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Adducins are Negative Regulators of Migration and Invasion of Normal Lung Epithelial Cells and Lung Cancer Cells

Parth Hitenbhai Amin

Parth Amin
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ADDUCINS ARE NEGATIVE REGULATORS OF MIGRATION AND INVASION OF NORMAL LUNG EPITHELIAL CELLS AND LUNG CANCER CELLS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

By

Parth Hitenbhai Amin

M.Sc. in Medical Biotechnology,

Sardar Patel University, 2012

Director: Dr. Andrei Ivanov

Associate Professor

Department of Human and Molecular Genetics

Virginia Commonwealth University

Richmond, Virginia

August 2016

ACKNOWLEDGEMENTS

Words are insufficient to express my deep sense of gratitude to Dr. Andrei Ivanov for giving me an opportunity to carry out my dissertation project in his laboratory. His constant support and guidance throughout the project have made this work possible.

I am highly indebted to my guide Dr. Susana Lechuga for her crucial contribution to this work. I would like to thank her for her constant support and guidance and for training me with all the necessary laboratory techniques. I would also like to convey my special thanks to my lab members Dr. Nayden Naydenov and Dr. Dong Dong Wang for helping me out in all the possible ways and for answering my tons of questions. I would also like to thank my lab colleague Faisal Shakir for helping me out in some or the other way. I am going to miss all the wonderful people of my laboratory and the time we enjoyed outside the lab. I will especially miss the endless conversations with Nayden Naydenov.

I would also like to thank all my committee members Dr. Jennifer Koblinski and Dr. Devanand Sarkar for their valuable suggestions.

A very special thanks to all my friends over here for creating unforgettable memories in Richmond.

Lastly, I would like to express my deep gratitude to my parents and family members for their endless love and support.

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LIST OF ABBREVIATIONS AND SYMBOLS

α------------------------------- Alpha

β------------------------------- Beta

γ------------------------------- Gamma

ADD1------------------------- Alpha Adducin

ADD2------------------------- Beta Adducin

ADD3------------------------- Gamma Adducin

ALS--------------------------- Amyotrophic Lateral Sclerosis

ATP--------------------------- Adenosine Triphosphate

BCA--------------------------- Bicinchoninic Acid assay

BSA--------------------------- Bovine Serum Albumin

Cas9-------------------------- CRISPR-associated protein 9

CRISPR---------------------- Clustered Regularly Interspaced Short Palindromic Repeats

DAPI-------------------------- 4',6-Diamidino-2-phenylindole

ECL--------------------------- Enhanced Chemiluminescence

ECM-------------------------- Extracellular Matrix

EDTA------------------------- Ethylenediaminetetraacetic acid

EGTA------------------------- Ethylene Glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

FA----------------------------- Focal Adhesions

FAK--------------------------- Focal Adhesion Kinase

FBS--------------------------- Fetal Bovine Serum

GAPDH--------------------- Glyceraldehyde 3-phosphate dehydrogenase

HEPES----------------------- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HGF------------------------- Hepatocyte Growth Factor

HRP------------------------- Horseradish peroxidase

IF---------------------------- Immunofluorescent staining

KO--------------------------- Knockout

MARCKS-------------------- Myristoylated Alanine-Rich C-Kinase Substrate

MDCK----------------------- Madin-Darby Canine Kidney Epithelial Cells

MEM------------------------- Minimum Essential Medium

MgSO$_4$----------------------- Magnesium Sulfate

MMPs----------------------- Matrix Metalloproteinases

NaCl-------------------------- Sodium Chloride

NMJ-------------------------- Neuromuscular Junctions

NSCLC---------------------- Non-Small Cell Lung Cancer

PFA--------------------------- Paraformaldehyde

PKA-------------------------- Protein Kinase A

PKC-------------------------- Protein Kinase C

RIPA------------------------- Radioimmunoprecipitation assay buffer

RPMI------------------------ Roswell Park Memorial Institute Medium

SDS-------------------------- Sodium Dodecyl Sulphate

siRNA----------------------- small inhibitory Ribonucleic acid

TBS-------------------------- Tris-buffered Saline

TBS-T ---------------------- Tris-buffered Saline-Tween

WB-------------------------- Western blotting

Abstract

ADDUCINS ARE NEGATIVE REGULATORS OF MIGRATION AND INVASION OF NORMAL LUNG EPITHELIAL CELLS AND LUNG CANCER CELLS

By- Parth Hitenbhai Amin, M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University

Virginia Commonwealth University 2016

Major Director: Dr. Andrei Ivanov, Associate Professor

Department of Human and Molecular Genetics

Cell migration is an important component of many physiological and pathological processes such as tissue and organ morphogenesis during development, wound healing, inflammatory immune response, and tumor metastasis. The actin cytoskeleton is the basic engine driving cell migration. In the present study, we elucidate the role of an important actin interacting proteins, Adducins, in motility of normal lung epithelium and lung cancer cells. Adducins are the family of cytoskeleton protein capping the fast growing end and facilitating the bundling of actin filaments. Adducins are encoded by the three closely related genes namely alpha (ADD1), beta (ADD2) and gamma (ADD3) Adducin. ADD1 and ADD3 are ubiquitously expressed, whereas ADD2 is most abundant

in brain and erythrocytes. Adducins are also involved in recruiting spectrin to the actin filaments forming spectrin-actin membrane skeletal network. Its role in cell motility remains controversial. In this study, we observed that CRISPR/Cas9 mediated stable knockout of ADD1 and ADD3 in 16HBE normal lung epithelium cells significantly increases transfilter migration of cells. On the other hand, stable overexpression of ADD1 in H1299 Non-Small Cell lung cancer cells significantly decreases wound healing, transfilter migration and Matrigel invasion of the cells. Importantly, the effects of Adducin depletion and overexpression on cell motility were not due to altered cell proliferation. ADD1 overexpressed H1299 cells were characterized by the increased adhesion and spreading on the collagen matrix. Fluorescence microscopy revealed alterations in their cortical actin cytoskeleton that was manifested in the assembly of peripheral F-actin bundles and formation of filopodia-like protrusions. These findings suggest that Adducins are negative regulators of motility of normal lung epithelial and lung cancer cells that act by altering the architecture of submembranous actin cytoskeleton and modulating cell adhesion to the extracellular matrix.

1. INTRODUCTION

Formation of epithelial tissues represents a key step in the evolution of multicellular organisms. Epithelia play important roles in not only forming a protective barrier that separates the interior of the body from the environment but also creating and maintaining the unique architecture and chemical composition of the internal organs. Epithelial barriers are composed of one or multiple layers of specialized cells that form tight contact with their neighbors and demonstrate orchestrated behavior. This organization is essential for the stability of epithelial layers and their ability to withstand mechanistic stress and other types of stress. However, epithelial barriers also possess a high level of plasticity that is essential for tissue morphogenesis and restitution. Cell migration is a key mechanism that mediates plasticity of epithelial layers under normal conditions and in the disease state. In the developing embryo, the coordinated migration of cells of different lineages to their strategic location plays a key role in the morphogenesis of organs and tissues [1]. In the normal adult tissue such as intestinal epithelium, cell migration plays a critical role in maintaining healthy intestinal epithelial lining by replacing the dead cells with the newly generated cells in the lower crypts [2]. Cell migration is essential for tissue repair and regeneration, triggering the effective immune response, progression of disease such as tumor metastasis [3, 4]. Because of essential functions of epithelial cell motility, it is important to understand the mechanism of this process.

Cell Migration is an outcome of coordinated regulation involving remodeling of the actin cytoskeleton, cell adhesion, cell contractility and dynamics of the plasma membrane. The role of

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actin cytoskeleton as a basic engine providing the essential force for driving cell migration is one of the key discovery made in the field of cell migration [5]. The overall dynamics of the actin cytoskeleton is regulated by actin-binding proteins and the associated signal transduction pathways [6]. Therefore, it is essential to study the actin-binding protein mediated regulation of cell motility.

1.1 Modes and the basic mechanism of epithelial cell migration

The different modes of epithelial cell migration are characterized based on the cell morphology, cytoskeletal organization, type of cell-matrix interaction and force generation, and modification of tissue structure imposed by migrating cells. Some cells migrate as individuals and some cell types migrate collectively in form of sheet, strands, tube or clusters [7]. The single cell migration has been studied extensively. The two major modes of cell migration are mesenchymal migration and amoeboid migration. Mesenchymal migration is characterized by elongated, fibroblast-like cell morphology with established cell polarity. It is driven by the actin-based protrusions formed at the leading edge of migrating cells and is dependent on the cell-ECM contact and degradation of ECM by soluble MMPs secreted out from migrating cells. On the other hand, amoeboid migration is characterized by rounded cell morphology and is independent of the requirement of cell-ECM contact and also the degradation of ECM by soluble MMPs. The cell motility is driven by the formation of plasma membrane blebs and contraction of cortical actin cytoskeleton which results into squeezing of cells through the gaps in ECM [8, 9]. Mesenchymal migration is the much more abundant type and shares mechanistic similarity with collective cell migration. Therefore, in the subsequent section, we will focus on the mechanisms of mesenchymal cell migration.

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The basic mechanism of cell motility is divided into four basic steps: polarization, protrusion, adhesion, and retraction. First of all, the cell gains asymmetric morphology with defined leading and trailing edges for efficient migration. Secondly, the polarized migrating cell forms protrusions at the leading edge in response to migratory and chemotactic stimuli. These protrusions are formed by the local polymerization of the cortical actin filaments. Thirdly, these protrusions are stabilized by the formation of the small nascent focal complexes which gradually grows into large organized focal adhesion complexes. These focal complexes consist of integrin receptors, actin filaments and a complex of cytoplasmic scaffold proteins clustered together. The focal adhesion complexes attach the migrating cell to the underlying substrate and serves as a point of traction over which the body of the cell moves. Finally, the coordinated contraction of the actin cytoskeleton and disassembly of the focal adhesions at the rear end results in translocation of the entire cell body in the direction of the leading edge of migrating cell [7, 10, 11, 12].
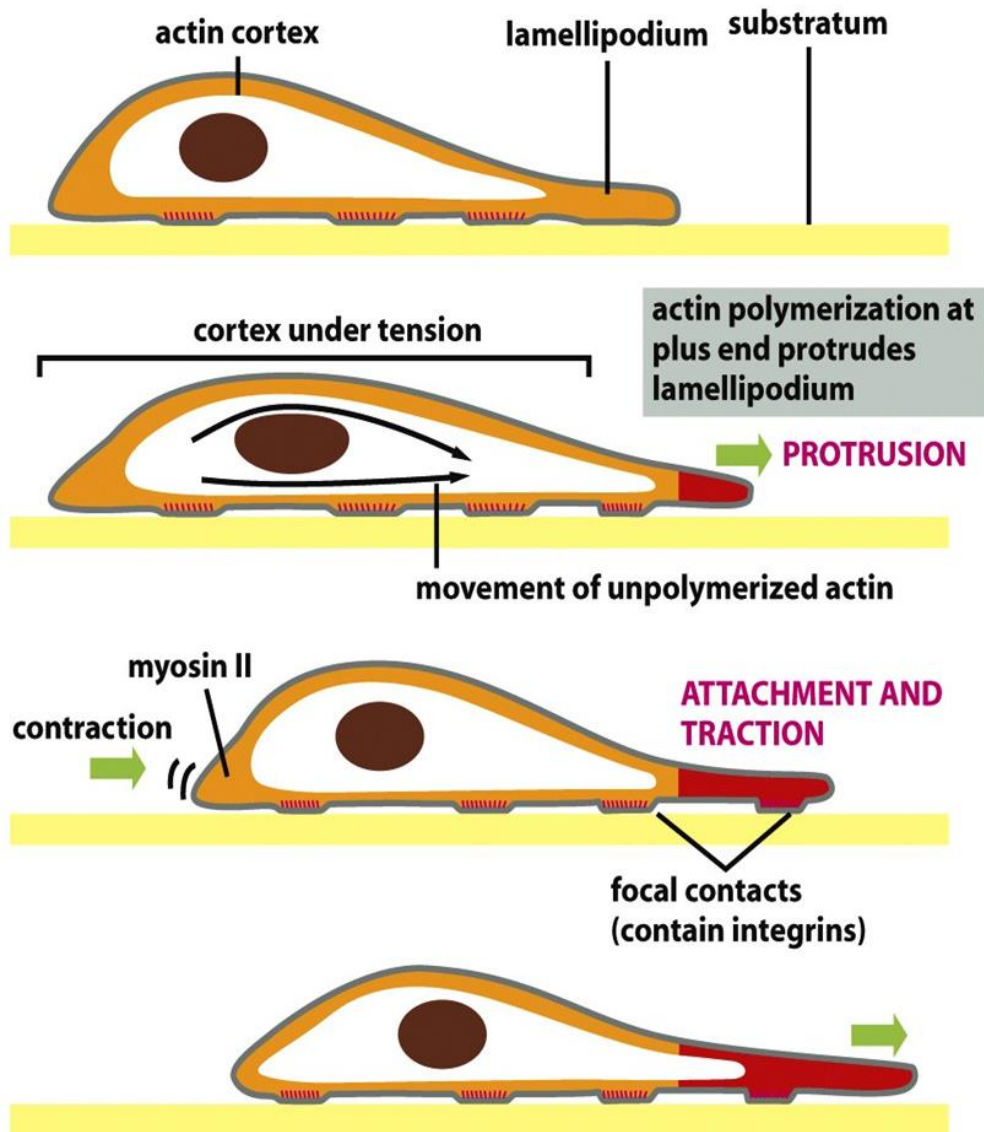
Figure 1: Cell migration cycle. Source-Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2010). Molecular biology of the cell. New York: Garland science; 2002.*Classic textbook now in its 5th Edition*.

1.2 Role of Actin Cytoskeleton in Cell Migration

The biochemical and mechanical properties of actin cytoskeleton drive cell motility. The building block of the actin cytoskeleton, actin filaments are formed by the polymerization of globular actin monomers. Actin filaments along with motor protein myosin serve as biological active spring in the cell which can exert or resist against force in a cellular environment [13]. The protrusions formed at the leading edge of the migrating cells are driven by polymerization of the cortical actin filaments. Lamellipodia, filopodia, invadopodia are the different types of protrusions formed in the migrating cells. Lamellipodia are the broad flat sheet-like membrane protrusions comprising of branched crosslink network of thin short actin filaments. Filopodia are long thin finger-like protrusions comprising of long unbranched parallel actin bundles. Invadopodia are not the leading edge, but basal protrusions that are characteristics only for the cancer cells that invade ECM [12, 13]. Also, contractile fibers are present throughout the cell except the leading edge consisting of unbranched bundles of actin filaments of mixed polarity and myosin II. The two different types of contractile fibers are ventral stress fibers and transverse arc. Ventral stress fibers run parallel to the direction of movement linking the focal adhesion sites facilitating retraction during cell migration. Transverse arcs are present behind lamellipodia and run parallel to the advancing leading edge. Transverse arcs help in actin disassembly. The contraction of cortical actin belt present below the plasma membrane helps in an amoeboid type of cell motility through blebs [13]. This indicates actin cytoskeleton as one of the major players in driving cell motility.

