

IDENTIFICATION OF A ROLE FOR INTEGRIN ALPHA 5 IN
COLONIC EPITHELIAL MORPHOGENESIS

By

Alina Starchenko

Dissertation

Submitted to the Faculty of the
Graduate School of Vanderbilt University
in partial fulfilment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cell and Developmental Biology

May 2017

Nashville, TN

Approved:

Robert J. Coffey, M.D.

Matthew Tyska, Ph.D.

Ian Macara, Ph.D.

Andreis Zijlstra, Ph.D.

Roy Zent, M.D.,Ph.D.

To my father, for teaching me how to think

To my mother, for teaching me how to learn

To my sister, for reminding me that work is not everything

To JW, for showing me how to be harder than the science

Acknowledgements

I must first thank Bob Coffey for accepting me into his lab and overseeing my scientific development. The Coffey lab has been a phenomenally supportive environment for me to learn the “hows” of science. The people in the lab, past and present, have truly made this a great experience. It would take too many pages to enumerate, but I would especially like to thank Jeff, Jennie, Galina, Qin and Jim for their technical teachings and assistance. I would like to thank Will, my first bench neighbour and reagent-recipe guru, for the countless protocols and California dome stories. To Michelle, Annie, and Yang: you all taught me so much about science and maybe a little too much about the joys of having a second x chromosome. To Christine and Emily, for forcing me into a gym habit – I look forward to seeing you in Boston. Thank you also to Jarred, Greg, Daniel, Jumpei and Eliot, for the mix of technical advice and happy hour gossip. I would like to acknowledge Bhumi, who has been there to answer all questions and guide my work forward. Thank you to Yuping, who not only taught me to handle a hoard of lab mice, but introduced me to a myriad of delicious and often strange asian treats – I will miss our constant texting. I would like to thank Meghan, who makes everything in the Coffey lab run smoothly. If the Coffey lab was the world, Ramona, you would be holding it on your shoulders like Atlas. Half the projects in this lab would not exist without your ideas, and I would certainly not be where I am today without your guidance and help.

I would like to thank my committee, Matt Tyska, Ian Macara, Andreis Zijlstra and Roy Zent. I would especially like to acknowledge Andreis, for his

help in guiding the early phases of my time here, and Roy, for his initial skepticism and suggestion of the experiments that ultimately opened the door to some interesting biology and ultimately my thesis project. I would also like to thank Jim Patton and Steve Hann, for all of their advice and hard work to make these programs run smoothly. To my former mentor, Cynthia Reinhart-King: your training enabled me to do the work that I have done here, and I would not have even considered joining a PhD program without the inspiration you provided.

Outside of lab, I would like to thank my family, for climbing both metaphorical and literal mountains with me. To my Russian family, Liza and Alicia: you are terrible at visiting but you still manage to be there for me, and I am so grateful for that. To my Nashville family, Diana, Cheryl and Courtney: I could not have asked for better partners in science and crime, and I am excited to see where the future takes all of us.

Table of Contents

	Page
ACKNOWLEDGEMENTS.....	iii
LIST OF FIGURES	vi
LIST OF ABBREVIATIONS AND ACRONYMS	viii
Chapter	
I: Introduction.....	1
I.1 Cell polarity.....	3
I.2 Integrins: Overview of structure and signaling.....	15
I.3 EGFR as a model receptor tyrosine kinase	31
I.4 Colorectal cancer.....	37
I.5 Rationale for thesis work	40
II: Characterization of the interaction between EGFR and Integrin β 1 in colorectal cancer	43
II.1 Introduction.....	43
II.2 Results	44
II.3 Discussion	49
III: Clustering of Integrin α 5 at the lateral membrane restores epithelial polarity in invasive colorectal cancer cells	58
III.1 Introduction.....	58
III.2 Results	59
III.3 Discussion	70
IV: Integrin β 1 clustering dampens morphologic response of colonic epithelial cells to RTK ligands.....	98
IV.1 Introduction	98
IV.2 Results	100
IV.3 Discussion.....	109
V: Conclusions and future directions	122
V.1 Overview	122
V.2 Future directions	125
VI: Materials and Methods.....	137
REFERENCES	144

List of Figures

Figure	Page
I-1: Cell polarity is an essential feature of all cells.	2
I-2: Overview of organization and junctions in a simple columnar epithelium.	5
I-3: Schematic of polarity protein signaling that drives epithelial polarization.	9
I-4: Integrin structure determines ligand binding ability.....	17
I-5: A schematic of integrin activation and signalling.	20
I-6: Overview of EGFR family members and signaling	34
I-7: Aberrant EGFR signaling acts as a driver of malignancy.	36
I-8: Overview of colonic crypt structure and CRC progression	39
II-1: Fibrillar type-1 collagen organization changes during colorectal cancer progression.....	52
II-2: Interaction between EGFR and integrin β 1 is increased in colorectal cancer.	53
II-3: Interaction between EGFR and integrin β 1 is increased in colorectal cancer.	54
II-4: CC/SC 3D model system recapitulates in vivo observations in vitro.....	55
II-5: EGFR ligands AREG and EGF elicit different phenotypic effects on CC cells in 3D.....	56
II-6: Treatment with AREG but not EGF increases abundance of EGFR/integrin β 1 interaction in 2D.....	57
III-1: Integrin β 1-activating mAb P4G11 abrogates invasive phenotype in colorectal cancer (CRC) cells in 3D.	76
III-2: P4G11 restores apico-basolateral polarity and epithelial cell-cell junctions in 3D.....	77
III-3: Differential processing of Integrin β 1 by two Integrin β 1-activating mAbs.....	78
III-4: P4G11 clustering of Integrin β 1 is necessary for rescue of epithelial cyst architecture in SC cells grown in 3D type-1 collagen.	79
III-5: P4G11 selectively induces membrane localization of integrin α 5 β 1.....	81

III-6: Integrin $\alpha 5$ is necessary for P4G11-mediated restoration of epithelial junctions in vitro.....	83
III-7: Integrin $\alpha 5$ clustering leads to fibronectin polymerization at the lateral surface in vitro.	84
III-8: Fibronectin is necessary and sufficient to induce re-distribution of integrin $\alpha 5$ and paxillin to the lateral membrane and induce TJ formation.....	86
III-9: Model depicting the process of P4G11- and fibronectin-mediated restoration of apico-basolateral polarity.....	87
III-10: Integrin $\alpha 5$ is present at the lateral surface in the more differentiated compartment of the normal human colon.....	89
III-11: P4G11 binds human integrin $\beta 1$ and activates integrin signaling pathways.	90
III-12: Treatment of SW480 cells with P4G11 restores apico-basolateral polarity.	91
III-13: Integrin $\alpha 5$ is necessary for P4G11-mediated rescue of epithelial junctions in vitro.....	92
III-14: Blockade of integrin $\alpha 5$ and FN interaction leads to loss of epithelial organization in 2D and 3D.	94
III-15: P4G11 treatment induces fibronectin polymerization in SW480 and SC.....	96
III-16. Integrin $\alpha 5$ is present at the lateral surface in adjacent normal colon but is absent from the surface in tumor.....	97
IV-1: Treatment of CC with EGF, NRG1 and HGF leads to an invasive phenotype in in 3D.....	113
IV-2: P4G11 blocks EGF, NRG1 and HGF-mediated invasion in 3D.....	114
IV-3: P4G11 blocks EGF-induced disorganization of mitotic spindle orientation.....	115
IV-4: Treatment with P4G11 dampens EGFR phosphorylation in response to EGF in CC.	116
IV-5: Treatment with P4G11 dampens EGFR endocytosis following EGF treatment in CC.....	117
IV-6: P4G11 clustering of integrin $\beta 1$ is required for blockade of EGF-mediated invasive colony morphology.....	118
IV-7: Treatment of CC with EGF, NRG1 and HGF leads to loss of membrane integrin $\alpha 5$	119
IV-8: Treatment of SC with P4G11 <i>in vivo</i> has subtle effects on tumor size and organization.....	120
IV-9: Treatment of invasive SC with P4G11 and Cetuximab results in smaller, better organized colonies in 3D.....	121

List of Abbreviations and Acronyms

3D	3-Dimensional
AJ	Adherens junction
AREG	Amphiregulin
APC	Adenomatous polyposis coli
aPKC	Atypical protein kinase C
Ca	Calcium
CC	Cystic clone 3 of HCA-7
Cdc42	Cell division control protein 42
CNA35	Collagen-binding adhesion protein 35
CRB2	Crumbs 2
CRC	Colorectal cancer
CTX	Cetuximab
DAPI	4',6-Diamidino-2-phenylindole, dihydrochloride
DLG	Discs large
DOX	Doxycycline
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular-signal regulated kinase
FERM	Initialism: Four-point-one, ezrin, radixin, moesin
FN	Fibronectin
Grb2	Growth factor receptor-bound protein 2
HGF	Hepatocyte growth factor
ILK	Integrin-linked kinase
JAM	Junction adhesion molecule
mAb	Monoclonal antibody
Mg	Magnesium
MMC	Monomeric collagen
Mn	Manganese
NRG1	Neuregulin 1
PALS1	Protein associated with Lin-7, 1

PATJ	PALS1-associated tight junction protein
Par3	Partitioning defective protein 3
Par6	Partitioning defective protein 6
PDZ	Initialism: PSD95/Dlg1/ZO-1
PI3K	Phosphoinositide 3-kinase
PLA	Proximity ligation assay
PTB	Phosphotyrosine-binding domain
PTEN	Phosphatase and tensin homolog
Rac	Ras-related C3 botulinum toxin substrate 1
Ras	Rat sarcoma protein
RGD	Arginylglycylaspartic acid
RhoA	Ras homology gene family, member A
RTK	Receptor tyrosine kinase
sFN	superfibronectin
SC	Spiky clone 1 of HCA-7
SH2	Src homology domain 2
TJ	Tight junction
TMA	Tissue microarray
TGF α	Transforming growth factor α
ZO-1	Zonula Occludens 1

Chapter I: Introduction

My dissertation aims to further characterize the contribution of integrin signaling to epithelial polarity, particularly in the context of colorectal cancer (CRC). Therefore, this introductory chapter will provide a background discussion of several areas relevant to my work. Specifically, this chapter will cover key aspects of cell polarity, integrin biology, and receptor tyrosine kinase signaling and how these topics overlap during CRC initiation and progression.

All bodily cavities of a multicellular organism are covered in a protective layer of epithelial cells, which serve as a barrier between the specialized internal components of the organism and the external environment. This organization allows for a regulated exchange of nutrients and waste products, while keeping out noxious agents and maintaining a properly hydrated and biochemically favorable internal environment. To maintain a well-organized monolayer, an individual epithelial cell forms highly regulated contacts with its neighbors and its underlying substrate. In cancer, the signaling networks that normally keep cells organized are deregulated, resulting in cells that gain the ability to metastasize and establish in distant areas of the body. The damage these metastatic cells inflict is at the root of cancer-related deaths. In my thesis work, I have joined the ranks of scientists trying to understand the processes that govern epithelial organization with the hope that these processes may eventually be harnessed to restore epithelial behavior in cancer cells and improve our ability to treat metastatic disease.

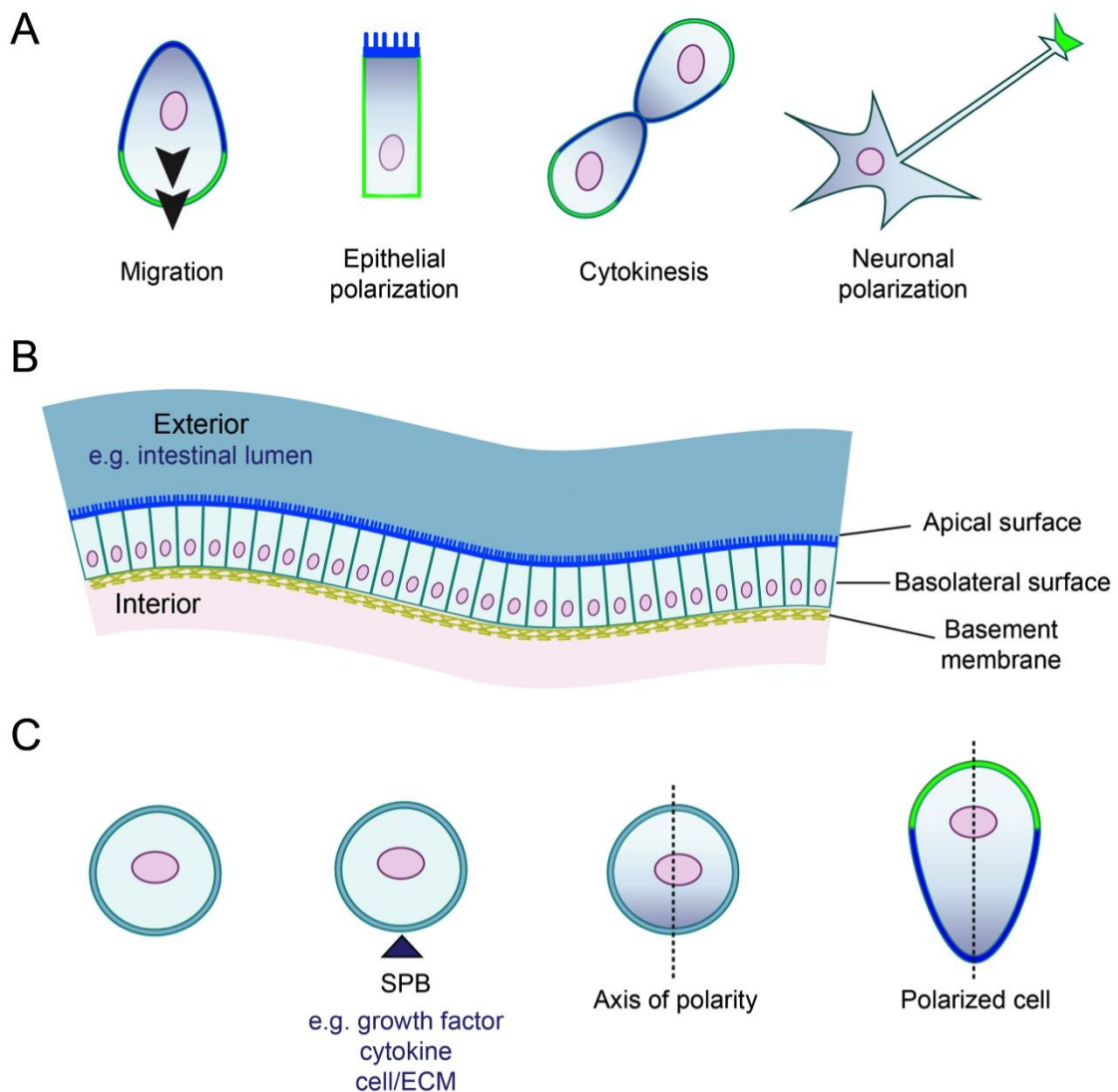


Figure I-1: Cell polarity is an essential feature of all cells.

(A) The ability to polarize allows for multiple cell functions including cell migration (chemotaxis), directional transport (epithelial polarity), cell division (cytokinesis) and neuronal polarity. (B) Epithelial cells line the interface between the interior and exterior milieus in an organism. The ability to polarize allows epithelial cells to directionally transport nutrients and waste in and out of the organism. (C) Polarity is established through an initial symmetry breaking process (SBP) that establishes a pole and an axis of polarity. The polarity machinery then uses this axis to define distinct cellular compartments. In epithelial cells these would be “apical” and “basolateral”

I.1 Cell polarity

Cell polarity is an essential feature of all cells, and is especially important for epithelial organization (Figure I-1A). The ability to polarize may have evolved to ensure proper inheritance during cell division and is an ancient property, conserved throughout the evolutionary tree [1]. Cell polarization allows a cell to orient itself relative to its neighbors, making this process necessary for the existence of multicellular organisms [2]. Of the different types of polarity, front-back cell polarity and apico-basolateral (epithelial) cell polarity (Figure I-1B) are most relevant in the context of tumorigenesis. Front-back cell polarity is used by migrating cells and establishes a front and a back of a cell, allowing for directional movement. Epithelial cells use mostly the same machinery (Figure I-1C) to establish an apico-basolateral polarity that allows them to differentiate between the interior and exterior sides of the organism and function as a semi-permeable barrier.

It has been speculated that apico-basolateral polarity functions as a tumor suppressor process [3]. Strong junctions that connect epithelial cells to one another and the underlying extracellular matrix (ECM) organize the epithelium and prevent tissue disorganization. Loss of organized epithelial junctions leads to loss of apico-basolateral polarity and paves the way for hyperproliferation, tissue disorganization and invasion in cancer.

Cells in epithelial tissues are bound together through specialized cell-cell and cell-matrix adhesions that allow for mechanical and biochemical communication. These junctions allow the cells comprising the tissue to function as a unit, which is required for epithelial integrity and, in turn, the maintenance of homeostasis in the interior compartment of the organism.

Epithelial junctions are characterized based on their function (Figure I-2). Anchoring junctions, including both cell-cell and cell-matrix adhesions, are responsible for the mechanical maintenance of the monolayer. These junctions anchor the cytoskeleton to the cell membrane, and allow cells to respond to mechanical cues sent by their neighbors and substrate. Occluding junctions, which seal gaps between individual cells, allow the epithelial sheet to function as a semi-permeable barrier. Gap junctions, which will not be discussed here, form pores between adjacent cells and allow for cell-cell communication through direct diffusion of certain solutes from the cytoplasm of one cell to another.

Intracellular adhering junctions provide monolayer integrity in response to the vast majority of forces encountered by epithelial cells [4-6]. Because many epithelia are only one cell thick, lateral cytoskeleton connections bear most of the tissue stresses and allow them to be distributed throughout the monolayer. The primary anchors of the cytoskeleton to the lateral cell membrane are adherens junctions (AJ), which anchor the actin cytoskeleton, and desmosomal junctions (DJ), which serve as anchorage sites for intermediate filaments. Specialized transmembrane adhesion proteins form the physical connections between the interior cytoskeletons of adjacent cells (Figure I-2). The cadherin superfamily is the major family of proteins that mediates epithelial cell-cell attachment. Members of this family participate in both adherens and desmosomal junctions. There are more than 180 members in the cadherin superfamily [7]. Arguably the most important of these, in the context of epithelial cell polarity, is E-cadherin.

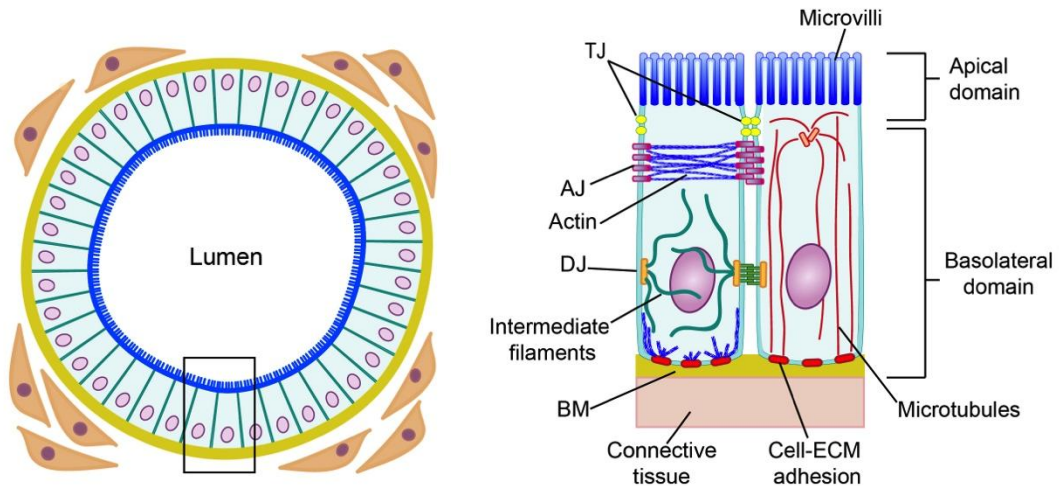


Figure 1-2: Overview of organization and junctions in a simple columnar epithelium.

Epithelial cells line the interior surface of many organs, which can be simplified into a tubular structure. The apical (blue) side of the cells faces the lumen while the basolateral side faces the interior of the organism and physically adheres to a basement membrane (BM) structure through integrin adhesions. In the colon, epithelial cells form apical microvilli to increase the absorptive surface area and for mechanical sensing. The apical and basolateral membrane domains are separated by tight junctions (TJ), which sit directly above adherens junctions (AJ) and anchor the cortical actin belt. Also in the basolateral domain are desmosomal junctions (DJ) that anchor the intermediate filaments and specialized adhesion sites for microtubules. Together, these junctions allow the monolayer of epithelial cells to function as a semi-permeable barrier.

Adherens junctions are composed largely of E-cadherin, which mediates cell-cell adhesion through calcium-dependent homophilic interactions with neighboring cells [8]. This protein has a small cytoplasmic domain, which serves as the assembly point for an actin-anchoring complex, and a large extracellular domain, which functions to bind the extracellular domain of another E-cadherin molecule, present on the surface of a neighboring cell. Many thousands of these interactions fasten together the membranes of adjacent cells in the epithelial monolayer in velcro-like fashion. In the canonical epithelial-to-mesenchymal (EMT) transition model, transcriptional silencing of the E-Cadherin gene is a key step of cancer progression [9, 10]. Although it is unclear that EMT occurs in human disease, loss of surface E-cadherin results in loss of cell-cell cohesion, which leads to loss of contact inhibition and over-proliferation. In human disease, many different mechanisms have been observed that all result in disassembly of membrane adherens junctions, suggesting that this is favorable for disease progression. Together, these observations show that cell-cell adhesion is an important mediator of epithelial tissue integrity and homeostasis.

Unlike the adhesive cell-cell junctions, occluding junctions, also called Tight Junctions (TJ) in vertebrates, support function as well as mechanical integrity of epithelia [11]. Tight junctions form a seal between cells and allow epithelial cells to form a uniform layer such that all diffusion must occur, in a regulated fashion, through the epithelium. Intestinal epithelial cells are a prime example of the importance of TJ. The major function of intestinal epithelial cells is to take up nutrients from the intestinal lumen. The cells then transport these nutrients through to their basolateral surface and secrete them into the

basolateral extracellular compartment where they diffuse into the bloodstream. For this to be efficient, these nutrients have to be prevented from leaking back out into the intestinal lumen. The TJ structures fulfil this role.

Structurally, TJs consist of rows of protein-protein contacts between adjacent cell membranes [12]. Core components of this structure include transmembrane barrier proteins, scaffolding proteins and cytoskeletal-linking proteins. The barrier function of the TJ complex is performed by homotypic interactions between the claudin, occludin and JAM families of transmembrane proteins. The cytoplasmic side of these proteins interacts with at least 40 different scaffold proteins including Zonula Occludens-1 (ZO-1), which I have used as a marker of TJ formation throughout this work. ZO-1 is a PDZ and SH3 domain protein that is part of the scaffolding complex between the transmembrane proteins and the actin cytoskeleton [11]. ZO-1 can directly bind actin, as well as other actin-binding proteins. How all of these proteins interact in the formation and maintenance of TJs remains to be determined. Importantly, the TJ structure physically prevents mixing of apical and basolateral domain components and, because of this, is required for the maintenance of polarity. Loss and translocation of TJ proteins has been reported in some cancers [13].

Interactions between epithelial cells and their surrounding ECM control signaling, differentiation and morphogenesis [14, 15]. Briefly, the integrin protein family is the major group of receptors that govern epithelial attachment to the extracellular matrix [16]. Due to their importance in the context of this work, the integrin family of proteins will be discussed in detail in

a subsequent section. Unlike their counterparts in intracellular adhesions, integrins form clusters on the cell surface that interact not with other integrins but with the surrounding structural ECM. In the context of epithelial cells, this would be the basement membrane. The adhesion structures formed by integrins also anchor the cytoskeleton, including both actin and microtubules and their associated binding proteins.

The adhesions in mature epithelia described above maintain monolayer organization and allow the tissue to perform as a unit. The formation of an epithelial tissue is complex, and is influenced by extracellular signaling from the microenvironment. Once formed, a polarized epithelial monolayer protects against cancer-driving processes [3]. Many of the molecular players in cancer also participate in epithelial polarity [3]. Although evidence that the polarity machinery is directly involved in cancer is just beginning to come to light, the removal of cell structures that stabilize an organized epithelium clearly facilitates the proliferative and invasive cellular processes that characterize cancer.

Crosstalk between epithelial junctions and polarity machinery governs epithelial polarization. Formation of a monolayer with apico-basolateral polarity depends on two interconnected mechanisms: one that allows cells to become individually polarized, and another that allows cells to orient their polarity axis in relation to surrounding cells and the basement membrane. Cell-cell and cell-ECM adhesion signaling is required for both processes, but it is not fully established how both pieces fit into the mechanism.

Three groups of evolutionarily conserved proteins mediate the establishment of individual cell polarity: Crumbs, the PAR system and the

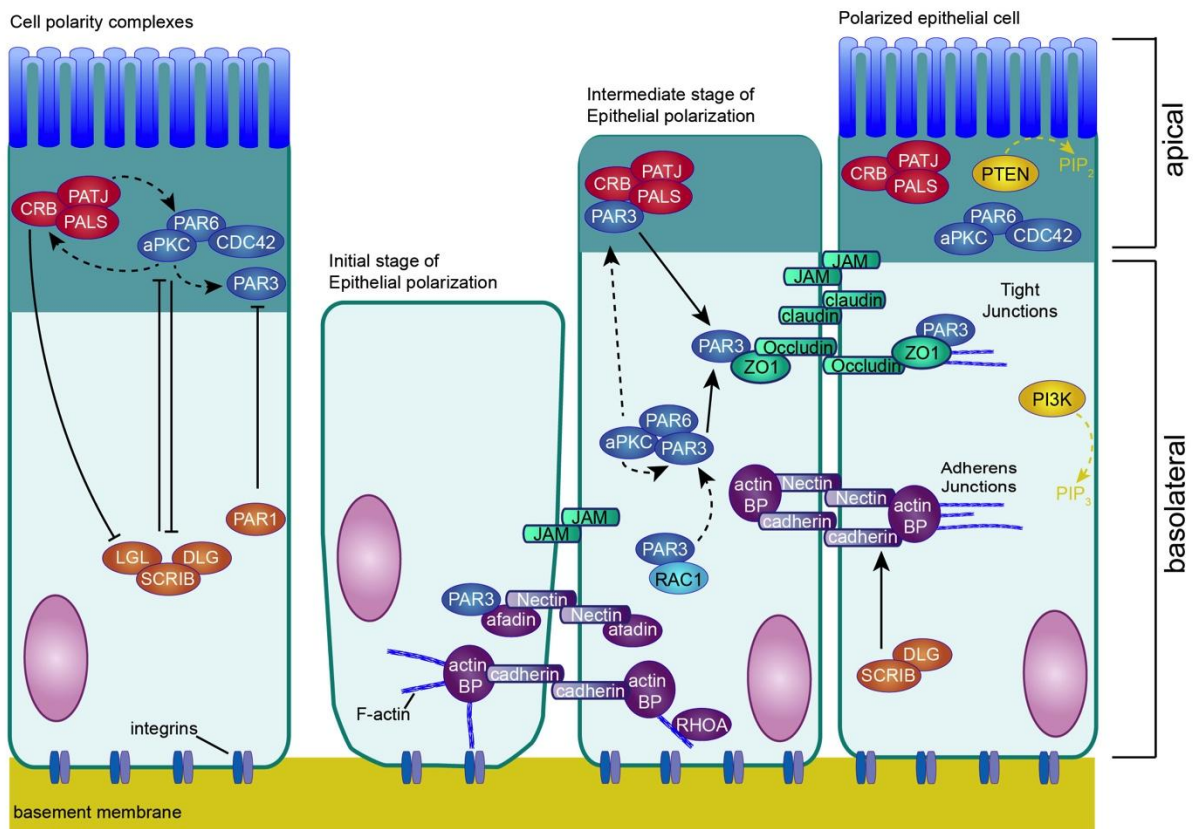


Figure I-3: Schematic of polarity protein signaling that drives epithelial polarization.

Mutually antagonistic signaling between three polarity complexes (crumbs, shown in red; scribble, shown in orange; PAR complex, shown in blue) establishes non-overlapping apical and basolateral membrane domains. These complexes participate in recruitment of the cell-cell adhesion molecules that stabilize and maintain polarity. Dashed lines indicate translocation to a different cellular compartment. Adapted from Martin-Belmonte *et al.*, 2012.

Scribble module (figure I-3). These are joined by the Rho family GTPases RhoA, Rac1 and Cdc42. Feedback loops of regulation between these families lead to segregation of these proteins to specific regions of the apical or basolateral domains [9, 17]. The Crumbs complex consists of the Crumbs, PALS1 and PATJ proteins and defines the apical domain. CRB3 localizes to the apical domain at the tight junction (TJ) region and recruits the PALS1 protein. PALS1 binds to the PDZ domain of CRB3 and recruits PATJ, a protein containing multiple PDZ domains that, in turn, interacts with Claudins and Zona Occludens (ZO) protein family members which make up the TJ. Cdc42/Par6 and aPKC form a functional unit and localize to the apical surface. The Scribble module, comprised of Scrib, Dlg and Lgl proteins, defines the lateral surfaces of the cell. Mutually antagonistic interactions and phosphorylation events between members of these three complexes are important for the formation of non-overlapping membrane domains, and are summarized in figure I-3 [17-19].

The origin of the initial polarization cue remains poorly understood. E-cadherin mediated cell-cell contact initiates recruitment of polarity proteins, leading to an early apical-basal asymmetry that is reinforced by expansion of the E-cadherin junction, which zips upwards as a discrete lateral surface is formed [20]. These early cell-cell adhesions begin as actin-rich membrane protrusions between neighbouring cells [21]. The initial contact is made by the nectin family of proteins, which then recruit E-cadherin and JAM-A to the site to form an adherens junction [17]. These, in turn, recruit claudins to their apical side to form tight junctions. Par3 is present at the early nectin adhesions, where it is necessary to form both adherens and tight junctions.

Scrib and Dlg, members of the Scribble complex, are recruited to the basolateral adherens junctions by E-cadherin. PALS1 may also help with the recruitment of E-cadherin to the region of cell-cell contact. aPKC is likely involved in maintenance of E-cadherin at the cell surface. Together, these observations suggest that early cell-cell contacts induce the recruitment of polarity proteins, which, in turn, promote the delivery of further adherens junction components, thus reinforcing adhesion and promoting AJ expansion [21].

Localized activation of Rho family GTPases control both front-back and apico-basolateral cell polarity [22, 23]. In the later case, cooperative signaling between the polarity proteins and the Rho GTPases regulates the formation of adherens junctions. In early cell-cell contacts, Cdc42 and Rac1 are activated and in turn activate the Par6/aPKC complex. Active aPKC is required for the formation of tight junctions. There is also increasing evidence that the PAR family proteins regulate a number of GTP exchange factors that modulate activities of both Rac and Cdc42 [24]. These observations provide insight into a role for the PAR family proteins in regulating actin dynamics in polarizing cells.

The specialized functions of the apical and basolateral domains rely on distinct lipid and protein compositions. Phosphatidylinositol phosphates are important determinants of cell polarity. Studies of epithelial cysts show enrichment of PIP_2 at the apical membrane and PIP_3 at the basolateral surface [9]. Insertion of PIP_2 into the basolateral membrane leads to re-localization of apical components into the basolateral surface. PIP_2 is generated by the lipid phosphatase PTEN, which also localizes apically during

the polarization process. The mechanism leading to phosphoinositide asymmetry is not well understood, but is thought to develop due to PTEN and PI3K recruitment and activity at apical and basolateral domains, respectively. Aberrant activation of PI3K, which is a downstream target of both growth factor and adhesion signaling, can contribute to loss of cell polarity in cancer; mutation of PTEN is also encountered in microsatellite unstable forms of CRC. Together, these observations identify membrane lipid composition and lipid phosphatase activities as important mediators of polarity.

Formation of a polarizing epithelial monolayer depends on cell-ECM interactions. The ability of a single cell to establish apico-basolateral polarity as described above is not sufficient to generate a well-formed epithelial tissue. To form a well-organized epithelium, each cell must orient its polarity along an axis that is appropriate for the tissue structure. This axis is defined by the cells' adhesions to the underlying ECM [15]. Mutations in proteins that mediate cell adhesion either to the ECM or to neighboring cells result in impaired epithelial morphogenesis, characterized by loss of an apical surface or the inability to generate a central lumen. In fact, engagement of an ECM ligand in the basement membrane is considered the initial step in epithelial polarization.

Adhesion signaling in response to the ECM is required for epithelial polarization *in vivo* and in 3D [15, 22, 25]. The ECM is a network of extracellular, crosslinked glycoproteins that provides structural support to a mass of cells. This matrix is dynamic, and is constantly remodelled by attached cells, even during homeostasis. The basal surface of an epithelial monolayer sits atop an ECM structure known as the basement membrane.

The basement membrane is secreted by epithelial cells and consists primarily of Collagen IV, laminins, nidogen and proteoglycans [26].

Cells interact with their surrounding ECM through a plethora of specialized adhesion receptors, which will be discussed in depth in the subsequent section. Briefly, the major ECM receptors on epithelial cells belong to the integrin family of proteins. Integrins are transmembrane proteins that bind ECM ligands with their extracellular portion and relay the resulting mechanical signals through assembly of a complex on their cytoplasmic domains.

Integrin signaling orients the location of the apical and basolateral surfaces in a layer of epithelial cells. Engagement of integrin $\beta 1$, an abundant integrin subunit in epithelial cells, with collagen ligand activates Rac1 and induces the formation of a laminin basement membrane. Failure to form this membrane, or the blockade of integrin $\beta 1$ signaling, results in the inversion of the cyst with the apical surface facing the ECM [14, 27]. These studies suggest that integrin-mediated interaction with the ECM is a general mechanism for the orientation of epithelial cell polarization.

It has been shown that adhesion to the basement membrane ECM induces strengthening of cell-cell contacts. A potential mediator of the proposed crosstalk between cell-cell and cell-ECM adhesions is the Ras family GTPase Rap1 [15]. Indeed, expression of a constitutively active Rap1 in MDCKII cells is able to rescue the polarity inversion phenotype in response to integrin $\beta 1$ blockade, even though it is not able to rescue the formation of a laminin basement membrane. Together, these data suggest that cell-ECM

signaling through integrin receptors is an early, if not the initial, polarizing cue in the apico-basolateral polarization process.

How does integrin signaling orient the apical surface? There have been many studies to examine whether integrins directly interact with the proteins that establish polarity and the temporal dynamics of when these interactions occur. It was shown that in 3D, inhibition of integrin β 1 signaling disrupted the interaction of the PAR3 with PAR6/aPKC leading to its mislocalization to the cytoplasm, thus abrogating its ability to define the apical boundary [15]. This likely occurs through integrin effects on RhoA GTPase, which restricts PAR1 to the basal surface of the cells and blocks its polarization-blocking phosphorylation of PAR3. Interestingly, these effects are tissue-dependent and, in some epithelial tissues, Integrin Linked Kinase (ILK), rather than the Rac/Rho axis determines the integrins' ability to influence polarity [28]. It has been speculated that the identity of the ECM underlying epithelia determines the downstream mediators of polarity through interactions with specific integrin heterodimers.

Beyond their role in polarity establishment, integrins also contribute to maintenance of polarity. Once established, properly oriented microtubules are necessary to maintain delivery of proteins to the appropriate membrane domain. Integrins participate in anchorage of the microtubule cytoskeleton to both matrix and cell-cell adhesions through interactions with ILK [28, 29]. In summary, formation of epithelial junctions is necessary for the formation of polarity. Continued maintenance of these junctions maintains the polarity machinery in place and thus allows for stable apico-basolateral polarity. However, this system is metastable. Thus, changes in adhesion dynamics can

trigger re-organization of the intracellular components and loss of polarity. How and whether integrin cell surface dynamics alter cell-cell adhesions to initiate, maintain, or lose apico-basolateral polarity remains unknown.

I.2 Integrins: Overview of structure and signaling

Extracellular matrix is produced, remodelled and degraded by cells. The matrix itself feeds back and exerts powerful regulatory effects on the cells. This cycle of inside-out and outside-in signaling is mediated by transmembrane ECM adhesion receptors. These receptors serve as a mechanical anchor between a cell and its surrounding microenvironment, but their ability to regulate cell behavior extends significantly beyond passive physical attachment. There are several families of proteins that have the ability to bind to and signal via ECM. The predominant regulators of cell/ECM interactions are the integrins.

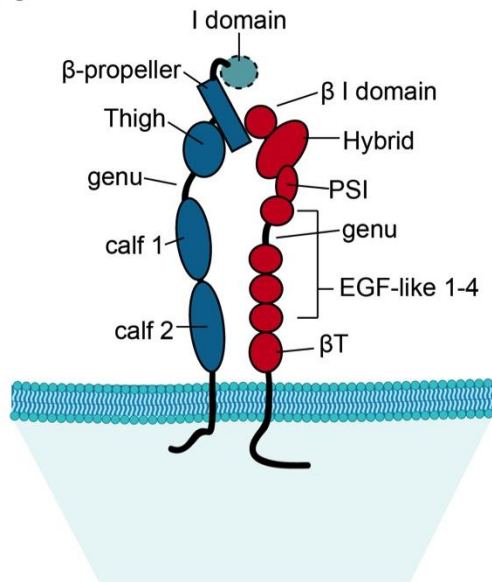
Integrins play an essential role in many physiological processes, including tissue morphogenesis, wound healing, and the regulation of cell growth and proliferation [30]. They are also heavily implicated in numerous pathologies such as thrombosis, cancer progression and metastasis [31, 32]. For many years, integrins were seen as bystanders of these processes. The ability of integrins, through their reciprocal interactions with the ECM, to drive aspects of disease is becoming increasingly appreciated. This is evident in the slow but steady increase in anti-integrin therapies in clinical use [33].

A growing body of literature suggests that within the well-understood types and roles of integrins lie subpopulations and regulatory mechanisms that, although important to cell behavior, are incompletely explored [34]. To

begin to understand how integrins contribute to such a plethora of processes, it is necessary to understand the basic aspects of how these molecules are structured, how their structure allows for force and signal transduction, and how these events are dynamically regulated to provide a constant flow of information about the external physical environment to the interior of a cell.

The ability of integrins to signal bi-directionally arises from their structure. An integrin receptor exists as non-covalently bound heterodimer consisting of an alpha- (α) and beta- (β) subunit. There are 18 α - and 8 β - known integrin subunits that combine to form at least 24 different heterodimers [16]. A single heterodimer has a large extracellular domain that binds to the ECM, a single membrane spanning region and a fairly short cytoplasmic tail (Figure I-4A). The extracellular domain of the α - and β - subunits is the largest portion of the protein and consists of several subdomains that are organized into a ligand binding N-terminal head domain that stands on a single-membrane spanning leg. The overall organization is shown in figure I-4. The α - extracellular domain consists of 4-5 subdomains, known as the β -propeller, a thigh and two calf domains. Nine of the α subunits have an additional I domain (figure I-4A, dotted line). The β -propeller contains the domains that bind metal ions, necessary to bind ligand. The structure of the β subunit consists of seven domains with flexible interconnections (figure I-4A). The binding of ligand takes place along the β -propeller/ β -I domain interface of the α and β subunits. Ligand binding is dependent on the presence of Mg^{++} and Ca^{2+} cations [35]. The transmembrane region of the integrin receptor regulates conformational changes in the receptor thereby controlling its activity. Association of the α and β transmembrane domains

A



B

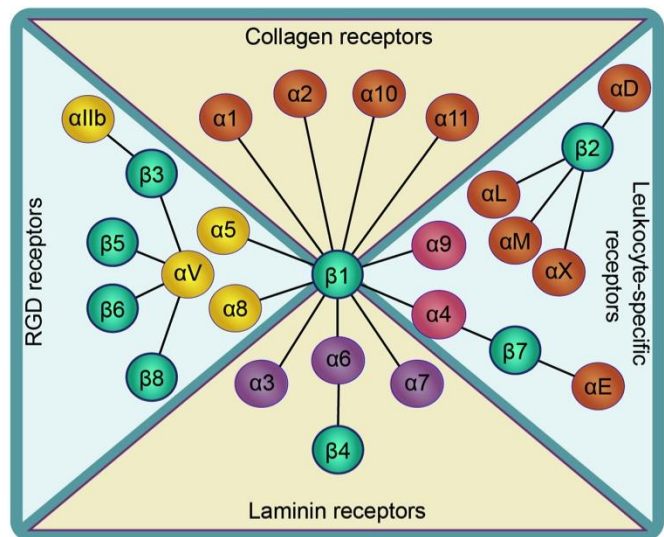


Figure I-4: Integrin structure determines ligand binding ability.

Integrins consist of an α - and β - subunit (blue and red, respectively) that heterodimerize to form an integrin receptor. The collagen binding α integrins possess an extra domain, the I domain, that is missing from the other α subunits. The same α and β subunits can heterodimerize with multiple partners to form a receptor with unique ligand binding ability.

results in an inactive receptor conformation [36] although this may not be the case in all integrins [37]. The cytoplasmic tail of the integrin serves as a platform for cytoplasmic adaptors, signaling proteins and cytoskeletal elements. Whether the tails of the integrin subunits interact with one another remains under debate, and NMR studies suggest a flexible integrin tail that becomes structured upon binding of cytoplasmic proteins like Talin [38]. Conformational re-arrangements in these regions define the function and activity of these receptors and allow a cell to respond to biochemical as well as mechanical ECM cues.

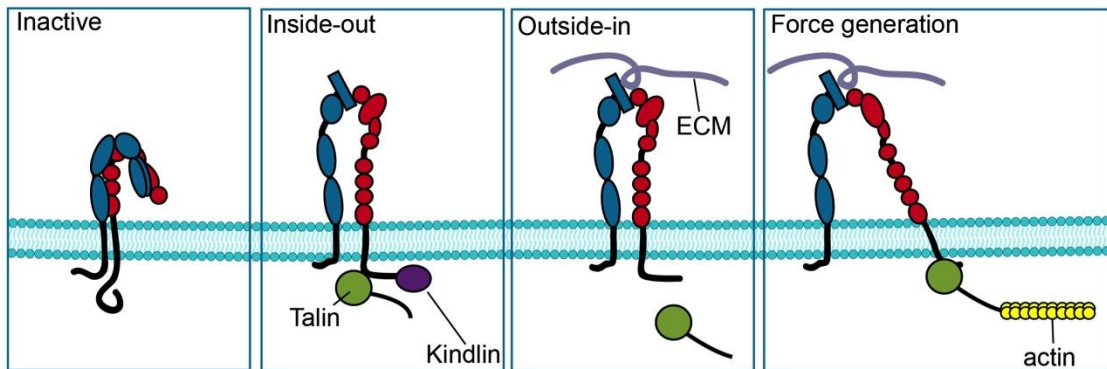
Each of the 24 heterodimeric integrin receptors has a specific, non-redundant function. This is illustrated partly by the specificity of each receptor to a particular ECM ligand (Figure I-4B), and partly by the phenotypes observed in knockout mice. Knockout mice have been generated for all the β and most of the α subunits. Notably, each phenotype is distinct and suggests a different role played by each integrin [39]. Clearly, the integrin family of proteins plays an important and multifaceted role in many biological processes. The manner in which they do this stems from differences in their ligand binding specificities.

