenopus* takes place at the animal caps of the embryo, at the cells of BCR during gastrulation which is easily accessible. The embryo animal caps are composed of a two-cell layer epithelium (the deep cell layer and the superficial cell layer) that are held together through cadherin interactions (Winklbauer and Stoltz, 1995; Winklbauer and Keller, 1996; Winklbauer, 1998; Davidson, Keller and DeSimone, 2004; Rozario *et al.*, 2009; Ninomiya *et al.*, 2012). The process of ECM matrix assembly and more precisely the formation of FN fibrils is a well-studied process initiating prior to gastrulation. The first FN secretion is observed at the animal cap of the embryo. This results in the formation of a thick network of FN fibrils (basement membrane) at the basal region of the deep cells (Winklbauer and Stoltz, 1995; Winklbauer and Keller, 1996; Winklbauer, 1998; Davidson, Keller and DeSimone, 2004; Rozario *et al.*, 2009; Ninomiya *et al.*, 2012). Initially we wanted to examine the localization of both integrins and cadherins in *Xenopus* embryos. *Xenopus* embryos were fixed at different developmental stages and animal cap explants were harvested and stained against integrin β1 and β-catenin. We observed that at stages where no FN fibrils are formed (early stage 9 embryos), active integrin β1 was colocalized (or localized in close association) with catenin. Precisely both receptors were co-localized at the lateral and apical areas of cells, along with the actin cytoskeleton (**Figure 42 A**). This suggested that similarly to in vitro setups, integrins are associated with cadherins *in vivo* in the embryo and this association is likely maintained through the actin cytoskeleton. This also suggests that our proposed actin trapping model also exists in the embryo. After initial FN fibril formation, we observed a gradual spatial separation of cadherins and integrins. Cadherins remained at the lateral parts of the cells while integrins were found localized at the basal regions of the cells, suggesting that upon the formation of the ECM, integrins are found at the cell contacts where their ligand is present (**Figure 42 A**). In an attempt to explore the possibility that the crosstalk between the two receptor families had effects on FN fibril formation we first examined the localization of FN fibrils at different gastrula stages. It has been previously reported that the formation of FN fibrils requires a free cell surface. Taking this into consideration and the observed distribution of integrins, we predicted that the initial fibril formation would take place only at the basal-most regions of the lateral sites of the cells. As shown, the first FN fibrils observed at

stage 9.5 are found at the basal area of cells where cell-cell junctions are located, suggesting that our *in vitro* observations agree to what happens *in vivo* during FN fibril formation (**Figure 42 B-C**).

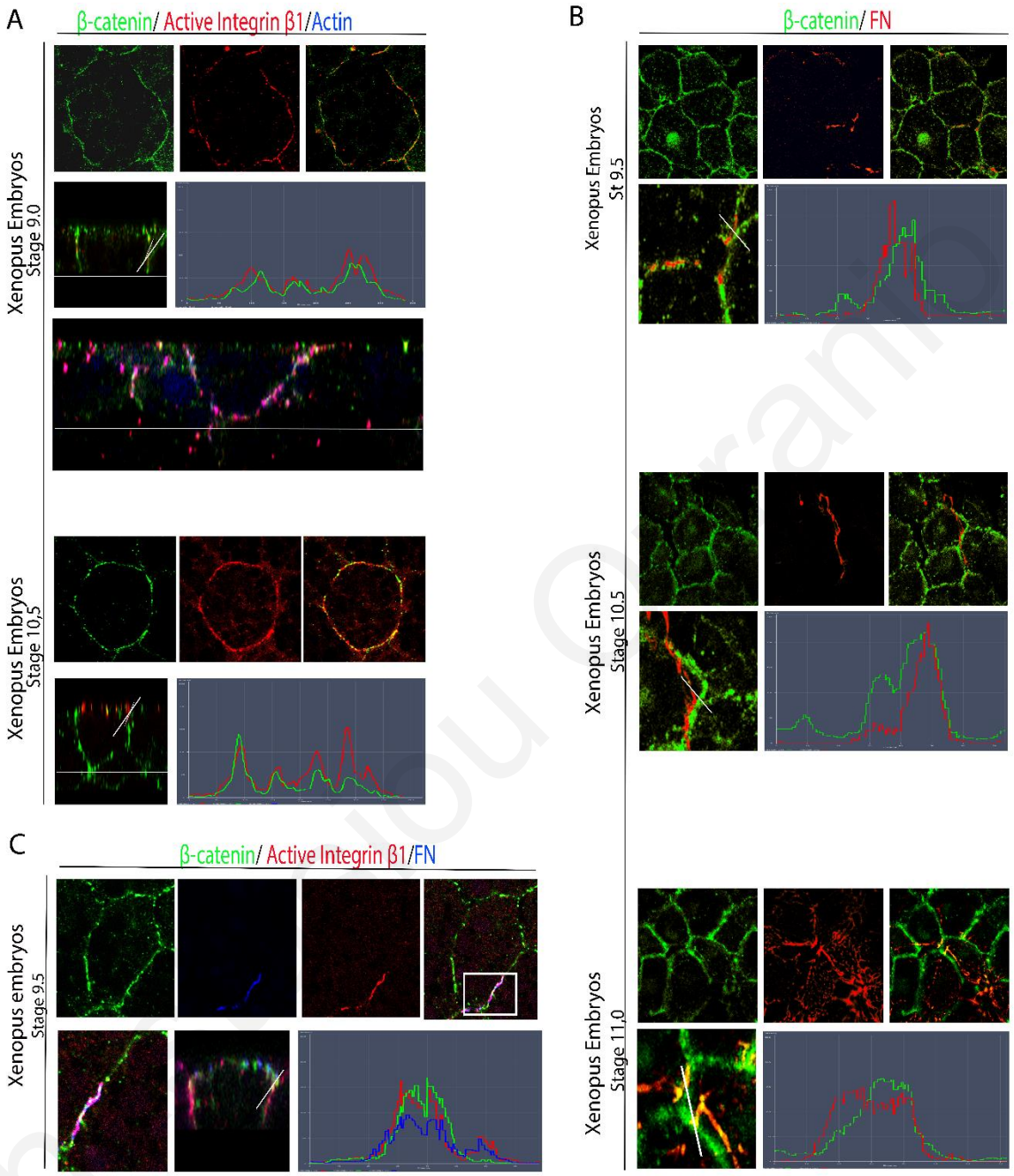


Figure 42: The spatial distribution of AJs determines the deposition topology of ECM in *Xenopus* embryos

A) Representative stacks from confocal images of *Xenopus* embryos cells at the animal cap before FN formation as stage 9.0. Embryos were stained with integrin β1 and β-catenin. Side projections of the cell the distribution of both integrin β1 and β-catenin in cells during this stage. The co-localization profile the spatial localization of these proteins. **B**) Representative stacks from confocal images of Xenopus en cells at the animal cap before and during FN-fibril formation at stages 9.5-11.0. Embryos were stained w and β-catenin. Side projections of the cell shows the distribution of both FN and β-catenin in cells duri stage. The co-localization profile shows the spatial localization of these proteins. **C**) Representative stack confocal images of *Xenopus* embryos cells at the animal cap before FN-fibril formation as stage 9.5. En were stained with FN, integrin β1 and β-catenin. Side projections of the cell shows the distribution of bo integrin β1 and β-catenin in cells during this stage. The co-localization profile shows the spatial localiza these proteins.

