Ligand-independent integrin beta1 signaling supports lung adenocarcinoma development

By

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DEDICATION

To my wife, the love of my life.

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I would like to thank my advisor, Dr. Roy Zent, for teaching me how to talk, think, and write like a scientist. I would also like to thank Dr. W. Kimryn Rathmell, who has always expressed unwavering confidence in me and made me believe in myself. I would also like to thank my thesis committee members Drs. Tim Blackwell and Ambra Pozzi for their many hours of discussion and insight as well as Dr. Pierre Massion, may he rest in peace.

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LIST OF ABBREVIATIONS

- μg: Microgram
- μm: Micrometer
- A: Alanine
- AKTi: AKT inhibitor
- AT2 cells: Alveolar epithelial type 2 cells
- BRDU: Bromodeoxyuridine
- CAF: Cancer-associated fibroblasts
- CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats / CRISPR-associated protein 9
- CST: Cell Signaling Technology
- DAB: 3,3'-Diaminobenzidine
- DAPI: 4',6-Diamino-2-phenylindole
- DEG: Differentially expressed gene
- Dox: Doxycycline
- ECM: Extracellular matrix
- EGF: Epithelial growth factor
- EMT: Epithelial-to-mesenchymal transition
- ERKi: ERK inhibitor
- FACS: Fluorescence-activated cell sorting
- FAK: Focal adhesion kinase
- FAKi: FAK inhibitor

- FBS: Fetal bovine serum
- IFNγ: Interferon gamma
- IL-32: Interleukin-32
- KEGG: Kyoto Encyclopedia of Genes and Genomes
- kg: Kilogram
- KO: Knock-out
- LUAD: Lung adenocarcinoma
- LSL: Lox-Stop-Lox sequence
- LOX: Lysyl oxidase
- MCT4: Monocarboxylase transporter 4
- mg: Milligram
- ml: Milliliter
- mm: Millimeter
- mM: Millimolar
- MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- mTOR: Mammalian target of rapamycin
- mTORC1: Mammalian target of rapamycin complex 1
- mTORC1: Mammalian target of rapamycin complex 2
- N: Asparagine
- NADPH: Reduced nicotinamide adenine dinucleotide phosphate
- ng: Nanogram
- OCT: Optimal cutting temperature compound
- OS: Overall survival

- P: Proline
- PBS: Phosphate-buffered saline
- PI3K: Phosphatidylinositol-3-kinase
- PIP3: Phosphatidylinositol-3,4,5-trisphosphate
- PROTAC: Proteolysis-targeted chimera
- PyMT: Polyomavirus middle T
- RBC: Red blood cell
- RCC: Renal cell carcinoma
- RFS: Recurrence-free survival
- RGD: Arginine-Glycine-Aspartate
- RPMI: Roswell Park Memorial Institute
- RNA-seq: RNA-sequencing
- SASP: Senescence-associated secretory phenotype
- SD: Standard deviation
- SEM: Standard error of the mean
- SPC: Surfactant protein C
- TAZ: Transcriptional co-activator with PDZ-binding motif
- TGFβ: Transforming growth factor β
- TKI: Tyrosine kinase inhibitor
- TMA: Tissue microarray
- TSP-1: Thrombospondin-1
- UMAP: Uniform manifold approximation and projection
- VEGF: Vascular epithelial growth factor

- WT: Wild type
- Y: Tyrosine
- YAP: Yes-associated protein (YAP)

CHAPTER 1

INTRODUCTION

Integrin Function and Structure

Integrins function as the principle extracellular matrix (ECM) receptor of the cell. The ECM is the non-cellular component of cells and tissues. It provides essential physical scaffolding for cells and tissues as well as biochemical and biophysical cues that are required for tissue morphogenesis, differentiation and homeostasis (1). Integrins span the plasma membrane and mediate anchorage of the cell to the ECM by forming a mechanical linkage between it and the cytoskeleton (**Figure 1**). This mechanical linkage is required for anchorage and anchorage-dependent cellular functions such as proliferation, migration, and invasion.

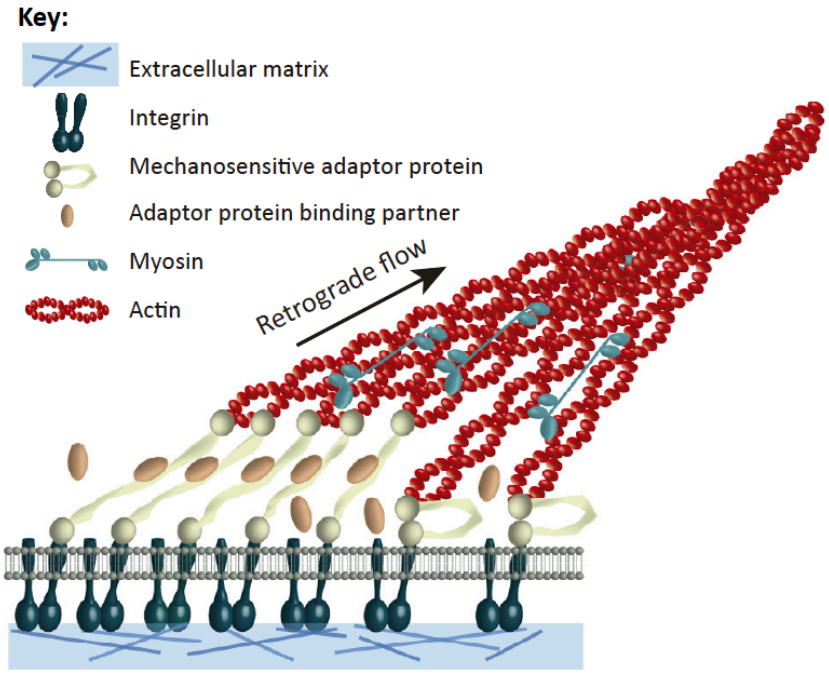

Figure 1: Integrins mediate cell adhesion by linking the extracellular matrix and the cytoskeleton. Integrins are transmembrane proteins that enable cell adhesion and adhesion-dependent functions by linking the cytoskeleton to the ECM. The figure was reproduced and adapted with permission (2); permission conveyed through the Copyright Clearance Center.

Integrins are composed of 18 α and 8 β subunits in mammals, which come together as 24 heterodimers that bind distinct ECM proteins (**Figure 2**) (3). These distinct heterodimeric pairs dictate substrate specificity. For example, integrin α1β1 forms a collagen receptor whereas integrin αvβ1 is an RGD receptor and integrin α3β1 is a laminin receptor. Integrin subunits are composed of three domains: ectodomain, a transmembrane domain, and a cytoplasmic tail (**Figure 3**). The ectodomain, located outside the cell, mediates ECM binding and binding-specificity. The transmembrane domain is composed of an α-helix that spans the phospholipid bilayer (classically the plasma membrane) (4-6). The cytoplasmic tails, except for integrin β4,

are short and mediate interactions with cytoskeletal and signaling proteins. Most integrin tail interactions with other cytoplasmic proteins occur via the β tails and it is unclear how αβ interactions of the integrin cytoplasmic domains regulate integrin specificity. The integrin β1 cytoplasmic tail consists of 47 amino acids and, like nearly all β integrins, has a membrane proximal NPxY motif (where x represents any amino acid) and a membrane distal NxxY motif (7). These motifs serve as the canonical recognition sequences for phosphotyrosine-binding domains and are binding sites for multiple integrin binding proteins (8). A Y-to-A mutation at these sites results in severe abnormalities in integrin function because it inhibits binding of cytosolic integrin binding proteins to integrin β1 tails (9-12).

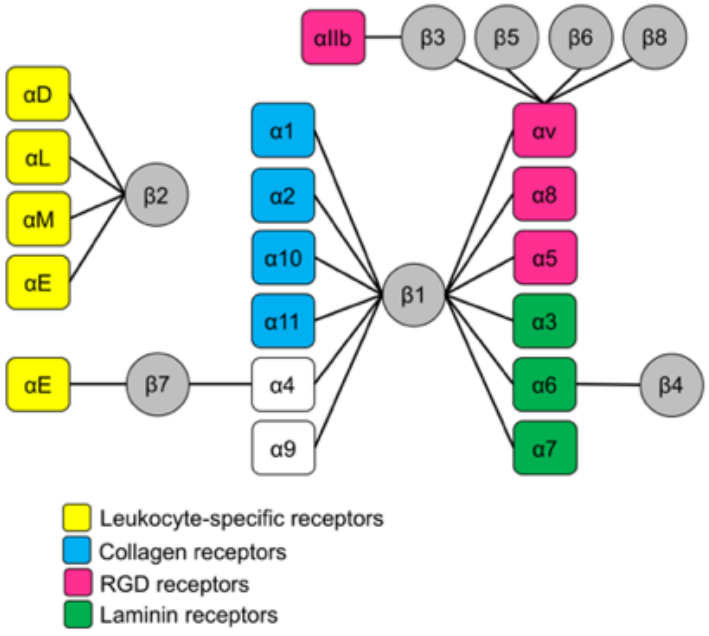

Figure 2: Integrins are heterodimeric receptors of the extracellular matrix. Integrins are transmembrane proteins composed of 18 α and 8 β subunits in mammals, which come together as 24 heterodimers that bind distinct ECM proteins.

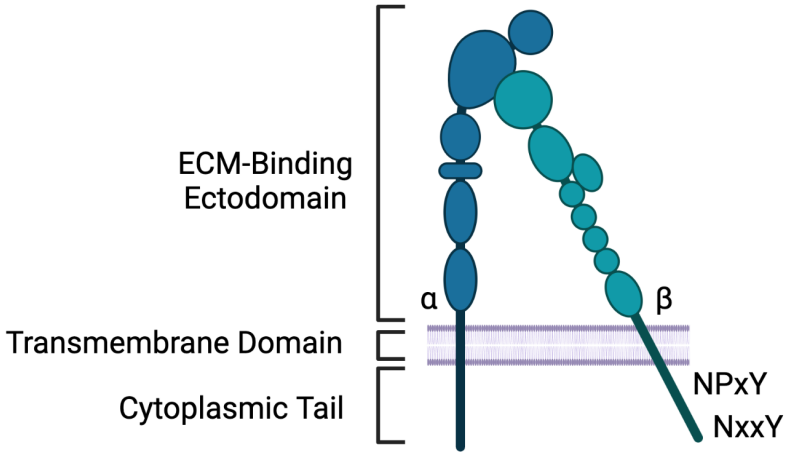

Figure 3: Integrin domains and cytoplasmic tail motifs. Integrins are composed of three domains: an ECM-binding ectodomain, a transmembrane domain, and a cytoplasmic tail. Most β subunit integrin tails contain a membrane proximal NPxY motif and a membrane distal NxxY motif that, upon phosphorylation, serve as binding sites for multiple integrin binding proteins and are critical to integrin function. "x" = any amino acid. Created using www.biorender.com.

Integrins in Epithelial Cell Biology

Integrins mediate epithelial cell adhesion to a specialized ECM structure called the basement membrane. The basement membrane is an amorphous, dense, sheet-like structure with multiple components including type IV collagen, laminin, and heparan-sulphate proteoglycans (1). Epithelial cell adhesion to the basement membrane is a key organizing principle for epithelial cells (**Figure 4, top**). The basement membrane serves as a demarcation line, separating epithelial cells from the underly stroma (1). In addition, epithelial cell survival requires correct

adhesion to the basement membrane. Epithelial cell detachment from the basement membrane typically results in apoptotic cell death. In this manner, epithelial cell adhesion to the basement membrane serves as an important regulator in multicellular organisms and misplaced cells are efficiently eliminated, preventing their disorganized growth in the wrong location (13). This apoptotic cell death due to loss or inappropriate adhesion is termed anoikis (13). Thus, epithelial cell adhesion to the basement membrane is critical for cell and tissue homeostasis.

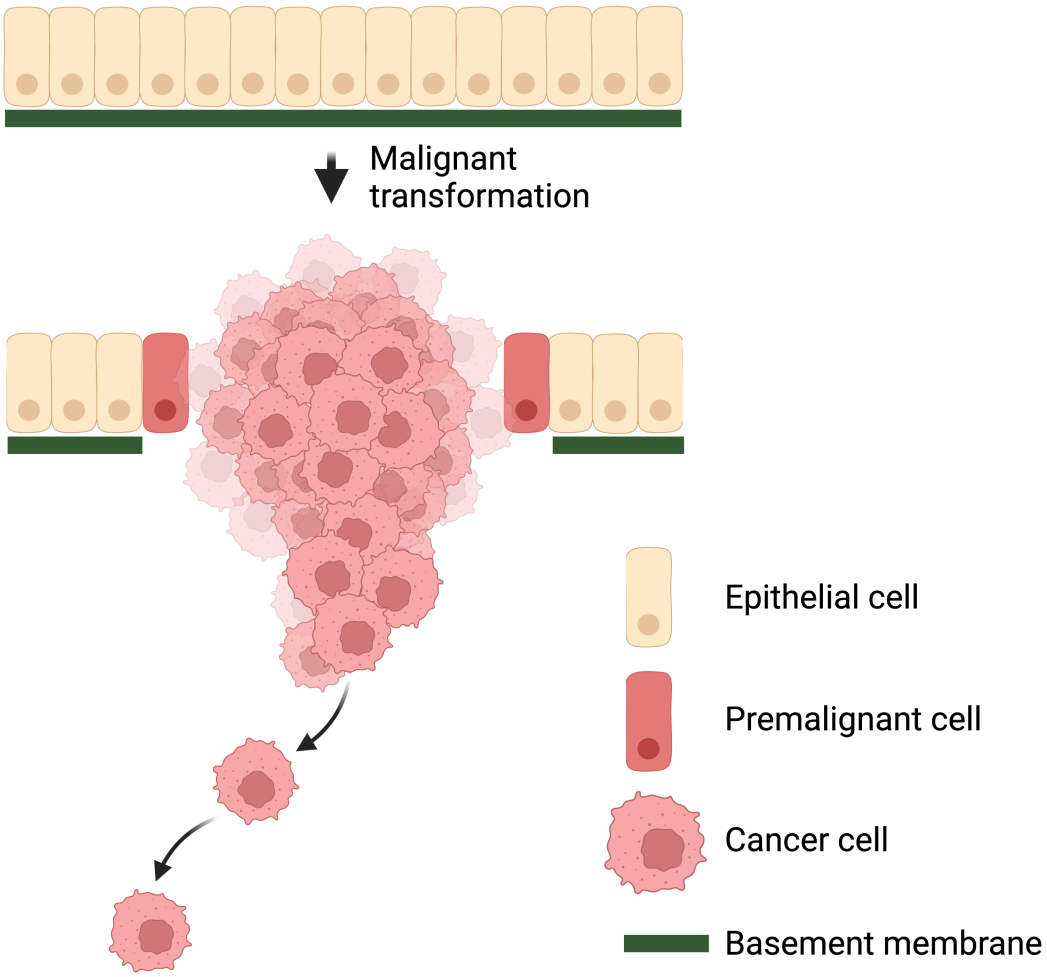

Figure 4: Basement membranes are a key organizing principle in epithelial tissues. (top) Integrins facilitate cell adhesion to the basement membrane in epithelial tissues. The basement membrane demarcates epithelial cells from the stroma. Non-transformed epithelial cells that lose adhesion to the basement membrane are typically eliminated. (bottom) The histopathologic definition of a malignant carcinoma includes invasion of carcinoma cells through the basement membrane and into the surrounding stroma. This invasion portends the ability of cells to metastasize to distant organs and cause the death of the organism. Unlike the epithelial cells from which they derive, carcinoma cells lose adhesion to the basement membrane. Created using www.biorender.com.

Integrins During Carcinogenesis

A key step in carcinogenesis is invasion of carcinoma cells through the basement membrane and into the surrounding stroma (**Figure 4, bottom**). This invasion is the defining histopathologic feature of malignant tumors and portends the ability of cells to metastasize to distant organs and cause the death of the organism. It also represents a major perturbation in the fundamental organization of epithelial tissues. In doing so, carcinoma cells no longer adhere to the basement membrane and, during hematogenous and lymphatic dissemination, could conceivably be devoid of all ECM adhesion. Thus, carcinoma cells must develop mechanisms whereby they can overcome the anoikis checkpoint inherent within the epithelial cells from which they derived. Integrin signaling in carcinoma cells likely contribute to overcoming this anoikis checkpoint.

Experimental data suggest many tumors retain their requirement for integrin signaling. For example, high integrin β1 expression in human lung adenocarcinoma correlates with decreased patient survival (**Figure 5**) (14). In addition, deletion of integrin β1 in breast epithelial cells resulted in decreased tumorigenesis in a mouse model of breast cancer (15). These data suggest that integrin signaling remains important in carcinoma cells despite their malignant transformation. Given the known role of integrins as ECM receptors, it has often been assumed that integrin contribution to tumor progression is dependent on the ECM-binding ectodomain. Indeed, treatment of cancer cells with antibodies targeting the ECM-binding ectodomain of integrins has improved survival in mice injected with cancer cells as well as decreased tumor growth (16). In addition, integrins are known to be critical during the metastatic cascade that

ultimately results in metastatic tumors growing in distant organs and organ failure (17). Such data spurred the development of pharmacologic inhibitors of the ECM-binding ectodomain of integrins as anti-neoplastic therapies. Unfortunately, these agents have not made a significant impact in the care of cancer patients. This was clearly demonstrated by the failure of cilengitide, a small molecule that inhibits integrin αvβ3 and αvβ5 binding to ECM, to improve outcomes of patients with glioblastoma when added to standard-of-care chemoradiation (18). Similarly, the αv integrin-targeting antibody abituzumab combined with standard-of-care chemotherapy failed to improve the primary endpoint of progression free survival in a randomized phase II trial in oxaliplatin-refractory, *KRAS* wild type colorectal cancer (19). These data suggest that, assuming integrins can function as tumor promoters, the best method to target these proteins remains undefined.

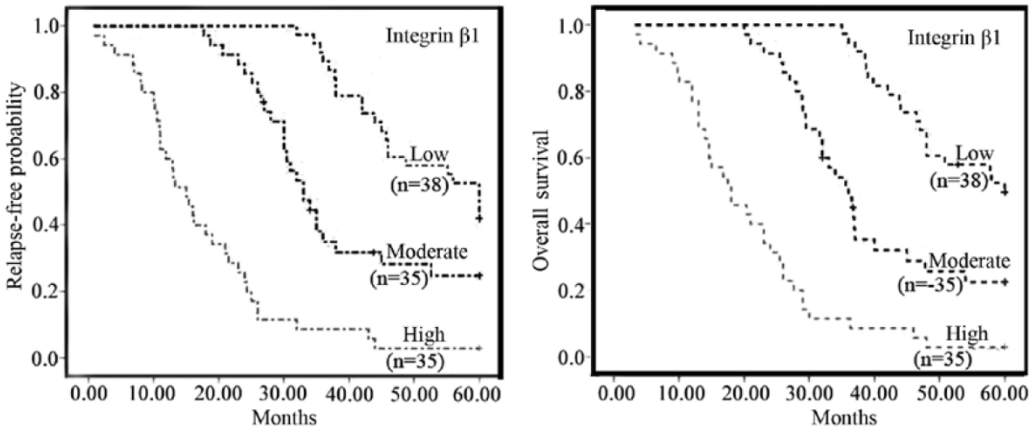

Figure 5: Integrin β1 expression in lung adenocarcinoma correlates with poor prognosis. Patients whose lung adenocarcinoma tumors express high levels of integrin β1 demonstrate worse (left) recurrence free survival and (right) overall survival relative to tumors with low or

moderate expression of integrin β1. The figure was reproduced and adapted with permission (14); permission conveyed via direct correspondence with the publisher.

CHAPTER 2

LITERATURE REVIEW

Prior to discussing our experimental approach and data exploring the role of integrin β1 in lung adenocarcinoma, we take this opportunity to perform a literature review to explore the various mechanisms whereby integrins can promote lung cancer development and progression. We chose to frame this discussion around the Hallmarks of Cancer, a classic review series within cancer biology.

Hallmarks of Cancer

Multicellular organisms are extraordinarily complex, with many cells forming elaborate tissues that work in coordination to form organs and sustain life. Some estimates place the number of cells in the adult human body at more than 3.0 x 10^13 cells (20). A tight organization and orchestration of these many cells is required for the organism to survive. Some cells need to proliferate rapidly, while others need to remain functional yet without further replication, while still others need to be eliminated for the organism to continue to thrive. Controls have developed overtime to regulate these many cell fates. When these controls break down, some cells may grow, survive and proliferate in an unregulated manner. Eventually, these uncontrolled cells may

travel to distant sites, locations where the cells would classically be unable to survive and continue its replicative stampede. This uncontrolled cell growth often proceeds in a way that destroys nearby cells, leading to impaired tissue function, organ failure, and ultimately the death of the organism. It is via this route that normal cells transform into cancer cells, a blight that continues to plague humanity across the globe.

The transition from a normal cell to a cancer cell is complex. However, Vogelstein and others have developed the "multihit" theory whereby this transition occurs not suddenly, but overtime via the accumulation of successive mutations (21). A single cell gradually accumulates mutations in its DNA overtime, and these mutational events facilitate each "step" towards a tumor with autonomous growth and metastatic potential. This gradual transition was first observed by pathologists, who noted the progression from normal cells to growths containing normal-appearing cells in excessive numbers, termed hyperplasia (22). As the cells continue to accumulate mutations, they may be observed as adenomas with not only increased numbers but abnormal appearance (22). A key step in this pathway is the transition to an invasive cancer or, in the context of epithelial cells, a carcinoma. This step is defined by invasion of cells through the basement membrane and into the adjacent stroma. At this point, a cancer is considered malignant with the ability to metastasize to distant tissues, eventually causing the death of the organism.

As discussed, there are many different cell types within the body with various functions, and many of these cell types are known to develop into cancers. These various, distinct cancers have been interrogated using a dizzying array of approaches, leading to an incredibly complex

field of cancer biology. Hanahan and Weinberg distilled this enormous data down into six "essential alterations in cell physiology that collectively dictate malignant growth": self-sufficiency in growth signals, insensitivity to growth-inhibitor signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (23). Ten years later, deregulation of cellular energetics and avoiding immune destruction were added as "emerging hallmarks" while genome instability and mutation and tumor-promoting inflammation were added as "enabling characteristic" that facilitate acquisition of core hallmarks (24). Finally, in the most recent edition, unlocking phenotypic plasticity and senescent cells were added as emerging hallmarks while nonmutational epigenetic reprogramming and polymorphic microbiomes were added as new enabling characteristics (25). In the following section, we will briefly introduce examples and mechanisms whereby integrins contribute to these many "Hallmarks of Cancer" (**Figure 6**) (23-25).