A common feature of most integrin receptors is their ability to bind a number of different ligands (figure I-4B). Many ECM ligands also possess the ability to bind to multiple integrin receptors [40]. From a molecular standpoint, this would suggest an evolutionary selection of common peptide motifs in ECM proteins that mediate integrin binding to the cation-pocket. The combinations of integrin-ECM partners can be placed into four classes. All of the α_v integrins, α_5 and α_8 containing β_1 integrins and $\alpha_{IIb}\beta_3$ recognize an

RGD peptide sequence. Interestingly, the $\alpha 5/\beta 1$ integrin contains a binding site for a synergy site in FN that binds to a site in the $\alpha 5$ beta-propeller. Integrins $\alpha 4\beta 1$, $\alpha 4\beta 7$, $\alpha 9\beta 1$, the $\beta 2$ subfamily and $\alpha e\beta 7$ all recognize an acidic LDV domain which is functionally related to RGD. The four alpha subunits that pair with $\beta 1$ and contain an additional alpha-I domain, $\alpha 1$, $\alpha 2$, $\alpha 10$ and $\alpha 11$ all bind the laminin/collagen subfamily. The remaining alpha subunits, $\alpha 3$, $\alpha 6$ and $\alpha 7$, are highly selective for laminin [41].

Integrin binding to ECM is accompanied by large conformational changes. Whether all integrins undergo large conformational changes is controversial. Those that do are thought to exist in two conformations: bent, and extended, although structural cryoEM work supports the existence of a number of transitional states [42]. The largest conformational shift occurs in the integrin extracellular domain. This region undergoes a large structural rearrangement that puts the integrin into a state with high affinity for ECM ligand, and is illustrated in figure I-5A. Under baseline levels, most integrins on the surface of a cell are thought to be in an inactive, bent, conformation. This has been confirmed through the use of conformation-specific antibodies whose epitopes lie in regions that are only exposed in certain conformational states [42, 43]. Using these antibodies to track integrin conformation after the addition of a ligand, or to analyze integrins in adhesions, suggests that integrins bound to ligand are in the extended, active conformation. The extracellular domains are not the only part of the integrin that undergoes conformational changes. When the integrin binds an ECM ligand and is stabilized in the extended state, the membrane and cytoplasmic domains separate, exposing binding sites necessary for adhesion mediated, outside-in,

A



B

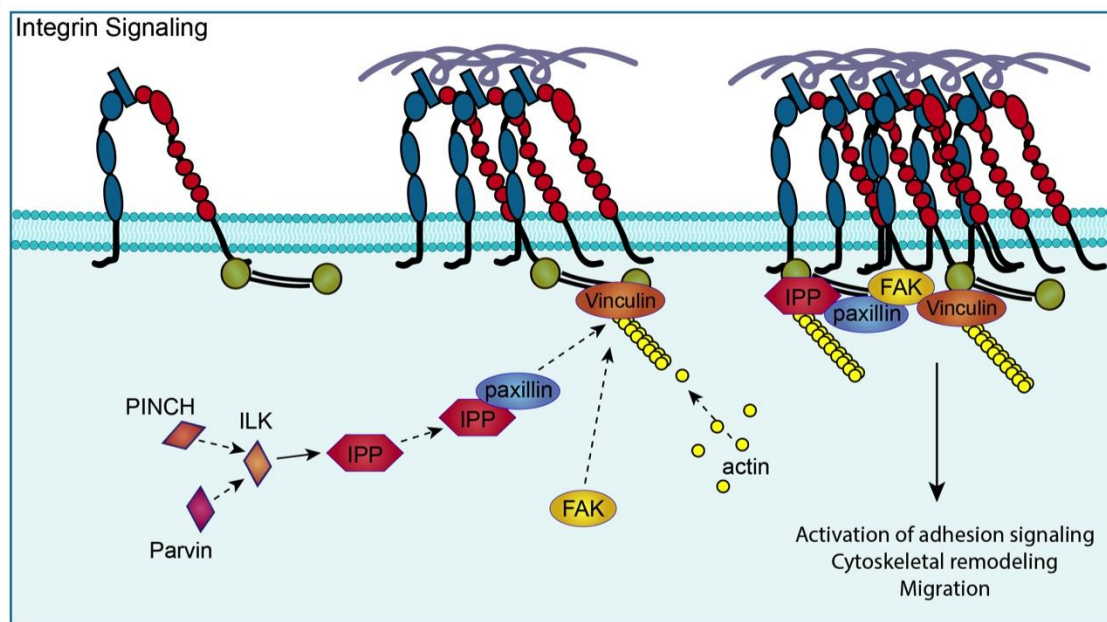


Figure I-5: A schematic of integrin activation and signalling.

(A) Integrins exist in several conformational states. They can be forced into a ligand-binding extended state through binding of talin to their cytoplasmic tail (inside-out) and are also thought to be activated into an extended state through binding of ligand (outside-in) although this is more controversial. Upon binding of both ligand and c-terminal actin binding proteins, the integrin is engaged by actin and placed under tension. (B) Integrins signal by forming a large, clustered, complex consisting of multiple integrins and many cytoplasmic adherensome components. Once assembled, these platforms mediate adhesion, motility and ECM remodeling.

signal transduction to take place. Additionally, it is thought that certain intracellular molecules can induce a strong binding of proteins that force the integrin into an extended conformation in the absence of ligand binding, resulting in the inside-out portion of the bidirectional signal [35].

The integrin-ligand interaction is thought to drive assembly of an integrin cluster. A large component of my thesis work depends on my ability to induce clustering of integrin $\beta 1$. Following recognition and binding of an ECM ligand, an integrin receptor assembles a clustered complex to induce a signaling output (figure I-5B) [44, 45]. Like the cadherin family explored previously, integrins function on the basis of a “velcro” principle. Many thousands of integrin receptors are at the surface, and the interaction of a single receptor with a ligand is not sufficient to produce a robust phenotype [44, 46]. In order for integrin signaling to occur, a number of integrins must form a cluster of ligand-bound, extended receptors. This cluster becomes sufficient to form an adhesive complex that is capable of generating and responding to force. Interestingly, the size of the cluster is not correlated with the amount of force that can be produced. In fact, the efficiency of integrin-ECM interaction to generate outputs tends to look like a bell-curve distribution [47]. Thus, while integrin affinity for a ligand determines the specificity of an interaction, the mechanical outputs are governed by the interactions and dynamics of many integrin receptors.

In summary, integrin activation occurs through a combination of conformational changes and complex formation that leads to signal transduction and alters cellular behaviors. Integrin activation is a contested process, and there are many ways to define it. Throughout this work, we have

focused on the effects of activating antibody and ligand binding to the extracellular domain on integrin distribution and signaling. Thus, we here define integrin activation in terms of eliciting an extended, ligand-binding conformation.

Adhesion signaling is induced through assembly of a multi-protein complex on the cytoplasmic tails of integrins which links the cytoskeleton to the ECM. Integrin engagement of ECM ligand activates adhesive signaling, which is necessary for migration, invasion and spreading [35]. Because a single integrin cannot generate a robust signal, clusters of integrins, ECM ligands, cytoskeletal proteins and a variety of scaffolding and signaling proteins assemble on both side of the membrane, forming a cell-matrix adhesion. There are several major types of adhesions. The most common are those that connect integrins to filamentous actin through mediating proteins like talin, paxillin, kindlin and vinculin. In 2D, there are distinct forms of actin-integrin adhesions: small, nascent focal complexes, larger focal adhesions and still larger, more mature, fibrillar adhesions that are characterized by fibronectin deposition [35]. In 3D, the components thought to be specific to the different 2D adhesions are intermixed and the structures that form are morphologically different than those in 2D [48, 49]. Some specialized cells form cell-matrix adhesions called hemidesmosomes, which links integrins to the intermediate fibers of the cytoskeleton.

The adhesion complex serves as a platform for cytoskeletal attachment and recruits multiple signaling molecules [50, 51]. The proteins that participate in this complex, and the interactions between them, have been dubbed the cellular adhesome [6]. The adhesome is a network comprising

150 components and 64 interactions. Including the associated proteins, the number of predicted interactions increases to over 6000. Some important players in this complex are summarized below and in figure I-5B.

Talin. Talin links the cytoplasmic domain of the integrin beta subunits to actin filaments. Binding of talin to the cytoplasmic integrin tail of the beta3 integrin subunit is thought to disrupt a salt bridge between it and its alpha subunit partner, which triggering a conformational change in the extracellular portion of the integrin that increases its affinity for ECM ligand [38, 52]. Talin is a large protein, consisting of a globular N-terminal head region and a flexible rod domain. A FERM domain present in the head region mediates binding to the tails of integrin beta subunits. This region also increases localized production of PIP₂, which can increase talin activation [53]. The tail region has binding sites for actin and vincullin. The calcium-dependent protease calpain can cleave talin, which allows for the turnover of cell-ECM adhesions [54]. In summary, talin plays an important role both in inside-out signaling as well as the connection of integrins to the actin cytoskeleton.

Vinculin. Vinculin is an adaptor protein with binding sites for multiple proteins including talin, F-actin and paxillin. As with talin, vincullin can be regulated by local PIP₂ levels. Vinculin is normally folded in an auto-inhibited conformation, stabilized by interactions between its head and tail regions. PIP₂ binding weakens these interactions, which exposes binding sites for talin and other proteins. Binding of vincullin to talin activates it [55]. Recent work utilizing a force-sensor model has determined that vinculin likely stabilizes integrin-based adhesions under tension [56]. Cells lacking vinculin have

significantly higher rates of adhesion turnover. Further, vinculin seems to modulate the interactions between paxillin and Focal Adhesion Kinase (FAK).

ILK, PINCH, and Parvins. These molecules are part of a multi-protein complex (sometimes referred to as the IPP complex) that regulates the actin cytoskeleton. Integrin-linked kinase (ILK) was originally identified as a serine-threonine kinase that interacts with integrin β 1 cytoplasmic tails [50]. More recent evidence suggests it is a pseudo-kinase, and functions as a scaffold, binding the PINCH and parvin proteins. Together, the proteins of the IPP complex regulate the actin cytoskeleton. Recent evidence suggests that the IPP also contributes to microtubule organization by capturing the growing ends of microtubules [57]. ILK plays an important role in the organization of the microtubule cytoskeleton in cell polarity [28], and is implicated in differentiation of multiple epithelial cell types [58].

Paxillin. Paxillin and its related family members are signaling adaptors that recruit a myriad of proteins to the adhesion. Paxillin is a member of the LIM-domain containing superfamily of proteins. This group of proteins is enriched at cell-ECM adhesions in response to tension [59]. There is a recent model that paxillin is specifically recruited to adhesions after they experience tension. Paxillin does recruit and link a number of key proteins to the adhesions, including vinculin, parvin and FAK [50].

FAK. FAK is a tyrosine kinase that is regulated by multiple processes including adhesion, receptor-tyrosine kinase signaling and a variety of oncogenes [51]. FAK consists of an N-terminal FERM domain that mediates membrane association, an SH2 domain that binds the oncogene Src, and a C-terminal domain that binds paxillin. FAK is recruited to cell-ECM adhesions,

and becomes linked between the plasma membrane and paxillin. Application of tension is through to induce conformational changes in FAK that leads to auto-phosphorylation, and the exposure of the Src binding region. FAK is required for the turnover of cell-matrix adhesion, and serves as an important node of crosstalk between the RTK and integrin pathways [50]. Whether or not FAK is truly mechanoregulated remains to be determined.

Cell-matrix interactions differ between 2D and 3D. Cellular interactions with the ECM are the foundation for many events during development and beyond, continuing to modulate homeostasis in most tissues through the lifetime of an organism. It is well established that the chemical composition of the extracellular milieu initiates a broad range of cellular responses, but the role played by the physical organization of a cell's microenvironment is just beginning to be understood.

To understand the origin of the differences between cell-ECM interactions in 2D and 3D, we should consider how a third dimension alters the rules of mechanical sensing and signaling. In 2D, a cell is exposed to a substrate through a single continuous surface. In 3D, a cell has two or more surfaces contacts with an ECM. The addition of just one extra adhesion dimension has been shown to alter cellular responses [60]. In a true 3D matrix, a cell is exposed to many simultaneous ECM contacts, and these might all have subtly different physical and chemical properties.

There are several model systems used to study cell behavior in 3D. Each has a specific purpose and physical properties, which must be taken into account when drawing conclusions about experimental results [61]. The most *in vivo*-like of these is known as cell-derived matrix (CDM). However,

due to their ease of use, by far the most common are the polymerizable 3D ECMs: type-1 collagen and Matrigel. Type-1 collagen gels consist of fibrillar collagen, which has been processed into small peptide pieces in a solution of acetic acid. Neutralization of this solution to pH 7.4 induces polymerization of these pieces into collagen fibrils. The physical properties of the resulting gel is highly dependent on experimental conditions [62, 63], and subtle fluctuations in temperature and diluting factors can alter fibril density, thickness and crosslinking. The benefit to collagen 1 gels is the relative purity of the sample, and there are few confounding growth factors and other ECM proteins present in the final product. Its fibrillar nature also allows cells to actively remodel and migrate within this matrix. Matrigel is derived from a mouse tumor that produces large amounts of ECM. It largely consists of laminins and type-4 collagen, and thus mimics the composition of the basement membrane. However, the ratio of these ECM components varies from batch to batch, as does the concentration of cell-secreted (and ECM-bound) growth factors and cytokines. In contrast to type-1 collagen, Matrigel polymerizes without fibril formation at the microscopic level and even fibroblasts cannot spread or form force-generating adhesions within this substrate [64].

There is currently no evidence that adhesion or mechanotransduction requires different molecular components in 2D and 3D. The organization of these components does, however, change. For example, 2D adhesions on fibronectin do not show colocalization of paxillin and integrin $\alpha 5$. In 3D, integrin $\alpha 5$ and paxillin are almost perfectly colocalized. An explanation for this may lie in the distribution of tension through adhesions in 2D and 3D. In 3D, almost all adhesions are under tension because of the biomechanical

properties of a 3D environment. In 2D, this is not the case [65]. Integrin $\alpha 5$ is usually seen in adhesions under tension. Thus, it would make sense that it would be more widely distributed in 3D. Other than this, it has been confirmed that integrin $\beta 1$, vinculin, paxillin and zyxin are present in 3D adhesion complexes, although their distribution is not as well defined [48]. Another notable difference is the signaling molecule FAK. In 2D, FAK autophosphorylation is high in response to adhesion formation. In 3D, both levels and dynamics of this process are changed [66]. Thus, although the composition of cell-matrix adhesions in 2D and 3D varies, the major players, and likely the mechanisms of mechanosensing, remain analogous [67].

Endocytosis and recycling regulates integrin signaling. Integrins exert tight spatiotemporal control over intracellular signaling pathways, particularly those that control cytoskeletal reorganization. The ability of an integrin to respond to ECM ligand is governed by their conformation and the formation of a cytoplasmic adhesion complex. To perform this function, an integrin has to be at the cell surface. Thus, cycles of integrin endocytosis and recycling control the availability of integrin heterodimers at the surface where they can bind and respond to ligand [68].

After endocytosis, an integrin heterodimer can undergo degradation or recycling back to the plasma membrane. It is thought that the entire surface pool of integrin is recycled within 30 minutes. However, the degradation turnover of integrins is slow, with a half-life of about 12-24 hours [69]. As such, the majority of integrins are recycled back to the cell surface through one of two processes: a long-loop and a short-loop [68]. The manner in which integrins are recycled determines the rate of adhesion assembly and

disassembly. Integrins are recruited into all three post-endocytotic fates through a variety of interactions with proteins that regulate trafficking, which determine recycling rates and thereby alter integrin signaling behaviors.

The fast short-loop recycling pathway controls integrin recycling after stimulation with growth factors [70]. This pathway is required for the incorporation of integrins into nascent adhesions and thereby mediates directionally persistent migration in 2D and 3D [71-73]. Further studies confirmed that Integrin β 1-containing integrins also follow the Rab4 pathway following growth factor stimulation. The long-loop route is characterized by Rab11 and Arf6-mediated recycling through the perinuclear compartment. In the absence of stimulation, many integrins are thought to recycle through this pathway. Integrin α 5 is recycled through this pathway through interactions with Rab11 effector RCP [74]. A switch to the faster recycling program of integrins is thought to be important for invasion during cancer progression [75].

Integrins remodel the ECM. The mechanical and biochemical environment that surrounds a cell directly modulates its behavior. The structural component of this microenvironment is called the extracellular matrix (ECM). This term is broad, and comprises large ECM structures, like bone and cartilage, and smaller specialized ones like the structural lining of blood vessels and other organ systems. The signaling capability of ECM is due to the ability of a cell to identify specific types of matrix and in the matrices' ability to bind and present a variety of growth factors and cytokines to the cell. Together, these properties make the ECM an important mediator of cell behavior in developing and adult tissues [76].

ECM provides a means of physical communication between cells. The structure of the ECM of a given tissue is highly organized and governed by the type and amount of ECM protein present. The most well-studied ECM proteins are large glycoproteins, secreted by cells and assembled into large structures [76]. Collagens are by far the most abundant ECM, making up a third of all protein in the human body. There are 28 different types of collagen, characterized as fibrillar or non-fibrillar, based on the type of matrix they form. Type-1 collagen is the most common, and is the main component in bone, skin and tendons. It also acts as the fibrillar structural component in most organ systems. Collagen IV is a less abundant form of collagen. It is secreted by epithelial cells and, along with laminins, makes up the structural component of the BM underlying the epithelium [30].

Fibronectin is a multidomain protein that serves as a linker and scaffold between cell surface receptors, collagens, other fibronectin molecules and growth factors. Fibronectin is secreted by cells as a dimeric glycoprotein, and exists in soluble and polymerized forms [77]. Soluble fibronectin, also called plasma fibronectin, was first isolated in blood. Cellular Fibronectin is present in many tissues as part of a fibrillar ECM. Humans have at least 20 splice variants of FN, which differ in the amount of V domain present [77]. These variants are translated into subunits that dimerize into different combinations in the ER. Most plasma dimers are composed of V₀ and V⁺ variants. In contrast, cellular FN consists mostly of V⁺ subunits. The identity of the subunit changes FN properties, especially in relation to clotting and polymerization. Multimeric FN fibrils are common in many tissues. Importantly, effects of soluble and cellular fibronectin on cells differ.

Assembly of FN dimers into fibrils is an integrin-mediated process. The basic assembly process depends on binding of FN to integrin receptors at the cell surface [77]. Binding of FN to integrin $\alpha 5\beta 1$ induces clustering of receptor and is thought to be the first step of the fibrillogenesis process. Application of tension to the FN dimer through the integrins results in the unfolding of the dimer and exposure of cryptic FN-FN binding sites [78, 79]. FN dimers begin to associate through their n-terminal ends [80]. Continuation of this process results in lengthening and thickening of fibrils. Over time, these fibrils are converted to a polymerized state, which is mediated through strong, non-covalent interactions. Although these fibrils are stable, they are constantly being remodeled and turned over through integrin $\beta 1$ -dependent mechanisms [77]. Although it has been previously detected in epithelial tissues and acts as a potent tumor suppressor [81, 82], the role of polymerized FN in epithelial tissues is not characterized.

Despite 20 years of study, integrins remain poorly understood. In part, this is due to dogmatic assumptions and a tendency to look at these molecules in very contrived settings. A lot is known about how a single flat cell moves along a glass surface, yet almost nothing is known about integrins in a monolayer of polarized epithelial cells, despite the fact that the latter is more representative of an *in vivo* scenario. In my thesis work, I have furthered the understanding of integrin signaling in epithelial cells. Although my work has led to more questions than answers, I hope that future work in this area will refine our understanding of how sub-populations of integrins work together in epithelial cells and perhaps expand the ability to treat integrins as clinical targets in human disease.

I.3 EGFR as a model receptor tyrosine kinase

Diagnostic criteria that differentiate a benign lesion from a life-threatening cancer include loss of organized epithelial structure, polarity and basement membrane attachment. Loss of epithelial cell polarity correlates with disintegration of cell-cell junctions and the loss of a basement-membrane. Thus, loss of apico-basolateral polarity enhances proliferative and migratory behaviors in epithelial cells [17]. Front-back cell polarity and epithelial cell polarity utilize many of the same molecular players. Because invasion utilizes aspects of front-back cell polarity, most of the core polarity proteins are retained during tumor progression. Thus, a cancer must carefully select mutation targets that lead to deregulation of polarity, but favor increased proliferation and invasion. In this section, I will use receptor tyrosine kinase (RTK) signaling through the Epidermal Growth Factor Receptor (EGFR) as an example of how this can be accomplished.

In the Coffey lab, it is our perspective that signaling through the EGFR family of RTKs is critical for epithelial homeostasis. In addition to this, EGFR and other members of the ErbB family can be aberrantly activated in cancer where it contributes to increased proliferation, survival and metastasis [83]. In normal human colon, EGFR ligands are present on the apical surface whereas the receptor is predominantly found on the basolateral surface. It is thought that if the monolayer is damaged, the EGFR ligands gain access to the receptor, thus promoting proliferation and migration to facilitate wound closure. We have previously shown that aberrant trafficking of EGFR ligands to the basolateral surface can lead to disruption of epithelial morphogenesis [84-86]. We have additionally shown a requirement for EGFR signaling during

early and late oncogenesis *in vivo* [87]. Anti-EGFR therapy is one of the only targeted therapies approved for late stage CRC, further supporting the clinical importance of this pathway.

EGFR is a member of the ErbB family of RTK proteins which contains three other members: ErbB2 (also Her2), ErbB3 and ErbB4. These proteins are widely expressed throughout the adult organism and have important roles in development [88]. Each member of the ErbB family has important structural and signaling commonalities with EGFR, coupled with important differences (Figure I-6). To signal, members of the ErbB family must dimerize. EGFR is able to dimerize with itself and the three other ErbB receptors. It might be more accurate, to refer to the EGFR signaling pathway as the ErbB signaling pathway, but I will adhere to tradition and will refer to it as the EGFR signaling pathway throughout this document [89].

There are eleven ligands that interact with the ErbB family of receptors, and seven of them can bind EGFR (figure I-6). In my thesis work, I have focused on EGF because it is the best-characterized high affinity ligand that binds specifically to EGFR. It is important to note that although all seven ligands bind EGFR, different ligands elicit different downstream effects *in vitro* and *in vivo*. The differing effects of EGF, TGF α and AREG on EGFR are a good example of this. All three ligands bind selectively to EGFR. All three result in phosphorylation of the receptor. However, EGF-bound receptor is targeted to the lysosome, TGF α -bound receptor is recycled back to the membrane and AREG bound receptor is endocytosed to a non-canonical Rab11-positive compartment [90, 91]. These differences are in part mediated by the dissociation constant between EGFR and the ligand. Further, crystal

structures of EGFR in complex with EGF or TGF α are distinct, which may contribute to the differences between phosphorylation and post-endocytic fate of the receptor, but provides little mechanistic explanation as to how these conformational differences alter signaling [83].

Large conformational changes in EGFR accompany ligand binding [92]. The EGFR extracellular region consists of four domains, which can exist in a tethered or extended conformation (figure I-6). It is thought that the extracellular domain fluctuates between the tethered and extended conformations stochastically, and is stabilized in the active, extended, conformation by the ligand binding (figure I-6). Binding of ligand to EGFR results in stabilization of the extended form of EGFR and exposes residues that favor dimerization between EGFR family members. Dimerization of two EGFR molecules stabilizes the cytoplasmic kinase domains in an active conformation and allows for transphosphorylation and autophosphorylation of key tyrosine residues on the cytoplasmic region of EGFR. These phosphorylated residues form a platform for interactions with downstream signaling molecules.

EGFR possesses at least 11 tyrosines in its non-catalytic cytoplasmic domain that can be phosphorylated and serve as a platform for interaction with other proteins. In addition, at least 6 serine and threonine residues have also been identified as putative phosphorylation sites. Some of these sites are autophosphorylated, and some are phosphorylated by recruited proteins. Phosphorylation of these residues creates binding sites for SH2 and PTB domains of nearby mitogenic signaling effectors [93]. These are summarized in figure I-6. Notable members of these interactions include PI3K and Src, as

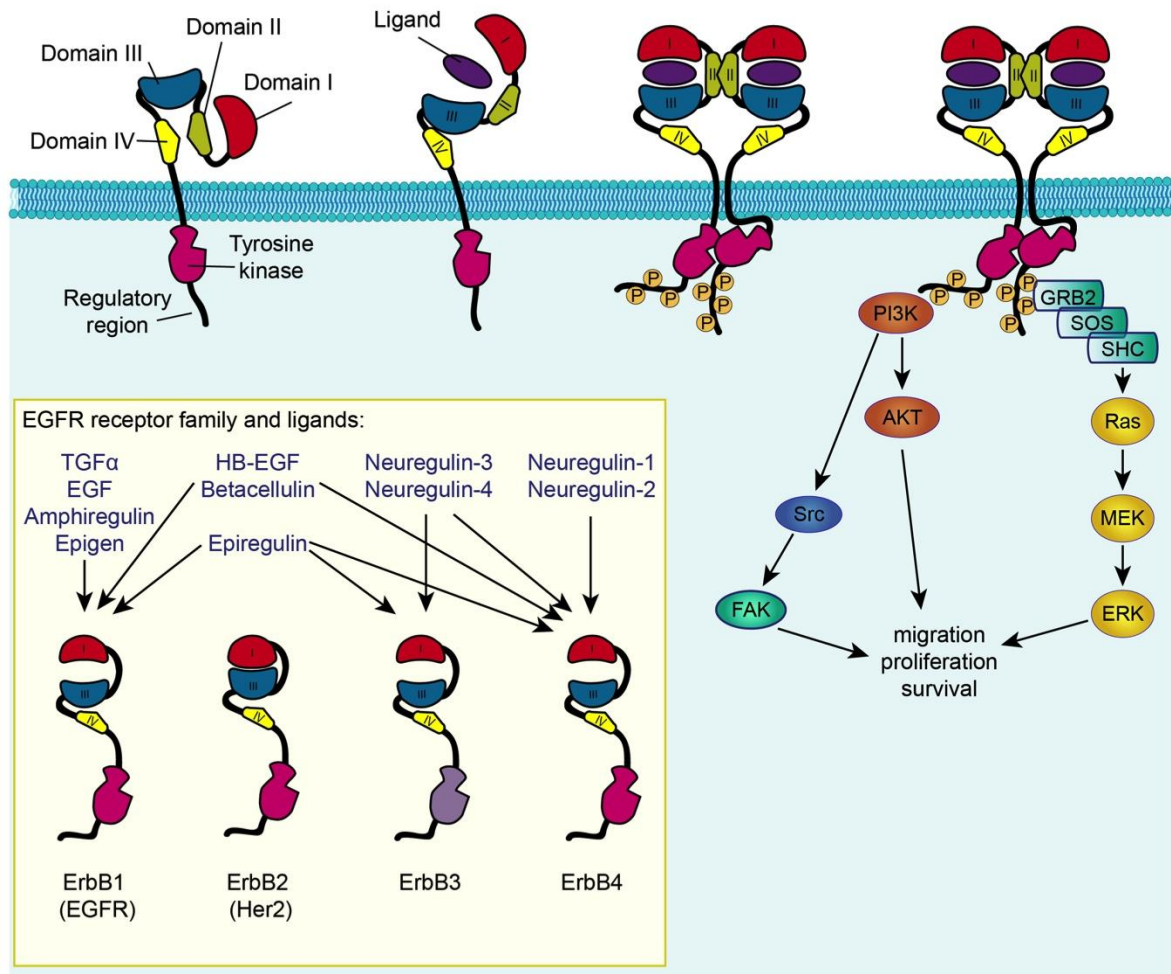


Figure I-6: Overview of EGFR family members and signaling

EGFR family members are single-pass transmembrane receptors consisting of four domains. To bind ligand, the receptor must be in an open conformation, which is then stabilized by the presence of ligand in the binding pocket. This allows for homo- or hetero-dimerization between EGFR family members and phosphorylation of the regulatory C-terminal tail region. Binding of cytoplasmic signaling proteins to the C-terminal tail of EGFR family members allows for signal transduction. Inset: there are 11 EGFR family ligands, which bind EGFR family receptors. Some can bind more than one receptor, while others can only interact with one of the four. The four receptors are structurally similar, but with several important differences: ErbB2 cannot bind ligand and exists constitutively in an active form and ready to dimerize and signal. ErbB3 is thought to have an impaired kinase domain, and thus it must heterodimerize to signal.

well as GRB2, which facilitates Ras activation downstream of EGFR. These proteins also mediate many of the malignancy-driving aspects of EGFR signaling.

Prolonged EGFR family activation leads to loss of apico-basolateral polarity through dissolution of epithelial cell-cell junctions [83]. Tight junctions in colon tumors are leakier than those in normal colon. Decreased expression of TJ components contributes to this, as does phosphorylation and mislocalization of TJ protein ZO-1. Phosphorylated ZO-1 can directly bind to EGFR, which decreases its TJ-associated function. Further, activation of ErbB2 causes it to bind the Par6/aPKC complex, which disrupts their ability to recruit Par3 to TJ, thus further compromising junction integrity. EGFR signaling also compromises adherens junction integrity by triggering internalization of E-cadherin. These disruptions of cell-cell junctions allow for further aberrant signaling through EGFR family members because dissolution of junctions allows the normally apical ligands to diffuse and activate the normally basolateral receptors. This leads to hyperproliferation and further tissue disorganization. At late stages, EGF can act in a cytokine-like fashion and organize an anterior-posterior axis, supported by activation of PI3K, Cdc42 and Rac1. The establishment of this axis primes cancer cells for invasion (figure 1.5). These data highlight the importance of RTK signaling as a target in cancer.

EGFR can control integrin signaling and invasion through its ability to regulate cell polarity and integrin activation [94]. In return, integrins alter how EGFR signaling is transduced. The complexity of this coordinate regulation is illustrated through interactions between EGFR, integrin $\alpha 2\beta 1$ and integrin

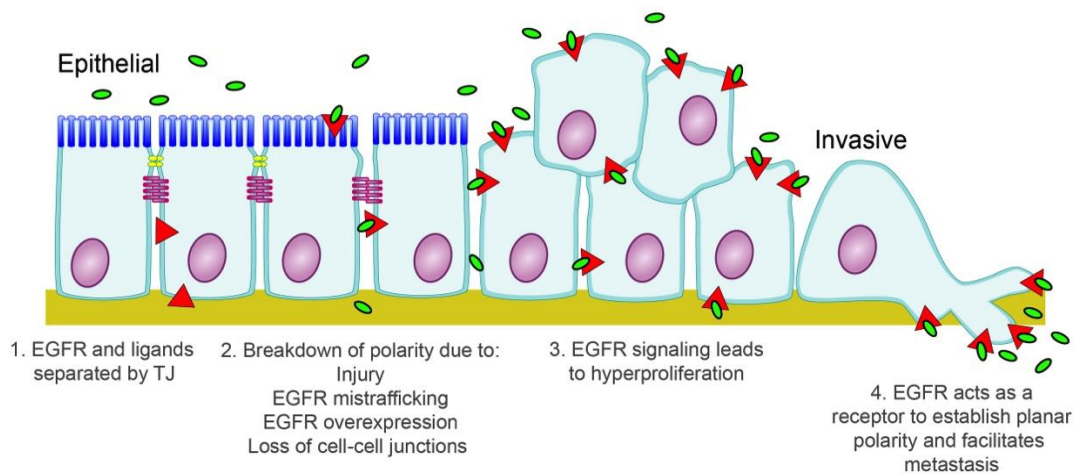


Figure I-7: Aberrant EGFR signaling acts as a driver of malignancy.

In normal colon epithelium, the EGFR and its ligand EGF are separated by the tight junction complex and thus EGF cannot signal. Disruption of tight junctions or the appearance of EGFR on the apical surface allows for sustained EGF-mediated signaling. This leads to progressive loss of epithelial polarity and hyperproliferation. In late stages of disease, EGF can induce front-back polarization and invasion by the epithelial cells into the underlying tissues.

$\alpha 5 \beta 1$. Integrin $\alpha 5 \beta 1$ negatively regulates EGFR signaling in a glycosylation-dependent manner. Specifically, integrin $\alpha 5$ blocks EGFR endocytosis by regulating its localization to lipid rafts [95]. Interestingly, EGFR over-activation blocks integrin $\alpha 5 \beta 1$ activation through ERK/p90RSK [96, 97]. At the same time, treatment with EGF causes cell spreading in an integrin $\alpha 2 \beta 1$ -specific manner [98]. From these observations, we can conclude that coordinate regulation between EGFR and integrins is not just integrin-specific, but also probably governed by post-translational modifications that the field has barely started to examine. It is likely that some of the contradictory observations in different tissues and contexts will be explained by these more refined studies.

I.4 Colorectal cancer

The human colon is under a constant onslaught of environmental damage from the food we eat. As such, it has evolved into a highly proliferative tissue wherein all epithelial cells turn over about once a week. To accomplish this, the colon maintains a large pool of stem cells that reside at the base of invaginations known as crypts of Lieberkuhn (figure I-8A). The stem cells divide and the daughter cells move up the crypt axis toward the luminal space, differentiating as they move [99]. By the time they reach the surface, the cells have become terminally differentiated epithelial cells that can perform a variety of specialized absorptive and secretory functions. As new cells migrate out of the crypt, older cells are sloughed off and excreted. This process is tightly regulated. Most of the well-characterized regulatory mechanisms involve signaling through soluble ligands, such as the Wnt family ligands, and juxtacrine signaling, through pathways such as Notch. Despite the abundance of ECM components around the epithelium, and the fact that

the intestinal epithelial cells are actively migrating throughout their lifetime, the direct contribution of adhesion signaling through the ECM to this process is unexplored.

Colorectal cancer progression involves a slow but persistent accumulation of mutations that drives over-proliferation and loss of polarity in the colon (figure 1.8B). Although each cancer is unique, a large subset of colon cancers are thought to develop as summarized by the “Vogelgram” [100, 101]. Briefly, CRC initiation begins with the loss or mutation of the Adenoma polyposis coli (APC) gene in a cell population that is retained in the crypt. Among several functions, APC stabilizes adherens junctions and contributes to maintenance of polarity. Expansion of colonic cells that lack APC results in hyper-proliferation and a loss of proper tissue organization in and around affected crypts. As the mass grows, further mutations arise and contribute to disease progression, culminating in invasion and metastasis (Figure 1-8B).

Growth factor signaling contributes to CRC progression. Many of the proteins that are mutated during CRC are mediators of growth factor signaling. These include activating mutations in PI3K, KRAS, BRAF and PTEN, as well as overexpression or mistrafficking of the growth factor receptors and their ligands. Recent, unpublished work in the Coffey group has also shown that changes in RTK signaling is a mechanism of resistance that develops during targeted therapy. Altogether, these observations highlight the importance of understanding how different signaling pathways come together to effect epithelial cell polarity and its loss in cancer.

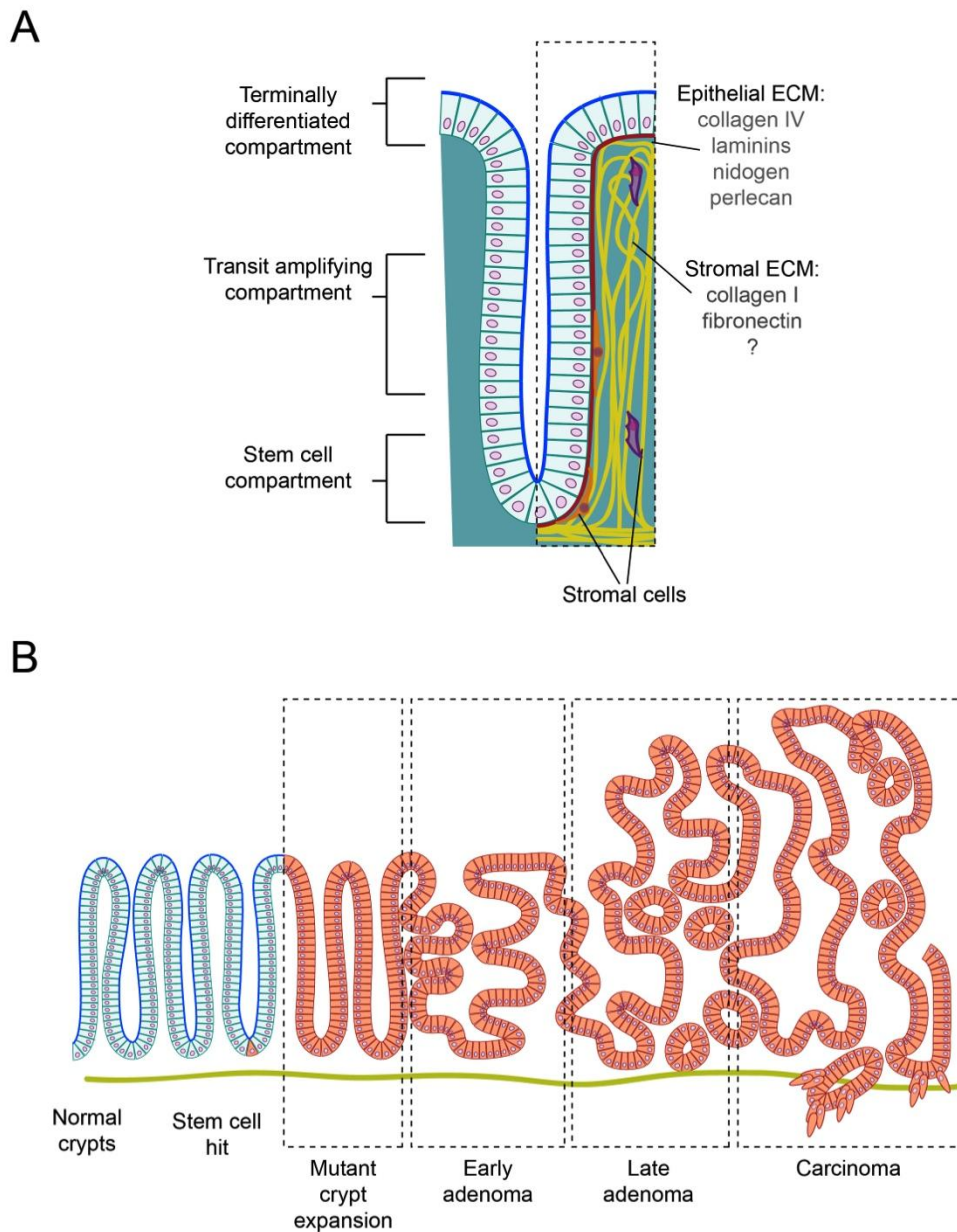


Figure I-8: Overview of colonic crypt structure and CRC progression

(A) Basic composition of a colonic crypt and surrounding ECM. (B) Colorectal cancer initiates as a single mutated cell that is able to proliferate and accumulate further mutations. These allow for disease progression and acquisition of a metastatic phenotype.

I.5 Rationale for thesis work

I have discussed some ways that integrins contribute to epithelial cell polarity. Integrin $\beta 1$ is required for epithelial organization and differentiation in the colon. The hyper-proliferative, poorly differentiated phenotype observed when it is knocked out in the intestinal epithelium during development *in vivo* is strong evidence to this end [102, 103]. Yet, further exploration of integrin $\beta 1$ in adult colon homeostasis, or in colon cancer progression, has not been performed.

Integrins are complicated proteins. They signal bidirectionally, exhibit conformational changes that alter both their affinity and avidity, and are rapidly and selectively recycled to and from the cell surface. Previous studies in epithelial cells focus on integrin $\beta 1$ silencing or blockade. Integrin $\beta 1$ is a member of 12 different receptors. Blocking or depleting $\beta 1$ ablates the function of all of them. I wanted to determine how the activity of endogenous integrin $\beta 1$ contributes to epithelial morphogenesis in the colon, with the hope that I could use this information to study how individual α -subunits, and therefore individual $\alpha\beta 1$ receptors, contribute to this system. All together, the work presented in the subsequent chapters has identified that integrin $\alpha 5/\beta 1$ recycling and activation dynamics contribute to colonic epithelial polarity and morphogenesis.

Chapter II provides a rationale as to why we chose to look at integrin $\beta 1$ signaling in CRC, particularly in the context of crosstalk with the EGFR. This chapter focuses on the questions: Are there gross changes in colon ECM during cancer progression? Do EGFR and integrin $\beta 1$ interact in the colon? Does this interaction contribute to ECM remodelling in the context of CRC? I

was able to show that fibrillar type-1 collagen is highly disorganized in human CRC. I was also able to show an increase in EGFR/integrin β 1 complex in the same tissues. To better understand how these observations may be interconnected, I validated a new 3D model for CRC for use in these studies. Although I was able to recapitulate the *in vivo* phenotypes with our model system, I was not able to technically perform the biochemical analyses that would have allowed us to ask mechanistic questions about the EGFR/integrin β 1 complex. However, the work presented here allowed us to confirm that interactions between EGFR, integrins and ECM are important to study in the context of CRC.

Chapter III has been submitted for publication in the Molecular Biology of the Cell journal. This chapter focuses on the following questions: Does modulation of integrin β 1 activity affect colony morphogenesis of CRC-derived cells? What role do particular integrin β 1 associated subunits play in the polarity of these cells? Can lateral integrins and deposition of ECM contribute to cell polarity? I address the effect of activation or blockade of integrin β 1 signaling to CRC-derived cell behavior and polarity and explore the mechanisms underlying observed differences in colony morphogenesis. From this work, we were able to conclude that integrin α 5 clustering at the lateral membrane contributes to epithelial polarity in the colon through fibronectin deposition. This work opens new questions about the role of integrins in modulating cell-cell interactions and identifies a new role for integrin signaling in colon epithelial cells.

Chapter IV is a summary of on-going work, describing my attempt to understand the interplay between integrin β 1 and EGFR in their ability to

regulate invasion and polarization in CRC cells. This research addresses the following questions: How does integrin $\beta 1$ activity change cell response to invasion-promoting RTK signals? Can clustering of integrin $\beta 1$ be a therapeutically useful strategy? This research has utilized and built on the work in chapter III in an attempt to study a new, membrane proximal, node of crosstalk between integrin $\beta 1$ and EGFR. Although this was the first project I worked on, an insufficient understanding of the contribution of integrins to the polarity process led me to focus on the work presented in chapter III. Armed with this knowledge, future work can focus on the contribution of integrin $\alpha 5$ and fibronectin polymerization to intestinal homeostasis and EGFR signal transduction in the colon and in CRC.

Chapter II: **Characterization of the interaction between EGFR and Integrin β 1 in colorectal cancer**

II.1 Introduction

Cancer progression is accompanied by large changes in the architecture of the extracellular matrix that surrounds the cancer cells at both primary and secondary sites. Re-organization of extracellular matrix is mediated by integrins, which are diverted from their role in maintenance of epithelial polarity to new roles in facilitating metastasis [17, 104]. Signaling through the receptor tyrosine kinase EGFR provides stimuli that favor invasion and migration [89]. Although downstream crosstalk between EGFR and integrin signaling is well established [94], the effects of membrane proximal interactions of EGFR and integrin β 1 on ECM remodelling during CRC progression are understudied.

The EGFR and integrin β 1 proteins are both transmembrane receptors that transmit information from the outside microenvironment to the inside of the cell. The EGFR transmits information about the soluble growth factors present, while integrin β 1 provides mechanical information about the surrounding extracellular matrix. These signals are then processed and integrated into a phenotypic response by the recipient cell. Signaling through integrin β 1 is necessary for a full cellular response to EGFR ligand [105]. Additionally, EGFR and integrins reciprocally regulate each other's recycling, which alters signal duration and intensity [106]. In the case of integrins, EGFR also stimulates adhesion turnover, which contributes to an invasive phenotype [96, 97, 107]. Whether a portion of this regulation occurs through the

formation of a complex containing integrin $\beta 1$ and EGFR at the cell surface is not well established.

