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## **ROLE OF ANILLIN IN REGULATION OF EPITHELIAL JUNCTIONS**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science  
in Human Genetics at Virginia Commonwealth University.

By

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May, 2014

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## LIST OF ABBREVIATIONS AND SYMBOLS

ANLN: Anillin.

AJ: Adherens Junction.

TJ: Tight Junction.

NM II: Non-muscle myosin II.

F-actin: Filamentous actin.

E-cad: E-cadherin.

N-cad: N-cadherin.

JAM-A: Junctional Adhesion Molecule-A

ZO: Zonula Occludens

SM-22: Smooth muscle marker 22

$\alpha$ -SMA: Alpha smooth muscle actin

$\alpha$ : Alpha

$\beta$ : Beta

$\gamma$ : Gamma

EMT: Epithelial Mesenchymal Transition

EMyT: Epithelial Myofibroblast Transition

TGF- $\beta$ : Transforming growth factor  $\beta$

ATP: Adenosine Triphosphate

GTP: Guanosine triphosphate

GAP: GTPase activating protein

GEF: GTP exchange factor

DMEM: Dulbecco's Modified Eagle Medium

RMLC: Regulatory myosin light chain

MLCK: Myosin light chain kinase

ROCK: Rho-associated protein kinase

TNF- $\alpha$ : Tumor necrosis factor alpha

MT: Microtubules

ERM: Ezrin-Radaxin-Moesin

FSGS: Focal segmental glomerulosclerosis

RCC: Renal cell carcinoma

## Abstract

### ROLE OF ANILLIN IN REGULATION OF EPITHELIAL JUNCTIONS

By Gibran Kamal Chadha.

A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Human Genetics at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Major Director: Andrei I Ivanov, Ph.D. Human Genetics.

Adherens junctions (AJs) and tight junctions (TJs) are characteristic features of differentiated epithelial cells and are critical for regulation of epithelial barriers and cell polarity. Integrity and remodeling of epithelial junctions depend on their interactions with underlying actomyosin cytoskeleton. Anillin is a multifunctional scaffold able to interact with different cytoskeletal proteins including F-actin and Myosin II. This project aimed to investigate roles of anillin in regulating epithelial AJs and TJs. Using A549 human lung epithelial and DU145 human prostate

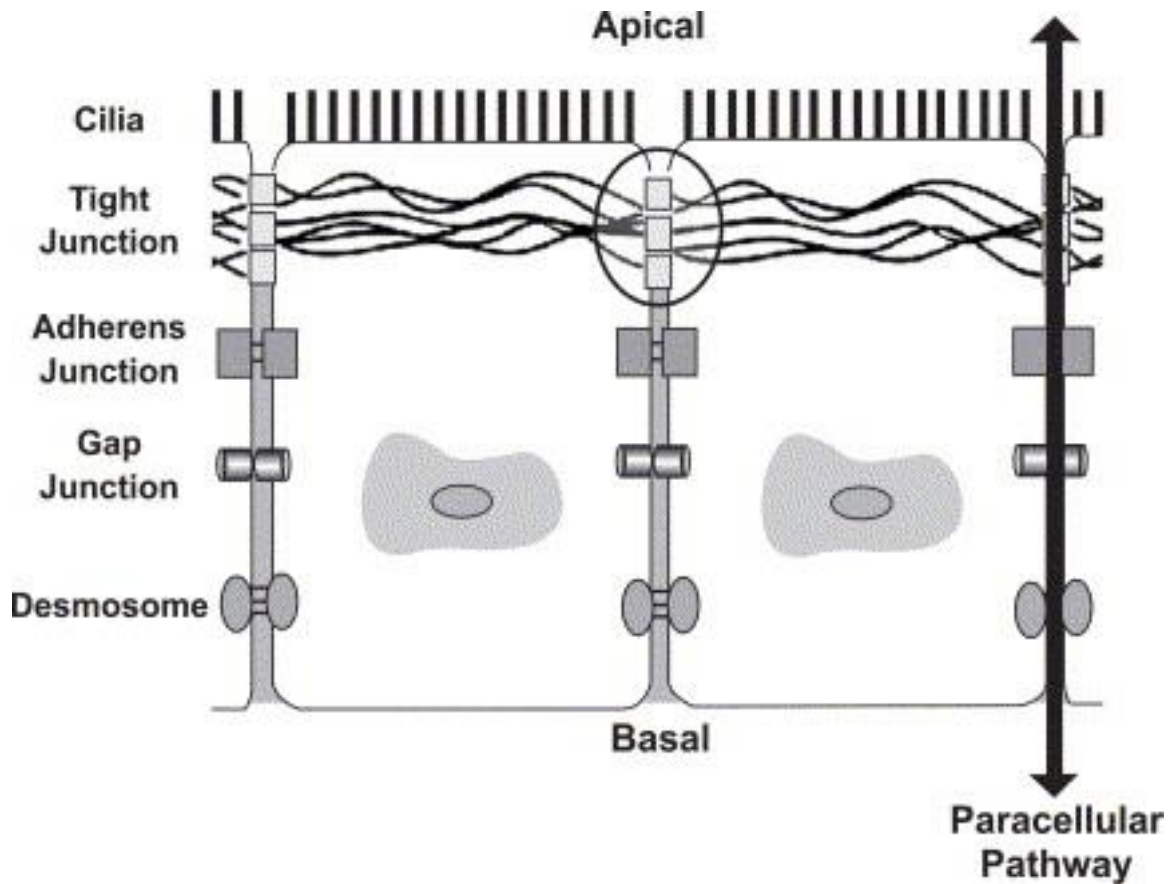
epithelial cells, we demonstrated the anillin depletion-induced loss of AJs and TJs. This was accompanied by disorganization of perijunctional actomyosin belt and disruption of the adducin-based membrane skeleton that links actin filaments to the plasma membrane and epithelial junctions. Depletion of anillin decreased protein levels of  $\gamma$ -adducin and downregulation of  $\gamma$ -adducin mimicked effects anillin knockdown on AJ and TJ integrity. These findings suggest a novel role for anillin in the assembly of epithelial junctions.

## INTRODUCTION

### **1. Role of Epithelial barrier**

Epithelium plays a vital role in the survival and development of multicellular organisms. The epithelial layers establish a barrier between the body's interiors and external environment, thus being crucial in maintaining the various internal compartments to preserve their unique architecture and chemical environment. (1) (2) Generally, simple epithelial linings in the reproductive, respiratory and renal systems are composed of a single layer of specialized cells (1) (3). The epithelium also serves as physical barrier between external pathogens and the immune system (4) (5) and is crucial in the process of inflammation as it serves as an interface between invading pathogens and the host immune system. (5) Inflammation causes this protective mechanism to become compromised due to stimuli that may arise on either side of the epithelial barrier, like pathogens on the apical side, which increase epithelial permeability to gain access to host tissue or active immune cells that induce barrier disruption via pro-inflammatory cytokines to facilitate their movement towards the site of pathogen invasion. Hence, understanding the mechanisms that control disruption of the epithelial barriers is key to identifying molecular targets (5).

The barrier properties and structural integrity of the epithelium is maintained by junctions, which are adhesive protein complexes that are present along the lateral plasma membrane. These junctions regulate epithelial intercellular adhesions as well as apico-basal polarity. The most important epithelial adhesive structures are known as tight junctions (TJs) and adherens junctions (AJs) (3, 6-8) that are positioned at the most apical aspects of the lateral plasma membrane and play crucial roles in regulating the integrity and barrier properties of epithelial layers (5). Figure 1 provides a representation of the arrangement of junctions in polarized epithelial cell. Both TJs and AJs are composed of transmembrane and cytosolic plaque protein complexes. The transmembrane protein interact with their partners on the opposing plasma membrane providing a mechanical link between epithelial cells and establishing a barrier to paracellular diffusion of fluid and solutes. The cytosolic plaque proteins bound to these transmembrane proteins on the cytosolic face are referred to as the cytosolic plaque proteins. (5) These plaque proteins can play multiple roles such as stabilization and anchoring of the transmembrane proteins, anchoring of signaling and trafficking molecules and linking these transmembrane proteins to the cellular cytoskeleton (3, 6-8 9, 10).



**Figure 1: Junctions in a polarized cell.**

The figure is a representative diagram of the organization and location of junctions in an apico-basolaterally differentiated cell. The point of cell-cell contact forms the intercellular junctional complex. This consists of tight junctions, the most apical, followed by adherens junctions, gap junctions and desmosomes, which are the most basally located. [Johnson LG. *Adv Drug Deliv Rev.* 2005; 57: 111-121]

## **2. Molecular composition of epithelial junctions.**

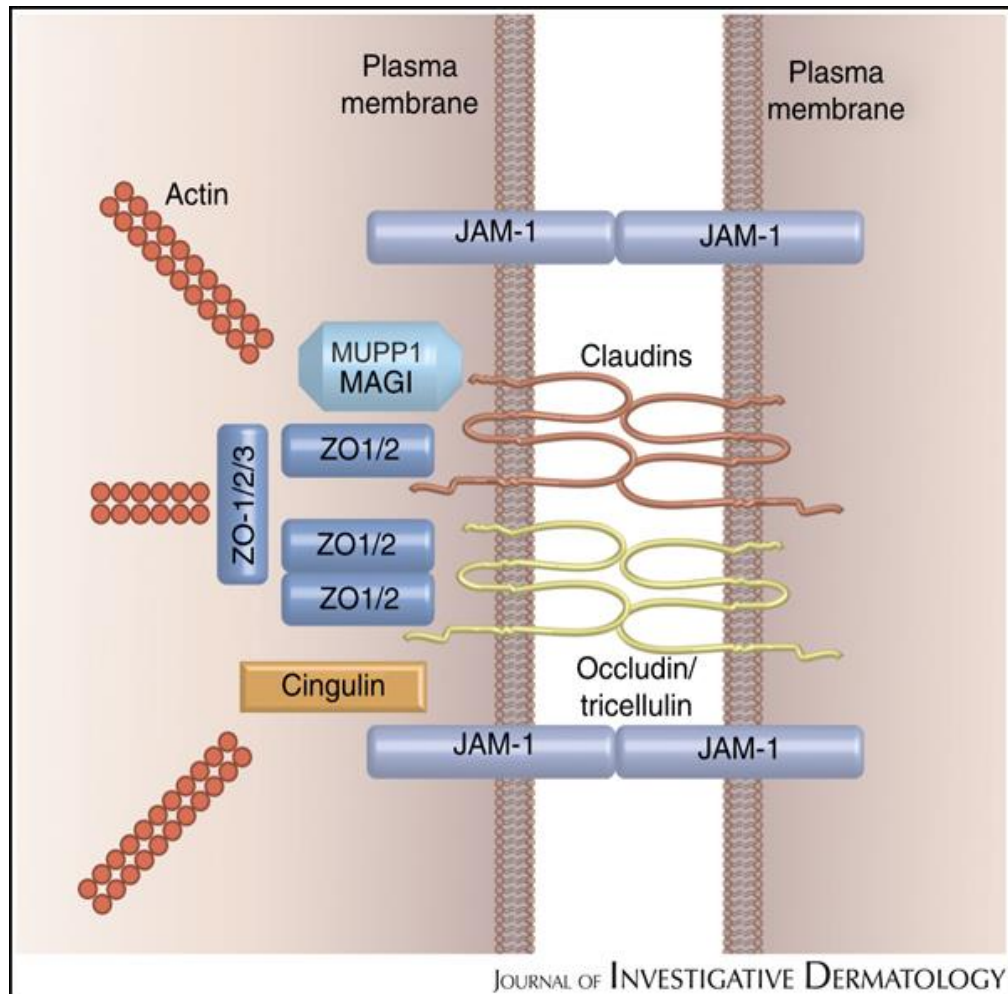
### **2.1 Tight Junctions**

TJs appear as a network of interconnecting fibrils that physically link the plasma membranes of two cells (4). These interconnecting fibrils are composed of integral and peripheral membrane proteins. The integral proteins are responsible for the cell-cell adhesions while the peripheral proteins form the TJ cytosolic plaque, responsible for stability and remodeling of the junction.

The transmembrane component is composed of Claudins, which are a family of tetraspan proteins that form the tight junctional fibrils, Occludin, members of the Claudin family and immunoglobulin-like molecules such as Junctional Adhesion Molecule-A (JAM-A) (Figure 2).

(4) The occludin and JAM-A molecules associate with the claudin based fibrils and regulate the assembly and barrier function of tight junctions. The cytosolic plaque of TJs consists of multiple proteins and has a high degree of complexity. The most studied components of the TJ cytosolic plaque are members of the zonula occludens (ZO) protein family, ZO-1, ZO-2 and ZO-3. The ZO family proteins possess multiple PDZ domains, allowing them to interact with other cytosolic plaque proteins as well as transmembrane components can bind, allowing the formation of scaffolding networks. (4, 11, 12, 13) These ZO family proteins also have actin-binding domains, which is likely to be responsible for association of TJs with underlying actin filaments. (5)





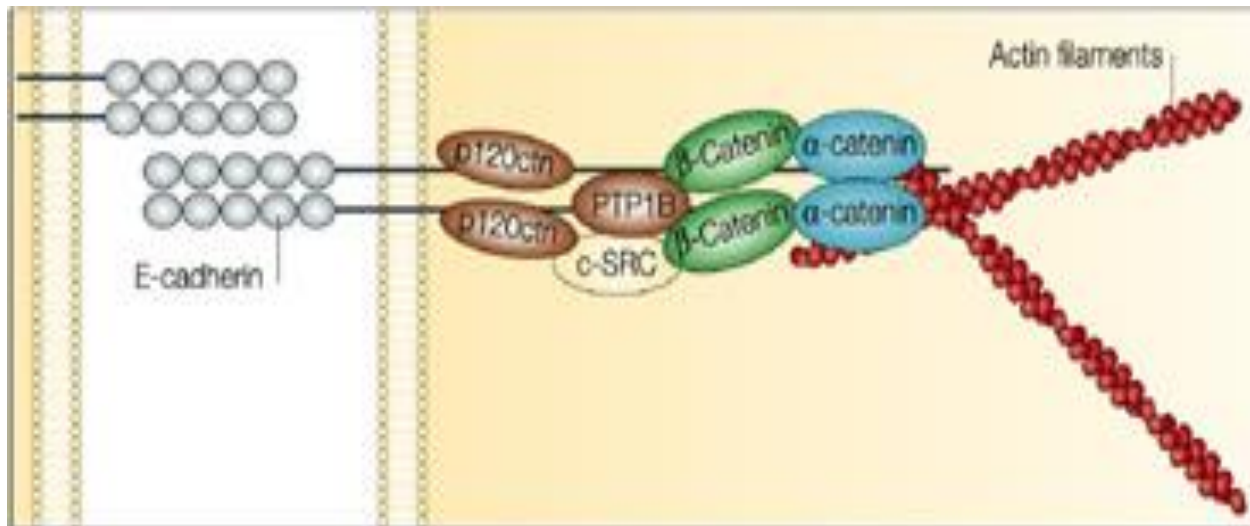
**Figure 2: Tight junction.**

ZO-1 or ZO-2 is important for clustering of claudins and occludin, resulting in the formation of tight junctional strands. The role of the other scaffolding proteins (ZO-3/MAGI/MUP1) is less clear. The ZOs and cingulin can provide a direct link to the actin cytoskeleton. **Tight Junctions/Adherens Junctions: Basic Structure and Function; Carien M Niessen**

## **2.2. Adherens Junctions**

Adherens junctions are generally positioned immediately below tight junctions and are characterized by two juxtaposing membranes. Their primary function is to mediate attachment of contacting cells. E-Cadherin and members of the Nectin family are the major transmembrane proteins of epithelial AJs (9, 10, 14). Figure 3 provides a representation of the different components of AJs. Cadherins are single spanned proteins with an extended extracellular fraction and a short cytoplasmic domain. The extracellular portion mediates calcium dependent adhesions with other cadherins, while the cytosolic domain interacts with cytoplasmic plaque scaffolding proteins. Several cadherins, including E-cadherin and N-cadherin, have in common an extracellular domain with five segments of repeated sequences and they regulate cell-cell adhesion in a homotypic manner through their association of amino-terminal extracellular domains. Calcium binding to the extracellular domain triggers a conformation that initiates the homotypic binding of cadherins between adjacent cells. These homotypic interactions are essential for cell recognition and separation during tissue morphogenesis. There are multiple classes of cadherin molecules and cells containing a specific cadherin sub-type tend to cluster together to the exclusion of other types. Thus it proves to be critical cell sorting and tissue morphogenesis (15, 16). The cytoplasmic plaque of AJs consists of the catenins, mainly  $\beta$ -catenin, p-120 catenin and  $\alpha$ -catenin (1, 9, 10).  $\beta$ - and p-120 catenin bind to the cytoplasmic domain of the cadherins, while  $\alpha$  catenins and other actin binding protein bind to  $\beta$ -catenin and p-120 catenin (17). They interact with the intercellular E-cadherin as well as actin-binding proteins like  $\alpha$ -catenin, vinculin and  $\alpha$ -actinin (1, 9, 10). Catenins stabilize the cadherin clusters at the plasma membrane and mediate the interactions between the transmembrane cadherins and

the cytoskeleton. Dissociation of p-120 results in ubiquitination of cadherins and hence removal from the cell surface via endocytosis (18).



### Figure 3: Adherens junctions.

Adherens Junctions facilitate cell-cell adhesion through homotypic binding between E-cadherin molecules on neighboring cells. The cytoplasmic plaque consists of proteins such as P-120,  $\alpha$ -catenin and  $\beta$ -catenin and they these proteins link the E-cadherin homodimers to the actin cytoskeleton. [A renaissance for SRC, Timothy J. Yeatman, *Nature Reviews Cancer* 4, 470-480 (June 2004)]

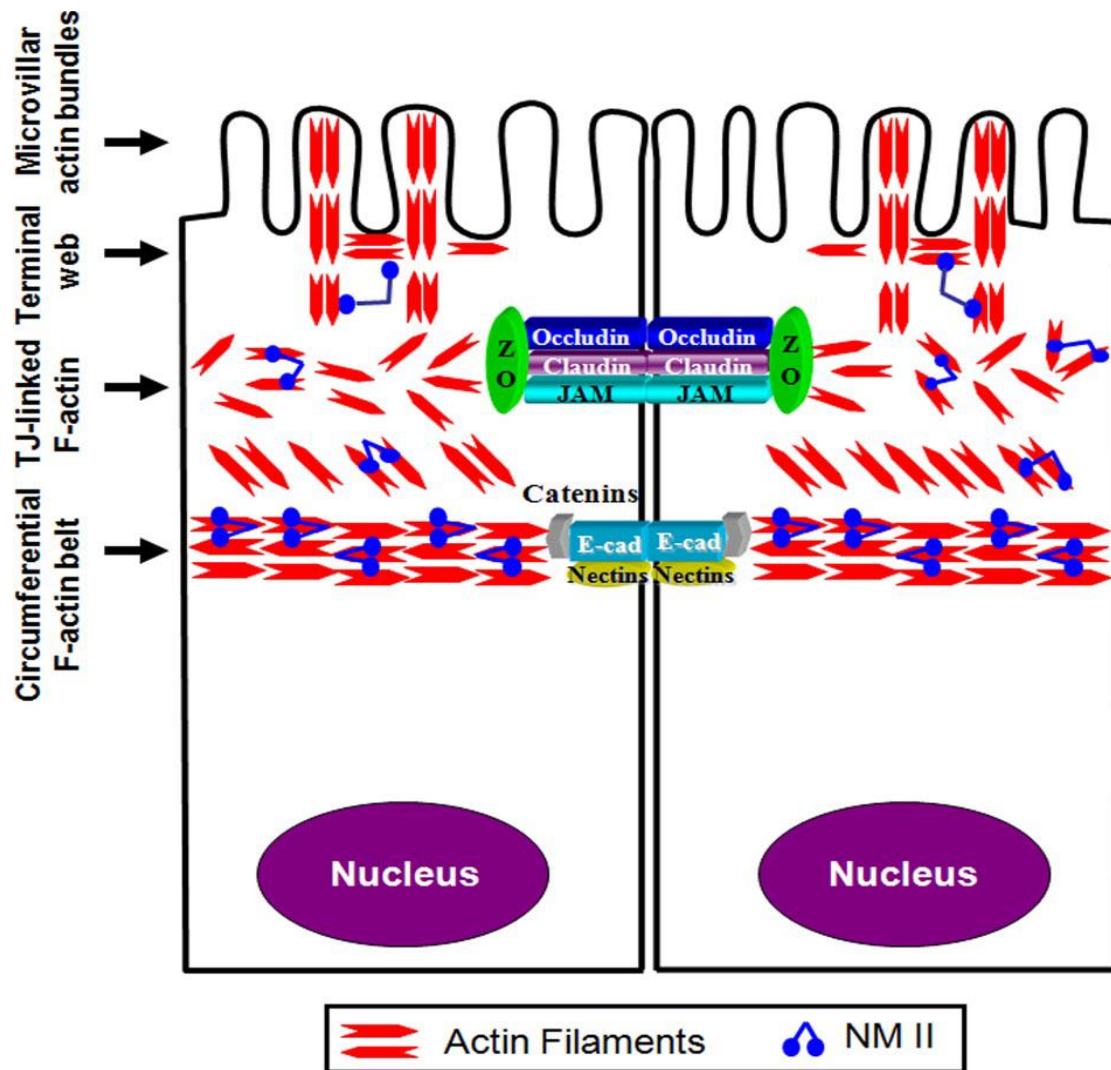
The molecular organization of the epithelial AJs and TJs relies on a delicate balance of adhesive strength and structural plasticity (19-22). Both these junctional complexes are highly dynamic structures that constantly undergo remodeling. This has been observed via multiple studies that have shown the cycling internalization of Claudins and E-cadherin from fully-formed TJs and AJs respectively (46, 47). This junctional plasticity is crucial to migration, expansion and changing of the shape of epithelial sheets without compromising the integrity and barrier properties (2, 32). A dynamic equilibrium between junctional disassembly and reassembly in normal epithelium is shifted towards disassembly under certain pathophysiological conditions

like inflammation and cancer (1). This combination of junctional strength and plasticity is determined by the interaction between junctions and the underlying actin cytoskeleton. (23-26).