In eukaryotic cells, Actin exists either in a monomeric G-actin form or in filamentous F-actin form. Actin filaments are double helical polymers of G-actin subunits all arranged head to tail giving filaments a molecular polarity. Actin filament possesses fast-growing barbed end (plus end) and

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slow growing pointed end (minus end). At steady state, a phenomenon called treadmilling is observed in which the assembly of the monomeric G-actin at the plus end equals the net disassembly of the subunit at the minus end. In the migrating cells, new actin filaments are formed by the polymerization of monomeric actin at the leading edge resulting into the formation of protrusions. The polymerization of actin consists of three steps: nucleation, elongation, and depolymerization. Treadmilling occurs at a very slow rate in *in vitro* conditions, whereas in *in vivo* a set of nucleation factors, elongation factors increases the rate. These nucleation and elongation factors are essential for the rapid filament growth in migrating cells. The new actin filaments are formed at the leading edge as a branch on the side of existing filaments. This new branches grows rapidly and push the membrane forward. The length of the new actin filaments is regulated by the binding of the capping proteins to the fast-growing end of actin filaments and preventing the further addition of the monomeric subunits. The bundling of the actin filaments into parallel and antiparallel bundles are required for the formation of filopodia and stress fibers in the cells. The single actin filament is not adequately stiff and cannot push the membrane, therefore, bundling of actin filaments provides the mechanical stability to filopodia and stress fibers further facilitating the migration of cells. This indicates capping and bundling of the filaments are essential for remodeling of the actin cytoskeleton during cell migration [14, 15, 16].
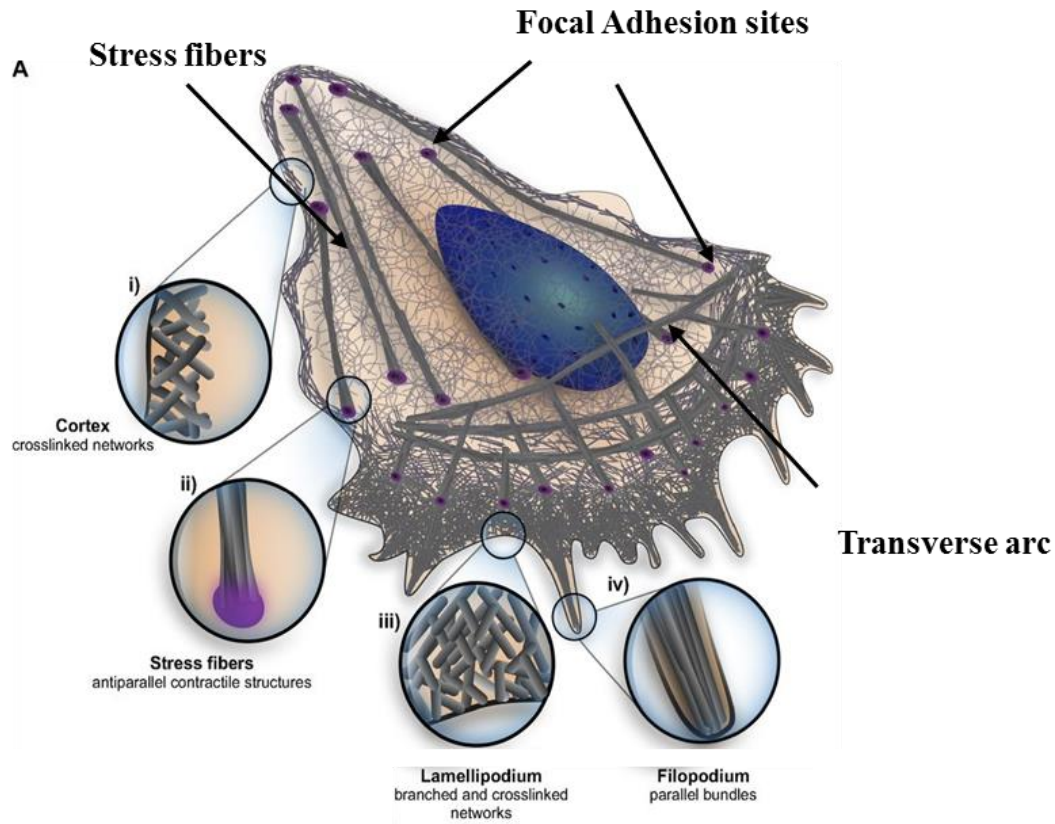
Figure 2: Overlay of actin architecture in migrating cells. Source- Blanchoin, L., Boujemaa-Paterski, R., Sykes, C., & Plastino, J. (2014). Actin dynamics, architecture, and mechanics in cell motility. *Physiological reviews*, *94*(1), 235-263.

1.3 Adducins: Important Actin Binding Proteins

Adducins are a family of cytoskeleton proteins encoded by three closely related genes namely α (ADD1), β (ADD2) and ɣ (ADD3) adducin which encodes polypeptides of 726, 713 and 674 amino acids respectively. Adducins are differentially expressed depending on the tissue type. α- and ɣ-adducin are ubiquitously expressed in almost all epithelial tissues, whereas the expression of β-adducin is limited to erythrocytes and brain. Various alternative spliced variants of adducins have been identified. The isolated form of adducins from the cells is always found either in heterodimers or heterotetramers form. Oligomerization is essential for adducins activities [17]. The interaction

between subunits of adducins stabilizes each other preventing their degradation in the cell. One of the *in vitro* studies showed downregulation of β- and ɣ- adducin upon siRNA-mediated downregulation of α-adducin in the cells and *vice versa*. This mutual downregulation of adducin subunits are at protein level without affecting the mRNA level. The similar mutual downregulation of adducin subunits was also observed in α-and ɣ- adducin null mice [18].

1.4 Structure of Adducin

All three adducins contain an N-terminal globular head domain, neck domain, and a C-terminal protease sensitive tail domain. The head domain and the neck domain possess oligomerization sites facilitating the formation of heterodimers and heterotetramers of adducins. The terminal end of the tail domain of all three adducins contains one of the highly conserved 22-residue MARCKS-related domain. This domain shares homology to MARCKS protein and possesses sites phosphorylated by PKA and PKC and also Ca^{2+}- Calmodulin binding site. MARCKS-related domain is required for the activities of Adducins and also potentially contributes to its folding. The phosphorylation sites of Rho kinase and PKA are present in the neck domain of Adducins [17]. In addition to this, the terminal tail domain and the neck region of α-Adducin possess nuclear localization signal and nuclear export signal respectively which helps in shuttling of Adducin between the nucleus and cytoplasm of the epithelial cells [19].

DOMAINS

| HEAD | NECK | TAIL |

proline rich

PKC
CALCIUM/CALMODULIN

+++

N

C

RHO KINASE

α: KSPSKKKKKFRTPSFLKKSKKKSDS 737
β: KSPSKKKKKFRTPSFLKKSKKKEKVES 726
γ: KSPSKKKKKFRTPSFLKKNKKKEKVEA 675

MARCKS related domain
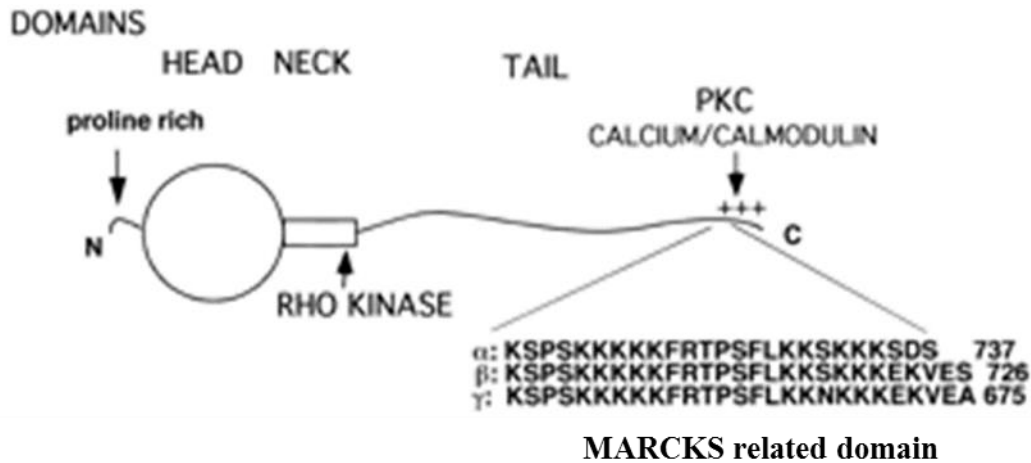
Figure 3: Model of structure of Adducin monomer. Source- Matsuoka, Y., Li, X., & Bennett, V. (2000). Adducin: structure, function and regulation. *Cellular and Molecular Life Sciences CMLS*, *57*(6), 884-895.

1.5 Functions of Adducins

The main functions of adducins as a regulator of actin cytoskeleton determined by *in vitro* assays are capping the fast-growing end of actin filaments, promoting the bundling of actin filaments and recruiting spectrin to the ends and sides of actin filaments facilitating the formation of spectrin-actin membrane skeletal network. The MARCKS-related domain is essential for the above mentioned activities of adducins [17].

Due to their participation in the assembly of the cortical actin cytoskeleton and the spectrin-based membrane skeleton, adducins have multiple functional roles in different eukaryotic cells. In neural tissue, adducins are involved in regulation of synaptic plasticity and synaptic contacts [20, 21]. Furthermore, the elevated level of phosphorylated adducin was shown to affect the synaptic stability at neuromuscular junctions (NMJ), which in turn influence pre and postsynaptic integrity in ALS [20]. The homozygous p.G367D point mutation in the oligomerization domain of γ-Adducin impairing the formation of heterotetramers is associated with inherited Cerebral Palsy

[22]. Adducins also regulates the diameter of both neuronal actin rings and axon as well as actin filament growth within actin rings [23]. In simple epithelia, adducins are involved in stabilization of adherens and tight junctions and regulation of epithelial junctional dynamics (disassembly and reassembly) [18]. In stratified epithelia, adducins regulate the desmosomal cohesion by indirectly regulating Desmoglein 3 (Dsg3) level [24]. Interestingly, the Gly460Trp polymorphism in α-adducin stimulates the activity of Na^+K^+ ATPase activity in renal tubular cells increasing the reabsorption of Na^+ and subsequently hypertension [25]. Finally, adducins appear to be essential for mitotic progression of cells as they are shown to associate with mitotic spindles and are crucial for proper spindle assembly and proper chromosomal segregation [26].
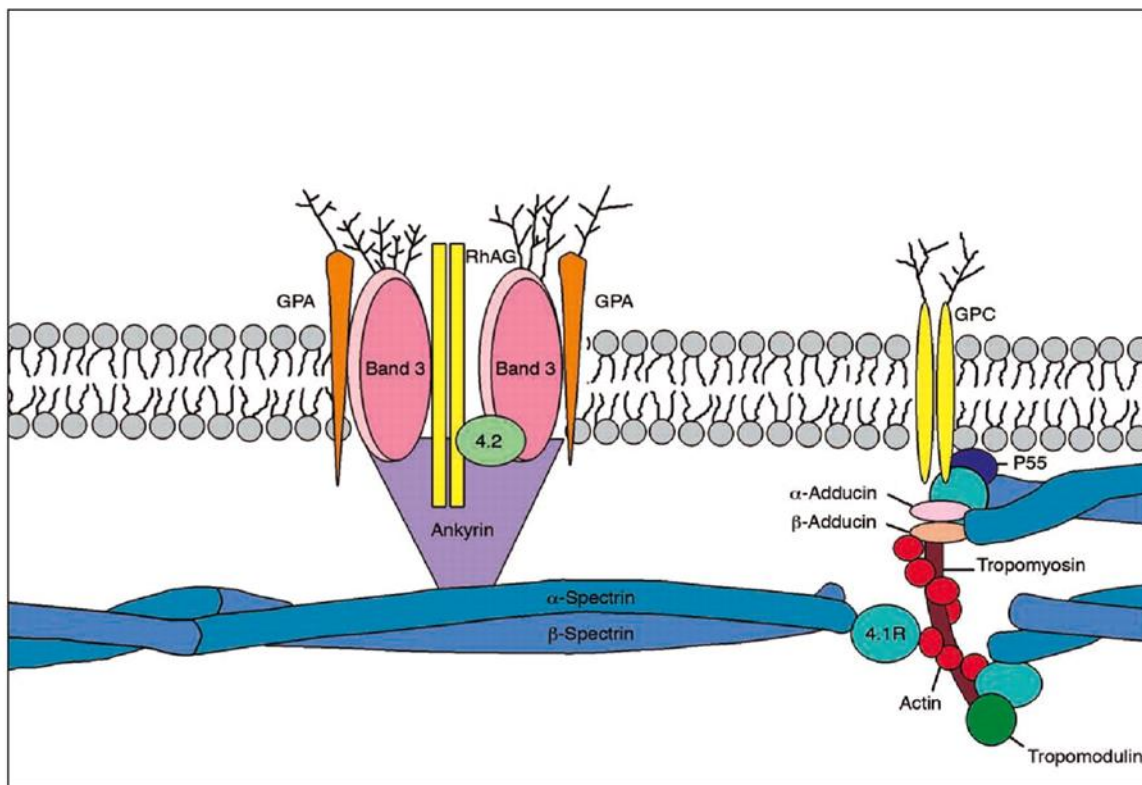
Figure 4: Schematic diagram of Adducin mediated spectrin-actin membrane skeletal network. Source-Iolascon, A., & Avvisati, R. A. (2008). Genotype/phenotype correlation in hereditary spherocytosis. *Haematologica*, *93*(9), 1283-1288.

1.6 Regulation of Adducins

The activities of adducins are regulated by mainly phosphorylation events and Ca^{2+} calmodulin binding. The phosphorylation of adducins at Ser726 and Ser713 in MARCKS-related domain by PKA and PKC inhibits both the actin capping and spectrin recruiting activities of adducins. On the other hand, phosphorylation of α-adducin at Ser408, Ser436 and Ser481 by PKA and at Thr445 and Thr480 by Rho kinase in the neck domain enhances adducin-actin interactions. Also, the binding of Ca^{2+} calmodulin at the sites located in the MARCKS-related domains of adducins inhibits the adducin-actin interactions [17].
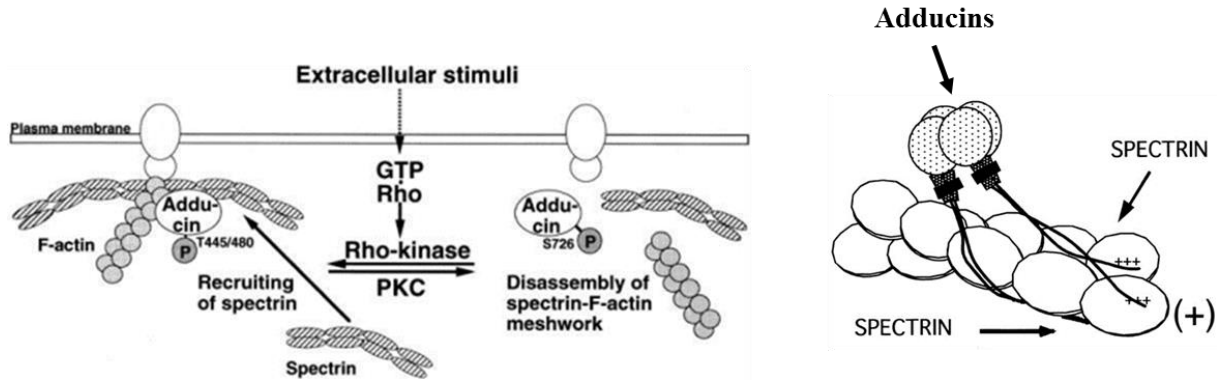

Figure 5: Model showing regulation of Adducins. Source- Matsuoka, Y., Li, X., & Bennett, V. (2000). Adducin: structure, function and regulation. *Cellular and Molecular Life Sciences CMLS*, *57*(6), 884-895.

1.7 Role of Adducins in Motility

Although, several lines of direct and indirect evidence have implicated adducins in the regulation of cell motility, the exact roles of these cytoskeletal proteins in migrating cells remains poorly understood. Only the role of α-adducin was previously investigated yielding controversial results with some studies describing α-adducin as a positive regulator while other suggesting it as a negative regulator of migration of different epithelial and cancer cells. The table shown below

summarizes the effect of downregulation, overexpression and phosphorylation of α-adducin on cell motility based on few studies. Only indirect evidence implicated ɣ-adducin in the cell migration. One of the studies showed the expression of ɣ-adducin is particularly associated with cell populations undergoing morphogenetic movements in the developing chick embryo [27]. There are two correlative studies indicating the role of ɣ-adducin in motility of glioma cells. The first study indicates ɣ-adducin as one of the direct target of miR-145 in human glioma cells, and this tumor suppressor miR-145 reduces invasion of glioblastoma cells by suppressing the activity of oncogenic ɣ-adducin protein [28]. The second study based on cDNA microarray analysis of glioma cell lines treated with motility inducing substrate shows strong down regulation of ɣ-adducin in migrating cells [29].