We moved on to examine if integrin activation at AJs and hence the guided ECM deposition that was driven mainly through tension *in vitro,* was taking place in the *in vivo* model as well. After removing the vitelline membranes of embryos at pre-gastrula stages (St8), tension was applied without altering the shape of the embryo. Embryos were fixed and stained against FN and catenin. As shown, control embryos at st10 display fibril formation at sites where AJs are generated (**Figure 43 A**). In contrast, embryos in which tension was applied, display progressed fibrillogenesis and formation of a FN matrix (**Figure 43 A**). This provides another indication that the FN deposition topology is dependent on the tension applied to the cell at the sites where AJs are formed. Next, we utilized the N-cadherin ΔCP mutant in an attempt to reduce actomyosin contractility at AJs and examine any potential effects on the FN fibril formation. The most abundantly expressed cadherins during gastrulation are E- and C-Cadherin, hence expression of the N-cadherin mutant could potentially lead to the enhancement of AJs, with no effects on tension reduction. In order to avoid that we decided to inject one out of 2 blastomeres of 2-cell stage embryos with the DNA encoding wild type N-cadherin and the other blastomere with the mRNA of the N-cadherin ΔCP mutant. DNA injections resulted in mosaic expression of the exogenous DNA and we expected that some cells eventually would express both constructs. This would presumably allow us to observe cells expressing both N-cadherin wt and the mutant and observe any defects on FN fibril formation. Embryos were fixed at stages known to display well-defined FN matrix (st10.5) and stained against FN and catenin. In order to visualize injected cells, we used Dextran as a lineage tracer. We observed that cells expressing the mutant displayed defective fibrillogenesis and side views of these cells revealed that no fibril formation was observed at the expected regions, in contrast to control cells displaying normal FN matrix formation (**Figure 43 B**). Overall, these experiments suggest that tension is what drives FN fibril formation at

those sites from the initial activation of integrin at AJs, in agreement with what we observed in the *in vitro* situation.

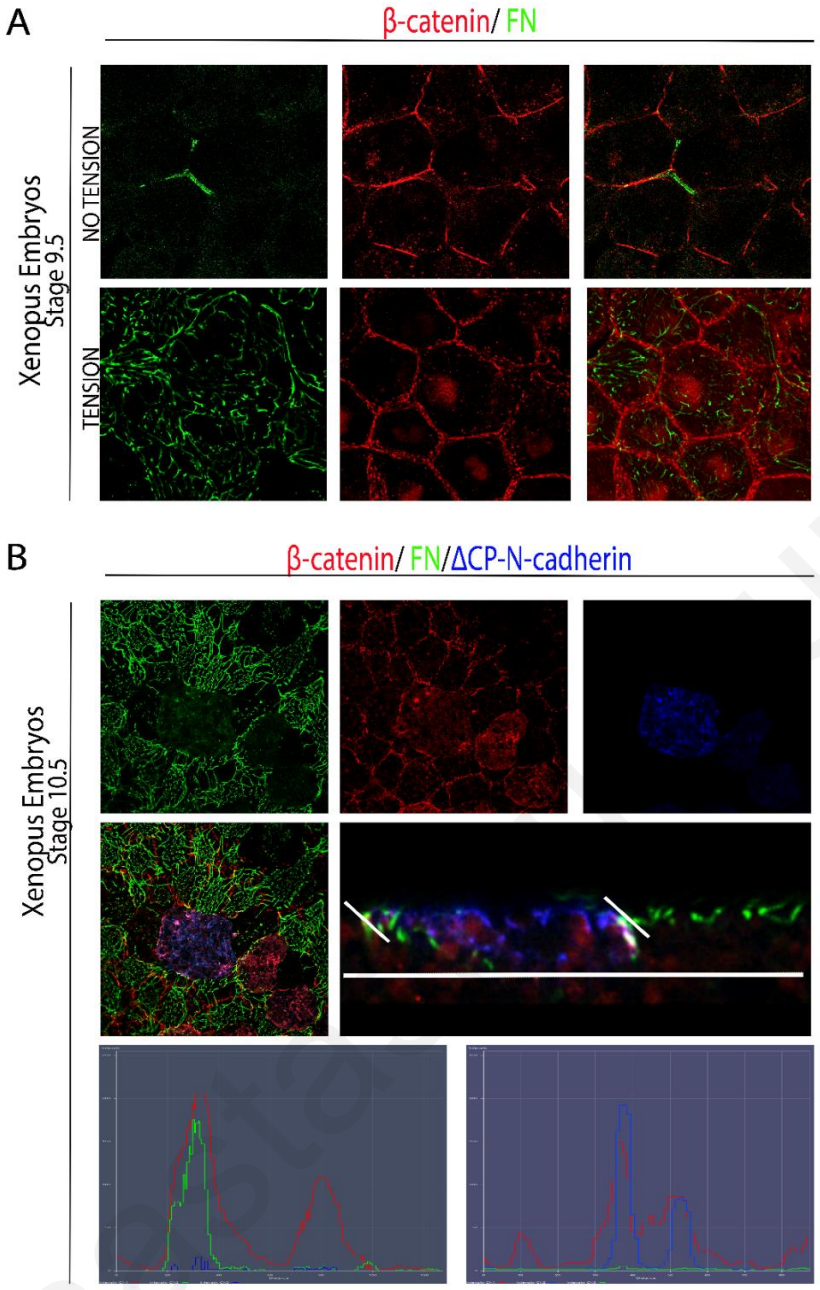


Figure 43: The distribution of AJS determines the deposition topology of ECM in *Xenopus* and is tension driven

A) Representative stacks from confocal image of *Xenopus* embryo cells at the animal cap during FN fibril formation at stage 10.5. At stage 8, prior fibrillogenesis, the tension was applied to the embryos. Embryos were stained with integrin β1 and β-catenin. Side projection of the cell shows the distribution of both β-catenin and FN in cells during this stage and co-localization profile showing the spatial localization of these proteins. **B**) Representative stacks from confocal images of *Xenopus* embryo cells at the animal cap during FN fibril formation at stages 10.5. Embryos were injected with N-cadherin wt GFP, N-cadherin ΔCP-GFP and stained with FN and β-catenin. Side projections of the cells show the distribution of both FN and β-catenin in cells during this stage and co-localization profile showing the spatial localization of these proteins.