### **3. Association of epithelial junctions with the actomyosin cytoskeleton.**

The apical region of polarized epithelial cells containing AJs and TJs is rich in elaborated network of actin filaments associated with their motor protein, non-muscle myosin II (NM II), as shown in Figure 4. The perijunctional actomyosin cytoskeleton can be divided into two components such as AJ and TJ-associated cytoskeleton. A functional connection between epithelial junctions and the actin cytoskeleton has been established via studies in multiple epithelial cell lines (1). Studies have reported that F-actin in the AJ region is organized into a circumferential ring composed of filaments that run parallel to the plasma membrane (1, 51, 54) and that within the ring, the actin filaments self-associate and are further cross-linked by myosin II or  $\alpha$ -actinin (1, 51, 53). These filaments occasionally branch out in the form of parallel bundles to allow insertion into the plasma membrane in the area of the AJ cytosolic plaque (1, 51, 54).

The TJ-associated actin filaments are organized similar to the AJ-associated F-actin belt and consists of F-actin filaments of mixed polarity running in parallel to the plasma membrane (1, 28). F-actin associated with TJs is reported to be loosely organized meshwork instead of the tightly backed belt as is observed in the case of AJs (1, 28, 47, 48). In addition, studies in intestinal epithelium suggest a physical and functional analysis between TJ and apical microvilli and (47, 49) and TJ- and AJ associated actin filaments form two connected zones in cross-sections of the small intestine (49).



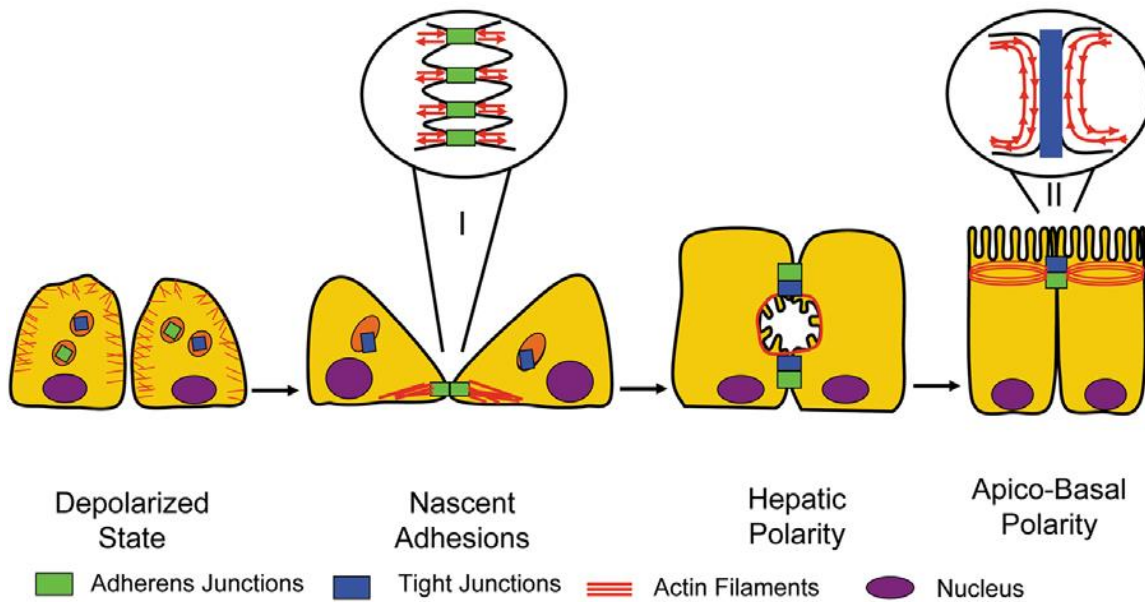
**Figure 4: Organization of the apical actin cytoskeleton in simple polarized epithelia.**

The apical actin cytoskeleton can be divided into 3 zones: Zone I has actin filament bundles that support apical microvilli; Zone II has an actin filament network associated with tight junctions and Zone III represents a perijunctional F-actin belt connected to the AJs.

Actin filaments associated with AJs and TJs consists of filaments of opposite polarity and are enriched in myosin II. [Actin motors that drive formation and disassembly of epithelial apical junctions. Andrei I Ivanov. *Frontiers in Bioscience* 13, 6662-6681, May 1, 2008]

The actomyosin belt that is oriented in parallel to the plasma membrane appears to be associated physically with AJs. The TJ –associated cytoskeleton is composed by a meshwork of F-actin bundles physically linked to TJs. This perijunctional actomyosin cytoskeleton is known to be critical regulator of stability and dynamics of epithelial junctions.

Figure 4 shows formation of mature junctional complexes occurs through two major steps; the initial AJ-like junction assembly followed by the establishment of TJs (14, 50, 45, 46). Each of the two steps is associated with F-actin cables at AJ-like junctions (50, 47-49) and with circumferential F-actin bundles at assembling TJs (42, 50). The F-actin cables appear at the initial stages of epithelial cell contact formation (50, 47-49), which are positioned perpendicular to the plane of intercellular contacts (47, 49). These actin cables co-localize with newly formed adhesion complexes, appearing as dot-like structures, at the site of intercellular contacts, called punctas (50, 47-49, 51). During maturation of these junctions, individual punctas cluster together to form a continuous adhesive belt and this is accompanied by F-actin bundling which results in the increase in density and formation of the perijunctional F-actin belt (50, 47-49, 60, 64). This puncta clustering is driven by actin bundling and indicates that actin filaments stabilize the junctions via bundling and formation of the perijunctional actomyosin belt.



## Figure 5: Reorganization of actin cytoskeleton during apical junction formation.

Formation of apical junctions involves 3 steps: Accumulation of parallel F-actin bundles in region of cell-cell contact and assembly of nascent adherens-like junctions; Second, Assembly of TJ encircling apical membrane. Final step; Transition from hepatic to apico-basal polarity, leading to formation of AJ and TJ and assembly of perijunctional F-actin bundles. [Actin motors that drive formation and disassembly of epithelial apical junctions. Andrei I Ivanov. *Frontiers in Bioscience* 13, 6662-6681, May 1, 2008]

Several studies indicate the crucial role of actin filaments in the regulation of AJs and TJs.

Pharmacological experiments have been conducted using several actin-depolymerizing toxins, like cytochalasins, which very effectively disrupt epithelial barriers. Cytochalasins bind to the barbed of actin filaments and prevent their elongation, blocking the association and dissociation of actin monomers. Another such pharmacological agent, Latrunculin B (Lat B), is known to specifically bind to and sequester monomeric actin, resulting in depolymerization of turning-over actin filaments (55). Exposure of cells to Lat B has been reported to result in a significant

decrease in actin localization as well as disintegration of  $\beta$ -catenin-based AJs in SK-CO15 cells (55).

In addition to pharmacologically induced actin depolymerization, several studies conducted have also revealed that downregulation of either  $\beta$ - or  $\gamma$ -actin isoforms in epithelial cells results in a marked impairment of barrier properties in intestinal epithelial cells (55). A decrease in TEER and an increase in permeability were observed on siRNA induced depletion of cytoplasmic actins. It was also observed that the loss of either actin isoform resulted in the redistribution of AJ proteins and TJ proteins from the areas of cell-cell contact, thereby disrupting junctional integrity; however, no change in expression levels of these junctional complex proteins was observed. F-actin depolymerization renders epithelial cells unable to remodel TJs and AJs (42, 50, 53). These pharmacological and molecular studies collectively suggest that Actin plays a very crucial role in not only the assembly by also the maintenance of epithelial junctions.

Given the physical association of actin with junctional complexes, it is not surprising that the remodeling of both sets of epithelial junctions is associated with a change in the framework of the perijunctional actomyosin skeleton. These changes have been extensively characterized using the 'calcium switch', a widely used *in vitro* junctional remodeling system, in which the removal of extracellular calcium triggers a fast and organized disassembly of both epithelial junction complexes. (51-56). This disruption is reversible and the re-introduction of calcium into the extracellular matrix results in the restoration of structurally and functionally normal AJs and TJs and multiple studies have been conducted using this model showing the associated change in skeletal organization along with remodeling of epithelial junctions. (42, 50). Actin has also been reported to stabilize junctions indirectly via suppressing junctional endocytosis. Latrunculin-A induced actin depolymerization has been reported to result in in Caveolae-mediated endocytosis



resulting in disruption of tight junction, as exhibited by the endocytosis of TJ proteins Occludin, ZO-1 and Claudin 4 (60).

The above discussed studies support of the idea that the actin cytoskeleton plays a dual role in regulation of epithelial junctions by stabilizing junctional complexes and creating efficient paracellular barriers in steady-state monolayers and by driving junctional remodeling (disassembly and reassembly) during breakdown and restitution of epithelial barriers. Given the complexity of regulatory mechanisms that build and maintain the cytoskeletal architecture, it remains a big challenge to understand the role of different modes of F-actin regulation in formation, maintenance and breakdown of epithelial junctions

#### **4. Nonmuscle myosin II as essential regulator of epithelial junctions.**

Organization and dynamics of the epithelial actin cytoskeleton is largely determined by nonmuscle myosin II (NM II). NM II is a cytoskeletal motor protein that converts chemical energy of ATP hydrolysis into mechanical forces that regulate static tension and contractility of the actin cytoskeleton. This motor exists as a heterohexamer of two heavy chains, two essential and two regulatory light chains (RLC) (57-60). The NM II heavy chain consists of a globular head which binds to actin and hydrolyzes ATP as well as an extended tail that coils with the heavy chain tails to form a rigid rod-like structure and these tails spontaneously self-associate which results in thick filaments. This complex structure serves as the actin motor protein and is crucial for its two major functions; to bind to actin and hydrolyze ATP, which is performed by the globular head of the heavy chains, which allows for actin filament sliding and renders the property of myosin-II dependent contractility. The other function is the bundling of actin filaments, which results in the formation of a rigid rod-like structure via coiling of two heavy

chain tails (57-59). Epithelial cells three different NM II isoforms, NM II A, II B and II C (61-63). The isoforms are highly similar in their amino acid sequence (42, 64), but vary in their functional properties (42, 61, 65-70). Despite these similarities, these isoforms are not functionally redundant and have multiple roles in cell motility, cytokinesis, regulation of cell and intercellular vesicular traffic (98).

Several studies have identified the activation of NM II as a major driving force for the disassembly of epithelial junctions. Extracellular calcium depletion (42, 51), treatment with proinflammatory cytokines and members of the TNF family (74-77) can all result in NM-II dependent disruption of epithelial junctions. Another study demonstrated that selective depletion of only NM-IIA sharply attenuates the formation of the contractile F-actin rings as well as TJ/AJ assembly in calcium-depleted SK-Co15 cells. (42).

While NM II plays a role in sealing of epithelial barriers, it also plays a role in the establishment of epithelial junctions. It is a multi-step process, with formation of AJs followed by the assembly of TJs, the latter coinciding with development of apico-basal cell polarity (14, 42, 45, 46). siRNA mediated knockdown of NM II-A dramatically reduces reassembly of the initial AJ-like junctions formed in colonic epithelial cells, while also showing an absence of radial F-actin cables that are found at the sites of initial cell-cell contacts (42). NM IIA knock-down results in a complete disappearance of radial F-actin cables in areas of initial intercellular contacts.

Collectively, this data indicates that NM II plays more of a structural than a motor protein role by bundling and cross-linking actin filaments.

NM II has also been shown to be critical in formation of TJs. The reassembly of TJs on inhibition of NM II using blebbistatin has been shown to be attenuated (42, 78, 79) or siRNA

mediated knockdown of NM IIA (42). This defect appears to be part of an effect of NM II inhibition on development of the normal epithelial phenotype. This defect in TJ assembly appears to be a subset of a broader effect of blebbistatin induced myosin II inhibition. During calcium depletion, cells inhibited for NM II do not acquire the columnar shape; fail to develop the normal apical plasma membrane domain and the perijunctional F-actin belt (34, 42, 50, 78). This unusual phenotype suggests that the cytoskeletal motor and intracellular machinery are linked and together regulate the establishment of cell polarity, and this regulation is through to be via the Rho GTPases (49, 79). The interplay between actin and NM II mediates the formation of apical actomyosin rings during the disassembly of junctions (51). This kind of interaction has also been implicated for other processes like wound healing and the formation of a cleavage furrow during the process of cytokinesis. (80-82)

Inhibition of the NM II motor has shown to increase the leakiness of the epithelial barrier, while over-activation of NM II by expression of either constitutively active RLC or MLCK also leads to increased epithelial permeability. This data indicates that for the tight epithelial barrier to be maintained, an intermediate level of NM II activity is required.

These pharmacological and molecular studies have shown that NM II is essential for the regulation and maintenance of junctions. However, NM II in turn has multiple binding partners that regulate its activity and it is very poorly understood how these proteins in turn might affect myosin function and by extension the maintenance of epithelial junction organization.

### **5. Rho GTPases signaling regulates the actomyosin cytoskeleton**

Due to its role in multiple processes, several mechanisms are in place to regulate the activity of NM II in epithelial cells. One of the more prominent mechanisms is the activatory

phosphorylation of the RLC, which is carried out by Rho-associated kinase (ROCK) and Myosin light chain kinase (MLCK). The activity of these two proteins is regulated by Rho GTPases (71-73).

Rho GTPases are small signaling G-proteins which are majorly involved in regulation of intracellular actin dynamics. They are also involved in cell movement and organelle development. Rho GTPases exist in two conformation states; GTP-bound when active, and GDP-bound when inactive. They have the ability to convert GTP to GDP via hydrolysis, however the process is slow. GTPases are very carefully regulated and via GAPs and GEFs. GTPases are activated via GTP exchange factors that exchange GDP for GTP and are inactivated by GTPase-activating proteins (GAP), which promote GTP hydrolysis. On the other hand, once GTP is hydrolyzed to GDP, the latter is switched out for another molecule of GTP via GTP Exchange Factor (GEF). Three members of the Rho GTPase family have been studied in detail, RhoA, Rac1 and Cdc42. RhoA is a known regulator of the actin cytoskeleton, involved in the formation of stress fibers. (83) One of the proteins that it acts on is the ROCK1. ROCK1 is a Ser/Thr kinase, which phosphorylates and activates LIM kinase, which in turn phosphorylates cofilin, thereby reducing depolymerization of actin.

ROCK increases the activating phosphorylation of NM II RLC by either directly or by inactivating the RLC phosphatase. ROCK dependent phosphorylation of NM II is crucial for regulating different contractile processes in adherent cells. ROCK has been shown to selectively phosphorylate RLC of NM II A.

RhoA and ROCK co-localize with both assembled and disassembling TJs in epithelial monolayers. Also, enhancing RhoA activity has been shown to induce disassembly of both tight

and adherens junctions. Also, disruption of apical junctions due to external stimuli is associated with an increase in Rho/ROCK stimulation and is attenuated by Rho and ROCK inhibition. (84)

Previously conducted studies have revealed the importance of RhoA in maintenance of adherens junctions. When carcinoma and non-tumorigenic epithelial cells were treated with cell botulinum C3 toxin, which selectively inactivates RhoA, it was observed that E-cadherin and  $\alpha$ -catenin localization was disrupted. It was also reported that inhibition of Rho, but not ROCK, interfered with the reformation of AJs after calcium-depletion induced disruption. In normal epithelial MDCK cells, inhibition of Rho but no ROCK sharply reduced the localization of  $\alpha$ - and  $\beta$ -catenin to sites of intercellular contact. Microinjection of constitutively active GTP-bound RhoA results in the mis-localization of  $\alpha$ -catenin in cells, suggesting further that re-regulated Rho-A activity results in the disruption of AJs (85).

Despite of the fact that mechanisms and functional effects of Rho-dependent activation of NM II are well understood, there are many other regulatory levels for both RhoA and NM IIA that involve a number of different Rho and NM II-interacting proteins. The next challenge is to understand which of these proteins can mediate the effects of Rho and NM II on epithelial junctions.

#### **6. Anillin as an important F-actin, NM II and Rho-binding protein.**

Anillin was originally isolated from *Drosophila* embryos and homologs were later found in vertebrates. It is a multi-domain protein that physically interacts with multiple cleavage furrow components as well as multiple structural and regulatory elements. The N-terminus contains two regions- One that binds to and bundles F-actin and the other that binds to phosphorylated Myosin II, while the C-terminus has a PH domain, involved in intracellular signaling. (88)

Anillin binds to F-actin, NM II as well as the septins, all of which are involved in filament-forming and enriched in the contractile ring. Anillin also binds to Rho GTPase-activating protein (GAP). This GAP can further bind to Rho Guanine exchange factor (GEF) Ect2. Hence, Anillin has the role of scaffold for regulatory proteins as well as for the actomyosin and microtubule cytoskeletons. (89)

There are multiple accounts of the association of Anillin with both Rho GTPases as well as myosin. Anillin regulates RhoA's equatorial cortical localization during cytokinesis and they closely co-localize in the contractile ring and in ingression furrow. (90) Depletion of Anillin from *Drosophila* spermatocytes dramatically reduces equatorial Rho and F-actin (89) while human Anillin is required for population of endogenous RhoA (90, 91). It has also been shown that the highly conserved AH domain is required for stabilization of RhoA localization *in vivo* and can bind directly to RhoA *in vitro* (90). Furthermore, Anillin and RhoA can not only be co-immunoprecipitated but over-expression of Anillin causes an increase in active RhoA. These results suggest a direct relation between the two molecules and contribution of Anillin in the activity of RhoA at the cleavage plane, thus contributing to regulating a critical function of the RhoA molecule.