Table 1: Effect of downregulation, overexpression, and phosphorylation of α-adducin on cell motility

Type of Adducin		Effect
ADD1	Overexpression	Increases MDCK cell spreading and transfilter migration [30]
ADD1	Overexpression	Decreases ovarian cancer cell Invasion through Matrigel and reduces cellular growth [31]
ADD1	Overexpression	Increases migration and invasion of lung cancer cell lines such as A549 and H460 [32]
ADD1	Knockdown	Decreases migration and invasion of lung cancer cell lines such as A549 and H460 [32]
ADD1	Phosphorylation by Rho Kinase at Thr 455 and Thr 480	Induces membrane ruffling of MDCK cells and wound induced migration in NRK49F cells [33]

1.8 Why should we study the roles of adducins in the migration of normal epithelial cells and lung cancer cells?

The overall goal of this study was to understand the roles of adducins in regulating motility of normal lung epithelial cells and non-small cell lung cancer (NSCLC) cells. There are several reasons for focusing on normal pulmonary epithelium and lung cancer. First, epithelial cell migration plays an essential role in repairing the pulmonary barrier injured during either viral infections or environmental insults (cigarette smoking) [34]. Impaired healing of the epithelial wound is thought to contribute to devastating lung diseases such as idiopathic pulmonary fibrosis [35]. On the other hand, NSCLC progression results in cancer metastasis, which is driven by the enhanced motility of tumor cells. The overall prognosis of the disease is poor as most of the patients present with advanced or metastatic lung cancer stage during the time of diagnosis. Second, α-adducin was shown to regulate lung epithelial cell differentiation and the establishment of the basolateral plasma membrane [36]. This may affect cell surface expression of matrix adhesion proteins and chemotactic receptors. Third, some clinical evidence suggests altered adducin expression and functions in NSCLC. For example, a recent study indicated that oncogenic transcription factor ZNF322A promotes lung cancer metastasis by upregulating the expression of α-Adducin [32]. The other study showed cMET/HGF signaling induces the phosphorylation of ADD1, ADD3, which may contribute SCLC invasion induced by functional cMET/HGF signaling [37]. Finally, the long alternative spliced isoform of ɣ-adducin has been reported in NSCLC as compared to normal lung tissue. Although this isoform is shown to be associated with metastasis in breast cancer, the association in NSCLC remains elusive [38].

2. MATERIALS AND METHODS

2.1 Antibodies and other reagents

The following monoclonal (mAb) and polyclonal (pAb) antibodies were used to detect cytoskeletal, focal adhesion and other proteins: anti-alpha adducin pAb (1:500 for WB and 1:200 for IF), gamma adducin mAb (1:200 for WB and IF) (Santa Cruz, Dallas, TX); anti-FAK mAb (1:1000), anti-paxillin mAb (1:3000) (BD Biosciences, San Jose, CA); anti p-FAK mAb (1:1000), p-paxillin pAb (1:1000 for WB and 1:200 for IF), GAPDH mAb (1:15000), anti-src pAb (1:1000) , p-src pAb (1:1000), anti-FLAG mAb (1:500) (Cell Signaling, Beverly, MA);anti-Ki-67 pAb (1:500) (Millipore, Billerica, MA). Alexa Fluor-488-conjugated donkey anti-rabbit (1:1000), Alexa Fluor-555-conjugated donkey anti-mouse secondary antibodies (1:1000), and Alexa Fluor-488 and 555-labeled phalloidin (1:1000) were obtained from Life Technologies. Horseradish peroxidase-conjugated goat anti-rabbit (1:1000) and anti-mouse secondary antibodies (1:1000) were obtained from Bio-Rad Laboratories. Note: Concentration of antibodies used are shown in parenthesis.

2.2 Cell Culture

HBEC3-KT, HBEC3-KTRL53Myc cells were obtained from Dr. John Minna, Hamon Center for Therapeutic Oncology, Internal Medicine and Pharmacology, UT Southwestern Medical Center. 16HBE 14o- cells were obtained from Dr. Dieter C. Gruenert, Department of Otolaryngology-Head and Neck Surgery, University of California, San Francisco. NCI-H1299 and NCI-H441 cells were acquired from American Type Culture Collection (Manassas, VA). NCI-H1573, HCC4019,

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NCI-H2030, NCI-H1703 and NCI-H23 cells were obtained from Dr. Samir Hanash, Department of Clinical Cancer Prevention, The University of Texas MD Anderson Cancer Center. 16HBE 14o- cells were cultured in MEM medium (Life Technologies) supplemented with 10% FBS, HEPES, and penicillin-streptomycin antibiotics. NCI-H441 cells were cultured in RPMI medium supplemented with 5%FBS, dexamethasone (250ug/ml, Sigma-Aldrich), insulin-transferrin-sodium selenite (ITS 100X, Life Technologies) and penicillin-streptomycin antibiotics. NCI-H1573, HCC4019, NCI-H1299, NCI-H2030, NCI-H1703 and NCI-H23 cells were cultured in RPMI medium (Life Technologies) supplemented with 10% FBS, HEPES (1M), pyruvate (100mM) and penicillin-streptomycin antibiotics. HBEC3-KT and HBEC3-KTRL53Myc cells were cultured in Keratinocyte serum-free medium (Life Technologies) without any antibiotics. The cells were grown in T75 flasks (BD Biosciences) and were seeded on collagen-coated coverslips or 6-well plastic plates for immunolabeling and functional experiments, respectively.

2.3 CRISPR/Cas9 mediated knockout of ADD1 and ADD3

The stable knockout of ADD1 and ADD3 in 16HBE cells was carried out using CRISPR Cas9 technology. The sequence of the four guide oligonucleotides used for knocking out of ADD1 and ADD3 are shown below in table 2. Guide oligonucleotides were phosphorylated, annealed and cloned into the BsmBI site of lentiCRISPR v2 vector (Addgene, 52961) according to the Zhang laboratory protocol [39, 40] (F Zhang lab, MIT, Cambridge, MA). All the constructs were verified by sequencing. The transfer plasmid possessing annealed guide oligonucleotides were cloned in recombinant deficient Stbl3 bacteria. The transfer plasmid was isolated from Stbl3 bacteria using midi prep plasmid isolation kit from Qiagen. The lentivirus was produced by transfecting HEK-

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293T cells with transfer plasmid lentiCRISPR v2 and packaging plasmids pLTR-G (Addgene, 17532) and pCD/NL-BH*DDD (Addgene, 17531). The lentivirus was collected at 48 hours and 72 hours after transfection by harvesting the media from HEK-293T cells. The lentiviral containing harvested media was filtered through 0.45µm filter to remove HEK-293T cells. 16HBE cells were grown in T-25 flask. At 70% confluency, 16HBE cells were infected with 1ml of lentiviral particle solution. After 24 hours of infection, the media containing lentivirus was replaced with the fresh medium containing puromycin. The puromycin will only allow the virus infected cells to grow. Before infecting the cells with lentivirus, the minimum concentration of puromycin that results in the complete death of 16HBE cells after 3-5 days was determined by antibiotic resistance curve. This concentration of puromycin was used for selecting the virus infected cells. Lastly, the cellular lysates were made from puromycin resistant 16HBE cells and the protein level of ADD1 and ADD3 was detected by Immunoblotting to check the efficiency of knockout.

Table 2: Sequence of guide oligonucleotides used for knocking out of ADD1 and ADD3 by CRISPR Cas9 technology

Gene	Oligo Name	Direction	Sequence (5'-3')
ADD1	Hs-aADD-1F	Forward	CACCGCCACAGCCCCTCACAAGGAG
	Hs-aADD-1R	Reverse	AAACCTCCTTGTGAGGGGCTGTGGC
	Hs-aADD-2F	Forward	CACCGGACCAGAGTGAACTCCGAGC
	Hs-aADD-2R	Reverse	AAACGCTCGGAGTTCACTCTGGTCC
	Hs-aADD-3F	Forward	CACCGCCTCTTGCCAATCTCCCCGG
	Hs-aADD-3R	Reverse	AAACCCGGGGAGATTGGCAAGAGGC
	Hs-aADD-4F	Forward	CACCGGAAAGCGCCTCCGGGGAGAT
	Hs-aADD-4R	Reverse	AAACATCTCCCCGGAGGCGCTTTCC
	Hs-gADD-1F	Forward	CACCGGAGGAGTGGTAATCACGCCT
	Hs-gADD-1R	Reverse	AAACAGGCGTGATTACCACTCCTCC

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ADD3	Hs-gADD-3F	Forward	CACCGACAAGTCTACAAGTCTGTAC
	Hs-gADD-3R	Reverse	AAACGTACAGACTTGTAGACTTGTC
	Hs-gADD-4F	Forward	CACCGGTGTTGAATAGATTGCAGCA
	Hs-gADD-4R	Reverse	AAACTGCTGCAATCTATTCAACACC
	Hs-gADD-5F	Forward	CACCGAAGGATCCCACATTTCATGG
	Hs-gADD-5R	Reverse	AAACCCATGAAATGTGGGATCCTTC

2.4 ADD1 overexpression by lentiviral gene delivery system

The stable overexpression of ADD1 in H1299 cells was carried out by lentiviral gene delivery system. The lentiviral expression vector pLKO.AS2.neomycin encoding FLAG-tagged wild type ADD1 was obtained from Dr Hong-Chen Chen laboratory, National Chung Hsing University, Taiwan. H1299 cells were grown in the T-25 flask. At 70% confluency, H1299 cells were infected with 1ml of lentiviral particle solution. After 24 hours of infection, the media containing lentivirus was replaced with the fresh medium containing neomycin. The neomycin will only allow the virus infected cells to grow. Before infecting the cells with lentivirus, the minimum concentration of neomycin that results in the complete death of H1299 cells after 3-5 days was determined by antibiotic resistance curve. This concentration of neomycin was used for selecting the virus infected cells. Lastly, the cellular lysates were made from neomycin resistant H1299 cells and the protein level of ADD1 was detected by immunoblotting using the anti-FLAG antibody to check overexpression of ADD1.

2.5 Total cell lysate and Immunoblotting

In order to obtain total cell lysates, cells grown in six-well plates were scraped and homogenized using Dounce homogenizer in RIPA buffer (20 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% sodium deoxycholate, 1% Triton X-100 (TX-100), and 0.1% SDS, pH 7.4) containing

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protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). The protein fraction in obtained lysates was cleared by centrifugation (20 min at $14,000 \times$ g). The protein concentration of total lysates was determined using a BCA protein assay kit. Samples were diluted with 2x SDS sample loading buffer and boiled. SDS-polyacrylamide gel electrophoresis was conducted using standard protocols with an equal amount of total protein loaded per lane (10 or 20 μg). The separated proteins were transferred to nitrocellulose membrane by electroblotting technique. The membranes were blocked with either 5% non-fat milk made in TBS-T to detect all non-phosphorylated proteins or 3% BSA made in TBS-T to detect all phosphorylated proteins made. The membrane was incubated overnight with primary antibodies. Next day, the membrane were washed three times with TBS-T for 10 minutes each and then incubated with HRP conjugated secondary antibody for 1 hour. After this, the membrane was washed three times with TBS-T for 5 minutes each and then washed two times with TBS for 5 minutes each. The membrane was finally developed using ECL from GE Healthcare. The images were obtained on standard X-ray film using SRX-101A image processor from Konica Minolta.

2.6 Wound Healing Assay or *In vitro* Scratch Assay

Cells grown to confluence were mechanically wounded by making a thin scratch wound in the monolayer using 200µl pipette tip. After creating the wound, the monolayer of cells was washed with complete medium. The wells were marked using the marker and replaced with the new complete medium. The images of cell-free area at the marked region were acquired at 0 and 9 hours after wounding using an inverted bright field microscope equipped with a camera. The percentage of wound healing was calculated using Image J software [41].

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2.7 Transfilter Migration Assay

Transfilter migration assay was performed using Transwells® with 8.0µm insert 24 well plate (Corning Incorporated). The membrane of transwell was coated with 50ul of collagen (3.9 mg/ml, Corning) and was allowed to dry overnight. Cells were trypsinized, resuspended in serum free medium, and added to the upper chamber of transwell at a concentration of $3X10^4$ cells per chamber. In the lower chamber, complete growth medium containing 10% FBS as a chemoattractant was added, and cells were allowed to migrate for 24h at 37^oC. Cells were fixed and permeabilized using 4% PFA and 0.5% Triton X-100 respectively. The non-migrated cells were removed from the top of the filter using cotton swabs. The cells at the bottom of the filter or membrane were stained using nuclear stain DAPI. The images were captured using fluorescence microscopy and the number of invaded cells were counted either manually or by using Image J software [41].

2.8 Matrigel Invasion Assay

The Matrigel invasion assay was performed using commercially available BD Biocoat invasion chambers (BD Biosciences). Cells were trypsinized. resuspended in serum-free medium, and added to the upper chamber of the transwell at a concentration of $2.5X10^4$ cells per chamber. In the lower chamber, complete growth medium containing 10% FBS as a chemoattractant was added, and cells were allowed to invade and migrate for 24h at 37^oC. Cells were fixed and permeabilized using 4% PFA and 0.5% Triton X-100 respectively. The non-migrated cells were removed from the top of the filter using cotton swabs. The cells at the bottom of the filter or membrane were stained using nuclear stain DAPI. The images were captured using fluorescence

microscopy and the number of invaded cells were counted either manually or by using Image J software [41].

2.9 Immunofluorescent staining

Cells were grown on the collagen coated coverslips in 24 well plates. Sparse or confluent cells grown were fixed with 4% PFA for 20 minutes at room temperature and after subsequent 3 washes with HANKS+ buffer (0.137M NaCl, 5.4mM KCl, 0.25mM Na_2HPO_4, 0.1g glucose, 0.44mM KH_2PO_4, 1.25mM $CaCl_2$, 1.0mM $MgSO_4$) were permeabilized with 0.5% Triton X-100 for 5 minutes. Fixed and permeabilized cells were washed 3 times with HANKS+ buffer and were blocked with HANKS+ buffer containing 1% BSA for 1 hour at room temperature. Next, cells are incubated with primary antibodies diluted in HANKS+ buffer containing 1% BSA for 1 hour. Cells were then washed for three times with HANKS+ buffer containing 1% BSA for 5 minutes each. Cells now are incubated with Alexa Fluor-conjugated secondary antibodies diluted in HANKS+ buffer containing 1% BSA for 1 hour. Cells were washed for three times with HANKS+ buffer containing 1% BSA for 5 minutes each and the coverslips were mounted using ProLong® Gold Antifade Mountant (Thermo Fisher Scientific). Fluorescently labeled cell monolayers were examined using a Zeiss LSM700 laser-scanning confocal microscope (Zeiss Microimaging, Thornwood, NY). The images were processed using Zen Lite software (Carl Zeiss Microscopy LLC).

2.10   Adhesion Assay

24 well plates were coated with 200µl of collagen type I and were allowed to dry overnight. Cells were trypsinized, harvested, counted with a hemocytometer, and resuspended in complete

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medium. 5×10^4 cells were seeded to each well and cells were allowed to adhere to collagen type I for 30 minutes at 37 °C. After incubation, unattached cells were removed and the wells were gently washed with HANKS+ buffer. The attached cells were fixed for 20 minutes at -20°C using methanol. After fixing, the cells were stained using a DIFF stain kit (IMEB Inc., San Marcos, CA). The images of adherent cells were captured using brightfield microscope and the number of adhered cells were manually counted.

2.11   Spreading Assay

Well plates were coated with 200µl of collagen type I and were allowed to dry overnight. Cells were trypsinized, harvested, counted with a hemocytometer, and resuspended in complete medium. 5×10^4 cells were seeded to each well and cells were allowed to attach and spread over collagen type I for 1 hour at 37 °C. After incubation, unattached cells were removed and the attached cells were fixed for 20 minutes at -20°C using methanol. After fixing, the cells were stained using a DIFF stain kit (IMEB Inc., San Marcos, CA). The images of attached cells were captured using brightfield microscope and spreading cells were manually counted.

2.12 Ki-67 proliferation Assay

Ki-67 proliferation assay was carried out which allows determination of the percentage of proliferating and non-proliferating cells based on the Ki-67 expression. Sparsely grown cells on collagen-coated coverslips were fixed and permeabilized with 4% PFA and 0.5% Triton X-100 respectively. The cells were immunolabeled for Ki-67. The coverslips were mounted using ProLong® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were captured using the fluorescent microscope and the percentage of Ki-67 positive cells were counted.