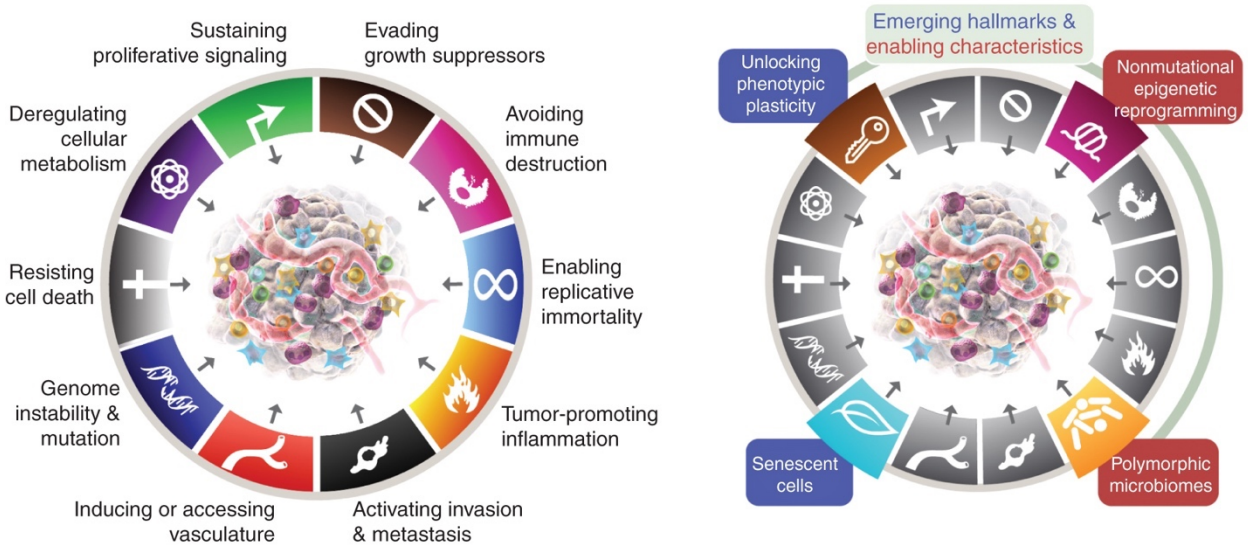

Figure 6: The Hallmarks of Cancer. (**left**) The Hallmarks of Cancer currently embody eight hallmark capabilities and two enabling characteristics. In addition to the six acquired capabilities - Hallmarks of Cancer - proposed in 2000 (23), the two provisional "emerging hallmarks" introduced in 2011 (24) - cellular energetics (now described more broadly as "reprogramming cellular metabolism") and "avoiding immune destruction"—have been sufficiently validated to be considered part of the core set. Given the growing appreciation that tumors can become sufficiently vascularized either by switching on angiogenesis or by co-opting normal tissue vessels (26), this hallmark is also more broadly defined as the capability to induce or otherwise access, principally by invasion and metastasis, vasculature that supports tumor growth. The 2011 sequel further incorporated "tumor-promoting inflammation" as a second enabling characteristic, complementing overarching "genome instability and mutation," which together were fundamentally involved in activating the eight hallmark (functional) capabilities necessary for tumor growth and progression. (**right**) The 2022 review incorporates additional proposed

emerging hallmarks and enabling characteristics involving “unlocking phenotypic plasticity,” “nonmutational epigenetic reprogramming,” “polymorphic microbiomes,” and “senescent cells.” The figure and legend was reproduced and adapted with permission (25); permission conveyed through the Copyright Clearance Center.

Sustained Proliferative Signaling

A core characteristic of tumor biology is its ability to proliferate in a sustained manner. Normal cells are in a state of homeostasis to preserve tissue architecture and function. Cancer cells, meanwhile, are often capable of proliferating independent of normal mitogens and sustain this proliferation irrespective of physiological checkpoints. Integrins can play an important role in this signaling. For example, when integrin α5β1 binds the ECM protein fibronectin in ovarian cancer cells, integrin α5β1 was observed to directly interact with the receptor tyrosine kinase c-Met (27). This association resulted in activation of c-Met independent of its ligand, hepatocyte growth factor, as well as downstream signaling molecules SRC and FAK. Thus, integrins can promote autonomous, mitogen-independent activation of proliferative signaling in cancer cells by cooperating with growth factor receptors.

In some instances, the role of integrins in promoting proliferative signaling is directly related to its role as a mechanoreceptor. Integrins bind both the ECM via its ectodomain as well as the cytoskeleton via its cytoplasmic tail and associated tail-binding proteins. Thus, the cell can directly infer the stiffness of the ECM via these connections and other adhesion-associated proteins (28). Many cancers are associated with increased deposition of ECM components (17),

resulting in a stiffer ECM. This process, termed desmoplasia, can promote cancer cell proliferation via integrin and adhesion protein signaling, such as SRC, FAK, p130Cas, and AKT (29,30). For example, ECM stiffness was shown to promote malignant progression of mammary epithelium by stabilizing vinculin, which mechanically couples integrin adhesions to actin at focal adhesions, and enhancing AKT activation (30). This colocalization of vinculin and activated AKT was strongest at the invasive border of breast tumors (where the ECM is stiffest). A stiff ECM sensed via integrin-mediated mechano-transduction is well known to activate other proliferative pathways as well. For example, two transcriptions factors that can promote tumorigenesis and that operate within the Hippo pathway, yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ) (31), demonstrate increased nuclear translocation and activation in response to a stiff ECM (32,33). Thus, integrin-mediated mechanosensing of stiff ECM directly results in activation of downstream growth, survival and proliferative signaling.

Finally, integrin-mediated adhesion has been shown to be necessary for progression through the cell cycle. Progression through the cell cycle is regulated by cyclin-dependent kinases (34), and cyclin D1 is among the first cyclins to be induced when cells enter G1 from quiescence (G0) (35). Growth factors and integrin-mediated cell adhesion leads to activation of the MAPK pathway and cyclin D1 expression (35). This process requires signaling via Rac1, a small GTPase in the Ras superfamily, positioning this enzyme as a key pro-proliferative effector of adhesion and integrin-mediated signaling (35). Overall, integrins mediate proliferative signaling via multiple mechanisms, including cooperation with growth factor receptors,

mechanotransduction of stiff ECMs leading to downstream activation of signaling proteins, and expression of cell cycle regulatory proteins such as cyclin D1.

Evading Growth Suppressors

Even when confronted with sustained proliferative signaling, normal cells have strong checkpoints to limit their functional impact. These checkpoints represent the "brakes", working to counteract excessive proliferative signaling or, in some contexts, even trigger cell death. Thus, cancer cells must develop mechanisms whereby they can disable these tumor suppressors to achieve the net elevated cellular proliferation rates necessary for tumor growth. Unlike many of the genetic mutations that "turn on" sustained proliferative signaling, which can promote the cancer phenotype with a mutation in a single gene allele, tumor suppressors typically require loss of both copies to sufficiently disable these negative feedback signals and thus allow excessive cell proliferation. Many such tumor suppressors have now been identified, often validated in genetically engineered tumor models where specific tumor suppressor genes have been mutated or inactivated (24).

Among the canonical tumor suppressors is the TP53 protein. TP53 functions as a safeguard of the cell and its genome. It receives input from many systems within the cell, and certain inputs can trigger a TP53-dependent halt to cell cycle progression or even apoptosis in response to overwhelming or irreparable damage to the cell and/or its genome. In addition to genomic damage, these input include nucleotide levels, mitogen signaling, glucose, and oxygen levels (24). Loss of a functional TP53 removes these safeguards and can allow progression of the

cell cycle irrespective to genomic damage or other signals. This unregulated cell proliferation is a key step in tumorigenesis. Integrin and cell adhesion signaling has been shown to decrease functional TP53 levels. For example, in melanoma cells, integrin αv was observed to inactivate TP53 as well as activate MEK and ERK, implicating integrin αv as a key regulator of melanoma cell survival and proliferation in certain contexts (36). Similarly, integrin α5β1 was shown to compromise chemotherapy induced TP53 activation in human glioblastoma cells, and depletion of integrin α5 increased TP53 activity and chemotherapy sensitivity (37). Furthermore, high levels of integrin αvβ1 were associated with glioma development, chemotherapy resistance via inhibition of TP53 activity, and decreased survival in patients with high grade glioma. The mechanism whereby integrins regulate TP53 levels and function remains incompletely described. However, some data suggest FAK, an integrin and growth factor-associated tyrosine kinase, may play a role. Investigators demonstrated that deletion of FAK results in TP53-mediated cell growth arrest. Furthermore, reconstitution of FAK resulted in increased TP53 turnover via MDM2-dependent TP53 ubiquitination. FAK facilitated TP53 turnover via a kinase-independent mechanism whereby it served as a scaffold, binding TP53 and MDM2 in the nucleus. Thus, integrin and its downstream signaling proteins play a direct role in evading growth suppressors, as illustrated by their effects on TP53 levels and function.

Enabling Replicative Immortality

Even in the context of sustained proliferative signaling and evasion of growth suppressors, safeguards remain in place that antagonize malignant transformation of the cell. Among these is the seemingly finite number of divisions an individual cell can undergo. Most

cells enter a period of senescence after a certain number of growth-and-proliferation cycles, marked by an irreversible entrance into a nonproliferative but viable state (24). For the few cells that escape from the senescence state, most then enter a crisis stage marked by genomic instability and cell death (24). A key factor in these senescence and crisis states are the continual erosion of telomeres with each cell cycle. Telomeres are series of hexanucleotide repeats at the end of chromosomes that progressively shorten with each cell cycle. As they shorten, they eventually lose their ability to protect the ends of chromosomes, resulting in unstable dicentric chromosomes, genomic instability, and cell death (24). Telomerase is a specialized DNA polymerase present in most immortalized cells that maintain and repair telomeres. Thus, data suggest that functional telomerase is necessary in many immortalized cells, including embryonal and cancer stem cells (38).

There are multiple examples of integrins defining stem cell populations and, at times, telomerase activity. Kunimura et al. identified the minority of cultured normal human epithelial cells with maintained telomerase activity (39). This subpopulation was predominantly composed of EGFR and integrin β1-positive cells. Similarly, a telomerase-positive population within the adult mouse testis was characterized by integrin α6 positivity. Why integrins correlate with telomerase activity in some cells is incompletely understood. However, some studies suggest that telomerase activity may directly regulate the expression of these integrins (40) and other cell adhesion-associated genes (41), suggesting that the stem cell phenotype is tightly regulated, in part, by a specific ECM niche.

Cancer stem cells, also known as tumor initiating cells, are a subpopulation of highly tumorigenic cancer cells capable of anchorage independent growth, self-renewal, and multi-lineage differentiation (42). They have been shown to play a role in metastasis, relapse, and treatment resistance (42-44). Just as in physiologic stem cells, integrins have been shown to serve as markers of cancer stem cells, including the β1, β3 and α6 subunits (45). However, integrins also have important functional roles in cancer stem cells. For example, integrin α6 is required for maintenance of glioblastoma stem cells and contributes to initiation of triple negative breast cancer via the regulation of NRP2, FAK and MAPK signaling (45,46). Thus, integrins play an important role in the biology of physiologic and cancer stem cells via telomerase-dependent and -independent mechanisms.

Resisting Cell Death

Programmed cell death represents another checkpoint that cells must overcome during malignant transformation. Several physiologic signals feed into the programmed cell death pathways, including excessive oncogenic signaling and DNA damage, as we have reviewed above (24). Prominent among these pathways is apoptosis. Apoptosis includes a signaling network that receives and processes signals from extracellular death-inducing signals (e.g., Fas ligand/Fas receptor) as well as an intrinsic program that processes intracellular cell death signals (24,47). TP53 is a major damage sensor that triggers apoptosis and has been shown to be regulated by cell adhesion and integrin signaling, as described above. Other pathways include autophagy, which allows for cellular material to be delivered to lysosomes for degradation resulting in basal or stress-induced turnover of cell components that can provide energy and

macromolecular precursors (48). Autophagy has been shown to be important in cancer development, where it can provide both tumor-promoting and tumor-inhibiting functions. Overcoming these programmed cell death pathways is necessary for tumorigenesis.

Integrin signaling can either promote cell survival or initiate programmed cell death, depending on the context and environmental cues (49). In response to platinum chemotherapy, both the ECM and cancer cell-intrinsic ECM signaling co-evolve to promote cancer cell survival and chemotherapy resistance in primary and metastatic ovarian cancer tumors. Specific ECM components (e.g., collagen 6) and matrix stiffness increase resistance to platinum-mediated, apoptosis-inducing DNA damage. This ECM remodeling promotes cell survival signaling via FAK and integrin β1-pMLC-YAP pathways (50). Similarly, primary and metastatic small cell lung cancer tumors are surrounded by extensive stroma, suggesting these tumors actively remodel their tumor microenvironment, which has been shown to promote tumorigenicity and treatment resistance via integrin β1-stimulated tyrosine kinase activation that suppresses chemotherapy-induced apoptosis. Integrin-mediated adhesion to ECM components in tumor cells has been shown to directly regulate endogenous regulators of apoptosis, including *BCL2* (51). Thus, integrin-ECM signaling in cancer cells can be important regulators of apoptosis.

While integrin binding to certain ECM components can provide anti-apoptotic and proliferative signaling, the impact of unligated integrins in cancer is more complex. As we have discussed previously, a defining feature of malignant transformation in carcinomas is invasion through the basement membrane into the adjacent stroma. These invading cells are thus no longer attached to the basement membrane, leaving them to adhere to other ECM proteins or

remain unligated. A similar unligated state exists for carcinoma cells during the process of metastasis, whereby they spread via hematogenous or lymphatic dissemination. Important cellular checkpoints typically trigger programmed cell death in cells with unligated integrins so as to prevent disorderly and potentially lethal cell migration. These pathways include both anoikis, apoptosis that occurs in response to cellular detachment from the ECM, as well as integrin mediated death, in which unligated integrins on adherent cells recruit and activate caspase 8 (49). In both instances, it is the lack of integrin-ECM ligation that triggers the pro-apoptotic signaling.

One mechanism whereby cancer cells can overcome this signaling is to promote unligated integrin clustering, similar to how they normally cluster in the context of ECM-induced focal adhesions, and subsequent downstream focal adhesion signaling. This can be achieved via the interaction of integrins with other molecules in the tumor microenvironment. For example, the extracellular domain of integrin α3β1, via its interactions with the tetraspanin scaffolding protein CD151 but independent of binding to laminin-332, can provide essential survival signals that control skin carcinogenesis (52). In addition, in tumor xenografts, unligated integrin αvβ3 interacts with galectin-3 at the plasma membrane, resulting in recruitment of KRAS and RalB. This ECM-independent clustering leads to the downstream activation of TBK1 and NF-κB, which regulates tumor initiation and anchorage independent growth (42). In these examples, integrins interact with CD151 or galectin-3 via their extracellular domain, thus promoting integrin signaling independent of binding to extracellular matrix. These interactions provide biochemical cues to the cell similar to those activated by integrin-ECM ligation and cell

adhesion, thus providing survival signaling and preventing cell death via anoikis or integrin mediated death.

Cancer cells have devised other mechanisms whereby they can activate integrin-dependent cell adhesion signaling in non-adherent cells and thus avoid cell death. One strategy is the cancer cells simply "take the ECM with them wherever they go". According to this hypothesis, during the invasion and degradation of the basement membrane during carcinogenesis (or ECM degradation and remodeling during the progression of established tumors), loss of ECM tension triggers uptake of ECM-ligated integrins into endosomes. These endosome-localized integrins continue to recruit cytosolic integrin binding proteins and adhesion molecules and thus form active signaling units irrespective of cell adhesion (17). There is significant data to support this theory, including localization of active integrins and ECM ligands within endosomes of cancer cells (53,54), active FAK localized to integrin β1-positive endosomes (55,56), and endosomal integrin signaling supporting anchorage-independent growth and metastasis in breast cancer cells (55). Whether cancer cells activate integrins via ligand-independent clustering or within the context of endosomal integrin signaling, both mechanisms rely on integrin signaling to provide "adhesion signaling" in non-adherent cells. These observations suggest that, just as integrin-dependent adhesion signaling is required for the survival of epithelial cells, carcinoma cells maintain this dependence (though at times this signaling is provided via unconventional mechanisms in nonadherent cancer cells).

Inducing or Accessing Tumor Vasculature

Tumors and normal tissue require oxygen and nutrients to survive and grow (57). Similarly, they require mechanisms to dispose of metabolic waste that would otherwise prove toxic to the growing tumor, such as carbon dioxide and lactic acid (24). Small tumors may be able to meet these metabolic needs via simple passive diffusion. However, when tumors grow to a volume greater than 2-3 mm^3, simple passive diffusion is typically insufficient to meet the metabolic needs of the growing tumor (58). Indeed, the observation that rapidly growing tumors are heavily vascularized while dormant ones are not led Judah Folkman to hypothesize that tumor angiogenesis was required for tumor progression (57,59). It is now widely accepted that tumor angiogenesis is required at the time of malignant transformation (60). This "angiogenic switch" requires endothelial cell proliferation, tubule formation and vessel branching. The overall rate of angiogenesis depends on a balance of factors, with angiogenesis inducers (e.g. VEGF) promoting these processes of the "angiogenic switch" and inhibitors (e.g., thrombospondin-1 or TSP-1) blocking these processes (24). Thus, by necessity, tumors have developed mechanisms to tilt the balance of angiogenesis inducers and inhibitors in a way that promotes sustained angiogenesis.

Many studies have evaluated the impact of integrin expression on tumor angiogenesis. Using integrin α1-null mice that express an oncogenic *KRAS* allele that spontaneously activates, investigators noted decreased incidence of non-small cell lung tumors and longer survival in the integrin α1-null mice relative to integrin α1-positive mice (61). Tumors in the integrin α1-null mice were smaller with decreased ERK activation and apoptosis but were also noted to be less

vascular. The decreased angiogenesis was attributed to increased levels of MMP9, which generates angiostatin with consequent inhibition of endothelial cell growth. Thus, in the context of non-small cell lung cancer, integrin α1 (which heterodimerizes with the integrin β1 subunit to form the collagen IV receptor α1β1) serves as a promoter of angiogenesis (61). Other studies have also identified integrin β1 heterodimers as important in tumor angiogenesis. For example, integrin β1 was observed to form a complex with CD93 and MMRN2 in endothelial cells, and deletion of CD93 resulted in diminished activation of integrin β1 and decreased organization of fibronectin into fibrillar structures during tumor vascularization (62). Thus, integrin α1β1 and other β1 heterodimers promote tumor angiogenesis via a variety of mechanisms.

Integrin β4 has also been shown to promote tumor angiogenesis. Deletion of a component of the cytoplasmic tail of integrin β4 in mice was sufficient to inhibit hypoxia- or FGF-induced angiogenesis (63). Further mechanistic work demonstrated that α6β4 promotes branching of medium- and small-size vessels into microvessels without affecting endothelial cell proliferation or survival. Subcutaneous injection of cancer cells into the integrin β4-null mice produced smaller and less vascularized tumors relative to control mice (63). Thus, endothelial cell integrins have been shown to be necessary for efficient tumor vascularization and maximal tumor growth.

Activating Invasion and Metastasis

The motility of cancer cells is a fundamental feature of malignant transformation and tumor progression. Even in the earliest stages of tumor development, cell motility can have a

profound impact on the growth rate of tumors. To estimate the impact of cell motility on tumor growth, investigators used mathematical modeling to evaluate the growth rate of tumors composed of highly motile cells versus those with decreased cell motility (64). Cell motility in growing tumors can function to alleviate steric hindrance and crowding of cells and thus serve to increase growth rate of tumors and can also contribute to the rapid onset of resistance to therapy (17,64). Likewise, cell motility is necessary for the invasion through the basement membrane and into the adjacent stroma – the defining feature of malignant transformation. Finally, cell motility is necessary during the metastatic cascade, a schematized multistep process whereby cancer cells invade through tissues, access the vasculature, disseminate hematogenously, and ultimately colonize in distant tissues (24,65). This metastatic cascade is what eventually results in failure of organs overwhelmed by metastatic tissue and patient death. Thus, cell motility is a critical feature of cancer cells across the spectrum of tumor development, progression and metastasis.

As the principal ECM receptor of the cell, integrins play an indispensable role in facilitating cell motility. Integrins, within the context of focal adhesions, serve as a mechanical linker between the extracellular matrix and the intracellular actin cytoskeleton. When this link is established, the force due to actin polymerization results in the slowing down of the retrograde flow, protrusion of the leading edge, and generation of rearward traction forces by which the cell can move forward – the so-called molecular clutch theory (66). Experimental data has supported this theory, demonstrating that integrin-mediated adhesion to the ECM as a necessary step in the promotion of cancer cell motility and migration (17). For example, investigators used a pooled CRISPR/Cas9 library screen to identify key regulators of small cell lung cancer metastasis.

These experiments identified two key members of an ubiquitin ligase complex that, when lost, stabilized integrin β1, activated downstream FAK and SRC signaling, and eventually drove small cell lung cancer metastasis (67). These data suggest that integrins are important in cancer cell migration, and this migration is necessary to achieve a malignant phenotype.

Integrins can also regulate cancer cell migration via mechanisms independent of the mechanical linkage of ECM and the actin cytoskeleton. For example, integrins can facilitate crosstalk between tumor cells and cancer-associated fibroblasts (CAFs) in breast cancer, where CAFs are both common and known to contribute to tumor progression. Investigators observed CAFs to produce abundant amounts of interleukin-32 (IL32), which contains an RGD motif. Integrin β3, which can heterodimerize with both integrin αv and αIIb to form RGD receptors, is up-regulated in breast cancer during epithelial-to-mesenchymal transition (EMT, a program whereby cells lose epithelial traits and acquire mesenchymal traits, resulting in increased cell motility, invasion and metastasis (68)). IL32:integrin β3 ligation resulted in downstream p38-MAPK activation in the cancer cells, expression of EMT markers, and increased tumor cell invasion (69). Therefore, in this context, integrins function to facilitate crosstalk between cancer cells and other cells within the tumor microenvironment, resulting in cancer cell EMT and increased tumor cell migration via an ECM-independent mechanism.