5.3 Discussion Chapter II

One of the most exciting findings of this project was that integrin β1 becomes activated at AJs in non-polar cells on N-cadherin Fc coated substrates. This finding was extremely intriguing since the two adhesion systems are known to be spatially segregated within cells and tissues and it was the first time that active integrin β1 was shown at cadherin-adhesion sites. Years of research have established that the integrin adhesions and AJs share a lot of major protein members. This sharing of components is a part of a cross-talk between different systems and is defined as the communication at the molecular level of different signaling pathways. The fact that both integrin- and cadherin-adhesion systems directly interact with Rho-GTPases, share components downstream of their adhesion receptors, together with the fact that they are both linked to actomyosin bundles (which modulate their stability) suggest that they are perfect candidates for a signaling crosstalk mechanism (Marsden and Douglas W. DeSimone, 2003; Weber, Bjerke and DeSimone, 2011; Chen *et al.*, 2012; Tseng *et al.*, 2012; Toh, Xing and Yu, 2015; Wang *et al.*, 2015, 2019; Langhe *et al.*, 2016; Mui, Chen and Assoian, 2016). For example, vinculin is the most well-characterized protein-member of both integrin adhesions (FAs) and AJs. At FAs, Vinculin directly interacts with Talin and is involved in bearing forces applied at FAs and therefore regulates FA dynamics. At AJs, upon tension application, vinculin binds to a-catenin and re-enforces the stability of these complexes. It was found that the phosphorylation of vinculin on Y822 is required for its association with AJs (Bays *et al.*, 2014). While, phosphorylation of vinculin on Y100 and Y1065 regulates the transition of force from the ECM to FAs and actin cytoskeleton (Auernheimer and Goldmann, 2014; Bays *et al.*, 2014). These phosphorylation requirements suggest a mechanism through which spatial regulation of vinculin, at FAs and AJs, is achieved. FAK, another FA protein, has been found associated with the AJs through the promotion of E-cadherin cell-cell adhesions and its association with VE-cadherin. This interaction was shown to be associated with the disruption of the β-catenin/VE-cadherin complex and lead to a decrease in AJ stability (Chen *et al.*, 2012). Paxillin in combination with FAK has also been associated with the assembly of N-cadherin dependent junctions which was shown to inhibit cell migration (Yano *et al.*, 2004). Studies showed that Src phosphorylates β-catenin at the AJs sites at Y654 and this phosphorylation was associated with the unbinding of catenin to E-cadherin leading to the disassembly of AJs (Gayrard *et al.*, 2018). Other studies showed that Src is a downstream target of E-cadherin and its activation leads to a positive loop through the PI3K signaling which was found to promote cell-cell contacts. Increased activity of Src was associated with negative results on AJs since it promoted their disruptions (McLachlan *et al.*, 2007). Cadherin-11 found co-localized with integrin β1 and paxillin and found to interact directly with syndecan 4, an FN binding protein unraveling a novel role of a cadherin family protein in the FAs and providing further evidence regarding the direct interaction between proteins of FAs and AJs (Langhe *et al.*, 2016). Lastly, FA proteins such as VASP, tes and Zyxin have been found to be implicated in AJs in a vinculin independent role and to modulate the dynamics of AJs (Oldenburg *et al.*, 2015). Besides the

interactions of FA proteins at AJs or AJ proteins at the FAs, studies also suggested a direct communication between integrins and cadherins in terms of their biochemical and signaling organization in cell and tissue level. This orchestrated cross talk was deriving from a shift in actomyosin contractility at bundles associated with both of these adhesion systems (Toh, Xing and Yu, 2015; Ng *et al.*, 2014; Mertz *et al.*, 2013; Ouyang *et al.*, 2013).

This project focused into identifying the mechanisms underlying integrin β1 activation at AJs. By taking advantage of previously characterized N-cadherin Fc coated substrates and micropatterns we observed that activation of integrin β1 is associated spatially with core-members of the AJs (β-catenin and N-cadherin). We observed that activation of integrin β1 takes place 1 hour after cell seeding on N-cadherin substrates and gets stronger over time. We also showed that this activation is present at AJ at sites where actin bundles are terminating. To preclude the possibility that this observation was a result of the artificial nature of AJs on the glass surfaces, we utilized PLL fused polyacrylamide gels and created cell doublets and showed that during doublet formation, active integrin β1 is observed that the cell-cell contacts where a central ring is formed. Thus, suggesting that the observation regarding integrin β1 activation is universal and not a result of the artificial nature of the *in vitro* system that we were using. In order to gain a better temporal understanding of the relation between integrin β1 and AJs we performed time-course experiments of Hela cells on N-cadherin Fc and observed that the activation of integrin β1 initiates as small puncta at points surrounding the AJs at the inside of the cell (away from cell labellum) at 45 minutes and they extend with time progression along the AJs at 60 minutes. This integrin activation found to be associated with the formation of AJs. Overall, this experiment suggests, that the spatial distribution of AJs determines the spatial activation of integrin β1. These results are supported by experiments performed using N-cadherin Fc striped micropatterns which showed that activation of integrin β1 appeared only at the regions where N-cadherin was concentrated (at the N-cadherin Fc stripes). Overall, these experiments show that activation of integrin β1 occurs at the sites where AJs. They also show that this activation is spatially associated with the AJ complexes and suggests that this is a stepwise process which is defined by the formation of AJs. In order to gain a better understanding of the spatial relationship of the receptors, deconvolution experiments using high resolution confocal microscopy experiments were performed. It was observed that integrins and cadherins within the same adhesion display distinct spatial relationships. Thus, proposing a mutually exclusive topology. This observation was supported by quantification of colocalization coefficient of integrin β1 activated with both N-cadherin and iβ-catenin at those sites. The fact that integrin activation is the first step to the connections of cell to the ECM, and the fact that integrin activation results to the recruitment of downstream FA protein members at the cell-ECM sides, led us to the question whether integrin activation at the sites of AJs was promoting other FA proteins as well. The plethora of studies showing the implication of FA proteins at AJs and their association with AJ components, was another reason why we wanted to examine the possibility that these proteins are recruited to these sites (Avizienyte *et al.*, 2002; Chen *et al.*, 2012; Oldenburg *et al.*, 2015; Wang *et al.*, 2019). To do that, cells on N-cadherin Fc at different