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2.13 Immunoprecipitation

Cells were homogenized in immunoprecipitation (IP) buffer (50 mM HEPES, 50 mM 1,4-piperazinediethanesulfonic acid, 1 mM EDTA, 2 mM $MgSO_4$, 1% TX-100, and 0.5% Igepal, pH 7.0) supplemented with proteinase inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3. Cells were homogenized and centrifuged, and supernatants were precleared with protein A – Sepharose beads (GenScript, Piscataway, NJ) for 1 hour at 4°C with constant rotation. Precleared lysates were then incubated overnight at 4°C with 1 µg of anti–ADD1 polyclonal antibodies or control rabbit immunoglobulin. Immunocomplexes were recovered by incubation with protein A–Sepharose beads for 1 hour at 4°C with constant rotation. The extensively washed beads were boiled with 2× SDS sample buffer and pelleted by centrifugation. Equal volumes of supernatants (25µl) were loaded into polyacrylamide gels and analyzed by immunoblotting as described.

3. RESULTS

3.1 Adducins are either downregulated or mislocalized in lung cancer cells with mesenchymal phenotype

In order to examine the role of adducins in the motility of lung epithelial cells, we first compared the expression level of ADD1 and ADD3 in 16HBE non-tumorigenic lung epithelial cell line and panel of lung cancer cells with either epithelial (NCI-H441, NCI-H1573, HCC4019) or mesenchymal (NCI-H1299, NCI-H2030, NCI-H1703, NCI-H23) phenotype. Immunoblotting results presented in Figure 6A shows that ADD1 expression does not depend on cell phenotype, whereas the level of ADD3 protein is significantly decreased in lung cancer cells with the mesenchymal phenotype. Next, we compared adducins expression in another non-transformed lung epithelial cell line (HBEC3-KT) and HBEC3 cells transformed by p53 knockdown and K-RAS/Myc overexpression [42]. Transformation of HBEC cells caused vivid epithelial-to-mesenchymal transition that was accompanied by significant downregulation of ADD3 expression (Figure 6B).
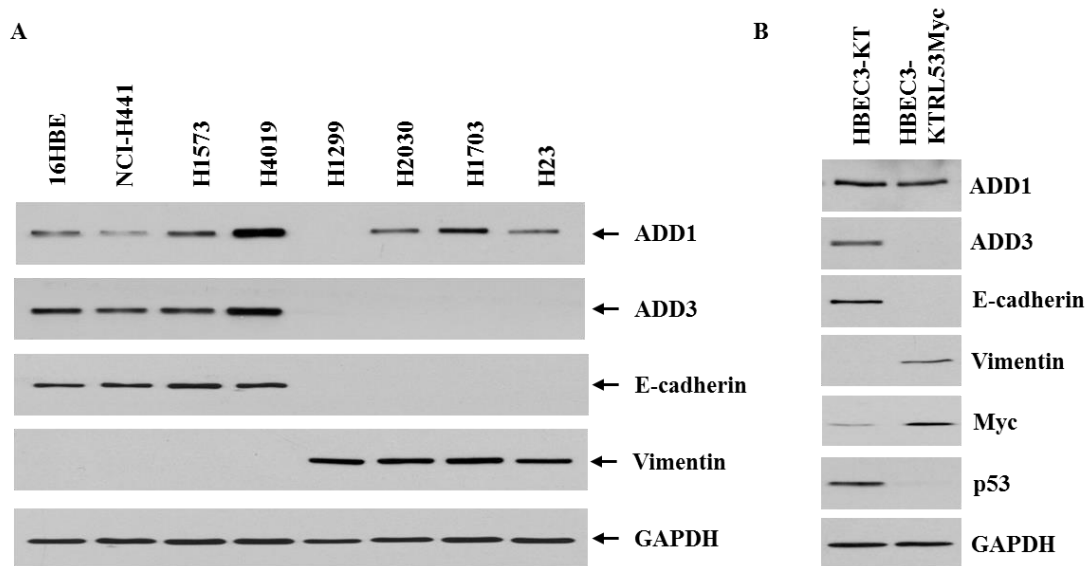
Figure 6: Downregulation of ADD3 in fully transformed lung cancer cells: A) B) Immunoblotting shows significant downregulation of ADD3 expression in fully transformed lung cancer cells with the mesenchymal phenotype. It also shows ADD1 expression does not change significantly in different cell types. Representative images of 2 independent experiments.

Since adducins are scaffold proteins, their activity depends on intracellular localization.

Next, we compared the intracellular localization of ADD1 and ADD3 in immortalized lung epithelial cells (16HBE, HBEC3-KT) and mesenchymal-type lung cancer cells (H1299 and HBEC3-KTRL53). Figure 7A and 7C shows the membrane localization of ADD1 and ADD3 in 16HBE and HBEC3-KT cells. By contrast, ADD1 appears to be redistributed from the membrane pool into the cytoplasmic pool in mesenchymal lung cancer cell lines (Figure 7B and 7D)**.** Consistently with our immunoblotting results, labeling intensity of ADD3 was dramatically decreased in H1299 and HBEC3-KTRL53 lung cancer cells. Together our data suggest that epithelial to mesenchymal transition that is characteristic of lung cancer progression is accompanied by either down-regulation (ADD3) or mislocalization (ADD1) of adducins. We hypothesize that the described alterations could impair normal functions of these cytoskeletal scaffold proteins.

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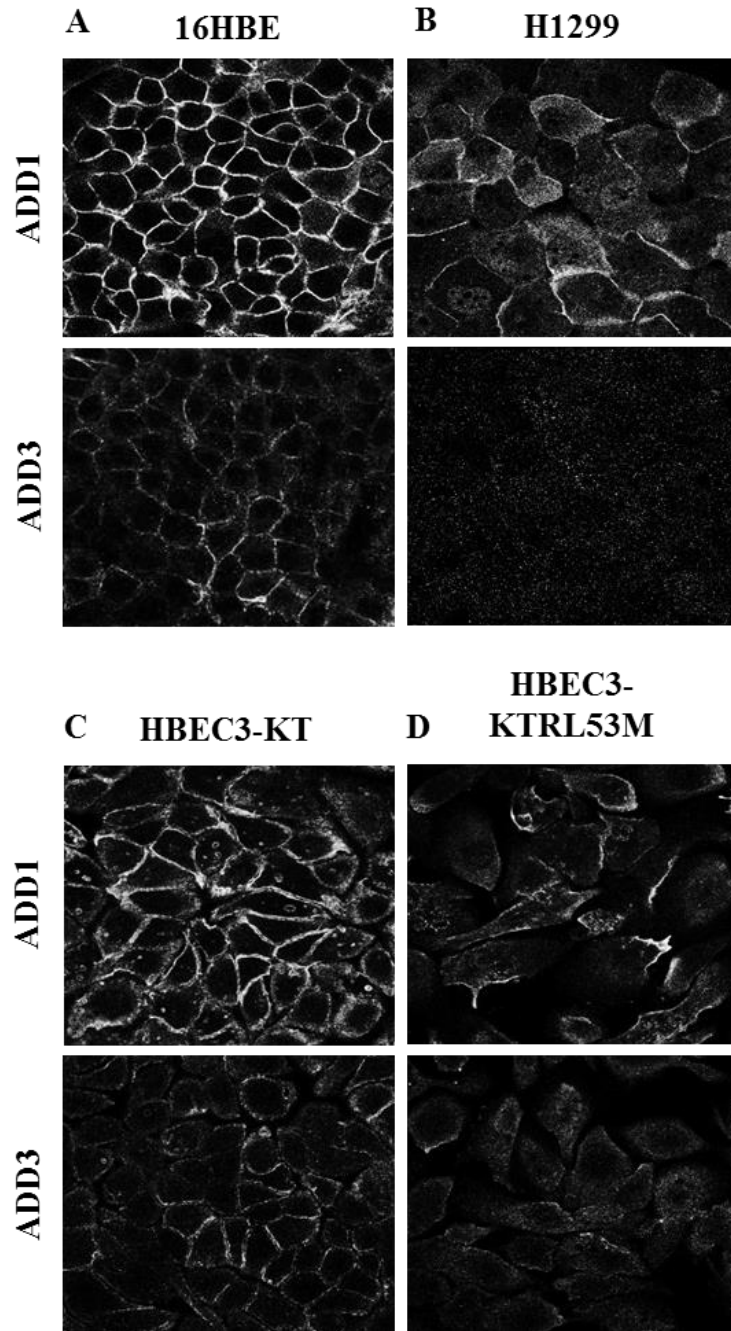
Figure 7: Mislocalization of ADD1 in fully transformed lung cancer cells: 16HBE, H1299, HBEC3-KT and HBEC3-KTRL53M cells were fixed and immunolabeled for ADD1 and ADD3. Immunofluorescence labeling and confocal microscopy shows membrane localization of ADD1 and ADD3 in 16HBE, HBEC3-KT **(A, C)** normal lung epithelial cells and diffuse cytoplasmic localization of ADD1 in H1299 and HBEC3-KTRL53Myc **(B, D)** fully transformed lung cancer cells. Representative images of 3 independent experiments.

3.2 Depletion of ADD1 enhances individual cell migration without affecting collective migration of 16HBE cells

In order to investigate the role of alpha-adducin (ADD1) in the regulation of cell motility, we created a stable ADD1 knockout in 16HBE cells using CRISPR-Cas9 technology. Figure 8A shows the efficiency of the protein knockout by four different guide oligonucleotides used for creating ADD1-deficient 16HBE cell lines. Interestingly, loss of ADD1 also caused the decrease in ADD3 protein level. This is consistent with previously described effects of ADD1 knockdown and knockout in cultured epithelial cells and mice and can be explained by hetero-oligomerization of ADD1 and ADD3 [18]. Because such hetero-oligomerization stabilizes adducins, loss of ADD1 results in proteolytic degradation of ADD3. As a result, ADD1-depleted 16HBE cell lines are characterized by a dual ADD1/ADD3 depletion. The stable 16HBE cell lines with the most efficient ADD1 knockout (oligo 3 and 4) were selected for our further experiments.

First, we performed a wound healing assay in order to determine the effect of adducins depletion on the collective migration of 16HBE cells. As shown in Figure 8B, no significant difference in the rate of cell migration was observed between control and ADD1 depleted 16HBE cells. This indicates that loss of adducins does not affect the collective migration of non-transformed lung epithelial cells. Next, we examined whether adducins may regulate motility of individual lung epithelial cells by using a Boyden Chamber transfilter migration assay. Figure 9A shows approximately 2.5 fold increase in transmigration of ADD1 depleted 16HBE cells as compared to control cells, thereby indicating enhanced motility of individual adducin-deficient 16HBE cells.

A)
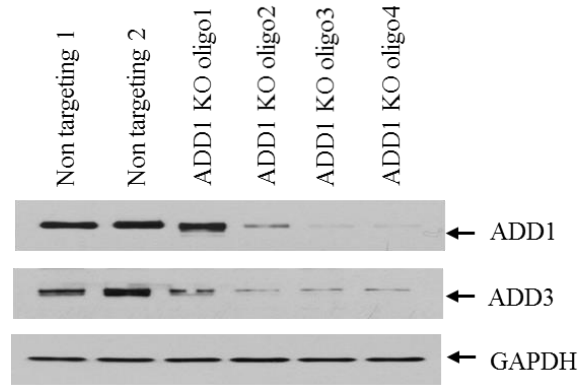
B)
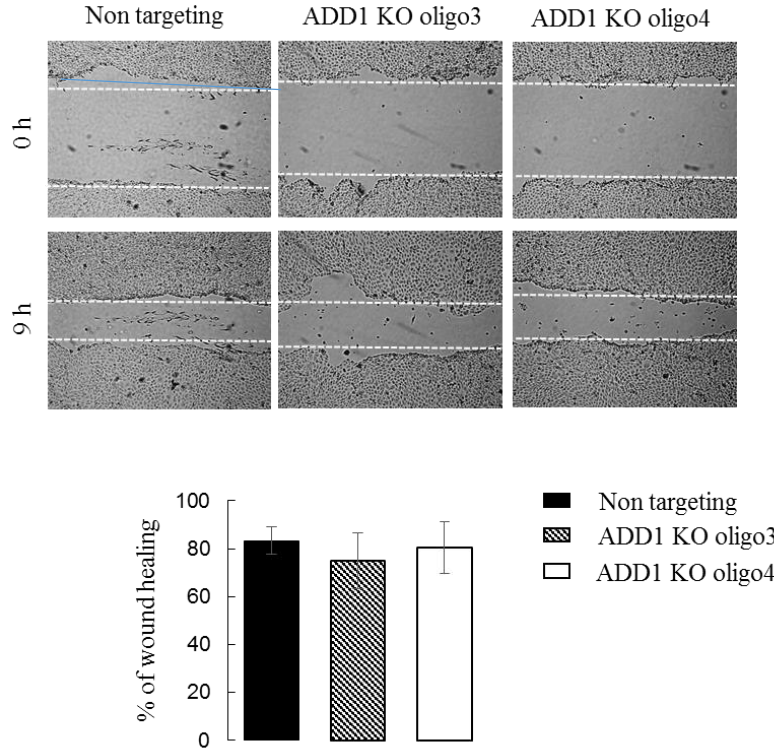
Figure 8: **Depletion of ADD1 does not affect collective migration of 16HBE cells.** Knock out of ADD1 was carried out in 16HBE cells using CRISPR/Cas9 technology. **A)** Immunoblotting shows the efficiency of ADD1 knockout using different CRISPR/cas9 oligos. **B)** Wound healing assay was performed as described in materials and methods using Control and ADD1 depleted 16HBE cells. The bar graph represents the percentage of wound healing between control and ADD1 depleted 16HBE cells. Data are presented as mean ± SE (n =6).
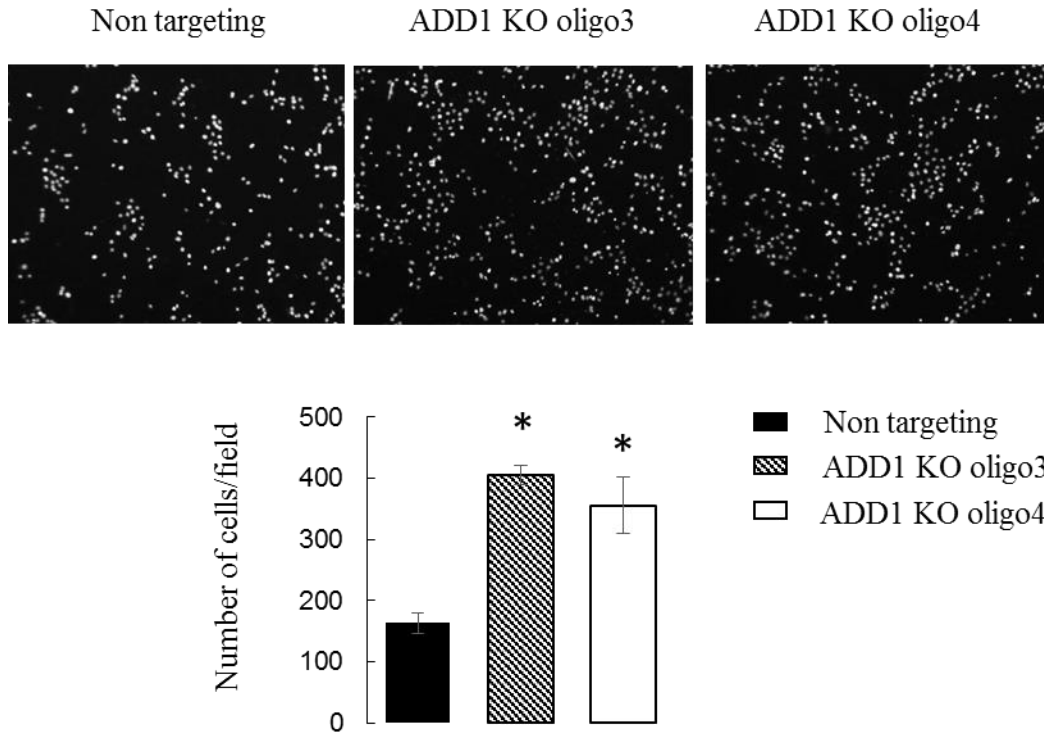
A)

Figure 9: Depletion of ADD1 enhances transfilter migration of 16HBE cells: Knock out of ADD1 was carried out in 16HBE cells using CRISPR Cas9 technology. **A)** Control and ADD1 depleted 16HBE cells were subjected to Transfilter Migration assay as described in materials and methods. The bar graph represents the number of cells migrated between control and ADD1 depleted 16HBE cells. Data are presented as mean $\pm$ SE (n =6); $p < 0.05$.