As mentioned earlier, Anillin has also been shown to exhibit correlation with motor protein Myosin. In vertebrates, *Drosophila* as well as *C.elegans*, Anillin and myosin are recruited independently (90-92). However, once recruited, the role of Anillin is to organize myosin (91). Depletion of *Drosophila* or human Anillin from cultured cells perturbs the temporal and spatial stability of myosin at the cell equator during cytokinesis. (88-90, 92, 93, 94)

Inhibition of Anillin by injection of an anti-Anillin antibody in mammalian cells slowed the rate of ring constriction throughout the course of ingression. (96) Depletion of Anillin from human or *Drosophila* cultured cells lead to lateral oscillations of the cytokinetic furrow followed by eventual failure. (88, 90, 92, 93, 95). Hence actomyosin contractility still occurs but cannot be accurately maintained at the division plane. These studies, cumulatively, indicate towards the regulatory as well as the scaffolding role of both Myosin as well as Rho GTPases, both of which are involved in the establishment of epithelial junctions.

While extensive studies have revealed that Anillin is crucial to the cytokinetic process, Anillin is also implicated in many cancers. A study conducted on Non-small cell lung cancer revealed that Anillin depletion using RNAi results in growth suppression and an increase in cell size and number of nuclei, and these cells subsequently died (97). However, another study conducted in renal cell carcinomas (RCC) revealed that anillin expression was observed in both the cytoplasm and nucleus in patients with RCC and cytoplasmic expression was associated with a favorable prognosis (98). Hence, while the upregulation of anillin is consistent in multiple cancers, there is a controversial understanding of its impact on patient survival. It has also been reported that the expression levels of anillin are positively correlated with the metastatic potential of tumors. As opposed to its well-studied role in cytokinesis, its role in interphase cells is very unclear. A few studies have reported that its over-expression can either promote the assembly and disruption stress fibers. Anillin has also been shown to be highly enriched in the Z discs of myocardial cells, where actin and myosin are stably anchored. Anillin has also been reported to be required for the stability of new, lateral plasma membranes, where anillin mutations resulted in the transformation of apposed plasma membranes into a line of vesicles over time. (98). In addition, anillin has also been reported to be crucial to cell-matrix adhesions. The R431C missense

mutation in anillin was reported to cause FSGS, which is a leading cause of kidney failure. Anillin was found to be upregulated in podocytes in specimens from individuals with FSGS and an overexpression of this anillin mutation in immortalized human podocytes resulted in enhanced podocyte motility. Although a disrupted glomerular function was observed, no evidence of podocyte detachment was observed. This could imply that there is a disruption in the junctional integrity of the glomerular basement membrane. In addition, ANLN depletion in zebrafish resulted in a loss of podocyte foot process effacement and an edematous phenotype (99). This is could be due to an inflammatory response, which has been previously associated with the endocytosis-associated with junctional disruption. From these studies, it is clear that anillin plays a myriad of roles outside of cytokinesis, especially in maintenance of epithelial junction integrity which remains largely uncharacterized. Due to this translucent understanding of the role of anillin, together with the fact that it is known to associate with actin, NM II and Rho GTPases, all of which play a critical role in junction formation, we decided to undertake a scientific endeavor in order to determine the role of Anillin in the formation, regulation and maintenance of epithelial intercellular junctions.



## MATERIALS AND METHODS

### Antibodies and reagents.

The following primary monoclonal (mAb) and polyclonal (pAb) antibodies were used to detect cytoskeletal, junction and signaling proteins: anti-Anillin pAb (Bethyl Laboratories; Montgomery, TX); anti-L-Caldesmon, p120-catenin, Ezrin, E-cadherin and Vimentin mAbs (BD Biosciences; San Jose, CA); anti- $\alpha$ -catenin mAb (Epitomic; Burlingame, CA); anti-total actin (MAB1501) (Millipore; Billerica, MA); anti-Myosin II-A/II-B/II-C pAbs (Covance; Princeton, NJ); anti-ZO-1 and Claudin 4 pAbs (Invitrogen; Carlsbad, CA); anti- $\beta$ -catenin and Fibronectin pAbs (Sigma-Aldrich; St. Louis, MO); anti-SM-22 and N-cadherin pAbs (Abcam; Cambridge, MA); anti- $\alpha$ -adducin, P-adducin (726), P-adducin (662) pAbs and anti- $\gamma$ -adducin mAb (Santa Cruz; Dallas, TX); anti-P-ERM and Moesin pAbs (Cell Signaling; Danvers, MA).

Alexa Fluor-488 and Alexa Fluor-555-conjugated donkey anti-mouse and goat anti-rabbit secondary antibodies and Alexa Fluor-555 phalloidin were obtained from Invitrogen.

Horseradish peroxidase conjugated, goat anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad Laboratories and Thermo Scientific (Rockford, IL). Latrunculin B was obtained from EMD-Millipore and TGF- $\beta$ 1 was purchased from R&B Systems (Minneapolis, MN). All other chemicals were obtained from Sigma-Aldrich. Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were acquired from Bio-Rad Laboratories.

### **Cell culture.**

A549 adenocarcinoma lung cancer cells, acquired from ATCC, were cultured in DMEM/F12 medium supplemented with 10% Fetal Bovine Serum and 5 ml Pen/Strep (from Invitrogen).

DU145 human prostate cancer cells, acquired from ATCC were cultured in DMEM medium supplemented with 10% Fetal Bovine Serum, 5 ml Pen/Strep, 7.5 ml HEPES and 5 ml pyruvate.

Both sets of cells were grown and cultured in T75 flasks (BD Biosciences) and were seeded on collagen-coated coverslips of collagen-coated (Costar). 6-well and 24-well plastic plates (Grenier Bio-One) were used to seed cells for biochemical experiments.

### **RNA interference.**

Expressional downregulation of proteins on interest in epithelial cells was achieved by using RNA interference (RNAi). Knockdown of Anillin was carried out using either siRNA Smartpool or individual siRNA duplexes from Dharmacon and Invitrogen. A549 and DU145 cells were transfected using DharmaFect 1 transfection reagent (Dharmacon) in Opti-MEM 1 medium (Invitrogen) according to the manufacturer's protocol with a final siRNA concentration of 50 nM. Dharmafect Non-targeting siRNA duplex #2 for siRNA from Santa Cruz and Dharmacon and non-coding Low GC content duplex 1 (Invitrogen) for siRNA duplexes from Invitrogen were used as controls. Cells were used for immunoblotting 72-80 hours and for qRT-PCR from 24-48 hours post-transfection.

### **Immunoblotting.**

Cells were homogenized in RIPA lysis buffer (20 mM Tris, 50 mM NaCl, 2mM EDTA, 2mM EGTA, 1% sodium deoxycholate, 1% Triton X-100 and 0.1% SDS. Ph. 7.4), containing protease inhibitor cocktail (1:100; Sigma), phosphatase inhibitor cocktails 1 and 2 (each at 1:200; Sigma-

Aldrich) and Pefabloc SC (Roche Diagnostics; Mannheim, Germany). Lysates were cleared by centrifugation (14000 g for 20 minutes), diluted two-fold with 2x SDS Laemmli buffer and boiled for 6 minutes. SDS-polyacrylamide gel electrophoresis was conducted using standard protocols with equal amount of total protein per lane (10 to 20 µg), followed by immunoblotting. Protein expression was quantified via densitometry using ImageJ software (NIH). Data is presented as normalized values using expression values in control siRNA-treated groups were at 100%. Statistical analysis was performed using Microsoft Excel.

### **qRT-PCR.**

Total RNA was isolated using the RNeasy mini kit (QIAGEN, Valencia CA) followed by DNase treatment to remove traces of genomic DNA. Total RNA (1 µg) was reverse transcribed using iScript cDNA synthesis kit (Biorad, Hercules CA). Quantitative real-time PCR was performed with 2 µg of cDNA per reaction using IQ SYBR green Supermix (Bio-Rad Laboratories) and Opticon DNA Engine Opticon thermocycler (MJ Research, Watertown, MA).

The following primers were used: human  $\alpha$ -SMA, forward-5'

AAAGACAGCTACGTGGGTGACGAA 3', reverse-5'

TTCCCATGTCGTCCCAGTTGGTGAT 3'; Human SM22, forward-5'

TCCAGGTCTGGCTGAAGAATGG 3' and reverse-5' CTGCTCCATCTGCTTGAAGACC 3';

Hs- $\beta$ -Adducin (NM\_017484.2); forward- TTCCCCTGTGATCTTGGGTGCCGGT reverse-

AGTGCCACAGGGGCCATCAGACAT; Hs- $\alpha$ -Adducin (NM\_176801.1), forward-

TCTGGGCTACAGAACTGGCT, reverse- TCTTCGACTTGGGACTGCTT and Hs- $\gamma$ -Adducin

(NM\_016824.3), forward- CACCTCCTCTCAGTCTTGGC, reverse-

GCTGTTGCAAGGGTATGGAT. In all cases, primers were designed to amplify all known

transcripts variants of the selected genes. Threshold cycle number for the gene of interest was

calculated based on the amplification curve representing a plot of the fluorescent signal intensity versus the cycle number. Delta threshold cycle number was calculated as a difference between threshold cycle numbers of anillin/adducin-siRNA- and control siRNA-transfected cells, and each value was normalized by the difference in the threshold cycle number for the housekeeping gene amplification in the same samples.

### **Cell motility assay.**

Motility of control and anillin-siRNA-transfected A549 and DU145 cells growing on standard 6-well culture plates was examined by using a wound healing assay. For that, a single linear wound was made through the cell monolayers with a sterile pipette tip. Each well was washed to remove cellular debris and was then placed in respective complete media. The positions at which wounds were to be measured were marked on the undersurface of the wells to ensure measurements taken across the different time points were taken at the identical points. Images of wounds were taken at 0 hours, 8 hours and 24 hours post-wounding on (Olympus IX70 Fluorescence Microscope). Measurements of the wound size were performed by using T-scratch imaging software. For each wound, four different measurements were along the length of the wound were averaged to determine the wound widths and distances that wound migrated into the wound space from both edges, i.e. extent of wound closure. The migrating distances of two different cell monolayers were measured and averaged in the experiment, and the same experiment was independently repeated three times for each experimental group.

### **Immunofluorescence labeling, confocal microscopy and image analysis.**

A549 and DU145 cell monolayers plated on collagen-coated coverslips were fixed in 100% methanol for 20 minutes at  $-20^{\circ}\text{C}$ . Fixed cells were blocked for 60 minutes at room temperature

in HEPES-buffered Hanks balanced salt solution (HBBS<sup>+</sup>) containing 1% Bovine Serum Albumin (BSA). After blocking, cells were incubated in appropriate concentrations of primary antibodies in blocking solution for 60 minutes. Cells were then washed and incubated with Alexa dye-conjugated secondary antibodies for 60 minutes, rinsed with blocking buffer and mounted on slides with ProLong Antifade medium (Invitrogen).

Immunofluorescence labeled cell monolayers were observed using Zeiss LSM 700 Laser Scanning Microscope (Zeiss Microimaging Inc.; Thornwood; NY). The Alexa Fluor 488 and 568 signals were imaged sequentially in frame-interlace mode to eliminate cross talk between channels. Image analysis was conducted using imaging software ZEN 2011 (Carl Zeiss Microscopy Inc.; Thornwood; NY) and Adobe Photoshop. Images shown are representative of at least 3 experiments. Multiple images were captured from each slide.

### **Statistics.**

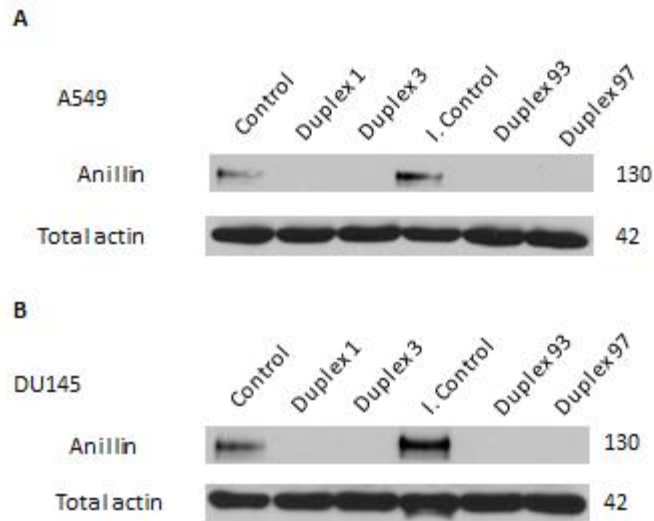
All numerical values from individual experiments were pooled and expressed as mean  $\pm$  standard error of the mean (S.E) throughout. Obtained numbers were compared by two-tailed Student's t-test, with statistical significance assumed at  $P < 0.05$ .

## RESULTS

### **Loss of anillin results in disruption tight junctions and adherens junction.**

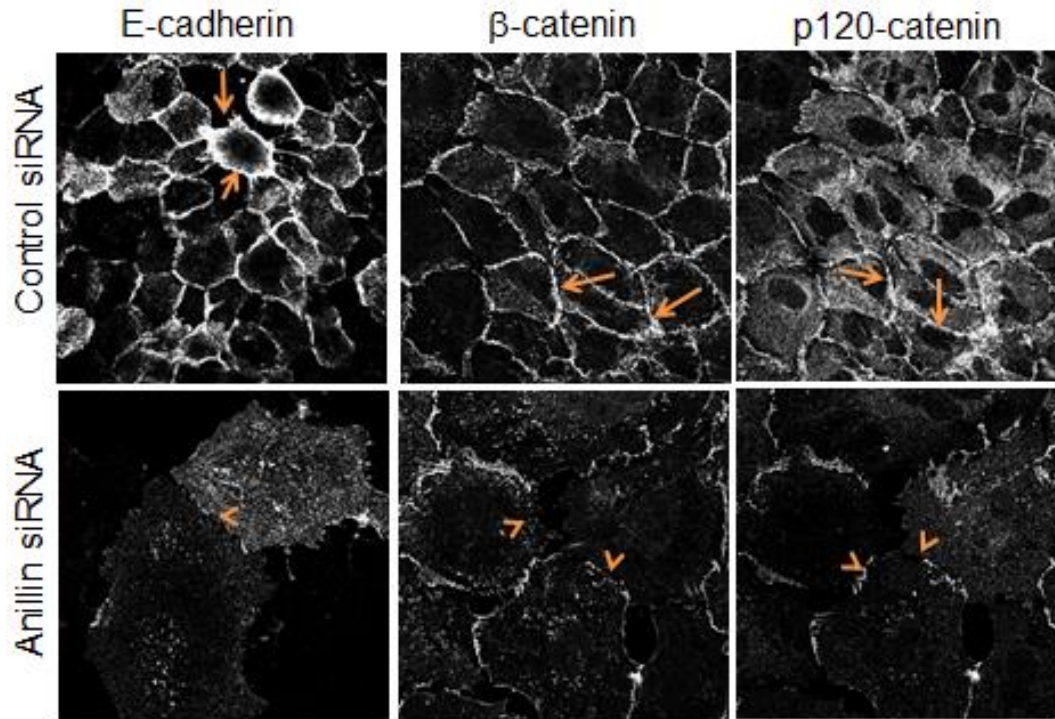
Despite extensive studies being conducted on the role of Anillin in mitotic cells, very little information is available on the role that anillin plays in interphase cells. Its role as a scaffold for myosin and Rho GTPases suggests that it is involved in cytoskeletal organization. Due to the fact that both RhoA and NM II play important role in establishment of junctions, we decided it would be interesting to see the effect of Anillin depletion on the integrity and organization of epithelial junctions. In order to test this, RNAi interference was used to knockdown Anillin in A549 human lung epithelial cells and DU145 human prostate epithelial cells. Although, A549 and Du145 cells are tumor-derived cell lines they preserve significant extent of epithelial differentiation and are characterized by well-formed cell-cell contacts. Another important feature of these cells is their responsiveness to different growth factors. For example, A549 cells are known to undergo EMT upon treatment with TGF-b, while DU145 cells respond to HGF with junctional disassembly and cell scattering. Hence these cell lines are good model to study dynamics of epithelial junctions in in vitro models of tissue fibrosis and tumor metastasis.

Transfection with the Smartpool Duplex from Dharmacon dramatically reduced Anillin expression in both A549 (Fig. 6A) and DU145 (Fig. 6B) cells.



**Figure 6: Anillin specific siRNAs result in a marked depletion of targeted protein.** Immunoblotting analysis shows that transfection of A549 (A) and DU145 (B) cells with two different sets of Anillin targeting siRNA duplexes results in substantial reduction in the expression levels of Anillin in both epithelial cell lines on day 4 post-transfection.

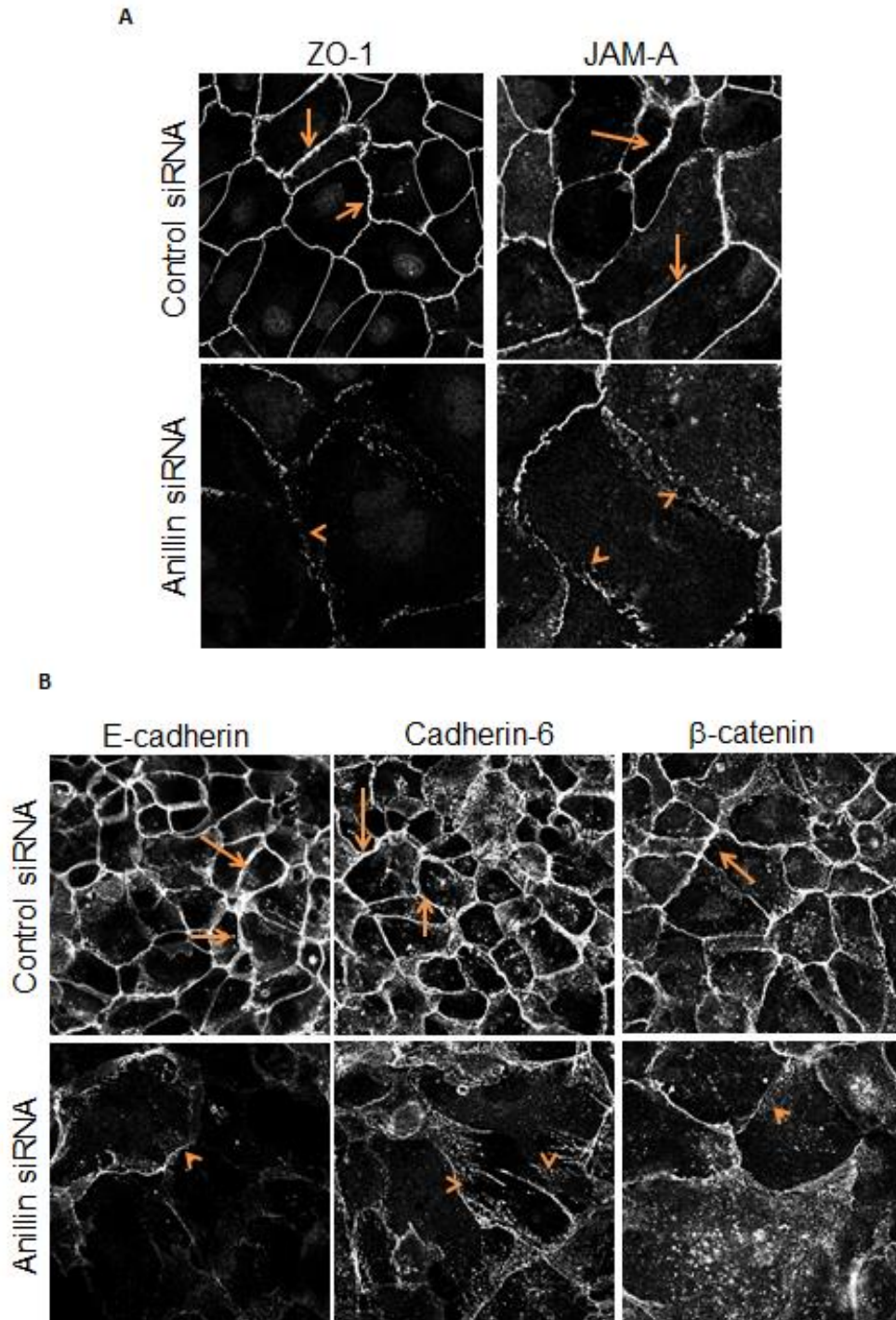
Immunolabeling and confocal microscopy of control siRNA-transfected A549 cell monolayers demonstrated predominant localization of E-cadherin, and  $\beta$ -catenin at the areas of cell-cell contacts (Fig. 7, arrows). p120 catenin was also accumulated at cell-cell junctions but was also present in the cytosol. Loss of anillin resulted in significant increase in cell size and AJ disassembly that was manifested by disappearance of E-cadherin,  $\beta$ -catenin and p120-catenin from the areas of cell-cell contacts (Fig. 7, arrows).