timepoints were stained against different proteins of the FA complexes (talin, vinculin, tensin, FAK and paxillin) and their localization with both active integrin β1 and AJ proteins was assessed. With the help of colocalization coefficient quantifications and the conduction of co-localization profiles using imaging software, we showed that all proteins are found at those sites upon the activation of integrin β1. However, the surprising finding was that the recruitment of vinculin and tensin was temporarily altered from their known localization at FAs. Something that suggests that the complex formed at those sites displays differences with the FA complex formation and so we called it HAs. This could presumably mean that the recruitment of these proteins at those sites is a result of integrin activation and not through their direct interaction with cadherin adhesion components. It could also mean that the complex formed upon integrin activation at AJ sites displays differences with the complex formed upon integrin activation at cell-ECM contacts. With the use of different α integrin subunits, we moved on to test the localization of major α integrin subunits at HAs. Different α integrin subunits were localized in close proximity with AJ components at HAs suggesting that the ligands of different integrin subunits may also be present at the AJs. Even though the cells were plated on cadherin substrates under serum free conditions and the time interval provided for their spreading was not sufficient for ECM ligand deposition, we wanted to further examine this possibility. With the use of different antibodies for the major integrin ligands, FN, laminin and collagen we observed that no localization is present at the HAs. This suggested that integrin activation at the AJs is not associated with any ECM deposited ligand. To further support this, we performed experiments using inhibitors of Golgi secretion and protein synthesis which showed that integrin activation on N-cadherin Fc was not affected. Overall this set of experiments clearly shows that the cadherin-based adhesion sites between adjacent cells elicit the activation and clustering of integrin and as a result leads to the formation of an FA-like complex, the HAs. Our results also show that the activation of integrin β1 at the sites of AJs and the subsequent formation of the HAs occurs in the absence of any detectable, deposited ligand.

Integrin activation exists in different conformations of the integrin molecules and each conformation is associated with different affinity for their ligands (Takagi and Springer, 2002; Wiesner, Legate and Fässler, 2005; Humphries, Byron and Humphries, 2006; Byron *et al.*, 2009; Anthis and Campbell, 2011; Campbell and Humphries, 2011; Elizabeth M Morse, Brahme and Calderwood, 2014; Kechagia, Ivaska and Roca-Cusachs, 2019). It has also been proven that integrins have the ability to become activated in the absence of deposited ligands through ECM, with through the application of tension (Maria and Ferraris, 2010; Petridou, Stylianou and Skourides, 2013; Ferraris *et al.*, 2014; Petridou and Skourides, 2016; Kim *et al.*, 2020). This mode of integrin activation was first observed during mitotic cell division and it was shown to be associated with the recruitment of other FA proteins at those sites leading to the formation of a so-called CMC. This complex was found indispensable for the proper orientation of mitotic spindle. The conformation of integrins at CMC was found to be significantly different from the one observed at the FA sites (Petridou and Skourides, 2016). Considering, that the recruitment of FA proteins at the sites of AJs display differences with

the recruitment observed at FAs and considering the fact that cells were spreading under serum-free conditions for a short period of time, we speculated that the mode of integrin activation at the AJs might display differences from the one observed at the conventional FAs. Thus, we moved on to examine the conformation of integrins at those sites and its similarities with integrin activation observed at the FAs sites. Antibodies recognizing distinct states of integrin activation are well characterized (Byron *et al.*, 2009; (Cormier *et al.*, 2018). Using these antibodies and comparing the cells on N-cadherin Fc with the cells on FN, we observed major differences in their signal intensities at the different complexes (AJs and FAs respectively). Antibodies recognizing the so-called extended head-piece close conformation of integrins were predominantly found at AJs. Antibodies recognizing the extended open head-piece conformation were found at FAs but they were absent from AJs. This extended open head-piece conformation has been associated with ligand binding (Cormier *et al.*, 2018). All these together showed that the active integrin β1 at the AJs displays a different conformation compared to the one observed at the FAs and suggested that the mode of integrin activation at AJs does not necessarily requires ligand binding. Since the tension driven activation was previously characterized in the CMC (Petridou and Skourides, 2016), we speculated that CMC and HAs share similarities in terms of the mode of integrin activation and the conformation of integrins. In order to clarify that, we moved and used conformational specific antibodies to compare the integrin conformation state observed at the HAs, FAs and at the mitotic cell cortex. Our results showed similarities between integrin conformation state at the mitotic cell cortex and HAs. In contrast to these, integrin conformation state at FAs varied greatly. This led us to the conclusion that integrin conformation state at HAs is different from the one observed at FAs and represents an intermediate state of activation, similarly to what has been described for integrin activation at the CMC (Petridou and Skourides, 2016). To further support this, we utilized a construct composed of integrin β1 tail fused to membrane binding sequence and GFP and observed its localization at AJs sites even in the absence of endogenous active integrin β1. This is a strong indication that the external ligand binding of integrins is not a prerequisite for the clustering of integrin β1 at the sites of AJs and suggests that actomyosin bundles terminate at AJs trap and cluster activated receptors at the vicinity of AJs. These results, in combination with the comparison of integrin activation state on interphase and mitotic cells, suggest that the state of integrin activation and the integrin conformation are different and occur in the absence of ligands. However, further characterization of the core-protein members of the CMC is required in order to compare the stoichiometry of CMC, HAs and conventional FAs. This could be achieved using proteomic approaches such as MS/MS analysis through which we could potentially identify the members of the distinct complexes and characterize each protein member separately. Besides, other commercially available antibodies can be used in order to explore the precise conformational state of integrin activation at the sites of AJs and compare it to the one observed at FAs and during mitosis. Experiments using confocal microscopy and deconvolution revealed that HAs and AJ-associated integrin activation are spatially segregated from AJ components. This suggests that integrin activation may play a role in the modulation of AJs'

dynamics. Experiments using characterization of the relationship over time of active integrin β1 and β-catenin, inhibitory antibodies against integrin and a well-characterized integrin inhibitory protein (SHARPIN) (Rantala *et al.*, 2011) agreed with our previous notion. These experiments also suggested that the role of integrin activation at those sites are associated with the disassembly of AJs and supported the notion that integrin activation modulates AJs dynamics.