Deregulated Cellular Metabolism

The uncontrolled cellular proliferation that drives tumor development requires corresponding changes in cellular metabolism to fuel that growth (24). Abnormal cellular

metabolism in cancer has been appreciated for decades. While virtually all cells metabolize glucose to pyruvate via glycolysis, most cells in the presence of oxygen shunt the pyruvate to the mitochondria for oxidative phosphorylation. However, Otto Warburg observed a preference for cancer cells to metabolize glucose to pyruvate via glycolysis, with very little being shunted to the mitochondria even in the presence of oxygen, so-called "aerobic glycolysis" (70,71). While being energy inefficient relative to oxidative phosphorylation, aerobic glycolysis does allow for shunting of glycolytic intermediates into anabolic pathways that produce macromolecules necessary for sustained cell growth and proliferation, such as the pentose-phosphate pathway (which produces ribose for nucleotides and NADPH for reductive biosynthesis) (72). The "Warburg Effect" is the archetypal metabolic derangement in cancer cells, though the field of cancer metabolism has flourished with many other significant alterations discovered. Indeed, recent work has shown that much of the glucose avidity observed in some tumors is the result of immune cell glucose consumption rather than tumor cell metabolism (73).

Integrin-dependent signaling and cell adhesion demonstrates significant crosstalk with the metabolic pathways of cancer cells. For example, integrin β1 activation upon matrix binding can activate mammalian target of rapamycin (mTOR) signaling (74). mTOR functions as a critical regulator of cell growth and metabolism (75) and is composed of two complexes, mTORC1 and mTORC2, each with distinct mechanisms of regulation and substrate specificity (76). mTORC1 is activated downstream of numerous growth factors as well as integrin:ECM ligation, resulting in the activation of phosphatidylinositol-3-kinase (PI3K), leading to the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3), which subsequently activates the serine/threonine kinase AKT (76). mTORC1 is also activated by amino acids and is thus a

mechanism of nutrient sensing. Thus, perhaps intuitively, activation of mTORC1 also enhances protein translation as well as other anabolic pathways, such as lipid biosynthesis when nutrients are plentiful. FAK can function as a critical mediator between integrin signaling and the PI3K/mTOR pathway. Integrin β1 activation by type I collagen results in activation of FAK, which subsequently results in the phosphorylation of the p85 subunit of PI3K and AKT. Inhibition of FAK attenuated PI3K and AKT activation whereas a constitutively active FAK restored this signaling (74). In addition, there is extensive evidence that metabolic signals can regulate expression, post-translational modification, trafficking, and degradation of integrins (77). Thus, integrin signaling and regulation of cellular metabolism are closely intertwined.

Similarly, integrins have been shown to regulate other signaling pathways and transcriptional programs, including the Hippo pathway and EMT, that directly regulate metabolic pathways (77). Other integrin-associated proteins, such as CD98 and monocarboxylase transporter 4 (MCT4), function directly as nutrient transporters (77-82). Cancer cell detachment from matrix has been observed to directly lead to a reduction in glucose uptake, ATP levels, and fatty acid oxidation as well as increased generation of reactive oxygen species. While further mechanistic work is required, particularly with *in vivo* models and patient samples, these data support the theory that integrin signaling functions to regulate nutrient uptake and metabolism in cancer.

Avoiding Immune Destruction

Evidence has accumulated supporting an active role for the immune system in monitoring and routinely eliminating immunogenic cancer clones, so-called "immunoediting" (24). For example, solid organ transplant recipients have a 3-fold excess risk of cancer relative to age- and gender-matched controls from the general population (83). Similarly, patients infected with HIV have similar increased incidence of cancer. In both transplant and HIV patients, much of this increased incidence is in viral-associated cancers, suggesting the cancer incidence may be due to decreased ability to clear viral infected cells. However, as HIV patients live longer with effective antiviral therapies, the cancer burden for common cancers not known to be associated with viral infections and are linked to aging, such as prostate cancer, is becoming higher than for virus-associated cancers (84). Further evidence for immunoediting comes from mouse studies. For example, when carcinogen-induced tumors develop in immunocompromised mice are then transferred to syngeneic, immunocompetent mice, they are often rapidly cleared. However, carcinogen-induced tumors developing in immunocompetent mice grow well regardless of the immune system of the recipient mice, suggesting these tumors have evolved mechanisms whereby they can bypass "immunoediting" (24,85,86). These data, coupled with the rampant success of drugs targeting immune checkpoint inhibitors in a diversity of cancers (87), provides strong support for the role of avoiding immune destruction during tumorigenesis.

Integrins and cell adhesion molecules are known to be critical for anti-tumor immune cell function. This includes regulating immune cell trafficking into tissues, activation and proliferation of immune effector cells in the tumor microenvironment, and formation of the

immunological synapse between immune cells and target cells (88). What has been less clear, until recently, is the impact on integrins and cell adhesion molecules on cancer cells in regulating the anti-tumor immune response. However, emerging data is suggesting that both the ECM and tumor cell ECM receptors (e.g., integrins) play an important role in regulating the immune tumor microenvironment. In terms of the ECM, lysyl oxidase (LOX) functions to cross-link collagen and fibronectin fibers in the ECM. In mouse models, inhibition of LOX resulted in decreased ECM content and stiffness as well as improved T-cell migration and efficacy of anti-PD-1 blockade (89). As for how ECM receptors regulate the anti-tumor immune response and immune tumor microenvironment, emerging data suggest the crosstalk can be substantial. For example, expression of the RGD receptor integrin αvβ3 was shown to correlate with basal and interferon-induced PD-L1 expression via a cytoplasmic tail-dependent STAT1 signaling (90). Interestingly, depletion of integrin β3 resulted in reduced PD-L1 expression, increased interferon-gamma signaling, and increased CD8+ cell infiltration. In contrast, integrin αvβ8 promotes tumor growth and immune escape via a distinct mechanism that is independent of PD-1/PD-L1 axis (91). Integrin αvβ8 binds and activates latent TGFβ. Inhibition of integrin αvβ8 using blocking antibodies results in potentiation of cytotoxic T-cell responses and recruitment of immune cells to the tumor presumably by inhibiting activation of latent TGFβ. Thus, integrin expression and tumor cell:ECM interactions can play an important role in the regulation of the anti-tumor immune response.

Unlocking Phenotypic Plasticity

Included in the most recent update of the Hallmarks of Cancer, unlocking phenotypic plasticity is a process whereby cancer cells avoid terminally differentiated states (25). During the development of organisms, organs and specialized tissues, there often exists a progenitor cell population with intact proliferative capabilities. As development and organogenesis proceeds and enters a homeostatic state, cells often become terminally differentiated with limited replicative abilities. This terminal differentiation represents another checkpoint that prohibits malignant transformation. To overcome this checkpoint, cancer cells often exhibit phenotypic plasticity that allows them to revert to a dedifferentiated state with intact proliferative ability. Alternatively, cancer cells may arise directly from progenitor cells that have not yet terminally differentiated, retaining their proliferative abilities and phenotypic plasticity. Cancer cells may also assume entirely distinct cellular differentiation pathways. In all these scenarios, the net result is a relatively dedifferentiated cancer cell capable of continued proliferation and adaptation.

Integrins have been shown to function as key regulators of cancer cell differentiation. For example, data from genetic models support the current paradigm that colonic stem cells are the cell of origin for adenocarcinoma of the colon (92). These colonic stem cells continually divide and replace more differentiated colonic epithelial cells, including goblet cells, enterocytes and neuroendocrine cells. Investigators working with a human colorectal cancer cell line with multipotent characteristics have shown that the collagen receptor integrin $\alpha2\beta1$ is required for differentiation of these cells, and function blocking antibodies targeting either the $\alpha2$ or the $\beta1$ subunits blocks these cells from developing into terminally differentiated epithelial-like cells

(93). Similarly, it has been observed in triple negative breast cancer cells that either blocking or knocking down integrin β1 results in TGFβ activation, increased expression of the EMT-associated transcription ZEB2, decreased expression of E-cadherin, and enhanced single cell motility (94). In both examples, ECM environmental cues sensed by the cell via integrin signaling resulted in enhanced cellular phenotypic plasticity (cells retaining stem cell-like properties or undergoing EMT-like changes). Thus, integrin dysregulation in cancer likely plays a significant role in regulating cancer cell plasticity in some cancer types.

Cellular Senescence

Cellular senescence is an arrest of cellular proliferation (95). Classically, this proliferation arrest is irreversible. Cellular senescence can be a part of embryonic development or a programmed cellular response to a variety of cellular stresses, such as telomere dysfunction, oncogene activation, DNA damage or nutrient deprivation (25). Given that oncogene activation and DNA damage are indeed "hallmarks" of cancer cells, it is then not surprising that, in some contexts, neoplastic cells may undergo senescence and stop proliferating, thus blunting tumorigenesis (96). Thus, in this context, cellular senescence can protect from tumorigenesis.

Interestingly, the impact of cellular senescence can extend beyond the individual senescent cell and into the microenvironment due to the associated secretion of senescence-associated secretory phenotype (SASP), which includes several components including bioactive chemokines, cytokines and proteases (95). This amplifying effect of cellular senescence is thought to mediate a variety of sequelae of increased cellular senescence, including impaired

tissue regeneration, chronic age-associated diseases, and organismal aging. This far-reaching effect of senescent cells on organ and organismal health was illustrated well by a model designed to eliminate naturally occurring senescent cells in aged mice (97). Impressively, this elimination of senescent cells resulted in preserved organ function, delayed tumorigenesis, and improved lifespan of these aged mice. Cellular senescence can adversely affect the organism via other mechanisms as well. For example, senescent cancer cells can undergo a transitory period of senescence, allowing them to survive cytotoxic chemotherapy and resume cell proliferation later, driving chemotherapy resistance and disease relapse (25). Likewise, other cells in the tumor microenvironment can undergo senescence and promote a pro-tumorigenic environment, such as cancer-associated fibroblasts (25). Thus, the role of cellular senescence on tumor development, growth and treatment response is complex and context dependent.

Likewise, the role of integrins and cellular adhesion in the regulation of cellular senescence is complex. Cellular senescence has been associated with increased cellular adhesion, including large focal adhesions, activated FAK, and decreased motility (98,99). This is further supported by the observation that treatment with integrin-blocking antibodies inhibits cellular senescence in human fibroblasts and mouse lungs *in vivo* (98). In a similar phenomenon, ligation of integrin $\alpha5\beta1$ in breast cancer cells with fibronectin in the bone marrow promotes a hypoproliferative and anti-apoptotic phenotype, allowing the cells to remain alive yet dormant for an extended period of time (100). However, the association between integrin:ECM ligation and cellular senescence is not absolute. Using a pancreatic adenocarcinoma mouse model, other investigators have shown that deletion of integrin $\beta1$ results in increased dissemination of tumor cells with decreased capacity for forming metastatic tumors in distant organs, largely due to

reduced cell proliferation and senescence of integrin β1-null cells (101). However, it should be noted that this study did not use the same definition of cellular senescence as other studies (namely, p16/INK4a expression) which may contribute to the contradictory conclusions. Regardless, a simple paradigm of "increased cell adhesion correlates with cellular senescence" is likely oversimplistic, and a more nuanced and contextual understanding is required in the study of integrins and cellular senescence in cancer.

Cancer Enabling Characteristics

Thus far, the hallmarks reviewed are functional attributes that cancer cells acquire that facilitate survival, proliferation and metastasis (24). These hallmarks may be acquired via distinct mechanisms at different time points during the multistep tumorigenesis of various tumors. However, Hanahan and Weinberg in the second and third edition of their "Hallmarks" review articles also highlighted attributes that do not directly contribute to the cancer phenotype, but rather facilitate the acquisition of other hallmarks (24). These so-called "enabling characteristics" include genomic instability and mutation, tumor promoting inflammation, non-mutational epigenetic reprogramming, and polymorphic microbiomes.

Genomic Instability and Mutation

"Cancer is, in essence, a genetic disease" is a quote attributed to the famous cancer biologist Bert Vogelstein. Indeed, cancer cells obtain the various hallmarks of cancer largely through mutations and/or alterations in their genome. The irony is that, given the high fidelity of

DNA replication in healthy normal cells, in most instances the time required for a cell to spontaneously acquire enough functional mutations to assume a malignant phenotype is exceedingly long (25). However, if a genomic alteration alters the stability of the genome, it may enable the accumulation of further mutations which subsequently hastens the accumulation of the hallmarks of cancer. We have already discussed examples, such as loss of telomeres leading to unstable dicentric chromosomes and genomic instability. Likewise, loss of TP53 disables a signal that would normally halt DNA replication in response to DNA damage, thus propagating genomic alterations to daughter cells that would otherwise have been "erased" when the cell is driven to apoptosis. Thus, genomic instability is a key feature of tumorigenesis and an important enabling hallmark of cancer that facilitates the acquisition of other hallmarks.

Integrins play an important role in cell adhesion and epithelial cell homeostasis, and disruption of integrin function can directly promote genomic instability by impairment of cell adhesion. This concept was well illustrated by Knouse et al., who demonstrated using organoid systems that tissue architecture, and specifically integrin function, is required for accurate chromosome segregation, suggesting that disruption of tissue architecture could underlie the widespread chromosome instability observed across carcinomas (102). While this manuscript demonstrates that integrin function is necessary for genomic stability, excessive integrin-mediated cell adhesion can also promote genomic instability. Using mammary epithelial cells, Rabie et al. demonstrated that stiff matrices and/or excessive integrin β1 clustering within focal adhesions promotes abscission failure and multinucleation of daughter cells (a sign of genomic instability, precursor to aneuploidy and a driver of neoplastic progression) (103). Thus, appropriate tissue architecture with the proper homeostatic balance of integrin-mediated cell

adhesion is required for normal cell division. Dysregulation of this system leads to genomic instability and enables tumorigenesis.

Tumor Promoting Inflammation

It has long been established that inflammatory cells are components of the microenvironment of many tumors. Indeed, Rudolf Virchow identified the presence of leukocytes within tumors in the 19th century (104). It is now appreciated that components of both the innate and adaptive immune system are important components of the tumor microenvironment. While the presence of these cells was initially attributed to an attempt by the immune system to clear the tumor, evidence has emerged implicating these cells as enablers of tumorigenesis as well (25). Indeed, the inflammatory response is now known to play an important role at various stages of tumorigenesis, including initiation, promotion, malignant conversion, invasion, and metastasis (104). One mechanism whereby immune cells can promote inflammation and drive tumorigenesis is through the supply of bioactive molecules to the tumor microenvironment. Hanahan provides several such examples in his latest review on the hallmarks of cancer, including growth factors that sustain proliferative signaling, survival factors that limit cell death, proangiogenic factors, ECM-modifying enzymes that facilitate angiogenesis and/or cell invasion, and factors that promote EMT (25). Immune cells can also promote mutagenesis through the release of reactive oxygen species and other promoters of genomic instability. Thus, tumor-associated inflammation is more than just a graveyard representing a failed anti-tumor immune response. It has emerged as an important promoter of tumorigenesis and enabler of cancer hallmarks.

The role of integrins in regulating tumor-associated inflammation is complex. Integrins are expressed on immune cells and play an important role in all immune cell functions, including immune cell trafficking into tissues, effector cell activation, proliferation, and the formation of the immunological synapse between immune cell and the target cell (105). Likewise, integrins are expressed on epithelial tissues that develop into carcinomas and integrin dysregulation can have a profound impact on the local immune microenvironment. For example, integrins are expressed on type 2 alveolar epithelial cells (the cell of origin of lung adenocarcinoma), and deletion of integrin β1 in aged mice resulted in development of emphysema, lymphoid aggregates and increased macrophage infiltration (106). Finally, integrins are expressed on tumor cells and downstream integrin signaling in tumor cells can modulate the immune tumor microenvironment. For example, in hepatocellular carcinoma cells, integrin α4β1 has been observed to interact with the matricellular protein SPON2, and the downstream integrin signaling promotes activation of RhoA and Rac1, increased F-actin reorganization, and M1-like macrophage recruitment (107). M1 macrophages classically are activated by IFNγ, express high levels of pro-inflammatory cytokines and major histocompatibility complexes, and are capable of priming anti-tumor immune responses (104). Thus, not surprising, expression of integrin α4β1 in HCC is associated with decreased metastasis and favorable prognosis (107). Integrins on tumor cells can also participate in inflammation-associated, tumor-promoting signaling. Albrengues et al. observed that lung inflammation induced by lipopolysaccharide or tobacco smoke led to extracellular matrix remodeling, exposing an integrin β1 ligand that subsequently triggered proliferation of otherwise dormant cancer cells (108). Thus, through a variety of mechanisms,

integrins are capable of regulating inflammation in tumor tissues and/or facilitating the response of cancer cells to inflammation.

Nonmutational Epigenetic Reprogramming

Mutations in genes whose protein products organize, modulate and maintain chromatin structure, and thereby regulate global gene expression, are now well-described (25). A prime example is spontaneous clear cell renal cell carcinoma (RCC), whose biology is driven largely by tumor suppressors. Among the top 5 most commonly mutated genes in clear cell RCC are three genes whose protein products are classically considered chromatin modifiers: *PBRM1, SETD2,* and *BAP1* (109). However, epigenetic reprogramming in a cell can be driven via mechanisms independent of genetic mutations (110), and this has now been widely observed within the context of cancer (25). For example, Snail-1 is a transcription factor and master regulator of EMT. Induction of Snail-1 in human mammary epithelial cells results in repression of epithelial-associated genes and expression of mesenchymal-associated genes (111). However, these dynamic changes in gene expression can largely be negated if cells are treated with pharmacologic inhibitors of histone acetylation and demethylation, suggesting these changes are highly dependent upon nonmutational epigenetic reprogramming. Thus, nonmutational epigenetic reprogramming is a physiologic process observed in healthy tissue as well as cancer tissue.

The mechanical and biochemical nature of a cell's microenvironment can have a profound influence on a cell's function and phenotype. As the principal ECM receptor of the

cell, integrins play an important role in sensing the cell's microenvironment and can transmit signals to the cell via nonmutational epigenetic reprogramming (25). For example, investigators have observed that stem-like tumor-repopulating cells sense mechanical signals and rapidly proliferate in soft matrices (112). However, in stiff matrices they enter a state of dormancy driven by an epigenetic program initiated by the translocation of Cdc42 into the nucleus, a cytosolic regulator of mechano-transduction (113). Cdc42 promotes the expression of Tet2, which epigenetically activates cell cycle-inhibiting genes p21 and p27 and downregulates integrin β3 to maintain dormancy. Thus, mechanosensing of the cell promotes epigenetic reprogramming that includes modulation of integrin expression and cellular dormancy. Similarly, cancer cell detachment from the ECM has been shown to increase expression and function of EZH2, a histone methyltransferase. Inhibition of EZH2 resulted in decreased cell proliferation, spheroid size and induction of apoptosis, suggesting that EZH2 expression in detached cancer cells may be a mechanism of overcoming anoikis (114). Thus, in both mechanisms, mechanical sensing of the microenvironment by the cell led to nonmutational epigenetic reprogramming that impacted the cancer phenotype.

Polymorphic Microbiomes

The role of oncogenic viruses is well-established, with approximately 10-15% of all cancers being associated with chronic viral infections (115). However, we are steadily gaining a better appreciation for how other microorganisms impact human health and disease. These microorganisms, predominantly bacteria and fungi, symbiotically colonize human tissues (predominantly barrier tissues of the body including epidermal, internal mucosa, gastrointestinal

tract, breast, and urogenital system) (25). These microorganisms make essential contributions to human health and well-being, and dysregulation of this microbiota (so-called dysbiosis) has been associated with many human diseases including allergy, diabetes, obesity, arthritis, inflammatory-bowel disease, and even neuropsychiatric disorders (116). This study has been accelerated by advancements in Next Generation Sequencing technology and bioinformatic techniques that allow for rapid detection and cataloging of this biodiversity (25). The role of the microbiome in human health and disease is becoming clearer as this field rapidly evolves.

In terms of cancer, there is mounting evidence that the microbiome plays an important role. Much of this work has been done in colorectal carcinoma, given the known role of the microbiome in colon physiology and pathophysiology. Indeed, investigators have established a role for the microbiome in colorectal carcinoma development and progression as well as modulating tumor response to different systemic therapies (117). Mechanistically, the microbiome has been shown to promote inflammation, modulate signaling pathways, and impact the anti-tumor immune response (117). Moving forward, investigators are making exciting attempts to modulate the microbiome and thus improve response to therapy. For example, Dizman et al. supplemented patients with metastatic renal cell carcinoma receiving immunotherapy with oral bifidobacteria products. The response rate in patients receiving standard of care was 20%, whereas those receiving the bifidobacteria improved to a whopping 58% (118). Thus, there is growing enthusiasm for modulating the gut microbiome to augment immunotherapy response in cancer.

Given that integrins are critical to leukocyte function, it is not surprising that modulation of their expression in these cells would impact the microbiome. For example, deletion of integrin β2 in mice (used as a model of the congenital disease leukocyte adhesion deficiency type 1) resulted in increased intestinal damage and increased systemic bacterial burden in a *Citrobacter rodentium* colitis model, likely due to decreased IL-22 production by integrin β2-null macrophages (119). In addition, enzymes produced by bacteria in the microbiome can modulate the ECM through post-translational modifications. Bacterial peptidyl-arginine deiminase expressed from *Porphyromonas gingivalis* may citrullinate collagen type II (120). This modification may affect fibroblast and stem cell interaction with collagen type II, leading to changes in fibroblast adhesion and migration (121). *P. gingivalis* oral infections were also associated with increased autoantibodies to type II collagens and citrullinated epitopes, both of which are associated with destructive autoimmune arthritis such as rheumatoid arthritis (122,123). Thus, these studies suggest a mechanistic link between oral *P. gingivalis* infections and rheumatoid arthritis, possibly via bacteria-mediated changes in the ECM. While it is clear that the microbiome can have a profound impact on cancer development and response to therapy, and the microbiome can modulate cell:ECM interactions in ways that are important to human health, further work is required to establish a mechanistic link between tumor:ECM interactions and the microbiome.