3.3 Depletion of ADD3 enhances individual cell migration without affecting collective migration of 16HBE cells

Next, we sought to investigate the role of ADD3 in the regulation of lung epithelial cell motility. We created 16HBE cell lines with stable CRISPR-Cas9 mediated knockout of ADD3. Figure 10A shows the efficiency of ADD3 knockout in four different 16HBE cell lines. Interestingly, loss of ADD3 induced modest downregulation of ADD1 expression, however, a significant fraction of ADD1 remained in ADD3-depleted cells. Since we did not detect ADD2 expression in 16HBE cells (data not shown), our data indicates the existence of two types of adducin oligomers: classical ADD1/ADD3 heterotetramers and ADD1/ADD1 homotetramers. Importantly, ADD3 knockout preserves the significant level of ADD1 protein, thereby allowing to study selective functions of ADD3 in lung epithelial cells. Based on our immunoblotting results, we selected two ADD3 depleted 16HBE cell lines created by oligo 4 and 5 for the subsequent experiments.

In order to determine the effect of ADD3 depletion on the collective migration of lung epithelial cells, we performed wound healing assay using 16HBE cell monolayers. As shown in Figure 10B, no significant difference in the rate of wound closure was observed between control and ADD3 depleted 16HBE cells. Next, we examined the effects of ADD3 depletion on transmigration of individual lung epithelial cells using the Boyden Chamber assay. Figure 11A shows that loss of ADD3 results in significantly (~ 2 fold) increase in transmigration of 16HBE cells. Collectively, our data suggest that adducins are dispensable for collective migration but negatively regulate individual motility of non-transformed lung epithelial cells.
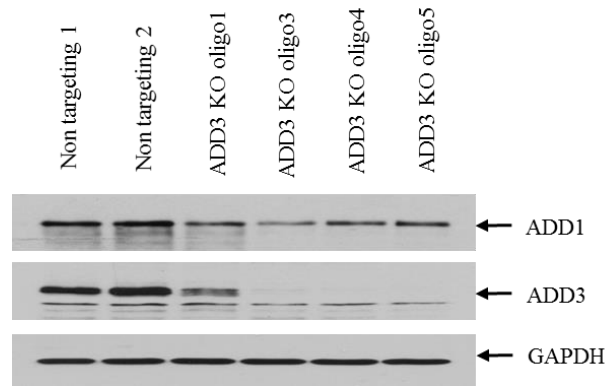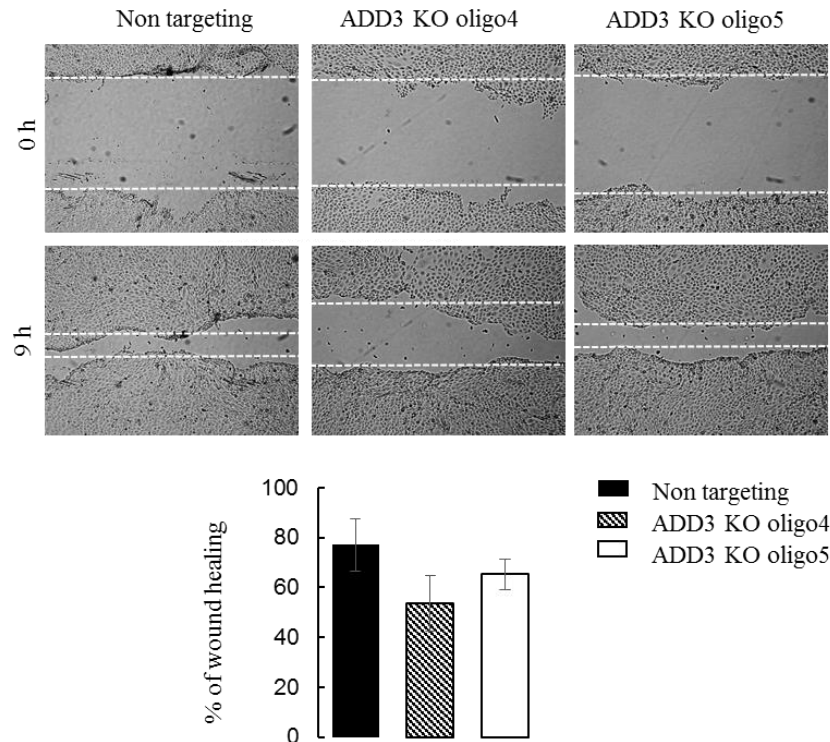
Figure 10: Depletion of ADD3 does not affect collective migration of 16HBE cells: Knock out of ADD3 was carried out in 16HBE cells using CRISPR Cas9 technology. **A)** Immunoblotting shows the efficiency of ADD3 knockout using different CRISPR cas9 oligos. **B)** Wound healing assay was performed as described in materials and methods using Control and ADD3 depleted 16HBE cells. The bar graph represents the percentage of wound healing between control and ADD3 depleted 16HBE cells. Data are presented as mean ± SE (n =6).
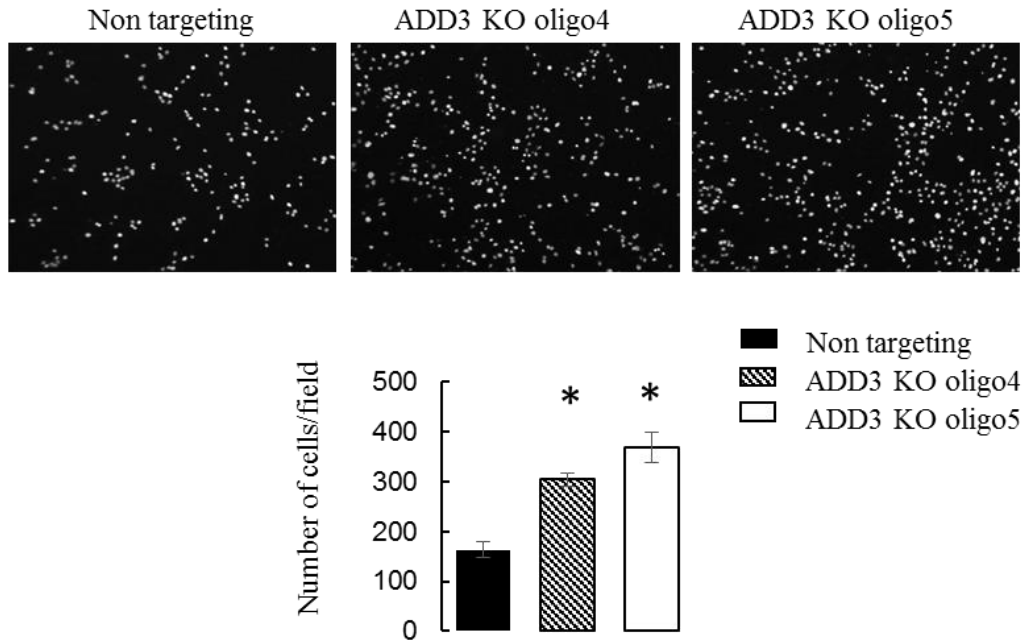
A)

Figure 11: Depletion of ADD3 enhances transfilter migration of 16HBE cells: Knock out of ADD3 was carried out in 16HBE cells using CRISPR Cas9 technology. **A)** Control and ADD3 depleted 16HBE cells were subjected to Transfilter migration assay as described in materials and methods. The bar graph represents the number of cells migrated between control and ADD3 depleted 16HBE cells. Data are presented as mean ± SE (n =6); $p < 0.05$.
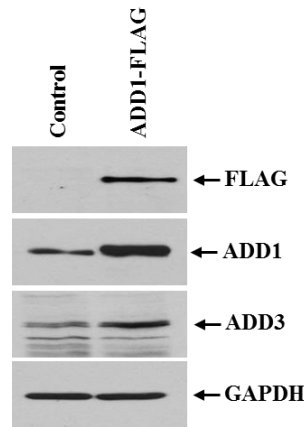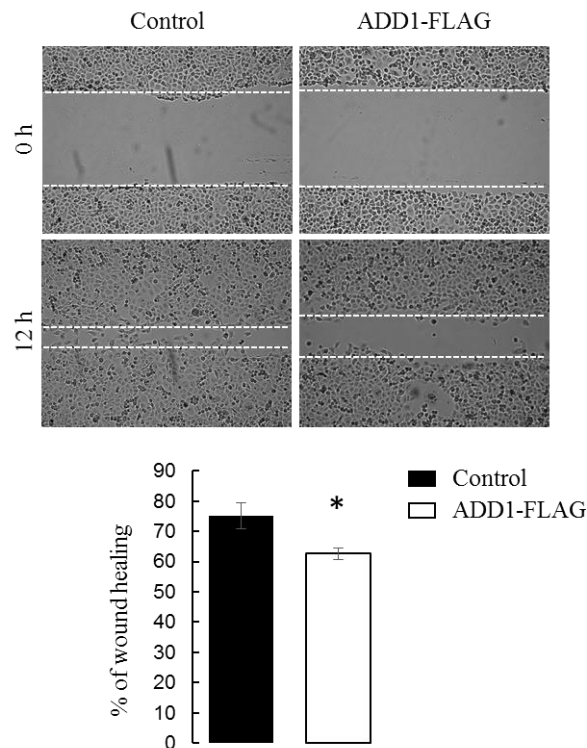
31

3.4 Overexpression of ADD1 decreases collective planar migration, transfilter migration and Matrigel invasion of H1299 cells

Next, we designed a gain-of-function experiments involving ADD1 overexpression in H1299 mesenchymal-type lung cancer cells that have the low level of endogenous ADD1 and ADD3 (Figure 6A). H1299 cells were transduced with lentiviral particles containing either Flag-tagged ADD1 or control plasmid with subsequent generation of stable cell lines. Immunoblotting analysis using anti-adducin or anti-Flag antibodies shows the high level of ADD1 overexpression (Figure 12A). The effects of ADD1 overexpression on lung cancer cell motility was investigated by using the wound healing assay, the Boyden Chamber transfilter migration assay, and a Matrigel invasion assay. We observed that ADD1 overexpression slightly, but significantly inhibited collective cell migration (Figure 12B) and had more marked inhibitory effect on either transfilter migration (Figure 13A) or Matrigel invasion (Figure 13B) of H1299 cells ($\sim$ 1.5 fold and 2 fold inhibition respectively). Overall, these results are consistent with the observed effects of adducins knockout and strongly suggest that adducins act as negative regulators of motility of non-transformed lung epithelial cells and lung cancer cells.

A)

B)

Figure 12: Overexpression of ADD1 decreases collective planar migration of H1299 cells. ADD1 was overexpressed in H1299 cells using lentiviral gene delivery system. **A)** Immunoblotting shows efficient flag tagged ADD1 overexpression in H1299 cells. **B)** Wound healing assay was performed as described in materials and methods using control and ADD1 overexpressed H1299 cells. The bar graph represents the percentage of wound healing between control and ADD1 overexpressed H1299 cells. Data are presented as mean ± SE (n =6); $p < 0.05$.
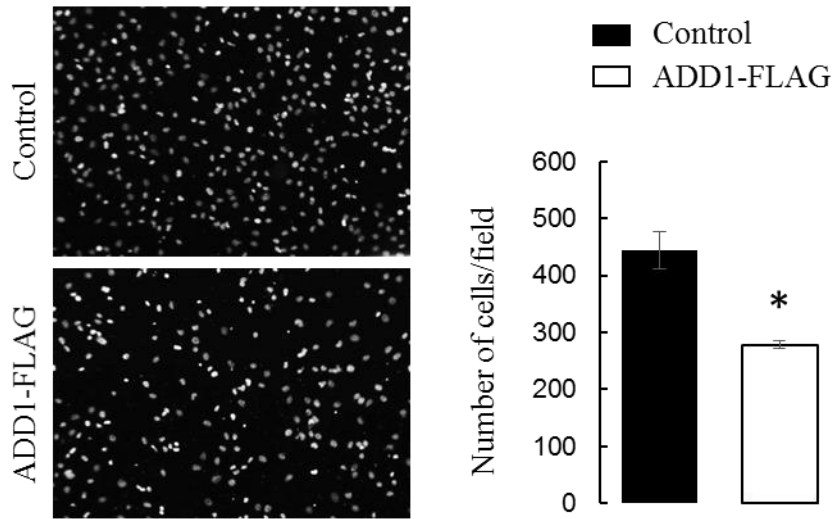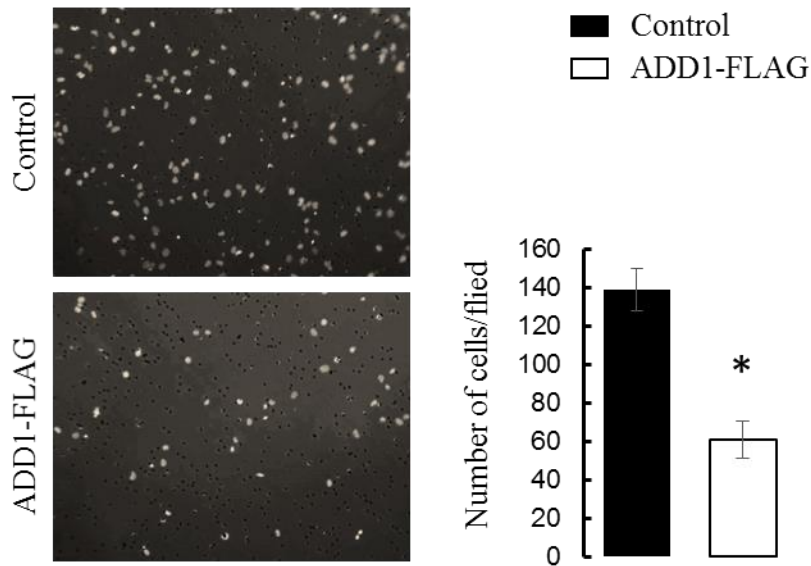
Figure 13: Overexpression of ADD1 decreases transfilter migration and Matrigel invasion of H1299 cells. ADD1 was overexpressed in H1299 cells using lentiviral gene delivery system. **A) B)** Control and ADD1 overexpressed H1299 cells were subjected to Transfilter migration assay and Matrigel invasion assay respectively as described in Materials and Methods. The bar graph represents the number of cells migrated and invaded between control and ADD3 depleted 16HBE cells. Data are presented as mean $\pm$ SE (n =6); $p < 0.05$.

Next, we sought to investigate the possible mechanisms responsible for the effect of adducins depletion and overexpression on cell motility. Several such mechanisms could be envisioned including effects of adducins on cell proliferation, cell-matrix adhesions and the actin cytoskeleton in migrating leading edge. In subsequent chapters, we describe experiments that were designed to address molecular mechanisms that may mediate the effects of adducins on cell motility.

3.5 Depletion of ADD1 and ADD3 and overexpression of ADD1 does not affect lung epithelial cell proliferation

Cell proliferation is known to affect the motility of different mammalian cells especially during relatively slow migration processes such as wound healing [43]. On the other hand, ADD1 was previously implicated in the regulation of epithelial cell proliferation by altering late stages of mitosis [19, 26]. Therefore we ask whether altered cell proliferation can underlie the observed effects of adducin depletion and overexpression on lung epithelial cell motility. To examine cell proliferation, we used immunolabeling of a known proliferative marker, Ki-67 [44]. Ki-67 is a protein expressed in the nucleus during the G2, S and M phases of the cell cycle and it is undetectable during the G0 and G1 (non-proliferating) phases. Sparse ADD1 and ADD3 depleted 16HBE cells and ADD1 overexpressing H1299 cells were fixed and immunolabeled for Ki-67. As shown in Figure 14A and Figure 14B, there is no significant difference in the percentage of Ki-67 positive cells between control cells and ADD1 and ADD3 depleted 16HBE cells. As shown in Figure 15A, there is no significant difference in the percentage of Ki-67 positive cells between control cells and ADD1 overexpressing H1299 cells. This indicates that the observed effect of adducin depletion and overexpression on epithelial cell motility is not due to altered cell proliferation.