In these studies, we sought to understand crosstalk between membrane proximal EGFR and integrins and the contribution of this complex to ECM remodelling and invasion in CRC. We examined the organization of ECM in varying stages of colon cancer utilizing a novel probe that binds selectively to fibrillar type-1 collagen and found that collagen fibrils are more fragmented in tumors and generally less organized than in normal colon. We also examined whether an interaction between EGFR and integrin $\beta 1$ increases in CRC tumors using proximity ligation assay, and found higher signal in tumor. To determine if we could study the interactions between ECM, EGFR and integrin $\beta 1$ *in vitro*, we utilized a recently developed 3D culture system to probe how altering EGFR signaling would alter colony morphology. We found that activation of EGFR with different EGFR family ligands elicit different extents of invasion and result in differing amounts of interacting EGFR/integrin $\beta 1$. All together, our results suggest that EGFR and integrin $\beta 1$ interact in CRC and understanding the basis for this interaction and its effects on cell behavior may open new avenues for clinical intervention.

II.2 Results

Colorectal cancer progression involves disorganization of type-1 collagen surrounding the cancer cells. To begin to understand the role that cell-ECM interactions play in CRC progression, we looked at the organization of type-1 collagen in CRC. We used fluorescently labeled CNA35, a peptide that selectively binds to fibrillar type-1 collagen, to examine the distribution of fibrillar collagen in human CRC. To get a broad view of collagen organization

in multiple tumors, we used a formalin-fixed paraffin-embedded tissue microarray (FFPE TMA). We utilized a heat and pressure-based antigen retrieval method, and subsequently stained the TMA with antibodies against cytokeratin, DAPI and CNA35. We qualitatively analyzed the TMA using fluorescence microscopy. We note that compared to normal colon tissues, the collagen in tumors is disorganized and fragmented (Figure 1A,B), and the extent of fragmentation and disorganization increases in later stage cancer. There also seem to be more instances of cytokeratin-positive colonic epithelium-derived cancer cells in direct contact with collagen fibrils in later stage disease (figure 1B). These data show that aberrant ECM organization, which is a hallmark in most forms of cancer, also occurs in CRC.

Higher levels of a EGFR/Integrin β 1-containing complex are present in colorectal cancer. We next asked if a direct EGFR and integrin β 1 interaction increased in cancer. It has been suggested that in addition to their downstream pathway crosstalk, EGFR and integrin β 1 interact directly at the cell surface in a manner that alters downstream signaling by one or both of these proteins. EGFR signaling contributes to integrin-mediated ECM remodelling [94] and we have previously implicated EGFR signaling as necessary for CRC progression *in vivo* [87]. To determine if an increase in EGFR-integrin β 1 interaction correlates with CRC progression and collagen disorganization, we looked for the presence of these two molecules in close proximity to one another in fixed CRC TMA tissues using a proximity ligation assay (PLA). PLA detects protein-protein interactions by producing signal when antibodies against the two target proteins are within 100nm of one another. We used an antibody against the intracellular portion of EGFR and

an antibody against the intracellular tail of integrin β 1, which we coupled with secondary antibodies linked to a probe. Places where EGFR and integrin β 1 interact produce a punctate fluorescent signal (Figure 2). We looked at the number of puncta present throughout the tissue. We noted an increase in PLA puncta in cancer (Figure 2,3), and particularly in more advanced tumors. Although qualitative, these data made us feel that further exploration of integrin β 1 and EGFR in the context of CRC is merited.

CC/SC model system recapitulates in vivo observations in a controlled 3D environment. In order to probe this further, we needed a model system with which we could study the interactions of EGFR and integrin β 1 in a controlled but *in vivo*-like setting. In the chapter I, I highlighted the differences between 2D and 3D cell culture, and between different 3D cell culture model systems. Because we were interested in studying invasion, we did not feel that Matrigel would be a good choice as cells are not known to form force-generating adhesions or canonical invasive protrusions in this matrix. Further, Matrigel, even the growth factor reduced version, has a plethora of growth factors and cytokines present which could affect our ability to study EGFR signaling [108]. Type-1 collagen has the ability to transmit force, does not contain significant soluble factors.

We recently developed a new 3D model system that recapitulates aspects of both a well-differentiated and an invasive CRC. This model, which we refer to as the CC/SC model system, consists of two cell lines which we derived from the parental HCA-7 cell line. This parental line is, in turn, derived from a well-differentiated, microsatellite unstable rectal cancer. HCA-7 cells were among many cell lines that we analyzed in 3D. What differentiated this

line from others is the appearance of two distinct colony morphologies that form when single HCA-7 cells are placed in type-1 collagen and allowed to grow for 15 days. Under these conditions, cells formed either cystic or invasive – “spiky” – colonies. Individual colonies were removed and expanded, until purified populations of “cystic” and “spiky” cells were generated. Throughout this document, these lines are referred to as CC (cystic clones) and SC (spiky clones). To determine whether these cells maintain these phenotypes *in vivo*, we performed a tumor xenograft study in nude mice. We find that following subcutaneous injection, CC form well differentiated, encapsulated tumors while SC form poorly differentiated tumors that invade into adjacent muscle. These observations and data led us to conclude that the CC/SC system would be a good tool to begin to dissect the role of membrane proximal EGFR and integrin β 1 signaling in CRC.

First, we wanted to determine if the CC and SC model recapitulates the differences we observed between collagen organization in normal and tumor tissues. We grew CC and SC for 15 days in 3D type-1 collagen and examined the organization of the collagen around the colonies using CNA35 and confocal microscopy. Figure 4 shows that collagen is organized mostly parallel to the CC colonies and is disorganized (both parallel and perpendicular) to cells in SC colonies. This is consistent with how collagen is aligned by epithelial cells versus tumor cells *in vivo* and corresponds with how other models of normal and malignant cell lines behave *in vitro* [109]. To test whether we see an increased amount of EGFR/integrin β 1 interaction in SC, we grew CC and SC in 3D type-1 collagen for 15 days and performed PLA analysis using antibodies against EGFR and integrin β 1. We find that there is

higher PLA signal in SC, suggesting higher levels of EGFR/integrin β 1 complex. These data led us to conclude that our 3D model system recapitulates key aspects of EGFR and integrin β 1 and ECM remodelling in CRC *in vitro*.

EGFR ligands have different effects on CC cell invasion and colony morphology. Next, we wanted to determine if stimulation of the EGFR pathway increases invasive CC cell behaviour in 3D. We plated single CC cells in 3D type-1 collagen and treated them with EGFR ligands AREG and EGF for 15 days. We found that the untreated CC cells form cysts while the colony morphology in treated CC depends on the ligand identity: treatment with EGF induces colony disorganization and invasion into the surrounding collagen (Figure 5A-B). Interestingly, this response is not seen when CC cells are treated with AREG (Figure 5A-B). From these data, we conclude that EGFR signaling affects CRC-cell-matrix interaction and behavior in 3D.

Because we noted different phenotypic effects of EGFR ligands EGF and AREG on colony morphology and cell/collagen interaction in 3D, we wanted to determine if these two ligands differentially affected the abundance of the EGFR/integrin β 1 complex. We grew CC cells on 2.5D fibrillar collagen gels and treated them with indicated ligands for 0, 5 or 30 minutes. Cells were fixed and analyzed by PLA using antibodies against the intracellular portions of EGFR and integrin β 1. We were surprised to find that the highest observed PLA signal was following AREG treatment (Figure 6). To confirm the presence of this interaction, we attempted to co-immunoprecipitate integrin β 1 and EGFR following EGF and AREG treatment. We were never able to reproducibly detect differences between treatments by this method. From

these data, we conclude that the EGFR and integrin β 1 interaction is ligand dependent and may display ligand-specific effects. Unfortunately, our inability to confirm this observation using a second method made us question our ability to move forward with the project, since an inability to detect the complex by immunoprecipitation and western blot would make it challenging to test further mechanistic details.

II.3 Discussion

It has been previously shown that EGFR signaling contributes to ECM disorganization *in vitro* [109]. It has also been suggested that EGFR and integrin β 1 may physically interact in cancer and that this interaction correlates with a more malignant state [110]. The contribution of EGFR to integrin-mediated ECM disorganization in CRC has not been well explored. In the work presented here, we show (qualitatively) that type-1 collagen disorganization correlates with CRC progression. We also show that an interaction between EGFR and integrin β 1 exists in CRC and also correlates with a more advanced disease state. Finally, we determine that our CC/SC model system can be used to study the interactions between integrin and EGFR in CRC invasion *in vitro*. Overall, these data provide a rationale for further exploration of the EGFR and integrin β 1 interaction in CRC.

In this section, we wanted to understand whether integrin β 1 and EGFR interact at the surface of CRC cells and how this contributes to disease progression. I had hypothesized that integrin β 1 and EGFR form a complex, which would promote cell-ECM adhesion and invasion. I was able to show that EGFR and integrin β 1 are in close proximity, possibly in a single complex or directly bound to one another, more often in human CRCs. I also showed

that in normal human tissue, the PLA signal is more prevalent in the terminally differentiated region than the stem cell region of normal colonic crypts. Together, these observations raised confusion. If EGFR interaction with integrin β 1 promotes more cancer-like behavior, I would expect it to be high in cancer and in the stem cell / proliferative compartment of normal colon crypts. If the interaction was inhibitory towards adhesion or EGFR signaling, I would expect it to be lower in cancer and higher in the terminally differentiated compartment. Thus, this observation led to more questions than answers. Is EGFR interacting with different integrin β 1-containing receptors in different regions of the colon? Is there an “epithelial” and a “malignant” set of EGFR-integrin interactions? There is evidence that constitutive knockdown of integrin β 1 during development leads to hyperplasia and impaired differentiation, which can be rescued through dampening of EGFR signaling. There is also evidence that specific integrin α subunits may differentially interact with EGFR [95, 98]. All together, these data suggest that the role on integrin β 1 in normal and disease colon states is nuanced and further study is necessary to understand its contribution and interactions with EGFR.

Using our CC/SC system I was able to corroborate the observations we made *in vivo*, although unfortunately I was not able to confirm my PLA observations via co-immunoprecipitation. This work was not wasted, though, as this analysis of the CC/SC model system allowed me to make some important hypotheses that we utilized as we moved forward into the two subsequent projects, which are discussed in chapter III and IV, and address some of the questions that were initially raised by these early PLA observations.

In summary, the data presented here provide rationale to further examine the relationship between EGFR and integrin β 1 and show that interplay contributes to colonic epithelial cell behaviour.

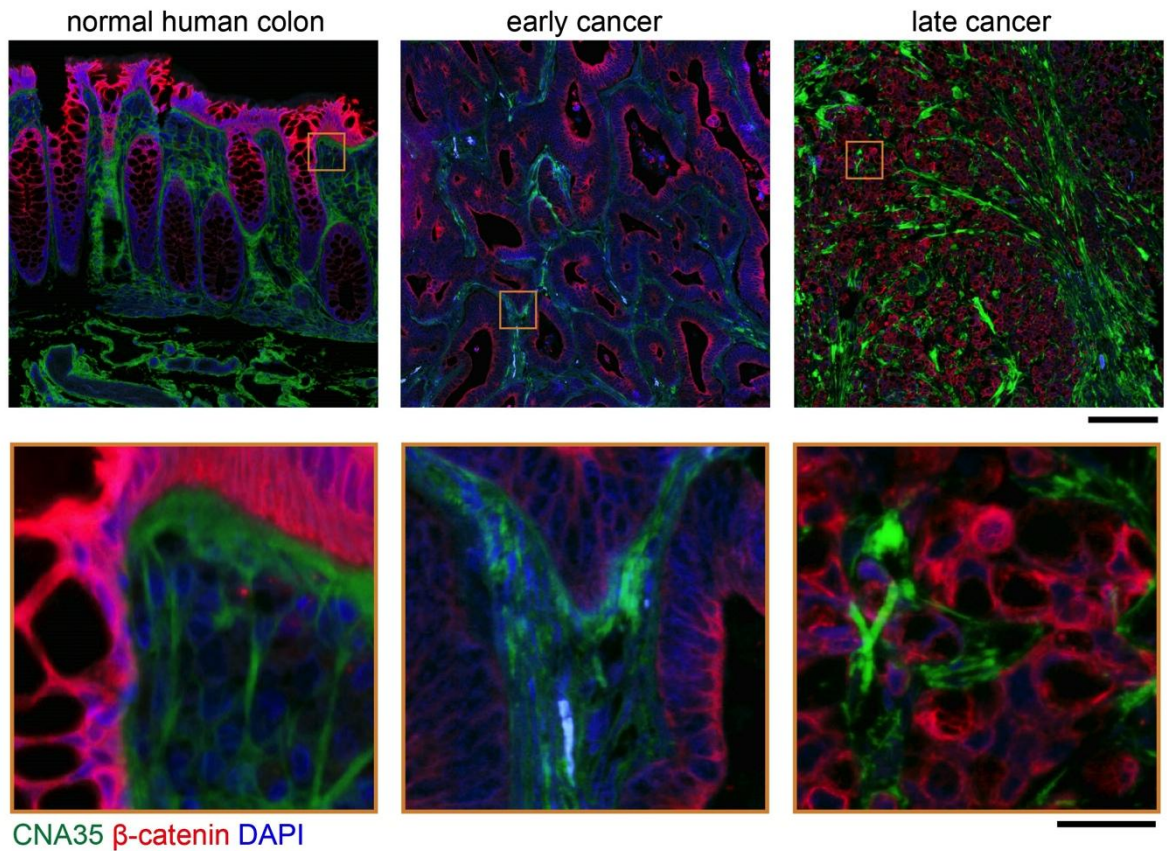


Figure II-1: Fibrillar type-1 collagen organization changes during CRC progression.

Representative normal and cancer cores were analyzed by confocal microscopy and stained with CNA35 (green), antibodies against β -catenin (red), and DAPI (blue). Scale bar, 100 μ m. Inset scale bar, 25 μ m.

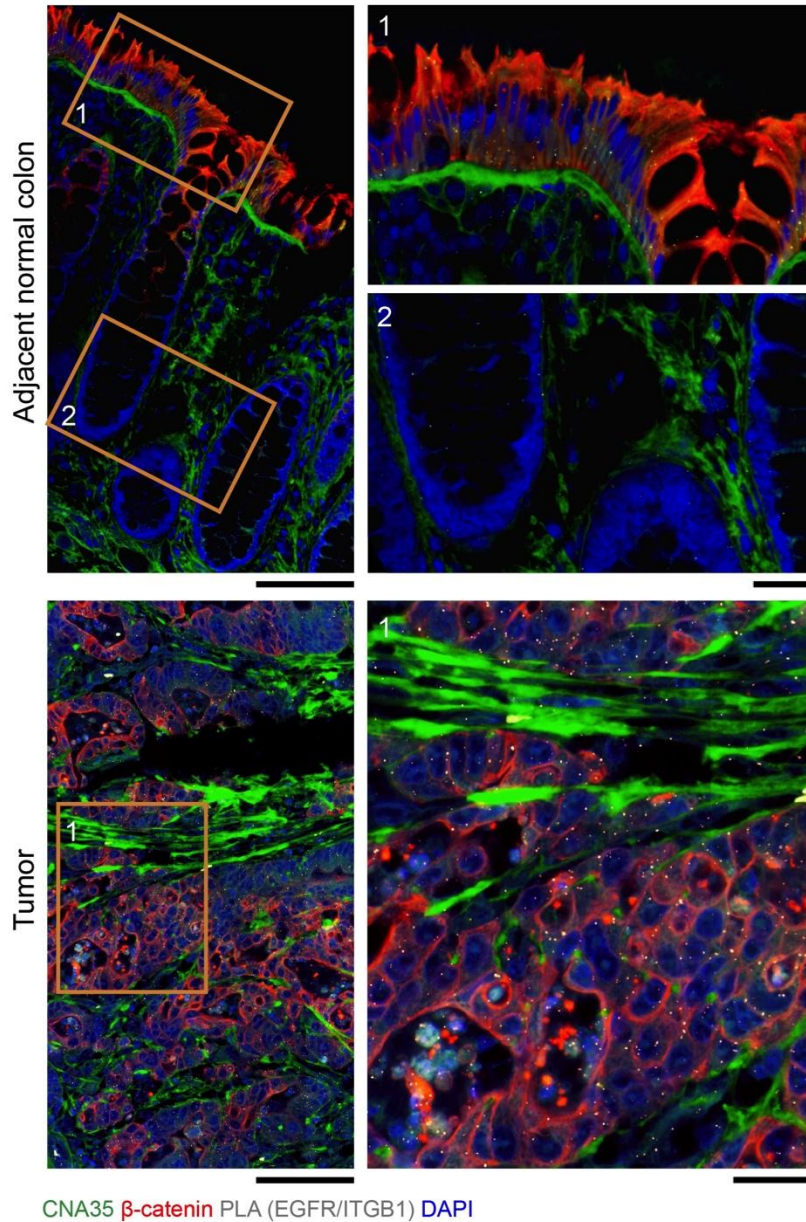


Figure II-2: Interaction between EGFR and integrin β 1 is increased in CRC.

Representative images of human normal and tumor tissue sections stained with CNA35 (green), an antibody against β -catenin (red), PLA using antibodies against EGFR and integrin β 1 (white), and DAPI (blue). Scale bar, 100 μ m. Inset scale bar, 25 μ m.

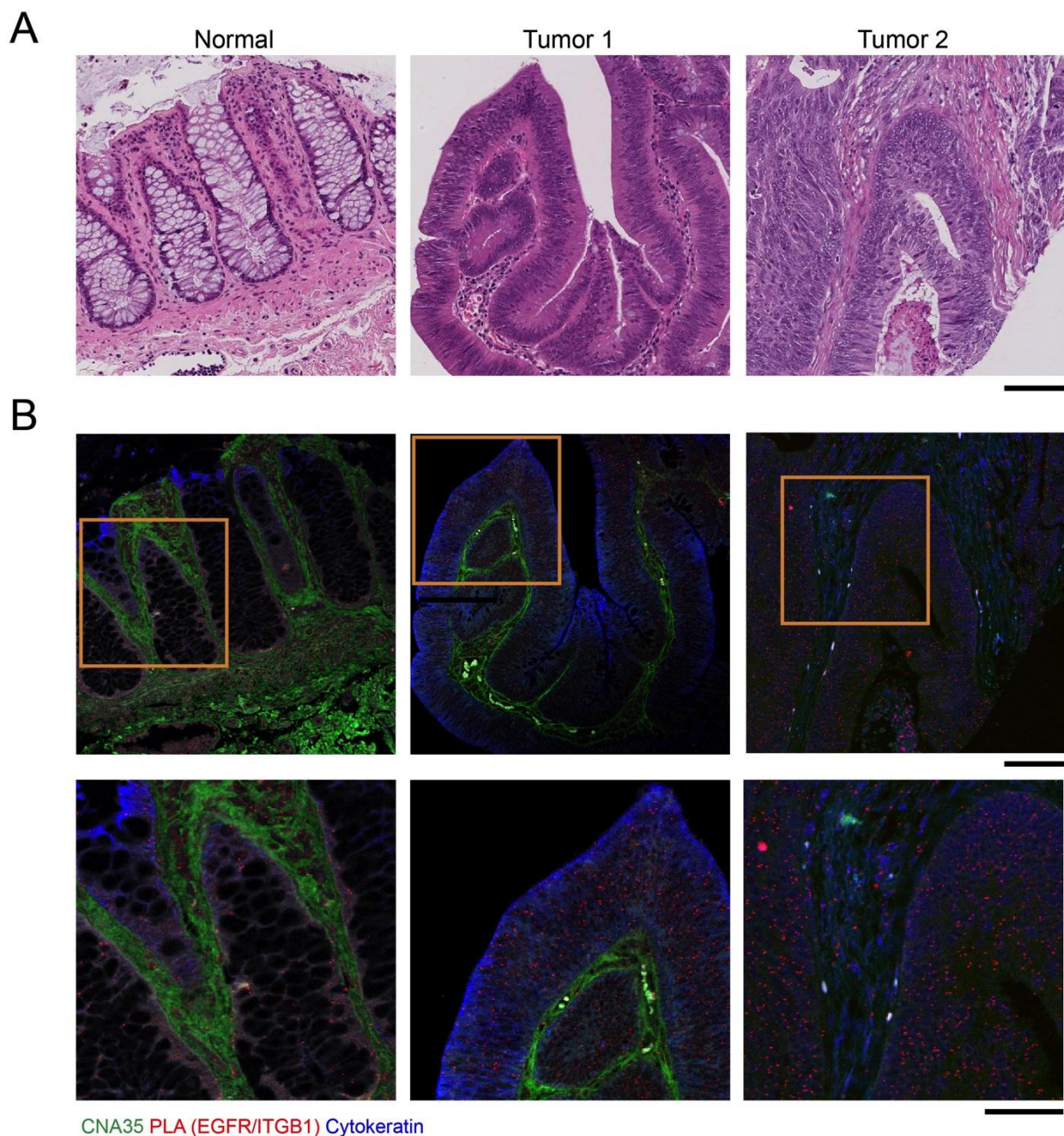


Figure II-3: Interaction between EGFR and integrin β 1 is increased in CRC.

Representative normal and tumor cores from CRC TMA. (A) Stained with hematoxylin and eosin. Scale bar, 100 μ m. (B) Stained with CNA35 (green), PLA using antibodies against EGFR and integrin β 1 (red), and an antibody against cytokeratin (blue). Scale bar, 100 μ m. Inset scale bar, 50 μ m.

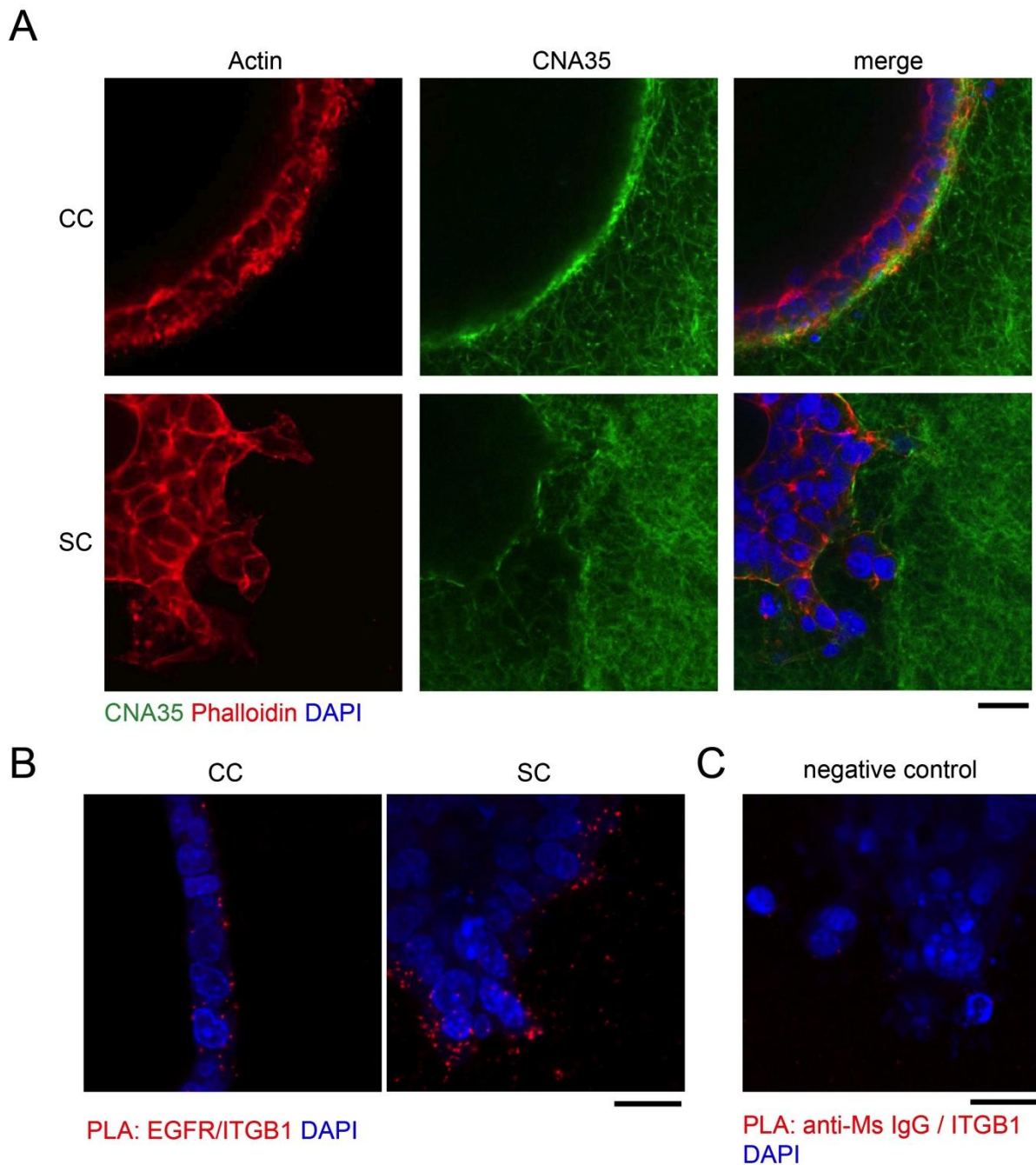


Figure II-4: CC/SC 3D model system recapitulates *in vivo* observations *in vitro*.

CC and SC colonies were grown in 3D type-1 collagen for 15 days. (A) Representative confocal image of CC and SC colonies stained with CNA35 (green), phalloidin (red), and DAPI (blue). Scale bar, 25 μ m. (B) Representative confocal image of PLA in CC and SC colonies utilizing antibodies against EGFR and integrin β 1. Scale bar, 25 μ m. (C) Representative confocal image of PLA signal in SC cells utilizing antibodies against integrin β 1 and an anti-Mouse secondary antibody. Scale bar, 25 μ m.

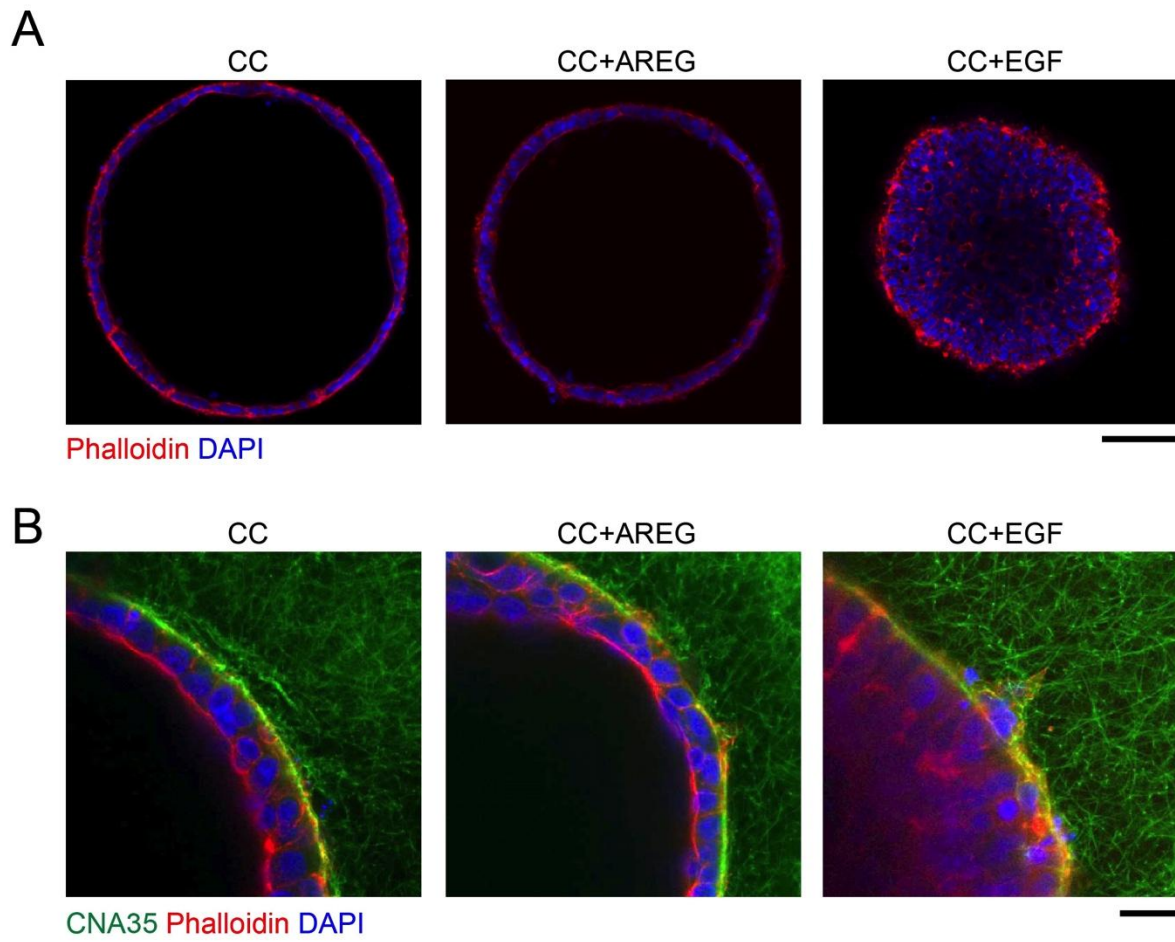


Figure II-5: EGFR ligands AREG and EGF elicit different phenotypic effects on CC cells in 3D.

CC grown in 3D type-1 collagen were treated with 20nM of AREG or EGF on days 8-15, fixed and stained. (A) Representative confocal image of CC colonies stained with phalloidin (red) and DAPI (blue). Scale bar, 100 μ m. (B) Representative confocal image of CC colonies stained with CNA35 (green), phalloidin (red), and DAPI (blue). Scale bar, 25 μ m.

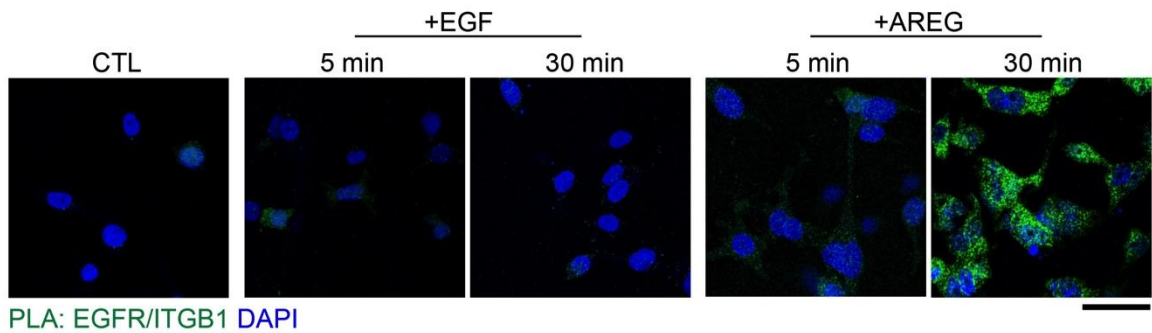


Figure II-6: Treatment with AREG but not EGF increases abundance of EGFR/integrin β 1 interaction in 2D.

SC cells grown on 2.5D type-1 collagen were treated for 5 and 30 min with 20nM EGF or AREG, fixed, and analyzed using the PLA protocol followed by confocal microscopy. Shown are the cells stained with DAPI (blue) and the PLA signal (green). Scale bar, 25 μ m.

Chapter III: **Clustering of Integrin $\alpha 5$ at the lateral membrane restores epithelial polarity in invasive colorectal cancer cells**

III.1 Introduction

Polarized epithelial cells line the boundary between the interior of an organism and its external environment. The ability of the cells to distinguish between their basolateral – internal -- and apical – external -- sides allows for regulated exchange of nutrients and their byproducts. Integrin engagement of extracellular matrix (ECM) ligands defines the basal cell surface and appears to be the first step in apico-basolateral polarization [5, 14, 28, 111].

Studies *in vivo* and *in vitro* show that integrin $\beta 1$ is required for epithelial morphogenesis [103, 112]. Apart from its role in orienting the apical surface [113], integrin $\beta 1$ signaling is thought to provide a cue for the recruitment of E-cadherin to nascent cell-cell contacts, stabilizing the extending lateral membrane and culminating in formation of tight junctions (TJ) [20]. *In vitro*, blockade or knockdown of integrin $\beta 1$ leads to cytoskeletal disorganization and loss of apico-basolateral polarity [28]. *In vivo*, elimination of integrin $\beta 1$ in the gut leads to defects in epithelial differentiation and proliferation, resulting in a disorganized tissue and post-natal lethality. The mechanisms whereby modulation of integrin $\beta 1$ signaling contributes to epithelial morphogenesis are incompletely understood.

Integrin $\beta 1$ heterodimerizes with one of 12 different α subunits to form a functional receptor [35]. The diverse developmental phenotypes in global integrin $\beta 1$ knockout mice suggest that these heterodimers have distinct

functions [39]. As colon-derived cells differentiate *in vitro*, they undergo a switch from integrin $\alpha 2$ expression to integrins $\alpha 3$ and $\alpha 5$ expression [114]. Interestingly, levels of both integrin $\alpha 3$ and $\alpha 5$ are reduced during human CRC progression [115]. The importance of integrin $\alpha 5$ in epithelial morphogenesis *in vivo* is further noted in systems that rely on branching morphogenesis in a fibronectin-dependent manner, although a mechanistic explanation for this is lacking [116]. The contribution of $\alpha 5$ subunits to epithelial polarity, particularly in the colon, also remains largely unexplored.

Here, examine the impact of integrin $\beta 1$ -activating monoclonal antibodies (mAbs) on CRC cells that exhibit an invasive phenotype when cultured in 3D in type-1 collagen. We have discovered that the Integrin $\beta 1$ -activating antibody, P4G11, blocks the invasive phenotype and restores apico-basolateral polarity. We have performed a detailed characterization of how P4G11 achieves this remarkable reversion. By stabilizing integrin $\beta 1$ at the lateral membrane, P4G11 induces clustering of integrin $\alpha 5$, which in turn leads to lateral deposition and polymerization of fibronectin. Both integrin $\alpha 5$ and polymerized fibronectin are required for the epithelial re-organizing effect of P4G11. Moreover, we detect integrin $\alpha 5$ and fibronectin at the lateral membrane of terminally differentiated colonocytes *in vivo* in the human colon, suggesting the possible physiological relevance of these findings.

III.2 Results

Integrin $\beta 1$ -activating antibody P4G11 reduces invasion and restores apico-basolateral polarity in invasive CRC cells. Integrin $\beta 1$ and type-1 collagen are reported to be important mediators of polarity in 3D [14, 117, 118]. We set out to determine if altering integrin $\beta 1$ function in invasive

CRC cells cultured in type-1 collagen might restore a more normal epithelial architecture. We recently developed a 3D system ideally suited to address this possibility. By placing single cells from a human CRC cell line, HCA-7, in 3D type-1 collagen, we have derived cell lines with two distinct morphological and functional properties: single-layered polarized cysts designated CC or solid spiky masses designated SC. Upon subcutaneous injection into athymic nude mice, CC form indolent, well-differentiated tumors whereas SC form locally invasive tumors [119]. To assess whether integrin β 1 activation might confer a less invasive morphology to SC, we plated single SC cells into 3D type-1 collagen and treated them with a panel of integrin β 1 function-altering monoclonal antibodies (mAbs) on days 1-15 [120]. We were surprised to find that SC colonies treated with P4G11 no longer formed invasive protrusions but rather formed cysts around a single central lumen (Figure 1A). Although treatment with another integrin β 1-activating mAb, 12G10, eliminated invasion and colonies exhibited lumen formation, the cells surrounding the lumen were multi-layered, and many colonies formed several lumens. A third integrin β 1-activating mAb, TS2/16, did not alter colony morphology, even at significantly increased concentrations (data not shown). Upon addition of an integrin β 1-blocking antibody, AIB2, SC colonies appeared as solid masses of cells. Thus, altering integrin β 1 activity shows profound effects on colony morphogenesis in 3D.

We next examined whether P4G11 might restore epithelial polarity in two other CRC cell lines (SW480 and LoVo) that exhibit an invasive morphology when cultured in 3D type-1 collagen. In this experiment, we also tested whether P4G11 might restore a more normal epithelial architecture to

established colonies, so P4G11 was not added until colonies had fully formed. SC, SW480 and LoVo cells were plated as single cells into type-1 collagen and allowed to grow for 8 days, at which time colonies were treated with P4G11 until day 15. Invasion was markedly reduced in all three lines (Figure 1B,C). Lumen formation was observed in SC and SW480 colonies but not in LoVo colonies (Figure 1B,D). Even though P4G11 was not administered to these cells until invasive colonies were fully formed, SC colonies still reverted to single-layered cysts with a central lumen as had occurred when P4G11 was added at the time of plating.

Having established that epithelial architecture is restored by P4G11, we examined its morphological effects on SC in more detail. Immunofluorescent analysis, using ezrin as an apical marker and integrin $\beta 1$ as a basolateral marker, showed that cells in P4G11-treated SC colonies exhibit apico-basolateral polarity (Figure 2A). Using transmission electron microscopy, we determined that P4G11 treatment induces formation of tight and adherens junctions beneath the apical surface (Figure 2B). To better track P4G11-mediated effects, we adopted a 2D system that was amenable to high magnification microscopy. We treated SW480 cells plated on monomeric collagen (MMC)-coated coverglass and found that P4G11 restored tight junction formation and polarity in these cells under these conditions (Figure 12). Thus, we conclude that P4G11-mediated activation of integrin $\beta 1$ restores epithelial junctions and features of apico-basolateral polarity to invasive CRC cells.

P4G11 induces retention of active integrin $\beta 1$ at the cell surface. In order to define the mechanism whereby P4G11 induced these phenotypic

effects, we first confirmed that P4G11 bound human Integrin β 1 using a mouse cell line stably expressing human integrin β 1 (Figure 11A). Treatment of cells with Mn^{2+} increases the amount of active integrin β 1 at the cell surface [121]. To confirm that P4G11 recognizes this active form of integrin β 1, we treated cells with Mn^{2+} and noted that this increased P4G11 binding (Figure 11B). P4G11 and 12G10 both increased the adhesion rate of cells from suspension to a collagen substrate. Further, P4G11 and 12G10 both increased FAK phosphorylation (Figure 11C-D). Thus, we conclude that P4G11 activates a population of integrin β 1.

Both P4G11 and 12G10 target ligand-induced binding sites (LIBS) on integrin β 1, but the effects of these antibodies on live cells are poorly understood. This led us to compare their effects on integrin β 1 localization and stability (Figure 3). We visualized 12G10 and P4G11 localization in SC after 15 days of exposure to these antibodies (10 μ g/ml) in 3D. P4G11 binding extended throughout the basolateral surface of a given cell in the cyst, while the 12G10 signal was primarily basal (Figure 3A). We used confocal microscopy to compare P4G11 and 12G10 internalization and their effect on integrin β 1 degradation. SC cells plated in 2D on MMC-coated coverglass were co-stained with the lysosomal marker LysoTracker and fluorophore-labeled P4G11 or 12G10. A substantial amount of P4G11 was bound at the lateral cell-cell interface at baseline and was still retained there at 24 hrs. 12G10 was detected at the cell surface initially, but appeared to be internalized and co-localized with LysoTracker at 24 hrs (Figure 3B). Immunoblot analysis of total integrin β 1 levels with increasing concentrations of 12G10 or P4G11 for 24 hrs on MMC-coated filters showed increased

integrin β 1 protein degradation in 12G10-treated cells (Figure 3C) compared to P4G11-treated cells. Loss of surface Integrin β 1 following 24 hr 12G10 treatment was further confirmed through cell-surface biotinylation (Figure 3D). From these studies, we conclude that P4G11 induces integrin β 1 retention at the cell surface.

P4G11 morphologic phenotype depends on integrin clustering.

Integrin function-altering mAbs are bivalent and can crosslink integrin β 1 in live cells in a manner that mimics cell-driven cluster formation [122, 123], which is a significant step in the formation of mature adhesion complexes and is critical for certain signaling events [45, 124]. To determine if P4G11 mediates its effects through induction of integrin β 1 clustering at the cell surface, we digested P4G11 into light/heavy chain F(ab)' fragments, which were subsequently conjugated to a fluorophore. P4G11 F(ab)' is capable of binding to integrin β 1 but is not retained at the lateral cell surface (Figure 4A,C). Addition of an antibody against mouse heavy and light chain should rescue bivalency of P4G11 F(ab)' and, as expected, it restores retention of cell-surface integrin β 1 (Figure 4C). To ascertain if the ability to cluster integrin β 1 is necessary for P4G11 to rescue an epithelial morphology in 3D, we treated SC with P4G11 F(ab)', as well as a combination of P4G11 F(ab)' and anti-mouse H+L. We found that loss of bivalency blocked the ability of P4G11 to reduce invasion in SC (Figure 4A,B). Rescue of bivalency through addition of the anti-Ms secondary antibody restored the ability of P4G11 F(ab)' to convert SC to a cystic morphology (Figure 4A,B) and induced basolateral retention of the F(ab)' fragment (Figure 4A, inset). Taken together, these results indicate that P4G11 bivalency is necessary for its ability to

restore apico-basolateral polarity and strongly suggest integrin β 1 clustering at the cell surface drives this P4G11-induced phenotypic switch.

P4G11 treatment results in lateral clustering of integrin α 5. Studies were next directed to determining which alpha subunit was partnering with integrin β 1 at the lateral membrane upon P4G11 exposure. Integrin α 5 expression correlates with a more differentiated state for CRC cells [114, 115]. To determine if integrin α 5 levels correlate with a differentiated cyst-morphology in our CC and SC system, we compared levels of various integrin subunits by immunoblotting. Of the integrin subunits tested, only integrin α 5 protein levels were different and higher in CC than SC. Treatment of SC with P4G11 did not increase integrin α 5 protein levels (Figure 5A). Since recycling and surface retention of integrins are key regulators of integrin signaling, we next asked if P4G11 alters integrin α 5 surface distribution in SC. SC cells were grown on MMC-coated Transwell filters for 5 days, treated with 10 μ g/ml P4G11 on days 5-6, and compared to CC cells grown for 6 days on MMC-coated Transwell filters. We used confocal microscopy to generate an X-Z plane reconstruction through the cell to examine basolateral integrin distribution. We noted higher levels of membrane integrin α 5 staining in CC than SC and an increase in integrin α 5 staining at the lateral membrane following P4G11 treatment (Figure 5B). We did not note obvious changes in lateral integrin β 1 staining (Figure 5B). We treated SC cells grown on MMC-coated Transwell filters with P4G11 over periods of time ranging from 30 min to 24 hrs and noted a gradual increase in surface integrin α 5 at the lateral cell membranes (Figure 5C). Using confocal microscopy, we determined that increased integrin α 5 staining was primarily lateral and brightest in the sub-

apical region (Figure 5B). To determine whether this re-localization was selective for integrin $\alpha 5/\beta 1$, we compared it to the distribution of integrin $\alpha 2$ (Figure 5D), another integrin $\beta 1$ binding partner. We detected integrin $\alpha 2$ along the entire basolateral membrane of SC cells with or without P4G11 treatment. The effect of P4G11 appeared to be selective for integrin $\alpha 5$ as it did not alter the overall distribution of the highly recycled transferrin receptor (Figure 5E). Next, we utilized cell-surface biotinylation to confirm a selective increase in surface integrin $\alpha 5$ in response to P4G11 treatment. SC colonies (treated with or without P4G11 overnight) were labeled with cell-impermeable biotin at 4°C. Cells were then lysed and subjected to streptavidin pull-down to isolate surface proteins followed by immunoblotting for integrins $\alpha 5$, $\beta 1$ and $\alpha 2$ (Figure 5F). Integrin $\alpha 5$ showed the most significant increase in surface levels following P4G11 treatment, whereas integrin $\beta 1$ surface levels increased slightly and integrin $\alpha 2$ surface levels were unchanged. Total cellular levels for all three proteins were unperturbed after P4G11 treatment. These data are recapitulated in 3D (Figure 5G-H). Altogether, these data suggests that P4G11 selectively increased levels of integrin $\alpha 5/\beta 1$ at the lateral cell surface of SC.