Integrins as Tumor Suppressors

We have just performed an extensive review on how integrin expression on cancer, immune and stromal cells can facilitate tumor development and growth by contributing to the

Hallmarks of Cancer. However, there are circumstances where integrin expression on cancer cells antagonize the cancer phenotype. For example, while many integrins are over-expressed in human cancers, the expression of others tends to be decreased. While such observations are merely correlative, they do suggest that not all integrins impact the cancer phenotype similarly. Indeed, the same integrin in different cancer types may impact the tumor in different ways. For example, expression of the integrin α7 subunit in esophageal squamous cell carcinoma is associated with poor differentiation, lymph node metastases and worse prognosis (17,124). However, truncating mutations of integrin α7 have been reported in glioblastoma and prostate cancer and forced expression of full-length integrin α7 in prostate and leiomyosarcoma cell lines resulted in suppressed tumor growth and metastasis *in vitro* and *in vivo* (17,125). Thus, patient and cell line data suggest that integrin α7 can function as either tumor promoter or suppressor.

Similarly, integrin β1 is likely capable of promoting or suppressing tumor growth depending on the context. This conclusion is supported by multiple genetic mouse models that demonstrate these opposite functions in various tumor types. For example, decreased tumor formation was observed in a polyomavirus middle T (PyMT)-driven breast cancer model with inducible knock out (KO) of integrin β1 in breast epithelium (15). However, integrin β1 can function as a tumor suppressor is the TRAMP prostate adenocarcinoma mouse model (126), where tumorigenesis is driven by prostate-specific expression of SV40 early T/t antigen genes. Thus, like integrin α7, the integrin β1 subunit is capable of either promoting or suppressing tumor growth.

In most circumstances, the biological and contextual cues that dictate whether an integrin functions as oncogene or tumor suppressor are unknown. However, the net effect of the cytokine transforming growth factor β (TGFβ) signaling plays an important role in some circumstances. TGFβ is a signaling molecule that participates in complex signaling networks and regulates a diverse array of developmental programs and cell behaviors including proliferation, differentiation, morphogenesis, tissue homeostasis and regeneration (127). In normal and premalignant tissue, the net effect of TGFβ signaling typically tilts towards tumor suppressor functions, including direct effects of the tumor cell promoting cytostasis, differentiation or apoptosis as well as tumor suppressive stromal effects (i.e., suppression of inflammation and stroma-derived mitogens) (128). However, as tumor development progresses, selective loss of the tumor suppressive effects may be lost, allowing tumor promoting effects to dominate in an unopposed fashion. These TGFβ-mediated tumor promoting effects include evasion of immune suppression, epithelial-to-mesenchymal transition, autocrine mitogen production, and cellular invasion (128). Integrins, including αvβ6, can function to activate latent TGFβ in the microenvironment as can the glycoprotein thrombospondin-1. Deletion of one or both genes in mice results in TGFβ-driven de novo tumorigenesis, highlighting the tumor suppressive role of TGFβ in normal or premalignant cells (129). However, in established tumors that have presumably "pruned" the tumor suppressive effects of TGFβ, integrin αvβ6 expression correlates with poor survival and metastasis and its inhibition can stop tumor growth (130). Thus, once again the contextual cues are of primary importance in determining the net effect of integrin expression on the cancer phenotype, though overall our understanding of these cues remains limited.

Summary

As this chapter details, integrins are involved in the initiation, development, growth, and metastasis of a variety of cancers. Integrins are integral to the cancer phenotype and play a role "every step of the way" as Drs. Hamidi and Ivaska state in the title of their review on this topic (17). Given the extracellular domain of integrins (which often portends "druggability" during drug development) and the well-described nature of the association between integrins (the receptor) and the extracellular matrix (the ligand), integrins emerged as a target for the development of anti-cancer therapeutics. However, the development of such therapeutics has been difficult and has yet to make an impact in the lives of most cancer patients. The reasons for this difficulty are many and have been suggested by the topics already covered, such as the dual roles integrins can play as either tumor promoters or tumor suppressors. In addition, emerging data has challenged a central tenet of integrin biology in the context of cancer, namely that integrin:ECM ligation is required for pro-tumorigenic integrin signaling. Based on this assumption, most integrin-targeted anti-neoplastic therapies have been designed to abrogate integrin:ECM interactions by targeting the ECM-binding extracellular domain of integrins. In chapter 3, we provide data suggesting that integrin β1 is capable of constitutive, ligand-independent oncogenic signaling in lung adenocarcinoma via its cytoplasmic tail. This is likely to be true for other integrins in other cancers as well. While this possibility partially explains some of the limited efficacy of integrin-targeted therapeutics in cancer, it also raises new challenges in designing effective integrin-targeted therapeutics in oncology.

CHAPTER 3

LIGAND-INDEPENDENT INTEGRIN BETA1 SIGNALING SUPPORTS LUNG ADENOCARCINOMA DEVELOMENT

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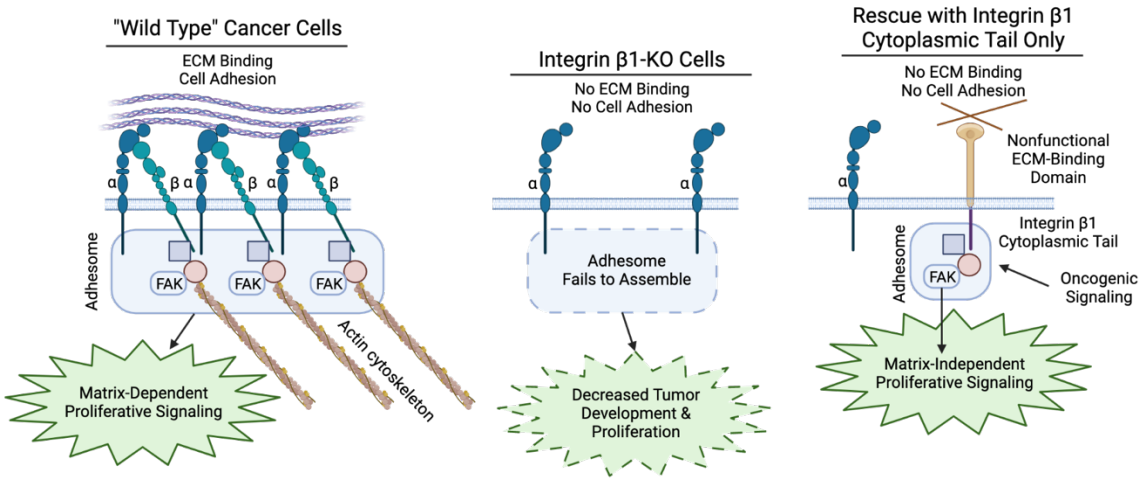

Figure 7: Graphical abstract. Panel made with assistance from www.biorender.com.

Introduction

We wished to understand the role of integrins during cancer development as well as why inhibition of the ECM-binding ectodomain has failed as a cancer therapeutic strategy. To address

these questions, we focused on *KRAS*-mutated lung adenocarcinoma, a subtype of non-small cell lung cancer. We chose to focus on this disease type because 1) non-small cell lung cancer is the top cause of cancer death in the US and world (132), 2) lung adenocarcinoma is the most common histology of non-small cell lung cancer, and 3) *KRAS*-mutations are the most common oncologic driver of lung adenocarcinoma (133). In addition, there are several high quality, well-established mouse models of *KRAS*-mutated lung adenocarcinoma (134). Finally, integrin β1 was selected as a focus of our studies for several reasons. Integrin β1 is highly expressed in lung epithelium and cancers. It also binds 12 distinct α integrin subunit. Thus, deletion of integrin β1 disrupts collagen, RGD and laminin receptors leading to a major perturbation of the cell's integrin repertoire of functional heterodimers despite deletion of only a single gene (**Figure 2**). In addition, integrin β1 expression is prognostic in non-small cell lung cancer (135) and specifically lung adenocarcinoma (14), suggesting it may play an important functional role in this cancer. These data and observations made the study of integrin β1 in the context of *KRAS*-mutated lung adenocarcinoma an ideal scenario in which to investigate the role of integrins during cancer development.

Materials and Methods

Urethane transgenic mouse model. We crossed integrin $\beta1^{f/f}$ mice on an FVB background with universal deleter Vasa-Cre mice to generate integrin $\beta1^{f/0}$ mice. We then crossed integrin $\beta1^{f/0}$ mice with mice with inducible Cre recombinase expression by the dox-inducible reverse tetracycline activator under control of the SPC promoter (106). AT2 deletion was introduced at four weeks of age using dox chow 200 mg/kg (Bio-Serv #S3888). Control $\beta1^{fl/0}$ mice were also

fed dox chow. Tumorigenesis was initiated with intraperitoneal urethane (ethyl carbamate, Sigma #U2500, 1.0 mg/kg) at eight weeks. Mice were sacrificed at approximately 42 weeks or upon reaching humane endpoints. All mice were obtained from Jax.com.

LSL-Kras-G12D; integrin $\beta 1^{f/f}$; SPC-CreER^{T2} tamoxifen inducible mouse model. We crossed the inducible LSL-Kras-G12D allele and the tamoxifen-inducible Cre recombinase allele onto integrin $\beta 1^{f/f}$ mice. All mice were C57/Bl6 background and obtained from Jax.com. Tamoxifen chow 400 mg/kg (Envigo #TD.130860) was started at four weeks of age (5 days tamoxifen chow, 2 days normal chow cycles). Mice were sacrificed approximately 16 weeks after tamoxifen chow or upon reaching humane endpoints.

Histology and tissue staining. Lungs were inflation fixed at 25 cm with 10% formalin, sectioned, and H&E stained. Integrin β1 immunohistochemistry of mouse tumors was performed on paraffin sections incubated with primary antibody (rabbit anti-integrin β1, Cell Signaling Technology (CST), Danvers, MA, USA #34971) and anti-rabbit secondary and DAB (#8114). For frozen sections, lungs were inflation fixed with a 2:1 mixture of PBS:OCT, embedded in OCT and flash frozen. Integrin β1 and pro-SPC were stained with primary antibodies (rat anti-integrin β1, Millipore, Burlington, MA, USA #1997; rabbit anti-pro-SPC, Abcam, Cambridge, UK #90716) and secondary antibodies (donkey anti-rat, Alexa 488, Life Technologies, Carlsbad, CA, USA #21208; donkey anti-rabbit, Alexa 555, Life Technologies #A31572) per manufacturer protocols. Images were obtained using a Nikon Spinning Disk TiE inverted fluorescence confocal microscope attached to an Andor DU-897 EMCCD camera (x60 objective). Three-dimensional super-resolution microscopy was performed using a Zeiss LSM 980 confocal

microscope with an Airyscan 2 detector and a 63x/1.40 Plan-Apochromat (Oil) objective. Stacks were acquired with 50-60 images per stack and ~1 image/0.15µm. Post-imaging processing (spectral unmixing) was performed using ImageJ/Fiji. 3D reconstructions and surface plots were created using Imaris Software.

Single-cell RNA-seq. Tumors and normal adjacent tissue of two WT and two integrin β1-KO mice were macrodissected and disassociated using the Miltenyi Biotec gentleMacs dissociator and the mouse tumor dissociation kit (Miltenyi Biotec #130-096-730). Tissue pellets were strained through 100 µm then 70 µm filters (Stemcell Technologies #27217 and 27216) and RBC lysis performed per manufacturer's protocol (RBC lysis buffer, Gibco #A10492-01ACK). Cells were stained with propidium iodide (Sigma #P4864). 10,000 viable cells were captured for each tissue. scRNA-seq libraries were prepared using the 10X Chromium Single Cell Platform (10X Genomics #1000006, 1000080, and 1000020) following the manufacturer's protocol. The libraries were sequenced using the NovaSeq 6000 with 150 base pair paired end reads. RTA (version 2.4.11; Illumina) was used for base calling and analysis was completed using 10X Genomics Cell Ranger software v2.1.1. The FASTQ and matrix files have been uploaded to NCBI Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/sra), series GSE175687.

Single-cell RNA-seq analysis. After alignment and demultiplexing, samples were jointly analyzed using a standard Seurat (136)/Scanpy (137) pipeline as previously described (138). Briefly, cells containing fewer than 500 genes, <0.5% or >10% mitochondrial reads were filtered and excluded from downstream analysis. Libraries were merged and jointly normalized and scaled using SCTransform (138) in Seurat v3.2 including "percent.mt" as a regression variable,

followed by principal components analysis using variable genes and graph-based clustering. Immune (*Ptprc*+), epithelial (*Epcam*+) and stromal (*Pecam1*+ or *Col1a1*+) cells were independently extracted, followed by recursive clustering, doublet exclusion (clusters containing nonphysiologic marker combinations), and cell-type annotation. Immune, epithelial and stromal objects were then merged, uniform manifold approximation and projection (UMAP)-embedded (139). Differential expression analysis was performed using the FindMarkers tool in Seurat using the Wilcoxon test. Visualization and presentation were performed using Scanpy v.1.51. Code used for these analyses is available at www.github.com/kropskilab/itgb1_tumor/.

Cell lines. A549 and H358 cell lines were a gift from Dr. Christine Lovly (Vanderbilt-Ingram Cancer Center, Nashville, TN, USA) and are available for ATCC. Cell lines were cultured in RPMI1640 (Gibco, #11875-093) with 10% FBS plus 1% antibiotic, routinely tested for mycoplasma contamination, and authenticated with short-tandem repeat analysis (Genetica, Burlington, NC, USA).

CRISPR. ITGB1 (integrin β1) was KO of the A549 and H358 cells using CRISPR/Cas9. We followed published protocols to engineer the CRISPR/Cas9 plasmids (140). Briefly, guide RNAs were designed to exon 2 of human integrin β1 gene. These included the guide RNA used in the integrin β1-KO (guide RNA set #1, top = 5'- CACCGTTACAACCAATTTTCTGGAT-3' and bottom = 5'- AAACATCCAGAAAATTGGTTGTAAC-3') and integrin β1-KO.1 cells (guide RNA set #2, top = 5'- CACCGTGAATTTACAACCAATTTTC-3' and bottom = 5'- AAACGAAAATTGGTTGTAAATTCAC-3'). These guide RNAs were cloned into the PX-458 vector purchased from www.addgene.com (140). Cells were transfected using Lipofectamine

2000 (Thermo #11668). Cells were stained with anti-integrin β1 primary antibody (rat anti-human AIIB2 clone, University of Iowa, Iowa City, Iowa, USA) and secondary antibody (Invitrogen, Waltham, MA, USA #A10545). Cells were sorted via fluorescence-activated cell sorting (FACS) and cells that stained negative for integrin β1 (henceforth referred to as integrin β1-KO) were collected.

Western blotting. Western blotting was performed as described in our previous manuscripts (10,141). Briefly, protein was extracted from cells, electrophoresed in a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. Membranes were blocked and incubated with primary antibody (anti-integrin β1, Millipore #AB1952; anti-pY397 FAK, CST #3283; total FAK, CST #3285; anti-pS473 AKT, CST #9271; total AKT, CST #9272; anti-pT202/4 ERK, CST #9101; total ERK, CST #9102; beta-actin, CST #3700). Membranes were incubated with secondary antibodies (goat anti-rabbit 800CW, Licor, Lincoln, NE, USA #926-32211 or donkey anti-mouse 680LT, Licor #926-68022). Signal was detected using a LI-COR Odyssey CLx Near-Infrared Western Blot Detection system.

Cell adhesion, migration, and proliferation assays. Cell adhesion and migration assays were performed as described previously (141). The migration assay used transwells with 8.0 μm pores (Costar #3422). BRDU-incorporation cell proliferation assays were used according to manufacturer protocols (Exalpha #X1327K2). These assays used plates and/or inserts coated with Matrigel (Corning, Corning, NY, USA #356230, 10 μg/mL), laminin I derived from Engelbreth-Holm-Swarm sarcoma (20 μg/mL, Invitrogen #23017-015), or vitronectin (0.5 μg/mL, Advanced Biomatrix, Carlsbad, CA, USA #5051).

Soft agar assays. 1,500 cells were suspended in 0.5 mL of 0.35% soft agar in RPMI (Difco, Waltham, MA, USA #214220) and plated in a 24 well plate on top of a base layer of 0.5 mL of 0.5% soft agar. Media was changed 3X/week and on day 21 the cells were stained with 0.5 mL of 2.5 mg/mL MTT (Sigma #M2128). The plate was imaged with GelCount colony counter (Oxford Optronix, Abingdon, OX, UK) and analyzed using GelCount software version 1.2.1.0. The FAK, AKT and ERK inhibitors were purchased from SelleckChem (Houston, TX, USA; FAK inhibitor VS-6063, #S7654; AKT inhibitor MK-2206, #S1078; ERK inhibitor SCH772984, #S7101).

Xenograft mouse model. Eight-week-old athymic mice (*Foxn1*^{nu}) were purchased from Jackson Laboratory (#002019-Nu/J). 1 x 10^6 cells were suspended in Matrigel (Corning, Corning, NY, USA #356230, 1 mg/mL) and injected into the left lung. At 45 days, mice were euthanized and heart/lungs resected en-bloc. Lungs were paraffin embedded, sectioned every 100 μm, and H&E stained. Images were obtained and tumor area per high power field was measured using ImageJ software (version 1.52). For bioluminescence experiments, cells were labelled with luciferase-positive lentivirus (System Biosciences, # BLIV713VA-1), mice were administered 30 mg/mL luciferin (Perkin-Elmer #122799) and bioluminescence measured using the Perkin-Elmer IVIS Spectrum bioluminescent and fluorescent imaging system prior to euthanasia.

Bulk RNA-seq and data analysis. RNASeq libraries were prepared using 300 ng of RNA and the NEBNext® Ultra™ II RNA Library Prep kit (NEB #E7760L) per manufacturer's instructions, with mRNA enriched via poly-A-selection using oligoDT beads. The RNA was then thermally

fragmented and converted to cDNA, adenylated for adaptor ligation and PCR amplifed. Individual libraries were assessed for quality using the Agilent 2100 Bioanalyzer and quantified with a Qubit Fluorometer. The adapter ligated material was evaluated using qPCR prior to normalization and pooling for sequencing.

RNASeq libraries were prepared using 300 ng of RNA and the NEBNext® Ultra™ II RNA Library Prep kit (NEB, Ipswich, MA, USA #E7760L) per manufacturer's instructions. The libraries were sequenced using the NovaSeq 6000 with 150 base pair paired end reads. The FASTQ files have been uploaded to NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra), BioProjectID SUB9677957.

The libraries were sequenced using the NovaSeq 6000 with 150 base pair paired end reads. RTA (version 2.4.11; Illumina) was used for base calling and data QC was completed using MultiQC v1.7 by the Vanderbilt Technologies for Advanced Genomics (VANTAGE) core (Vanderbilt University, Nashville, TN). The FASTQ files have been uploaded to NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra), BioProjectID SUB9677957.

RNA-seq analysis. Dragen pipeline was run on Basespace to perform QC and analyze the RNA-seq fastq files to generate read counts for each of the samples (142). Genes with very low counts (sum across samples <=10) were excluded. DESeq2 was used to perform two sets of differential gene expression analysis: 1) comparing integrin β1-KO vs. WT, and 2) identifying genes associated with the rescue phenotype: by comparing KO.ITGB1, KO.Tacβ1 and KO.YYAA versus integrin β1-KO. Pathway enrichment analysis was performed using KEGG pathways

from MSigDB (msigdbr package (143)) using Fisher's exact test. Heatmaps were plotted using ComplexHeatmap package (144). These analyses were performed using R version 4.0.3.

Integrin β1 expression in integrin β1-KO cells. The KO.ITGB1, KO.Tacβ1 and KO.YYAA A549 cells were engineered by re-expressing full integrin β1 or its various constructs in the integrin β1-KO cells. Full-length integrin β1 was expressed in the integrin β1-KO cells to create the KO.ITGB1 cells. The Tacβ1 chimeric gene was expressed in the integrin β1-KO cells to create the KO.Tacβ1 cells. Integrin β1 containing Y-to-A cytoplasmic tail mutations at residues Y783 and Y795 was expressed in integrin β1-KO cells to create the KO.YYAA cells. These genes were cloned using standard molecular biology techniques into the PB-CMV-MCS-EF1α-GreenPuro *piggyBac* transposon vector (SBI #PB513B-1). All vector sequences were confirmed with DNA sequencing. Cells were transfected with both the expression vector and pCMV-m7pB transpose vector (145). Cells were collected via flow cytometry that expressed either the integrin β1 ectodomain (KO.ITGB1 and KO.YYAA) or the Tac domain (KO.Tacβ1). A mouse anti-human IL-2R PE-conjugated antibody was used to stain the KO.Tacβ1 cells (R&D Systems #FAB1020P).

Patient data and tissue microarray staining. The tissue microarray is composed of tumors spotted in duplicate from 65 deidentified patients from Vanderbilt University Medical Center (Nashville, TN, USA). Slides were placed on the Leica Bond Max IHC stainer. All steps besides dehydration, clearing and cover slipping were performed on the Bond Max. Slides are deparaffinized. Heat induced antigen retrieval was performed on the Bond Max using their Epitope Retrieval 1 solution for 20 minutes. Slides were incubated with anti-integrin β1 (CST

#34971) or isotype control. The Bond Polymer Refine system was used for visualization. Slides were the dehydrated, cleared and cover slipped.

TCGA data analysis. TCGA lung adenocarcinoma (LUAD) data was analyzed for *ITGB1* (integrin β1) gene expression and its association with the expression of other genes/pathways and survival data. TCGA LUAD data was downloaded from www.cbioportal.org. Integrin β1 gene expression data was modeled as a mixture of gaussians to identify a high versus low expression groups using the mixtools package in R. Survival analysis was performed to identify the association of the integrin β1 expression groups versus overall survival. Correlation analysis was performed to identify genes significantly correlated with integrin β1 expression (adjusted p-value < 0.001). Geneset enrichment was performed using KEGG pathways from msigdbr R library.

Statistical analyses. Statistical analyses, unless stated otherwise, were performed with GraphPad Prism version 9.0.0. Please see methods for details of statistics used in the analysis of single cell RNA-seq data, bulk RNA-seq data, and clinical data. An unpaired, two-tailed *t* test was used single comparisons and Sidak's multiple comparisons test for multiple comparisons. A P value of <0.05 was considered significant. Error bars represent standard error of the mean (SEM).

Study approval. All animal experiments were approved by the Vanderbilt University Medical Center Institutional Animal Care and Use Committee. Mice were housed in an AAALAC-accredited facility with a standard 12-hour light/dark schedule and fed regular chow diet, unless stated otherwise.