A)
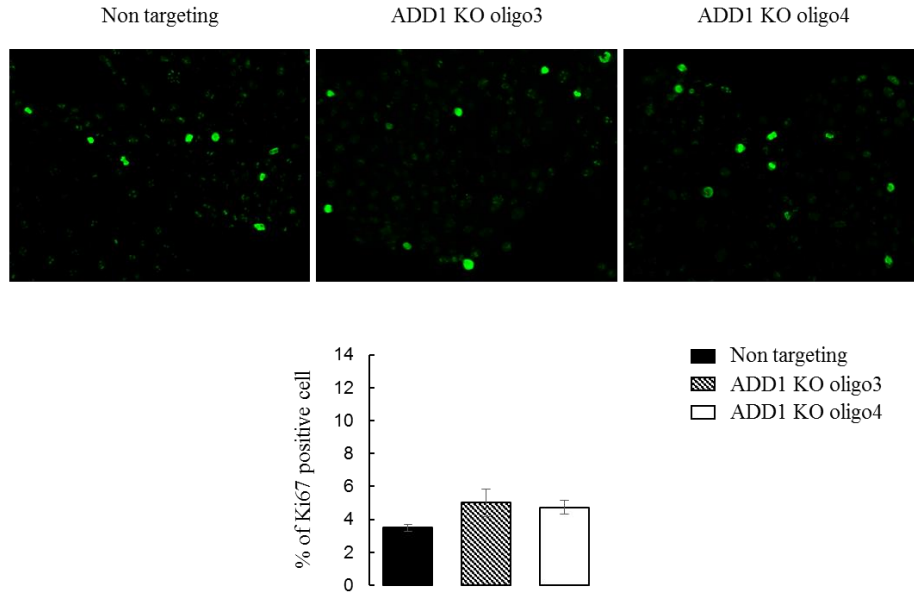
B)
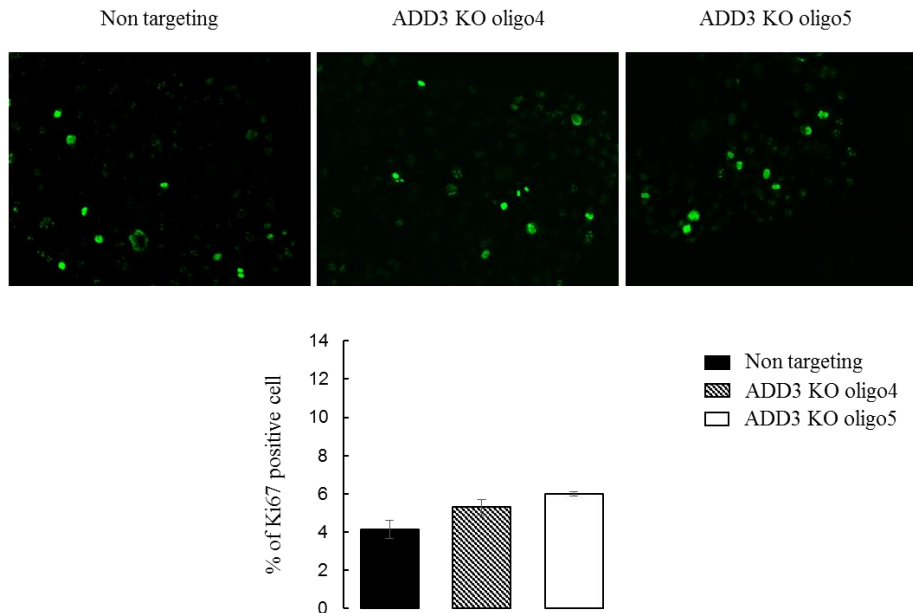
Figure 14: Depletion of ADD1 and ADD3 does not affect proliferation of 16HBE cells:
Sparse active proliferating ADD1 and ADD3 depleted 16HBE cells were fixed and immunolabeled for Ki-67. **A) B)** Immunofluorescence shows no significant difference in the percentage of Ki-67 positive cells between control and ADD1 and ADD3 depleted 16HBE cells. Data are presented as mean $\pm$ SE (n =3).

A)
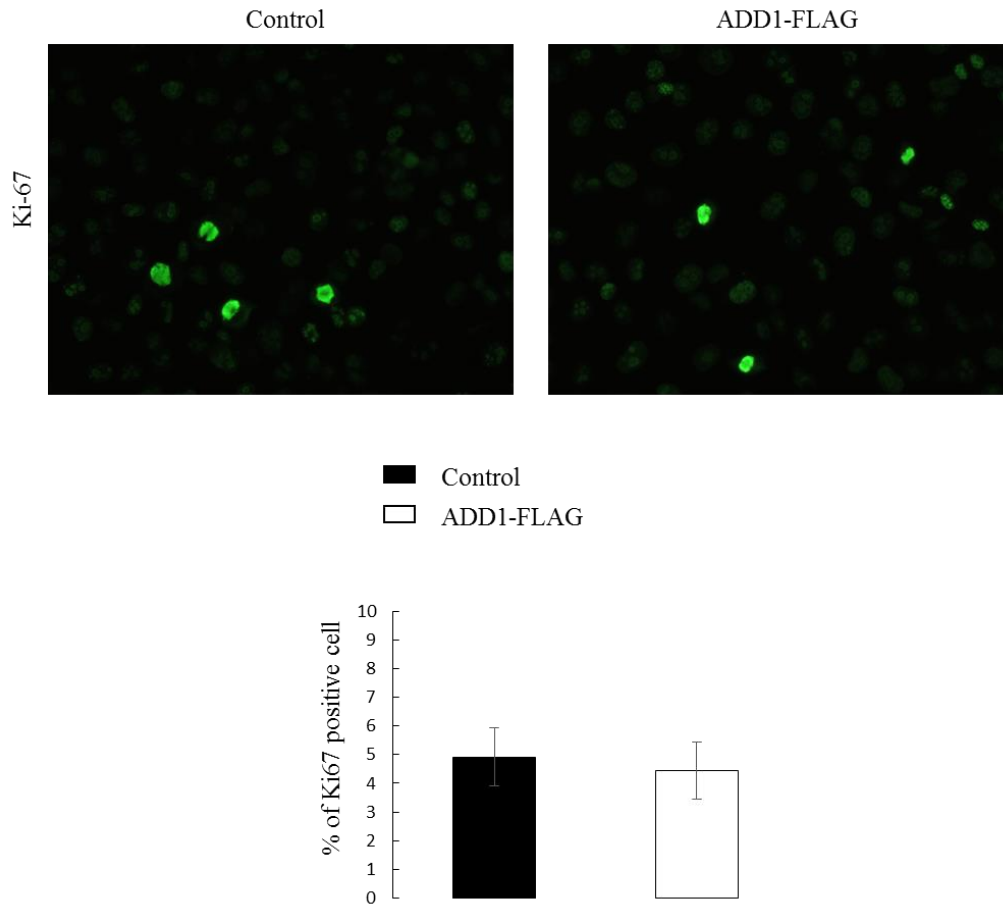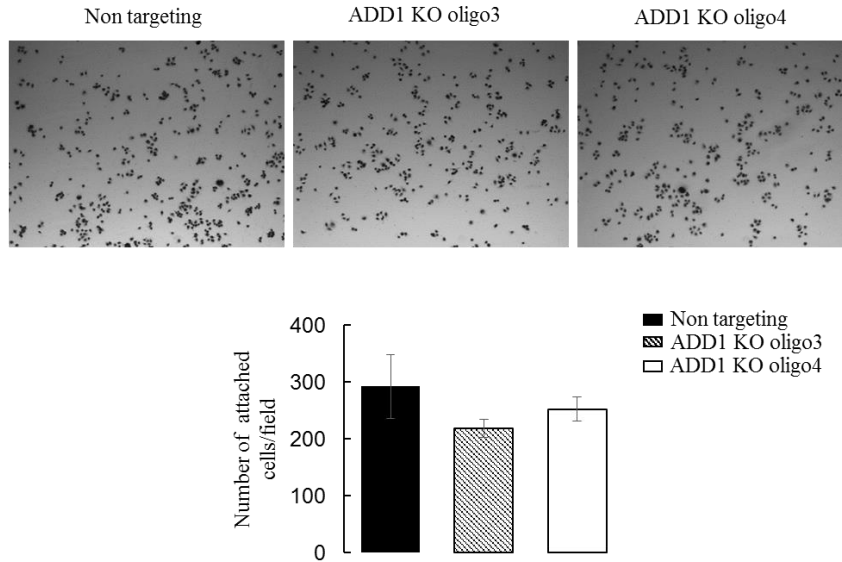

Figure 15: Overexpression of ADD1 does not affect proliferation of H1299 cells: Sparse active proliferating ADD1 overexpressing H1299 cells were fixed and immunolabeled for Ki-67. **A)** Immunofluorescence shows no significant difference in the percentage of Ki-67 positive cells between control and ADD1 overexpressing H1299 cells. Data are presented as mean ± SE (n =3).

3.6 Overexpression of ADD1 increases cell-matrix adhesion and spreading of lung cancer cells

Adhesion to the ECM and spreading of adherent cells represent two key early events of planar cell migration. Interaction with ECM triggers cytoskeletal rearrangement leading to the assembly of the migrating leading edge. Furthermore, cell attachment to ECM provides traction forces that are required for the forward movement of the cell body. Given the importance of these events for cell motility, we sought to investigate if modulation of ADD1 and ADD3 expression could affect ECM adhesion and spreading of lung epithelial cells. Figure 16A shows that depletion of ADD1 does not affect adhesion of 16HBE cells to the collagen type I matrix. Further, Figure 16B shows that ADD1 depletion does not affect spreading of 16HBE cells over collagen type I matrix. Similarly, Figure 17A and 17B shows that ADD3 depletion does not affect cell matrix adhesion and spreading of 16HBE cells. Surprisingly, ADD1 overexpression affected ECM adhesion and spreading of lung cancer cells. Thus, Figure 18A shows that ADD1 overexpression resulted in approximately 2 fold increase in the number of H1299 cells attached to collagen I as compared to control H1299 cells. Furthermore, Figure 18B shows approximately 2 fold increase in spreading of ADD1 overexpressed H1299 cells. These data may have several interpretations. First is the existence of the protein expression threshold below which epithelial cell adhesion and spreading are ADD1-independent. The increase in the ADD1 level above such a threshold activates some new mechanisms that promote ECM adhesion and cell spreading. Our data suggest that the increased adhesiveness of ADD1-overexpressing H1299 cells may represent one of the mechanisms that leads to the decrease in planar migration, individual cell migration, and invasion of these cells.

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A)

B)
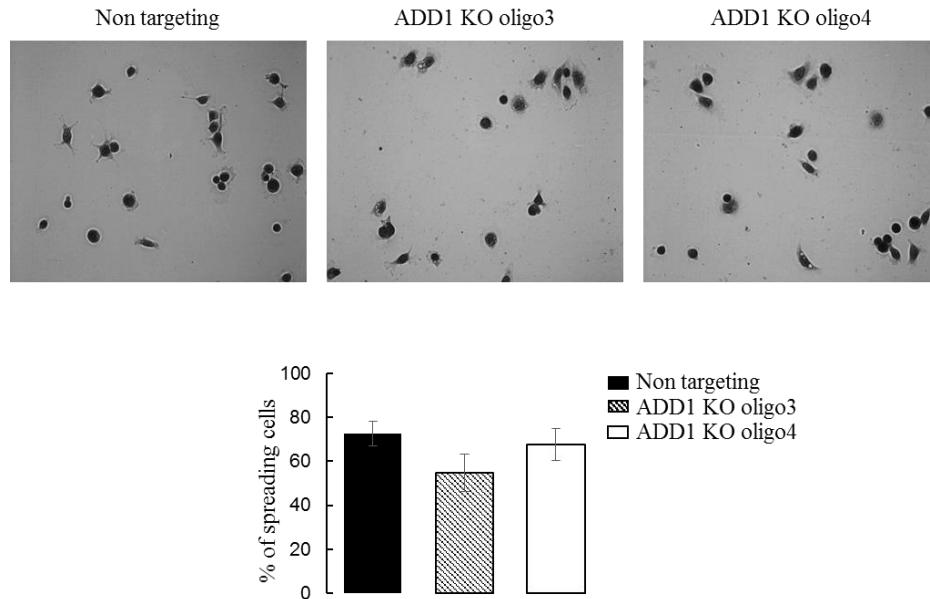
Figure 16: Depletion of ADD1 does not affect cell-matrix adhesion and spreading of 16HBE cells: Adhesion and spreading assay was performed using control and ADD1 depleted 16HBE cells as described in materials and methods. **A) B)** Depletion of ADD1 does not affect attachment and spreading properties of 16HBE cells to collagen type I extracellular matrix. Data are presented as mean ± SE (n =6).

39

A)

B)

Figure 17: Depletion of ADD3 does not affect cell-matrix adhesion and spreading of 16HBE cells: Adhesion and spreading assay was performed using control and ADD3 depleted 16HBE cells as described in materials and methods. **A) B)** Depletion of ADD3 does not affect attachment and spreading properties of 16HBE cells to collagen type I extracellular matrix. Data are presented as mean ± SE (n =6).

A)

Control   ADD1-Flag

B)