Integrin $\alpha 5$ is necessary for apico-basolateral polarity in CRC cells. Given the dramatic difference in integrin $\alpha 5$ membrane localization, we wanted to directly examine its contribution to the P4G11-mediated re-organization process. To this end, we generated a doxycycline (Dox)-inducible integrin $\alpha 5$ knockout system in which an anti-integrin $\alpha 5$ shRNA was induced upon addition of Dox. To lower the likelihood of non-specific observations, we compared the phenotypes of two shRNA constructs, sh1

and sh3, which target different portions of the integrin $\alpha 5$ mRNA. SC and SW480 cells expressing both constructs were generated and the ability of Dox to induce silencing of integrin $\alpha 5$ was confirmed (Figure 6A, Figure 13A). We did not observe any obvious compensatory changes in levels of either integrin $\beta 1$ or integrin $\alpha 2$, despite having to treat with Dox for 4 days prior to plating due to the long half-life of integrin $\alpha 5$ (Figure 6A, Figure 13A). SC-A5sh1 and SW480-A5sh1 cells were grown in 3D type-1 collagen for 15 days in the presence of Dox to determine the contribution of integrin $\alpha 5$ to cell growth and survival in 3D. Silencing integrin $\alpha 5$ resulted in a 70% reduction in SC colony number (data not shown). Cells that survived retained low but detectable integrin $\alpha 5$ (Figure 13A), complicating the analysis of SC in this assay. Thus, in subsequent experiments, we focused on SW480 cells; however, all the trends noted in SW480 were also observed in SC (Figure 13A,B).

We hypothesized that the P4G11-induced epithelial reorganization requires integrin $\alpha 5$. To test this, we examined colony morphology after P4G11 treatment in cells in which integrin $\alpha 5$ expression was silenced. We found that A5sh1- and A5sh3-expressing cells, in the absence of Dox, respond to P4G11 like parental cells (Figure 6B,C). In marked contrast, when integrin $\alpha 5$ was depleted with Dox-induced shRNA A5sh1 or A5sh3, we did not see a reduction in invasion following P4G11 treatment (Figure 6B,C). To better determine if integrin $\alpha 5$ is required for cell polarity and tight junction formation in response P4G11, we treated SW480-A5sh1 cells grown on MMC-coated coverglass with P4G11 in the presence or the absence of Dox. We examined the distribution of ZO-1, a marker of tight junctions after 24 hr exposure to P4G11. We find that P4G11-treated SW480 exhibit some polarity

in the presence of integrin $\alpha 5$ with well formed tight junctions and a general increase in lateral cell/cell interaction (Figure 6D-F). When integrin $\alpha 5$ is depleted by A5sh1, however, this polarization is absent in both P4G11-treated and untreated cells (Figure 6D, F). SW480 cells expressing integrin $\alpha 5$ A5sh3 mirror these phenotypes (not shown). These data suggest that integrin $\alpha 5$ is necessary for P4G11-mediated tight junction formation and epithelial re-organization.

To examine this further, we treated SC, which polarize on Transwell filters and have residual integrin $\alpha 5$ on the cell surface (Figure 5F), for 6 days with JBS5, a mAb that should blocks any integrin $\alpha 5$ signaling by inhibiting the integrin $\alpha 5$ and fibronectin interaction [125]. Cells were fixed and their organization was assessed by confocal microscopy. We find that blockade of integrin $\alpha 5$ signaling abrogates the ability of SC cells to form a polarized monolayer and results in profound actin disorganization accompanied by decreases in E-levels of Cadherin and ZO-1 (Figure 14A-D). Treatment of CC in 3D with JBS5 on days 1-15 results in significantly fewer well-organized colonies (Figure 14E-D). Taken together, these data suggest that integrin $\alpha 5$ contributes to epithelial morphogenesis in these CRC cells. Moreover, we propose that localization and activity of integrin $\alpha 5$, rather than simply net protein levels, mediate these effects.

Lateral deposition and polymerization of fibronectin is necessary and sufficient to induce epithelial polarity. Integrin $\alpha 5$ has been shown to increase cell-cell cohesion through fibronectin deposition [126]. Thus, we asked if lateral integrin $\alpha 5$ clustering induces epithelial polarization through changes in fibronectin localization and polymerization. We treated SW480

cells cultured on MMC-coated coverglass with P4G11 for 48 hrs and probed for lateral ECM deposition. We found that P4G11-treated cells formed large fibronectin fibrils at the cell surface (Figure 7A-C) while the distribution of laminin, which is bound by integrin β 1 paired with integrin α 3 and integrin α 6, is unchanged (Figure 7A). Fibronectin fibrils co-localized with lateral integrin α 5 with the latter residing underneath the TJ marker, ZO-1 (Figure 7A,F). To determine if fibronectin in these fibrils is polymerized, we performed a 1% DOC solubility assay on P4G11-treated SW480 cells, as previously described [127]. While levels of DOC-soluble fibronectin monomer are similar in treated and untreated cells, treatment with P4G11 leads to higher levels of DOC-insoluble fibronectin (Figure 7D). To confirm that integrin α 5 is responsible for the observed fibronectin deposition, we eliminated integrin α 5 in SW480-A5sh1 cells by administration of Dox, and then treated these cells with P4G11. P4G11 no longer induced fibronectin deposition in these integrin α 5 knockdown cells (Figure 7E). SC cells showed a similar re-distribution of fibronectin to the lateral surface after P4G11 treatment (Figure 15C). Together, these data led us to conclude that lateral clustering of integrin α 5 induces fibronectin polymerization.

Polymerized fibronectin has biological properties distinct from its monomeric counterpart. Thus, we wanted to characterize the role of polymerized fibronectin in the restoration of apico-basolateral polarity. It has been shown that serum-derived, soluble fibronectin is necessary to initiate fibronectin polymerization [128]. We find that the fibrils formed by P4G11-treated SW480 cells contain both bovine fibronectin and human fibronectin (Figure 15B). To test whether serum fibronectin is required to initiate fibril

formation and restoration of apico-basolateral polarity, we treated SW480 cells with P4G11 in medium containing fibronectin-depleted serum. We continued to observe lateral integrin $\alpha 5$ clustering, but fibronectin-fibril formation and paxillin re-localization were both disrupted, as was TJ formation. Addition of bovine fibronectin recues fibronectin deposition and TJ formation (Figure 8A). We next blocked fibronectin polymerization using the inhibitor peptide, pUR4B, paired with the control III-11C peptide, as previously described [129]. Concurrent treatment with P4G11 and pUR4B, but not the control peptide, abrogates the ability of P4G11 to induce fibronectin polymerization, lateral paxillin recruitment and tight junction formation (Figure 8B). These data led us to propose a model that lateral, integrin $\alpha 5$ -mediated, fibronectin polymerization contributes to epithelial morphogenesis (Figure 9).

If this model is correct, we expect addition of polymerized fibronectin, but not monomeric fibronectin to recapitulate the P4G11 phenotype in the absence of antibody. We treated SW480 cells with monomeric fibronectin or polymerized superfibronectin on MMC-coated coverglass in the absence of P4G11. Treatment with polymerized superfibronectin, but not monomeric fibronectin, is sufficient to trigger integrin $\alpha 5$ clustering, appearance of lateral fibronectin fibrils and ZO-1 recruitment to TJ (Figure 8C). Overall, these data show that polymerization of fibronectin is not only necessary but sufficient to drive the integrin $\alpha 5$ -induced process leading to epithelial polarity. To assess a possible physiological role for these findings, we examined the distribution of integrin subunits and fibronectin in the human colon. We note that integrin $\alpha 5$ levels are highest at the lateral surface of terminally differentiated epithelial cells, where it co-localizes with fibronectin (Figure 10). Further, we compared

integrin $\alpha 5$ localization in a small number of CRC tumors ($n=3$). We note a lack of membrane integrin $\alpha 5$ in tumor tissue compared to adjacent normal (Figure 16). These findings suggest an *in vivo* role for lateral integrin $\alpha 5$ in human colonic epithelial homeostasis.

III.3 Discussion

It has long been appreciated that integrin signaling is required for the formation of a polarized epithelial monolayer [14, 112, 113]. By binding to the basal ECM, integrin $\beta 1$ is activated. This provides a cue for cell-cell junctions to be established, resulting in the development and maturation of adherens junctions and tight junctions. Recent work has shown that the contribution of integrin $\beta 1$ to apico-basolateral polarity is more complex than this simplified view [28, 34]. Herein, we have discovered that integrin $\beta 1$ -activating mAb, P4G11, is able to restore apico-basolateral polarity to invasive disorganized CRC cells. Using P4G11 as a tool, we show that activation of integrin $\beta 1$ at the lateral membrane leads to clustering of integrin $\alpha 5/\beta 1$. Lateral integrin $\alpha 5$, in turn, results in local polymerization of fibronectin. We demonstrate that inducible knockdown of integrin $\alpha 5$ blocks the epithelial-reorganizing effect of P4G11, and addition of polymerized fibronectin is sufficient to confer this effect.

Antibodies that alter the function of integrin $\beta 1$ have different biological effects in live cell studies. These antibodies bind the integrin $\beta 1$ extracellular domain and elicit conformational changes that either activate or inhibit signaling [120]. Each antibody has a different epitope, many of which are mapped. Recent work has confirmed the existence of a conformationally

inactive form of integrin $\alpha 5\beta 1$ and highlighted differences in receptor conformation in complex with different mAbs [42].

Most of the work characterizing these function-altering antibodies has been related to mapping epitopes and using these to detect conformational changes in the receptor. A notable exception has been work utilizing these mAbs to track how integrin $\beta 1$ activation alters its interaction partners and its rate of endocytosis [130-132]. Herein, we have provided a detailed characterization of the effects of two activating antibodies on integrin $\beta 1$ dynamics. We and others show that P4G11, like 12G10, is a LIBS antibody, thought to bind to and stabilize integrin $\beta 1$ in the extended conformation. Structural studies of 12G10 and TS2/16 have confirmed this activating effect of 12G10 on integrin $\beta 1$ conformation [42]. Based on our findings, we predict that P4G11 has a similar effect on the conformation of bound integrin $\beta 1$. Because they affect receptor conformation in similar ways, the effects of different LIBS antibodies on integrin $\beta 1$ function are thought to be alike. However, this is not the case. Three different integrin $\beta 1$ activating antibodies, 12G10, TS2/16, and P4G11, have distinctly different effects on colony morphology in 3D. Further analysis of 12G10 and P4G11, which both bind high-affinity integrin, show that P4G11 induces receptor clustering at the surface, while 12G10 induces receptor internalization and degradation. Importantly, we do not know if the F(ab)' fragment retains function that is unrelated to restoration of polarity. Overall, these data highlight the need for an in-depth understanding on how integrin $\beta 1$ function-altering mAbs work in live cells. Further, our work identifies P4G11, a rarely used reagent, as a

useful tool to study integrin $\beta 1$ recycling dynamics and activation in epithelial cells.

Integrin $\alpha 5$ is required for epithelialization *in vivo* and is sufficient to increase cell-cell cohesion in suspension *in vitro* [133-135]. The cellular basis for its ability to regulate cell cohesion in the absence of a basal ECM substrate is unclear. Integrin $\alpha 5$ has largely been studied in a variety of fibroblast-like cells, which do not exhibit robust cell-cell interaction. In these cells, integrin $\alpha 5$ regulates multiple cellular processes including mitosis, motility, and proliferation. Integrin $\alpha 5$ can form a complex with ZO-1 and direct its localization, although this also has not been confirmed in epithelial cells [136, 137]. Recent work in colon-derived cells showed that integrin $\alpha 5$ expression correlates with a more epithelial phenotype and negatively regulates signaling pathways associated with disorganization and malignancy [114, 138]. Our data suggests a cellular mechanism for these discrepancies. We show that retention of integrin $\alpha 5$ at the lateral cell-cell interface restores polarity in CRC cells. We do not observe differences in integrin $\alpha 5$ protein level during this transition, suggesting that recycling and activation dynamics are the primary drivers of this process. This effect is integrin $\alpha 5$ specific, as we do not see differences in localization of integrin $\alpha 2$, another integrin $\beta 1$ binding partner. Further, depletion of integrin $\alpha 5$ blocks the polarity process and negatively affects the ability of cells to survive in 3D collagen, in the absence of any effects on levels of integrin $\alpha 2$ and integrin $\beta 1$. Inhibition of integrin $\alpha 5$ signaling in polarizing cells blocks their ability to establish a polarized monolayer. Together, these data lead us to speculate that integrin

$\alpha 5$ signaling is context-dependent and that lateral integrin $\alpha 5$ signaling is required for the epithelialization process.

Interaction of fibronectin with cellular integrin $\alpha 5$ regulates many aspects of cell behavior including migration, growth, and differentiation [116, 139-141]. It is increasingly appreciated that polymerized forms of ECM proteins elicit phenotypic changes distinct from their monomeric forms [81, 82, 128]. Fibronectin assembly into a fibrillar structure is an integrin-mediated process [78, 79, 142]. In fibroblast cells, this occurs through integrin $\alpha 5$ activation, binding of soluble fibronectin, and clustering. This unfolds the fibronectin molecules and allows their N-terminal ends to interact, initiating fibril assembly and polymerization. We show that integrin $\alpha 5$ can mediate a similar process on the lateral surface of epithelial cells. As in fibroblasts, we note deposition of fibronectin in regions of integrin $\alpha 5$ membrane localization, which, over time, results in formation of DOC-insoluble fibronectin. We also note the recruitment of paxilin, previously reported to participate in fibronectin assembly [79], to the regions of fibril deposition. There is some controversy over the ability of other RGD integrins to mediate fibronectin assembly. Our integrin $\alpha 5$ depletion studies support the model that integrin $\alpha 5$ is necessary for this process. To our knowledge, this is the first time that lateral, integrin $\alpha 5$ -mediated, polymerization of fibronectin has been observed in epithelial cells.

Increases in intercellular fibronectin deposition positively correlate with cell-cell cohesion [126]. The cell-cell adhesion proteins that contribute this increase in adhesion have not been identified. Although these previous data are derived from non-polarizing cells, our data supports a fibronectin-

mediated increase in cell-cell interaction. In intestinal cells, lateral deposition of fibronectin at cell-cell junctions has been observed *in vitro* [143]. We note that fibronectin polymerization laterally between cells alters cell morphology and restores tight junction formation and cell polarity. Polymerized fibronectin has anti-metastatic effects and can cluster surface integrin $\alpha 5$ [82, 144]. Addition of polymerized fibronectin to disorganized cells is sufficient to induce integrin $\alpha 5$ clustering and TJ formation, further supporting our model. All together, these observations suggest a role for polymerized fibronectin in epithelial cell-cell adhesion. The dynamics of this process, as well as the proteins that mediate the increased intercellular cohesion, have yet to be characterized.

Integrins and ECM proteins are differentially expressed along the crypt-villus axis of intestinal crypts [145]. Integrin $\beta 1$ surface levels are higher in the proliferative progenitor compartment at the crypt base [102]. Integrin $\beta 1$ is required for proper intestinal organization. Deletion of integrin $\beta 1$ in the intestinal epithelium during development *in vivo* resulted in intestinal epithelial cell hyperproliferation, impaired differentiation, and, ultimately, early post-natal lethality [103]. Intestinal epithelial cells in these animals show impaired endocytosis and a deregulation of pathways that normally regulate intestinal homeostasis. An inducible depletion of integrin $\beta 1$ in the adult gut has not been performed [146].

Little is known about the contribution of integrin $\beta 1$ -binding alpha integrins to intestinal development and homeostasis. We detect a complex of integrin $\alpha 5\beta 1$ /fibronectin at the lateral membrane of the post-mitotic terminally differentiated luminal compartment of the colonic epithelium. Interestingly,

cell-cell connections are known to be tighter at the luminal surface than at the crypt base. These data lead us to propose a model wherein a lateral integrin $\alpha 5$ /fibronectin complex participates in tightening of cell-cell junctions during formation of the well-differentiated compartment of intestinal colonic crypts. Epithelial integrin $\alpha 5$ levels decrease in CRC. It will be noteworthy to determine if loss of lateral integrin $\alpha 5$ contributes to cancer progression and whether retention of integrin $\alpha 5$ at the lateral membrane can halt certain malignant processes.

In summary, our results show that integrin $\alpha 5\beta 1$ clustering at the lateral membrane can induce fibronectin polymerization, and this restores apico-basolateral polarity in invasive, disorganized CRC cells. This adds to a growing body of research supporting the view that Integrin $\beta 1$ and its associated alpha integrins contribute much more to cell behavior than anchoring Integrin $\beta 1$ to the basal ECM.

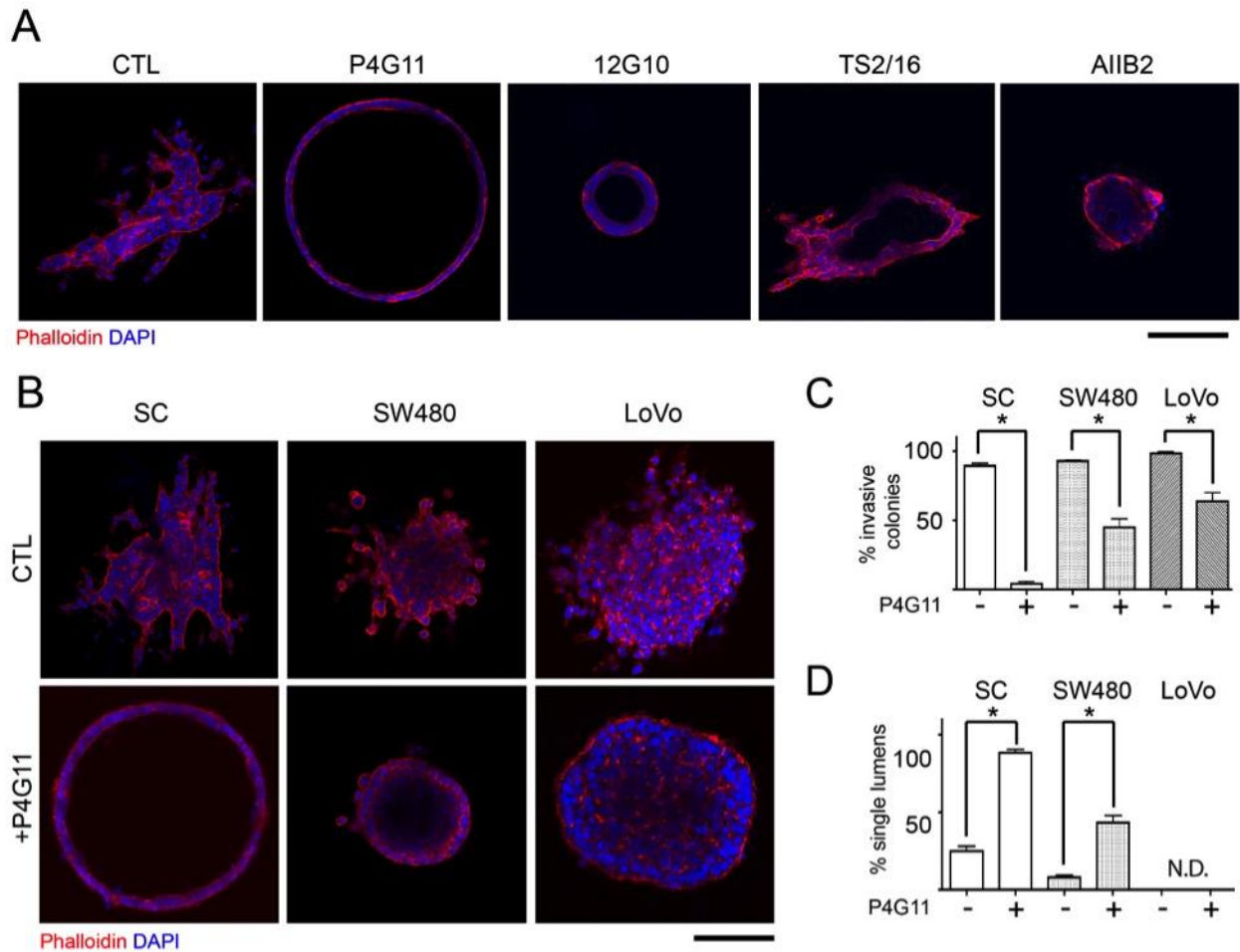


Figure III-1: Integrin β 1-activating mAb P4G11 abrogates invasive phenotype in CRC cells in 3D.

(A) SC cells were plated in 3D type-1 collagen as single cells in the presence or absence of integrin β 1 mAbs (10 μ g/ml) as indicated. The medium was replaced every 2-3 days until day 15 when colonies were fixed and stained with phalloidin (red) and DAPI (blue). Scale bar, 100 μ m. (B) SC, SW480, and LoVo cells were plated as single cells in type-1 collagen and medium was replaced every 2-3 days. At day 8, P4G11 (10 μ g/ml) was added and medium was again changed every 2-3 days until day 15 when colonies were processed as in (A). Scale bar, 100 μ m. (C) Quantification of invasive phenotype of colonies growing in type-1 collagen in the presence or absence of P4G11 (mean \pm SEM; $n > 400$ colonies from three separate replicates). (D) Quantification of percentage of colonies forming a single central lumen in (B) (mean \pm SEM; $n > 400$ colonies in each of three separate replicates). N.D., not detected.

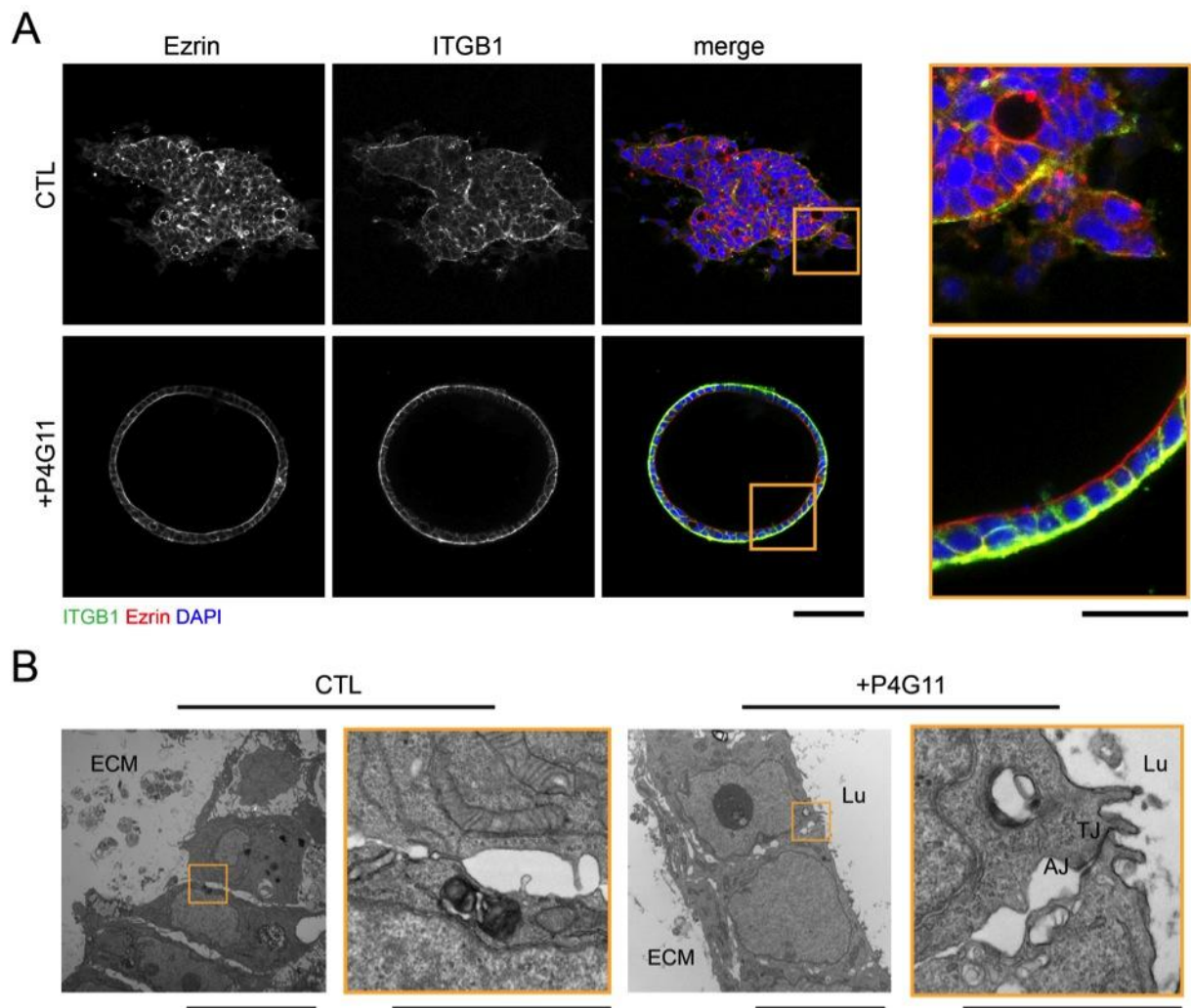


Figure III-2: P4G11 restores apico-basolateral polarity and epithelial cell-cell junctions in 3D.

(A) SC cells were plated as single cells in type-1 collagen and medium was replaced every 2-3 days. At day 8, P4G11 (10 $\mu\text{g}/\text{ml}$) was added and medium was again changed every 2-3 days until day 15 when colonies were fixed and stained with antibodies against Integrin $\beta 1$ (green), ezrin (red) and DAPI (blue). Representative confocal cross section through the equatorial plane of SC colonies are displayed here. Scale bar, 100 μm , 25 μm insets. (B) Representative transmission electron microscopy (TEM) images of SC colonies treated with P4G11. Highlighted sections are displayed at higher magnification on the right of each morphology. Lu, lumen; ECM, extracellular matrix; AJ, adherens junction; TJ, tight junction. Note the appearance of AJ and TJ in the magnified region in SC colonies treated with P4G11. Scale bars, 5 μm ; 2.5 μm insets.

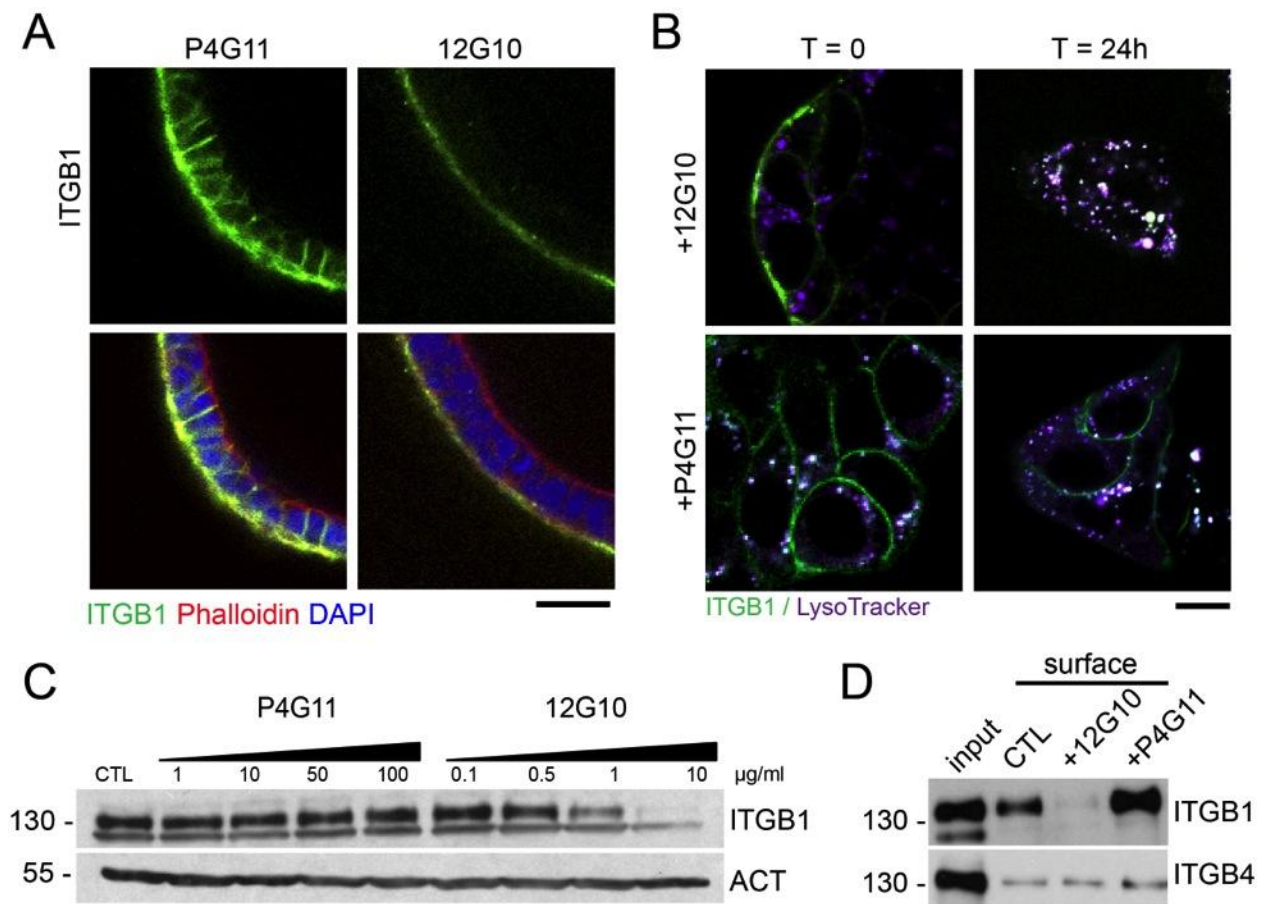


Figure III-3: Differential processing of Integrin β 1 by two Integrin β 1-activating mAbs.

(A) Representative confocal images of SC colonies grown as in Figure 1A, fixed and stained with DAPI (blue), phalloidin (red) and anti-Ms-488 (green) to determine localization of Integrin β 1-bound P4G11 or 12G10 after treatment with antibody on days 1-15. Scale bar, 25 μ m. (B) Representative confocal image of SC grown on MMC-coated coverglass for 48 hrs and stained with LysoTracker-FarRed (LysoTracker) prior to 24 hr treatment with primary labeled P4G11 or 12G10. Initial and final localization of the antibodies show different patterns in the cells, with substantial P4G11 remaining at the cell/cell surface and 12G10 being largely internalized and co-localized with LysoTracker-positive vesicles. Scale bar, 10 μ m (C) SC cells grown on MMC-coated Transwell filters were treated with different concentrations of P4G11 or 12G10 for 24 hrs. The cells were examined by immunoblot analysis with antibodies to Integrin β 1 and phalloidin. Treatment of cells with 12G10 caused decreased cellular levels of Integrin β 1 at highest concentrations (1 and 10 μ g/ml). Data presented are from one of at least three similar experiments with independent SC preparations. (D) Surface levels of Integrin β 1 in SC cells grown on MMC-coated Transwell filters after a 24 hr, 12G10 or P4G11 treatment (10 μ g/ml) were analysed using cell-surface biotinylation and streptavidin immunoprecipitation followed by immunoblot analysis with antibodies against Integrin β 1 and integrin β 4 as a control.

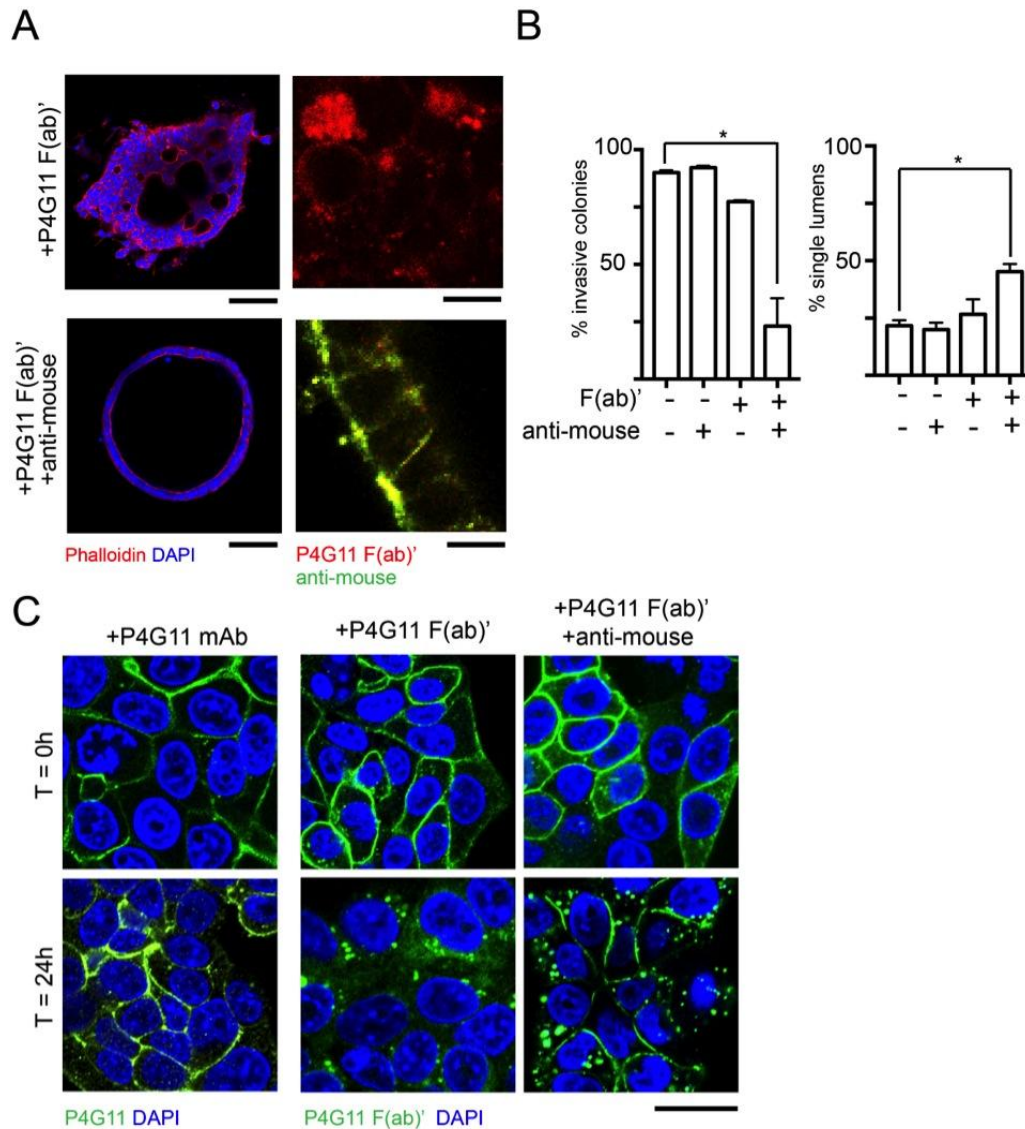


Figure III-4: P4G11 clustering of Integrin β 1 is necessary for rescue of epithelial cyst architecture in SC cells grown in 3D type-1 collagen.

(A) Representative confocal image of SC grown as in Figure 1A. At day 1, P4G11 f(ab)[']-dy594 fragment at 10 μ g/ml in the presence or absence of a 488-conjugated anti-mouse secondary antibody. Colonies were grown until day 15, fixed and stained with phalloidin (red), DAPI (blue). Scale bar, 100 μ m. Inset shows localization of F(ab)[']-dy594 alone (red) or in the presence of anti-Ms (green). Scale bar, 10 μ m. (B) Quantification of the percentage of colonies exhibiting an invasive morphology as well as a single lumen with F(ab)[']-dy594 fragment and with anti-Ms rescue (mean \pm SEM; n > 400 colonies from three separate replicates). (C) Representative confocal image through the midline of SC grown on MMC-coated coverglass and treated with 10 μ g/ml P4G11 mAb, F(ab)[']-dy594, or F(ab)['] with anti-mouse (H+L) chain antibody at 4 $^{\circ}$ C (time = 0). Cells were then incubated at 37 $^{\circ}$ C for 24 hrs. Shown are cells treated at T=0 and T=24 hrs. Note long term membrane retention of F(ab)[']-dy594 in the presence of anti-mouse secondary antibody. Scale bar, 20 μ m.

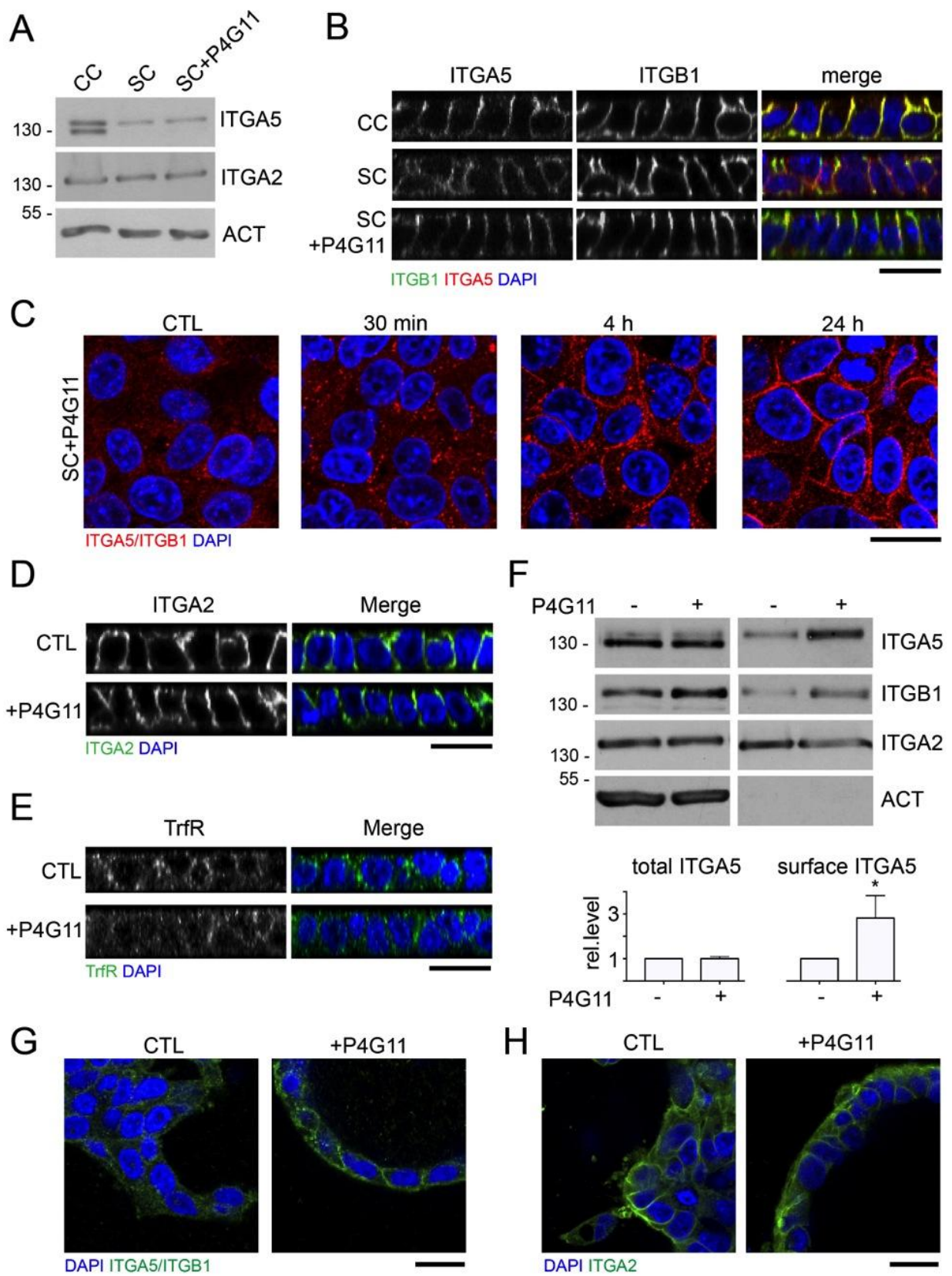


Figure III-5: P4G11 selectively induces membrane localization of integrin $\alpha 5\beta 1$.

(A) CC, SC, and SC treated with P4G11 were grown in 3D type-1 collagen as in Figure 1A. Immunoblot analysis of total levels of integrin $\alpha 5$, integrin $\alpha 2$, and actin are shown (B) Representative XZ plane reconstruction of CC, SC, and SC treated with P4G11 grown on MMC-coated Transwell filters for 5 days and treated with P4G11 on days 5-6 stained with antibodies against integrin $\alpha 5\beta 1$ (red), integrin $\alpha 2$ (green), and DAPI (Blue). Scale bar, 20 μm . (C) Representative confocal image of SC cells treated with P4G11 for indicated lengths of time stained with antibody against integrin $\alpha 5\beta 1$ (red) and DAPI (blue). Scale bar, 20 μm . (D, E) Representative XZ plane of SC cells grown on Transwell filters for 5 days stained with antibodies against integrin $\alpha 2$ (H, green), TrfR (I, green), and DAPI (blue). Scale bar, 20 μm . (F) SC grown on Transwell filters as in (B) underwent surface biotinylation at 4°C followed by lysis and streptavidin pull-down of biotinylated protein. Relative levels of integrin $\alpha 5$, Integrin $\beta 1$, integrin $\alpha 2$, and actin in total and post SA-pull down were analyzed in each fraction by immunoblotting. Levels of integrin $\alpha 5$ were quantified using ImageJ (mean \pm SEM; n=3). (G) Representative confocal image of SC treated with P4G11 in 3D type-1 collagen as in Figure 1A stained with antibody against integrin $\alpha 5\beta 1$ (green) and DAPI (blue). Scale bar, 20 μm . (H) Representative confocal image of SC treated with P4G11 in 3D type-1 collagen stained with an antibody against integrin $\alpha 2$ (green) and DAPI (blue). Scale bar, 20 μm .

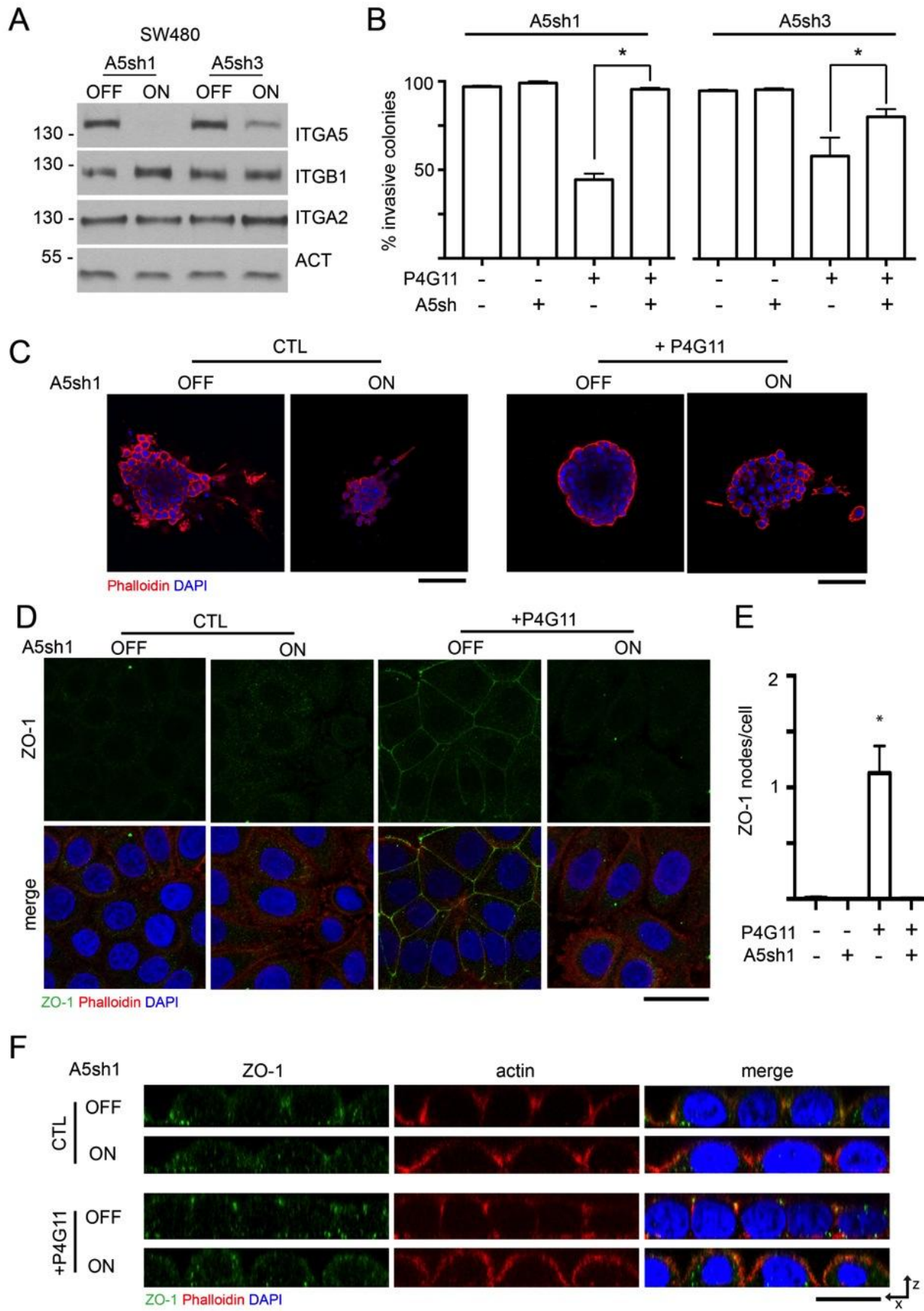


Figure III-6: Integrin $\alpha 5$ is necessary for P4G11-mediated restoration of epithelial junctions *in vitro*.