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Results

Deletion of integrin β1 in type 2 alveolar epithelial cells reduces tumorigenesis. We set out to define the role of β1 integrins on lung cancer initiation and progression using the LSL-Kras-G12D mouse strain, which carries a Lox-Stop-Lox (LSL) sequence followed by the Kras G12D point mutation allele commonly associated with human cancer. When this mouse is bred to a strain expressing Cre recombinase under control of tissue specific promoters, the Cre recombination deletes the LSL cassette and allows expression of the mutant KRAS oncogenic protein. To study the role of integrin β1 in lung cancer, we crossed these mice with integrin $\beta1^{f/f}$ and SPC-$CreER^{T2}$ mice. These mouse crossings were designed to simultaneously induce the LSL-Kras-G12D mutation and delete the integrin β1 subunit in type 2 alveolar (AT2) cells, the cell of origin for lung adenocarcinoma (146), in an inducible fashion. Unfortunately, tumor initiation occurred in mice never exposed to tamoxifen, suggesting constitutive activation of Cre. This precluded the use of this model from further study (**Figure 8**).

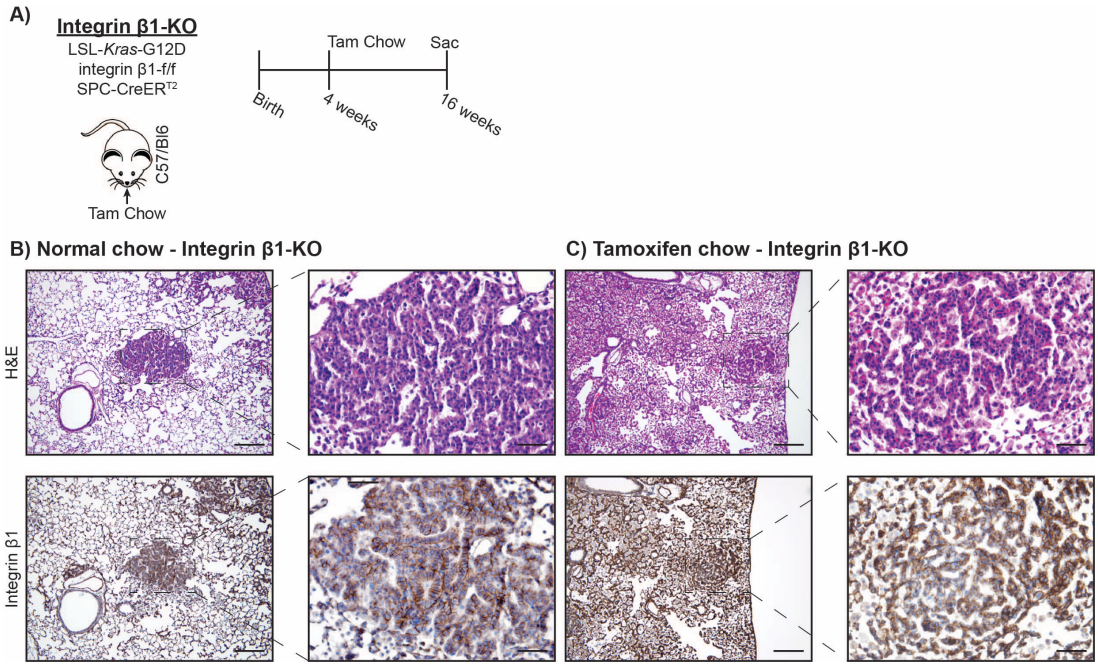

Figure 8: LSL-Kras-G12D tamoxifen model. A) LSL-*Kras*-G12D; integrin β1$^{f/f}$; SPC-CreER^{T2} were bred and the model was set up as depicted in the diagram. **B)** Mice died prematurely due to tumor formation in the lungs of tamoxifen-naïve mice. These tumors were integrin β1 positive (lower panel). **C)** Tumor formation was increased even further in tamoxifen-exposed mice, which also stained positive for integrin β1 (lower panel). These data suggested this Cre was extremely leaky, so no further experiments were not attempted with this model.

We then made use of a urethane-induced lung cancer model in mice where the integrin β1 subunit was deleted in AT2 cells. These mice were generated by crossing integrin β1-floxed mice (β1$^{f/0}$) mice with a doxycycline (dox) inducible Cre recombinase under control of the surfactant protein-C promoter (SPC rtTA;TetO-Cre) (**Figure 9A, Figure 10**). Although we previously showed efficient integrin β1 deletion in this model (106) we verified this again in mice fed dox chow by staining frozen sections for pro-SPC to identify AT2 cells and integrin β1.

Under low magnification, AT2 cells were identified with robust, often circumferential integrin β1 staining in $\beta1^{f/0}$ (control) mice fed dox chow (**Figure 11A**, left panels, white arrows). The integrin β1 staining was markedly decreased in pro-SPC-positive cells in SPC rtTA;TetO-Cre;$\beta1^{f/0}$ (here called integrin β1-KO) mice fed dox chow (**Figure 11A**, right panels, white arrows). This staining was examined in more detail and quantified using three-dimensional super-resolution microscopy with reconstructions and surface plots for integrin β1 (**Figure 11B**). There was more than a 2X decrease in integrin β1 staining in integrin β1-KO AT2 cells (**Figure 11B**).

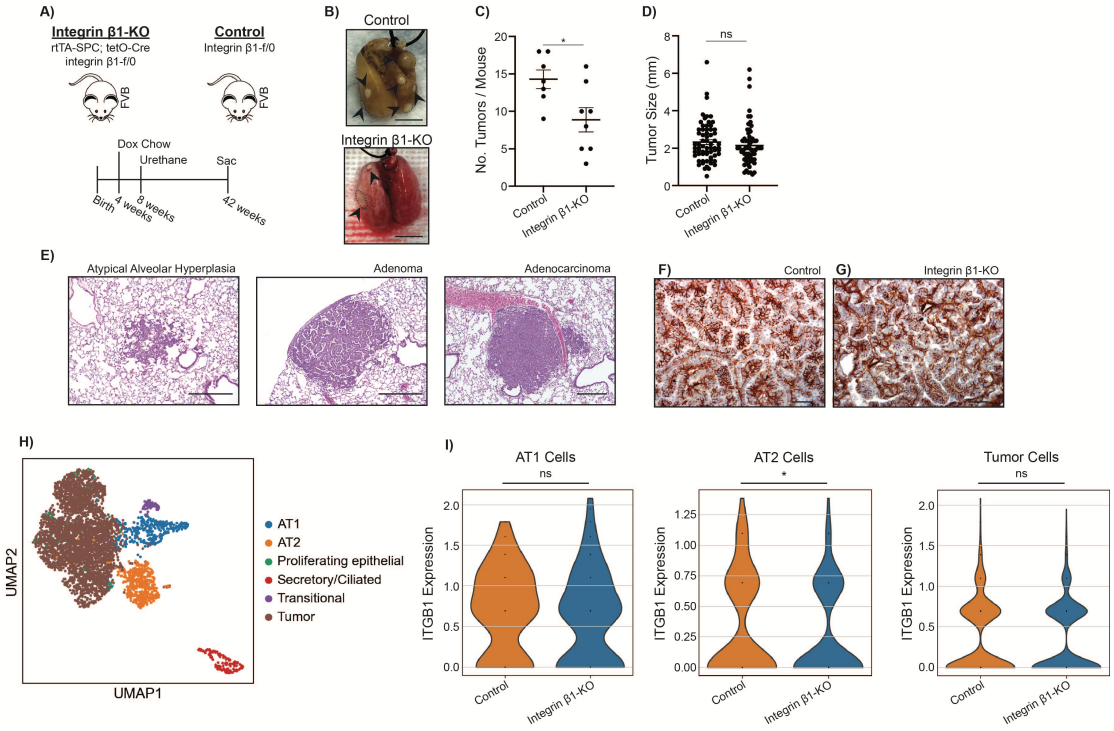

Figure 9: Deletion of integrin β1 in type 2 alveolar epithelial cells results in development of fewer tumors. A) Tumorigenesis was initiated with urethane in integrin $\beta1^{f/0}$ mice without (control, n=7) and with (integrin β1-KO n=8) dox-inducible SPC rtTA;TetO-Cre. **B)**

Representative photograph of formalin-inflated lungs (removed en-bloc with heart/mediastinum) demonstrating fewer tumors in the integrin β1-KO mice relative to the control mice (arrow heads = tumor, scale bar = 1 cm); **C)** quantitation of tumor count across the entire cohort (mean +/- SEM). **D)** Longest diameter of all tumors from integrin β1-KO and control mice is graphed (mean +/- SEM). Both control and integrin β1-KO mice developed lesions across the spectrum of disease, with representative photomicrographs shown of **E)** atypical alveolar hyperplasia, adenomas, and adenocarcinomas (scale bar = 500 mm). Lesions that developed in the WT and integrin β1-KO mice were histologically indistinguishable, and the lesions shown are representative of those that developed in either strain of mouse. FFPE tumors from **F)** control and **G)** integrin β1-KO mice were stained for integrin β1 with representative photomicrographs shown (n=5, scale bar = 50 mm). **H)** Single cell RNA-seq was performed on tumors and adjacent normal tissue (tissue was pooled for n=2 mice from each genotype). Uniform manifold approximation and projection (UMAP) depicting epithelial-like cells isolated from tumors or adjacent tissue from integrin β1-KO and control mouse lungs are shown. **I)** Relative levels of integrin β1 (*Itgb1*) gene expression is shown for AT1, AT2 and tumor cells. *$p<0.05$; ns = $p>0.05$ by unpaired, two-tailed *t* test.

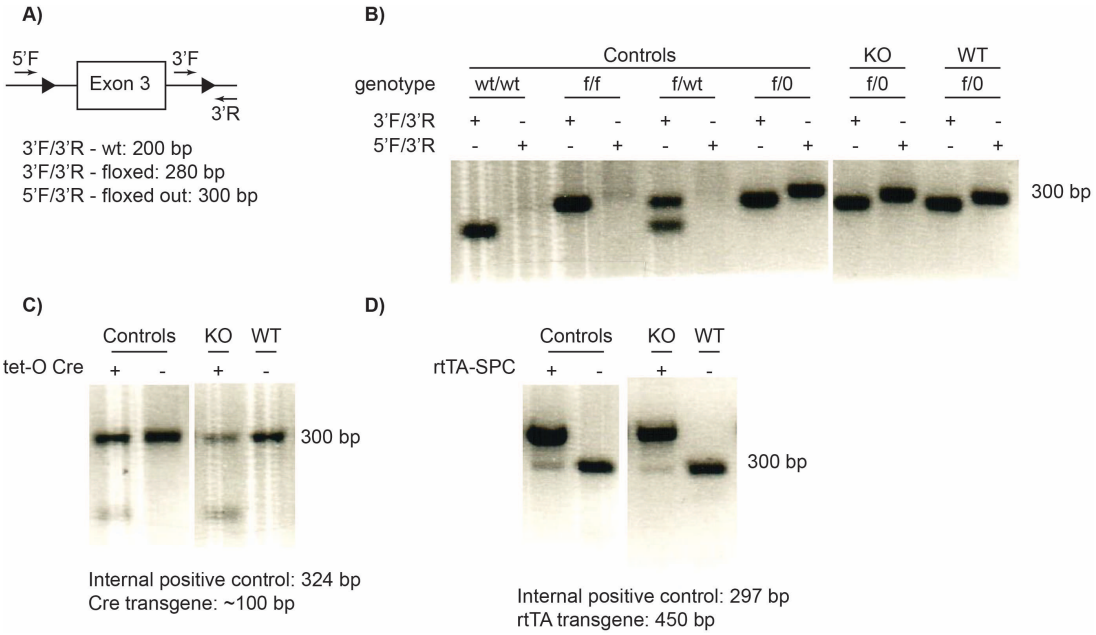

Figure 10: Genotyping results for SPC rtTA; TetO-Cre; integrin β1$^{f/0}$ mouse. The integrin β1$^{f/0}$ mice were selected for "WT" mice. The SPC rtTA; TetO-Cre; integrin β1$^{f/0}$ mice were selected for the integrin β1-KO or "KO" mice.

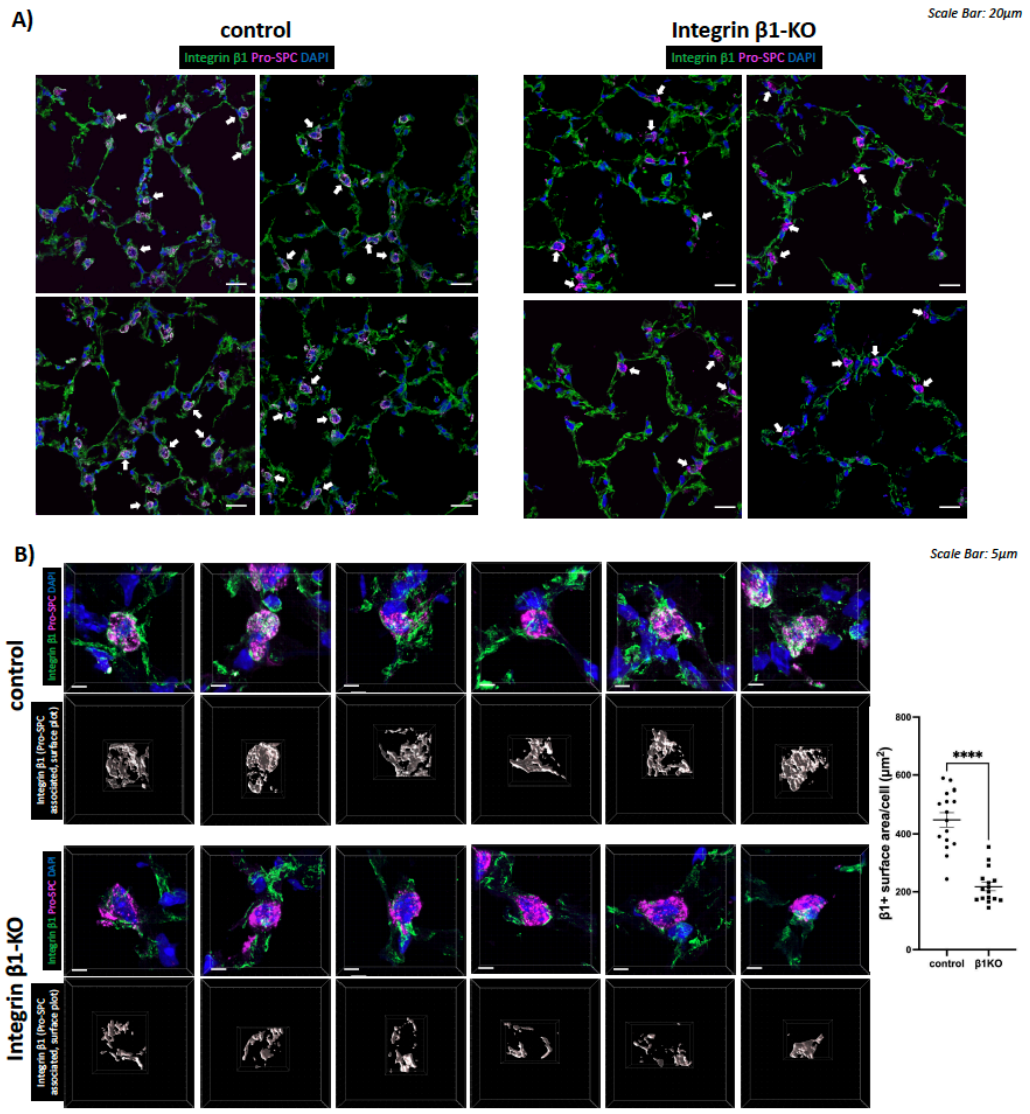

Figure 11: Integrin β1 is deleted in type 2 alveolar epithelial (AT2) cells in mice. A) Immunostaining for pro-SPC (magenta) and integrin β1 (green) in integrin $\beta1^{f/0}$ mice without (control) and with SPC rtTA;TetO-Cre (integrin β1-KO). Scale bar = 20 μm. White arrows mark examples of SPC-positive cells. **B)** Three-dimensional super-resolution microscopy was performed on SPC-positive cells of control and integrin β1-KO lungs (upper panels). These images were used for three dimensional reconstructions and surface plots for integrin β1

associated with pro-SPC were rendered (lower panels). Quantification of integrin β1-positive surface areas was compared. Scale bar = 5 μm. Graph shows mean +/- SEM, n=3 mice for each genotype, only 3D conformations graphed. ***p<0.0001 by unpaired, two-tailed *t* test.

For tumor induction, mice were started on dox chow at 4 weeks of age, given intraperitoneal urethane at 8 weeks and then aged to approximately 42 weeks. Significantly fewer tumors developed in SPC rtTA;TetO-Cre;$\beta1^{f/0}$ (here called integrin β1-KO) mice than $\beta1^{f/0}$ (control) mice (**Figure 9B-C**). There were no size differences in the tumors (**Figure 9D**) and both control and integrin β1-KO mice developed lesions across the spectrum, including atypical alveolar hyperplasia, adenomas, and adenocarcinomas (**Figure 9E)**. As the tumors in both cohorts of mice were similar in size and appearance, we investigated whether there were differences in integrin β1 expression. Although integrin β1 is significantly decreased in most AT2 cells within the normal lung of the integrin β1-KO mice (**Figure 11**), integrin β1 was expressed in all tumors assessed by immunostaining (**Figure 9F-G**). To verify that integrin β1 expression was similar in tumors irrespective of the genotypes, we assessed gene expression data from single cell RNA-sequencing (seq) of tumor and adjacent normal lung epithelial cells in control and integrin β1-KO mice (**Figure 12, Figure 9H**). Normal AT2 cells demonstrated a significant decrease in gene expression of *Itgb1* (integrin β1) in integrin β1-KO mice, while tumor cells from both cohorts demonstrated similar levels of integrin β1 (**Figure 9I),** suggesting only AT2 cells that escaped integrin β1 deletion were able to develop into tumors. The single cell RNA-seq data also demonstrated robust expression of AT2 cell specific *Sftpc* (the gene encoding surfactant protein C) and *Sftpa1* (the gene encoding surfactant protein A) genes in cells labeled as tumor cells (**Figure 12**). We further confirmed that the tumors developed from AT2

cells as they stained positive for the AT2 cell marker pro-SPC (**Figure 13**). Taken together these data suggest that integrin β1 expression is required for tumorigenesis in this carcinogen-induced lung cancer model.

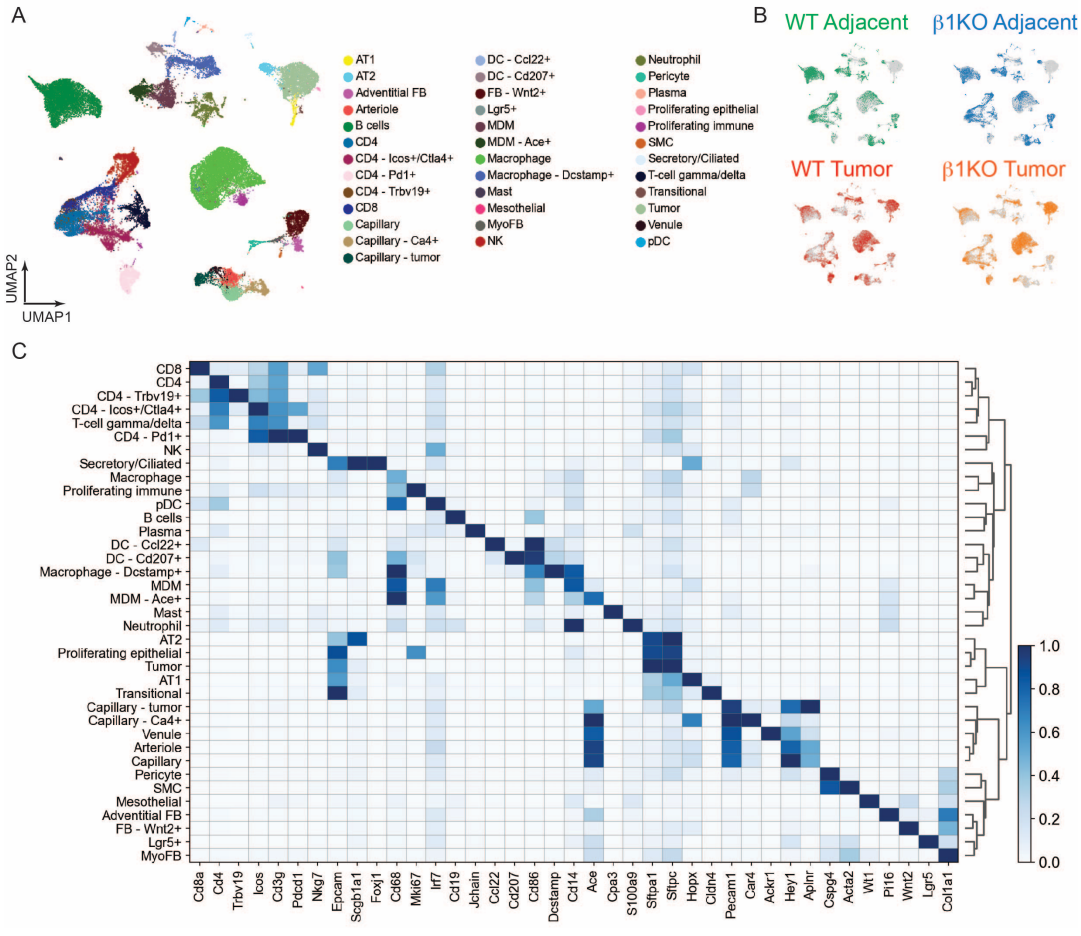

Figure 12: Cell types and discriminating marker expression identified in single cell RNA-seq experiment. A) Uniform manifold approximation and projection (UMAP) depicting 41,494 cells isolated from tumors or adjacent tissue from β1-KO and control mice after urethane. **B)** Corresponding cell types are shown in relative proportion in control and integrin β1-KO mice for

both tumor and adjacent normal tissue. **C)** Heatmap demonstrating key discriminating marker expression across cell types.