**Figure 7: Anillin depletion results in disruption of adherens junctions in A549 lung epithelial cells.** A549 cells plated on coverslips were transfected with either control or Anillin-specific siRNAs and on day 4 post-transfection were immunolabeled for adherens junction proteins E-cadherin,  $\beta$ -catenin and p-120 catenin. Note the enrichment of all three adherens junction proteins at the areas of cell-cell contacts in control cells (arrows) and disappearance of AJ protein from the intercellular contacts of Anillin depleted cells (arrows).

A major difference between A549 and DU145 cells is that the latter cells developed prominent TJ that were visualized by immunolabeling of ZO-1 and JAM-A in addition to AJ composed by E-cadherin, Cadherin-6 and  $\beta$ -catenin (Fig. 8). Immunolabeling for ZO-1 and JAM-A reveals a continuous high-intensity staining pattern in the areas of cell-cell contacts of control cells. Loss of anillin transformed this labeling into weak, discontinuous staining pattern that is indicative of TJ disassembly (Fig. 8A).





**Figure 8: Loss of Anillin results in disassembly of TJs and AJs in DU145 prostate epithelial cells.** DU145 cells plated on coverslips were transfected with either control or Anillin- specific siRNAs. On day 4 post-transfection, the cells were immunolabeled for protein markers of TJs (A) and AJs (B). Note a prominent localization of AJ and TJ proteins at well-aligned intercellular contacts in control cells (arrows) and distortion of intercellular contacts accompanied by loss of junctional localization of both TJ and AJ proteins in Anillin depleted cells (arrowheads).

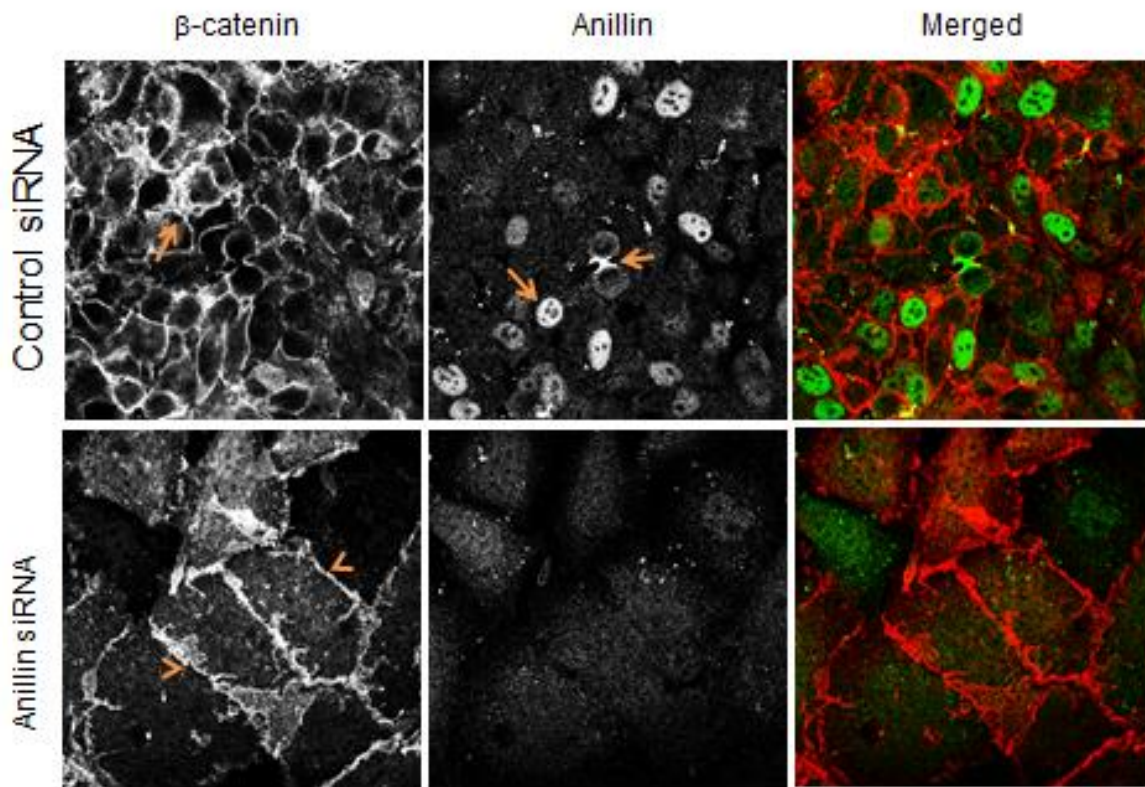
Additionally, loss of anillin disrupted junctional labeling of junction proteins E-cadherin, Cadherin 6 and  $\beta$ -catenin. As shown in Figure 8B, control cells reveal sharp localization of these AJ proteins at the cell-cell contacts. Anillin depletion results in the deformation of intercellular contacts accompanied by the loss of junctional localization of the AJ proteins. E-cadherin is diffused from the intercellular junctions while Cadherin 6 and  $\beta$ -catenin show organizational disruption and internalization into the cell.

Hence, our study reveals that the depletion of Anillin results in the disruption of intercellular contacts in both A549 and DU145 cells, showing that the phenomena of junctional disruption is not a cell-specific effect and is common for different cell lines. These data suggest that anillin is critical in maintaining the proper organization of epithelial adherens and tight junctions.

#### **Anillin is not accumulated at cell-cell contacts in interphase cells.**

Next we sought to investigate molecular mechanisms underlying the effects of anillin knockdown on epithelial junctions. Given previously published data that anillin can physically interact with different regulators of the actomyosin cytoskeleton, one can suggest that anillin can accumulate at the areas of cell-cell contacts and stabilize junction-associated F-actin filaments. In order to examine this possibility, we conducted co-immunolabeling of anillin in A549 cell monolayers. The staining pattern observed indicated that Anillin is primarily localized in the nucleus of interphase epithelial cells (Fig. 9). Interestingly, in dividing cells anillin was exclusively localized in the cleavage furrow, which is consistent with its role in cytokinesis (Fig. 9). Such cleavage furrow localization of anillin in mitotic cells highlight specificity of anillin

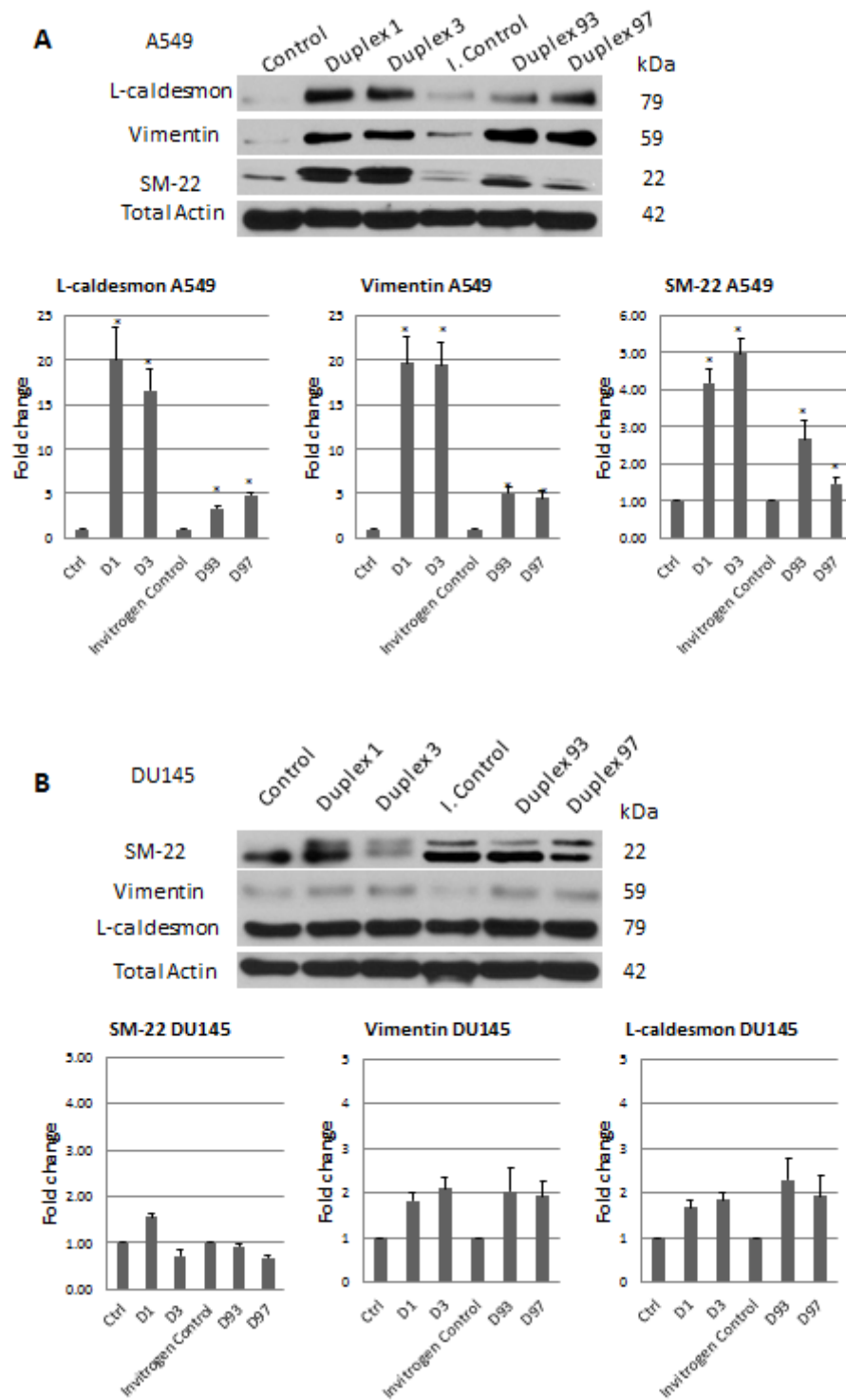
antibody used for immunolabeling and suggests that its nuclear localization in interphase cells does not represent a staining artefact. This is also supported by the lack co-localization of anillin with AJ protein  $\beta$ -catenin. Overall, this data rules out direct association of Anillin with intercellular junctions in confluent cell monolayers and suggest that loss of anillin affect junctional integrity via some indirect mechanisms.

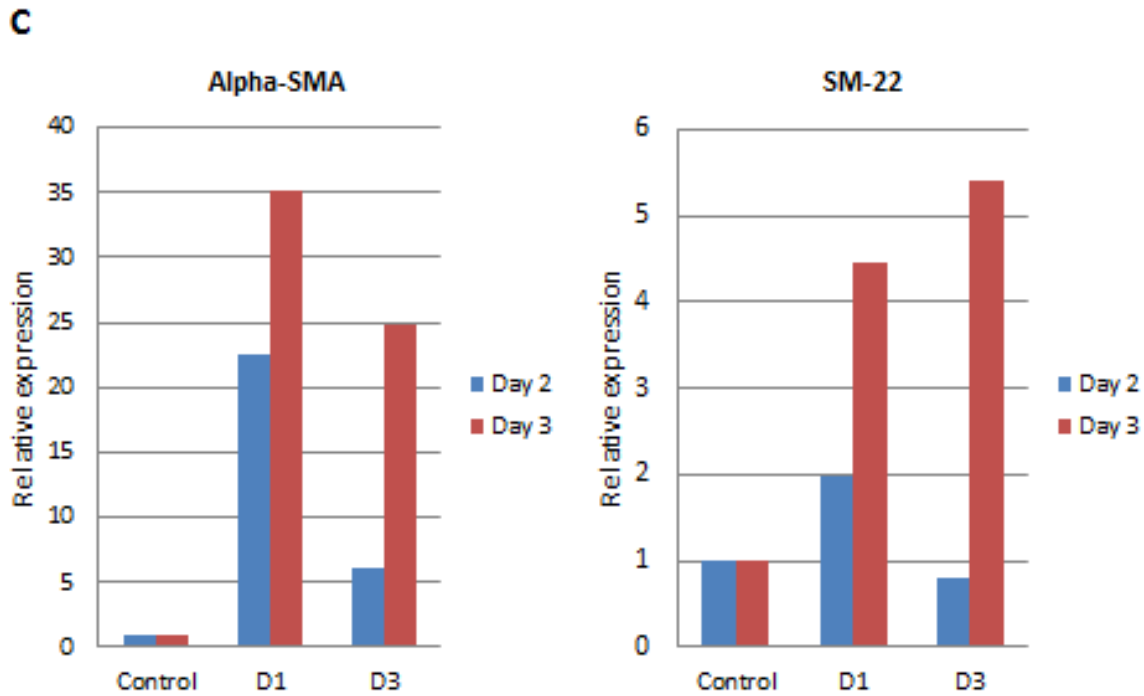


**Figure 9: Anillin does not localize at intercellular contacts in interphase cells.**

Immunostaining and confocal microscopy show predominantly nuclear localization of Anillin in interphase A549 cells and its selective recruitment to the cleavage furrow in mitotic A549 cells (arrows).  $\beta$ -catenin localizes at site of cell contacts in control siRNA transfected cells and this signal is lost from intercellular contacts when anillin is depleted.

**Anillin depletion induces EMT-like phenotype in A549 cells, but not in DU145 cells.**





**Figure 10: Anillin depletion selectively induces epithelial-to myofibroblast transition (EMT) in A549 cells.** A549 (A) and DU145 (B) epithelial cells were transfected with two sets of anillin siRNA duplexes: Dharmacon duplexes 1 and 3 and Invitrogen duplexes 93 and 97 along with their appropriate controls. Cell lysates were collected 4 days post-transfection and subjected for immunoblotting analysis of different EMT markers. Note a strong up-regulation of all tested EMT markers in A549 cells (A) but no consistent changes in EMT marker expression in DU145 cells (B). Quantitative real time RT-PCR analysis conducted in A549 cells on days 2 and 3 post-transfection reveals a dramatic increase in mRNA expression of  $\alpha$ -SMA and SM-22 in anillin-depleted cells (C). Data are presented as mean  $\pm$  SE (n=3); \*p < 0.05.

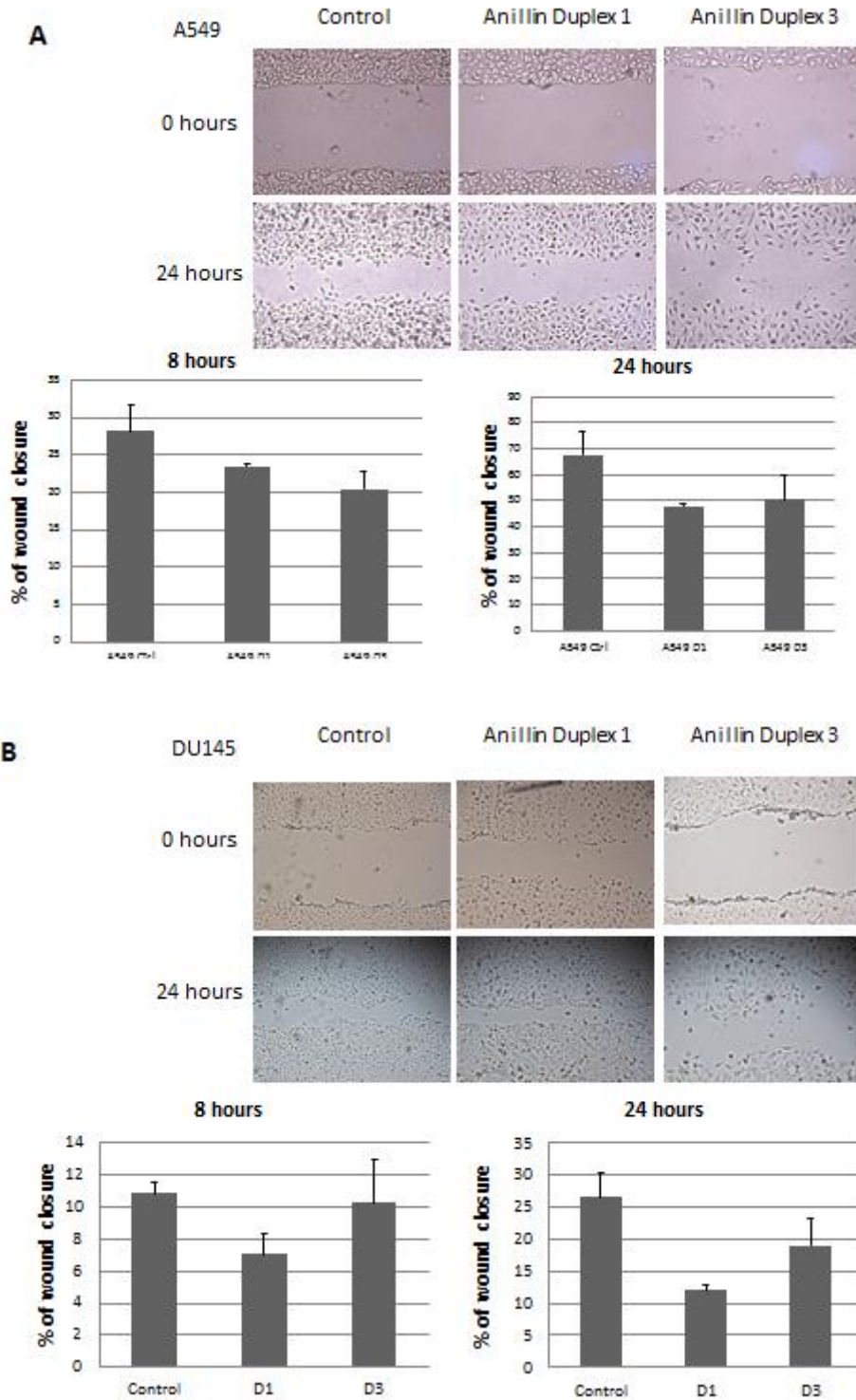
While examining the effects of anillin depletion on epithelial junctions, we noticed that loss of this protein markedly increased size of A549 and Du145 cells (Figs 7 & 8). Furthermore, anillin depletion appears to alter the shape of A459 cells from an orthogonal to a spindle-like. These changes resembled alteration of A549 cell morphology during the Epithelial Mesenchymal Transition (EMT) triggered by incubation with transforming growth factor beta (TGF $\beta$ ) (data not shown). Given fact that TGF $\beta$ -induced EMT readily disrupts epithelial cell-cell contacts, we

hypothesized that AJ and TJ disassembly in A459 and DU145 cells can be due to induction of EMT. To test this hypothesis, we examined expression levels of common EMT markers, L-caldesmon, Vimentin and SM-22, in control and Anillin depleted A549 and DU145 cells. Anillin depletion was achieved by using specific siRNA duplexes obtained from both Dharmacon and Invitrogen. Figure 10A shows that all anillin-specific siRNA consistently upregulated expression of 3 markers of EMT in A549 cells. By contrast, no consistent induction of EMT markers was observed in DU145 cells (Fig. 10B) where SM-22 expression was increased by only one set of anillin siRNAs, while no significant upregulation of L-caldesmon or Vimentin was observed. These results suggest that Anillin depletion-induced EMT phenotype is a cell-specific phenomenon, occurring only in A549 cells and not in DU145 cells

Since EMT induction involves transcriptional reprogramming of the cell we next decided to investigate if upregulation of EMT marker in A549 cells was due to increase in their mRNA expression. This was investigated by qRT-PCR analysis of myofibroblast markers SM-22 and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). Cell lysates were collected at two separate time points (days 2 and 3 post-transfection) in order to determine if the increased transcription was at an earlier or later time point. As is observed in Figure 10C,  $\alpha$ -SMA transcription is significantly increased as early as day 2 and continues to increase going into day 3 and a more prominent increase in SM-22 transcription on day 3 as compared to day 2. These results suggest that anillin knockdown causes transcriptional reprogramming of A549 epithelial cells toward increased expression of proteins characteristics for mesenchymal and myofibroblast cells. Furthermore, our data suggest that junctional disassembly in anillin-depleted epithelial cells is likely to be independent of EMT induction.