It is well documented that the disassembly of AJs is largely dependent on endocytosis and endocytic vesicles which in turn depend mainly on the intact microtubule network (Kamei *et al.*, 1999; Lu *et al.*, 2003; Bryant and Stow, 2004; Chilov *et al.*, 2011; Byron *et al.*, 2015). Microtubule network has been shown to target FA complexes but not AJs at cells attached on N-cadherin Fc (Kamei *et al.*, 1999; Lu *et al.*, 2003; Bryant and Stow, 2004; Chilov *et al.*, 2011; Byron *et al.*, 2015). Taking these into account, in combination with our evidence showing that integrin activation is associated with the disassembly of AJs, we wanted to explore the possibility that the MT network and endocytosis have a role in AJs' disassembly. Using antibodies against MT network, we showed that the targeting of microtubules at AJs occurs after integrin activation which presumably means that this connection is involved in the turnover of AJs. This assumption was further supported by the inability of cells to disassemble their AJs upon Nocodazole D treatment which disrupted the MT network. These experiments provided further support to the notion that integrin activation at AJs guides AJ disassembly and suggested that this disassembly requires an intact MT network. Since the intact microtubule network is a prerequisite for the turnover of AJs we moved on to examine a precise role of endocytosis in this process. Experiments using markers of endocytic vesicles showed that the endocytic vesicles are localized at the sites of AJs only upon integrin activation. This suggested that integrin activation guides the endocytosis at the sites of AJs. This suggestion was further supported with the use of an endocytosis inhibitor which led to the strengthening of AJs and their inability to disassemble. It would be interesting to carry out experiments that will reveal proteins associated with the disassembly of AJs downstream of β1 and the precise mechanisms of endocytosis and/or degradation in the disassembly of AJs. It also would be interesting to examine the role of clathrin mediated endocytosis in this process. It has been proposed that E-cadherin can be internalized through different endocytic pathways depending on the cellular context (Brüser and Bogdan, 2017). It has been also shown that E-cadherin contains an AP-2 motif which is associated with clathrin and mutations at this domain have been associated with prevention of clathrin endocytosis of E-cadherin (Brüser and Bogdan, 2017). It would be extremely interesting to examine the possibility that this endocytic pathway is also involved and/or associated with the turnover and disassembly of AJs. Apart from these, it has been proposed that p120-catenin acts as an inhibitor of cadherin endocytosis (Kiss, Troyanovsky and Troyanovsky, 2008; Bulgakova and Brown, 2016). Experiments in cultured cells have shown that in the absence of p120-catenin, cadherins are internalized rapidly and are degraded (Xiao *et al.*, 2005). We could perform experiments using p120-catenin mutants in order to explore any possible relation between the removal of p120 catenin from these sites and the activation of integrin which eventually will lead to the disassembly of AJs. The role of proteins such as Calpain

and Hakai in AJ disassembly has been proven before. Using different approaches, we could explore further their association with AJ driven integrin activation at those sites. This could be achieved with experiments using Calpain inhibitors or DN constructs for both proteins. Apart from these, disrupting the function of Calpain downstream targets could presumably allow us to identify the precise mechanism behind Calpain-driven AJ disassembly and examine the effects of integrin activation under these conditions. The fact that HAs displayed major differences with FAs in terms of protein recruitment, integrin conformational state and integrin mode of activation had driven us to try and understand the mechanism through which integrin becomes activated at HAs. To do that, different mechanistically approaches were used. Cadherin Fc micropatterns showed that the clustering of cadherins was guiding integrin activation. This observation is further supported by experiments using mutants of cadherins lacking the cytoplasmic domain and hence the ability to bind to the actin cytoskeleton, an inhibitor of actin polymerization known as Cytochalasin D and FRAP experiments. These experiments collectively showed that integrin activation was lost from the sites of AJs at a faster rate than the one of cells on FN suggesting that the integrin activation is dependent mainly through the actin cytoskeleton at those sites. These results agree with our previous results observed with integrin tail mutant. These experiments also suggest that the clustering of cadherins is not sufficient to maintain integrin activation at HAs. Overall, these results show that when the immobilized ligand is present at the sites of FAs, then the clustering and activation of integrins is mainly depended on the ligands and not the actin cytoskeleton. All these together strengthen our notion that the integrin activation on AJs is not based on a deposited ligand.

Previous studies including work from our laboratory, showed that integrins can become activated through the application of force in the absence of ligands (Maria and Ferraris, 2010; Ferraris *et al.*, 2014; Petridou and Skourides, 2016; Kim *et al.*, 2020). This notion was also supported by later data showing that deformation of the membrane through force application leads to the activation of integrins (Kim *et al.*, 2020). Taking these into consideration, we wanted to explore the possibility that integrin activation at AJs, where high tension is applied, depends on force application. The suggestion that the activation of integrin at AJs is associated with tension and actin trapping mechanism underlined from mechanistic approaches where tension was eliminated from the sites of AJs. With the utilization of a conformational catenin sensor and the elimination of the tension applied to these sites through ROCK inhibitor we showed that the activation of integrins at AJs is highly associated with the tension applied to those sites. The fact that cells on FN displayed slower rates of integrin (talin) removal from those sites also suggests that the integrin activation at FAs sites is based on the deposition of ligands while on AJs is mostly through force and/or soluble undetectable ligand. These results are further supported by experiments performed on micropatterned surfaces composed of regions coated with N-cadherin Fc adjacent to regions coated with FN. These experiments showed that the activation of integrin was following spatially the clustering of cadherins and both of them were associated with the same actomyosin bundles terminating at the sites of AJs. The fact that AJ sites are highly associated with tension is generally known, considering this and in combination with

the differences observed in the activation of integrin at sites of AJs with FAS we can conclude that during AJ formation and cadherin clustering, the actomyosin bundles terminating at those points and the actin cytoskeleton-associated at those sites is what guides to the integrin activation. More precisely, we suggest that a pool of activated integrins becomes trapped through this thick actin network and localized to these sites in the absence of any detectable deposited ligand. This pool of integrins becomes active upon tension application at the AJs. This tension derives from the thick actomyosin bundles that are present in these regions.

The formation of ECM matrix is crucial for normal development and mutations at genes coding for proteins of the ECM matrices are associated with a wide variety of diseases such as cancer and different rare syndromes (Bateman, Boot-Handford and Lamandé, 2009; Jansen, Atherton and Ballestrem, 2017). A well-studied ECM matrix is the FN matrix which is observed early during *Xenopus* development at the cells of the BCR (Winklbauer *et al.*, 1992; Winklbauer and Stoltz, 1995; Winklbauer and Keller, 1996; Winklbauer, 1998; M. Marsden and DeSimone, 2001; Davidson, Keller and DeSimone, 2004; Nagel *et al.*, 2004; Rozario *et al.*, 2009; Schwarzbauer and DeSimone, 2011). Both integrins and cadherins have been reported to be implicated in the formation of this matrix both *in vitro* and *in vivo* however, the mechanisms through which these two families of receptors communicate between them and their precise synergistic or antagonistic roles during this process remain highly unknown. It has been proposed that integrin α5β1 heterodimers are associated with each other on neighboring cells when integrins are in the closed, inactive conformation. N-cadherin was shown to be a major factor for the stabilization of this inactive form of integrins and the inhibition of FN fibril formation. Downregulation of N-cadherin resulted in activation of integrins and subsequent formation of FN matrix (Marsden and Douglas W DeSimone, 2003; Davidson *et al.*, 2006; Jülich *et al.*, 2015). These data suggested that the differential molecular interactions and differential strength of adhesion are what gives cadherins these different roles during FN matrix assembly. Experiments by Dzamba et al. suggested that the non-canonical Wnt/PCP pathway has a role in this process (Dzamba *et al.*, 2009). This work, together with experiments performed in *Zebrafish* suggested a model for FN fibril formation, in which changes in cell-cell adhesion from cadherins, result in the reorganization of the actin cytoskeleton. This event, was shown to be dependent on Rac and Pak and found indispensable for the translocation of integrins to bound FN at cell-cell contact sites where the matrix formation is initiated. It was also proposed that adherens junctions have a role similar to the role of FAs in this procedure and generate tension on integrins, necessary to expose binding sites within FN (LaFlamme, Akiyama and Yamada, 1992; Dzamba *et al.*, 2009). Taking into consideration the fact that integrin activation at AJs occurs in a deposited ligand independent but tension dependent manner, leads to the consequent recruitment of FA proteins and to the disassembly of AJs, we suggest that the spatial deposition of the ECM is affected by this process. With the use of exogenous FN-GFP construct and patterned micropatterns we showed that the spatial deposition topology of ECM is guided by the AJ spatial distribution. This agrees with *in vivo* studies that show the association of both cadherin and integrins in the formation of ECM matrix