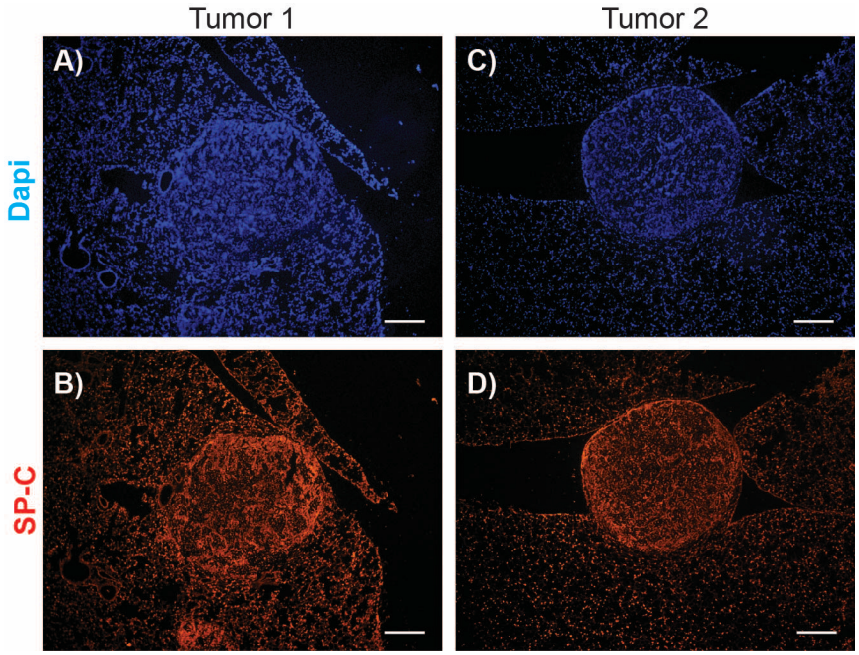

Figure 13: Urethane-induced tumors in integrin β1-KO mice express pro-SPC. Frozen sections from urethane-induced tumors that developed in SPC rtTA; TetO-Cre; integrin $\beta1^{f/0}$ mice treated with doxycyline (i.e., integrin β1-KO mice) were stained with **A & C)** DAPI and for **B & D)** SPC. Tumors express pro-SPC, which is consistent with the cells of origin being SPC-positive type 2 alveolar epithelial cells.

Human lung adenocarcinoma cells require expression of integrin β1 to form colonies and tumors. As the urethane model suggested integrin β1 is required for lung tumor initiation, this was investigated further in lung adenocarcinoma cell lines where integrin β1 was genetically downregulated. We utilized the *KRAS*-mutated human lung adenocarcinoma cell lines A549 and

H358 and deleted *ITGB1* (integrin β1) using CRISPR/Cas9. The results obtained were similar in both cell lines, thus we show data for the A549 cells in the main figures (**Figure 14**) and H358 cells in **Figure 15**. Deletion of integrin β1 was confirmed by Western blot (**Figure 14A**). Classical integrin β1-dependent functions such as adhesion, migration, and proliferation were maintained in the WT but not integrin β1-KO A549 cells on the integrin β1-dependent matrix laminin I (**Figure 14B-D)**. WT and integrin β1-KO A549 cells behaved similarly when they were plated on the integrin β1-independent matrix vitronectin (**Figure 14B-D)**. Surprisingly, the integrin β1-KO A549 cells also demonstrated decreased colony formation in the adhesion-independent soft agar assay, suggesting that non-adherent cancer cells required integrin-dependent signaling to form colonies (**Figure 14E**). Next, we injected the A549 cells into the lung parenchyma of athymic mice. The integrin β1-KO A549 cells demonstrated decreased tumor formation in the lungs when evaluated by bioluminescence (**Figure 14F**) and histology (**Figure 14G**). These data indicate that integrin β1 signaling is required for tumor development in an orthotopic model of lung cancer.

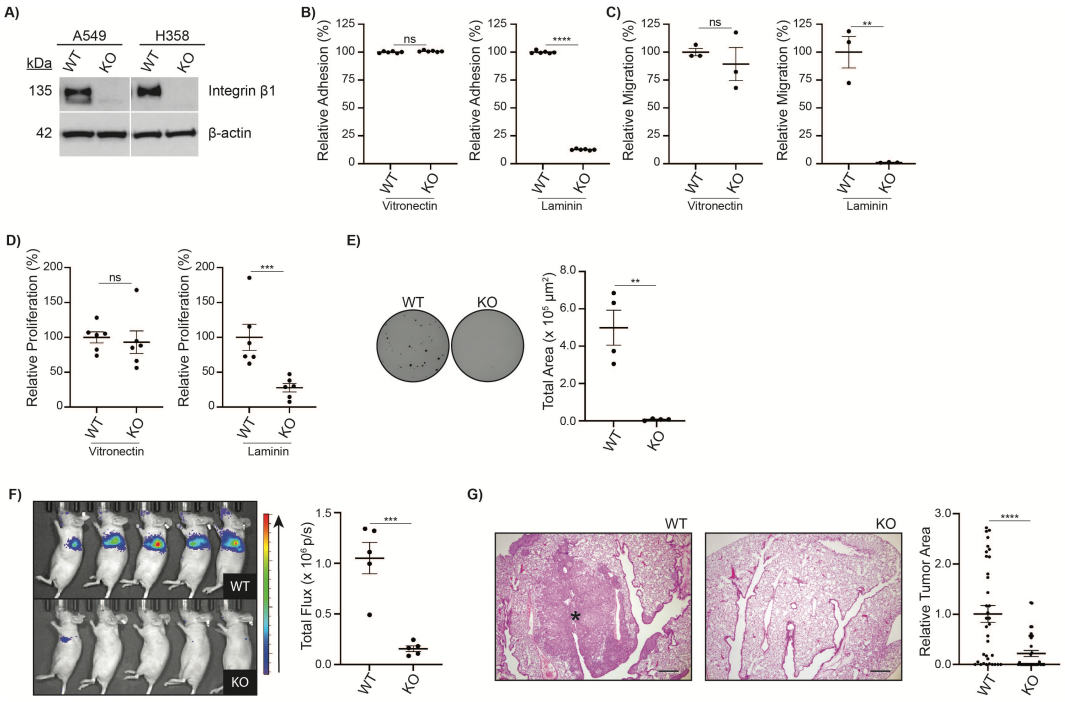

Figure 14: Deletion of integrin β1 in A549 human lung cancer cells results in decreased colony formation and tumor development. Integrin β1 is deleted in A549 and H358 human lung adenocarcinoma cells using CRISPR/Cas9 (KO). **A)** Lysates from wild type (WT) and KO were analyzed by Western blot for levels of integrin β1. The solid white lines represent lane splicing from the same gel. The WT and KO A549 cells were plated on integrin β1-independent (vitronectin) and -dependent (laminin I) matrices. Relative **B)** adhesion, **C)** migration and **D)** proliferation measured as BRDU-incorporation is graphed for WT and integrin β1-KO cells (n=3 replicates) (mean +/- SEM). **E)** The WT and integrin β1-KO A549 cells were plated in soft agar. Representative photomicrographs of the wells and colony surface area quantification are shown (n=3 replicates) (mean +/- SEM). Luciferase-tagged A549 WT and integrin β1-KO cells were injected into the left lung of athymic mice. After 3 weeks, tumor burden was quantified via **F)** luciferin injection and measurement of bioluminescence (mean +/- SEM) and **G)** relative surface

area as measured by microscopy (representative photomicrographs shown, asterisk denotes tumor, scale bar = 20 mm, n=5 mice for each genotype, left lung from each mouse was sectioned every 100 mm X 7 sections) (mean +/- SEM). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns = p>0.05 by unpaired, two-tailed *t* test.

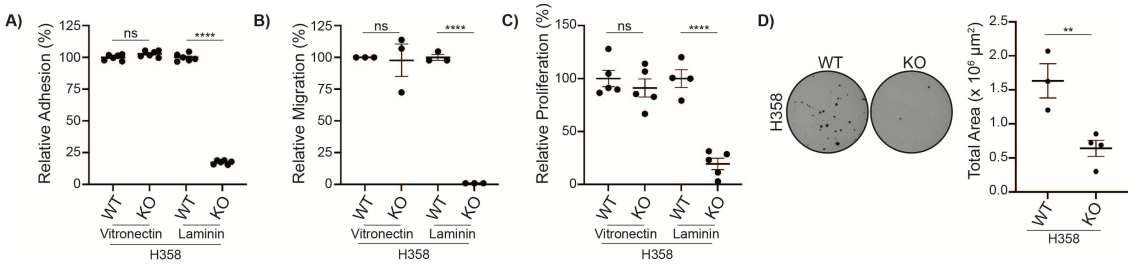

Figure 15: Deletion of integrin β1 in H358 human lung cancer cells results in decreased adhesion, migration, proliferation, and colony formation. The WT and integrin β1-KO H358 cells were plated on integrin β1-independent (vitronectin) and -dependent (laminin I) matrices. Relative **A)** adhesion, **B)** migration, **C)** BRDU proliferation and **D)** colony formation (representative photomicrographs and quantification) are graphed for WT and integrin β1-KO cells (n=3 replicates). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns = p>0.05. Graphs show mean +/- SEM.

Integrin β1 regulates gene expression in matrix-dependent and -independent manner. To understand why integrin β1 is necessary for tumor development, we performed RNA-seq on WT and integrin β1-KO A549 cells. Cells were plated on either Matrigel that allows integrin-dependent cell adhesion in WT integrin β1-positive cells but not integrin β1-KO cells, or vitronectin that allows integrin αv-dependent adhesion of both WT and integrin β1-KO cells.

The gene expression of WT and integrin β1-KO cells were compared, and differentially expressed genes (DEGs) were identified ($p<0.01$). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed on these DEGs from cells plated on Matrigel or vitronectin and ranked (top = most significant, bottom = less significant, **Figure 16A**). Many of the significantly different gene sets found in integrin β1-KO cells plated on Matrigel were no longer different when the cells were plated on vitronectin, suggesting that adhesion via αv integrins is sufficient to normalize these gene expression changes (**Figure 16A**). There were also differentially expressed gene sets in cells plated on either Matrigel or vitronectin, suggesting expression of these genes requires integrin β1 expression but not integrin β1-dependent ECM interactions (black boxes). To explore this biology further, we identified DEGs shared by cells plated on Matrigel and vitronectin (657 matrix-independent DEGs) and DEGs exclusive to cells plated on Matrigel (6,832 matrix-dependent DEGs) (**Figure 16B**). Pathways associated with cell proliferation, including KEGG_CELL_CYCLE, KEGG_DNA_REPLICATION, KEGG_PYRIMIDINE_METABOLISM, and KEGG_PURINE_METABOLISM (arrows, **Figure 16C**) predominated in the matrix-dependent DEGs and a heatmap for KEGG_CELL_CYCLE demonstrated that gene expression was decreased in the integrin β1-KO cells (**Figure 16D**). When we examined the pathways enriched in a matrix-independent manner, the major DEGs included those pertaining to the ECM and cell adhesion (KEGG_ECM_RECEPTOR_INTERACTION, KEGG_FOCAL_ADHESION, KEGG_ADHERENS_JUNCTION, arrows, **Figure 16E**) and the heatmap for KEGG_FOCAL_ADHESION demonstrated robust changes in several ECM-associated genes in the integrin β1-KO cells (**Figure 16F**). These data implicate integrin β1 in the regulation of

cancer relevant genes via mechanisms that are both dependent and independent of integrin-mediated cell adhesion to ECM.

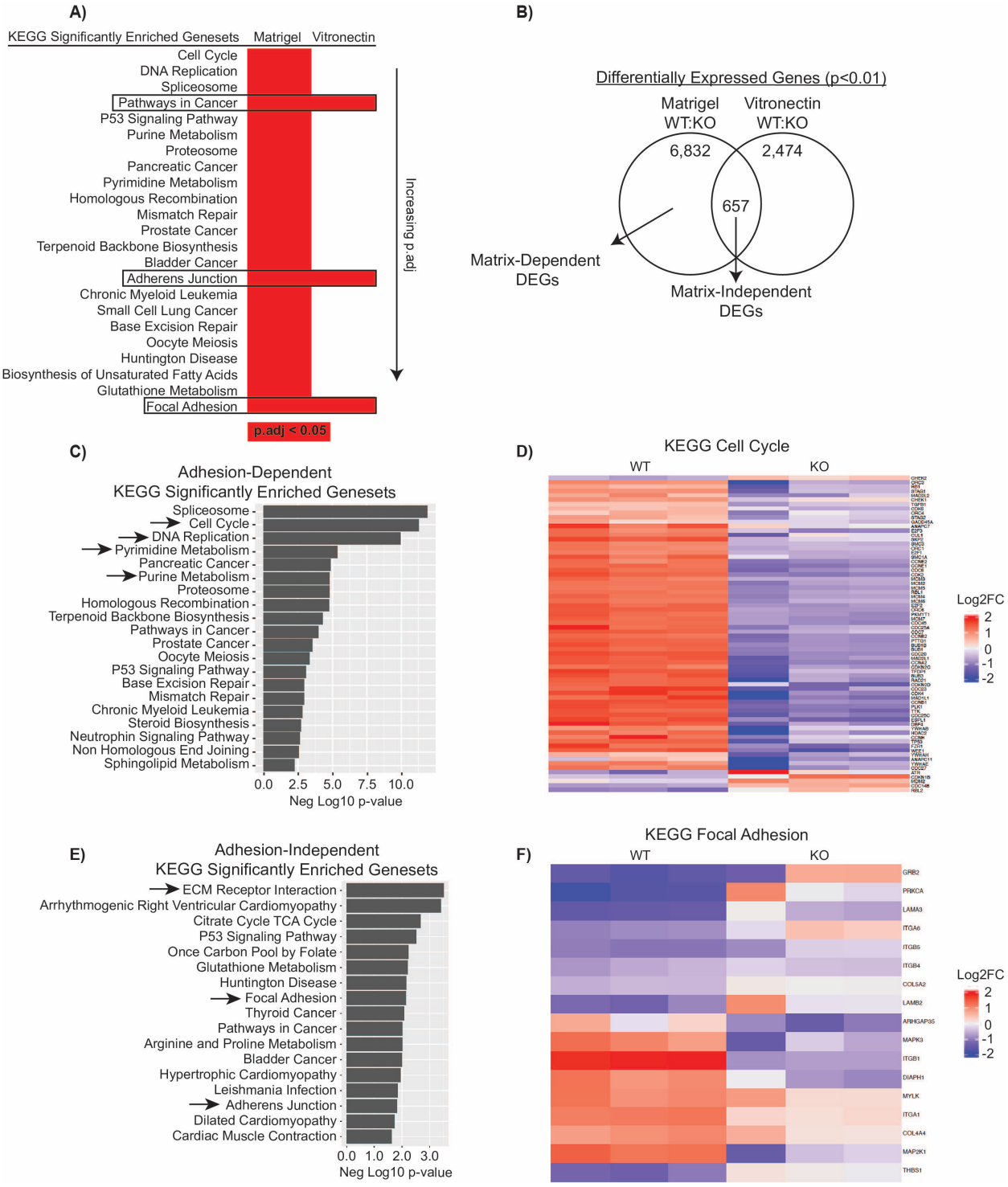

Figure 16: Differential gene expression in wild type and integrin β1-knock out A549 cells. **A)** WT and integrin β1-KO A549 cells were plated on the integrin β1-dependent matrix Matrigel or the integrin αv-dependent matrix vitronectin and gene expression measured by RNA-seq (n=3 replicates for each cell line). Gene set enrichment analysis was performed using differentially expressed genes (DEGs, $p<0.01$). Shown are the significant pathways (p adjusted or p.adj <0.05) from cells plated on Matrigel and corresponding p.adj values for cells plated on vitronectin (red square = p.adj <0.05, white square = not significant). **B)** Venn diagram demonstrates DEGs exclusive to cells plated on Matrigel that normalize in cells plated on vitronectin (matrix-dependent DEGs) as well as DEGs shared by cells plated on either matrix whose directionality aligns (e.g., increased in integrin β1-KO cells relative to WT cells on both matrices; matrix-independent DEGs). **C)** Gene set enrichment analysis was performed on matrix-dependent DEGs, demonstrating several significant gene sets including proliferation-associated gene sets (arrows). **D)** Representative heat map of significant gene set from the matrix-dependent genes (KEGG_CELL_CYCLE). **E)** Gene set enrichment analysis was also performed on matrix-independent DEGs, demonstrating several significant gene sets including cell adhesion and ECM-related genes (arrows). **F)** Representative heat map of significant gene set from the matrix-independent genes (KEGG_FOCAL_ADHESION).

Integrin β1 regulates growth factor-dependent signaling required for colony formation. We next tested whether integrin β1 regulates cancer cell proliferation signaling pathways as suggested by the gene expression data. We utilized EGF as it is a well-known growth factor that drives lung tumorigenesis and activating mutations in the EGF receptor are driver mutations in

some lung tumors (147,148). We treated WT and integrin β1-KO A549 cells plated on Matrigel with EGF and then measured activation of key cell proliferation signaling molecules, AKT and ERK, as well as FAK, a known downstream target of integrin β1 that is stimulated in adherent proliferating cells (**Figure 17A-D**). Interestingly, there was decreased phosphorylation of FAK and AKT in integrin β1-KO A549 cells prior to treatment with EGF, suggesting that integrin β1 signaling plays a role in the basal activation of these pathways in *KRAS*-mutated lung cancer cells. We further noted that EGF treatment of WT A549 cells resulted in increased FAK, ERK and AKT phosphorylation, which was less robust in the integrin β1-KO A549 cells. As the difference in basal and EGF-induced FAK activation between the cell lines was highly significant, we suspected this was a major mechanism whereby integrin β1 regulates tumor cell growth and proliferation. To test this hypothesis, we treated WT and integrin β1-KO A549 cells plated in soft agar with the FAK tyrosine kinase inhibitor (TKI) defactinib, as well as inhibitors to AKT and ERK (cell signaling proteins that commonly transmit important mitogen signaling in cells). First, we confirmed that these inhibitors were active in WT A549 cells treated for 1 hour by evaluating the phosphorylation status of the target kinases (**Figure 18**). Next, drug doses were selected that result in a robust, significant decrease in colony formation (**Figure 19**). Finally, WT A549 cells were treated with single inhibitor or combinations (**Figure 17E-F**). FAK inhibition failed to completely inhibit colony formation, and the addition of either the AKT or ERK inhibitor reduced colony formation further. These data suggest that FAK provides oncogenic signaling independent of AKT and ERK.

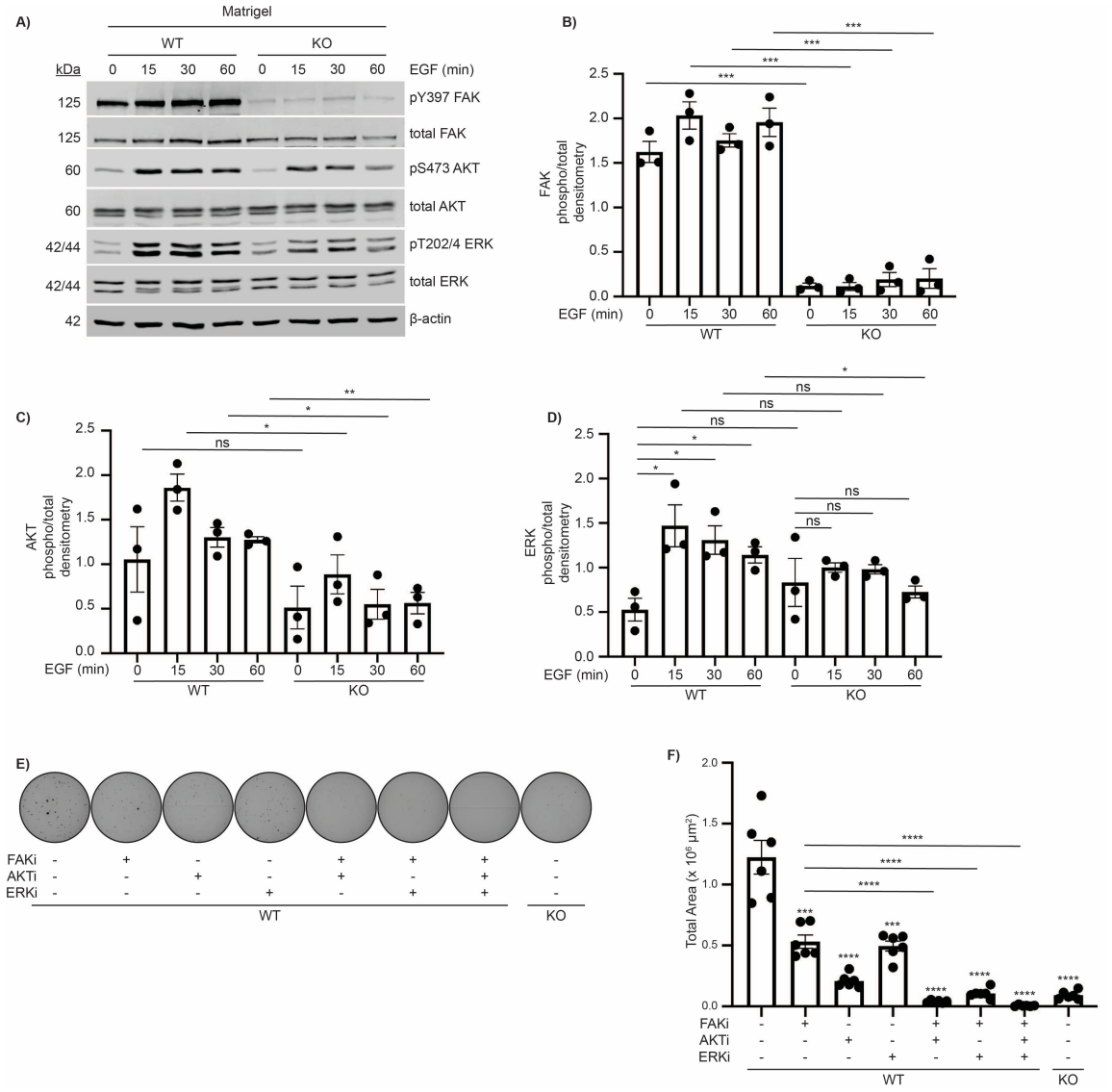

Figure 17: Deletion of integrin β1 results in decreased growth factor-dependent signaling. **A)** A549 cells were plated on the integrin β1-dependent matrix Matrigel and stimulated with EGF (1 ng/ml) for 0, 15, 30 or 60 minutes and then lysates were analyzed by Western blot for levels of total and activated FAK, AKT and ERK. **B-D)** Results were quantified via densitometry and quantified (average of n=3 replicates). WT A549 cells were treated with inhibitors of FAK (defactinib, 1.0 mM), AKT (MK-2206, 0.1 mM) and ERK (SCH772984, 0.01 mM) alone or in

combinations. Integrin β1-KO cells included as negative control. Representative **E)** photomicrographs and **F)** surface area quantification of colonies are shown (n=3 replicates, each replicate consisting of 6 wells, representative data from one replicate shown). All comparisons include DMSO-treated cells (i.e., those not treated with FAKi, AKTi or ERKi) unless comparison otherwise marked by bar. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns = p>0.05 by Sidak's multiple comparison test. Graphs show mean +/- SEM.