Control   ADD1-Flag

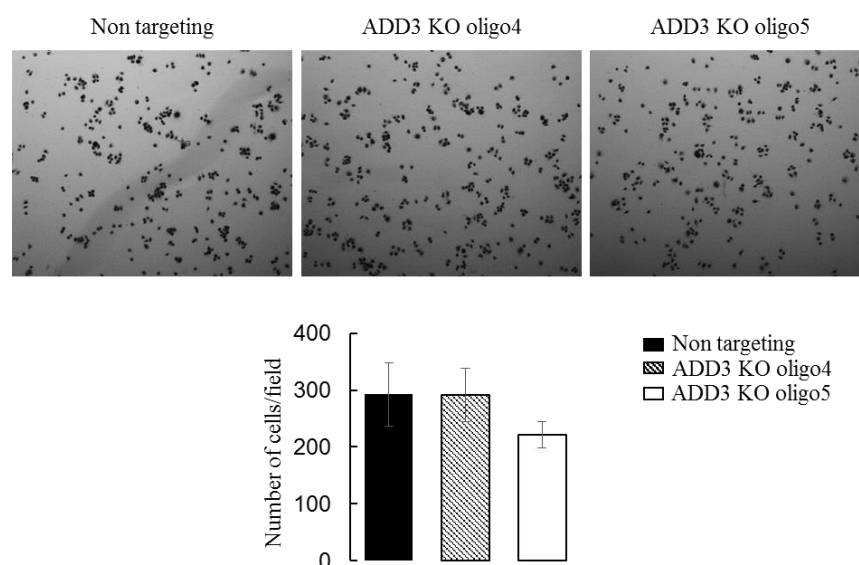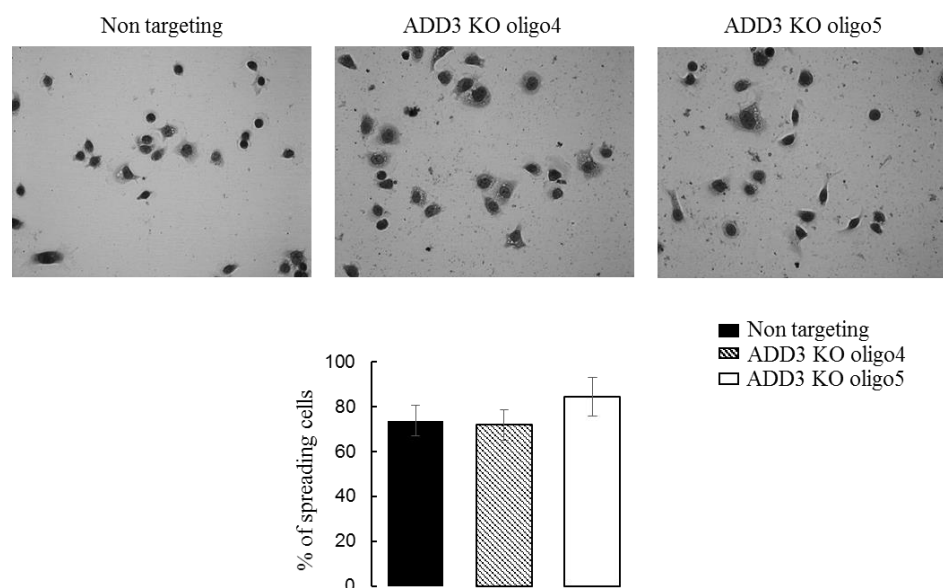**Figure 18: Overexpression of ADD1 enhances cell-matrix adhesion and spreading of 16HBE cells:** Adhesion and spreading assay was performed using control and ADD1 overexpressed H1299 cells as described in materials and methods. **A) B)** Overexpression of ADD1 increases attachment and spreading properties of 16HBE cells to collagen type I extracellular matrix. Data are presented as mean ± SE (n =6); $p < 0.05$.
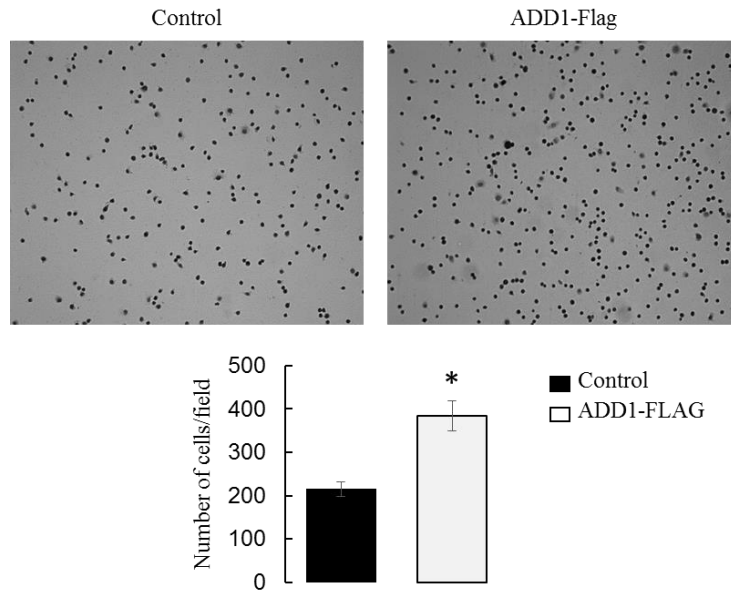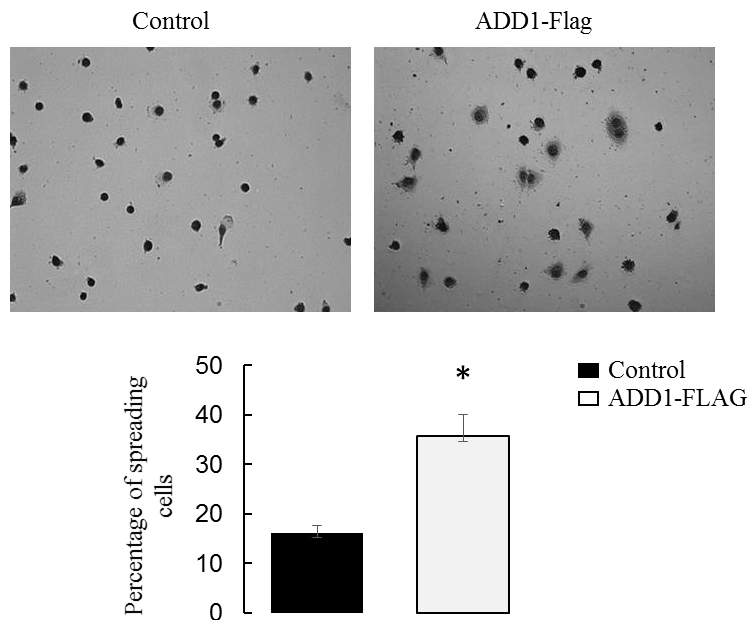
3.7 ADD1 overexpression leads to the formation of large adhesion complexes

Given our results that ADD1 overexpression increases ECM adhesion and spreading of H1299 cells (Figure 18A and 18B), we sought to dissect molecular mechanisms underlying these functional effects of ADD1 overexpression. ECM adhesions are mediated by specialized structures formed at the base of attached cells that are called 'focal adhesions' (FA). FA are composed of transmembrane receptors, integrins that mediate cell attachment to the ECM as well as the large cytoplasmic plaque that mediates integrin attachment to the associated actin cytoskeleton [45]. The cytosolic plaque of FA contains a large number of scaffolding, signaling and actin-binding proteins, such as paxillin, vinculin, talin, FAK, c-Src, etc. Phosphorylation of these signaling and scaffolding proteins serves as the reliable indicator of FA assembly.

In order to examine the effects of ADD1 overexpression and depletion on the organization of FA, we carried out immunolabeling for phosphorylated (p) paxillin. Figure 19A shows that ADD1 overexpression resulted in the assembly of relatively large p-pax-based FA that was confined to the periphery of spreading H1299 cells. By contrast, control H1299 cells possess smaller FA that is broadly distributed throughout the cell base. Surprisingly, the immunoblotting analysis revealed lower expression of total paxillin and p-paxillin, unchanged FAK phosphorylation and increased Src phosphorylation in ADD1 overexpressing H1299 as compared to control cells (Figure 19B). The observed effects are unusual since a canonical FA signaling involves a cascade of sequential phosphorylation events that starts with Src autophosphorylation and involved increased phosphorylation of FAK and paxillin [46]. The mechanisms underlying the observed effects of ADD1 overexpression on the molecular composition of FA remain to be investigated.

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We also examined the effects of ADD1 Depletion on FA morphology and composition in 16HBE cells. Figure 20A shows that loss of ADD1 did not affect the density and size of FA; it also did not alter expression and phosphorylation of several key FA proteins (Figure 20B). Overall this data is consistent with our functional study, which shows no effect of ADD1 knockout on ECM adhesion of 16 HBE cells (Figure 16A).

43
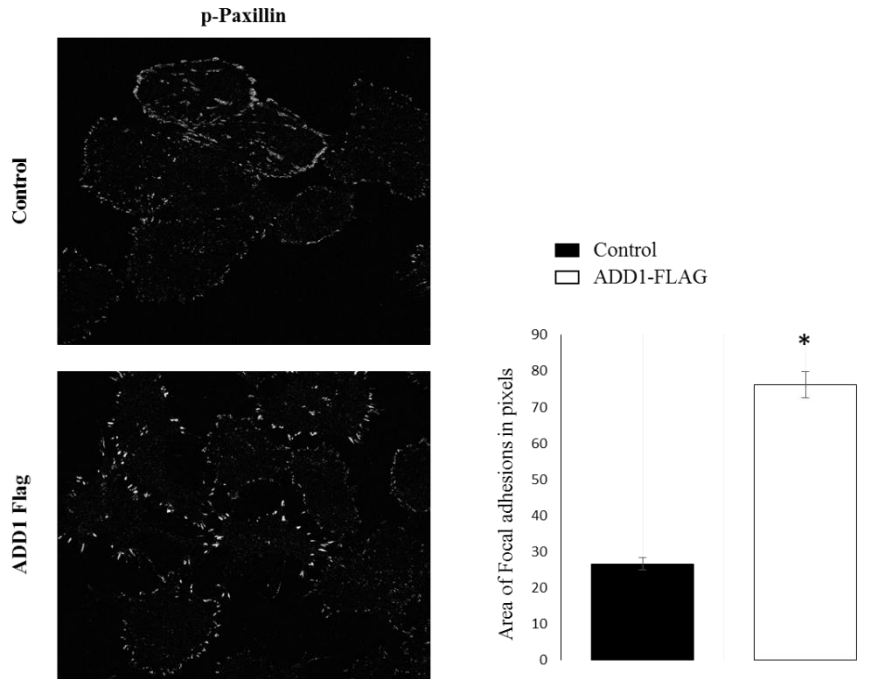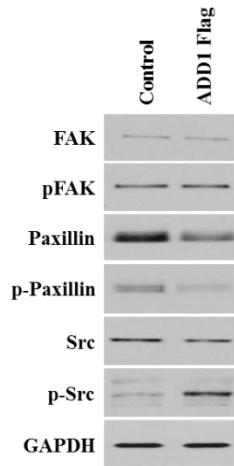
Figure 19: ADD1 overexpression leads to the formation of large focal adhesion complex: Sparse control and ADD1 overexpressed H1299 cells were immunolabeled for p-paxillin. **A)** Immunofluorescence labeling and confocal microscopy show large focal adhesion complexes in ADD1 overexpressed H1299 cells compared to control H1299 cells. The area of focal adhesions is calculated using Image J software. Data are presented as mean ± SE (n =3); p < 0.05. **B)** Immunoblotting shows the decrease in expression of focal adhesion protein paxillin and p-paxillin in ADD1 overexpressed cells as compared to control H1299 cells. Representative images of 3 independent experiments.

44

A)
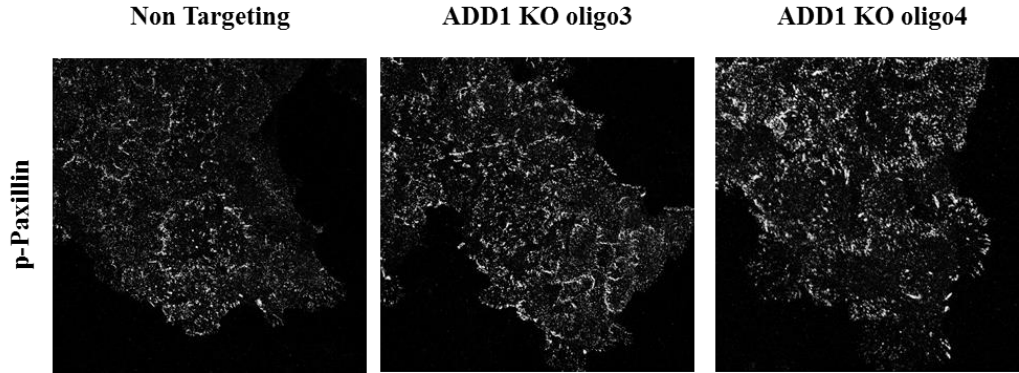
B)
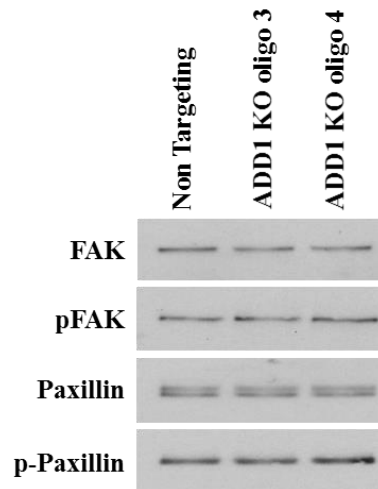
Figure 20: ADD1 depletion does not affect the formation of focal adhesion complex: Sparse control and ADD1 depleted 16HBE cells were immunolabeled for p-paxillin. **A)** Immunofluorescence labeling and confocal microscopy show no effect on the density and size of FA between control and ADD1 depleted 16HBE cells. Representative images of 2 independent experiments. **B)** Immunoblotting shows no alteration in expression and phosphorylation of key FA proteins between control and ADD1 depleted 16HBE cells. Representative images of 3 independent experiments.

3.8 ADD1 knockout and overexpression alter the organization of cortical actin cytoskeleton

Adducins are actin-binding proteins that may affect the organization of the actin cytoskeleton by two different mechanisms such as capping and bundling of actin filaments [17]. Given key roles of the actin cytoskeleton in regulating cell spreading and migration, we sought to examine how manipulation of ADD1 expression may affect the organization of actin filaments in lung epithelial and lung cancer cells. Actin filaments were visualized in fixed ADD1 depleted 16HBE cells and ADD1 overexpressing H1299 cells by using fluorescently (Alexa Fluor 555) labeled phalloidin. Control 16HBE cells demonstrated two types of F-actin rich structures at the migrating edge of small cell colonies (Figure 21A). One is small lamellipodia containing F-actin bundles that run perpendicularly to the cell edge (arrows). The other structure was prominent cortical F-actin arcs that run in parallel to the migrating cell edge. Loss of ADD1 significantly altered such F-actin organization. Specifically, it resulted in the disappearance of parallel F-actin arcs and increased the size of protruding lamellipodia (Figure 21A). We also imaged architecture of the actin cytoskeleton in the middle of migrating 16HBE cell monolayers. In control monolayers, F-actin was assembled as prominent perijunctional actin belt associated with intercellular contacts (Figure 21B). The labeling intensity of this belt was significantly lower in ADD1-depleted 16HBE cells, which indicates decreased assembly of the perijunctional actin filaments. ADD1 overexpression in H1299 cells also resulted in significant changes of the cortical actin cytoskeleton. These changes were manifested by the appearance of multiple filopodia like protrusions on the cell periphery that contained tightly-packed F-actin bundles (Figure 22A).

Finally, we attempted to detect physical interactions between ADD1 and actin filaments in 16HBE cells by using immunoprecipitation analysis. We successfully immunoprecipitated ADD1 using an ADD1-specific polyclonal antibody (Figure 23A) and were able to detect ADD3 isoform in

ADD1 pull-down. This result indicates the strong interaction between ADD1 and ADD3 in lung epithelial cells. Surprisingly, we did not detect either beta cytoplasmic actin or gamma cytoplasmic actin in ADD1 pull-down. This negative result may have at least two different explanations. One is that ADD1-actin interactions are weak and transient and are easily disruptable by detergent-based cell lysis. Another possibility is that the complexes are detergent insoluble and are lost after detergent-induced solubilization of the cell content. Further studies are required to dissect mechanisms of adducins interactions with the actin cytoskeleton in epithelial cells.

Overall, our data suggest that both knockout and overexpression of ADD1 result in significant alterations in the organization of the actin cytoskeleton in migrating lung epithelial cells and lung cancer cells. The altered organization of the actin cytoskeleton is likely to contribute to ADD1-dependent effects on cell migration. We will describe possible mechanisms and functional consequences of these cytoskeletal alterations in the Discussion chapter of this Thesis.
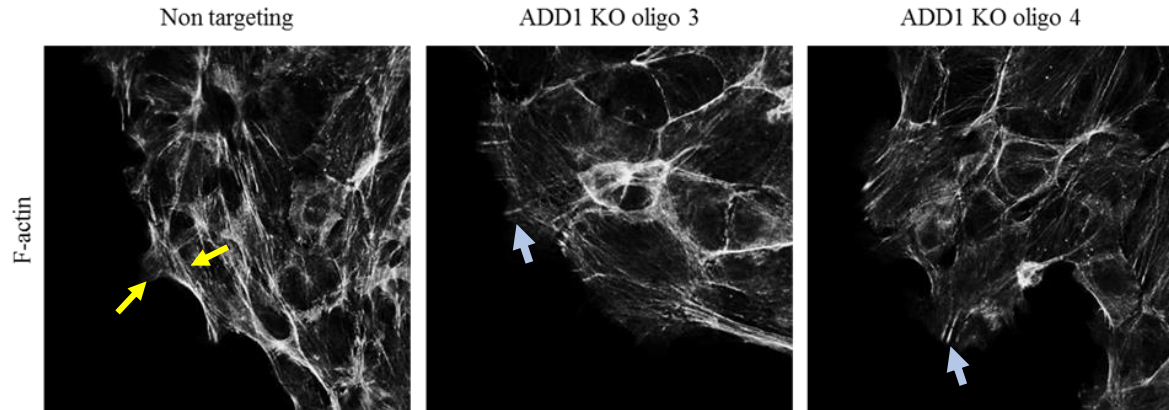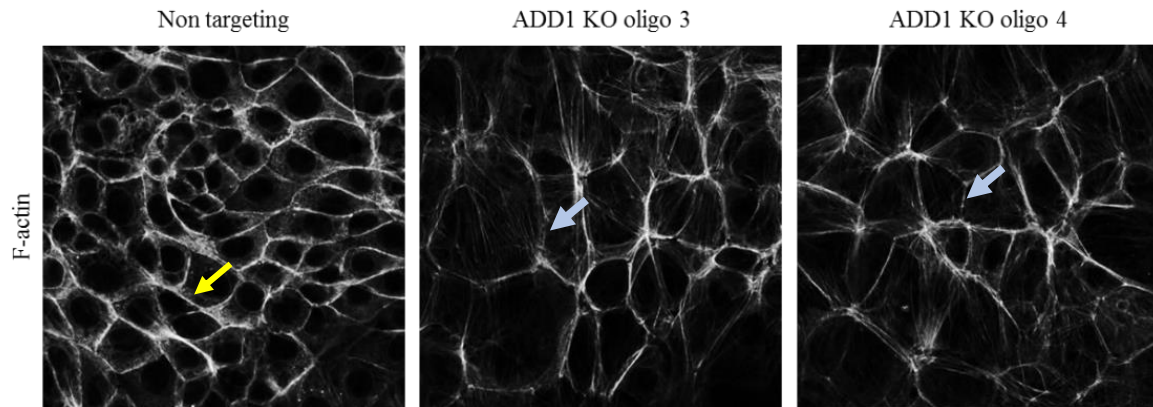
Figure 21: Depletion of ADD1 alters the organization of cortical actin cytoskeleton of 16HBE cells: Sparse and confluent control and ADD1 depleted 16HBE cells were labeled with fluorescent Alexa Fluor 555 Phalloidin. **A)** Fluorescence labeling and confocal microscopy of sparse cell colonies show the disappearance of parallel F-actin arcs and increased size of protruding lamellipodia at migrating leading edge of ADD1 depleted 16HBE cells compared to control 16HBE cells. Representative images of 2 independent experiments. **B)** Fluorescence labeling and confocal microscopy of confluent cell monolayers show decreased assembly of perijunctional actin filaments at cell-cell contact region in ADD1 depleted 16HBE cells as compared to control cells. Representative images of 3 independent experiments.