### **Anillin depletion does not increase cell motility.**

It is well-appreciated that disruption of epithelial junctions and induction of EMT frequently results in enhanced cell motility. In addition to the increased cell size and altered cell shape, another thing observed as part of the EMT phenomena is an increase in cell motility. Keeping this in mind, we decided to investigate if the loss of Anillin, which alters cell shape and size, also results in an increase in cell motility. In order to examine this effect, we down-regulated Anillin in A549 and DU145 cells using the RNAi approach. Cells were mechanically wounded and cell migration into the wound was examined at 8 and 24 h after wounding. As is observed in Figure 6A-B, no increase in cell motility is observed in Anillin depleted cells on comparison of multiple time points, i.e. comparison between 0 hours and 8 hours and between 0 hours and 24 hours in both A549 (Fig 11A) and DU145 (Fig 11B). Both sets of values obtained for the graph were obtained by subtraction of values obtained at later time point by values obtained at earlier time points. All data is representative of three identical experiments conducted. Hence, Anillin depletion does not result in any increase in cell motility in A549 and DU145 cells. The fact that anillin knockdown does not increase cell migration but upregulates EMT markers in A549 cells suggests that loss of anillin induces not a classical EMT but rather a subset of EMT known as Epithelial to Myofibroblast transition (EMyT). EMyT is characterized by induction of cytoskeletal/contractile proteins that are enriched in smooth muscle of myofibroblast cells without stimulating cell motility.

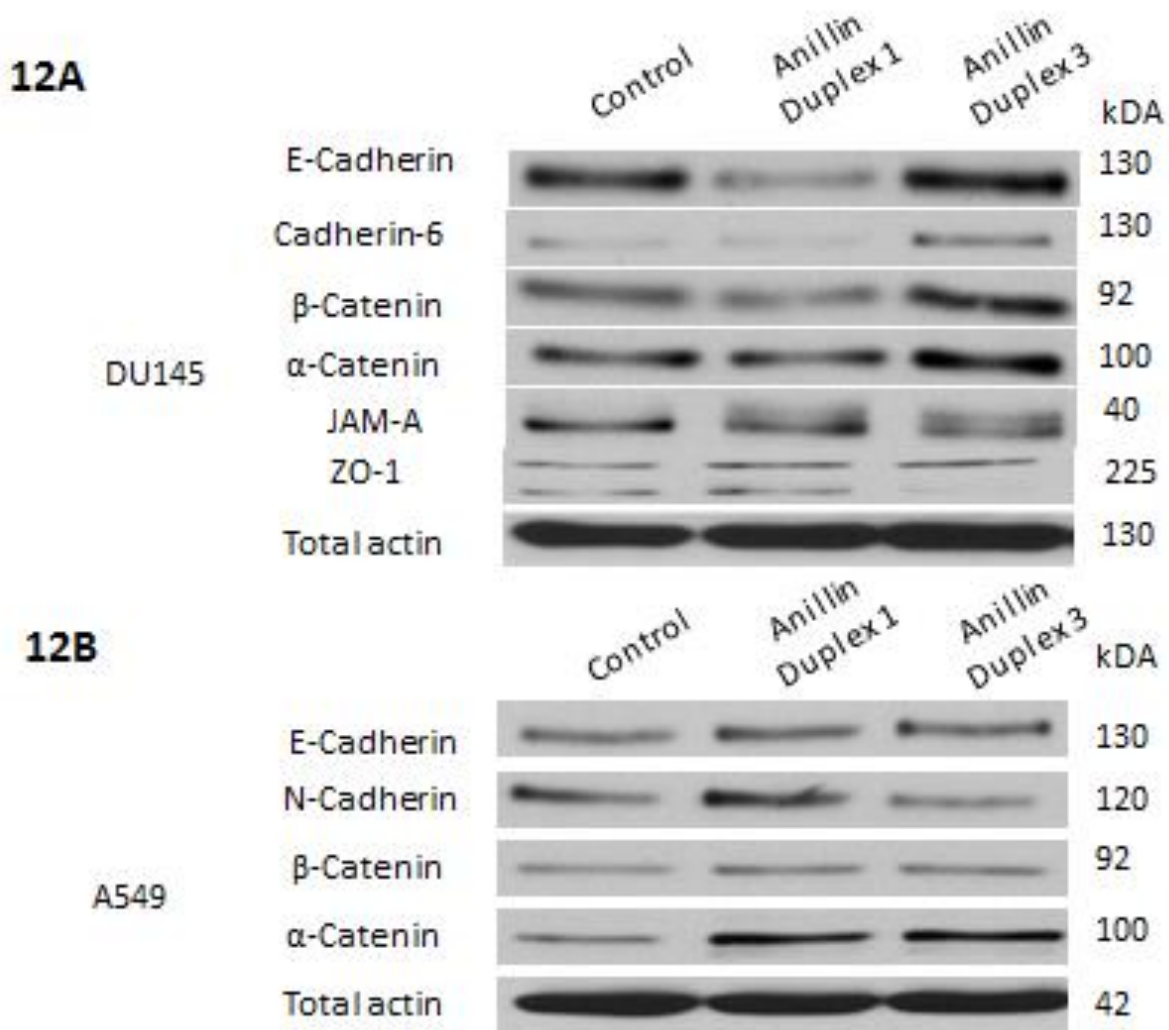


**Figure 11: Anillin depletion does not increase motility of epithelial cells.** Control or anillin siRNA transfected A549 (A) and DU145 (B) cell monolayers were mechanically wounded on day 4 post-transfection and cell migration into the wound area was examined at 0, 8 and 24 h after wounding. Data is presented as mean SE of three independent measurements.



### **Anillin depletion does not affect expression of different AJ and TJ proteins.**

Anillin depletion results in disruption of junctional complexes. A likely explanation for this disruption of intercellular junctions might be the downregulation of junctional protein expression, hence causing the loss of localization of AJ and TJ proteins from the site of intercellular contact and deformation of cell-cell contacts. In order to test this hypothesis, we investigated the effect of anillin knockdown on expression of different AJ and TJ proteins in Du145 and A459 cells. Immunoblotting analysis of total cell lysates demonstrated that loss of anillin did not affect expression of AJ proteins E-cadherin, Cadherin 6,  $\alpha$ - and  $\beta$ -catenin or TJ protein JAM-A and ZO-1 in DU145 cells. Similarly, anillin depletion did not downregulate expression of AJ proteins in A549 cells and even caused a slight increase in  $\alpha$ -catenin expression (Fig 12B); these results strongly suggest that AJ and TJ disassembly in anillin-depleted cells cannot be explained by the decreased expression of different junctional proteins.

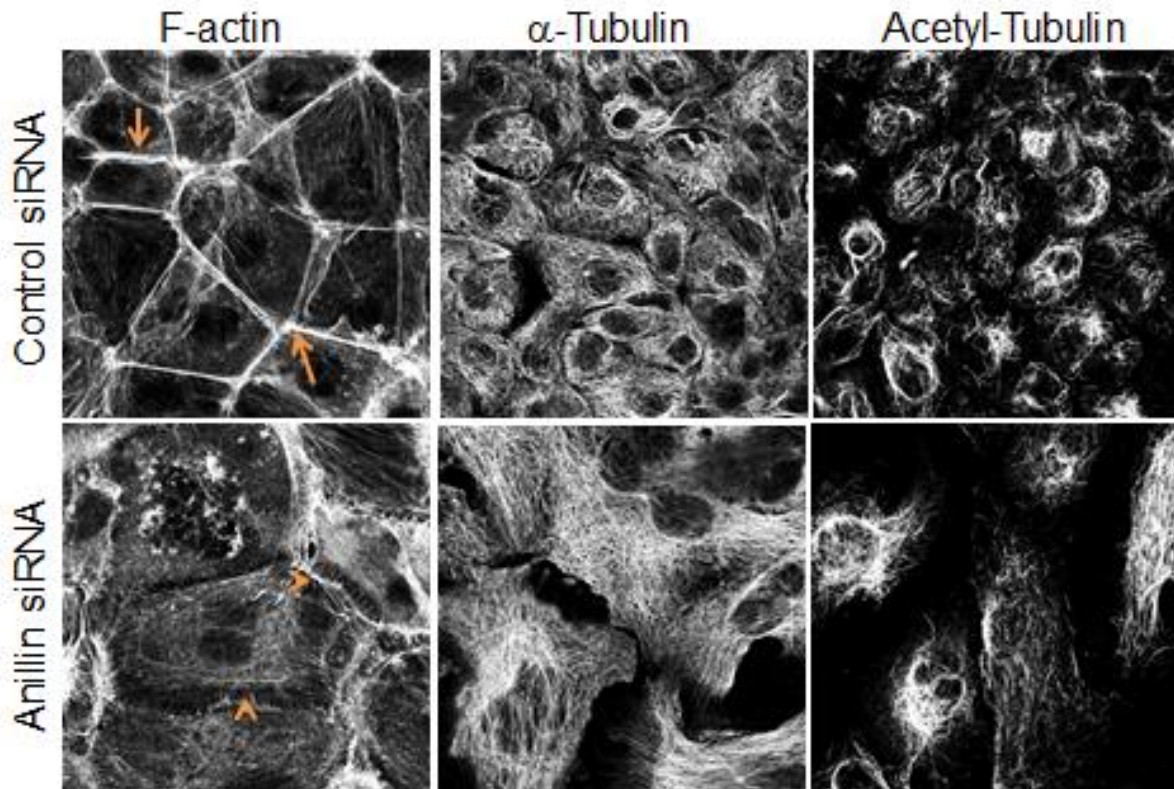


**Figure 12: Effect of anillin knockdown on expression levels of AJ/TJ proteins in DU145 and A549 cells.** Immunoblotting analysis was conducted on cell lysates collected from control and Anillin depleted DU145 (A) and A549 (B) on day 4 post-transfection. Anillin depleted DU145 cells do not show consistent changes in the expression level of any examined AJ or TJ protein as compared to the control group. By contrast, anillin depleted A549 cells show substantial increase in  $\alpha$ -catenin expression and slight increase in E-cadherin and  $\beta$ -catenin levels.

### **Anillin depletion induces disorganization of the perijunctional F-actin belt in DU145 cells.**

The absence of any effect of anillin knockdown on junctional protein expression suggests the involvement of an alternative mechanism. Given a critical roles of the cytoskeleton in regulation stability and remodeling of epithelial junctions, we next investigated the effects of anillin knockdown on the integrity of two major cytoskeletal elements such as actin filaments and microtubules. This indicates that Anillin may be affecting the integrity and organization of epithelial junctions via modulation of some other proteins. Since Anillin has been shown to be a scaffold for multiple cytoskeletal proteins, including actin, the next logical step was to determine the underlying mechanism of junctional disruption was to explore the possibility that the structural organization of the peri-junctional actomyosin belt is being affected. In addition to observing the effects of Anillin depletion on the actin cytoskeleton, we also wanted to determine if there were any effects on the organization of other cytoskeletal networks like the microtubule (MT) network. Confluent DU145 cell monolayers were used since they have well-defined and more consistent junctions and so serve as a suitable system for conducting these studies. Staining was conducted for F-actin,  $\alpha$ -tubulin and stable post-translationally modified Acetylated tubulin. Figure 13 shows a well-organized actin cytoskeleton with a sharp localization of actin at the intercellular contacts, representing the peri-junctional F-actin belt and a normally organized  $\alpha$ -tubulin and Acetyl-tubulin staining pattern. Anillin depletion in these cells reveals complete disorganization of the actin network within the cell as well as disruption of the peri-junctional F-actin belt. However, no change is observed in the general organization of  $\alpha$ -tubulin or acetylated tubulin. This data indicates that anillin depletion induces the disorganization of the perijunctional F-actin belt in DU145 cells. Since the actin cytoskeleton is critical to maintaining the

shape and size of the cell, the Anillin-induced disruption might explain the distorted shape and increased size of Anillin depleted cells and account for the breaking of cell-cell contacts.

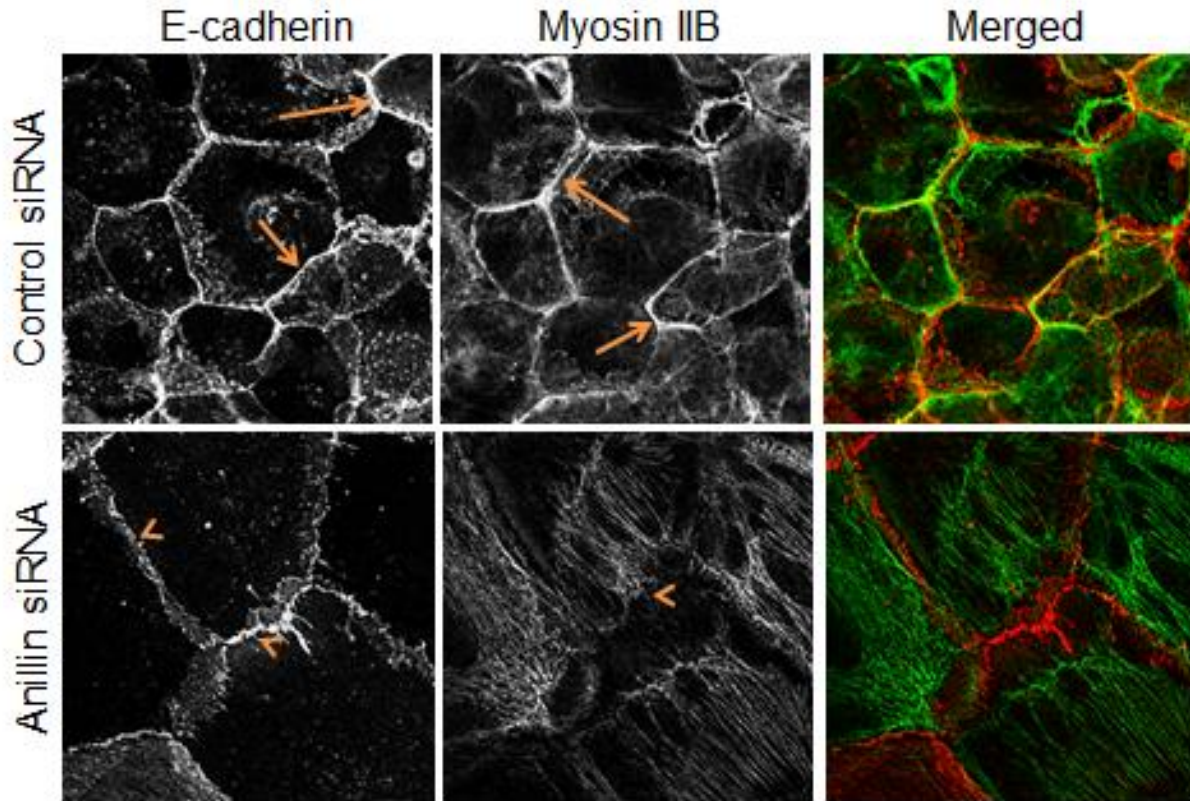


**Figure 13: Anillin depletion induces disorganization of perijunctional F-actin belt in DU145 cells.** Du145 cells were transfected with either control or anillin-specific siRNAs. Cells were fixed on day 4 post-transfection and were labeled for filamentous (F) actin using fluoresceinated phalloidin and for microtubules using antibodies against  $\alpha$ -Tubulin or acetylated tubulin. Note that anillin knockdown disrupts a characteristics perijunctional F-actin belt but has no effect of general organization of total and acetylated microtubules.

## **Anillin depletion induces mislocalization of the peri-junctional nonmuscle myosin II.**

Myosin serves as the motor protein for actin, rendering the property of dynamism to the cytoskeleton structure. Previous data has shown siRNA mediated depletion of NM IIA results in complete disappearance of radial F-actin in the areas of initial cell-cell contacts and loss of F-actin bundles is observed in NM-IIA deficient fibroblasts and epithelial cells (103,105).

Previously published data has suggested that at early stages of junctional assembly NM II works as an important actin-bundling/cross-linking protein. In addition, NM II has also been reported to be critical for TJ formation. Studies have reported TJ attenuation after blebbistatin treatment (55, 119,136) or siRNA mediated knockdown of NM IIA (71). Since junctions are affected on depletion of Anillin, an alteration in myosin localization or organization might serve to explain the junctional disruption. In order to investigate this hypothesis, we conducted dual immunolabeling for E-cadherin and Myosin II-B on control and Anillin depleted monolayers of DU145 cells. Figure 14 shows co-localization of E-cadherin and Myosin IIB at the site of cell-cell contacts in control cells where NM IIB is organized in the perijunctional actomyosin belt. Since NM II plays a critical role actin bundling protein in junctional assembly, its mislocalization on anillin depletion may account for the loss of actin from the perijunctional region.

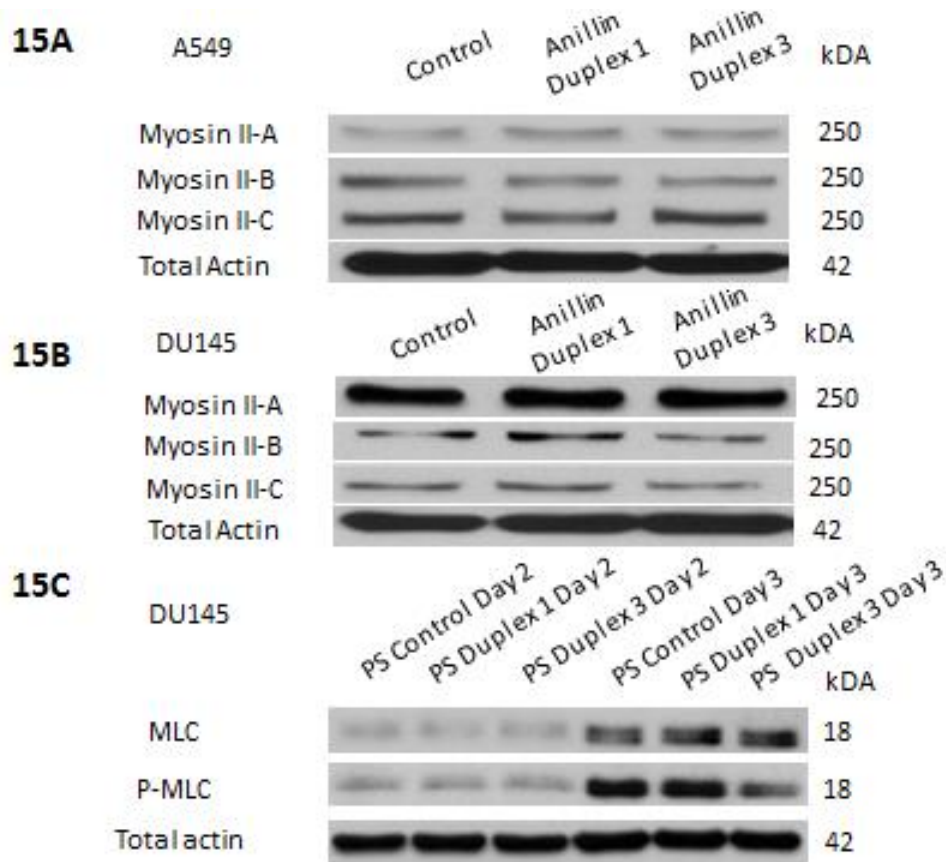


**Figure 14: Anillin depletion induces mislocalization of perijunctional NM II motor.**

DU145 cells were transfected with either control or anillin-specific siRNAs and on day 4 post-transfection were subjected to a dual labeling for JAM-A (red) and non-muscle myosin IIB (NM IIB, green). Note prominent accumulation of NM IIB in the perijunctional actomyosin belt in control cells and loss of perijunctional NM IIB in anillin-depleted cells.