(A) Immunoblot analysis of total levels of integrin $\alpha 5$, Integrin $\beta 1$, integrin $\alpha 2$, and Actin in SW480 cells engineered to produce anti-integrin $\alpha 5$ shRNA in the presence (ON) or absence (OFF) of doxycycline. Two different shRNAs targeting different parts of the integrin $\alpha 5$ gene were compared to reduce likelihood of a non-specific phenotype (A5sh1 and A5sh3). (B) Quantification of the percentage of invasive SC colonies present when cells in (A) were grown in 3D type-1 collagen and treated with P4G11 in the presence (shA5 +) or absence (shA5 -) of anti-integrin $\alpha 5$ shRNA. Note lack of response to P4G11 in colonies expressing anti-integrin $\alpha 5$ shRNA (mean \pm SEM; n > 300 colonies from three separate replicates). (C) Representative confocal image of cells in (A) grown in 3D type-1 collagen as in Figure 1A and treated with P4G11 in presence (ON) or absence (OFF) of anti-integrin $\alpha 5$ shRNA (A5sh1 shown) stained with phalloidin (red) and DAPI (blue). Scale bar, 100 μ m. (D) Representative maximum intensity projections of SW480 cells grown on MMC-coated coverglass stained with phalloidin (red), DAPI (blue), and an antibody against ZO-1 (green). Scale bar, 20 μ m. (E) Quantification of number of cells exhibiting ZO-1 localization of cell-cell membranes through ImageJ analysis as described in materials and methods (mean \pm SEM; n > 5 20x fields of view from three separate replicates). (F) Representative XZ plane reconstruction of cells in (A) stained with antibodies against ZO-1 (green), phalloidin (red), and DAPI (blue). Scale bar, 20 μ m.

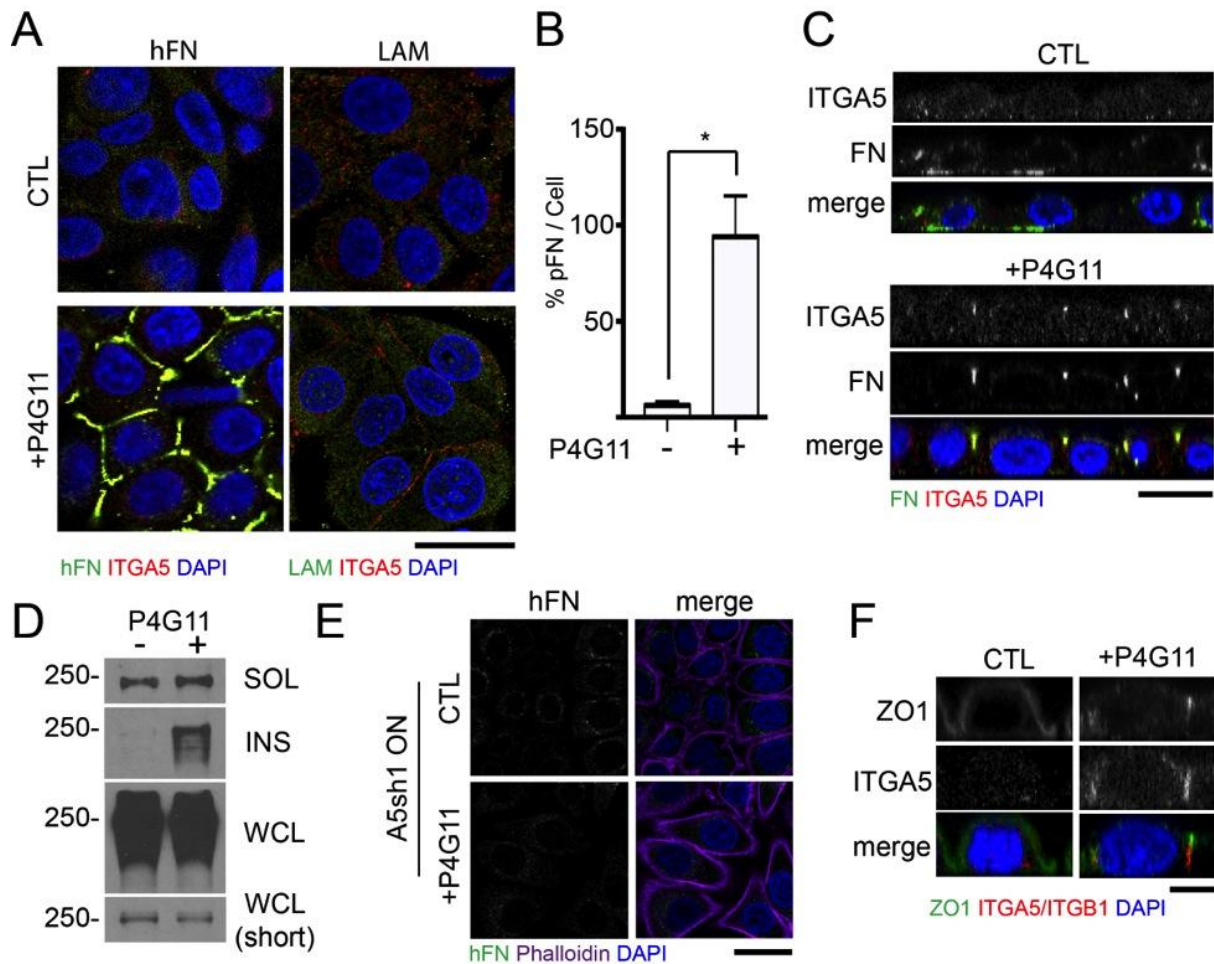


Figure III-7: Integrin $\alpha 5$ clustering leads to fibronectin polymerization at the lateral surface *in vitro*.

(A) SW480 cells were grown on MMC-coated coverglass and treated with P4G11 for 48 hrs. Representative confocal image of SW480 cells stained with antibodies against DAPI (blue), integrin $\alpha 5\beta 1$ (red), and either human fibronectin or laminin (green). Note that only fibronectin is assembled and deposited laterally in response to P4G11 treatment. Scale bar, 20 μm . (B) Quantification of amount of fibronectin fibrils per field of view normalized to cell number as in (A) (mean \pm SEM; $n > 5$ fields of view from three separate replicates). (C) Representative XZ plane reconstruction of SW480 cells stained with antibodies against integrin $\alpha 5\beta 1$ (red), human fibronectin (green), and DAPI (blue). Scale bar, 20 μm . (D) Analysis of fibronectin polymerization in SW480 cells grown as in (A) by 1% DOC solubility assay. (SOL = soluble in 1% DOC; INS, insoluble in 1% DOC; WCL, whole cell lysate) followed by immunoblotting with an antibody against fibronectin. (E) Representative confocal cross section of SW480 A5sh1 cells treated with P4G11 in the presence of Dox and stained with antibodies against human fibronectin, phalloidin (purple), and DAPI (blue). Scale bar, 10 μm . (F) Representative XZ plane reconstruction of SW480 cell-cell junction stained with antibodies against ZO-1 (green), integrin $\alpha 5\beta 1$ (red), and DAPI (blue). Scale bar, 5 μm .

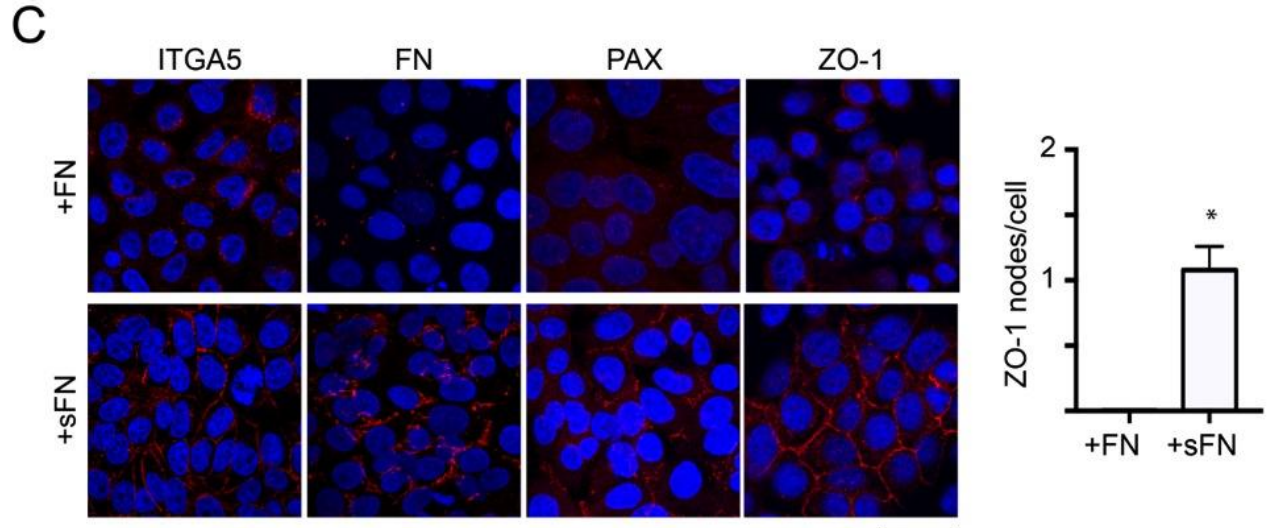
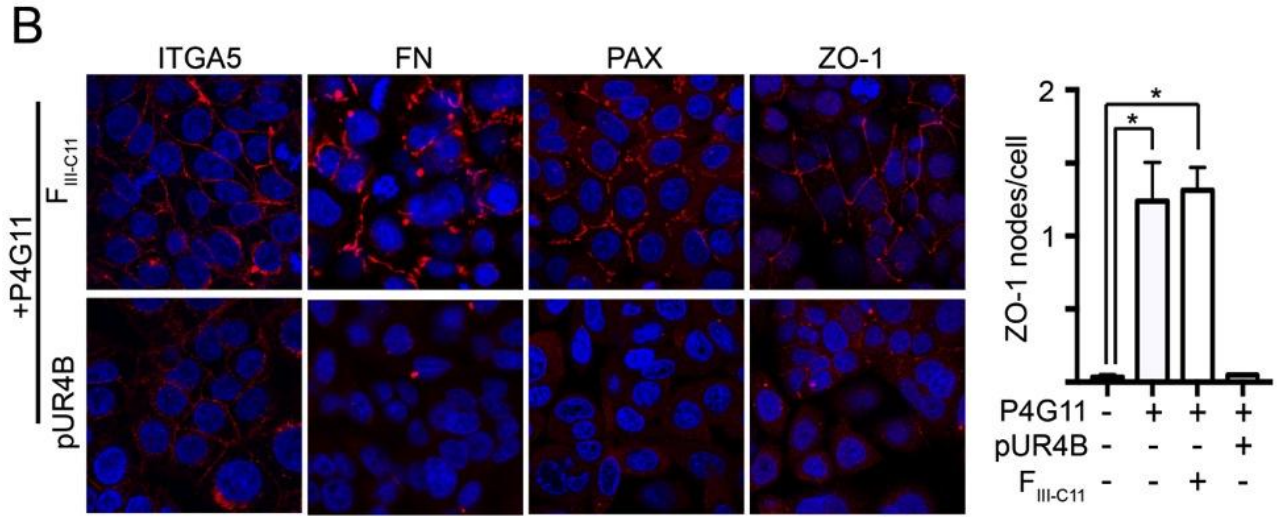
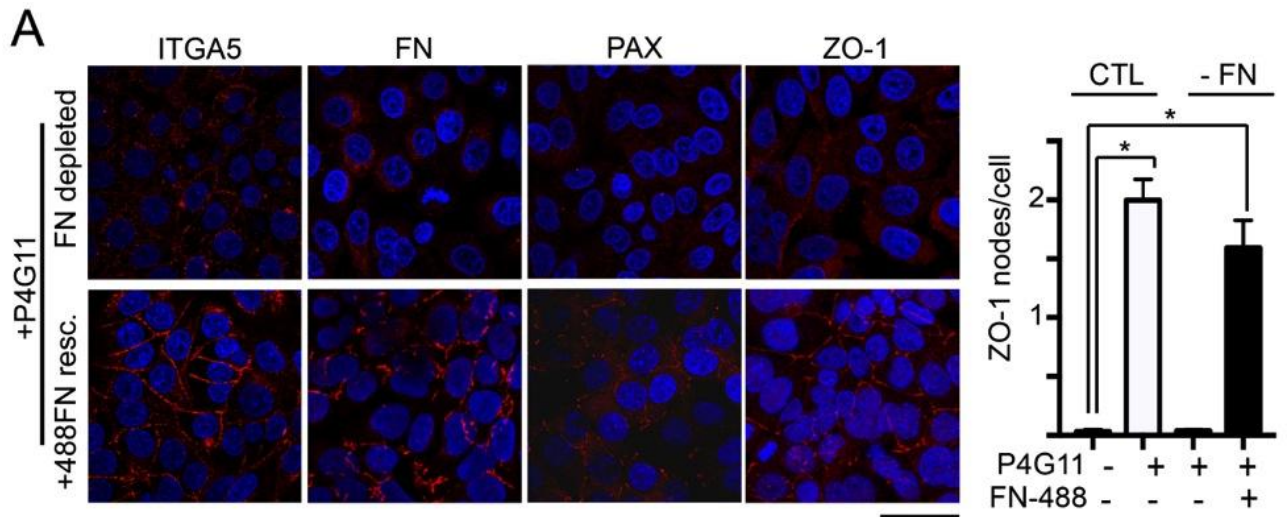


Figure III-8: Fibronectin is necessary and sufficient to induce re-distribution of integrin $\alpha 5$ and paxillin to the lateral membrane and induce TJ formation.

SW480 cells were grown on MMC-coated coverglass and treated for 48 hrs with indicated conditions and stained with antibodies against integrin $\alpha 5$, human fibronectin, paxillin, or ZO-1 (all shown in red) and DAPI (blue). Shown are representative maximum intensity projections generated using confocal optical sectioning and quantification of number of cells exhibiting ZO-1 localization of cell-cell membranes through ImageJ analysis as described in materials and methods (mean \pm SEM; n > 5 20x fields of view from three separate replicates). Scale bars, 50 μ m. (A) Cells were grown in medium depleted of serum fibronectin, treated with P4G11 for 48 hrs with or without addition of 488-bovine fibronectin. (B) SW480 cells were treated with P4G11 for 48 hrs in the presence of fibronectin polymerization-blocking peptide pUR4B or control peptide F_{III-C11}. (C) SW480 cells were treated with polymerized superFibronectin or an equivalent amount of 488-bovine fibronectin for 48 hrs.

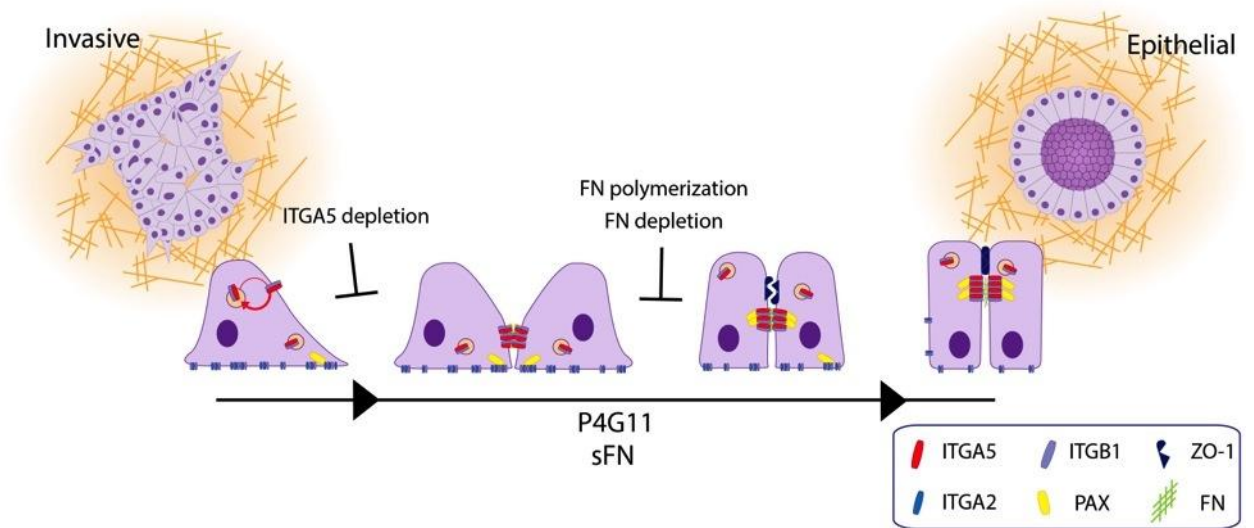


Figure III-9: Model depicting the process of P4G11- and fibronectin-mediated restoration of apico-basolateral polarity.

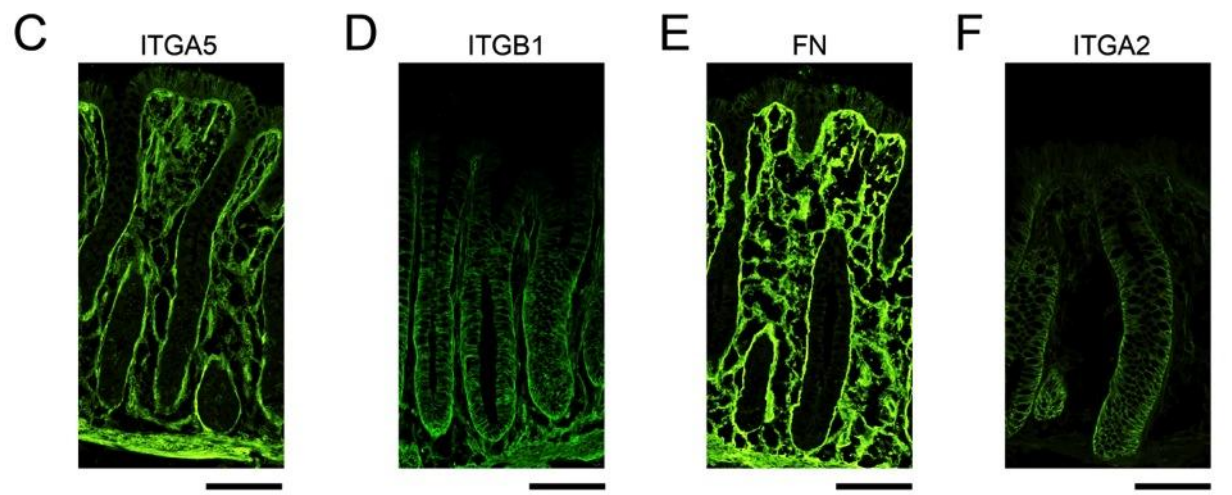
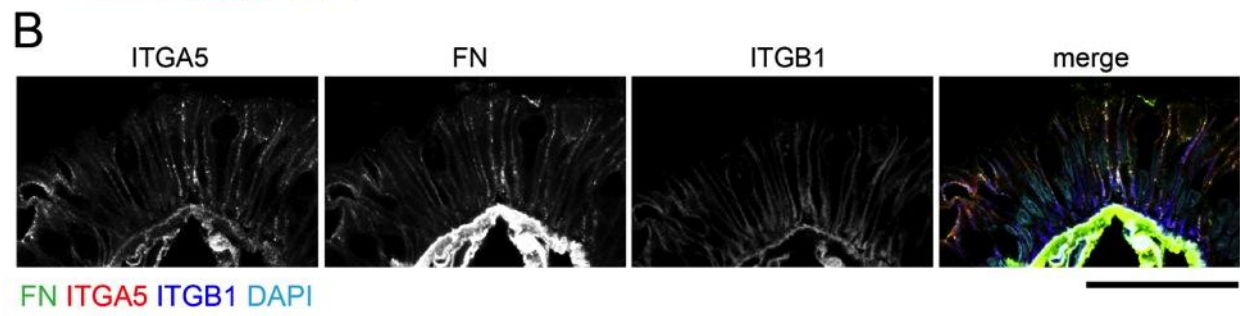
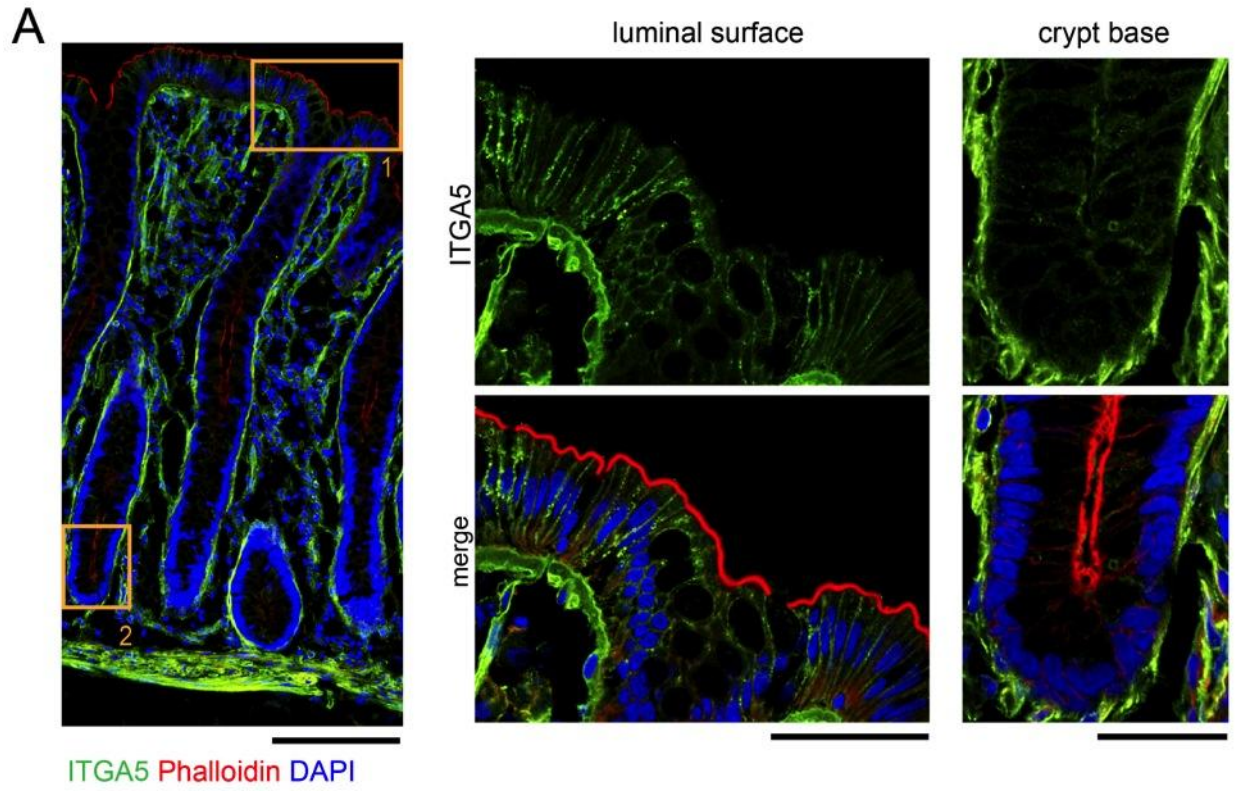


Figure III-10: Integrin $\alpha 5$ is present at the lateral surface in the more differentiated compartment of the normal human colon.

(A) Representative confocal images of a normal human colon section stained with antibody against integrin $\alpha 5$ (green), phalloidin (red) and DAPI (blue) scale bar, 100 μm . High magnification view of differentiated (1) and progenitor (2) regions of crypt as labeled 1 and 2. Scale bar, 50 μm . (B) High magnification of the epithelial cells at the luminal surface on the colon stained with antibody against integrin $\alpha 5$ (red), fibronectin (green), integrin $\beta 1$ (blue) and DAPI (teal) (C-F). Sections of normal human colon stained with antibodies against integrin $\alpha 5$ (C), integrin $\beta 1$ (D), fibronectin (E) and integrin $\alpha 2$ (F). Scale bars, 50 μm .

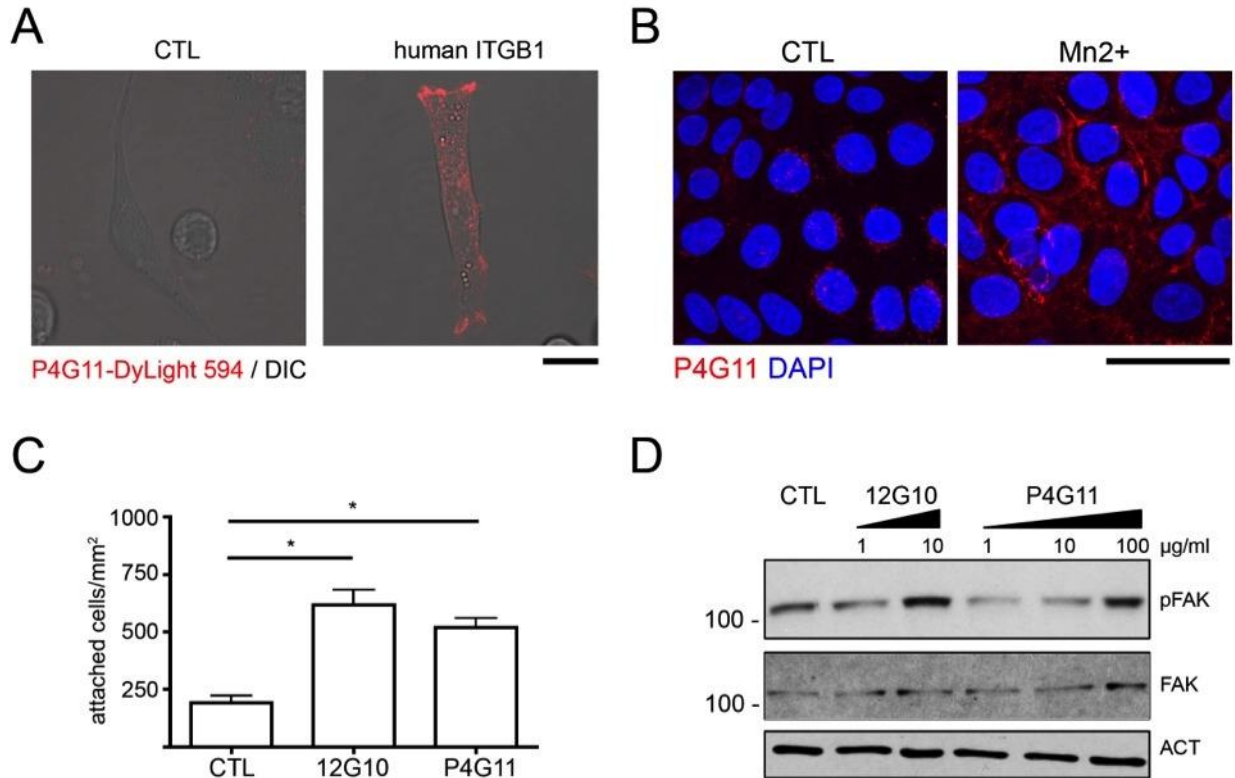


Figure III-11: P4G11 binds human integrin β 1 and activates integrin signaling pathways.

(A) Representative confocal images of P4G11-594 (red) binding pattern in MDCK cells expressing murine integrin β 1 (CTL) or human integrin β 1 with merged DIC (grayscale). Scale bar, 10 μ m. (B) Representative confocal maximal intensity projection of SC cells grown on MMC-coated coverglass and treated with 1 mM Mn^{2+} for 5 min prior to fixation. Fixed cells were permeabilized and stained with P4G11 (red) and DAPI (blue). Scale bar, 20 μ m. (C) Quantification of average number of SC cells adherent to a MMC-coated coverglass surface after 30 min in the presence of indicated mAb (mean \pm SEM, n = 5 fields of view from each of three separate biological replicates). (D) Levels of FAK activation in SC cells grown on MMC-coated coverglass measured by immunoblot analysis of phosphorylated Y392 after a 15 min treatment with indicated concentration of indicated mAb. Analysis was performed using antibodies against pY392 FAK, total FAK, and actin. Notably, changes in pFAK with P4G11 were only detected at 10-fold higher concentrations than used for 3D type-1 collagen rescue and conversion experiments.

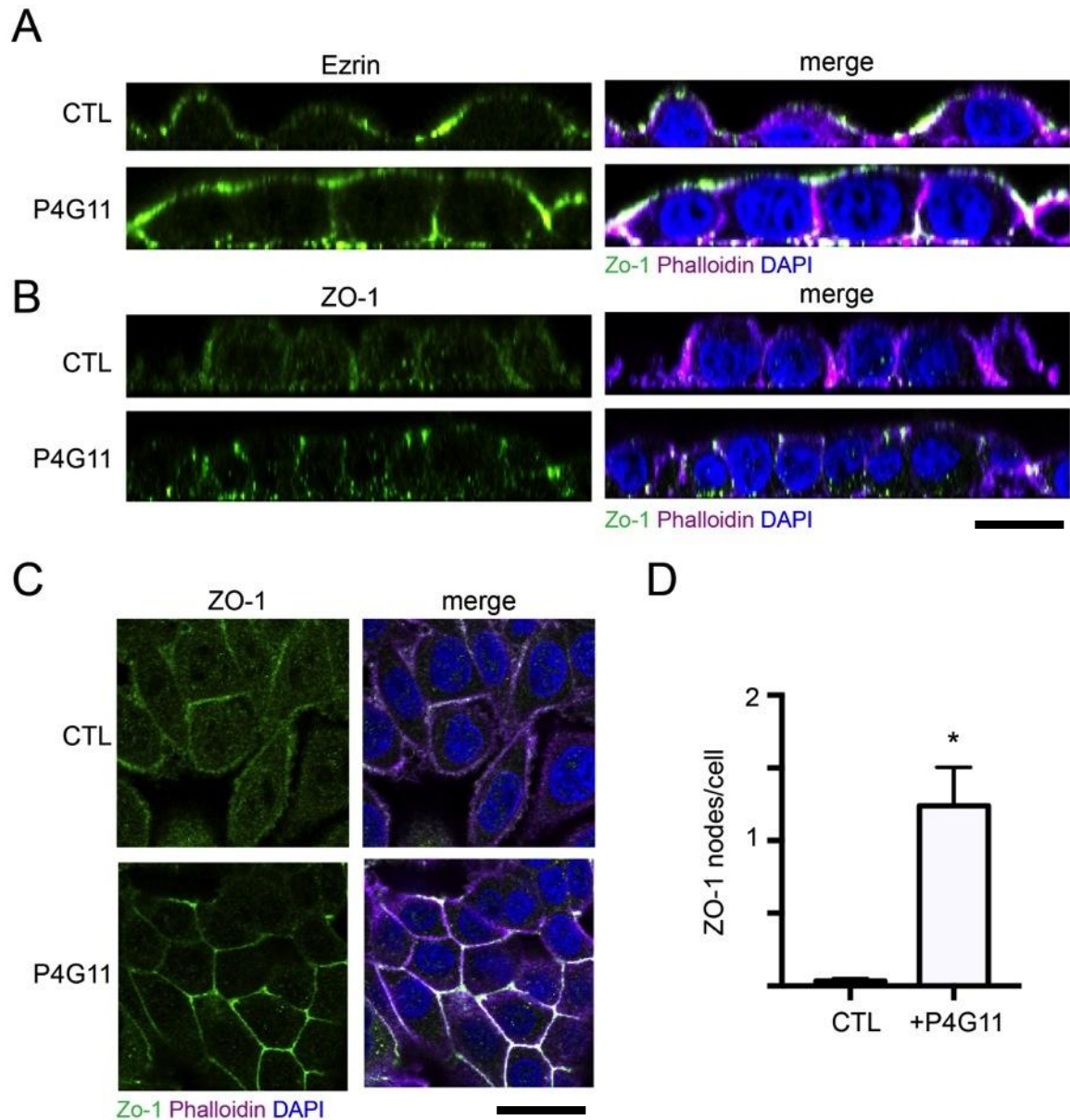


Figure III-12: Treatment of SW480 cells with P4G11 restores apico-basolateral polarity.

SW480 cells were grown on MMC-coated coverglass for 24 hrs and treated with P4G11 for 48 hrs. Cells were fixed and analyzed by confocal microscopy. (A) X-Z plane confocal reconstruction of SW480 cells stained with anti-Ezrin antibody (green), phalloidin (purple), and DAPI (blue). Scale bar, 25 μ m. (B) X-Z plane confocal reconstruction of SW480 cells stained with anti-ZO-1 antibody (green), phalloidin (purple), and DAPI (blue). Scale bar, 25 μ m. (C) Representative confocal cross-section through SW480 cells stained with anti-ZO-1 antibody (green), phalloidin (purple), and DAPI (blue). Scale bar, 25 μ m. (D) Quantification of the number of ZO-1 nodes formed by SW480 cells with and without P4G11 treatment (mean \pm SEM, N = 5, 20x fields of view from three separate biological replicates).

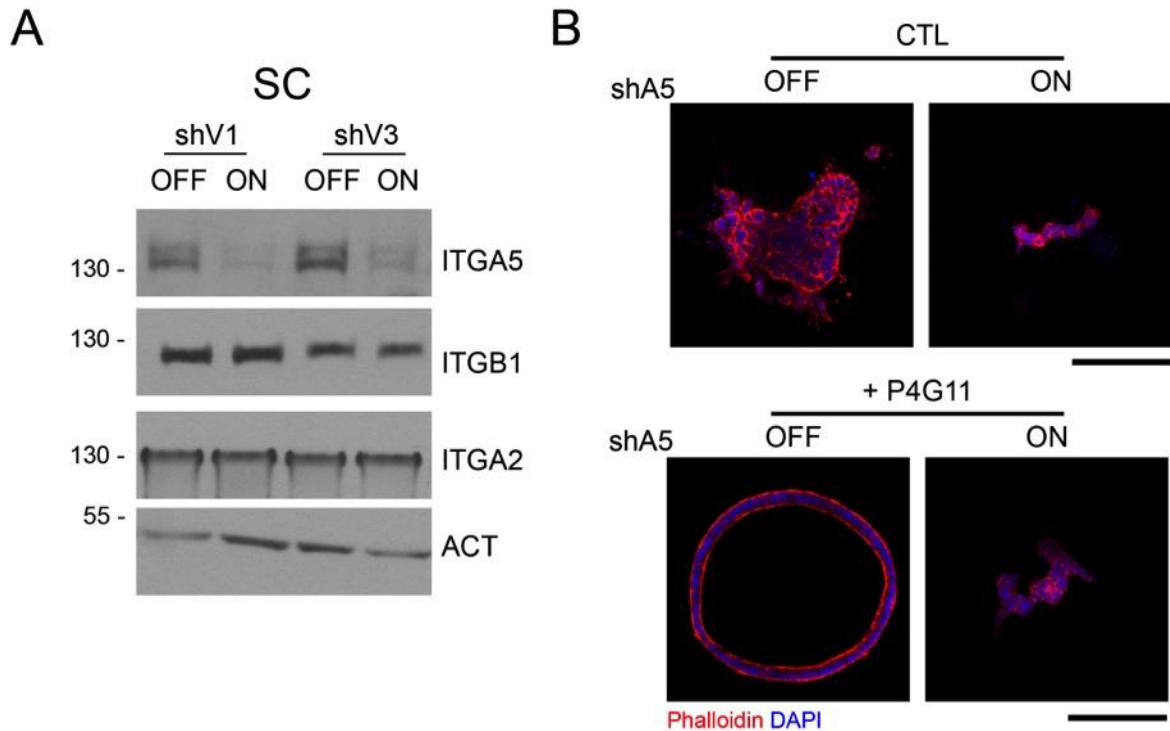


Figure III-13: Integrin α 5 is necessary for P4G11-mediated rescue of epithelial junctions *in vitro*.

(A) Immunoblot analysis of total levels of integrin α 5, integrin β 1, integrin α 2 and Actin in SC cells engineered to produce anti-integrin α 5 shRNA in the presence (ON) or absence (OFF) of doxycycline. Two different shRNAs targeting different parts of the integrin α 5 gene were compared to reduce likelihood of a non-specific phenotype (A5sh1 and A5sh3). (B) Representative confocal image of SCshV1 cells grown as in Figure 1A in presence (ON) or absence (ON) of anti-integrin α 5 shRNA (sh1 shown) stained with phalloidin (Red) and DAPI (blue). Scale bar, 100 μ m.

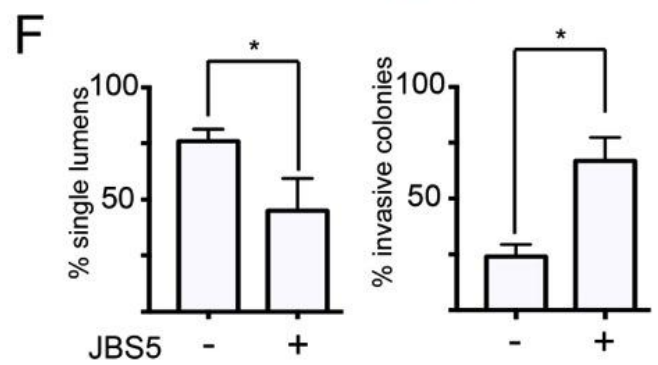
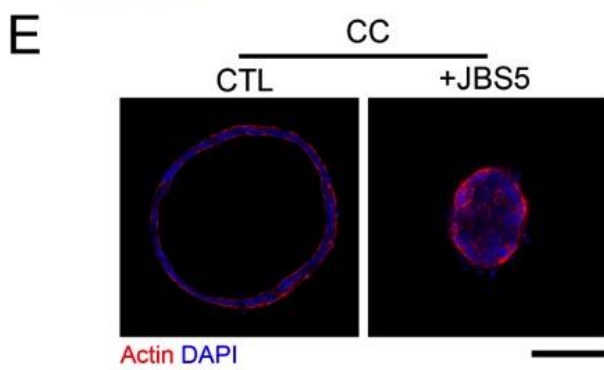
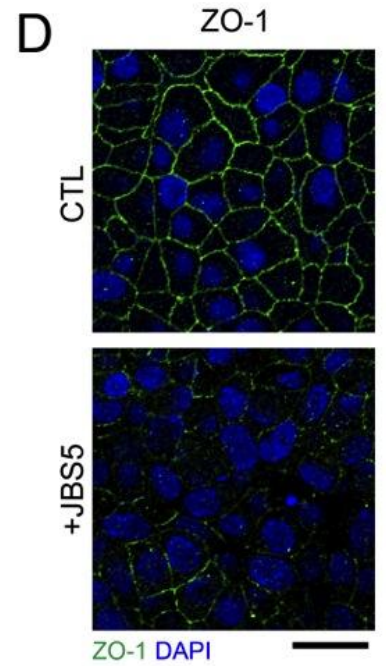
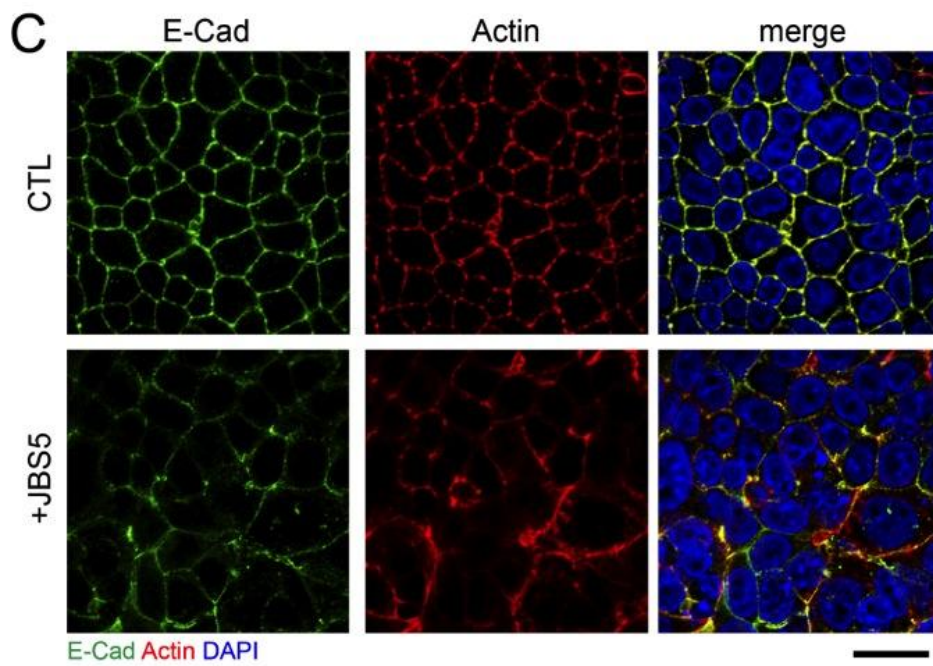
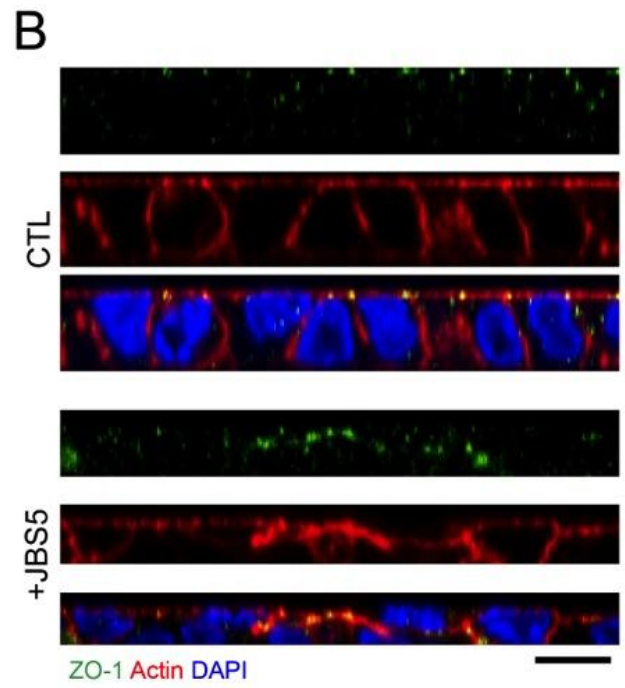
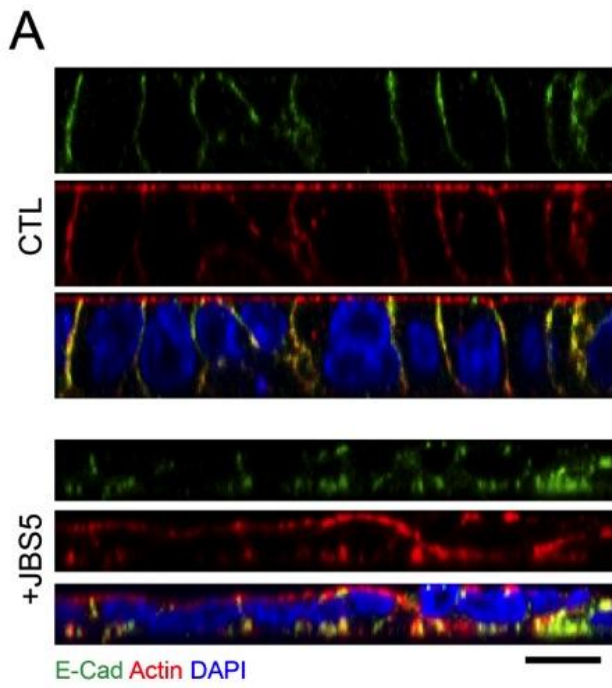


Figure III-14: Blockade of integrin $\alpha 5$ and FN interaction leads to loss of epithelial organization in 2D and 3D.