A)
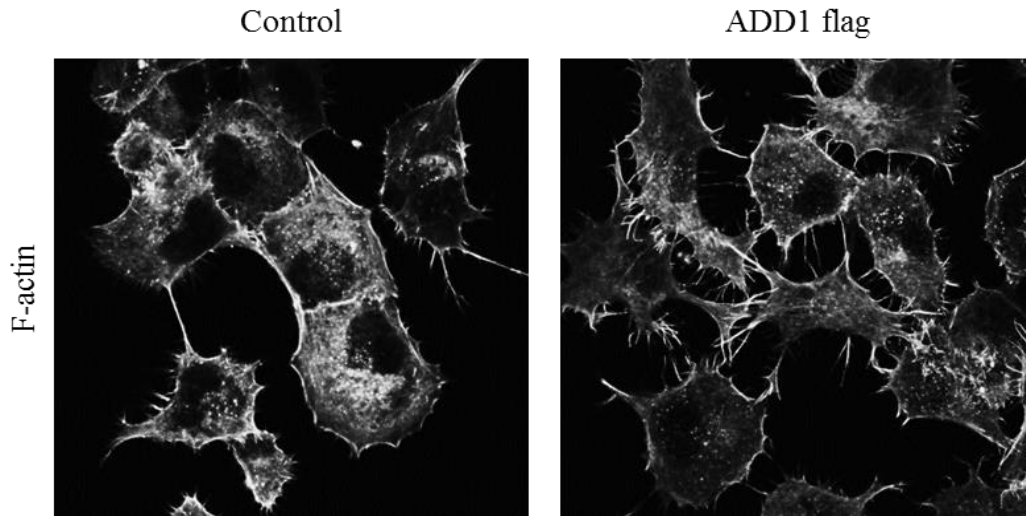
Figure 22: ADD1 overexpression also alters the organization of cortical actin cytoskeleton of H1299 cells. Sparse control and ADD1 overexpressing H1299 cells were labeled with fluorescent Alexa Fluor 555 Phalloidin. **(A)** Fluorescence labeling and confocal microscopy of sparse cell colonies show multiple filopodia like protrusions on the cell periphery in ADD1 overexpressing H1299 cells compared to control H1299 cells. Representative images of 3 independent experiments.

A)
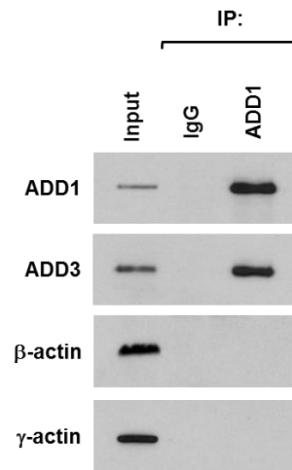
Figure 23: ADD1 interacts with ADD3 *in vivo*: Co-immunoprecipitation of ADD3, β-actin and γ-actin with ADD1 from 16HBE cell extract. Immunoprecipitation of ADD1 with anti-ADD1 polyclonal antibody from whole cell extract, then immunoprobed with an anti-ADD3, β-actin and γ-actin antibody **A)** Immunoblotting shows ADD1 interacts with ADD3, but the interaction of ADD1 with beta and gamma actin was not detected. Representative images of 3 independent experiments.

4. DISCUSSION

Adducins are important scaffold proteins that play an essential role in the organization of the cortical cytoskeleton [47]. This role is based on a dual activity of adducins that serve as F-actin capping and bundling proteins, as well as linkers between actin filaments and submembranous spectrin oligomers. Since the remodeling of the cortical cytoskeleton drives cell motility, it is important to understand what role adducins may play in this process. Surprisingly, this important question remains underexplored and previous studies yielded descriptive and controversial data regarding the effects of ADD1 on the migration of different epithelial and cancer cells [30, 31, 32, 33]. The present study is designed to determine the roles of adducins in the regulation of motility of lung epithelial cells and non-small cell lung cancer (NSCLC) cells and to dissect underlying molecular mechanisms. Our studies have identified that adducins act as negative regulators of migration and invasion by mechanisms involving modulation of the actin cytoskeleton and cell-matrix adhesions.

4.1 Downregulation and mislocalization of adducins in lung cancer cells with mesenchymal phenotype

An initial interesting finding of our study demonstrates the effects of NSCLC cell phenotype on adducin expression and localization. A recent study revealed significant phenotypic heterogeneity of NSCLC cells, which can be grouped into three broad categories: epithelial, mesenchymal and epithelial-mesenchymal hybrids [48]. The former category includes cells that preserve characteristics of well-differentiated normal pulmonary epithelium such as high E-cadherin

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expression, assembly of robust intercellular junctions and poor invasiveness. By contrast, mesenchymal-type NSCLC cells loss cell-cell contact and acquired high invasiveness [48]. We observed that ADD3 expression was significantly downregulated in NSCLC cells with a highly invasive mesenchymal phenotype (Figure 6A). While we did not find differences in ADD1 expression between epithelial and mesenchymal NSCLC cells, its localization was different in the cells with two different phenotypes. In lung epithelial cells, ADD1 was predominantly localized at the lateral plasma membrane at the areas of cell-cell contacts (Figure 7A and 7B), which is consistent with its membrane localization in other types of well-differentiated epithelial cells [18]. By contrast, a significant fraction of ADD1 was accumulated intracellularly in invasive mesenchymal lung cancer cell lines. Similar effects were observed in the pair of primary and fully-transformed HBEC lung epithelial cell lines. A non-transformed, epithelial-type HBEC3-KT cells show lateral membrane localization of ADD1 and relatively-high level of ADD3 protein, whereas fully-transformed mesenchymal HBEC3-KTRL53Myc cells were characterized by mislocalization of ADD1 and downregulation of ADD3 expression (Figure 6B, 7B and 7C). Our results are consistent with a previous study that demonstrated downregulation and mislocalization of ADD1 and ADD3 during progression from normal renal epithelium to dedifferentiated renal carcinoma [49]. While this suggestion remains to be proven, we hypothesize that epithelial to mesenchymal transition that occurs during NSCLC progression *in vivo* might be also accompanied by downregulation of ADD3 expression and mislocalization of ADD1. Since adducins are cytoskeletal scaffold proteins, their activity is limited to the sites of their localization. Hence not only downregulation of ADD3 but also mislocalization of ADD1 should result in adducin dysfunctions in advanced NSCLC with the mesenchymal phenotype. Such adducin dysfunctions may play causal role in lung cancer dissemination and metastasis.

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4.2 Adducins are the negative regulators of cell motility

The results of this study strongly suggest that adducins act as negative regulators of motility of normal lung epithelial cells and NSCLC cells. This conclusion is supported by two lines of evidence. One is a significant increase in migration of individual ADD1 and ADD3 depleted 16HBE cells (Figure 9A and 11A), and the other is the decreased collective cell migration, individual migration and Matrigel invasion of ADD1-overexpressing H1299 cells (Figure 12B, 13A and 13B). Lack of effects of ADD1 or ADD3 knockout on collective motility of 16HBE cells is intriguing and it most likely reflects compensatory/equalizing effects of two processes occurring at the migrating leading edge and inside the cell monolayer as described in a subsequent chapter of the Discussion. Our findings agree with a published study that shows inhibited invasiveness of ovarian cancer cells after ADD1 overexpression [31], but contradicts to the reported acceleration of transfilter migration of ADD1-overexpressing renal epithelial cells [30]. Since binding properties and localization of adducins is regulated by several key signaling pathways (ROCK, PKC, Src) it is possible that these cytoskeletal scaffolding proteins may differently regulate cell motility in a cell type and context-dependent fashion.

4.3 Alteration in cell-matrix adhesion and cortical cytoskeleton are the possible mechanisms responsible for effect of adducins on cell motility

In the present study, we not only characterized functional consequence of manipulation of adducins expression on migration and invasion of lung epithelial and NSCLC cells but also attempted to dissect molecular mechanisms that mediate adducin-dependent cell motility. We found that these mechanisms do not involve altered cell proliferation (Figure 14A, 14B and 15A), which suggests that previously reported role of ADD1 in spindle assembly and cell mitosis is not

a universal function of this protein [26]. However, our data suggests that the effects of adducin overexpression and knockout on cell migration and invasion involve alterations of cell-matrix adhesions and the actin cytoskeleton. The overexpression of ADD1 increases the ECM adhesion and spreading of H1299 cells (Figure 18A and 18B). Cell matrix adhesion is one of the important determinants of cell migration. The relationship between cell migration and cell adhesion is biphasic. Cell migration occurs most efficiently when cells are attached to ECM at intermediate adhesion strength, whereas too weak or too strong adhesion to ECM inhibits cell migration [50, 51]. Therefore, we believe that increased adhesiveness of ADD1-overexpressing H1299 cells significantly contributes to the decreased migration and invasion of these cells. How does ADD1 overexpression stimulate ECM adhesion of NSCLC cells? Our immunofluorescence imaging indicates that this effect is mediated by the enlargement of focal adhesions (Figure 19A), although examining the expression and activation of key FA proteins yield some paradoxical results. For example, we found dramatic activation of Src in ADD1 overexpressing cells as compared to control cells (Figure 19B). However, such Src activation did not result in activation (hyperphosphorylation) of its downstream protein effectors such as FAK and paxillin (Figure 19B). Oppositely, paxillin phosphorylation was even diminished in ADD1-overexpressing NSCLC cells most likely due to decreased total expression of this FA protein. These results indicate that ADD1 overexpression activates some non-canonical signaling events leading to the enlarged FA and increased ECM adhesion. Our findings created several unresolved questions. One question is related to the mechanisms of Src activation in ADD1-overexpressing cells. A recent study demonstrated that Src directly interacts and phosphorylates β-adducin in neutrophils [52]. It is tempting to speculate that ADD1 can also interact with Src and that such interaction stimulates Src activity. Another question is how can activated Src increase the strength of ECM adhesions,

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bypassing FAK activation? Several scenarios could be envisioned. For example, Src can stimulate FA assembly by phosphorylating other scaffolding proteins such as p130Cas [53]. Another mechanism may involve indirect effects of Src, which could modulate organization of FA by regulating the assembly of the FA-associated actin cytoskeleton. Indeed, an actin-binding protein cortactin, which plays a key role in regulating lamellipodia and invadopodia in migrating cells, is known to be a bona fide substrate of Src [54]. The notion that increased adhesiveness of ADD1-overexpressing NSCLC cells can be due to rearrangement of the actin cytoskeleton is supported by our data showing that high level of ADD1 triggers multiple F-actin rich spikes or filopodia-like protrusions on the cell surface (Figure 22A). This result is in agreement with a previous study that reports a specific assembly of cortical F-actin bundles after ADD3 overexpression in zebrafish cardiomyocytes [55]. Since adducins are able to bundle actin filaments, it is likely that high level of submembranous ADD1 promotes the assembly of cortical F-actin bundles. When such F-actin bundling occurs at the cell base, it would stabilize/enlarge FA thereby increasing cell adhesion to ECM.

However, the described adhesion-dependent mechanism appears to work only at conditions of adducin overexpression, since the loss of ADD1 and ADD3 did not affect ECM attachment of 16HBE cells (Figure 16A and 17A). Nevertheless, loss of adducins also resulted in significant rearrangements in the cortical actin cytoskeleton that were manifested by the assembly of large protruding lamellipodia on the migrating cell edge and disappearance of parallel F-actin arcs at the edges of cell colonies (Figure 21A). We believe that the enhanced formation of lamellipodia can explain the increased migration and invasion of adducin-depleted lung epithelial cells. Why does adducin knockout promote lamellipodia assembly? Adducins are known actin-capping proteins

[17]. They bind to the faster-growing barbed end of actin filaments and inhibit filament growth. Hence, loss of adducins should exert a de-capping effect on actin filaments and stimulate actin polymerization. When these events happen at the migrating cell edge, accelerated F-actin polymerization leads to lamellipodia formation and accelerated cell motility. Interestingly, the motility promoting effects of adducins knockout was observed only during transfilter migration of individual (or small clusters) lung epithelial cells, but not during collective migration of 16HBE cell monolayers. The difference between these types of cell motility is that collective migration of differentiated epithelial cells depends on the assembly of intercellular junctions and the perijunctional F-actin cytoskeleton that transduces forces and coordinates directional movement of the entire cell monolayer. Destabilization of cell-cell adhesions is known to decrease collective cell migration [56, 57]. However, previous studies demonstrated that knockdown of adducins in epithelial cell monolayers decreases the strengths of cell-cell adhesions [18, 24]. Furthermore, in the present study, we found that knockout of ADD1 decreases the intensity of F-actin bundles in the areas of cell-cell contact, thereby indicating destabilization of the perijunctional F-actin cytoskeleton (Figure 21B). Based on this data we hypothesize that knockout of adducins results in two major effects in migrating lung epithelial cell monolayers. One effect is accelerated lamellipodia assembly at the migrating wound edge that should promote cell movement. The other effect is weakening of cell-cell contacts that decrease mechanical forces responsible for pulling forward the cells positioned inside the monolayer. This effect should decrease movement of the monolayer. As a result, these two effects of adducin knockout compensate each other resulting in the lack of significant effects on collective migration of lung epithelial cells.

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5. CONCLUSION

Our study suggests that adducins are important regulators of cell motility. The gain of functions and loss of functions experiments indicates that adducins negatively regulates cell migration and invasion of normal lung epithelial cells and lung cancer cells. The mechanisms through which adducins regulates cell motility are the combinatorial effect on cell-matrix adhesion and the cortical actin cytoskeleton. Finally, we conclude that the downregulation and mislocalization of adducins may be responsible for the transition of less invasive epithelial to the more invasive mesenchymal phenotype of NSCLC cells resulting into metastasis.

6. FUTURE DIRECTIONS

In order to investigate the effect of ADD3 overexpression on cell motility, we will create stable ADD3 overexpressing H1299 cells and use this cells will perform all the mentioned assays in the thesis. We are also interested to study the effect of adducin depletion and overexpression on dynamics of the actin cytoskeleton, therefore, we will be performing G-actin incorporation assay and FRAP assay using LifeAct-GFP actin sensors. We are also interested in *in vivo* metastasis study, therefore we will be inoculating control and ADD1 and ADD3 overexpressing H1299 cells in mice by intracardiac injection and will monitor tumor growth and metastasis.

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VITA

Parth Hitenbhai Amin was born on September 2nd, 1989, in Ahmedabad, India. He received his Master of Science degree in Medical Biotechnology from Sardar Patel University, Gujarat, India in December 2012.