### **Knockdown of Anillin does not affect expression and activation status of NM II motor.**

One of the explanations for the mislocalization of the peri-junctional NM II could be the Anillin induced down-regulation of NM II isomers or myosin regulatory light chain. The mislocalization of NM II may also be due to the altered activation status of the NM II motor. In order to test this hypothesis, we conducted immunoblotting analysis to determine any change in the expression levels of NM II as well as the activation status of NM II via examining MLC phosphorylation. As is observed in Figures 15 A-B, no change is observed in the expression levels of multiple isoforms of Myosin II, i.e., IIA, B and C in either DU145 (Fig 15A) or A549 (Fig 15B) cells. Figure 15C shows no change in the expression levels or phosphorylation of MLC, suggesting that Anillin has no effect on the expression or activation status of the NM II motor and that this is unlikely to be the mechanism underlying mislocalization of NM II from the site of cell-cell contacts.

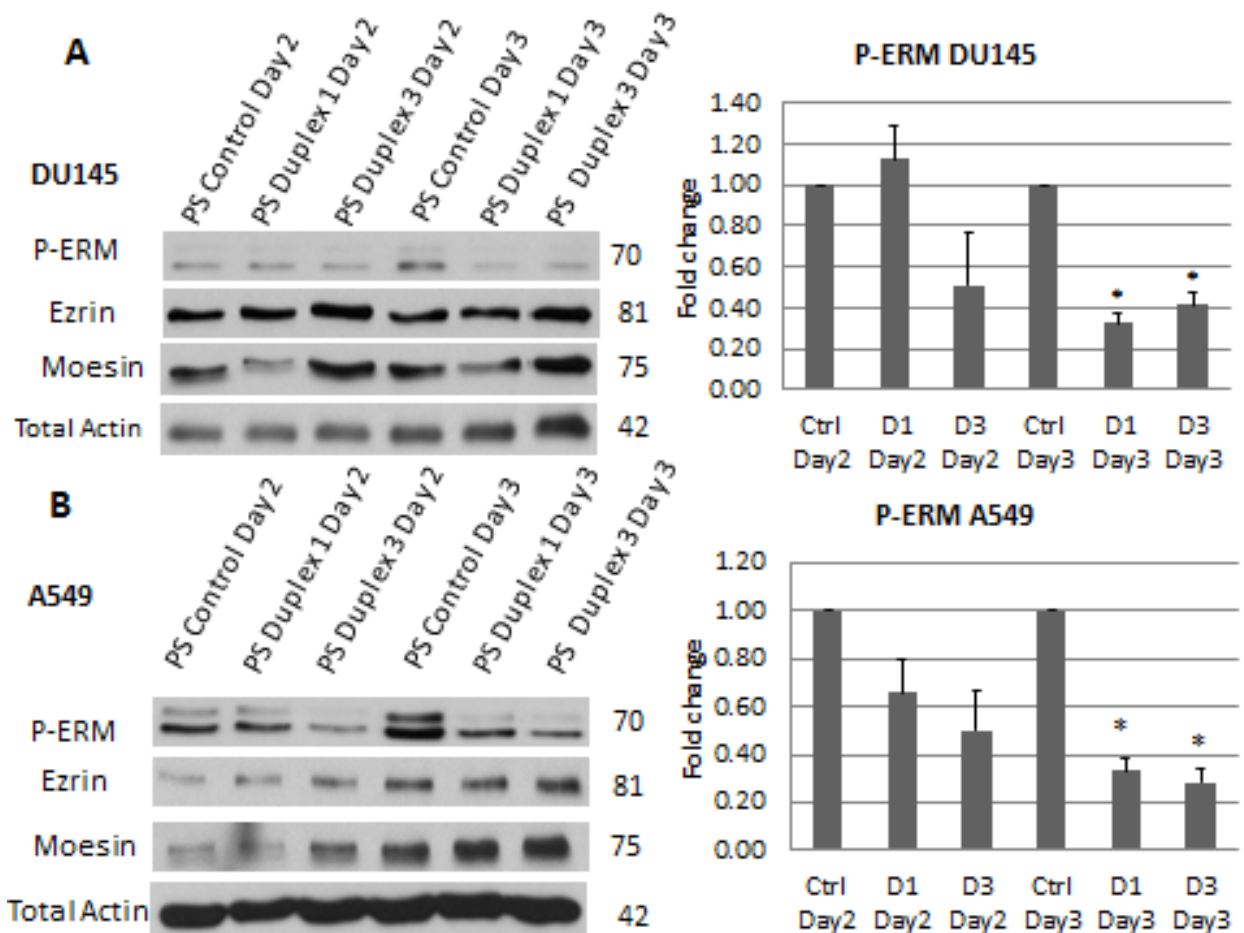


**Figure 15: Knockdown of Anillin does not affect expression and activation status of NM II motor.** Immunoblotting analysis of cell lysates collected from control and Anillin depleted DU145 (A) and A549 (B) cells demonstrates no significant change in the expression levels of different NM II heavy chain isoforms when compared to control groups. (C) Immunoblotting analysis show neither altered expression of myosin light chain (MLC), nor significant changes in MLC phosphorylation after anillin knockdown in DU145 cells.



### **Anillin depletion results in dephosphorylation of ezrin-radixin-moesin (ERM) proteins.**

In addition to the reorganization of perijunctional actin filaments, another mechanism of that can drive the epithelial junction reorganization involves remodeling of the plasma membrane. The ERM family of proteins serves as linkers between the actin cytoskeleton and the plasma membrane proteins and as signal transducers in response to remodeling of the cytoskeleton (104). We hypothesized that loss of cytoskeletal organization due to anillin depletion may be due to alteration in the expression levels/activation status of the ERM complex. Figure 16 shows no change in the total Ezrin or Moesin levels. Interestingly, there is a decrease in the level of phosphorylated ERM, indicating ERM becomes more active when Anillin is depleted. Previous studies have demonstrated that the ERM complex has to be dephosphorylated to perform its linking function. Our results indicate that more of the ERM complex is available in its functionally active confirmation and hence make it unlikely to be the underlying mechanism for actin cytoskeleton disorganization and subsequent disruption of epithelial junctions.



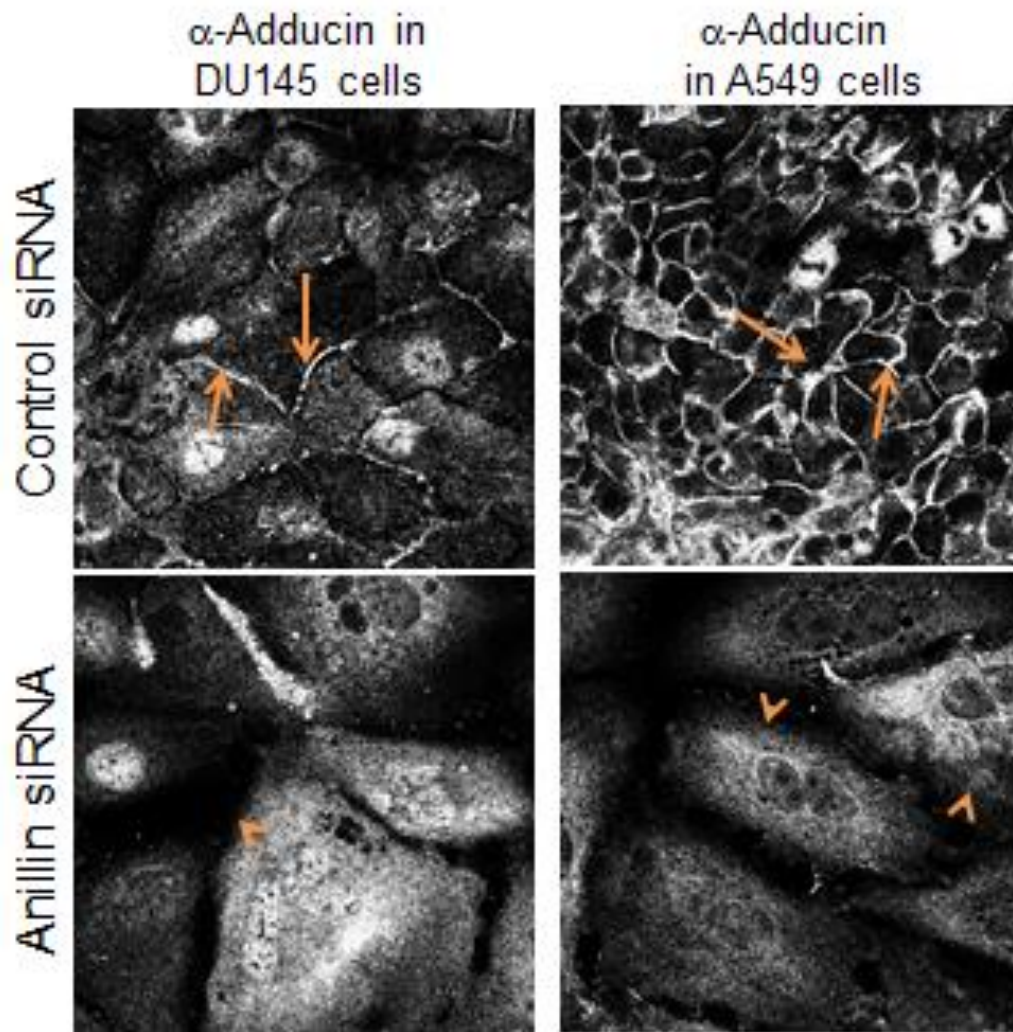
**Figure 16: Anillin depletion results in dephosphorylation of ezrin-radixin-moesin (ERM) proteins.** DU145 (A) and A549 (B) epithelial cells were transfected with either control or anillin specific siRNA duplexes D1 and D3. Cell lysates collected on days 2 and 3 post-transfection were analyzed for expression and phosphorylation of ERM proteins. Note that anillin knockdown triggers significant dephosphorylation of ERM proteins on day 3 post-transfection without affecting expression of total ezrin or moesin. Data are presented as mean  $\pm$  SE (n=3); \*p < 0.05.

## **Loss of anillin disrupts the adducin-based membrane skeleton at epithelial cell-cell contacts.**

Another set of linker proteins is the adducins, which recruit spectrins to actin filaments and promote assembly of the spectrin lattice at the plasma membrane, allowing interaction between actin cytoskeleton and the plasma membrane. Adducin has also been reported to be an actin-bundling and capping protein. As previously observed, there is a disruption in the architecture of the perijunctional actomyosin skeleton. Since adducin forms a crucial link between the actomyosin skeleton and the plasma membrane and junction complexes, we decided to examine the localization and expression of  $\alpha$ -adducin since any change in either the localization or expression might explain the disruption of the perijunctional actomyosin.

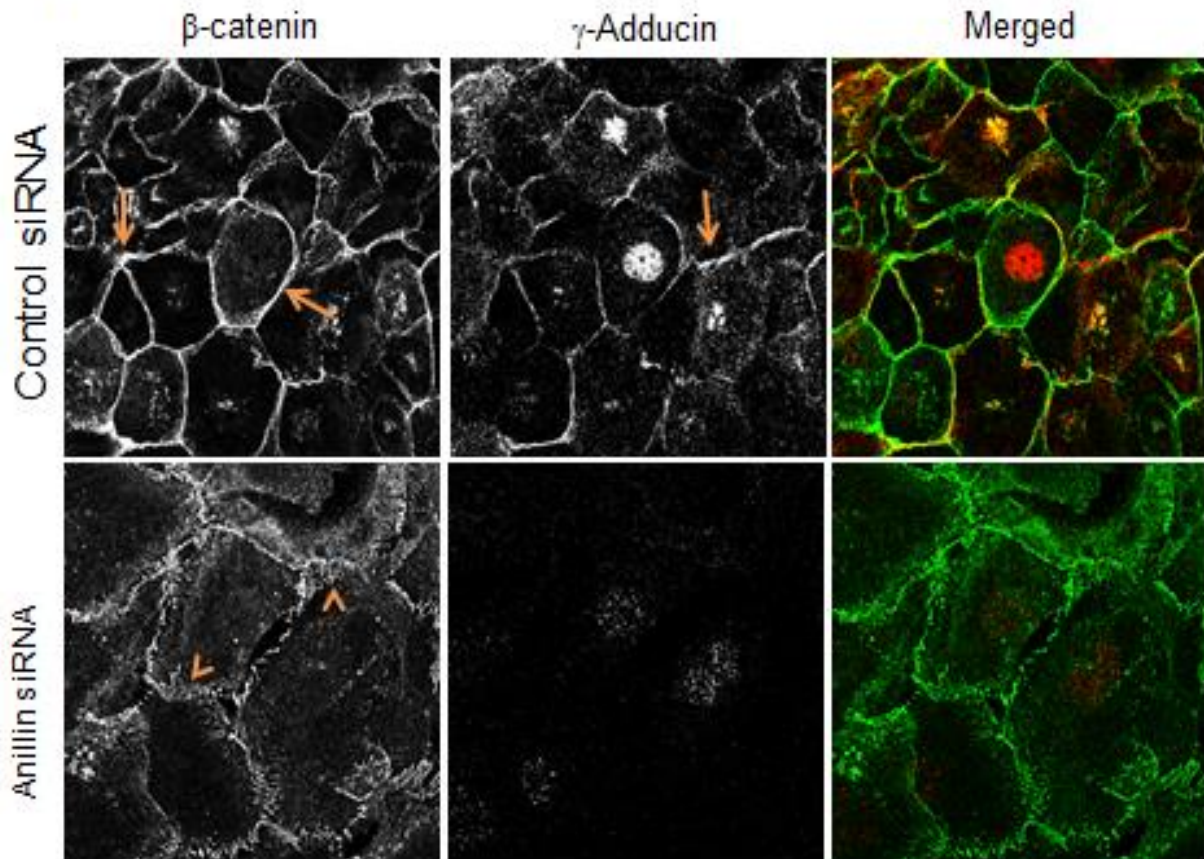
Figure 17 shows a significant  $\alpha$ -adducin signal at the site of cell-cell contacts in control cells and a loss of signal from intercellular junctions in anillin depleted cells. In addition, Figure 18 shows significant localization of both  $\beta$ -catenin and  $\gamma$ -adducin at the site of intercellular contacts in control cells and a significant loss of signals for both proteins in anillin depleted cells. These results indicate that the localization of adducin is disrupted on knockdown of anillin.

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**Figure 17: Loss of anillin disrupts the adducin-based membrane skeleton at epithelial cell-cell contacts.** A549 and DU145 epithelial cells plated on coverslips were transfected with either control or Anillin- specific siRNAs. On day 4 post-transfection, the cells were immunolabeled for  $\alpha$ -adducin, a protein component of the membrane skeleton. Note a prominent localization of  $\alpha$ -adducin at the areas of intercellular contacts in control cells (arrows) and disappearance of this membrane skeleton protein from cell-cell junctions in Anillin depleted cells (arrowheads).

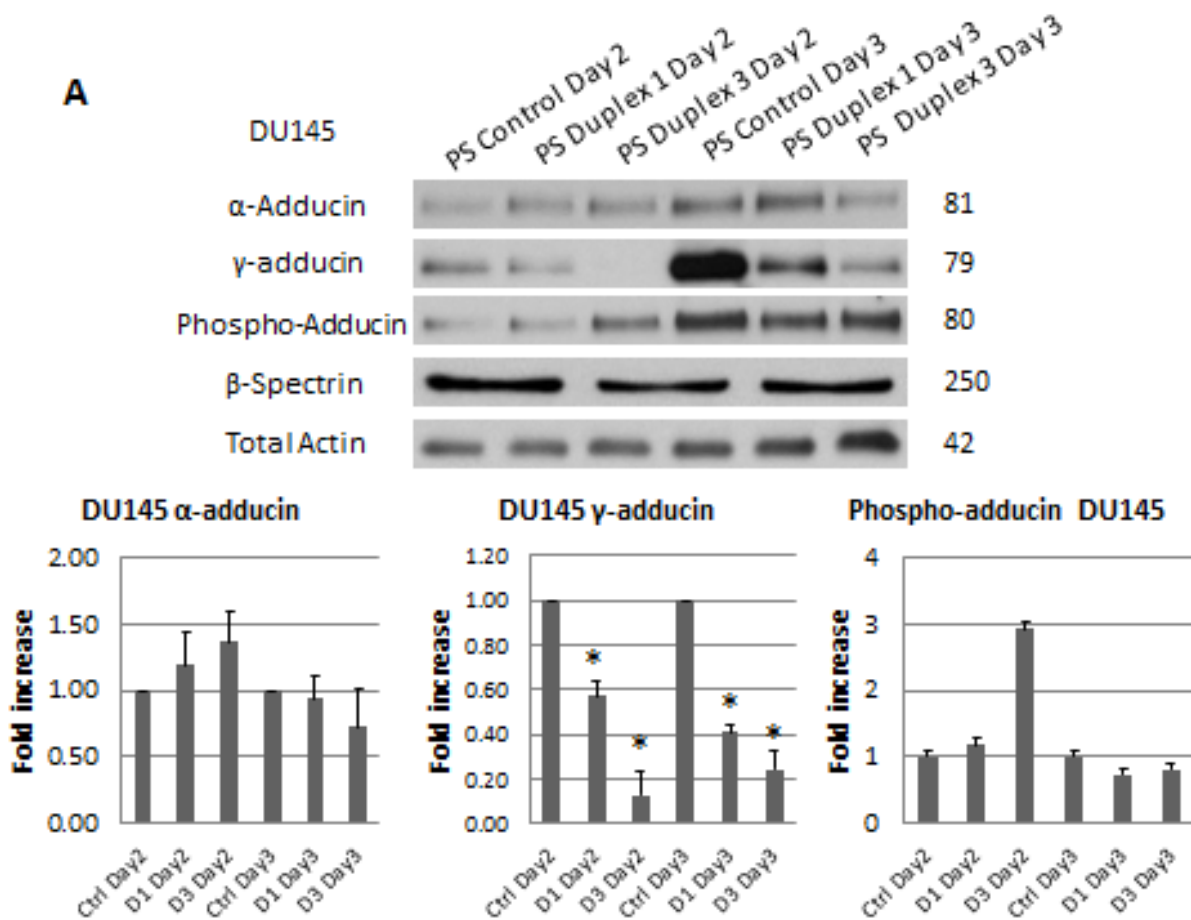
18

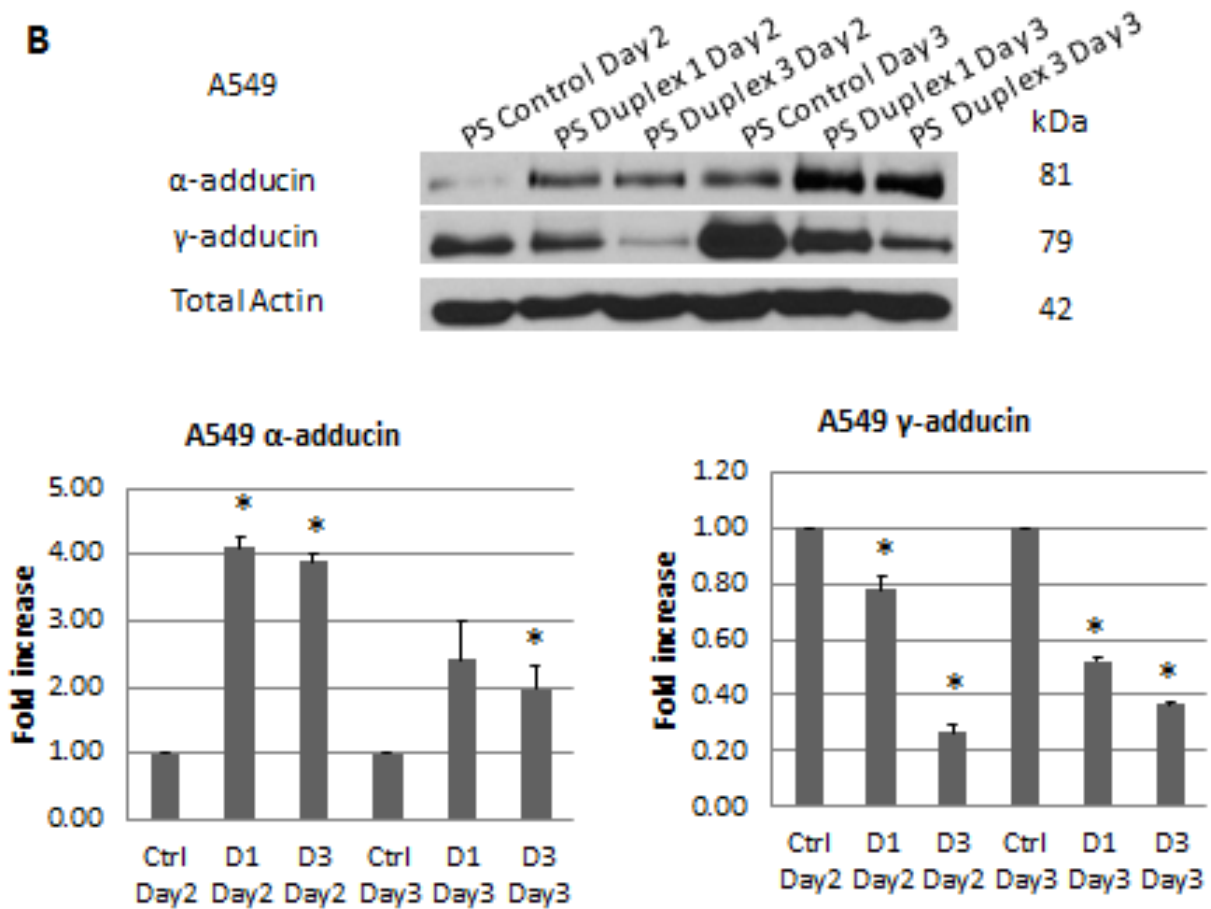


**Figure 18: Loss of anillin disrupts proper localization of  $\gamma$ -adducin.** DU145 epithelial cells plated on coverslips were transfected with either control or Anillin- specific siRNAs. On day 4 post-transfection, the cells were co-immunolabeled for  $\beta$ -catenin and  $\gamma$ -adducin. Note the prominent localization of  $\beta$ -catenin and  $\gamma$ -adducin at sites of intercellular contacts (arrows) and the significant loss of both proteins from cell-cell junctions in anillin deplete cells (arrowheads).