and more precisely the FN fibrin formation during *Xenopus* development. Using *in vivo* approaches with *Xenopus* embryos we moved on to show that the precise sites of initial FN fibril formation follow the AJs spatial distribution. We also showed that integrin activation is firstly observed at sites where cell-cell exist. Upon FN fibril formation, integrins are found at regions where their ligands are present. This provides further evidence to our notion that the initial activation of integrin at cell-cell contact sites of AJs is associated with actin cytoskeleton. This led to us to the conclusion that similarly to what observed *in vitro*, the first FN fibrils and the deposition of the ECM are guided by the clustering of integrins which is in turn driven by the clustering and formation of AJs. Our data also show that the ECM deposition topology is guided by the spatial distribution of AJs. The use of tension application and cadherin mutant lacking cytoplasmic tail also showed that the fibril formation is affected by disruption of tension and actin connections suggesting that similarly to the *in vitro* model the tension through actomyosin bundles is crucial for this process.

5.4 Conclusions Chapter II

In this section we showed that integrin β1 becomes activated at the sites of AJs. This activation occurs in a stepwise fashion, initiating as intracellular small punctuated clouds forming away from the cell labellum and in vicinity with AJs and extend overtime forming linear structures along pre-existing AJs. This activation was found to be spatially segregated from AJs and the spatial relation between the two structures varied within individual adhesions. The fact that integrin β1 became activated at AJs, led to the suggestion that, like in FAs, downstream FA protein members might be recruited at those sites. This was shown using confocal microscopy and co-localization profile generation and quantifications. However, the recruitment of proteins in response to the spatial distribution of active integrin β1 displays significant differences with what is observed at FAs. Further experiments using inhibitors for Golgi secretion, Protein synthesis and antibodies against ligands of ECM showed that the activation of integrin at those sites was not associated with the presence of deposited ligand displaying similarities to the mode of activation and integrin conformation that was observed at the cell cortex during mitosis (Petridou and Skourides, 2016; Maria and Ferraris, 2010; Ferraris *et al.*, 2014; Petridou and Skourides, 2016; Kim *et al.*, 2020). These observations were further supported with experiments using different conformational specific antibodies. We moved on and identified the precise role of integrin β1 activation at those sites through microtubule targeting, integrin inhibition ans inhibition of endocytosis. We showed that upon integrin activation, MTs are targeted to the AJs, endocytic caveolin-1-based vesicles localize at AJs and AJs disassemble. Additionally, we used different mechanistic approaches and identified the precise mechanism through which AJ-associated integrin β1 activation was taking place at those sites. We confirmed that upon cadherin clustering, integrin becomes activated at those sites and this activation was based on the intact actin cytoskeleton and actomyosin bundles which apply tension to these sites. We also provided further evidence regarding the differences of integrin-binding at those sites in comparison with integrins present at the FAs. We showed that integrin activation at those sites is associated and based on cadherin clustering and occurs through the formation of a thick actomyosin bundle network at the sites where AJs are terminating. This network found to lead to the trapping of integrins, their clustering and their activation at AJs. This process was shown to be tension driven and suggesting that deposition of ligands is not a prerequisite for this process. Lastly, we showed that the activation of integrins at those sites led to the guidance of the ECM deposition both *in vitro* and *in vivo* in *Xenopus* embryos. The FN matrix assembly was found to depend on the tension applied at those sites during development in agreement with our results in our *in vitro* setups.

The fact that HAs display major similarities with the CMC complex observed during mitotic cell division is a fascinating suggestion since it would provide evidence regarding the roles of the protein members in processes that are adhesion independent. It would be extremely interesting to examine through proteomic analysis the components of the CMC complex identify them and explore their similarities with HAs. Preliminary data also suggested that some members of the CMC are also

present during apical constriction in *Xenopus* and it would be fascinating to find an association and a precise involvement of the protein members of the CMC there with the proteins at the HAs. During AC in *Xenopus*, extreme force application and cadherin enrolment guide this morphogenetic movement. The cells acquire a bottle-shaped shape displaying apicobasal polarity and shrink. It was previously shown that this morphogenetic movement is a result of cell-autonomous and asynchronous contraction pulses followed by cell-autonomous Ca^{2+} pulses. These events were found to be driven by a contractile actin pool, and hence tension application in those cells. It was also shown that the contraction of a cell increased the probability for a neighboring cell to contract suggesting that a mechanical cross-talk between the cells of the neural plate was taking place. Preliminary data suggest that CMC may have a role in this process since integrin β1 was found to increase in both cells undergoing constriction and their neighboring cells. Taking into consideration the relation between actin, integrin activation and AJs shown in this project and the fact that phosphorylation of CMC core protein members found at those cells it would be interesting to identify if the complex involved in all these processes that are a result of an increased applied tension to the cells and tissue is same. It would also be of high interest to expand these observations in other mammal model organisms like mouse in order to compare the process of ECM deposition and the major protein players. This will provide us with a better understanding of this procedure and acquire knowledge regarding the conservation of the complexes involved in it.

6. Future Work

This project is separated in two major chapters. The first one, shows that the mitotic cell responses to substrate topological cues are independent of the molecular nature of adhesion and highlights the role of the CMC in this process. The second one, connects the two major metazoan adhesion systems; FAs and AJs. It suggests that AJs under high tension lead to localized activation of integrins in the vicinity of the AJs. It also suggests that integrins at AJs becomes clustered and activated via an actin trapping mechanism. Lastly, it shows that integrin activation leads to the subsequent recruitment of additional FA proteins and leads to AJ disassembly. This disassembly results in ECM deposition which is determined by the spatial distribution of AJs.