(A-D) SC cells grown on MMC-coated Transwell filters and treated with integrin $\alpha 5$ blocking antibody JBS on days 2-6 and analyzed by confocal microscopy (A) shown is a representative confocal z-reconstruction of SC stained with anti-E Cadherin antibody (green), phalloidin (red), and DAPI (blue). Scale bar, 20 μm . (B) Representative confocal z-reconstruction of SC stained with antibody against ZO-1 antibody (green), phalloidin (red) and DAPI (blue). Scale bar, 20 μm . (C) Representative confocal image of SC in (A) seen in the XY plane stained with antibody against E-Cadherin (green), phalloidin (red), and DAPI (blue). Scale bar, 20 μm . (D) Representative confocal image of SC in (B) seen in the XY plane stained with antibody against ZO-1 (green), phalloidin (red), and DAPI (blue). Scale bar, 20 μm . (E) Single CC cells grown into mature colonies using a collagen 1 sandwich assay were treated with JBS5 on days 1-15 and stained with phalloidin (red) and DAPI (blue). Scale bar, 100 μm . (F) Quantification of number of CC colonies exhibiting a single central lumen or an invasive phenotype in (E) (mean \pm SEM; n = 3 biological replicates).

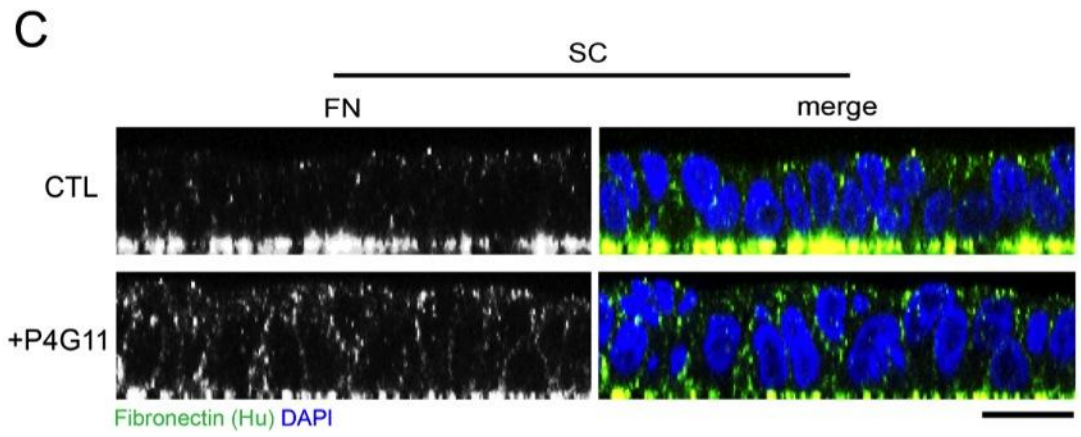
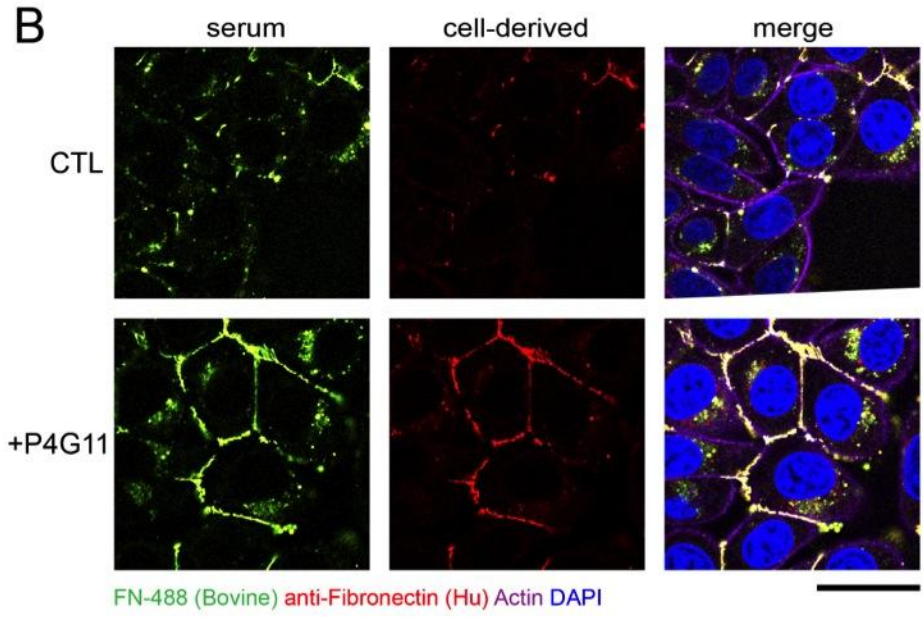
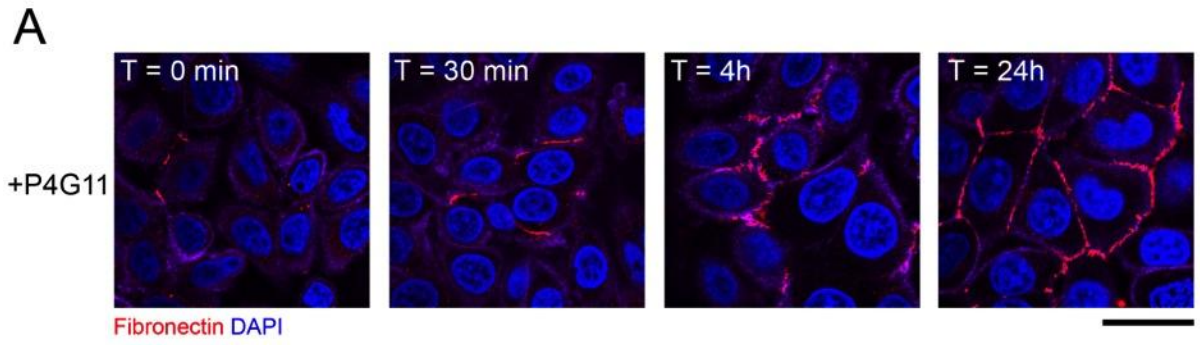


Figure III-15: P4G11 treatment induces fibronectin polymerization in SW480 and SC.

(A) A representative confocal cross-section of SW480 cells grown on MMC-coated coverglass, treated with P4G11 and fixed at different time points were stained with phalloidin (purple), DAPI (blue) and an anti-fibronectin antibody (red). Scale bar, 25 μm . (B) Representative confocal cross-section of SW480 cells grown on MMC-coated coverglass, treated with P4G11 in the presence of 488-fibronectin in the serum for 48 hrs. Cells were stained with an antibody against human fibronectin (red) and DAPI (blue) and fluorescence of serum-derived 488-fibronectin (green) can also be seen. Note that there is significantly more 488-fibronectin signal than that arising from the human fibronectin. Scale bar, 25 μm . (C) SC cells grown on filters for 5 days on MMC-coated Transwell filters were treated with P4G11 days 5-6 and stained with an antibody against fibronectin (green) and DAPI (blue). Scale bar, 20 μm .

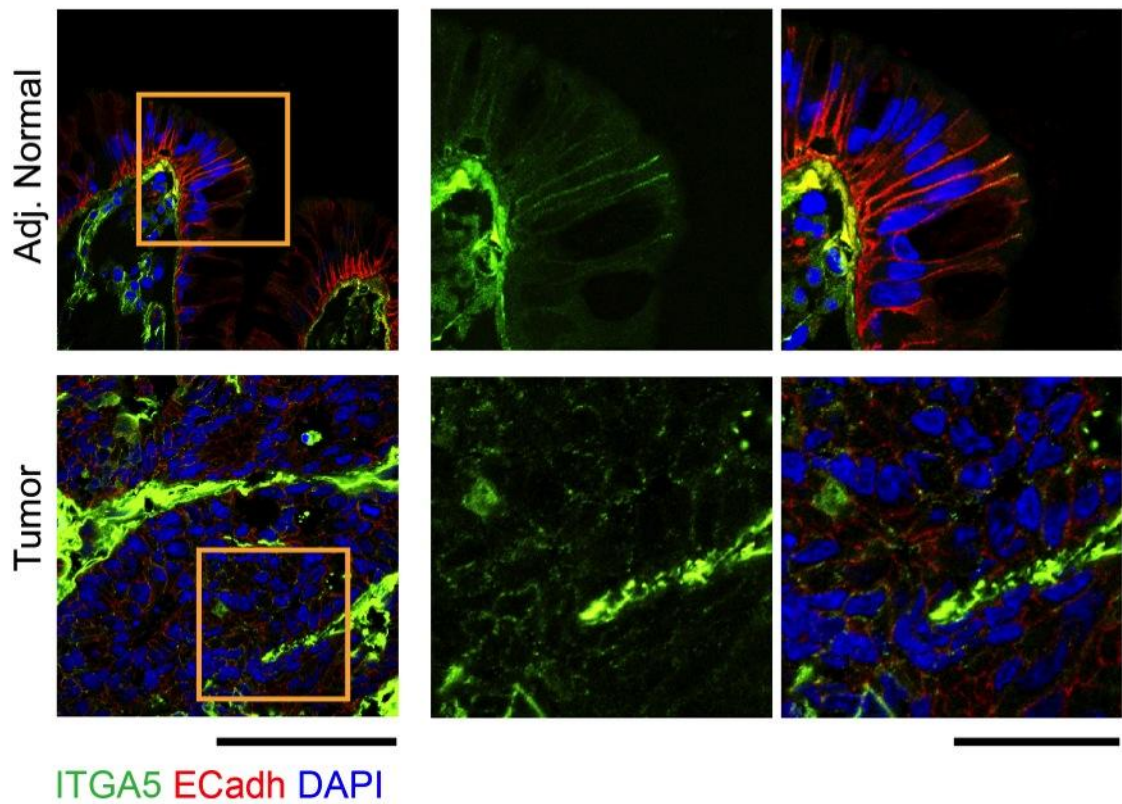


Figure III-16. Integrin $\alpha 5$ is present at the lateral surface in adjacent normal colon but is absent from the surface in tumor.

Representative confocal images of a tumor and an adjacent normal human colon section stained with antibody against integrin $\alpha 5$ (green), e-cadherin (red), and DAPI (blue) scale bar, 100 μm . High magnification view of adj. normal and tumor sections. Scale bar, 50 μm .

Chapter IV: Integrin $\beta 1$ clustering dampens morphologic response of colonic epithelial cells to RTK ligands

IV.1 Introduction

Interactions between cancer cells and their surroundings trigger signaling cues that determine cell behavior and contribute to malignancy [62, 76, 109]. The extracellular environment provides growth factors that govern proliferation, differentiation and motility. Adhesion signaling through the integrin family of ECM receptors has profound effects on how a cancer cell responds to these soluble cues. Recent work suggests that in addition to downstream nodes of crosstalk, direct interactions between integrin $\beta 1$ and EGFR have profound effects on EGFR signaling [147]. How integrin $\beta 1$ surface dynamics and activation play into this process remains an open question.

Receptor tyrosine kinase signaling occurs through binding of a ligand to the extracellular portion of a single pass transmembrane receptor [88, 89]. This causes a conformational shift that results in phosphorylation of specific tyrosine residues on the cytoplasmic domain of the protein. Phosphorylated receptor serves as a platform for activation of signaling coordinators like Ras and PI3K. These signals are further amplified through a downstream cascade of protein-protein interactions, culminating in a broad array of cellular responses. In cancer, sustained signaling through the EGFR and HGF families of receptors supports hyperproliferation and disease progression [83].

Recent work suggests a new, direct, interaction of some RTK with integrin $\beta 1$. This interaction can be both stimulatory as well as inhibiting, and seems to depend heavily on the identity of the integrin $\beta 1$ heterodimerization partner. For instance, integrin $\alpha 5$ seems to negatively regulate EGFR while integrin $\alpha 2$ may be stimulatory [95, 98, 148]. These observations are also tissue specific and studies often have contradictory results. An emerging model suggests that much like RTK ligands stimulate integrin recycling, integrins can, in turn, alter the endocytosis and recycling of RTK receptors in a manner which controls RTK signaling outputs.

In this work, we have begun to explore how cell surface clustering of integrin $\beta 1$ contributes to CRC-derived cell response to RTK ligands EGF, NRG1 and HGF. We show that treatment of cyst-forming CC cells with these ligands in 3D stimulates colony disorganization and invasion, and that this phenotypic response can be blocked using integrin $\beta 1$ activating antibody P4G11. We show that the ability of P4G11 to cluster integrin $\beta 1$ mediates its ability to block both invasion and the ability of EGF to disorganize the mitotic spindle orientation, rescuing epithelial monolayer formation. Clustering of integrin $\beta 1$ at the cell surface results in impaired EGFR endocytosis and phosphorylation in response to EGF stimulation, suggesting a mechanism to explain the P4G11 effect. We hypothesize that integrin $\alpha 5$ membrane retention induced by P4G11 mediates these effects. We further show that treatment of invasive CRC-cell derived xenograft tumors with P4G11 may result in a more glandular organization *in vivo*. Further, we see an additive effect of simultaneous treatment of P4G11 with Cetuximab in 3D. These data suggest a role for integrin $\beta 1$ clustering in dampening of RTK-mediated

signaling. Although these results are unfinished, together they suggest that integrin $\beta 1$ surface activity strongly affects RTK signal transduction and that targeting integrin $\beta 1$ clustering may be a therapeutic approach in CRC.

IV.2 Results

Antibody-mediated activation of integrin $\beta 1$ blocks RTK-mediated disorganization and invasion in CRC cells. In the previous chapter, we showed that anti-integrin $\beta 1$ antibody P4G11 is able to convert invasive CRC cells to a more polarized morphology through integrin $\alpha 5$. We wondered if P4G11-mediated integrin $\beta 1$ activation could also overcome the malignant phenotypes associated with increased signaling through EGF, NRG1 and HGF. First, we wanted to ascertain the ability of these ligands to induce invasion in CC. Thus, we grew single CC cells in type-1 collagen for 8 days and then treated these cells with ligand for 24 hrs. Cells were fixed and analyzed by confocal microscopy (Figure 1). Treatment with EGF, NRG1 and HGF induces invasion in CC cells within this time frame. To confirm that these were invasive protrusions, we looked for the formation of actin and paxillin rich tips in invading cells by immunofluorescence. We find that invading cells, but not control cells, possess these invasive structures (Figure 1B). To determine the effect of these ligands on overall colony organization, we grew CC for 8 days in type-1 collagen and then treated the colonies with 20ng/ml EGF, NRG1 or HGF on days 8-15. After 15 days, colonies were fixed and their morphology was analyzed by confocal microscopy. Figure 2A shows that treatment with EGF, NRG1 or HGF leads to the formation of colonies that lack a central lumen. These colonies also exhibit varying number of cells that either protrude into the surrounding 3D matrix or invade into it as single cells.

These data suggest that all three ligands stimulate an invasive as well as disorganizing program in CC cells in 3D.

Our observation in Chapter III that P4G11 is able to restore polarity to invasive CRC cells made us ask if P4G11 could be protective against the disorganizing effects of EGF, NRG1 or HGF. We co-treated 8 day old CC colonies with 20ng/ml of indicated ligand and 10 μ g/ml P4G11 until day 15. Colonies were fixed and colony morphology was analyzed using confocal microscopy. Figure 2B shows that P4G11 blocks both the disorganizing and invasion-promoting effects of EGF, NRG1 or HGF in CC cells in 3D (Figure 2B,C). Because P4G11 is thought to stabilize integrin β 1 in an active state, we wondered what effect blocking integrin β 1 would have on CC colony morphology with and without concurrent EGF treatment in 3D. We grew CC as described above and treated CC colonies with AIB2 with or without EGF on days 8-15 (Figure 2D). We find that AIB2 treated cells do not form cysts, but rather clusters of cells similar to SC treated with AIB2 (chapter III). Like in Chapter III, AIB2 blocks invasion in CC cells treated with EGF in 3D. From these data we conclude that P4G11 has a dominant effect on CC morphology and dampens the disorganizing and invasion-promoting effects of RTK ligands to favor an epithelial organization program.

Integrin β 1 activation blocks EGF-mediated mitotic spindle misorientation. We observed that CC co-treated with P4G11 and ligand not only maintain a non-invasive phenotype, but also the ability to organize into a monolayered cyst. Thus, we next wanted to determine how P4G11 dampening of EGF, HGF and NRG1 signaling blocked loss of a central lumen and cyst morphology in CC cells. We decided to focus on EGF because

EGFR is our receptor of interest and EGF binds specifically to EGFR whereas NRG1 interacts with ErbB3, which adds complexity into the system. To get a better view of the system, we first wanted to determine whether the phenotypic effects of long term EGF and P4G11 treatment of CC extended to Transwell filters. We plated CC on MMC-coated Transwell filters and grew them for 5 days in the presence of 20ng/ml EGF with and without 10ug/ml P4G11. Cells were fixed, stained with DAPI and Phalloidin and analyzed by confocal microscopy. We find that untreated CC cells form a well-organized monolayer while cells treated with EGF do not. Co-treatment of CC with EGF and P4G11 rescues the formation of a well-organized monolayer (Figure 3A).

We next wanted to determine the effects of EGF and P4G11 on mitotic spindle orientation in CC, because this is critical for the formation of an epithelial monolayer. It was previously shown that EGF deregulates the orientation of the mitotic spindle in epithelial cells [149]. Normally, epithelial cells orient their mitotic spindle parallel to the substrate (Figure 3A,B). Treatment with EGF causes the spindle to orient randomly, resulting in cell multilayering and disorganization (Figure 3A-B). It is also well established that integrin $\beta 1$ participates in the orientation process. To confirm that untreated CC cells grown on Transwell filters divide primarily parallel to the filter, we grew CC cells for 4 days on filters and then fixed cells for immunofluorescence. To determine the angle of the spindle, we used the methodology outlined in figure 3D. Briefly, antibodies against gamma tubulin, alpha tubulin and DAPI were used to localize the location of the spindle microtubule organizing center (MTOC) and kinetochote components. A line was drawn between the gamma-tubulin-rich MTOC poles, and its smallest

angle with respect to the filter substrate was measured (figure 3D). Angles close to zero suggest a parallel plane of division compatible with epithelial monolayer formation. Angles distributed between 0 and 90° suggest disorganization. As expected, the angle of division in untreated CC cells is near 0°. Treatment of CC with EGF on days 4-5 of culture on the filter result in angles ranging from 0 - 90°, confirming that EGF causes spindle disorganization in our cells. Co-treatment of CC with P4G11 and EGF rescues the parallel orientation of the spindle (Figure 3C, E). Thus, activation and clustering of integrin β 1 promotes cell division that promotes monolayer formation in CC.

P4G11 dampens EGFR phosphorylation and endocytosis following EGF treatment. The ability of P4G11 to dampen both invasion and spindle disorganization made us ask if P4G11 had a broad dampening effect on EGFR. Thus, we tested the effect of P4G11 on EGFR phosphorylation and endocytosis in response to EGF in CC. It is well established that EGFR phosphorylation and signaling is mediated by integrin-based cell adhesion. To determine if integrin β 1 clustering dampens EGFR phosphorylation, we pre-treated CC cells with P4G11 for 4 hrs and then applied a 5 min EGF treatment (20 ng/ml). Cells were lysed and levels of phosphorylated EGFR were analyzed by immunoblotting. We found that pre-treatment of CC with P4G11 results in a lower level of phosphorylation of Y1173 on EGFR (Figure 4).

We hypothesized that P4G11-mediated retention of integrin β 1 at the surface impairs the endocytosis of EGFR and leads to lower levels of phosphorylation. It has been previously reported that integrin β 1 controls EGFR recycling [94] and we previously showed (chapter III) that P4G11

induces surface clustering of integrin $\beta 1$. To test if P4G11 decreases EGFR endocytosis post EGF treatment, we grew CC cells on Transwell filters, pre-treated cells with P4G11 for 4 hrs and then treated with EGF for 5 min. We used confocal microscopy to determine EGFR localization following treatment. We find that in control CC cells treated with EGF, the EGFR is internalized following EGF treatment. In cells pre-treated with P4G11, we note less loss of EGFR membrane staining following EGF treatment (Figure 5A-B). These data led us to speculate that surface retention of integrin $\beta 1$ decreases EGFR phosphorylation in response to EGF through impaired endocytosis of the EGFR.

Integrin $\beta 1$ clustering is necessary to block EGF-mediated disorganization. We previously showed in chapter III that P4G11 mediates epithelial polarization through its ability to cluster integrin $\beta 1$ at the cell surface. We wanted to determine if P4G11-mediated clustering of integrin $\beta 1$ is necessary for its ability to dampen EGF-mediated CC colony disorganization in 3D. We treated CC cells in 3D type-1 collagen with EGF in the presence or absence of P4G11 F(ab)' on days 1-15. We fixed the colonies and analyzed their morphology by confocal microscopy. We found that the F(ab)' fragment was not able to rescue EGF-induced disorganization and invasion (Figure 6A-B). These data led us to conclude that P4G11-induced clustering of integrin $\beta 1$ is necessary to block EGF-induced colony disorganization and invasion in 3D type-1 collagen.

P4G11 blocks loss of membrane ITGA5 following EGF, NRG1 and HGF treatment. Based on our work in chapter III, we predicted that physical interactions between P4G11-clustered integrin $\alpha 5$ and EGFR mediates

dampening of EGFR phosphorylation and endocytosis. Integrin $\alpha 5$ has been identified both as a positive and a negative regulator of EGFR, seeming in a tissue-dependent manner. Direct interaction of integrin $\alpha 5$ and EGFR regulates EGFR endocytosis and activity. We hypothesized that integrin $\alpha 5$ and EGFR coordinately regulate one another's recycling. Thus, increased RTK signaling would cause integrin $\alpha 5$ endocytosis along with the associated RTK while surface clustering of integrin $\alpha 5$ would retain the associated RTK at the cell surface.

We first wanted to determine if RTK signaling induces loss of membrane integrin $\alpha 5$. We grew CC cells on filters for 5 days in the presence of EGF, NRG1 and HGF. Cells were fixed and localization of integrin $\alpha 5$ was determined by confocal microscopy. We do note a decrease in membranous integrin $\alpha 5$ in treated cells (Figure 7A). To determine if P4G11 treatment blocks the loss of integrin $\alpha 5$ from the lateral membrane, we performed the previously described experiment in the presence of P4G11. We find that P4G11 treatment prevents loss of integrin $\alpha 5$ from the lateral cell membrane (Figure 7B). These data led us to conclude that retention of integrin $\alpha 5$ at the cell surface correlates with the ability of P4G11 to maintain epithelial polarity in EGF, NRG1 and HGF treated cells. Because we have previously shown that surface clustering of integrin $\alpha 5$ is sufficient to induce epithelial polarity in CRC cells, we hypothesize that surface clustering of integrin $\alpha 5$ is a dominant determinant of polarity and overrides the disorganizing signaling from EGF, HGF and NRG1. Further work directly testing whether surface integrin $\alpha 5$ is necessary and sufficient to dampen RTK signaling and promote an epithelial morphology is necessary to test this hypothesis. It will also be useful to

determine whether integrin $\alpha 5$ directly interacts with and negatively regulates EGFR as has been reported, and whether these effects extend to ErbB3 and MET.

P4G11 treatment of SC tumors in vivo does not decrease tumor size but does result in better organized tumors. Having established that P4G11 is able to dampen colony disorganization and cell invasion in CC cells in 3D, we wanted to test its ability to do so in vivo. We chose to use SC cells as our model tumor forming cell line, because they have previously been shown as invasive *in vivo* and because they have the strongest response to P4G11. In chapter III, we show that of several CRC lines that form disorganized colonies and are invasive *in vivo*, SC undergo the largest switch from a disorganized colony to one exhibiting apico-basolateral polarity. We injected 1 million SC cells in PBS subcutaneously into nude mice (8 total) and allowed the cells to form a palpable tumor, which took about 7 days. At day 7, we began administration of 150ug/animal P4G11 in PBS or PBS alone through intraperitoneal (IP) injection (4 treated, 4 untreated). Injections were performed every 2-3 days for three weeks. The overall health of the mice was observed during this time, and they were weighed daily. We did not notice any adverse effects of P4G11 treatment on the overall health of the mice. At the conclusion of 3 weeks, the tumors were excised, weighed, split in half and fixed in 4% PFA, for frozen section analysis, and formalin, for paraffin embedding. We did not notice a significant change in the final size of the tumors (Figure 8A,B). These data were not surprising, as we have never observed an effect of P4G11 on proliferation in 3D.

We next wanted to determine if P4G11 treatment resulted in decreased invasion or a more glandular tumor organization. In human tumors, a more glandular organization would be defined as a tumor where cells are still organized in recognizable epithelial structures. These tumors tend to have a better prognosis. We first needed to confirm penetration of P4G11 into the tumor. Because nude mice do not produce their own antibodies, we were able to stain with an anti-mouse secondary antibody that detected the mouse-derived P4G11. Although there is some cross-reactivity with what looks like matrix components in both groups (Figure 8C), we note membrane staining at the lateral surface of SC cells in P4G11-treated tumors which is absent in control tumors (Figure 9C).

We wanted to know if the P4G11 tumors were better organized. We examined H&E sections of treated and untreated tumors and noted that there were more lumens and that the lumens appeared larger. Additionally, the P4G11-treated tumors were generally better encapsulated with ECM (Figure 8D). To determine if this correlated with increased polarity in the cells surrounding these lumens, we examined the localization of E-cadherin and actin in sections of these tumors. We noted that there were more cells with apical actin and basolateral E-cadherin in P4G11-treated tumors (Figure 8E). We had hoped to gauge the ability of P4G11 to block invasion *in vivo*. Unfortunately, the SC tumors formed were not strongly invasive in either group, and therefore we could not make any deductions on this topic. From this data, we concluded that P4G11 is sufficient to induce a more polarized phenotype, but that treatment with P4G11 alone is not sufficient to make a significant impact on tumor *in vivo*. Repeating this experiment with a larger

sample size, different temporal organization, or different CRC cell lines may yield stronger results.

P4G11 and Cetuximab co-treatment results in smaller, less invasive, colonies in 3D. Adhesion signaling through the integrin family of receptors is an important mediator of receptor tyrosine kinase (RTK) signal transduction. We wanted to determine if altering integrin $\beta 1$ function in CRC-derived epithelial cells would alter the way these cells respond to RTK inhibition. We utilized the CC system described previously to address this question. We plated CC as single cells suspended in 3D type-1 collagen for 15 days and grew them with or without EGFR blocking antibody Cetuximab (CTX) for 15 days, replacing media every 1-3 days. We noted a >90% reduction in colony number after 15 days of culture. Interestingly, treatment of CC cells with identical concentrations of CTX in standard 2D culture does not result in a similar reduction of growth and survival (PNAS paper). CC cells plated on 2D plastic and treated with CTX on days 1-4 of culture proliferate and reach confluency. This observation led us to conclude that integrin $\beta 1$ and EGFR regulate survival and proliferation in 3D.

These data made us hypothesize that increased integrin $\beta 1$ signaling sensitizes CC cells to EGFR inhibition. Thus, we wanted to determine if treatment of SC with P4G11 would sensitize these cells to CTX. We grew SC cells in 3D type-1 collagen as described previously. Cells were treated with CTX and P4G11 alone and together. Looking at the morphology of SC under these conditions, we observed that SC grown in 3D type-1 collagen and treated with CTX on days 1-15 maintain their disorganized, invasive morphology, although they seem to be smaller. P4G11 treatment of SC on

days 1-15 results in the restoration of polarity, but we have not been able to detect an effect on proliferation in 3D. The combination of CTX and P4G11 treatment (days 1-15) does not alter the number of colonies formed, but does result in the formation of significantly smaller, non-invasive colonies (Figure 9). These data suggest that targeting EGFR and integrin β 1 pathways in CRC may have a beneficial effect clinically.

IV.3 Discussion

An epithelial cell uses external growth factor and mechanical signals to regulate its polarity [9]. Nodes of crosstalk between these pathways facilitate their integration into a single phenotypic response. For example, adhesion to a substrate by integrins is necessary for robust RTK signaling and activation of RTK signaling alters integrin adhesion [94] and cell motility. Research into crosstalk between the two pathways has mostly focused on downstream mediators. Recent work has shown that integrin regulation of EGFR and other RTK receptors is more complex than this, and that cell surface interactions between RTK and integrins add an additional layer of reciprocal regulation to the signal transduction system [94]. Here, we have discovered that induction of integrin β 1 clustering using an anti-integrin β 1 activating mAb P4G11 blocks EGF-, NRG1- and HGF-induced invasion and disorganization. We show that clustering of integrin β 1 dampens EGFR endocytosis and phosphorylation, and blocks the ability of EGF to induce spindle misorientation and integrin α 5 internalization. We found that P4G11 treatment confers a more glandular tumor organization *in vivo* and that coupled inhibition of EGFR with P4G11 results in smaller, well-organized

colonies *in vitro*, suggesting a clinical significance for our findings. Further work is necessary to link some of these observations into a single story.

Members of the EGFR and MET families are important targets in cancer, and their activities correlate with tumor induction and maintenance. Less is known about the role of integrin $\beta 1$ in tumor progression, although it is required for growth and motility of tumor cells *in vitro*. The results we present here suggest that integrin $\beta 1$ dynamics regulate cell response to RTK stimulation. Cooperation between integrin and RTK, particularly EGFR, signaling in tumor progression has been extensively reported, but has focused on levels rather than recycling or activation dynamics on integrin $\beta 1$. Our work suggests that redistribution, rather than changes in protein level, of the integrin is sufficient to drive changes in cellular response to RTK ligand stimulation.

Formation of a surface complex containing both EGFR and integrin $\beta 1$ facilitates crosstalk and coordinate recycling of the two proteins [74]. Depletion of integrin $\beta 1$ impairs baseline EGFR recycling, which is necessary for signal transduction. Conditional deletion of integrin $\beta 1$ *in vivo* impaired both EGFR signaling as well as general recycling from the basolateral surface [151], suggesting that integrins may be dominant mediators of this process in epithelial cells. This corresponds with our observations that clustering of integrin $\beta 1$ affects signaling through broad range of RTK receptors, including EGFR, ErbB3, and MET which bind the ligands EGF, NRG1 and HGF. Together, these observations suggest that dynamics of integrin $\beta 1$ recycling may function to regulate dynamics of endocytosis and recycling of RTK and thus regulate their ability to transduce signals to the interior of the cell.

Integrin $\alpha 5$ directly binds to and controls signaling activities of EGFR [95,97,151]. Integrin $\alpha 5$ has long been known to control proliferative and migratory responses to EGFR ligand EGF. It has been suggested that active EGFR forms a complex with integrin $\alpha 5$. This interaction has been reported as stimulatory in some cases and inhibitory in others. Integrin $\alpha 5$ is required for the pro-survival Akt signaling induced by EGF. Simultaneously, interaction of integrin $\alpha 5$ with EGFR through a specific set of glycosylation moieties acts to block its endocytosis and phosphorylation following EGF treatment [95]. Our work suggests that these discrepancies might be resolved through observation of integrin $\alpha 5$ dynamics rather than expression levels. We hypothesize that EGFR and integrin $\alpha 5$ are coordinately recycled. Activation and endocytosis of EGFR causes removal of surface integrin $\alpha 5$ and vice versa. Thus, in a system where integrin $\alpha 5$ is rapidly recycled, such as in a mesenchymal cell, it may act to enhance EGFR signaling. In epithelial cells where integrin $\alpha 5$ is stably at the surface as we propose in chapter III, it lowers EGFR endocytosis and thus acts in an inhibitory fashion. We propose that P4G11 mimics the later case by clustering integrin $\alpha 5$ at the lateral surface and thus dampening EGFR signaling. The ability of P4G11 to also dampen effects of HGF and NRG1 suggests that integrin $\alpha 5$ plays a larger role in RTK signal regulation that previously recognized.

Our data suggests that integrin $\beta 1$ clustering can be clinically useful in some disease settings. We show that treatment of tumors with P4G11 in nude mice results in better differentiated tumors and that combining P4G11 with EGFR inhibition has synergistic effects on colonies in 3D. Optimization of the P4G11 target would be necessary to improve its potency *in vivo*.

In summary, our results indicate that integrin $\beta 1$ clustering has profound effects on RTK signal transduction in CRC cells. Although the connection between EGFR and integrin $\alpha 5$ in our system remains untested, we are confident that further analysis in this direction will uncover a novel and broad mechanism of RTK signaling regulation by integrin surface dynamics.

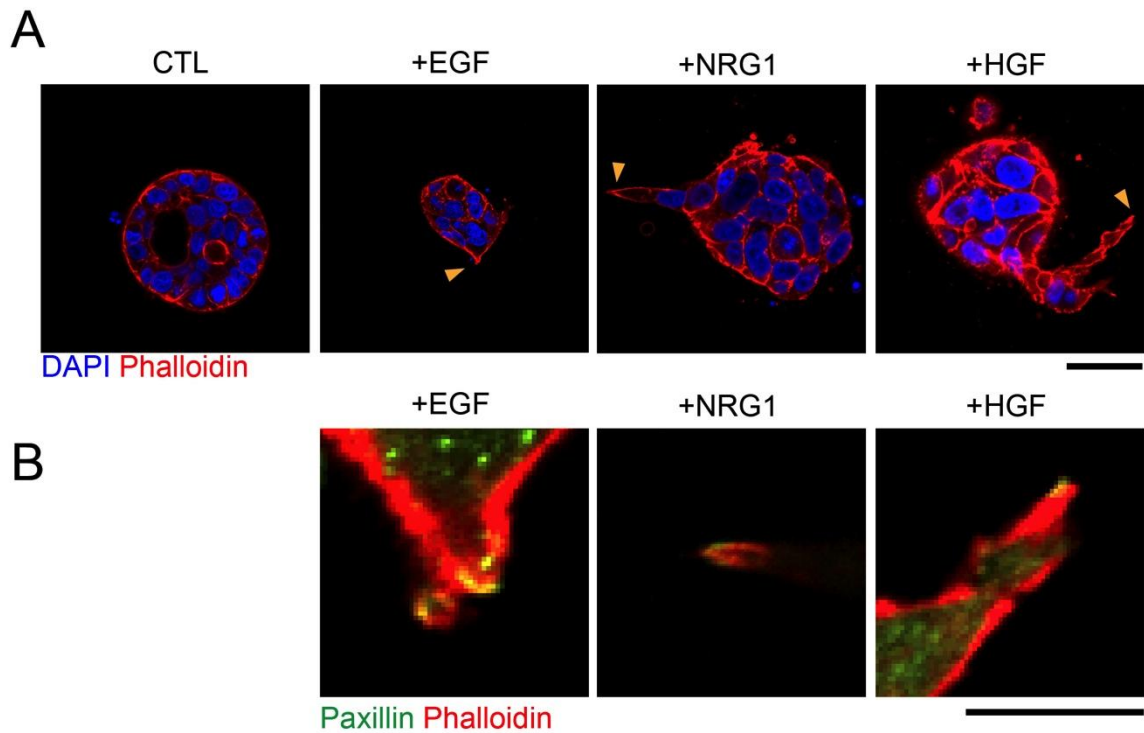


Figure IV-1: Treatment of CC with EGF, NRG1 and HGF leads to an invasive phenotype in in 3D.

(A) CC cells were plated in 3D type-1 collagen as single cells until day 8. At day 8, the indicated RTK ligands (20 nM) were added to the grown media. The medium was replaced every 2-3 days until day 15 when colonies were fixed and analyzed using confocal microscopy. Colonies with stained with phalloidin (red) and DAPI (blue). Scale bar, 25 μm (B) Regions of colonies indicated with arrows in (A) showing localization of paxillin (green) and phalloidin (red). Scale bar, 10 μm .

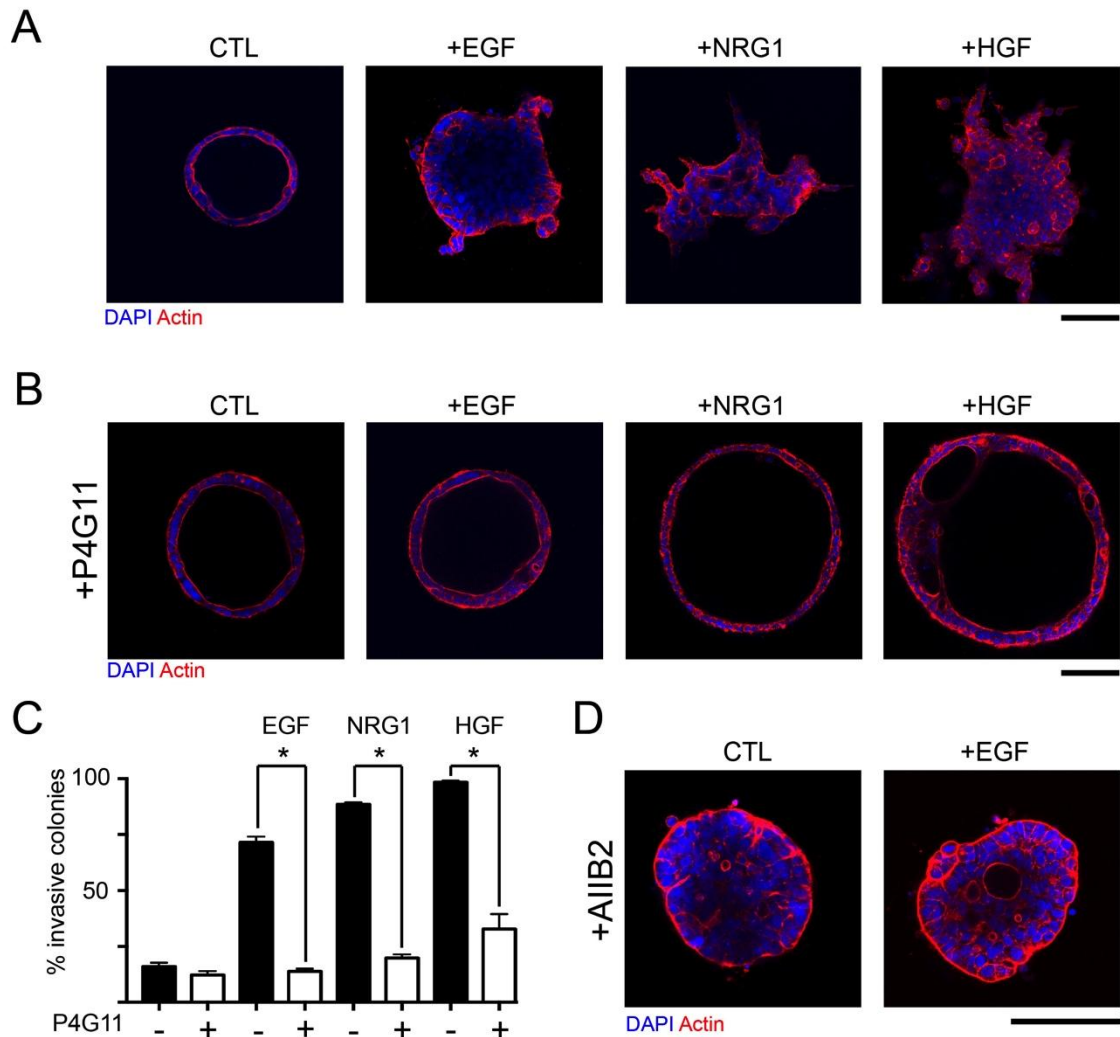


Figure IV-2: P4G11 blocks EGF, NRG1 and HGF-mediated invasion in 3D.

(A) CC cells were plated in 3D type-1 collagen as single cells until day 8. At day 8, the indicated RTK ligands (20 nM) were added to the grown media. The medium was replaced every 2-3 days until day 15 when colonies were fixed and analyzed using confocal microscopy. Colonies were stained with phalloidin (red) and DAPI (blue). Scale bar, 100 μ m (B) CC cells were grown as in (A) and treated with ligand (20 nM) and P4G11 (10 μ g/ml) on days 8-15. Colonies were stained with phalloidin (red) and DAPI (blue). Scale bar, 100 μ m. (C) Quantification of number of colonies exhibiting an invasive phenotype in (A,B) (mean \pm SEM; n>300 from 3 biological replicates). (D) Treatment of CC with integrin β 1 blocking antibody AIB2 on days 8-15. Colonies were stained with phalloidin (red) and DAPI (blue). Scale bar, 100 μ m.

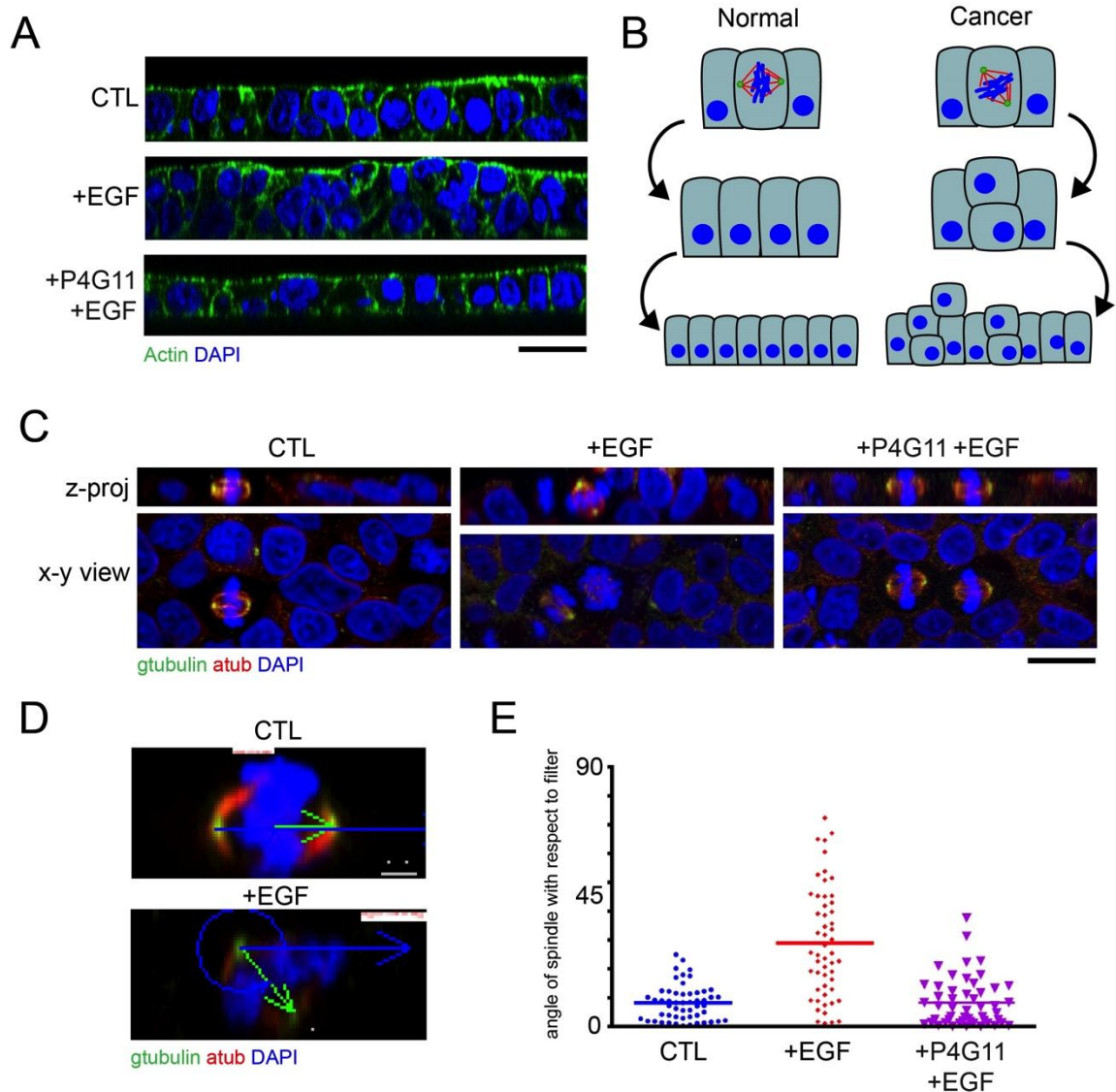


Figure IV-3: P4G11 blocks EGF-induced disorganization of mitotic spindle orientation.