One mechanism for the observed delocalization may be decreased expression levels of adducin.

Figure 19A-B reveals a selective downregulation in the expression levels of  $\gamma$ -adducin and no change in  $\alpha$ - or  $\rho$ -adducin levels is observed. We also observe a small reduction in the expression levels of  $\beta$ -spectrin, which may be due to the loss of adducins from the site of actin cytoskeleton-plasma membrane contact.





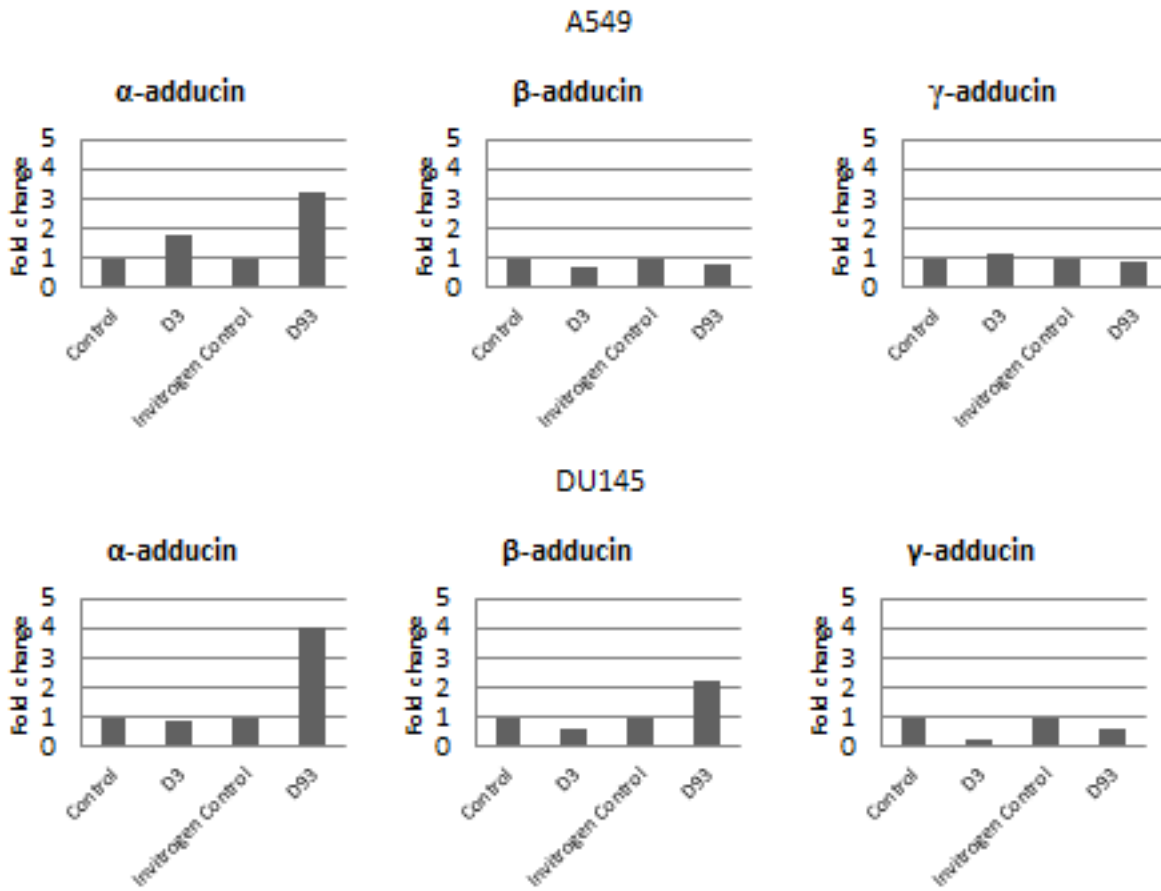
**Figure 19: Anillin depletion results in selective downregulation of  $\gamma$ -adducin.** DU145 (A) and A549 (B) epithelial cells were transfected with either control or anillin specific siRNA duplexes D1 and D3. Cell lysates collected on day 4 post-transfection were analyzed for expression and phosphorylation of different adducin isoforms. Note that anillin knockdown results in a selective down-regulation of  $\gamma$ -adducin expression without significantly affecting expression and phosphorylation of  $\alpha$ -adducin or spectrins. Data are presented as mean  $\pm$  SE

Together, these results suggest that Anillin depletion results in the disruption of the cortical membrane skeleton as well as the selective downregulation of  $\gamma$ -adducin in two different cell lines. Anillin depletion induced downregulation of  $\gamma$ -adducin may be the underlying mechanism for loss of organization of both the cortical membrane skeleton and perijunctional actomyosin belt.

### **Anillin depletion does not affect adducin expression at transcription level.**

Our current study revealed a selective downregulation of  $\gamma$ -adducin on depletion of anillin. It was important to determine if anillin depletion induced adducin knockdown at a transcriptional level and if any compensatory upregulation of other isoforms was observed. This was investigated by qRT-PCR analysis for all three isoforms ( $\alpha$ ,  $\beta$  and  $\gamma$ ) of adducin. We observed no consistent changes between control and anillin depleted samples for any of the three adducin isoforms (Figure 20). In addition, no compensatory upregulation of either  $\alpha$ - or  $\beta$ -adducin was observed. It is unlikely that downregulation of adducins is at a transcriptional level and loss of  $\gamma$ -adducin observed on knockdown of anillin may be due to post-translational degradation.



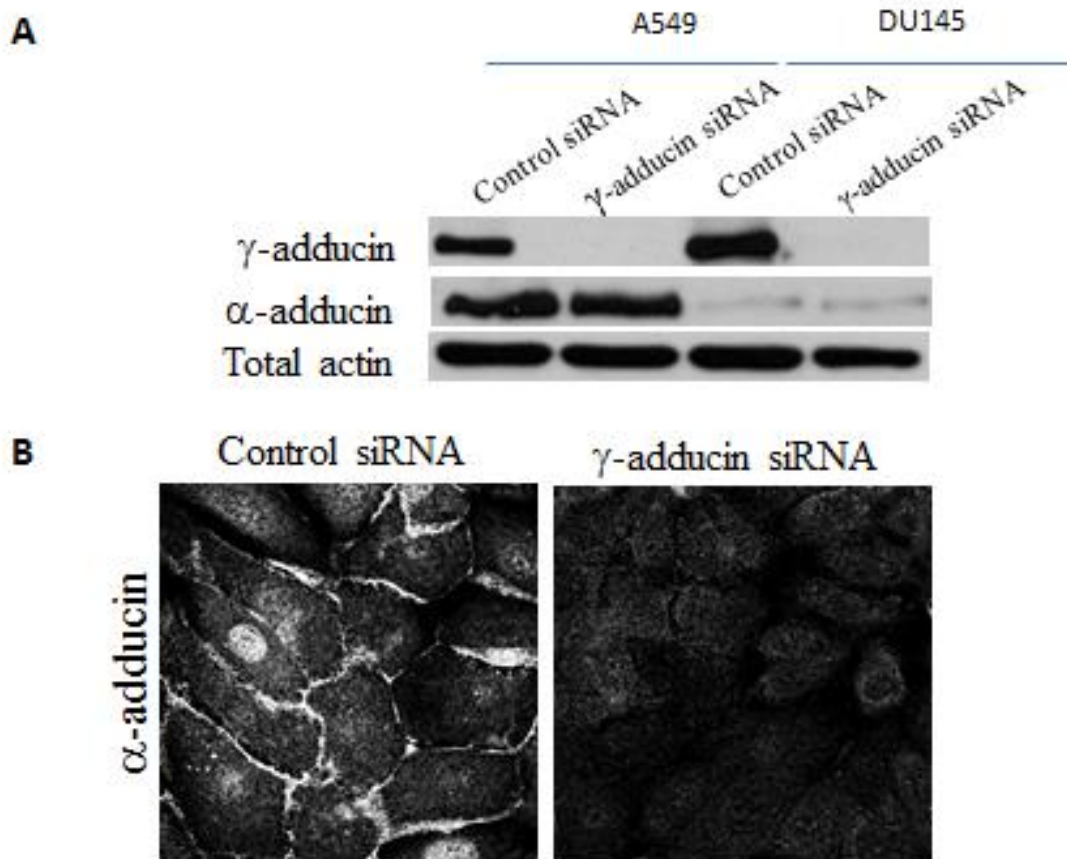


**Figure 20: Anillin depletion does not affect adducin expression at transcription level.**

A549 and DU145 epithelial cells were transfected with two anillin siRNA duplexes: Dharmacon duplexes 3 and Invitrogen duplexes 93 along with their appropriate controls. Cell lysates were collected 4 days post-transfection. Quantitative real time RT-PCR analysis conducted in both cell lines reveals on consistent change in expression levels of  $\alpha$ -,  $\beta$ - or  $\gamma$ -adducin in anillin depleted cells.

### **Loss of $\gamma$ -adducin displaces $\alpha$ -adducin from the site of intercellular contacts.**

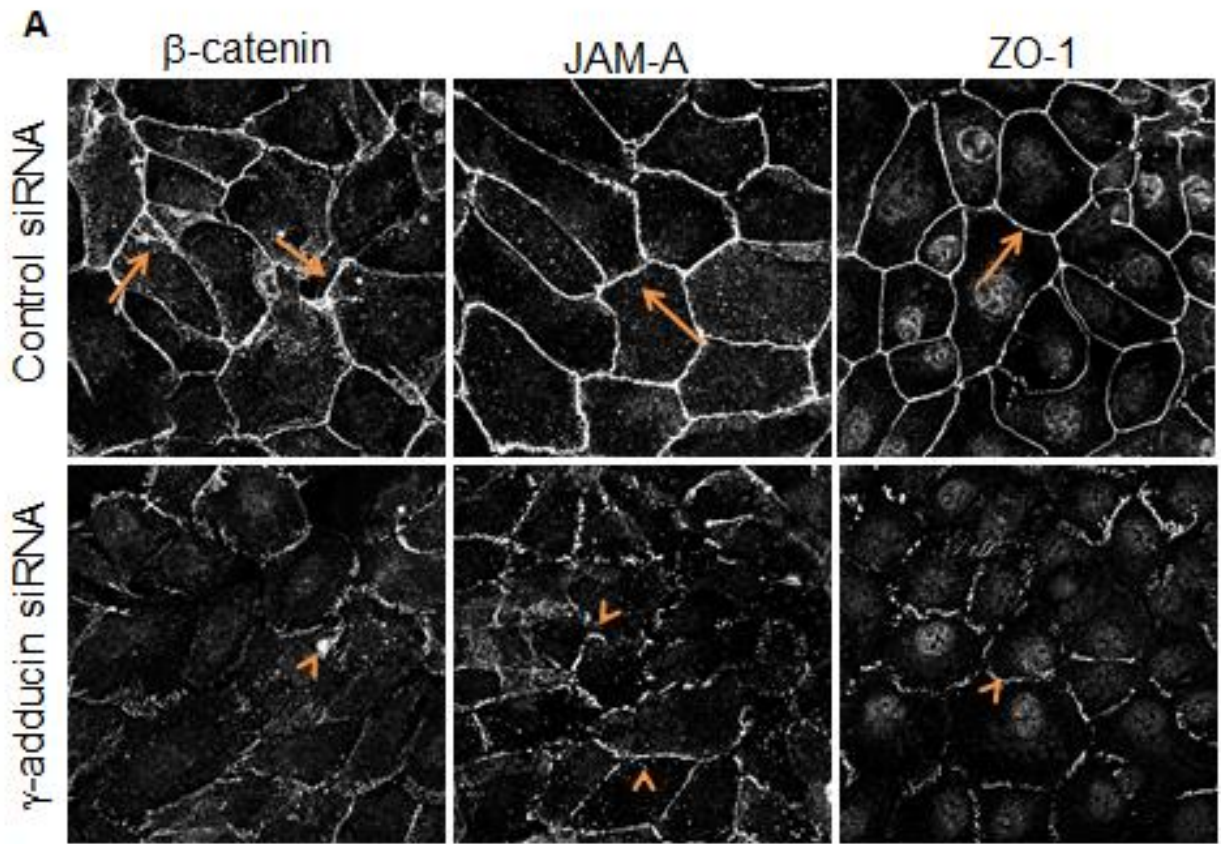
Our previous results revealed a selective down-regulation of  $\gamma$ -adducin along with delocalization of  $\alpha$ -adducin from the site of cell-cell contacts. To confirm this hypothesis, DU145 cells transfected with control and  $\gamma$ -adducin were also immunolabeled for  $\alpha$ -adducin. Figure 21 shows  $\gamma$ -adducin knockdown results in the loss of  $\alpha$ -adducin localization from intercellular contacts. However, immunoblot analysis reveals that  $\gamma$ -adducin knockdown has no effect on expression levels of  $\alpha$ -adducin. This confirms the hypothesis that the loss of  $\gamma$ -adducin results in the displacement of  $\alpha$ -adducin from the site of cell-cell contact. As  $\alpha$ - and  $\gamma$ -adducin act as a dimer, this may account for the displacement of one component when the other is depleted from its functional position in the cell.

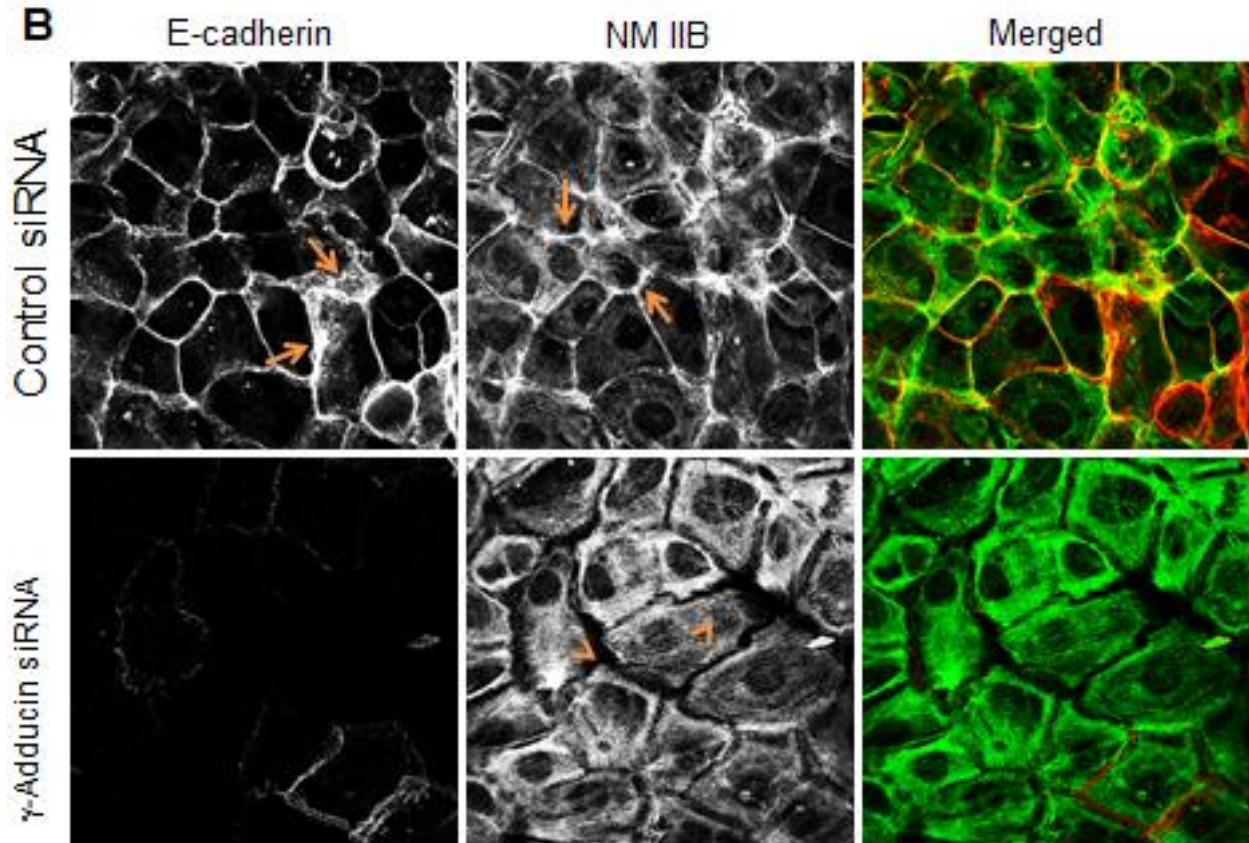


**Figure 21: Loss of  $\gamma$ -adducin displaces  $\alpha$ -adducin from the areas of cell-cell contacts.** DU145 and A549 cells were transfected with either control or  $\gamma$ -adducin specific siRNAs. (A) Cell lysates collected on day 4 post-transfection were analyzed for expression of different adducin isoforms. (B) DU145 cells were labeled using  $\alpha$ -adducin antibody. In DU145 cells, loss of  $\gamma$ -adducin results in disappearance of  $\alpha$ -adducin from the areas of cell-cell contacts.

### **Loss of $\gamma$ -adducin disrupts epithelial junctions.**

Our previously shown results strongly suggest that the loss of  $\gamma$ -adducin has an effect on the stability of epithelial junctions. Figure 22 shows a loss  $\beta$ -Catenin, JAM-A, ZO-1, E-cadherin and NM IIB signal from the site of cell-cell contacts in cells depleted for  $\gamma$ -adducin. These results reveal that the loss of  $\gamma$ -adducin contributes to the disruption of epithelial junctions and the disorganization of NM IIB. This data, together with previous results, strongly indicates that the loss of anillin results in the selective loss of  $\gamma$ -adducin and this may be the underlying mechanism for the disorganization of the perijunctional actomyosin belt and hence disrupt epithelial junctions.