Numerous different experiments can be proposed in order to further characterize and understand these two distinct processes. Some of them are briefly described in this section:

a) **Characterization of the CMC proteome and comparison to FAs and/or HAs:**

It was previously stated that the CMC complex is composed of bona-fide FA protein members. It would be extremely interesting if we moved on to compare the FA proteome with that of the CMC and determine any possible context dependent variations. This will allow us to understand this complex and gain an in depth understanding of its assembly and function. Initially, we need to separate proteins participating in the formation of the CMC from the ones at FAs. CMC has been shown to be assembled upon integrin β1 activation. We will take advantage of this to immunoprecipitate the complex. We will apply double thymidine block in order to synchronize Hela cells during mitosis and/or during interphase (to separate the 2 complexes) and will allow cells to attach and spread on different substrates. Cells will be seeded on substrates in which integrins can be activated in a force and ligand-dependent manner (FN), and on substrates on which they can be activated in a force dependent but ligand-independent manner (N-cadherin Fc). Substrates on which cells remain inactive (polylysine) will be used as controls. We will keep the cells under serum free conditions in order to avoid ECM deposition. After attachment, cells will be crosslinked either with formaldehyde or through the use of photo amino acids. Lysates will be generated from mitotic and interphase cells and will subsequently be immunoprecipitated using β1 specific antibodies. This approach will allow enrichment of immunoprecipitated fractions of proteins participating in the CMC from mitotic cells. Immunoprecipitated proteins will then be digested and analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS). The data that will derive from this analysis will be assessed in terms of their ability to directly interact with integrin in the CMC. The amount of proteins derived from this analysis, will be assessed and the predominant proteins are going to be validated. The initial validation of the proteins is going to be performed using immunofluorescence experiments. Mitotic cells on FN are going to be used for these experiments and the ability of the candidate proteins to localize at the cortex of the mitotic cells is going to be assessed. Finally, we aim

to compare the proteome of the CMC in mitotic cells to the proteome of the AJ-guided CMC and the FA proteome. Both the CMC and the HAs are formed upon membrane tension. The conformational state of integrins at the two complexes displays similarities. Their stoichiometry in respect to the already characterized CMC proteins, also shares similarities. Thus, we aim to compare the proteinic composition of these complexes with the complexes at the conventional FAs in respect to the newly identified proteins. All these proteins will be examined with respect to their recruitment to linear AJs (HAs) using immunofluorescent experiments. The identified proteins will be presented on a map and compared to the already existing FA proteomes.

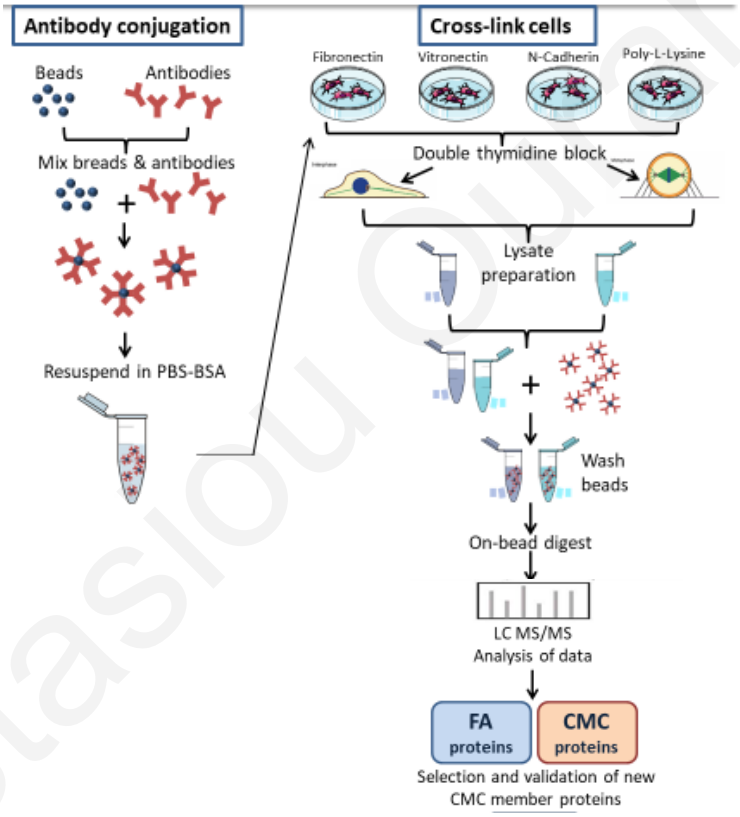


Figure 44: Flow chart of the strategy of CMC proteome determination

Schematic representation of the major stages for the CMC proteome determination

b) Investigate the mechanism through which CMC affects spindle capture on the cortex.

As stated above, the precise mechanism by which this CMC polarization at the spindle capture sites regulates the capturing of the spindle and its alignment with external forces is still unknown. Preliminary data from our group (Unpublished data) and work from Fink. et al. showed that a subcortical actin clouds (SAC), have the ability to reorient upon retraction force distribution (Fink *et al.*, 2011). Recently, Kwon et al. showed that these SAC regulate spindle orientation through astral microtubule capturing, in a myosin10 depended manner

(Kwon *et al.*, 2015). Rac1 is a well-documented actin cytoskeleton regulator and previous studies have implicated Rac1 in spindle orientation. These data together with the evidence that Rac1 is activated upon p130Cas phosphorylation might provide a solid indication that SAC is depended or regulated by the CMC. This suggest that a possible mechanism, through which CMC controls proper positioning of the mitotic spindle, through the formation of this Rac1 depended SAC, which guides the capture of the spindle through myosin 10 mediated interactions with astral microtubules.

Initially we aim to investigate the role of CMC proteins in the regulation of the SAC observed in mitotic cells. We will take advantage of integrin β1 blocking antibodies, like AIIB2, in order to eliminate any possible recruitment of the CMC proteins in these cells. Previous data from our lab suggest that these antibodies affects the spreading of cells and so cells will be seeded on N-cadherin chimeric protein as previously described. Hela cells stably expressing an F-actin marker (life-act Ruby) and a chromosome marker (Histone-CFP), will be treated with the blocking antibody. Untreated cells will be used as a control. Both control and treated cells will be visualized live using an upright Zeiss Axioimager equipped with a heated stage, and the SAC will be quantified for the effects on actin pool. We next plan to examine how the CMC proteins individually affect the SAC. In order to do this, cell lines lacking p130cas and FAK will be transfected with the markers mentioned above and analyzed following the same approach. The role of Rac1 in the formation of this pool will be evaluated using commercially available inhibitors of this protein and a FRET sensor. A similar procedure as described above will be followed, using stably expressing cells in the presence or absence of the inhibitor, and the role of Rac1 in the pool formation will be examined. We will take advantage of the Raichu FRET Rac sensor which will allow us to investigate the spatiotemporal activation of Rac1 during mitosis, as well as the effects of force in this activation. For this, Hela cells stably expressing histone-mkate will be transfected with the sensor along with an actin marker (Utrophin mcherry). They will be seeded on FN micropatterned CS and imaged live on a Zeiss LSM710 spectral confocal microscope. Using PDMS membranes we will be able to apply uniaxial stretch on mitotic cells and the effects of force in Rac1 activation will be analyzed. If the appearance of SAC is associated with the Rac1 activation we aim to explore the effects of CMC in Rac1 activity. In order to do this, we will use cells lacking CMC proteins p130Cas and FAK, as well as the AIIB2. Cells will be transfected with the FRET sensor and a marker for actin, and will be seeded on FN micropatterns. The cells will be compared to reconstituted cells in the presence or absence of the inhibitory antibody and the effects of CMC on Rac1 activity will be assessed. Lastly, we will attempt to examine if the Rac1 localized activation elicits the SAC formation and influence the spindle capture sites at the cortex. To do so, we will take advantage of a photoactivable construct for Rac1 (PA-Rac1) which can be activated using 458 nm light or deactivated using 473 nm light. Hela cells will be transfected with the construct along with