(A) Confocal z-reconstruction of CC that were grown on Transwell filters for 6 days and treated with EGF with or without P4G11 on days 1-6. Cells were fixed and stained with phalloidin (green) and DAPI (blue). Scale bar, 25 μ m. (B) Schematic of how mis-orientation of the mitotic spindle can lead to monolayer disorganization (C) Representing confocal z-reconstructions and accompanying xy-plane image of CC grown on Transwell filters for 4 days and treated with 20nM EGF on days 4-5. Cells were fixed and stained with antibodies against gamma-tubulin (green), alpha-tubulin (red) and DAPI (blue). Scale bar, 25 μ m. (D) Example image of parallel and misoriented CC spindle angle determination. CC were grown and stained as in (C). (E) Quantification of the distribution of angles of CC cells grown as in (C). (mean; n=50, points from three separate biological replicates are shown)

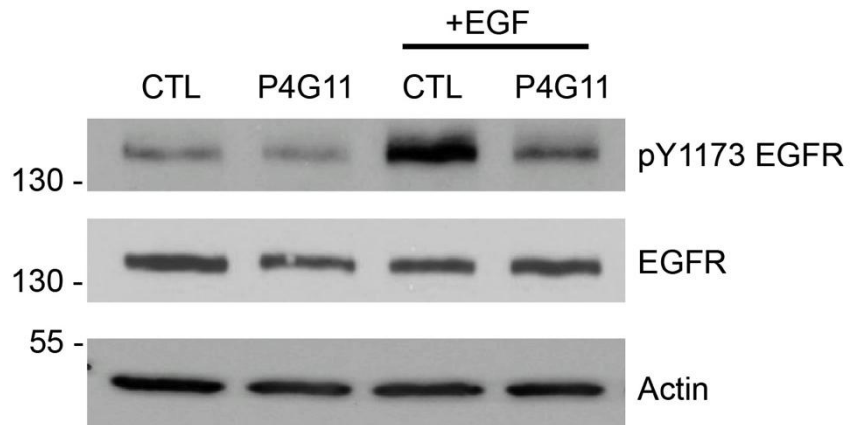


Figure IV-4: Treatment with P4G11 dampens EGFR phosphorylation in response to EGF in CC.

CC were grown on Transwell filters for 6 days and treated with P4G11 for 4 hrs prior to a 5 min treatment with EGF in serum-free media. Cells were lysed and levels of pY1173 EGFR, total EGFR and actin were analyzed by immunoblotting.

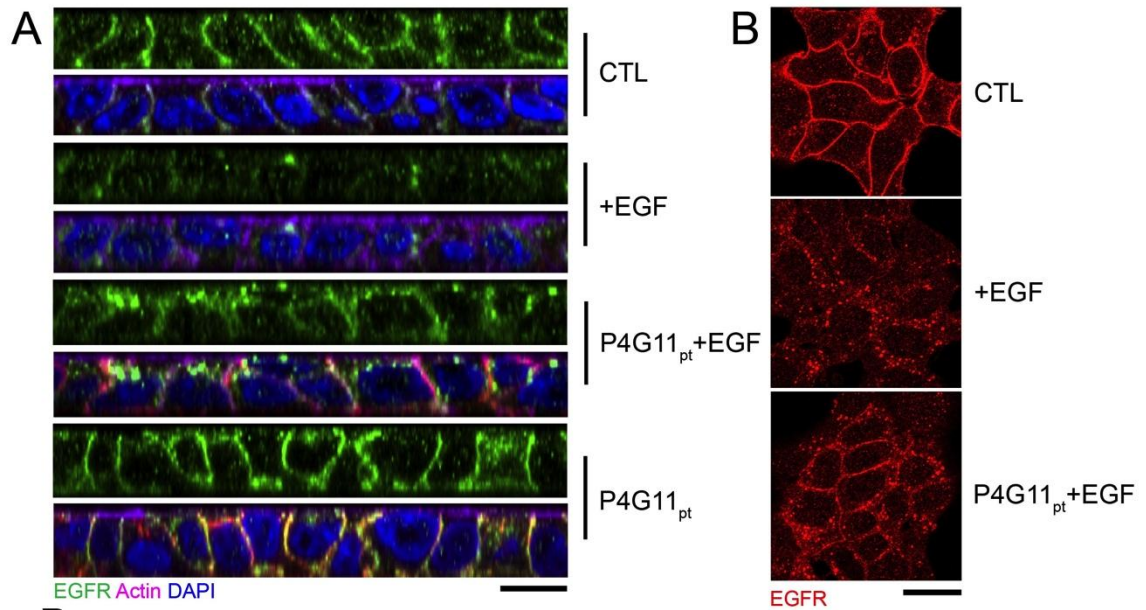


Figure IV-5: Treatment with P4G11 dampens EGFR endocytosis following EGF treatment in CC

(A) CC cells were grown as in figure 4. After 5 min EGF treatment, cells were fixed and stained with antibodies against EGFR (green), phalloidin (purple), and DAPI (blue). Scale bar, 25 μm . (B) Representative confocal XY view of CC cells grown as in figure 4, fixed and stained with an antibody against EGFR (red). Scale bar = 25 μm .

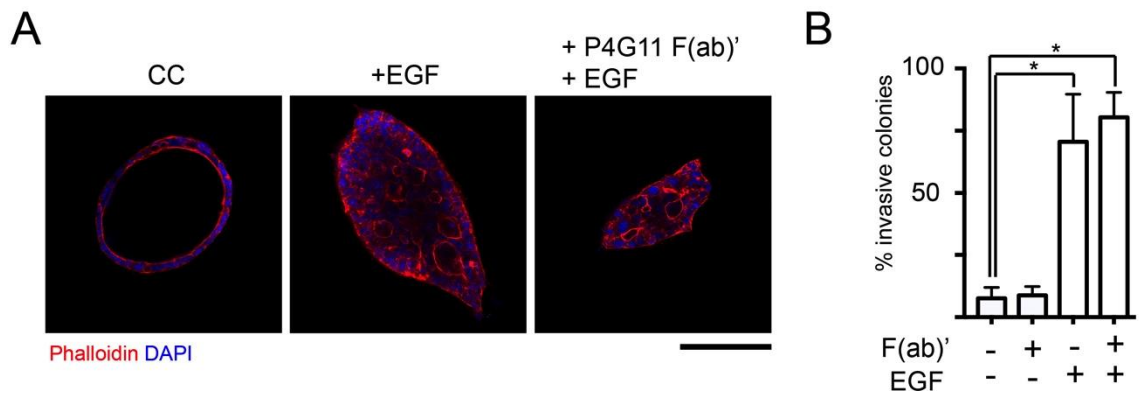


Figure IV-6: P4G11 clustering of integrin β 1 is required for blockade of EGF-mediated invasive colony morphology.

(A) CC were grown in 3D type-1 collagen for 15 days and treated with EGF with and without the P4G11 F(ab)' fragment. Colonies were fixed and stained with phalloidin (red) and DAPI (blue). Scale bar, 100 μ m. (B) Quantification of the number of invasive colonies in (A) (mean \pm SEM; n>300 in three biological replicates)

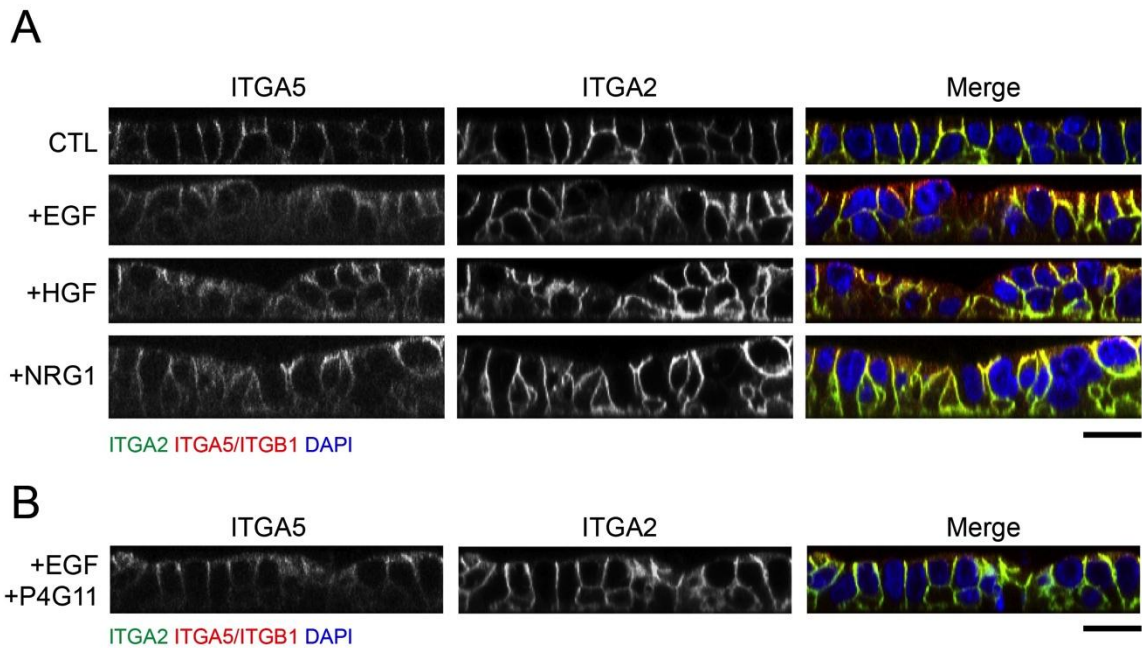


Figure IV-7: Treatment of CC with EGF, NRG1 and HGF leads to loss of membrane integrin $\alpha 5$

(A) CC were grown on filters for 6 days and treated with 20 nM indicated ligand on days 2-6. Cells were fixed and stained with antibodies against integrin $\alpha 2$ (green), integrin $\alpha 5$ (red), and DAPI (blue). Scale bar, 25 μm . (B) SC cells grown as in (A) treated with EGF and P4G11 on days 2-6 were fixed and stained with antibodies against integrin $\alpha 2$ (green), integrin $\alpha 5$ (red), and DAPI (blue). Scale bar, 25 μm .

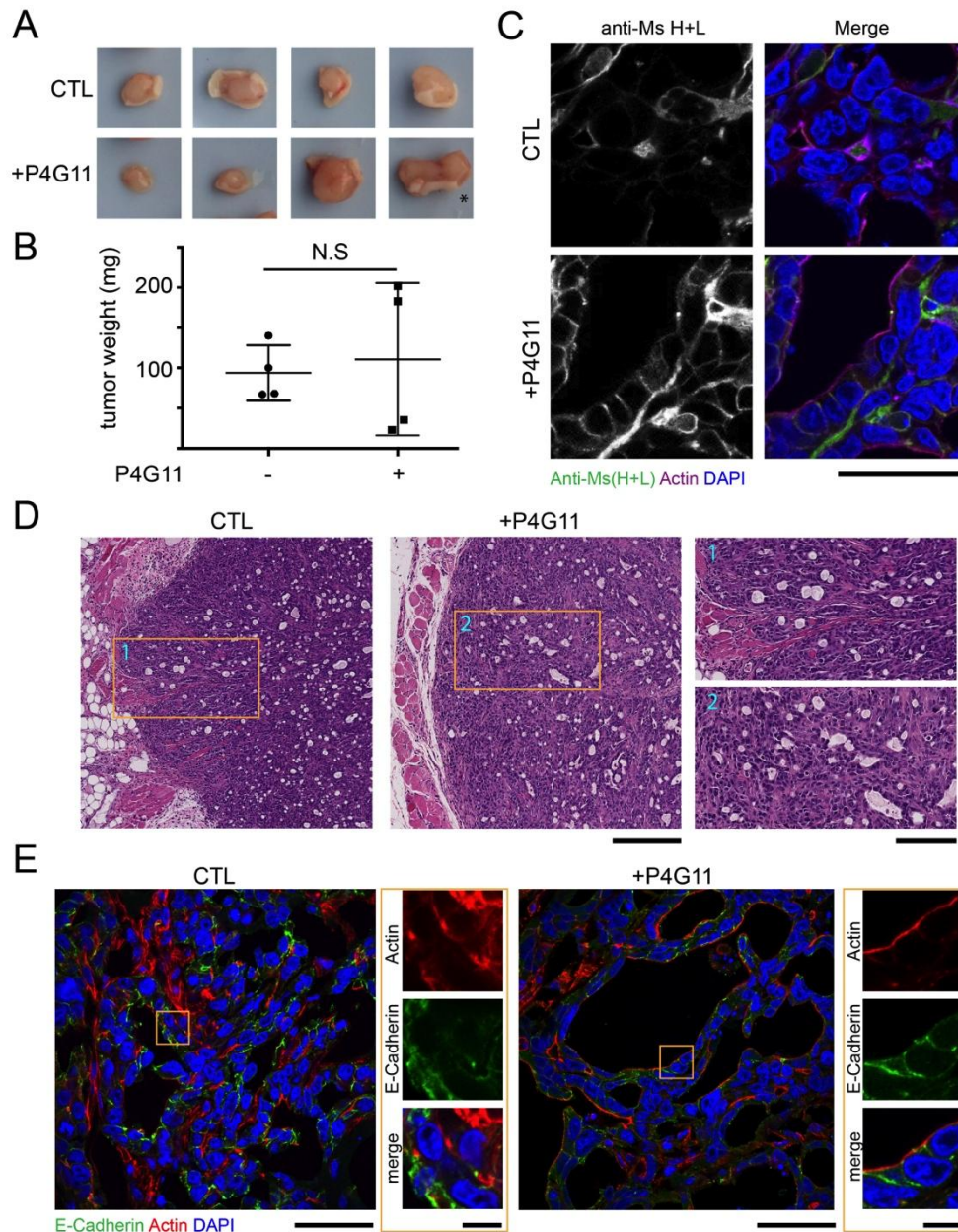


Figure IV-8: Treatment of SC with P4G11 *in vivo* has subtle effects on tumor size and organization.

SC were injected subcutaneously into nude mice and grown until tumors became palpable. P4G11 (100 μ g/ml final) was administered through intraperitoneal injection every 2 days for 21 days. Tumors were harvested and analyzed. (A) Images of tumors (B) tumor weight. Shown is mean \pm SEM. N=4 animals per group. (C) Representative confocal image of control and P4G11 treated tumor tissue stained with antibody against mouse H+L chain (green), phalloidin (purple) and DAPI (blue). Scale bar, 50 μ m. (D) H&E analysis of control and treated tumors. Scale bar, 250 μ m, inset scale bar, 50 μ m. (E) Representative confocal image of control and treated tumor sections stained with antibody against E-cadherin (green), phalloidin (red) and DAPI (Blue). Scale bar, 25 μ m. Inset scale bar, 10 μ m.

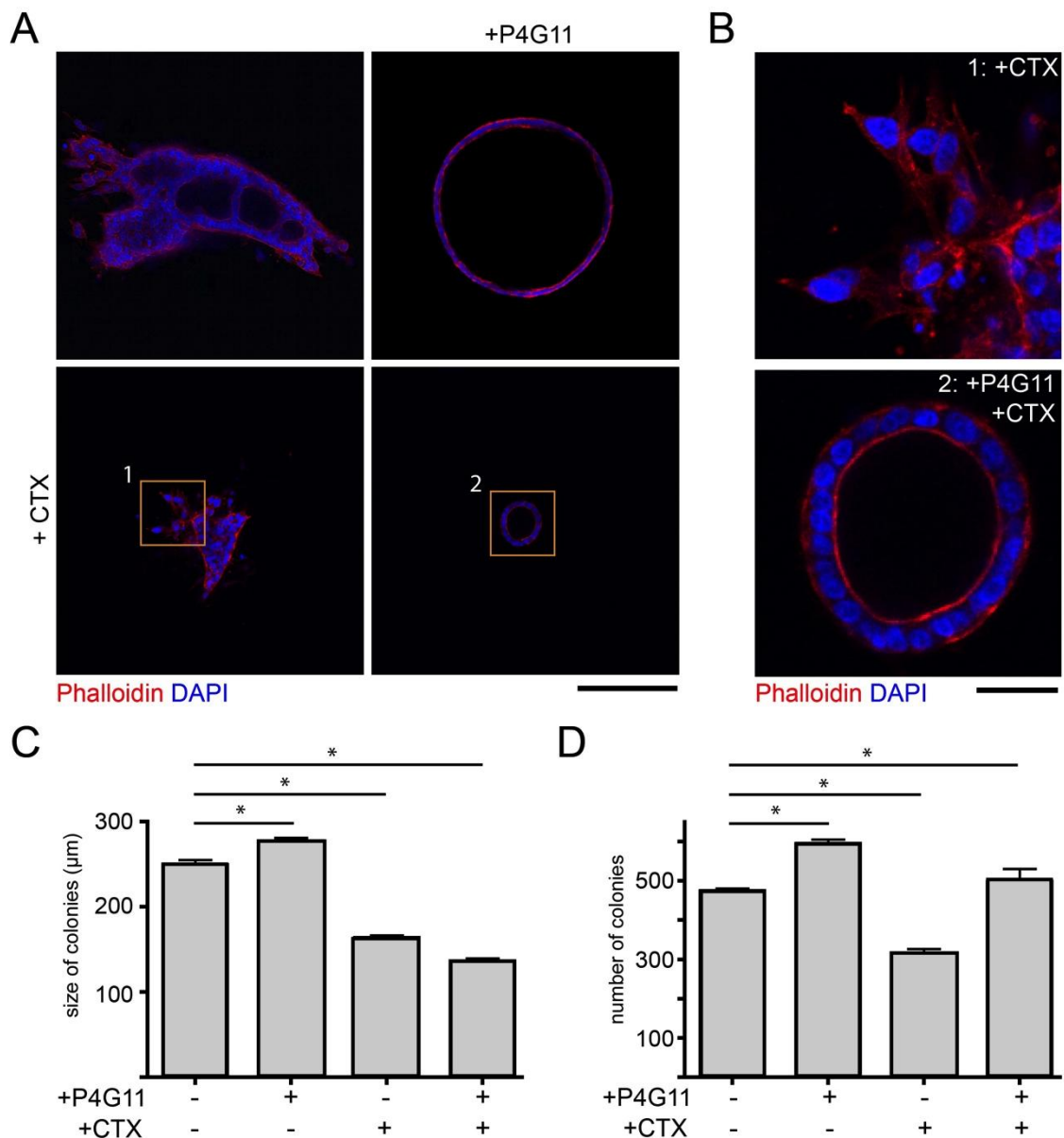


Figure IV-9: Treatment of invasive SC with P4G11 and Cetuximab results in smaller, better organized colonies in 3D

(A) SC were grown in type-1 collagen for 15 days and treated with P4G11, cetuximab or both on days 1-15. Colonies were fixed and stained with phalloidin (red) and DAPI(blue). Scale bar, 100 μm . (B) Magnified regions 1 and 2 in (A) showing colony organization. Phalloidin (red), DAPI (blue). Scale bar, 50 μm . (C) Quantification of colony size in (A) (mean \pm SEM; n>300 from three biological replicates) (D) Quantification of colony number in (A) (mean \pm SEM; n>300 from three biological replicates)

Chapter V: **Conclusions and future directions**

V.1 Overview

Integrins regulate formation and maintenance of human tissues through their ability to orchestrate cytoskeletal and ECM remodeling. Cancer hijacks this ability to facilitate its aberrant growth and metastasis. CRC is characterized by progressive loss of apico-basolateral cell polarity and gain of invasive characteristics. Integrin $\beta 1$ is required for both normal epithelial morphogenesis and cancer-associated migration, but whether it directly regulates the transition is not well understood.

To form a functional receptor, integrin $\beta 1$ pairs with one of 12 different α -subunits. The result is 12 integrin $\beta 1$ -containing receptors with unique *in vivo* functionalities. To date, most studies have focused on the global contribution of integrin $\beta 1$ to epithelial polarity, without differentiating between its α -subunit partners. In the human intestine, levels of integrin $\alpha 5$ are decreased in cancer. Integrin α subunits are also under tight spatial and temporal control in various models of colonic epithelial cell differentiation [115, 150]. However, the contribution of specific integrin α subunits to colonic epithelial cell behavior has remained unexplored. The work presented here details a role for integrin $\beta 1$ partner integrin $\alpha 5$ in regulating colonic epithelial cell morphogenesis.

Previous studies in our lab have focused on the role of EGFR and its ligands in CRC. We have shown that EGFR signaling is required for

establishment and progression in CRC [87]. We have additionally shown that mistrafficking of EGFR ligands leads to loss of epithelial polarity and may also play a role in CRC tumorigenesis [84-86]. Recently, we have turned our focus to enhance the understanding of resistance to anti-EGFR therapies, like Cetuximab, in CRC. These studies led us to observe an increase in sensitivity of CRC-derived cells to Cetuximab in 3D collagen versus 2D plastic. These results were surprising to us and made us wonder how increased integrin signaling altered cell behavior and EGFR signaling in CRC cells in 3D.

Our first study, detailed in chapter II, sought to identify a physical interaction between integrin $\beta 1$ and EGFR in CRC. Using PLA, we confirmed the existence of such a complex, but technical difficulties made it unfeasible to continue to explore the EGFR and integrin $\beta 1$ interaction from this angle. Chapters III and IV describe the use of monoclonal antibodies that alter the function of integrin $\beta 1$ as an alternate way to explore how integrin $\beta 1$ activity contributes to CRC cell behavior *in vitro*. Previously, studies have focused on integrin knockdown or blockade rather than activation. Pioneering studies in 3D cyst-formation assays showed that blockade of integrin $\beta 1$ signaling led to loss of polarity and inversion of the cyst phenotype with the apical surface facing the ECM [14, 113]. Notably, blockade of integrin $\beta 1$ also results in the loss of a central lumen, which is often overlooked [21, 26]. Thus, loss of integrin $\beta 1$ signaling results not only in misorientation of polarity, but in loss of cyst architecture. In our work, integrin $\beta 1$ blockade results in a similar loss of a central lumen but we do not notice an obvious inversion phenotype. Interestingly, antibody-mediated activation of integrin $\beta 1$ restores epithelial polarity to otherwise invasive CRC cells. This occurs through the ability of

mAb P4G11 to cluster integrin $\beta 1$ at the cell surface. Analysis of the α -subunits within this system identified integrin $\alpha 5$ as the only integrin protein that became differentially localized to the membrane with P4G11 treatment. We knocked down integrin $\alpha 5$ and determined that it was necessary for the epithelial polarity-inducing effect of P4G11. Blockade of integrin $\alpha 5$ in polarizing cells led to a loss of polarity, suggesting that integrin $\alpha 5$ may be a mediator of polarization in CRC cells. We determined that integrin $\alpha 5$ clustering leads to lateral deposition and polymerization of FN, which leads to establishment of polarity and formation of epithelial junctions. From this work, we proposed a model that lateral integrin $\alpha 5$ can form a clustered complex that leads to deposition and remodeling of FN and then this leads to the assembly of a later adhesion integrin $\alpha 5$ /FN complex that can recruit paxillin. We were able to observe lateral integrin $\alpha 5$ and FN in terminally differentiated epithelial cells, suggesting that a lateral integrin $\alpha 5$ /FN interaction may play a role in colonic epithelial cells *in vivo*.

Our next aim was to determine how integrin $\beta 1$ clustering could promote epithelial morphogenesis during CRC. The contribution of integrin $\beta 1$ to EGFR and other RTK signal transduction pathways to cancer is well known [94]. Recent studies have identified integrin $\alpha 5$ as a negative regulator of EGFR through membrane proximal interactions that dampen EGFR phosphorylation and endocytosis [147, 151]. Therefore, our goal was to determine if P4G11-mediated integrin $\alpha 5\beta 1$ clustering could decrease malignancy-promoting RTK signals in CRC. We showed that co-treatment of P4G11 with multiple RTK ligands resulted in maintenance of an epithelial phenotype. We tested the effect of P4G11 on RTK-mediated invasion in 3D

collagen and mitotic spindle misorientation and found that P4G11 blocks both processes. For EGFR, this occurred through a blockade of phosphorylation and endocytosis almost identical to that attributed to integrin $\alpha 5$ in [147]. We tested P4G11 *in vivo* and determined a promising effect on tumor organization. However, a larger study with more invasive cell lines, like SW480 would be necessary to fully conclude that P4G11 has clinical potential. We also present preliminary evidence that combined integrin $\beta 1$ clustering therapy with P4G11 and EGFR blockade using Cetuximab in 3D results in smaller, better organized colonies. Because of its effect on both WT and mutant K-Ras cell lines, it may be clinically relevant in the future to determine if anti-integrin $\beta 1$ drugs can be an adjuvant therapy for standard CRC treatments.

In summary, our studies investigating the role of integrin signaling in CRC have identified a role for lateral clustering of integrin $\alpha 5$ in colonic epithelial morphogenesis and signal transduction. These findings suggest a physiological role for a previously ignored population of laterally localized integrins and highlight the importance of characterizing contributions of particular $\alpha\beta$ integrin receptors to homeostasis and cancer progression in epithelial tissues.

V.2 Future Directions

Richard Hynes once pointed out that the explosion of multicellular organisms corresponded with a great expansion in types of ECM [76]. How these different forms contribute to cell and tissue specification is unknown, although evidence is mounting that temporally regulated integrin-ECM interactions are critical for development, disease and homeostasis. In the

work presented here, I have shown that integrin $\alpha 5$, which promotes motility in some cell types, supports a stationary, polarized phenotype in colonic epithelial cells. How is this possible?

A cell is basically just a network of peptides suspended in a bag of lipids. How those peptides interact with one another determines whether that cell is a motile mesenchymal stem cell or a stationary, differentiated, epithelial cell. The overall set of peptides, or proteins, is nearly identical in every human cell. So, how can the human body contain so many thousands of cell types, with vastly different behaviors and functions?

One might speculate that subtle differences in the number and local concentrations of individual proteins can have profound effects on the function of other proteins in the network due to basic principles of thermodynamics. Specifically, increasing the number of proteins in an “active” conformation would certainly alter the likelihood of certain protein-protein interaction events, favoring some over others, and could lead to large changes in the overall network. In this way, increasing surface clustering of an integrin may profoundly change the function of any surface protein in its vicinity, completely altering downstream signaling. For example, we show a subtle decrease in EGFR phosphorylation with P4G11 treatment (Chapter IV). Since signals from the membrane to the nucleus are usually amplified by their cytoplasmic mediators, it is easy to imagine that subtly changing how EGFR is internalized might have large effects on the ability of phosphatases or EGFR tail-binding proteins to act. This would alter the magnitude of the downstream signal as it propagates and therefore dampen the responsiveness of the cell. Alternatively, deposition of lateral ECM that binds lateral integrin between two

neighboring cells (Chapter III) could stabilize neighboring membranes in a manner that could increase the likelihood of the formation of canonical cell-cell interaction proteins like cadherins and occludins to form bonds.

Of course, how P4G11 actually alters the signaling state of the recipient cell, as well as the consequences of integrin $\alpha 5$ redistribution on the protein-protein interaction network should be further defined. This way, we can understand the role of lateral integrin clustering in governing cell behavior, which will lead to a better understanding of a novel way by which the ECM can govern development and homeostasis through tightly controlled integrin signaling.

V.3 Future directions: Experimental approach

Further studies should focus on investigating the mechanisms through which integrin $\alpha 5$ clustering at the lateral surface influences apico-basolateral cell polarity in the colon. There are three specific avenues that require further exploration: *in vitro* studies focusing on the integrin $\alpha 5$ and FN contribution to polarity, *in vivo* studies to determine how integrin $\alpha 5$ contributes to homeostasis and cancer in adult colon, and *in vitro* studies to identify the protein machinery that regulates surface levels of integrin $\alpha 5$.

The first goal should be to identify the molecular links between integrin $\alpha 5$, fibronectin and apico-basolateral polarity. Chapters III and IV document the ability of surface integrin $\beta 1$ to drive apico-basolateral polarization and dampen RTK signaling through interactions with integrin $\alpha 5$ and ECM ligand FN. These studies raise an important question: How do recycling dynamics of integrin $\alpha 5\beta 1$ contribute to epithelial polarity?

Throughout this project, we have utilized P4G11 as a tool to study integrin signaling. We have done so blindly, because the epitope for this antibody has not been mapped and we have little idea how P4G11 affects integrin conformation or induces integrin clustering. If we want to continue working with this reagent, its effect on integrin $\beta 1$ needs to be characterized. There are several ways to approach this. We could begin by expressing different segments of integrin $\beta 1$ to determine which portion of the protein P4G11 binds and use this to map an epitope. I suspect this will not work because P4G11 seems to be very particular about its binding conditions and integrin $\beta 1$ conformation. Although this is not in the purview of the Coffey lab, it would be fascinating to determine what an integrin $\alpha 5/\beta 1$ complex with P4G11 looks like using an approach similar to that used by the Springer group to determine differences in integrin $\alpha 5/\beta 1$ conformation when bound to either 12G10 or TS2/16 [42]. Ideally, we would compare the complex of integrin $\alpha 5/\beta 1$ with either P4G11 or sFN, as this would clarify whether the P4G11 phenotype simply mimics a bivalent ligand with high binding affinity or if it alters integrin conformation in a different way. This analysis would also show which portion of the integrin P4G11 binds and whether it stabilizes a unique conformation of integrin $\alpha 5\beta 1$. A structural analysis of this sort is absolutely necessary if we were to consider using P4G11 as a therapeutic strategy, since it would allow for intelligent refinement of a P4G11-like reagent to increase potency and reduce side effects.

Our model in chapter III demands an analysis of the effects of P4G11 and sFN on the rate of integrin $\alpha 5$ recycling. Previous work surrounding whether increased integrin $\alpha 5$ is prognostically good or bad in the context of

cancer is controversial. In mesenchymal cells and their cancer equivalents, integrin $\alpha 5$ contributes to invasion, increased proliferation and survival. In more epithelial-like systems, integrin $\alpha 5$ dampens EGFR and Her2 signaling, decreases proliferation rate, and is lost during cancer progression [138, 152]. Reconciling these data has been challenging, but the system we have presented here provides us with a set of tools to do so. We have shown that increasing the level of integrin $\alpha 5$ at the surface through induction of clustering (using P4G11 or sFN) is required for the rescue of epithelial polarity in CRC cells. But, how are we really affecting integrin $\alpha 5$ signaling? First, we could be imparting on $\alpha 5$ an epithelial-promoting function by clustering and activating it at the surface. Second, we could be blocking an invasion-promoting integrin $\alpha 5$ signal that depends upon rapid recycling. The third, and in my mind most likely, possibility is a combination of the first two: we could be converting integrin $\alpha 5$ from a promoter of invasion to a promoter of epithelial polarity by altering its recycling rate and surface distribution. To begin to parse between these possibilities, we can generate a fluorescently labelled integrin $\alpha 5$ protein and determine its recycling rates at baseline and in the presence of both organizing and disorganizing signals. It can be difficult to generate fluorescently labelled integrins, but a validated integrin $\alpha 5$ -GFP construct exists [153]. This construct can be paired with confocal microscopy and fluorescence recovery after photobleaching (FRAP) studies to determine the turnover rate of membrane integrin $\alpha 5$ protein as previous described [154]. We can then determine how P4G11 and sFN affect endocytosis and recycling of integrin $\alpha 5$. Based on our model in chapter III, we might predict that P4G11 activation and clustering of integrin $\alpha 5$ would slow its endocytosis.

The ability to watch integrin $\alpha 5$ surface dynamics will also allow us to track the spatiotemporal interactions of FN and integrin $\alpha 5$ in polarizing epithelial cells. Lateral FN has been previously detected in colon-derived cells [143]. In chapter III, we see a similar accumulation of FN at the lateral surface between colon-derived epithelial cells *in vitro* and at the lateral surface of terminally differentiated epithelial cells in human colon tissue. In both cases, we show a static image of lateral FN. Whether the FN is deposited laterally and is retained at the cell-cell interface indefinitely or is part of a transient process as in branching morphogenesis [155-157] is unknown. Further, the interactions of FN-bound integrin $\alpha 5$ with components of adherens and tight junctions have not been determined. We have detected a lateral ring of paxillin that co-localizes with the FN fibrils in SW480 cells. Paxillin is recruited to adhesions that are under tension, and suggests that the lateral complex is contributing to the force balance between the cytoskeleton, cell surface adhesion proteins, and the neighbouring cell. We hypothesize that the lateral integrin-ECM interaction stabilizes interactions between membranes of neighbouring cells to facilitate formation of nascent cell-cell junctions that ultimately lead to cell polarity. To begin to test this we will have to determine how lateral FN polymerization contributes to the ability of the cell-cell junction to oppose tensile force. There have been a number of microscopy-based techniques that estimate various parameters of the physics of cell-cell and cell-ECM interactions [158, 159]. Recently, several of these have been extended to estimate forces involved in cell-cell junctions [160]. If we determine that FN increases the strength of lateral cell-cell interactions

through integrin $\alpha 5$, then we can begin to ask questions about the cytoplasmic side of this complex.

Integrin adhesions serve as anchors for both the actin and microtubule cytoskeleton [161]. If it is true that the lateral FN/integrin $\alpha 5$ complex is under tension, as the presence of paxillin and preliminary liveCell analysis suggests, then it must be connected to the cytoskeleton. During the establishment of epithelial polarity and for its maintenance, the cytoskeleton of a non-polarized cell undergoes large changes that allow for proper force distribution and protein trafficking throughout the epithelial monolayer [162]. We do not currently know which component(s) of the cytoskeleton the lateral integrin $\alpha 5$ effects. We have preliminary data suggesting that RhoA inhibition blocks the ability of P4G11 to restore polarity to SW480 cells. RhoA is part of the group of GTPases that control both front-back and apico-basolateral polarity [161]. We do not currently know if RhoA inhibition blocks the P4G11-mediated polarization because P4G11, through its ability to cluster integrin $\alpha 5$, directly activates RhoA, or just because RhoA activation is a necessary downstream component of cell polarity. Because RhoA can be activated by FN/integrin $\alpha 5$ association and is required for FN polymerization [24, 129, 140, 163, 164], we hypothesize that RhoA is activated by the lateral integrin $\alpha 5$ /FN complex and induces signaling that promotes apico-basolateral polymerization. This would have to be tested by a determination of whether RhoA is localized to the integrin $\alpha 5$ /FN complex and whether it is activated there. It has been previously reported that localized activation of RhoA at the lateral epithelial cell surface occurs during the polarization process [165, 166]. In the future, a similar approach could be undertaken to determine the effect

of lateral integrin $\alpha 5$ clustering on RhoA activation in epithelial polarization and the contribution of FN polymerization to this process.

If RhoA is a direct target of the lateral integrin $\alpha 5$ cluster, then we can begin to decipher how agents that induce lateral integrin $\alpha 5$ clustering contribute to polarity. Rho GTPases Cdc42, RhoA and Rac all contribute to formation and expansion of early cell-cell junctions that lead to formation of apical and basolateral domains [165]. Rho signaling stabilizes E-cadherin at early cell-cell junctions through myosin IIA. In addition, Rho GEFs promote AJ integrity through regulation of myosin II and stabilization of the cortical actin ring. RhoA is also a regulator of TJ formation. Inhibition of Rho in intestinal epithelial cells reduces TJ function [23, 24, 162, 167]. Rho GEFs control activity of multiple polarity-associated proteins including Patj, Cdc42 and LKB1 [165]. It has additionally been shown that integrin $\alpha 5$ can directly control ZO-1 localization within the cell [137]. A determination of the effects of lateral integrin $\alpha 5$ clustering on the early and late distribution of these proteins will shed light onto the physiological role of this complex. Understanding how lateral integrin signaling fits into the known polarity machinery in colonic epithelial cells would be an important step to ascertain that lateral integrin and lateral ECM have a distinct mechanical function away from the basal cell-basement membrane interface.

The final *in vitro* question that remains to be addressed is how does integrin activation alter RTK signaling. In chapter II, we show that an interaction between EGFR and integrin $\beta 1$ may exist in CRC. In chapter IV we show that activation of integrin $\beta 1$ dampens the invasive and disorganizing effects of EGF, NRG1 and HGF. There is compelling evidence that integrin

β 1-rich adhesions contain many growth factor receptors [94], including EGFR, MET and ErbB3 and that direct interactions between integrin β 1-containing receptors and RTKs alter their signaling. Based on these observations, we might predict that integrin α 5 interacts with and modulates the activity of MET and ErbB3 (receptors for ligands HGF and NRG1) in a similar manner as it has been reported to bind to and negatively regulate the activity of EGFR [95]. In this case, increased surface integrin α 5 would increase the net amount of negative RTK regulation at the surface and dampen the signal in response to ligand. This model also provides an explanation for the observation that polarized cells seem generally less susceptible to disorganizing signals than their non-polarized counterparts [17, 168].

All together, these experiments will shed light on how the lateral integrin α 5 contributes to epithelial polarity and will broaden our understanding of integrin signaling in epithelial cells.

The second goal should be to probe the role of integrin α 5 in colonic epithelium in vivo. An immediate question is to understand the contribution of integrin α 5 to colonic epithelial cell behavior *in vivo*. Specifically, we would ask how integrin α 5 knockdown affects colonic crypt structure and whether it contributes to intestinal tumor progression. To address this question, we will first determine whether integrin α 5 knockdown in adult murine colon affects colonic homeostasis. There are several ways to ask this question. The most straightforward is to observe colon structure in an inducible model of villin-driven integrin α 5 loss. To do this, a *VillinCre; α 5^{fl/fl}* mouse can be generated. The Coffey lab has utilized the *Villin* driver model previously and the integrin α 5 floxed allele has been developed and

extensively characterized by the Hynes group. In this conditional mutant mouse, integrin $\alpha 5$ will be genetically removed from all *villin*-expressing cells, which encompasses the entire epithelial compartment in the colon. This model is a good place to start since this promoter has been previously used to drive constitutive integrin $\beta 1$ silencing with an observable phenotype [103, 146]. We can use this model to determine whether integrin $\alpha 5$ is necessary for colonic homeostasis in adults. Based on where we observed lateral integrin $\alpha 5$ /FN in human colon, we might expect to see impaired epithelial organization near the top of the crypt. Importantly, the expression levels of integrin $\alpha 5$ in the colon has not been examined extensively, therefore it is possible that while membrane integrin $\alpha 5$ is enriched in terminally differentiated cells, it is still expressed and has a function in other regions of the colonic crypt. Thus, it is likely that *villin*^{cre}; $\alpha 5$ ^{fl/fl} will show defects in differentiation or proliferation in stem/progenitor cells in addition to the terminally differentiated compartment. If we confirm integrin $\alpha 5$ loss (e.g. by immunostaining) and do not see an observable phenotype, then layering in a damage cue (e.g. DSS) to may be informative to determine if integrin $\alpha 5$ contributes to damage repair.

It has been reported than the level of integrin $\alpha 5$ decreases as colon cancer progresses. In chapter III, we observed loss of lateral integrin $\alpha 5$ from the surface of poorly organized cancers. In chapter IV, we showed that treatment of SC tumors *in vivo* with P4G11 results in better tumor organization. Based on these observations, we hypothesize that reduction or knockout of integrin $\alpha 5$ in a murine model of CRC might lead to increased malignancy. To test this, we will introduce an integrin $\alpha 5$ knockout into an

existing model for CRC. Two possibilities are to cross an integrin $\alpha 5^{fl/fl}$ into the existing $Lgr5^{CreER/+}; Apc^{fl/fl}$ or $LrigCre^{ER/+}; Apc^{fl/+}$. These mouse models have several differences, but the primary one in relation to this experiment would be the temporal arrangement of integrin $\alpha 5$ knockdown and tumor initiation. In the first model, integrin $\alpha 5$ and *APC* will be removed simultaneously. In the second, integrin $\alpha 5$ will be knocked out with a single copy of *APC* but the second allele of *APC* would occur stochastically. Thus, in the first case we would be asking how integrin $\alpha 5$ contributes to tumor initiation and progression, whereas in the second we would also observe whether integrin $\alpha 5$ knockdown somehow sensitizes the system to the loss of the second *APC* allele. Standard parameters, like tumor size and molecular and pathological analyses will be used to determine the contribution of integrin $\alpha 5$ to tumorigenesis *in vivo*.

The long term goal should be to identify factors determine integrin $\alpha 5$ surface levels. The two routes of investigation outlined above are questions that need to be addressed to confirm that the integrin $\alpha 5$ complex we observed in chapter III has a function *in vivo*. The *in vitro* studies proposed will provide further insight into what that function is. If we find that lateral surface levels of integrin $\alpha 5$ significantly impact epithelial polarity in the colon, then the question arises: what cellular players control integrin $\alpha 5$ surface levels? Although similar questions have been asked often, nature has thus far not been forthcoming with an answer. Integrins seem to be endocytosed through every possible process [169, 170], and although we can detect when integrins switch from a short to a long recycling path, and we show that it is important, a master regulator or switch within the cell for the

process has not been identified. One might speculate that proteins associated with integrin endocytosis, like Rab25, Dab2, Numb, and similar mediators are plausible targets [171]. A long-term goal to understand integrin recycling will require a sensitive model system and a systematic approach. One could imagine using some sort of large expression/stimulation panel, with SC morphology as a quantitative readout, to identify cellular determinants of integrin $\alpha 5$ surface levels, signaling and recycling.

Chapter VI: **Materials and Methods**

Cell Culture. All cell lines were maintained in DMEM containing 10% bovine growth serum, non-essential amino acids, L-glutamine, and penicillin/streptomycin. For 3D cultures, type-1 collagen was diluted in DMEM containing 10% fetal bovine serum. Stable TET-ON anti-integrin $\alpha 5$ shRNA expressing SW480 and SC cells were generated using the SMARTvector inducible lentiviral shRNA construct. shRNA sequences (GATTCTCAGTGGAGTTTTA) sh1, targeting exon 1, and (AAGGGAACCTCACTTACGG) sh3, targeting exon 9, were selected by immunoblotting as having best knockdown efficiency. Cells were transfected with lentivirus, selected with, and subsequently induced using 1 μ g/ml doxycycline (dox). High (top 10%) GFP-expressing populations were fluorescently sorted. Cells were propagated in absence of Dox using DMEM containing 10% TET-screen FBS. Levels of integrin $\alpha 5$ were comparable in uninduced cells. Silencing upon addition of Dox was confirmed by immunoblotting. Due to the long half-life of integrin $\alpha 5$, cells were grown in Dox for at least 72 hrs prior to plating for an experiment.