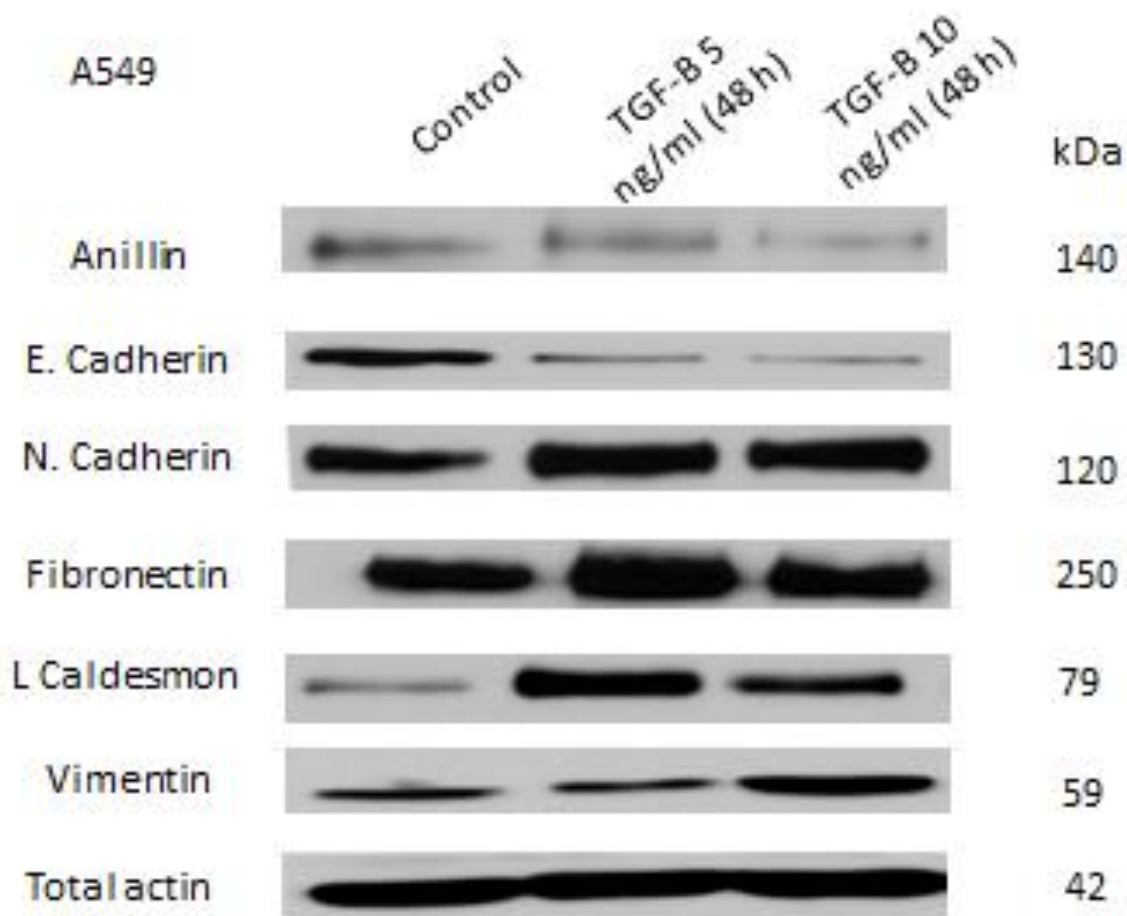




**Figure 22: Loss of  $\gamma$ -adducin disrupts epithelial junctions:** DU145 cells plated on coverslips were transfected with either control or  $\gamma$ -adducin - specific siRNAs. On day 4 post-transfection, the cells were immunolabeled for protein markers of AJs and TJs. Note a prominent localization of  $\beta$ -catenin, JAM-A, ZO-1 (22A) and E.cadherin and NM IIB proteins (22B) at well-aligned intercellular contacts in control cells (arrows) and distortion of intercellular contacts accompanied by loss of junctional localization of AJ, TJ and NM IIB proteins in  $\gamma$ -adducin depleted cells (arrowheads).

## **TGF- $\beta$ treatment downregulates Anillin expression and disrupts epithelial junctions in A549 cells.**

Our current study reveals that anillin depletion results in a phenomenon which is a subset of classical EMT, i.e. Epithelial Myofibroblast Transition (EMyT). It would be reasonable to suggest that TGF- $\beta$  treatment would result in decreased anillin expression. Figure 23 reveals a downregulation of anillin expression in samples treated with 10 ng/ml as well as a consistent upregulation of multiple EMT markers like N-cadherin, Fibronectin, L-caldesmon and Vimentin, coupled with the downregulation of E-cadherin expression. These results are in tandem with previous reports of TGF- $\beta$  induced EMT in A549 cells. In addition, our studies reveal that anillin expression is downregulated on treatment with TGF- $\beta$ . The loss of anillin might be contributing to the EMT phenotype and a more in-depth analysis would need to be conducted to elucidate how anillin might affect the induction of EMT.



**Figure 23: TGF- $\beta$  treatment down-regulates anillin expression and disrupts epithelial junctions in A549 cells.** Confluent A549 monolayers were treated with either vehicle, or two concentrations of TGF- $\beta$  (5 and 10) ng/ml for 48 hours, following which cell lysates were collected for immunoblotting analysis. Note that TGF- $\beta$  induces a classical EMT that was manifested by down-regulation of E-cadherin and up-regulation in mesenchymal markers, N-cadherin, Fibronectin, vimentin and L-caldesmon. Such EMT is accompanied by down-regulation of anillin.



## DISCUSSION

Simple epithelial linings in the gastrointestinal, respiratory, renal and reproductive systems are composed of a single layer of epithelial cells (1, 2). These cells exhibit unique morphological features, like tight lateral contacts with neighboring cells and prominent apico-basal cell polarity. These features are crucial for maintenance of a paracellular barrier as well as determine the directionality of absorptive and secretory process in differentiated epithelia (1, 2) and are regulated by epithelial junctions. There is a lot of evidence that points to the highly dynamic structures of epithelial junctions that constantly undergo remodeling (21, 22) and this observed plasticity is crucial for epithelial morphogenesis. Disruption in the dynamic equilibrium between junction disassembly and reassembly has been reported to significantly contribute to two pathophysiological conditions in particular: inflammation and cancer (99-101). The integrity and remodeling of epithelial junctions in both normal and pathophysiological conditions including altered phosphorylation and expression of junctional proteins, vesicle trafficking of junctional components and the reorganization of perijunctional actin filaments and microtubules. (8, 30, 33-37). However, despite of intensive studies in last decades, mechanisms that regulate assembly and functions of epithelial junction remain incompletely understood. The present study demonstrates that a known F-actin binding protein anillin plays a previously unanticipated role in assembly of epithelial junctions.

### **Anillin is critical for maintenance of junctional integrity.**

The major finding of our study revealed anillin depletion resulted in the disruption of tight and adherens junctions in two separate cell lines (A549 and DU145 cells). Our study provides the first evidence of anillin regulating the integrity of epithelial junctions. There has been previous evidence of indirect regulation of epithelial cell-cell contacts by anillin. For example, loss of anillin was shown to induce excessive dynamics of lateral cell-cell contacts in *Drosophila* epithelium which can be indicative of destabilization of intercellular junctions (102).

Furthermore, anillin knockdown in zebrafish resulted in the breakdown of the glomerular epithelial barrier, although integrity of epithelial junctions under these experimental conditions has not been investigated (102).

### **Anillin promotes epithelial phenotype in A549 cells.**

Besides regulating cell-cell adhesions, anillin appears to have additional functions in epithelial cells. Our study shows that anillin controls the epithelial transcription program, as its loss results in the induction of an EMT-like phenotype in A549 cells. The phenotypical change does not strictly resemble classical EMT since anillin knockdown did not cause cadherin switching (down-regulation of E-cadherin and upregulation of N-cadherin). Furthermore, anillin depletion did not stimulate motility of A549 cells as would be expected in case of classical EMT.

Together, this data suggests that anillin depletion induced epithelial-myofibroblast transition (EMyT), a subset of EMT which is characterized by upregulation of contractile/cytoskeletal proteins abundant in smooth muscle and myofibroblasts (121). Since loss of anillin expression resulted in enhanced transcription of  $\alpha$ SMA and SM22 mRNA (Fig.10C) and anillin localization was restricted to the interphase nucleus (9), we speculate that anillin acts as a transcriptional

repressor that inhibit expression of a subset of genes involved in mesenchymal/myogenic cell differentiation. Mechanisms of such repression remain to be investigated, although a large scale proteomic analysis of protein-protein interactions provides some ground for speculation. This analysis identified two putative binding partners for anillin, a SNW domain containing 1 (SNW1) protein as well as TAF10 RNA polymerase II. These proteins are known to regulate gene expression and can act as enhancers or repressors of different genes. It is tempting to speculate that SNW1 and/or TAF10 can mediate the effects of anillin on transcription programming of epithelial cells. It should be noted that EMyT induction was not observed in DU145 cells where loss of anillin disrupted epithelial junctions. This indicated that junctional disassembly and induction of EMyT represent two independent effects of anillin knockdown in epithelial cells.

#### **Anillin regulates organization of the perijunctional actomyosin belt.**

Lack of effect of anillin knockdown on expression of different AJ and TJ proteins (Fig.12) prompted us to search for alternative mechanisms of junctional disassembly in anillin-depleted cells. Since anillin is known scaffolding protein interacting with both actin filaments and NM II (1) it is logical to suggest that it may regulate integrity of epithelial junctions by controlling assembly of the actomyosin bundles at the area of cell-cell contacts. Indeed, our immunolabeling data support this conclusion by demonstrating that loss of anillin results in disappearance of the perijunctional actomyosin belt (Fig. 13, 14). Interestingly, we did not find any effects of anillin depletion on organization of microtubules, which highlights a selective role of anillin in regulating the actin cytoskeleton.

Our data about mislocalization of NM IIB from the site of cell-cell contacts indicates loss of contractile cortical cytoskeleton in anillin-depleted epithelial cells. This result is in a good agreement with previous pharmacological inhibition or RNAi studies demonstrated that inhibition of NM II activity dramatically affects integrity of the epithelial barrier and remodeling of AJs and TJs at different experimental conditions (26, 34 76, 77). Interestingly, loss of anillin did not significantly affect a total amount of active NM II in the cells as was indicated by unchanged level of myosin light chain phosphorylation (Fig 15C). This indicates that anillin is likely to control a specific pool of the actomyosin cytoskeleton that is associated with intercellular junctions. Given nuclear localization of anillin in interphase cells, such a control of perijunctional cytoskeleton should be indirect and is likely to involve modulation of some signaling cascades and expression of different regulators of the actomyosin cytoskeleton.

### **Anillin depletion disrupts the membrane skeleton.**

In a search for mechanisms that may mediate disassembly of the perijunctional actomyosin cytoskeleton in anillin depleted cells, we examined specific protein scaffolds that attach actin filaments to the plasma membrane. Such actin-membrane interactions are essential for the assembly of cortical F-actin (104). Furthermore, they are critical for stabilizing epithelial cell-cell contacts and formation of adherens and tight junctions (103-105). We rationalized that loss of physical attachments of actin filaments with the lateral plasma membrane in anillin depleted cells would prevent formation of the perijunctional actomyosin belt, which in turn will increase membrane dynamics and trigger AJ/TJ disassembly. This hypothesis is supported by a previous study in *Drosophila* epithelium where loss of anillin destabilized lateral plasma membranes at the areas of cell-cell contacts eventually causing membrane vascularization and endocytosis. (86).

Two major mechanisms are known to link the actin cytoskeleton to the plasma membrane. One involves so called ERM (ezrin-radixin-moesin) proteins and the other involves a spectrin-adducin-ankyrin membrane skeleton. While ERM proteins directly link actin filaments to phospholipid layers of the plasma membrane, the membrane skeleton attach actin filaments to cytosolic domains of several plasma membrane proteins (106). Importantly, some components of the spectrin-adducin-ankyrin cytoskeleton can bind AJ and TJ proteins such as E-cadherin, ZO-1 and  $\alpha$ -catenin and therefore can link the actin filaments directly to junctional complexes at the plasma membrane. Activity of ERM and membrane skeleton proteins can be regulated at different levels which includes protein synthesis, posttranslational modification and degradation. These proteins are phosphorylated by major protein kinases such as Rho associated kinase and protein kinase C and such phosphorylation of ERM proteins or adducins is known to negatively regulate their functions (106, 124). For example, phosphorylation of ERM proteins keep them into auto-inhibited conformation that becomes active via dephosphorylation (124).

We investigated the effects of anillin knockdown on expression and activation status of ERM proteins and the membrane skeleton. Our data revealed no change in the expression levels of ezrin or moesin \ reduced P-ERM levels in anillin depleted DU145 cells (Fig.16). Such dephosphorylation most likely reflects ERM activation which should increase association of these proteins with lipid membranes and actin filaments. We concluded that the observed ERM activation cannot be responsible for disruption of the perijunctional actomyosin cytoskeleton in anillin depleted cells. Contrary to activation of ERM proteins, loss of anillin disrupted organization of the adducin-based membrane skeleton in A459 and DU145 epithelial cells. Such disruption was manifested by disappearance of  $\alpha$ -adducin from the region of cell-cell contacts along with a selective downregulation of  $\gamma$ -adducin expression. Importantly siRNA-mediated

knockdown of  $\gamma$ -adducin disrupted integrity of AJs and TJs in DU145 cells thereby mimicking effects of anillin depletion on junctional integrity (Fig. 22).

The spectrin-adducin based membrane skeletal network mediates the most abundant interactions of actin filaments with the cytosolic face of the plasma membrane, the major component of which is the spectrin tetramer (107-109). Spectrin rods only weakly associate with actin filaments and this association is significantly increased by accessory proteins, most notably by adducins (107, 110). Adducins are a class of proteins that recruit spectrins to actin filaments and promote assembly of spectrin lattice at the plasma membrane (106). This protein family consists of three homologous proteins termed  $\alpha$ -,  $\beta$ - and  $\gamma$ -adducins and can readily form heterodimers of heterotetramers of either  $\alpha/\beta$  or  $\alpha/\gamma$  subunits (107, 110)

In addition to linking actin filaments to spectrin oligomers, adducins can act as actin filament bundling and capping proteins (Mische *et al.*, 1987; Taylor and Taylor, 1994; Kuhlman *et al.*, 1996). Adducin blocks filament elongation and depolymerization from the barbed ends (114) which slows down F-actin treadmilling and increases filament stability. Hence, loss of adducins may have two different effects on perijunctional actin cytoskeleton. One effect is direct destabilization of actin filaments via their de-bundling and de-capping. The second effect is displacement of actin filaments from junctional complexes and the plasma membrane due to loss of interactions with the membrane skeleton and junctional proteins.

Our data on AJ/TJ disassembly in adducin-depleted cells agrees with previously published studies.  $\alpha$ - or  $\gamma$ -adducin knockdown showed delay of reassembly of both AJs and TJs in SK-CO15 cells that were subjected to calcium switching model for remodeling of epithelial junctions. Although the cells were able to eventually form morphologically normal cell-cell

contacts, even in the absence of adducin, they appeared to be less stable than those found in normal cells. Previous data also indicates that adducin does regulate the assembly of F-actin cytoskeleton in epithelial cells. The crucial role of perijunctional actin belt in supporting the structure of epithelial junctions, we can suggest that the defective F-actin assembly due to loss of adducin may be the underlying mechanism for the impaired formation of AJs and TJs in anillin depleted cells.

Spectrins, another component of the membrane skeleton, have also been implicated in regulation of epithelial apical junctions.  $\beta$ II spectrin depletion attenuates formation of AJs in human intestinal and bronchial epithelial cells (112, 113).  $\alpha$ -II spectrin and perijunctional F-actin belt was found to be spatially segregated in polarized epithelial cells with intact junctions. In contrast, spectrin became enriched in apical contractile actin rings which are known to drive junction disassembly in calcium-depleted cells and interestingly, adducin was also found accumulated at these contractile rings with fragments of disassembled AJs (114). This data suggests that spectrin-adducin mediated membrane attachments of the peri-junctional F-actin belt may stabilize the junctional structures by limiting diffusion of AJ/TJ proteins within the plasma membrane (114).

An intriguing observation of the present study is a selective decrease in  $\gamma$ -adducin protein expression following anillin depletion (Fig. 21). No change in mRNA level of any of the three adducin isoforms ( $\alpha$ ,  $\beta$  and  $\gamma$ ) was observed suggesting posttranscriptional downregulation of  $\gamma$ -adducin in anillin depleted cells (Fig 20). At present, we do not know mechanism of such posttranscriptional suppression which may involve inhibition of protein synthesis or increased  $\gamma$ -adducin degradation. Interestingly, loss of  $\gamma$ -adducin in anillin depleted cells was not accompanied by expressional downregulation of  $\alpha$ -adducin isoform, although this isoform was

not able to accumulate at intercellular junctions (Fig.21). This result is consistent with previously published data on  $\gamma$ -adducin knockout mice (115) and indicates that epithelial adducins can exist as homotypical ( $\alpha$ -subunit only) and heterotypical ( $\alpha/\gamma$ -isoform oligomers) complexes with different localization and functions.

### **Involvement of anillin cancer and tissue fibrosis.**

The effects observed due to loss of anillin may have several pathophysiological implications. mRNA levels of anillin is increased during tumor progression in breast, ovarian, kidney, colorectal, hepatic, lung, endometrial and pancreatic tumors non-small cell lung cancer (NSCLC) and pancreatic carcinomas (116). However, the exact roles of anillin upregulation in tumor development remain unknown. We demonstrated that anillin is likely to serve as a positive regulator of epithelial phenotype. Hence upregulation of anillin in cancer may have effect on cell proliferation but would unlikely regulate tumor progression which is frequently accompanied by loss of epithelial differentiation. Is it possible however, that some tumor promoters would decrease anillin expression thereby inducing disruption of cell-cell contacts, EMT-like transition and dissemination of tumor cells. Further studies are needed to test this hypothesis. Studies in lung (117), breast (118) and pancreatic (119) cancers demonstrate overexpression of anillin as a poor prognosis of the disease and show that overexpression in NSCLC increases cell motility (97). We can speculate that due to a pro-proliferative effect of anillin expression. However, anillin expression in renal cell carcinoma has been reported as a marker of favorable prognosis, which can be due to enhanced epithelial differentiation and suppression of tumor progression. Our current study revealed that a loss of anillin resulted in the induction of EMT phenotype in lung epithelial cells. Furthermore, we observed that anillin was downregulated on TGF- $\beta$



treatment along with induction of EMT in A549 cells. The induction of the EMT or EMyT is a common manifestation of tissue fibrosis, which is often seen in chronic inflammation. Fibrosis involves the loss of normal cellular elements and extracellular matrix of damaged tissues, replaced by scar tissue accumulation and loss of normal tissue function. It is characterized by the excessive deposition of collagen deposited as fibers, leading to failure of normal organ function (101). The process of fibrosis involves EMyT, wherein fibroblasts, epithelial, endothelial and other cell types undergo transdifferentiation to myofibroblasts and this process is generally induced by injury or pro-inflammatory mediators like TGF- $\beta$  (120). TGF- $\beta$  is a potent pro-fibrotic cytokine and it regulates fibroblast to myofibroblast differentiation, resulting in upregulation of contractile genes such as  $\alpha$ -smooth muscle actin and SM-22 (121). (121-123). Our results revealing downregulation of anillin on TGF- $\beta$  treatment suggests that the loss of anillin may contribute to myofibroblast transformation during tissue fibrosis driven TGF- $\beta$  and/or other growth factors. It is also possible that anillin expression can be downregulated by other proinflammatory mediators, which would result in weakening cell-cell adhesions and disruption of epithelial barriers during tissue inflammation. Further investigation is required to examine roles and regulation of anillin under different pathophysiological conditions.

## CONCLUSION

In conclusion, our study reveals a novel role of anillin in the regulation of intercellular junctions in lung and prostate epithelial cells. We observed that anillin is critical for assembly of both adherens and tight junctions by mechanisms independent of junctional protein expression. The effects of anillin depletion on epithelial junctions are likely to involve disorganization of the perijunctional actomyosin cytoskeleton and adducin-based membrane skeleton. A specific expressional down-regulation of  $\gamma$ -adducin in anillin-depleted cells is likely to mediate such cytoskeletal disruption and junctional disassembly. We hypothesize that function of anillin could be important for remodeling of epithelial junctions during normal embryonic morphogenesis and tissue rejuvenation. Furthermore anillin-dependent alterations in epithelial differentiation may also play roles in the development of tissue fibrosis and tumor progression.

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## Vita

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