a marker for actin and histone as described above, the cells will be seeded on charged coverslips and will undergo live imaging using confocal microscopy. The formation of SAC will be quantified and assessed in respect to the local activation of Rac1. Following this, Hela cells expressing the PA-Rac1 and the actin marker, will be transfected with EMTB construct (spindle visualization), will be seeded on FN micropatterned coverslips and imaged live. These experiments are going to be performed in *Xenopus* embryos too using fluorescently tagged proteins and MOs for the CMC proteins. Overall these experiments will allow us to explore the mechanism through which the CMC complex guides spindle orientation through the formation of the SAC observed in mitotic cells via the activation of Rac1.

c) Examine the role of the CMC in *Xenopus* embryos.

As described above integrin β1 is activated upon force application at the lateral cortex of mitotic cells both in adherent cells and in embryonic epithelia. It has been previously shown that this activation as well as the polarized distribution of active β1 are both necessary for correct spindle orientation in cultured cells. This relationship has not been established in the embryo. The Xenopus outer epithelium offers a unique system to do this since it is a polarized epithelium but its polarity does not depend on integrin signaling Petridou and Skourides, 2016; Petridou and Skourides 2014). It has been previously shown that FAK is necessary for sensing forces that orient the spindle. In addition, the functional determinants of FAK are identical with those in cultured cells i.e both the FAT domain and interaction with paxillin are required while the FERM domain and the kinase activity are dispensable. We could take advantage of FRET sensor approaches to image β1 activation in the live embryo as well as use integrin β1 DN and MO to explore the role of integrin β1 and the newly discovered CMC members that will derive from the MS/MS analysis. This will include laser ablation experiments to determine how integrin β1 responds to changes of forces exerted on the cell cortex as well as loss of function experiments to address the individual roles of CMC proteins in force sensing and spindle orientation. Beta 1 activation will be tracked using a GFP-beta 1 donor and mCherry-Talin FRET acceptor. This FRET pair can reliably report beta 1 activation based on talin binding on beta tails a ubiquitous step in integrin activation (Rantala et al. 2011).

d) Examine the role of the CMC in *Xenopus* neural tube closure.

Previous work from our lab suggested that CMC plays a role in the mechanical cross talk of apically constricting cells of the neural plate. Since both CMC and HAs are formed upon high tension application and since apical constriction is a morphogenetic movement characterized by high tension requirements, we could potentially explore the possibility that the CMC is implicated in this process. This could be achieved through experiments using

inducible approaches. To do so we will take advantage of photo morpholinos and specifically ablate the function of each CMC protein. This will allow us to monitor the spatial and temporal expression and function of these proteins. If any phenotypes derive we could then proceed to generate Crispr Cas9 for CMC proteins in order to identify the precise effects of these proteins during neural tube closure. We can also examine how blocking CMC function affects contraction pulses, constriction patterning and the mechanical cross talk between cells of the neural plate. This can be achieved using markers like GECO-Red which is a genetically coded Ca2+ indicator in combination with the mutants of the proteins of interest. Briefly the MOs of the CMC protein members will be co-injected with that of GECO-RED into one or two dorsal blastomeres at 4-cell stage embryos. This will allow targeting of the neural plate. Embryos will be allowed to develop to stage 14 and imaged for neural tube closure using confocal microscopy and time lapse imaging. Effects on neural tube closure, contraction pulses, constriction patterning and the mechanical cross talk between cells will be assessed.

7. References

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8. Abbreviations

AC: Animal Cap

AMTs: Astral Microtubules

AJs: Adherens Junctions

AP: Animal pole

A-P: anterior-posterior

aPKC: atypical PKC

BCR: Blastocoel Roof

BSA: Bovine Serum Albumin

Cas: Crk-associate substrate

CAMs: Cell Adhesion Molecules

CMC: Cortical Mechanosensory Complex

CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats

D-V: Dorsal-Ventral

DMZ: Dorsal Marginal Zone

DM: Dominant Negative

ECM: Extracellular Matrix

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial to Mesenchymal Transition

FAs: Focal Adhesions

FB: Fibrillar Adhesion

FBS: Fetal Bovine Serum

FC: Focal Complex

FN: Fibronectin

FRAP: Fluorescence Recovery After Photobleaching

GFP: Green Fluorescence Protein

hGG: Human Chorionic Gonadotropin

ICAM-4: Intercellular Adhesion Molecule

ILK: Integrin Linked Kinase

LGN: Leu-Gly-Asn repeat-enriched protein

LIM: Lin11, Isl-1, Mec-3

MAPK: Mitogen Activated Protein Kinase

MEFs: Mouse embryonic Fibroblasts

MLC: Myosin Light Chain

MMR: Marc's Modified Ringer's

MO: Morpholino

MZ: Marginal Zone

NuMA: Nuclear Mitotic Apparatus

PDMS: Polydimethylsiloxane

PFA: Paraformaldehyde

PIP: Phosphoinositides

PIP_2: Phosphatidylinosito-4,5-biphosphate

PIP_3: Phosphatidylinosito-4 phosphate 5-kinase type 1γ

PLCγ: Phospholipase Cγ

PLL: Poly-L-Lysine

PTEN: Phosphatase and Tensin homolog

RFs: Retraction Fibers

RFP: Red Fluorescence Protein

RGD: Arg-Gly-Asp

ROCK: Rho-associated protein Kinase

RT: Room Temperature

SAC: Sub-cortical Actin Clouds

SBD: Src Binding domain

SD: Substrate domain

SEM: Standard Error of Mean

SH: Src Homology

TALEN: Transcription Activator-Like Effector Nuclease

u-PAR: Urokinase-type Plasminogen Activator Receptor

VASP: Vasodilator-stimulated Phosphoprotein

VBS: Vinculin Binding Sites

VN: Vitronectin

VP: Vegetal Pole

Wt: Wild Type

9. Publications

The spatial distribution of AJs formation and clustering leads to tension driven activation of integrins and guides extracellular matrix deposition topology.

Ouranio Anastasiou, Rania Hadjisavva and Paris A. Skourides

(Manuscript in Preparation)

Mitotic cell responses to substrate topological cues are independent of the molecular nature of adhesion

Ouranio Anastasiou, Rania Hadjisavva, and Paris A. Skourides

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