Reagents. PureCol bovine type-1 collagen was purchased from Advanced Biomatrix, San Diego, CA. All cell culture components were purchased from Hyclone Laboratories. Protein G agarose and Rhodamine-phalloidin were purchased from Life Technologies. 488-conjugated Fibronectin was purchased from Cytoskeleton. Fibronectin-free serum was a kind gift from Dr. Alissa Weaver. CNA35 was a kind gift from Dr. Erin Rericha,

Antibodies. A1B2 and P4G11 hybridomas were purchased from the Iowa Developmental Studies Hybridoma Bank. Antibodies were produced and purified by the Vanderbilt antibody core facility (VAPR). P4G11 was used at 10 μ g/ml in all studies unless otherwise indicated. DyLight-594 conjugated P4G11 was produced by the VAPR antibody core. Monovalent P4G11 F(ab)' fragments were produced using the Ficin digestion kit from Millipore and conjugated to DyLight-594 by VAPR. Total Integrin β 1 antibody P5D2, activating antibody 12G10, and 12G10-Alexafluor488 were purchased from Abcam. Integrin β 1 activating mAb TS2/16 and integrin α 5-blocking JBS5 were purchased from Thermofisher. Antibodies for ezrin, fibronectin, paxillin, EGFR, p1173 EGFR, ZO-1, integrin α 2 and TrfR were purchased from Abcam. Rat monoclonal anti-integrin α 5 β 1 was purchased from Thermofisher. All secondary antibodies were purchased from Invitrogen.

3D Type-1 Collagen Cultures. Briefly, assays were set up using three layers of type-I collagen. Top and bottom layers were 2 mg/ml collagen alone while the middle layers consisted of 2 mg/ml collagen plus cells at 5,000 cells/ml in single cell suspension. All three layers contained 400 μ l volume per well of a 12-well culture dish. Medium (400 μ l) with or without reagents was added on top and changed every 2 to 3 d. Colonies were observed and counted after 14-17 d. All antibodies were used at 10 μ g/ml. All growth factors were used at 20ng/ml.

2.5D type-1 collagen cultures. Bovine collagen was neutralized and plated onto the bottom of a pre-warmed plate/dish/coverslip. Enough collagen solution is added to coat the surface with ~30 μ m layer of polymerized collagen. Thickness was estimated by confocal microscopy.

Monomeric collagen cultures. Tissue culture surfaces were coated in a 1:10 mixture of PBS and bovine collagen, incubated for 30 min, and rinsed 2x with PBS prior to plating of cells.

Colony Counting. Colonies were counted using GelCount (Oxford Optronix) with identical acquisition and analysis settings and represented as mean from triplicates \pm SEM. For cystic and spiky morphology, counts were performed manually from three individual wells and represented as mean \pm SEM.

Protein Isolation from 3D culture for immunoblotting. To isolate cells from 3D collagen, 1 ml collagenase solution (1 mg/ml collagenase I dissolved in complete medium) was added to one well of the 12-well plate and incubated at 37 °C until gels dissolved (between 1-2 h). Then cells were collected by centrifugation, washed twice with PBS and lysed in NP40 buffer containing protease (complete Protease Inhibitor Cocktail Tablets from Roche) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets from Roche).

Immunoblotting. Cell lysates were generated from 3D collagen cultures as described above. Briefly, the middle layer was removed and placed into 100 μ l RIPA buffer for 30 min at 4 °C. Sample was then centrifuged at 14,000 rpm for 10min to get rid of solid collagen pellet. Supernatant was diluted with 4X Laemmli buffer and 10% BME, boiled for 5 min, and separated on a 8% SDS-PAGE reducing gel. Lysates were then transferred overnight onto a nitrocellulose membrane, blocked for 30 min in 5% milk, and incubated overnight with antibodies at 4 °C. Membranes were washed and developed with secondary HRP-linked antibodies. Cells grown on MMC coated plastic or glass were lysed in sample buffer with 10% BME, briefly sonicated to shear

nucleic acids and boiled for 5 min prior to running on an SDS-PAGE reducing gel.

1% DOC solubility assay. For analysis of fibronectin polymerization, cells were lysed in 1% DOC (sigma) as previously described. Lysates were centrifuged at 20k g for 10 min. Supernatant and pellet were separated and diluted in 4X sample buffer and analyzed by immunoblotting.

Immunofluorescence. 3D IF: Collagen sandwich was fixed in 4% paraformaldehyde for 30 min at room temperature (RT). Middle layer was removed and placed into IF buffer (1% BSA, 1% Triton X-100 in PBS) overnight. 568-phalloidin and DAPI were added for 4 h at 4 °C prior to wash and confocal microscopy on Nikon A1R. IF: Primary antibodies were added at 1:500 overnight in IF buffer. Samples were then washed, and secondary antibody was added at 1:1000 for 4 h at 4 °C. Samples were washed and whole mounted onto a #1.5 glass coverslip and subsequently analyzed using confocal microscopy on a Nikon A1R. Tissue section (FFPE): Fresh slides from FFPE blocks derived from SC and CC nude mouse xenografts were deparafinized and re-hydrated; subjected to high pressure antigen retrieval for 40 min, blocked with DAKO tissue block and incubated with primary antibody overnight at 4°C. Slides were washed in PBS and Alexafluor-linked secondary antibody was added, along with DAPI. Slides were washed and mounted with pro-long and analyzed with confocal microscopy using a Nikon A1R. (Frozen): Tumor xenografts or human tissues were fixed in 4% PFA, placed overnight in 30% sucrose, and frozen in OCT compound (TissueTech). Frozen blocks were cryosectioned into 6 µm sections, permeabilized with 1% Triton and blocked with glycine (0.3M, 30min) and DAKO (1h) protein block. Primary

antibody was added overnight. Secondary antibodies from Invitrogen (AlexaFluor-linked) were added 1:1000 for 30 min. Slides were washed, mounted in Prolong (Life Technologies), and analyzed with confocal microscopy using a Nikon A1R or SIM (as indicated).

Proximity ligation assay. This assay was performed using the Duolink kit (mouse/rabbit) and protocol and was adapted to our experimental setup in 3D: the collagen sandwich was separated and prepared for standard IF as described above. Samples were incubated with the selected primary antibody pair, followed by the DuoLink negative and positive strand secondary antibodies. The DuoLink ligation solution was added and allowed to hybridize at 37C. Enzyme and amplification mix was added for 2h at 37C. Visualization solution was added for 30 min at 37C. Samples were extensively washed and whole-mounted in 50% glycerol/PBS. In tissue. Tissues were prepared as for FFPE IF, and incubated with primary antibody pair overnight. Duolink protocol was followed as prescribed in DuoLink protocol. Samples were mounted in 50%glycerol/PBS and maintained at 4C prior to microscopy.

Transmission Electron Microscopy. Cells were removed from the middle collagen layer, washed 2X with EM wash buffer and fixed in fixation buffer for 1 h. Specimens were processed for TEM and imaged in the Vanderbilt Cell Imaging Shared Resource-Research Electron Microscopy facility. Embedding: Collagen matrix with cysts was initially rinsed with 0.1 M sodium cacodylate buffer, fixed in 2.5% gluteraldehyde in 0.1 M cacodylate buffer, pH 7.4 at room temperature (RT) 1 h, then transferred to 4 °C overnight. The samples were washed in 0.1 M cacodylate buffer and then incubated 1 h in 1% osmium tetroxide at RT, followed by additional washing with 0.1 M cacodylate buffer.

Subsequently, the samples were dehydrated through a graded ethanol series and then three exchanges of 100% ethanol, followed by two exchanges of pure propylene oxide (PO). Samples were then infiltrated with 25% Epon 812 resin and 75% PO for 30 min at RT. Next, they were infiltrated with Epon 812 resin and PO [1:1] for 1 h at RT, then overnight at RT. The samples were subsequently infiltrated with resin for 48 h then allowed to polymerize at 60 °C for 48 h. Sectioning and Imaging: Thick sections (0.5-1 µm) were collected using a Leica Ultracut microtome, contrast stained with 1% toluidine blue, and imaged with a Nikon AZ100 microscope. Ultra-thin sections (70-80 nm) were cut, collected on 300-mesh copper grids, and post-stained with 2% uranyl acetate and then with Reynold's lead citrate. Samples were subsequently imaged on the Philips/FEI Tecnai T12 electron microscope at various magnifications.

ZO-1 quantification. The ImageJ angiogenesis analyzer was used to count the number of nodes in cells that had been fixed and stained for ZO-1 localization. The number of ZO-1 nodes in each field was normalized to the number of DAPI-labelled nuclei in each field, as counted by ImageJ.

Animal studies. Nude mice were injected with 200k SC cells in 100ul PBS subcutaneously in their right flank. Tumors were allowed to establish (become palpable) for 7 days before treatment was started. Treatment consisted of intraperitoneal injection of P4G11 or vehicle for a final *in vivo* concentration of roughly 100ug/ml (assuming 1.5ml blood per animal). Injections were performed every 2-3 days for 21 days, at which point animals were sacrificed and tumors were removed. Tumors were cut in half and fixed for both frozen

(4% PFA, 4C, 2h) and FFPE (Formalin, RT, overnight then transfer to ethanol) analysis.

Statistical Analyses. Two-tailed, two-sample t-tests were used to determine statistical significance. p-values of less than 0.05 were considered significant. Calculations were performed using Prism for Mac (64-bit for Mac).

Acknowledgements. We acknowledge the support of Vanderbilt University's Cell Imaging, Translational Pathology, and Flow Cytometry Shared Resources. We thank Janice A. Williams for help with the transmission electron microscopy. We thank Jane Sottile for her generous gift of the pUR4B and control plasmids and Alissa Weaver for her generous gift of fibronectin-free serum. We would also like to thank Roy Zent for his technical expertise and Erin Rericha for the gift of labelled CNA35.

Chapter VII: References

1. Macara, I.G. and S. Mili, *Polarity and Differential Inheritance-Universal Attributes of Life?* Cell, 2008. **135**(5): p. 801-812.
2. Li, R. and B. Bowerman, *Symmetry Breaking in Biology*. Cold Spring Harbor Perspectives in Biology, 2010. **2**(3).
3. Royer, C. and X. Lu, *Epithelial cell polarity: a major gatekeeper against cancer?* Cell Death Differ, 2011. **18**(9): p. 1470-7.
4. Flitney, E.W., et al., *Insights into the mechanical properties of epithelial cells: the effects of shear stress on the assembly and remodeling of keratin intermediate filaments*. Faseb Journal, 2009. **23**(7): p. 2110-2119.
5. Yeaman, C., K.K. Grindstaff, and W.J. Nelson, *New perspectives on mechanisms involved in generating epithelial cell polarity*. Physiological Reviews, 1999. **79**(1): p. 73-98.
6. Bazellieres, E., et al., *Control of cell-cell forces and collective cell dynamics by the intercellular adhesome*. Nature Cell Biology, 2015. **17**(4): p. 409-+.
7. Baum, B., J. Settleman, and M.P. Quinlan, *Transitions between epithelial and mesenchymal states in development and disease*. Seminars in Cell & Developmental Biology, 2008. **19**(3): p. 294-308.
8. Meng, W.X. and M. Takeichi, *Adherens Junction: Molecular Architecture and Regulation*. Cold Spring Harbor Perspectives in Biology, 2009. **1**(6).
9. Tanos, B. and E. Rodriguez-Boulan, *The epithelial polarity program: machineries involved and their hijacking by cancer*. Oncogene, 2008. **27**(55): p. 6939-6957.
10. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition*. Journal of Clinical Investigation, 2009. **119**(6): p. 1420-1428.
11. Van Itallie, C.M. and J.M. Anderson, *Architecture of tight junctions and principles of molecular composition*. Seminars in Cell & Developmental Biology, 2014. **36**: p. 157-165.
12. Colegio, O.R., et al., *The role of claudin extracellular domains in tight junction fibril architecture and paracellular charge selectivity*. Molecular Biology of the Cell, 2002. **13**: p. 286a-286a.
13. Martin, T.A. and W.G. Jiang, *Loss of tight junction barrier function and its role in cancer metastasis*. Biochimica Et Biophysica Acta-Biomembranes, 2009. **1788**(4): p. 872-891.

14. Yu, W., et al., *Beta1-integrin orients epithelial polarity via Rac1 and laminin*. Mol Biol Cell, 2005. **16**(2): p. 433-45.
15. Lee, J.L. and C.H. Streuli, *Integrins and epithelial cell polarity*. J Cell Sci, 2014. **127**(Pt 15): p. 3217-25.
16. Hynes, R.O., *Integrins: Bidirectional, allosteric signaling machines*. Cell, 2002. **110**(6): p. 673-687.
17. Martin-Belmonte, F. and M. Perez-Moreno, *Epithelial cell polarity, stem cells and cancer*. Nature Reviews Cancer, 2012. **12**(1): p. 23-38.
18. Goldstein, B. and I.G. Macara, *The PAR proteins: Fundamental players in animal cell polarization*. Developmental Cell, 2007. **13**(5): p. 609-622.
19. Martin-Belmonte, F. and K. Mostov, *Regulation of cell polarity during epithelial morphogenesis*. Current Opinion in Cell Biology, 2008. **20**(2): p. 227-234.
20. Schreider, C., et al., *Integrin-mediated functional polarization of Caco-2 cells through E-cadherin-actin complexes*. J Cell Sci, 2002. **115**(Pt 3): p. 543-52.
21. Roignot, J., X. Peng, and K. Mostov, *Polarity in Mammalian Epithelial Morphogenesis*. Cold Spring Harbor Perspectives in Biology, 2013. **5**(2).
22. Yu, W., et al., *Involvement of RhoA, ROCK I and myosin II in inverted orientation of epithelial polarity*. EMBO Rep, 2008. **9**(9): p. 923-9.
23. Terry, S.J., et al., *Spatially restricted activation of RhoA signalling at epithelial junctions by p114RhoGEF drives junction formation and morphogenesis*. Nature Cell Biology, 2011. **13**(2): p. 159-U120.
24. Van Aelst, L. and M. Symons, *Role of Rho family GTPases in epithelial morphogenesis*. Genes & Development, 2002. **16**(9): p. 1032-1054.
25. Bryant, D.M., et al., *A molecular switch for the orientation of epithelial cell polarization*. Dev Cell, 2014. **31**(2): p. 171-87.
26. Kenny, P.A. and M.J. Bissell, *Tumor reversion: correction of malignant behavior by microenvironmental cues*. Int J Cancer, 2003. **107**(5): p. 688-95.
27. Ojakian, G.K. and R. Schwimmer, *Regulation of epithelial cell surface polarity reversal by beta 1 integrins*. J Cell Sci, 1994. **107** (Pt 3): p. 561-76.
28. Akhtar, N. and C.H. Streuli, *An integrin-ILK-microtubule network orients cell polarity and lumen formation in glandular epithelium*. Nat Cell Biol, 2013. **15**(1): p. 17-27.
29. Meads, T. and T.A. Schroer, *Polarity and nucleation of microtubules in polarized epithelial cells*. Cell Motil Cytoskeleton, 1995. **32**(4): p. 273-88.

30. Bonnans, C., J. Chou, and Z. Werb, *Remodelling the extracellular matrix in development and disease*. Nature Reviews Molecular Cell Biology, 2014. **15**(12): p. 786-801.
31. Desgrosellier, J.S. and D.A. Cheresh, *Integrins in cancer: biological implications and therapeutic opportunities (vol 10, pg 9, 2010)*. Nature Reviews Cancer, 2010. **10**(12): p. 890-890.
32. Tamkun, J.W., et al., *Structure of Integrin, a Glycoprotein Involved in the Transmembrane Linkage between Fibronectin and Actin*. Cell, 1986. **46**(2): p. 271-282.
33. Ley, K., et al., *Integrin-based therapeutics: biological basis, clinical use and new drugs*. Nature Reviews Drug Discovery, 2016. **15**(3): p. 173-183.
34. Elias, B.C., et al., *The Integrin beta 1 Subunit Regulates Paracellular Permeability of Kidney Proximal Tubule Cells*. Journal of Biological Chemistry, 2014. **289**(12): p. 8532-8544.
35. Campbell, I.D. and M.J. Humphries, *Integrin Structure, Activation, and Interactions*. Cold Spring Harbor Perspectives in Biology, 2011. **3**(3).
36. Zhu, J.Q., et al., *Requirement of alpha and beta subunit transmembrane helix separation for integrin outside-in signaling*. Blood, 2007. **110**(7): p. 2475-2483.
37. Lu, Z., et al., *Implications of the differing roles of the beta1 and beta3 transmembrane and cytoplasmic domains for integrin function*. Elife, 2016. **5**.
38. Critchley, D.R. and A.R. Gingras, *Talin at a glance*. Journal of Cell Science, 2008. **121**(9): p. 1345-1347.
39. Bouvard, D., et al., *Integrin inactivators: balancing cellular functions in vitro and in vivo*. Nature Reviews Molecular Cell Biology, 2013. **14**(7): p. 430-442.
40. Garcia, A.J. and D. Boettiger, *Integrin-fibronectin interactions at the cell-material interface: initial integrin binding and signaling*. Biomaterials, 1999. **20**(23-24): p. 2427-33.
41. Humphries, J.D., A. Byron, and M.J. Humphries, *Integrin ligands at a glance*. Journal of Cell Science, 2006. **119**(19): p. 3901-3903.
42. Su, Y., et al., *Relating conformation to function in integrin alpha5beta1*. Proc Natl Acad Sci U S A, 2016. **113**(27): p. E3872-81.
43. Lu, C., et al., *Locking in alternate conformations of the integrin alphaLbeta2 I domain with disulfide bonds reveals functional relationships among integrin domains*. Proc Natl Acad Sci U S A, 2001. **98**(5): p. 2393-8.
44. Lepzelter, D., O. Bates, and M. Zaman, *Integrin Clustering in Two and Three Dimensions*. Langmuir, 2012. **28**(12): p. 5379-5386.
45. Hato, T., N. Pampori, and S.J. Shattil, *Complementary roles for receptor clustering and conformational change in the adhesive and*

- signaling functions of integrin alpha(IIb)beta(3)*. Journal of Cell Biology, 1998. **141**(7): p. 1685-1695.
46. Kornberg, L., et al., *Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase*. J Biol Chem, 1992. **267**(33): p. 23439-42.
 47. Engler, A., et al., *Substrate compliance versus ligand density in cell on gel responses*. Biophysical Journal, 2004. **86**(1): p. 617-628.
 48. Harunaga, J.S. and K.M. Yamada, *Cell-matrix adhesions in 3D*. Matrix Biology, 2011. **30**(7-8): p. 363-368.
 49. Doyle, A.D. and K.M. Yamada, *Mechanosensing via cell-matrix adhesions in 3D microenvironments*. Experimental Cell Research, 2016. **343**(1): p. 60-66.
 50. Hytonen, V.P. and B. Wehrle-Haller, *Mechanosensing in cell-matrix adhesions - Converting tension into chemical signals*. Experimental Cell Research, 2016. **343**(1): p. 35-41.
 51. Parsons, J.T., *Focal adhesion kinase: the first ten years*. Journal of Cell Science, 2003. **116**(8): p. 1409-1416.
 52. Priddle, H., et al., *Disruption of the talin gene compromises focal adhesion assembly in undifferentiated but not differentiated embryonic stem cells*. Journal of Cell Biology, 1998. **142**(4): p. 1121-1133.
 53. Di Paolo, G., et al., *Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin*. Nature, 2002. **420**(6911): p. 85-9.
 54. Franco, S.J., et al., *Proteolysis of talin by calpain is required for efficient disassembly of adhesion complexes*. Molecular Biology of the Cell, 2004. **15**: p. 3a-3a.
 55. Carisey, A. and C. Ballestrem, *Vinculin, an adapter protein in control of cell adhesion signalling*. European Journal of Cell Biology, 2011. **90**(2-3): p. 157-163.
 56. Li, Z., H. Lee, and C. Zhu, *Molecular mechanisms of mechanotransduction in integrin-mediated cell-matrix adhesion*. Exp Cell Res, 2016. **349**(1): p. 85-94.
 57. Ghatak, S., J. Morgner, and S.A. Wickstrom, *ILK: a pseudokinase with a unique function in the integrin-actin linkage*. Biochem Soc Trans, 2013. **41**(4): p. 995-1001.
 58. Rooney, N., et al., *The Integrin-Mediated ILK-Parvin-alpha Pix Signaling Axis Controls Differentiation in Mammary Epithelial Cells*. Journal of Cellular Physiology, 2016. **231**(11): p. 2408-2417.
 59. Schiller, H.B., et al., *Quantitative proteomics of the integrin adhesome show a myosin II-dependent recruitment of LIM domain proteins*. Embo Reports, 2011. **12**(3): p. 259-266.
 60. Beningo, K.A., M. Dembo, and Y.I. Wang, *Responses of fibroblasts to anchorage of dorsal extracellular matrix receptors*. Proceedings of the

- National Academy of Sciences of the United States of America, 2004. **101**(52): p. 18024-18029.
61. Dutta, R.C. and A.K. Dutta, *Cell-interactive 3D-scaffold; advances and applications*. Biotechnology Advances, 2009. **27**(4): p. 334-339.
 62. Carey, S.P., et al., *Biophysical control of invasive tumor cell behavior by extracellular matrix microarchitecture*. Biomaterials, 2012. **33**(16): p. 4157-4165.
 63. Mason, B.N., et al., *Tuning three-dimensional collagen matrix stiffness independently of collagen concentration modulates endothelial cell behavior*. Acta Biomater, 2013. **9**(1): p. 4635-44.
 64. Hakkinen, K.M., et al., *Direct Comparisons of the Morphology, Migration, Cell Adhesions, and Actin Cytoskeleton of Fibroblasts in Four Different Three-Dimensional Extracellular Matrices*. Tissue Engineering Part A, 2011. **17**(5-6): p. 713-724.
 65. Friedland, J.C., M.H. Lee, and D. Boettiger, *Mechanically Activated Integrin Switch Controls $\alpha(5)\beta(1)$ Function*. Science, 2009. **323**(5914): p. 642-644.
 66. Cukierman, E., et al., *Taking cell-matrix adhesions to the third dimension*. Science, 2001. **294**(5547): p. 1708-1712.
 67. Doyle, A.D., et al., *Micro-environmental control of cell migration - myosin IIA is required for efficient migration in fibrillar environments through control of cell adhesion dynamics*. Journal of Cell Science, 2012. **125**(9): p. 2244-2256.
 68. Caswell, P.T., S. Vadrevu, and J.C. Norman, *Integrins: masters and slaves of endocytic transport*. Nature Reviews Molecular Cell Biology, 2009. **10**(12): p. 843-853.
 69. Lobert, V.H., et al., *Ubiquitination of $\alpha 5 \beta 1$ integrin controls fibroblast migration through lysosomal degradation of fibronectin-integrin complexes*. Dev Cell, 2010. **19**(1): p. 148-59.
 70. Tuloup-Minguez, V., et al., *Autophagy modulates cell migration and $\beta 1$ integrin membrane recycling*. Cell Cycle, 2013. **12**(20): p. 3317-3328.
 71. Woods, A.J., et al., *PKD1/PKC μ promotes $\alpha v \beta 3$ integrin recycling and delivery to nascent focal adhesions*. Embo Journal, 2004. **23**(13): p. 2531-2543.
 72. Christoforides, C., et al., *PKD Controls $\alpha v \beta 3$ Integrin Recycling and Tumor Cell Invasive Migration through Its Substrate Rabaptin-5*. Developmental Cell, 2012. **23**(3): p. 560-572.
 73. White, D.P., P.T. Caswell, and J.C. Norman, *$\alpha V \beta 3$ and $\alpha 5 \beta 1$ integrin recycling pathways dictate downstream Rho kinase signaling to regulate persistent cell migration*. Journal of Cell Biology, 2007. **177**(3): p. 515-525.

74. Krishnan, M., et al., *Rab25 regulates integrin expression in polarized colonic epithelial cells*. *Molecular Biology of the Cell*, 2013. **24**(6): p. 818-831.
75. Onodera, Y., et al., *Rab5c promotes AMAP1-PRKD2 complex formation to enhance beta 1 integrin recycling in EGF-induced cancer invasion*. *Journal of Cell Biology*, 2012. **197**(7): p. 983-996.
76. Hynes, R.O., *The extracellular matrix: not just pretty fibrils*. *Science*, 2009. **326**(5957): p. 1216-9.
77. Singh, P., C. Carraher, and J.E. Schwarzbauer, *Assembly of fibronectin extracellular matrix*. *Annu Rev Cell Dev Biol*, 2010. **26**: p. 397-419.
78. Mosher, D.F., *Assembly of Fibronectin into Extracellular-Matrix*. *Current Opinion in Structural Biology*, 1993. **3**(2): p. 214-222.
79. Sechler, J.L. and J.E. Schwarzbauer, *Coordinated regulation of fibronectin fibril assembly and actin stress fiber formation*. *Cell Adhesion and Communication*, 1996. **4**(6): p. 413-424.
80. Ohashi, T. and H.P. Erickson, *Domain unfolding plays a role in superfibronectin formation*. *J Biol Chem*, 2005. **280**(47): p. 39143-51.
81. Morla, A., Z. Zhang, and E. Ruoslahti, *Superfibronectin is a functionally distinct form of fibronectin*. *Nature*, 1994. **367**(6459): p. 193-6.
82. Pasqualini, R., et al., *A polymeric form of fibronectin has antimetastatic effects against multiple tumor types*. *Nature Medicine*, 1996. **2**(11): p. 1197-1203.
83. Normanno, N., et al., *Epidermal growth factor receptor (EGFR) signaling in cancer*. *Gene*, 2006. **366**(1): p. 2-16.
84. Singh, B., et al., *Induction of lateral lumens through disruption of a monoleucine-based basolateral-sorting motif in betacellulin*. *Journal of Cell Science*, 2015. **128**(18): p. 3444-3455.
85. Singh, B., G. Bogatcheva, and R.J. Coffey, *Apical mistrafficking of epiregulin can be a driver event in epithelial cancers*. *Molecular Biology of the Cell*, 2014. **25**.
86. Singh, B., G. Bogatcheva, and R.J. Coffey, *A 3D system to elucidate a direct role for epiregulin mistrafficking in epithelial transformation*. *Cancer Research*, 2015. **75**.
87. Roberts, R.B., et al., *Importance of epidermal growth factor receptor signaling in establishment of adenomas and maintenance of carcinomas during intestinal tumorigenesis*. *Proceedings of the National Academy of Sciences of the United States of America*, 2002. **99**(3): p. 1521-1526.
88. Herbst, R.S., *Review of epidermal growth factor receptor biology*. *International Journal of Radiation Oncology Biology Physics*, 2004. **59**(2): p. 21-26.
89. Lemmon, M.A. and J. Schlessinger, *Cell signaling by receptor tyrosine kinases*. *Cell*, 2010. **141**(7): p. 1117-34.

90. Baldys, A., et al., *Essential Role of c-Cbl in Amphiregulin-Induced Recycling and Signaling of the Endogenous Epidermal Growth Factor Receptor*. *Biochemistry*, 2009. **48**(7): p. 1462-1473.
91. Roepstorff, K., et al., *Differential Effects of EGFR Ligands on Endocytic Sorting of the Receptor*. *Traffic*, 2009. **10**(8): p. 1115-1127.
92. Kovacs, E., et al., *A Structural Perspective on the Regulation of the Epidermal Growth Factor Receptor*. *Annual Review of Biochemistry*, Vol 84, 2015. **84**: p. 739-764.
93. Lee, N.Y., T.L. Hazlett, and J.G. Koland, *Structure and dynamics of the epidermal growth factor receptor C-terminal phosphorylation domain*. *Protein Science*, 2006. **15**(5): p. 1142-1152.
94. Ivaska, J. and J. Heino, *Cooperation Between Integrins and Growth Factor Receptors in Signaling and Endocytosis*. *Annual Review of Cell and Developmental Biology*, Vol 27, 2011. **27**: p. 291-320.
95. Hang, Q.L., et al., *Integrin alpha 5 Suppresses the Phosphorylation of Epidermal Growth Factor Receptor and Its Cellular Signaling of Cell Proliferation via N-Glycosylation*. *Journal of Biological Chemistry*, 2015. **290**(49): p. 29345-29360.
96. Vial, D. and P.J. McKeown-Longo, *Role of EGFR expression levels in the regulation of integrin function by EGF*. *Molecular Carcinogenesis*, 2016. **55**(6): p. 1118-1123.
97. Vial, D. and P.J. McKeown-Longo, *Epidermal Growth Factor (EGF) Regulates alpha 5 beta 1 Integrin Activation State in Human Cancer Cell Lines through the p90RSK-dependent Phosphorylation of Filamin A*. *Journal of Biological Chemistry*, 2012. **287**(48): p. 40371-40380.
98. Leabu, M., et al., *Integrin alpha 2 beta 1 modulates EGF stimulation of Rho GTPase-dependent morphological changes in adherent human rhabdomyosarcoma RD cells*. *Journal of Cellular Physiology*, 2005. **202**(3): p. 754-766.
99. Clevers, H., *The Intestinal Crypt, A Prototype Stem Cell Compartment*. *Cell*, 2013. **154**(2): p. 274-284.
100. Markowitz, S.D. and M.M. Bertagnolli, *Molecular origins of cancer: Molecular basis of colorectal cancer*. *N Engl J Med*, 2009. **361**(25): p. 2449-60.
101. Kinzler, K.W. and B. Vogelstein, *Lessons from hereditary colorectal cancer*. *Cell*, 1996. **87**(2): p. 159-70.
102. Fujimoto, K., R.D. Beauchamp, and R.H. Whitehead, *Identification and isolation of candidate human colonic clonogenic cells based on cell surface integrin expression*. *Gastroenterology*, 2002. **123**(6): p. 1941-1948.
103. Jones, R.G., et al., *Conditional deletion of beta 1 integrin in intestinal epithelium*. *Gastroenterology*, 2005. **128**(4): p. A99-A99.
104. Canel, M., et al., *E-cadherin-integrin crosstalk in cancer invasion and metastasis*. *Journal of Cell Science*, 2013. **126**(2): p. 393-401.

105. Wang, F., et al., *Reciprocal interactions between beta 1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: A different perspective in epithelial biology*. Proceedings of the National Academy of Sciences of the United States of America, 1998. **95**(25): p. 14821-14826.
106. Morello, V., et al., *beta 1 integrin controls EGFR signaling and tumorigenic properties of lung cancer cells*. Oncogene, 2011. **30**(39): p. 4087-4096.
107. Ning, Y., T. Buranda, and L.G. Hudson, *Activated epidermal growth factor receptor induces integrin alpha 2 internalization via caveolae/raft-dependent endocytic pathway*. Journal of Biological Chemistry, 2007. **282**(9): p. 6380-6387.
108. Hughes, C.S., L.M. Postovit, and G.A. Lajoie, *Matrigel: A complex protein mixture required for optimal growth of cell culture*. Proteomics, 2010. **10**(9): p. 1886-1890.
109. Cox, T.R. and J.T. Erler, *Remodeling and homeostasis of the extracellular matrix: implications for fibrotic diseases and cancer*. Disease Models & Mechanisms, 2011. **4**(2): p. 165-178.
110. Petras, M., et al., *Molecular interactions of ErbB1 (EGFR) and integrin-beta 1 in astrocytoma frozen sections predict clinical outcome and correlate with Akt-mediated in vitro radioresistance*. Neuro-Oncology, 2013. **15**(8): p. 1027-1040.
111. Ojakian, G.K. and R. Schwimmer, *The Polarized Distribution of an Apical Cell-Surface Glycoprotein Is Maintained by Interactions with the Cytoskeleton of Madin-Darby Canine Kidney-Cells*. Journal of Cell Biology, 1988. **107**(6): p. 2377-2387.
112. Chen, J.C. and M.A. Krasnow, *Integrin Beta 1 Suppresses Multilayering of a Simple Epithelium*. Plos One, 2012. **7**(12).
113. O'Brien, L.E., et al., *Rac1 orientates epithelial apical polarity through effects on basolateral laminin assembly*. Nature Cell Biology, 2001. **3**(9): p. 831-838.
114. Halbleib, J.M., et al., *Transcriptional modulation of genes encoding structural characteristics of differentiating Enterocytes during development of a polarized epithelium in vitro*. Molecular Biology of the Cell, 2007. **18**(11): p. 4261-4278.
115. Stallmach, A., et al., *Diminished expression of integrin adhesion molecules on human colonic epithelial cells during the benign to malign tumour transformation*. Gut, 1992. **33**(3): p. 342-6.
116. Williams, C.M., et al., *Fibronectin expression modulates mammary epithelial cell proliferation during acinar differentiation*. Cancer Res, 2008. **68**(9): p. 3185-92.
117. Park, C.C., et al., *Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo*. Cancer Res, 2006. **66**(3): p. 1526-35.

118. Howlett, A.R., et al., *Cellular growth and survival are mediated by beta 1 integrins in normal human breast epithelium but not in breast carcinoma*. J Cell Sci, 1995. **108 (Pt 5)**: p. 1945-57.
119. Li, C.X., et al., *Excess PLAC8 promotes an unconventional ERK2-dependent EMT in colon cancer*. Journal of Clinical Investigation, 2014. **124(5)**: p. 2172-2187.
120. Byron, A., et al., *Anti-integrin monoclonal antibodies*. Journal of Cell Science, 2009. **122(22)**: p. 4009-4011.
121. Dransfield, I., et al., *Divalent cation regulation of the function of the leukocyte integrin LFA-1*. J Cell Biol, 1992. **116(1)**: p. 219-26.
122. Kornberg, L.J., et al., *Signal Transduction by Integrins - Increased Protein Tyrosine Phosphorylation Caused by Clustering of Beta-1 Integrins*. Proceedings of the National Academy of Sciences of the United States of America, 1991. **88(19)**: p. 8392-8396.
123. Jewell, K., et al., *Stimulation of Tyrosine Phosphorylation of Distinct Proteins in Response to Antibody-Mediated Ligation and Clustering of Alpha(3) and Alpha(6) Integrins*. Journal of Cell Science, 1995. **108**: p. 1165-1174.
124. Calderwood, D.A., S.J. Shattil, and M.H. Ginsberg, *Integrins and actin filaments: Reciprocal regulation of cell adhesion and signaling*. Journal of Biological Chemistry, 2000. **275(30)**: p. 22607-22610.
125. Mould, A.P., et al., *Defining the topology of integrin alpha5beta1-fibronectin interactions using inhibitory anti-alpha5 and anti-beta1 monoclonal antibodies. Evidence that the synergy sequence of fibronectin is recognized by the amino-terminal repeats of the alpha5 subunit*. J Biol Chem, 1997. **272(28)**: p. 17283-92.
126. Robinson, E.E., R.A. Foty, and S.A. Corbett, *Fibronectin matrix assembly regulates alpha5beta1-mediated cell cohesion*. Mol Biol Cell, 2004. **15(3)**: p. 973-81.
127. Mckeownlongo, P.J. and D.F. Mosher, *Binding of Plasma Fibronectin to Cell-Layers of Human-Skin Fibroblasts*. Journal of Cell Biology, 1983. **97(2)**: p. 466-472.
128. Sottile, J. and D.C. Hocking, *Fibronectin polymerization regulates the composition and stability of extracellular matrix fibrils and cell-matrix adhesions*. Molecular Biology of the Cell, 2002. **13(10)**: p. 3546-3559.
129. Shi, F., et al., *Fibronectin Matrix Polymerization Regulates Smooth Muscle Cell Phenotype through a Rac1 Dependent Mechanism*. Plos One, 2014. **9(4)**.
130. Arjonen, A., et al., *Distinct recycling of active and inactive beta1 integrins*. Traffic, 2012. **13(4)**: p. 610-25.
131. Humphries, J.D., et al., *Dual functionality of the anti-beta1 integrin antibody, 12G10, exemplifies agonistic signalling from the ligand binding pocket of integrin adhesion receptors*. J Biol Chem, 2005. **280(11)**: p. 10234-43.

132. Araki, E., et al., *Clustering of Syndecan-4 and Integrin beta 1 by Laminin alpha 3 Chain-derived Peptide Promotes Keratinocyte Migration*. *Molecular Biology of the Cell*, 2009. **20**(13): p. 3012-3024.
133. Koshida, S., et al., *Integrin alpha5-dependent fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos*. *Dev Cell*, 2005. **8**(4): p. 587-98.
134. Brafman, D.A., et al., *Regulation of endodermal differentiation of human embryonic stem cells through integrin-ECM interactions*. *Cell Death Differ*, 2013. **20**(3): p. 369-81.
135. Robinson, E.E., et al., *Alpha5beta1 integrin mediates strong tissue cohesion*. *J Cell Sci*, 2003. **116**(Pt 2): p. 377-86.
136. Hamalisto, S., et al., *A ZO-1/alpha5beta1-integrin complex regulates cytokinesis downstream of PKCepsilon in NCI-H460 cells plated on fibronectin*. *PLoS One*, 2013. **8**(8): p. e70696.
137. Tuomi, S., et al., *PKCepsilon regulation of an alpha5 integrin-ZO-1 complex controls lamellae formation in migrating cancer cells*. *Sci Signal*, 2009. **2**(77): p. ra32.
138. Kuwada, S.K., J. Kuang, and X. Li, *Integrin alpha5/beta1 expression mediates HER-2 down-regulation in colon cancer cells*. *J Biol Chem*, 2005. **280**(19): p. 19027-35.
139. Pimton, P., et al., *Fibronectin-mediated upregulation of alpha5beta1 integrin and cell adhesion during differentiation of mouse embryonic stem cells*. *Cell Adh Migr*, 2011. **5**(1): p. 73-82.
140. Brennan, J.R. and D.C. Hocking, *Cooperative effects of fibronectin matrix assembly and initial cell-substrate adhesion strength in cellular self-assembly*. *Acta Biomater*, 2016. **32**: p. 198-209.
141. Hsia, H.C., M.R. Nair, and S.A. Corbett, *The fate of internalized alpha5 integrin is regulated by matrix-capable fibronectin*. *J Surg Res*, 2014. **191**(2): p. 268-79.
142. Wu, C.Y., et al., *Integrin Activation and Cytoskeletal Interaction Are Essential for the Assembly of a Fibronectin Matrix*. *Cell*, 1995. **83**(5): p. 715-724.
143. Quaroni, A., K.J. Isselbacher, and E. Ruoslahti, *Fibronectin synthesis by epithelial crypt cells of rat small intestine*. *Proc Natl Acad Sci U S A*, 1978. **75**(11): p. 5548-52.
144. Schwartz, M.A., C. Lechene, and D.E. Ingber, *Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin alpha 5 beta 1, independent of cell shape*. *Proc Natl Acad Sci U S A*, 1991. **88**(17): p. 7849-53.
145. Beaulieu, J.F., P.H. Vachon, and S. Chartrand, *Immunolocalization of extracellular matrix components during organogenesis in the human small intestine*. *Anat Embryol (Berl)*, 1991. **183**(4): p. 363-9.

146. Xu, C., et al., *Regulation of sonic hedgehog expression by integrin beta1 and epidermal growth factor receptor in intestinal epithelium*. IUBMB Life, 2014. **66**(10): p. 694-703.
147. Hang, Q., et al., *Integrin alpha5 Suppresses the Phosphorylation of Epidermal Growth Factor Receptor and Its Cellular Signaling of Cell Proliferation via N-Glycosylation*. J Biol Chem, 2015. **290**(49): p. 29345-60.
148. Hang, Q., et al., *N-Glycosylation of integrin alpha5 acts as a switch for EGFR-mediated complex formation of integrin alpha5beta1 to alpha6beta4*. Sci Rep, 2016. **6**: p. 33507.
149. Banon-Rodriguez, I., et al., *EGFR controls IQGAP basolateral membrane localization and mitotic spindle orientation during epithelial morphogenesis*. Embo Journal, 2014. **33**(2): p. 129-145.
150. Basson, M.D., N.J. Emenaker, and M.A. Sanders, *Alpha integrin subunits regulate human (Caco-2) intestinal epithelial proliferation and phenotype*. Cell Physiol Biochem, 2000. **10**(1-2): p. 27-36.
151. Kuwada, S.K. and X. Li, *Integrin alpha5/beta1 mediates fibronectin-dependent epithelial cell proliferation through epidermal growth factor receptor activation*. Mol Biol Cell, 2000. **11**(7): p. 2485-96.
152. Drivalos, A., et al., *Expression of alpha5-integrin, alpha7-integrin, Epsilon-cadherin, and N-cadherin in localized prostate cancer*. Urol Oncol, 2016. **34**(4): p. 165.e11-8.
153. Laukaitis, C.M., et al., *Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells*. Journal of Cell Biology, 2001. **153**(7): p. 1427-1440.
154. Cluzel, C., et al., *The mechanisms and dynamics of alpha v beta 3 integrin clustering in living cells*. Journal of Cell Biology, 2005. **171**(2): p. 383-392.
155. Jiang, S.T., W.J. Chuang, and M.J. Tang, *Role of fibronectin deposition in branching morphogenesis of Madin-Darby canine kidney cells*. Kidney International, 2000. **57**(5): p. 1860-1867.
156. Roman, J., E.C. Crouch, and J.A. McDonald, *Reagents That Inhibit Fibronectin Matrix Assembly of Cultured-Cells Also Inhibit Lung Branching Morphogenesis In vitro - Implications for Lung Development, Injury, and Repair*. Chest, 1991. **99**(3): p. S20-S21.
157. Sakai, T., M. Larsen, and K.M. Yamada, *Fibronectin requirement in branching morphogenesis*. Nature, 2003. **423**(6942): p. 876-881.
158. Polacheck, W.J. and C.S. Chen, *Measuring cell-generated forces: a guide to the available tools*. Nature Methods, 2016. **13**(5): p. 415-423.
159. Liu, Z.J., et al., *Mechanical tugging force regulates the size of cell-cell junctions*. Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(22): p. 9944-9949.
160. Ng, M.R., et al., *Mapping the dynamics of force transduction at cell-cell junctions of epithelial clusters*. Elife, 2014. **3**.

161. Lamouille, S., J. Xu, and R. Derynck, *Molecular mechanisms of epithelial-mesenchymal transition*. Nature Reviews Molecular Cell Biology, 2014. **15**(3): p. 178-196.
162. Nelson, W.J., *Remodeling Epithelial Cell Organization: Transitions Between Front-Rear and Apical-Basal Polarity*. Cold Spring Harbor Perspectives in Biology, 2009. **1**(1).
163. Julich, D., et al., *Cross-Scale Integrin Regulation Organizes ECM and Tissue Topology*. Dev Cell, 2015. **34**(1): p. 33-44.
164. Hocking, D.C., J. Sottile, and K.J. Langenbach, *Stimulation of integrin-mediated cell contractility by fibronectin polymerization*. Journal of Biological Chemistry, 2000. **275**(14): p. 10673-10682.
165. Mack, N.A. and M. Georgiou, *The interdependence of the Rho GTPases and apicobasal cell polarity*. Small GTPases, 2014. **5**(2): p. 10.
166. Yu, H.H., et al., *p120-catenin controls contractility along the vertical axis of epithelial lateral membranes*. J Cell Sci, 2016. **129**(1): p. 80-94.
167. Nusrat, A., et al., *Rho-Protein Regulates Tight Junctions and Perijunctional Actin Organization in Polarized Epithelia*. Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(23): p. 10629-10633.
168. Rundhaug, J.E., et al., *Changes in Responsiveness of Rat Tracheal Epithelial-Cells to Transforming Growth Factor-Beta-1 with Time in Culture*. Journal of Cellular Physiology, 1992. **152**(2): p. 281-291.
169. Pellinen, T., et al., *Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta 1-integrins*. Journal of Cell Biology, 2006. **173**(5): p. 767-780.
170. Bridgewater, R.E., J.C. Norman, and P.T. Caswell, *Integrin trafficking at a glance*. Journal of Cell Science, 2012. **125**(16): p. 3695-3701.
171. De Franceschi, N., et al., *Selective integrin endocytosis is driven by interactions between the integrin alpha-chain and AP2*. Nature Structural & Molecular Biology, 2016. **23**(2): p. 172-+.