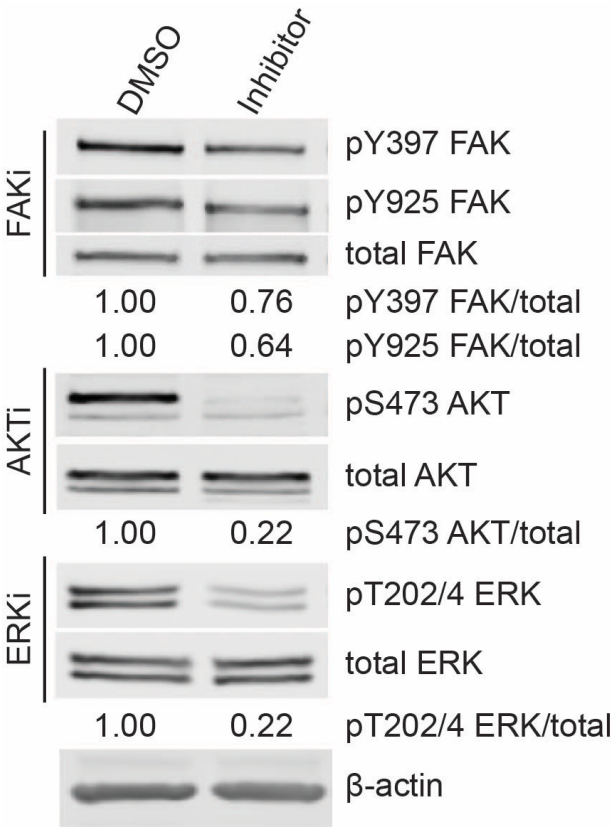

Figure 18: Inhibitors to FAK, AKT and ERK result in decreased kinase phosphorylation. **A)** A549 cells were treated with inhibitors (1 μM) of FAK (defactinib), AKT (MK-2206) and ERK (SCH772984) for 1 hour. Phosphorylation of the kinases were measured by western blot. Shown are the ratio of densitometry values for the phosphorylated and total protein.

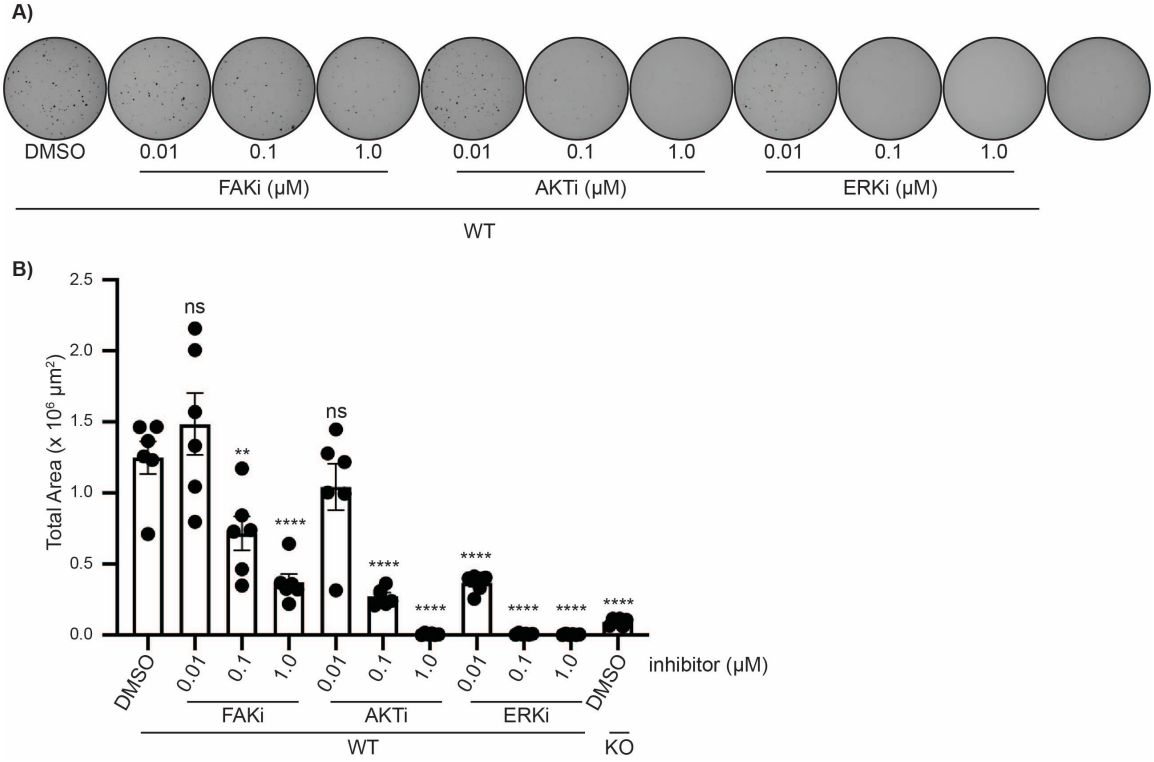

Figure 19: Inhibition of FAK, AKT and ERK in A549 human lung adenocarcinoma cells reduces colony formation in soft agar assay. WT and integrin β1-KO A549 cells were treated with inhibitors of FAK (defactinib), AKT (MK-2206) and ERK (SCH772984) at the specified doses. Representative **A)** photomicrographs and **B)** surface area quantification of colonies are shown. All comparisons include DMSO-treated cells. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns = p>0.05 by Sidak's multiple comparison test. Graphs show mean +/- SEM.

The integrin β1 cytoplasmic tail is necessary and sufficient for tumor formation. Since integrin β1 is required for colony formation in soft agar, it likely mediates its effects by an adhesion-independent mechanism. We therefore generated integrin β1-KO A549 cells where we

introduced either the full length integrin β1 subunit (KO.ITGB1), a chimeric protein consisting of an integrin β1 cytoplasmic tail fused to the extracellular and transmembrane domains of the interleukin 2 receptor (KO.Tacβ1) (10) or an integrin β1 subunit with Y-to-A cytoplasmic tail mutations at residues Y783 and Y795 that disrupt integrin signaling (KO.YYAA) (**Figure 20A**) (9,10,149,150). These cells were flow sorted to achieve cell populations with comparable surface expression of these proteins (**Figure 20B**). As expected, the KO.ITGB1, KO.Tacβ1, and the KO.YYAA cells demonstrated similar adhesion, migration, and proliferation on the integrin β1-independent matrix vitronectin (**Figure 20C-E**). By contrast, the KO.Tacβ1 and KO.YYAA cells demonstrated decreased adhesion, migration, and proliferation relative to the KO.ITGB1 cells when plated on the integrin β1-dependent matrix laminin I (**Figure 20C-E**). Despite the inability to bind ECM, the KO.Tacβ1 cells formed robust colonies in soft agar and tumors in mice. The KO.YYAA cells formed almost no colonies in soft agar and significantly less tumor burden in mice (**Figure 20F-G**). These data support the conclusion that a functional integrin β1 cytoplasmic tail promotes colony and tumor formation, irrespective of cell adhesion.

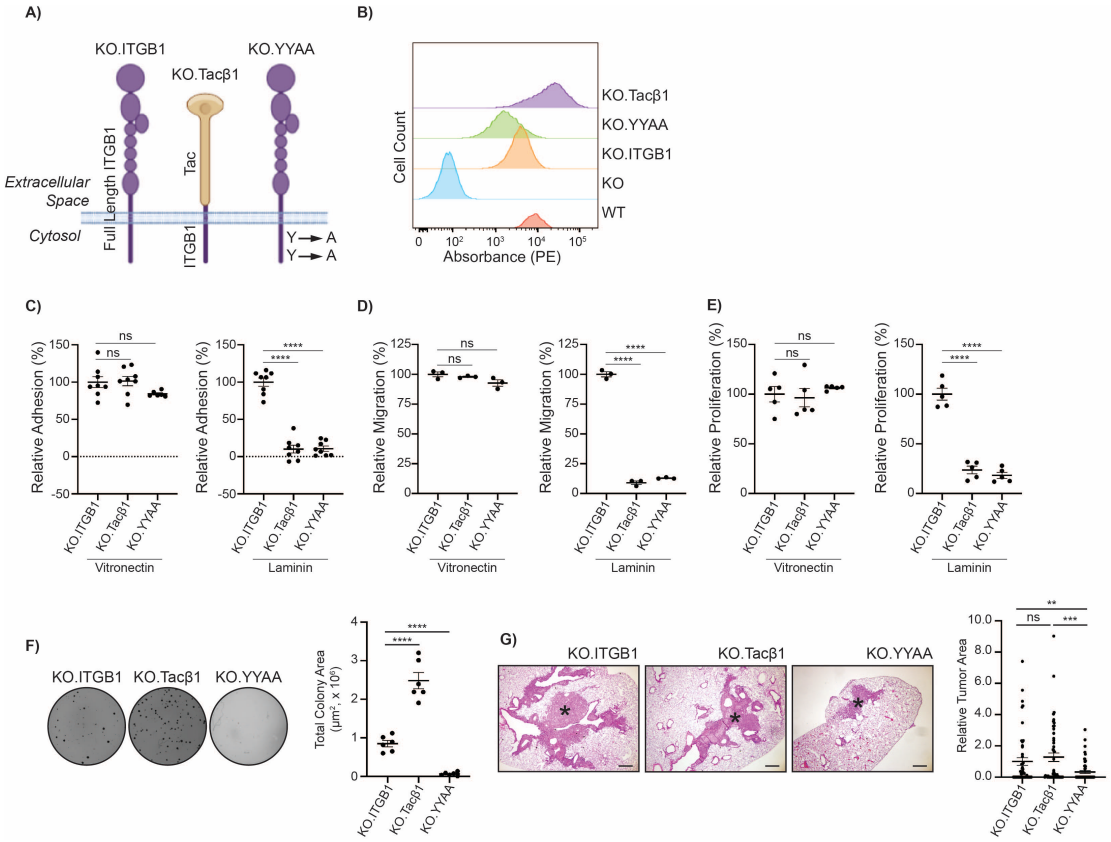

Figure 20: Expression of the integrin b1 cytoplasmic tail in cells lacking endogenous integrin β1 restores colony and tumor formation. A) Full-length integrin β1 (ITGB1), the cytoplasmic domain of integrin β1 fused to the extracellular domain of Tac (Tacb1), and full-length integrin β1 with Y783A and Y795A mutations (YYAA) was re-expressed in integrin β1-KO A549 cells. Panel made with assistance from www.biorender.com. **B)** Surface expression of transfected proteins was measured via flow cytometry by targeting the extracellular domain of integrin β1 (KO.ITGB1, KO.YYAA) or Tac (KO.Tacβ1). Cells were evaluated for **C)** adhesion, **D)** migration, and **E)** proliferation on vitronectin and laminin I (n=3 replicates). **F)** A soft agar colony formation assay was performed using the KO.ITGB1, KO.Tacβ1, and KO.YYAA cells. Representative photomicrographs are shown and data quantified (n=3 replicates, each replicate

consisting of 6 wells, representative data from one replicate shown). **G)** Cells were injected into the left lung of athymic mice (KO.ITGB1 n=10 mice, KO.Tacβ1 n=10 mice, KO.YYAA n=11 mice). Mice were sacrificed and histologic evaluation performed to determine whether cells formed tumors (representative photomicrographs shown, asterisk denotes tumor, scale bar = 20 mm, left lung from each mouse was sectioned every 100 mm X 5 sections). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns = p>0.05 by Sidak's multiple comparison test. Graphs show mean +/- SEM.

The integrin β1 cytoplasmic tail is sufficient for proliferative gene expression signatures and FAK activation. We next assessed whether the integrin β1 tail was sufficient to reconstitute the gene expression profile of cells with full length integrin β1 when plated on the integrin β1-dependent matrix Matrigel. A549 cells with a functional integrin β1 cytoplasmic tail (KO.ITGB1, KO.Tacβ1) demonstrated similar expression patterns for the top 50 differentially expressed genes (**Figure 21A**). These cells demonstrated higher expression of genes from the KEGG_CELL_CYCLE gene set than the cell lines lacking a functional integrin β1 cytoplasmic tail (integrin β1-KO, KO.YYAA) (**Figure 21B**). They also demonstrated higher levels of FAK phosphorylation than the KO.YYAA cells and integrin β1-KO cells (**Figure 21C-D**). Together, these data suggest that a functional integrin β1 cytoplasmic tail is sufficient to restore expression of matrix-dependent cell cycle-related genes and activate FAK in integrin β1-KO lung adenocarcinoma cells lacking integrin-mediated adhesion to ECM.

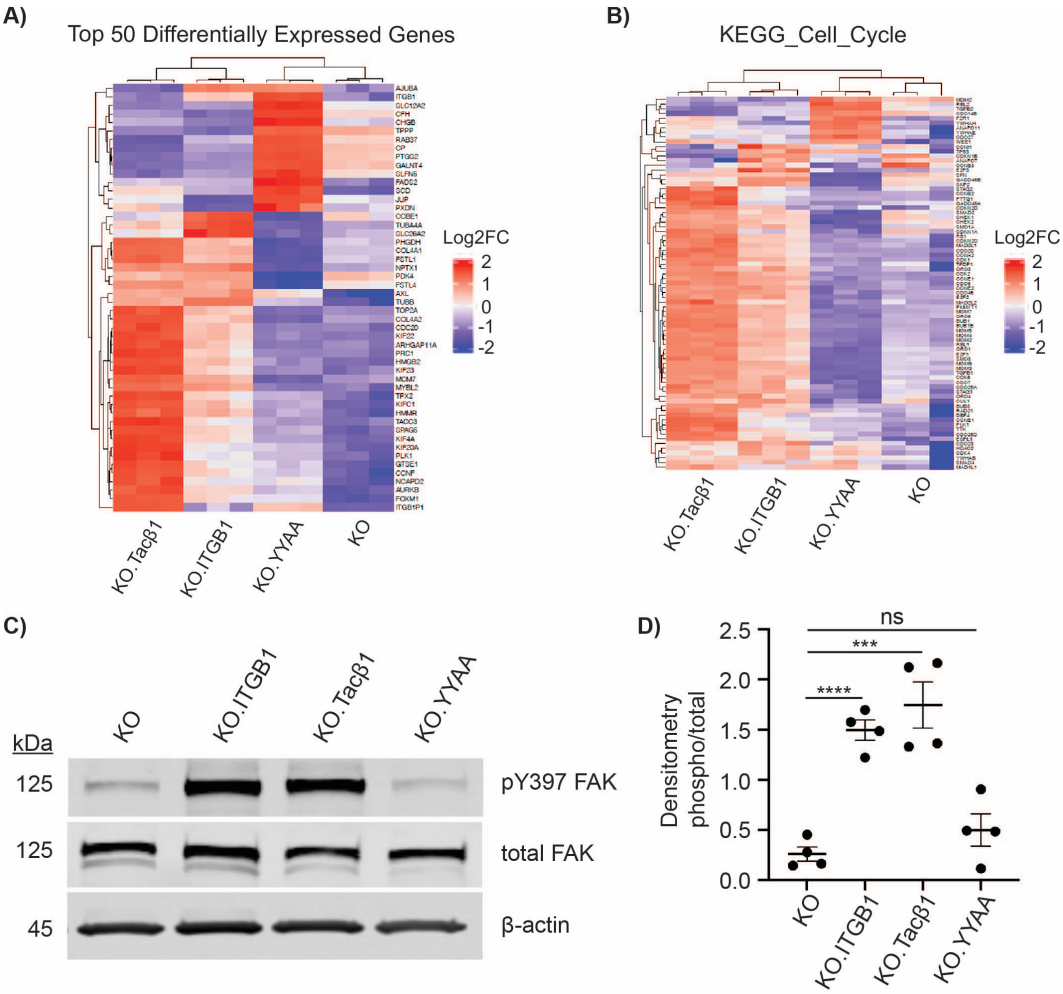

Figure 21: Integrin β1 cytoplasmic tail expression promotes proliferative gene expression signatures and FAK activation. A) Transcriptomic gene expression was evaluated in KO, KO.ITGB1, KO.Tacβ1 and KO.YYAA cells plated on Matrigel and unsupervised clustering performed (n=3 replicates for each cell line). **B)** Expression of genes in the KEGG_CELL_CYCLE gene signature are increased in the KO.ITGB1 and KO.Tacβ1 cell lines relative to the KO and KO.YYAA cell lines. **C-D)** Cell lysates were analyzed by Western blot for levels of total and pY397 FAK and quantified by densitometry (n=4 replicates). The solid

white lines represent lane splicing from the same gel. ***p<0.001; ****p<0.0001; ns = p>0.05 by Sidak's multiple comparison test. Graph shows mean +/- SEM.

Integrin β1 expression in human lung tumors correlates with tumor size, survival, and cancer-associated gene signatures. The data gathered from our mouse and human cell models of lung adenocarcinoma suggest that integrin β1 is important for tumor development. We therefore assessed its relevance to human health by determining whether a similar correlation is observed in human tumors. Immunohistochemistry for integrin β1 was performed on a tissue microarray (TMA) consisting of 65 clinically annotated human lung adenocarcinomas. Tumor and patient characteristics are summarized (**Table 1)**. The stained TMA was reviewed by a pathologist and staining intensity scored on a scale of 0-3 (**Figure 22A**). Integrin β1 was expressed in all molecular subtypes of lung adenocarcinoma, though expression was lower in *EGFR*-mutated tumors (n=17, mean 1.4 +/- standard deviation (SD) 0.7) relative to *KRAS*-mutated tumors (n=40, mean 1.9 +/- SD 0.8, p=0.03) and all other tumors (n=8, mean 2.1 +/- SD 0.5, p=0.04) (data not shown). Like previous studies where integrin β1 expression correlates with recurrence-free survival (RFS) and overall survival (OS) in lung adenocarcinoma (14), our study demonstrated a trend towards improved RFS and OS survival in patients with low (tumors scored as 0-1 staining intensity) integrin β1-expressing tumors (**Figure 23**). In addition, tumors with relatively higher (tumors scored as 2-3 staining intensity) integrin β1 expression were larger than those with lower integrin β1 expression (**Figure 22B**). Thus, integrin β1 expression correlates with large tumors and worse outcomes in patients.

Demographic Characteristics	
N (Total)=	71
AGE at Block Collection (SD)	66.5(8.5)
Gender	
M	22(31%)
F	49(69%)
Race	
White	69(97%)
Black	2(3%)
BMI (SD)	26.9(5.8)
Smoking status	
Current	13(18%)
Former	47(66%)
Never	11(16%)
Age Started (SD)	18.8(6.5)
Age Quit (SD)	50.1(15.4)
Pack years of smoking (SD)	44.7(29.8)
Asbestos Exposure	
Yes	5(7%)
No	66(93%)
Prior Cancer	
Yes	39(55%)
No	32(45%)
Nodule Size (mm) (SD)	25.9(14.6)
Nodule Location	
LUL	16(23%)
LLL	12(17%)
RUL	19(27%)
RML	4(6%)
RLL	20(28%)
PET avidity (suv)	
Positive	49(69%)
Negative	16(23%)
N/A	4(6%)
Not Available	2(3%)
Diagnosis Age, years (SD)	66.5(8.5)
Performance Status at dx (ECOG)	
Grade 0	61(86%)
Grade 1	7(10%)
Not Available	3(4%)
Path Stage	
Stage IA	28(40%)
Stage IB	18(25%)
Stage IIA	7(10%)
Stage IIB	6(8%)
Stage IIIA	11(16%)
Stage IIIB	1(1%)
Primary Treatment	
Surgery	71(100%)
FEV1% (AVG ± STDV)	(86.8 ± 18.8)

Table 1: Tissue microarray characteristics. The lung adenocarcinoma tissue microarray was designed with 71 patients, though no tumor was available for analysis for 6 patients. All patient tissue was procured at Vanderbilt University Medical Center.

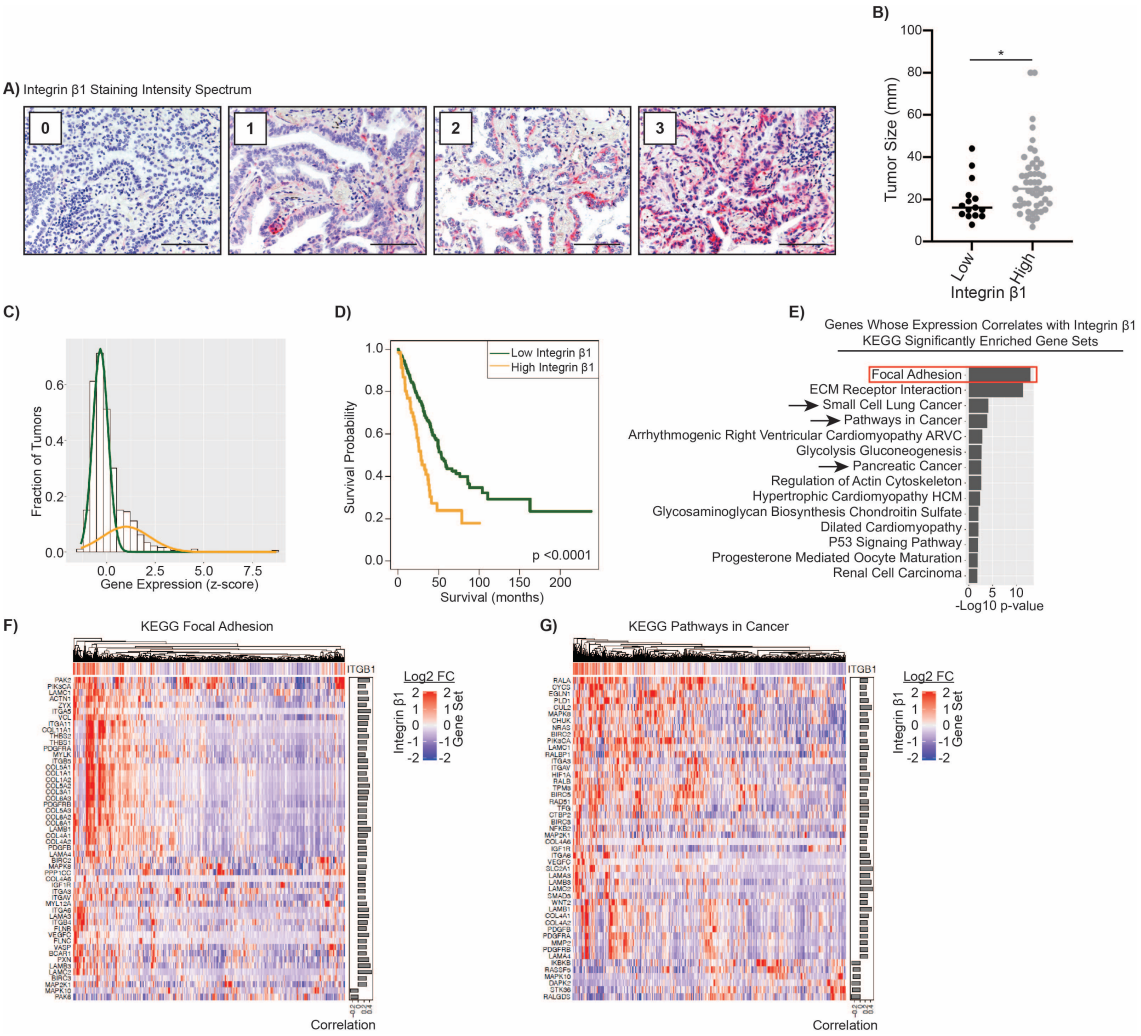

Figure 22: Increased integrin β1 protein and gene expression correlates with increased tumor size, poor survival, and increased expression of cancer-associated gene sets in human lung adenocarcinoma. A) Tissue microarray including 65 human lung adenocarcinomas was stained for integrin β1 (red) and expression quantified by a pathologist (0-3). **B)** The size of tumors with high (score 2-3 staining intensity) versus low integrin β1 protein expression (score 0-1 staining intensity) was compared (mean is graphed for each group). **C)** Gaussian mixture modelling was performed and identified tumor groups with increased (orange) and decreased (green) *ITGB1* (integrin β1) gene expression. **D)** Overall survival was evaluated via Kaplan-

Meier curve analysis in the integrin β1-high and -low groups. **E)** Genes were identified whose expression correlates with integrin β1 expression. Pathway enrichment analysis of integrin β1-correlated genes demonstrates strong correlation with several gene expression signatures, including focal adhesion (**Figure 22E** red box, **Figure 22F**) and cancer-associated pathways (**Figure 22E** black arrows, **Figure 22G**).

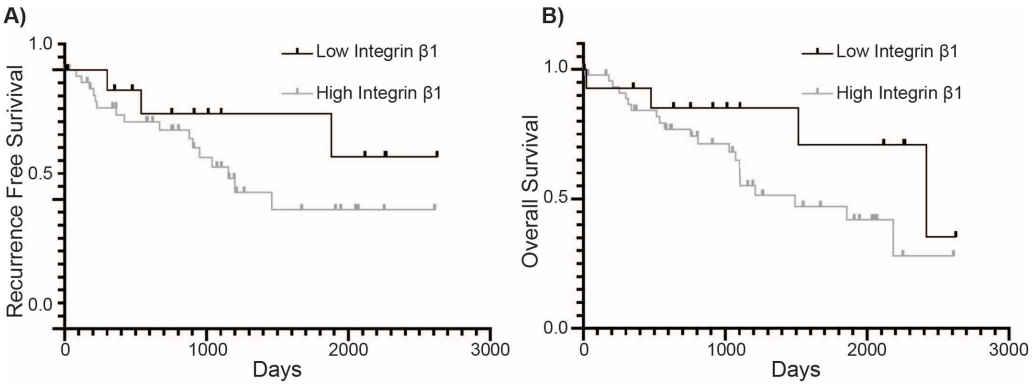

Figure 23: Tumors with high integrin β1 protein expression trend towards inferior recurrence free survival and overall survival. A) Recurrence free and **B)** overall survival was compared in patients with high (score 2-3 staining intensity) versus low integrin β1 protein expression (score 0-1 staining intensity) using a Kaplan-Meier curve.

We next performed Gaussian mixture modelling on the lung adenocarcinoma TCGA RNA-seq data to evaluate gene expression patterns in human tumors (147). Both *ITGB1* (integrin β1)-high and integrin β1-low populations of tumors were identified (**Figure 22C**) and the integrin β1-high tumors exhibit decreased survival (consistent with other similar studies (135)) (**Figure 22D**). Genes whose expression correlated with integrin β1 (Spearman's correlation >0, q-value <0.001) were identified. When we

performed KEGG gene set enrichment analysis, pathways that reflect classical integrin adhesion-dependent biology such as KEGG_FOCAL_ADHESION (**Figure 22E** red box, **Figure 22F)** were enriched with integrin β1-correlated genes. In addition, gene sets associated with aggressive and highly proliferative cancers that were enriched in the integrin β1-expressing cancer cell lines, including KEGG_SMALL_CELL_LUNG_CANCER, KEGG_PATHWAYS_IN_CANCER and KEGG_PANCREATIC_CANCER, were correlated with integrin β1 expression in the human tumors (**Figure 22, Figure 22E** black arrows, **Figure 22G**). Consistent with our findings in mouse and cell line models, these studies suggest that integrin β1 promotes tumor growth in human lung adenocarcinoma.

CHAPTER 4

DISCUSSION

The mechanism whereby integrins promote aggressive tumor biology has classically focused on integrin-ECM binding, which facilitates cell adhesion and proliferative signaling. However, integrin-targeted therapeutics that inhibit integrin-ECM binding have failed to improve clinical outcomes in cancer patients. In the current study, we deleted the integrin β1 subunit (resulting in no expression of integrin β1 heterodimers) in both chemical carcinogen and cell line-based models of lung adenocarcinoma. We demonstrate that integrin β1 signaling is necessary for tumor development in mice. Next, we showed that the integrin β1 cytoplasmic tail is sufficient for integrin β1-dependent FAK activation, gene expression and tumor development. Thus, we conclude that integrin β1 is a signaling hub for lung tumor development and

proliferation that utilizes its cytoplasmic tail by mechanisms that do not require integrin-ECM binding. These data suggest that future strategies to inhibit integrins in cancer should target cytoplasmic tail-dependent signaling.

When we tested the role of integrin β1 during lung tumorigenesis by developing an autochthonous mouse model using the Cre-Lox system and the carcinogen urethane, we found that the few tumors that developed in the integrin β1-KO mice invariably expressed integrin β1. The most likely explanation for this observation is that the AT2 cells with incomplete deletion of integrin β1 exposed to urethane undergo clonal expansion and develop into lung adenocarcinomas. This results in fewer integrin β1 expressing tumors in integrin β1-KO mouse that are a similar size to those that develop in control mice. A similar phenomenon of "breakthrough" carcinogenesis in integrin β1-KO mice has been described in a breast cancer model, resulting in integrin β1-positive tumors developing in integrin β1-KO mice (15). Another explanation for the formation of tumors in integrin β1-KO mice is that the tumors develop from SPC-negative cells, however our data show that the tumor cells express SPC protein making this possibility unlikely. Another possible but even more unlikely explanation for the formation of tumors in integrin β1-KO mice is that integrin β1 is dispensable for tumor formation. In this case we would have expected to observe some integrin β1-null tumors, however every tumor that developed in mice expressed integrin β1. As "Cre-escape" was a limitation of this model, we utilized a xenograft model with complete integrin β1 deletion in tumor cells to demonstrate the requirement of integrin β1 in lung tumor development.

Our findings that integrin β1 is required for tumor formation in both chemical-induced and human cell line lung adenocarcinoma models are consistent with other studies demonstrating that integrin β1 promotes tumor formation. For example, KrasLA2 mice, which carry an oncogenic mutation in *Kras* that spontaneously activates and leads to lung tumor formation, were crossed with integrin α1-null mice resulting in deletion of integrin α1β1 (61). The integrin α1-null mice demonstrated improved survival and the integrin α1-null tumor cells demonstrated decreased cell adhesion, ERK-activation, and tumorigenicity relative to controls due to decreased classical integrin-mediated signaling upon collagen binding. There was also decreased tumor formation in a polyomavirus middle T (PyMT)-driven breast cancer model with inducible knock out (KO) of integrin β1 in breast epithelium (15). Integrin β1 was also necessary for tumor development and growth in a mouse model of pancreatic neuroendocrine tumor (101). The only mouse model suggesting integrin β1 can function as a tumor suppressor is the TRAMP prostate adenocarcinoma mouse model (126), where tumorigenesis is driven by prostate-specific expression of SV40 early T/t antigen genes. Deletion of integrin β1 in the prostate epithelium resulted in an increased percentage of prostate gland involved by tumor and increased tumor cell proliferation, though the mechanisms are not known. Our results with the integrin β1-null A549 and H358 cells were more dramatic than those seen in our chemical-induced lung adenocarcinoma model, likely due to the complete integrin β1 deletion achieved in the cell lines. While our data are consistent with the role of integrin β1 in mediating cancer cell line invasion, migration and metastasis on ECM (3,151,152), the inability of the integrin β1-KO cells to form colonies in soft agar suggests an adhesion-independent mechanism as well.

The integrin β1-KO lung cancer cells exhibited decreased FAK, AKT and ERK phosphorylation when compared to control cells and this difference was most prominent for FAK. Pharmacologic inhibition of FAK also inhibited colony formation of WT cancer cells in soft agar. FAK is a nonreceptor protein tyrosine kinase downstream of integrins that regulates cell signaling and gene transcription, which in turn controls cell adhesion, migration, proliferation and survival (153). FAK regulates gene expression via its kinase-dependent function in focal adhesion complexes localized to the plasma membrane or endosomal complexes (154), and it also translocates to the nucleus where it regulates gene expression independent of its kinase activity (153). FAK is frequently overexpressed in tumors and promotes several important malignant features including cancer stemness, epithelial-to-mesenchymal transition, and resistance to anti-cancer therapies (153). Increased phosphorylated FAK is observed in both non-small cell and small cell lung cancer relative to normal lung (155). In mice with mutant *Kras* and deletion of *Cdkn2a* in lung epithelial cells, lung tumors develop with activation of ERK, RHOA and FAK and subsequent deletion or pharmacologic inhibition of FAK resulted in tumor regression (156). Treatment of A549 cells in this study with FAK inhibitors in combination with either AKT or ERK inhibitors resulted in decreased soft agar colony formation relative to treatment with a single inhibitor, suggesting that FAK provides oncogenic signaling that may be independent of AKT and ERK. There are several candidate pathways that could be contributing to FAK-dependent colony formation via AKT/ERK-independent mechanisms. For example, pharmacologic inhibitors of FAK have been shown to promote its translocation to the nucleus (157), where FAK promotes ubiquitylation and degradation of TP53 and restriction of TP53 tumor suppressive functions (158-160). Thus, our study is consistent with prior work that identifies FAK activation as a key component that

promotes lung adenocarcinoma development. While there may be many scenarios whereby FAK is activated in cancers, our models suggest that the integrin β1 cytoplasmic tail is necessary for FAK activation.

The integrin β1 cytoplasmic tail restored tumor formation, signaling (including FAK phosphorylation) and gene expression patterns to those seen in integrin β1-positive cells. The integrin β1 cytoplasmic tail was previously shown to be sufficient to reconstitute cell functions like paracellular transport in β1 null kidney epithelial proximal tubule cells (10). Other studies with Tacβ1 have been performed in the cells with retained endogenous integrin β1 expression, and their results have been inconsistent. For example, mouse fibroblasts cells engineered to express Tacβ1 exhibit constitutive, adhesion-independent FAK activation (consistent with our results) (161) whereas in CHO cells the Tacβ1 chimeric protein inhibited cell spreading and decreased SRC and FAK phosphorylation due to sequestration of integrin β1 cytoplasmic tail binding proteins (162). In addition, others demonstrated that FAK tethered to the plasma membrane is activated and primed for autophosphorylation (163). However, none of these previous studies demonstrated the ability of the integrin β1 cytoplasmic tail to restore a cell's ability to form tumors independent of its extracellular domain thus making the current study novel.

Adhesion-independent integrin signaling can promote tumor survival and growth. For example, the extracellular domain of integrin α3β1, via its interactions with CD151 but independent of binding to laminin-332, can provide essential survival signals that control skin carcinogenesis (52). In addition, in tumor xenografts, unligated integrin αvβ3 interacts with

galectin-3 at the plasma membrane, resulting in recruitment of KRAS and RalB. This ECM-independent clustering leads to the downstream activation of TBK1 and NF-κB, which regulates tumor initiation and anchorage independent growth (42). In these examples, the extracellular domains of the integrins interact with CD151 or galectin-3, thus promoting integrin signaling independent of binding to extracellular matrix. In contrast, our data suggest a mechanism of integrin β1 cytoplasmic tail signaling that can be propagated independent of the extracellular domain. Work by others has demonstrated that increased integrin expression in suprabasal skin epithelial cells not in contact with the basement membrane (and presumably not in contact with other ECM components) can lead to increased tumor formation in a mouse skin carcinogenesis model (164). However, these tumors arise from basal cells, suggesting that the mechanism is altered communication between the suprabasal and basal skin cells, possibly via a TGFβ-dependent mechanism (164). Thus, this is a distinct mechanism from that proposed in this manuscript.

There remain other important questions regarding integrin signaling in cancer. For example, Macias-Perez et al. previously demonstrated that integrin α1 was required for development of *Kras*-mutated lung adenocarcinoma, and deletion of integrin α1 improved mouse survival. However, in this model, integrin β1 was unperturbed. Thus, how does one reconcile our observation that integrin β1 is sufficient for lung tumorigenesis with their observation that α1-null/β1-positive cells demonstrate markedly decreased tumor development? One possibility is that the Tacβ1 construct is "artificially active" and in more physiological systems the most critical integrin heterodimer in lung

adenocarcinoma development is α1β1. Thus, deletion of either integrin α1 or integrin β1 is sufficient to abrogate tumor development.

We found that cells containing Y-to-A mutations at residues Y783 and Y795 in the cytoplasmic tail of integrin β1 (KO.YYAA) produced no colonies in soft agar, significantly less tumor burden in mice and failed to restore FAK phosphorylation and gene expression patterns seen in the WT cells. This is consistent with studies where these Y-to-A mutations rendered phenotypes that were similar to an integrin β1-null phenotype in constitutive knock in models (165) as well as tissue-specific knock in models targeting the skin (165) and collecting system of the kidney (9). The tyrosines in these motifs are important in facilitating induced-fit protein-protein interactions of multifunctional integrin-binding proteins, suggesting that integrin β1 function is mediated by these integrin binding proteins. Thus, identification of these integrin binding proteins is a key step in understanding the mechanism whereby integrin β1 enables tumor development and growth. Much work has been completed in this area, which is summarized in several high-quality review articles (5,166). Classically, talin proteins bind the membrane proximal NPxY motif of integrin β cytoplasmic tails. Talins are ~270 kDa proteins, components of adhesion plaques and key regulators of integrin affinity for ligand (5). In addition, recent work has demonstrated direct binding between talin and the master cell cycle regulator cyclin-dependent kinase 1 (CDK1) during integrin-mediated adhesion (167). If such interactions were to be demonstrated in cancer cells to be adhesion-independent, it would strongly suggest a partial mechanism whereby integrin β1 can promote tumorigenesis independent of its ectodomain. Meanwhile, kindlin proteins classically bind the membrane distal NxxY motif of integrin β cytoplasmic tails (5). Kindlins are 77 kDa proteins that are essential components of the

integrin adhesion complex and also regulate integrin affinity for ligand. All three kindlins localize to integrin-dependent adhesion sites, though tissue expression differs amongst the three. In addition to talin and kindlin, other proteins bind to these regions of integrin β cytoplasmic tails. Understanding these protein complexes that facilitate integrin β1 cytoplasmic tail-dependent signaling is critical for the rational design of new integrin-targeted therapeutics.

We found that increased integrin β1 expression in human lung adenocarcinoma tumors is significantly associated with increased tumor size, which supports our data that integrin β1 provides signaling that promotes tumor growth. In addition, the lung adenocarcinoma TCGA cohort, where increased integrin β1 expression correlated with cancer associated gene expression pathways, supports the association between integrin β1 expression and aggressive cancer in humans. These observations are consistent with other studies where integrin β1 overexpression was shown to be an independent prognostic factor for lung adenocarcinoma and its expression correlates with an aggressive lung adenocarcinoma phenotype (14,135,168,169).

Classically integrins are thought to be activated by intracellular signaling, after which they bind to a multivalent ECM ligand leading to integrin clustering and focal adhesion formation (170). The focal adhesions are a hub that informs a cell about the physical and biochemical nature of its surroundings, facilitates cell adhesion, and enables numerous well described integrin-dependent cell functions. In addition, integrin-dependent focal adhesion formation is required for maximum activation of growth factor receptors and consequent cell proliferation (171). Integrin β1 mutants that promote tumor formation have been shown to *increase* integrin affinity for ECM components, leading to increased, non-specific ligand binding

that would presumably mimic ligand-dependent integrin signaling (172,173). These classic integrin functions fail to explain why the integrin β1 cytoplasmic tail can restore the malignant phenotype in cells lacking endogenous integrin β1. One possibility is that the integrin β1 cytoplasmic tail is activated oncogenic Ras proteins that facilitate integrin-dependent signaling independent of cell adhesion (170,174). Alternatively, the integrin β1 cytoplasmic tail may play a role in transmitting oncogenic Ras signaling. Thus, deletion of integrin β1 breaks a necessary signaling network resulting in decreased cancer cell proliferation and tumor formation.

In conclusion, our data suggest that just as non-transformed epithelial cells require integrin-mediated adhesion signaling for survival, *KRAS*-mutated lung adenocarcinomas maintain this requirement for cell survival and proliferation. We further show in cancer cells this signaling can be provided independent of cell adhesion or integrin β1:ECM ligation via the integrin β1 cytoplasmic tail, thus facilitating the malignant phenotype independent of integrin:ECM ligation. These findings suggest that anti-integrin cancer therapies need to target the cytoplasmic tail to be successful.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

Integrins play a critical role in the biology of epithelial cells, which require integrin signaling for survival. Carcinoma cells that evolve from epithelial cells do not lose this requirement. They too require integrin signaling for survival and proliferation, as evidenced by

the data presented in Chapter 3 and elsewhere (17,49,170,175). Even in carcinomas where certain integrins have been shown to function as tumor suppressors, that does not mean that those tumors can survive if devoid of all integrin signaling from all subunits. Thus, the reason that there has been limited success in integrin-targeted therapeutics for the treatment of cancer is not because carcinomas have evolved to a state where integrin signaling is superfluous or unnecessary. Rather, cancer cells have likely evolved mechanisms whereby integrin signaling is active in a constitutive, ligand-independent manner. Thus, integrin-targeted therapies that target the extracellular domain and seek to abrogate integrin:ECM ligation is likely to be insufficient for the treatment of cancer when used in isolation.

Mechanisms of Ectodomain-Independent Integrin Signaling

The mechanism whereby integrin β1 can promote tumor development and growth independent of the ECM-binding ectodomain remains poorly understood despite the data presented in Chapter 3. In this section, we consider critical mechanistic details of integrin β1 cytoplasmic tail-dependent signaling in cancer, the extent of our current knowledge, and discuss future experiments that could better resolve these details.

Identifying Critical Integrin β1-Containing Heterodimers

In Chapter 4, we discussed our observation that the integrin β1 cytoplasmic tail is sufficient for lung tumorigenesis in the context of previous observations that α1-null/β1-positive cells demonstrate markedly decreased tumor development. Superficially, these

observations appear to contradict each other and reconciliation requires careful consideration. One possibility is that, among the many integrin β1-containing heterodimers, integrin α1β1 is uniquely critical in facilitating lung adenocarcinoma development. Thus, deletion of either integrin α1 or integrin β1 is sufficient to significantly inhibit tumor development. This hypothesis could be tested in a functional screen. For example, if we were to design a CRISPR screen whereby A549 human lung adenocarcinoma cells were treated with a CRISPR library that includes guide RNAs targeting integrin β1 and all 12 of integrin β1's binding partners (i.e., integrin α1, α2, α3, etc.), based on this hypothesis we would predict that only deletion of integrin α1 and β1 would result in decreased tumor development. While this result is possible, other results are perhaps more likely. For example, what if deletion of integrin subunits α1 and β1 resulted in decreased tumor development but so too did deletion the integrin subunits α2 and α3? These data would suggest that integrin "dose" is important and if net integrin expression and/or signaling falls below a certain threshold, tumor development cannot proceed. In this scenario, deletion of integrin α1 and/or β1 results in loss of tumor formation not because of a unique status of integrin α1β1 but rather because these subunits are expressed at sufficient levels that deletion crosses a critical threshold whereby tumor development cannot proceed.

Integrin Binding Proteins in Ectodomain-Independent Integrin Signaling

The tyrosines in the NxxY and NPxY motifs of the integrin β1 cytoplasmic tail are required for lung adenocarcinoma development, suggesting that integrin β1 function is mediated by the integrin binding proteins that bind these sites. Most of these proteins contain phosphotyrosine binding domains, suggesting they directly bind at the NxxY and NPxY motifs

(**Figure 24**). To narrow the list of candidates of key integrin binding proteins mediating the tumor promoting effects of integrin β1, the integrin β1-KO lung adenocarcinoma cells should be forced to express an integrin β1 protein with a single tyrosine-to-alanine mutation in NPxY or NxxY motif. If only one of the two sites is required for tumorigenesis, we would learn important information regarding which amino acid residues in the cytoplasmic tail and protein binding partners are required to mediate the tumor promoting effect of integrin β1. If both single mutants fail to form tumors, then one can conclude that integrin binding proteins interacting at both the NPxY and the NxxY sites are required for tumorigenesis. Even in this scenario, we could move forward with a CRISPR-based screen targeting genes corresponding to the ~30 potential integrin binding proteins, a reasonable size for such an approach (**Table 2**). The basic workflow includes cloning the guide (g)RNAs targeting the integrin binding proteins into a lentiviral vector (four gRNA/gene), infecting integrin β1-KO cells, placing the cells into either soft agar or a mouse, allowing colony and/or tumor formation, and then sequencing the colonies/tumors to determine which clones emerged (**Figure 25**). Appropriate statistical analysis is then employed to determine whether a particular clone is over- or under-represented. One would expect that genes corresponding to key integrin binding proteins responsible for transmitting pro-tumorigenic integrin β1 signaling to be underrepresented in tumors developing from cells treated with such a screen. Such data could thus identify novel mechanistic details regarding integrin β1 signaling in *KRAS*-mutated lung adenocarcinoma.

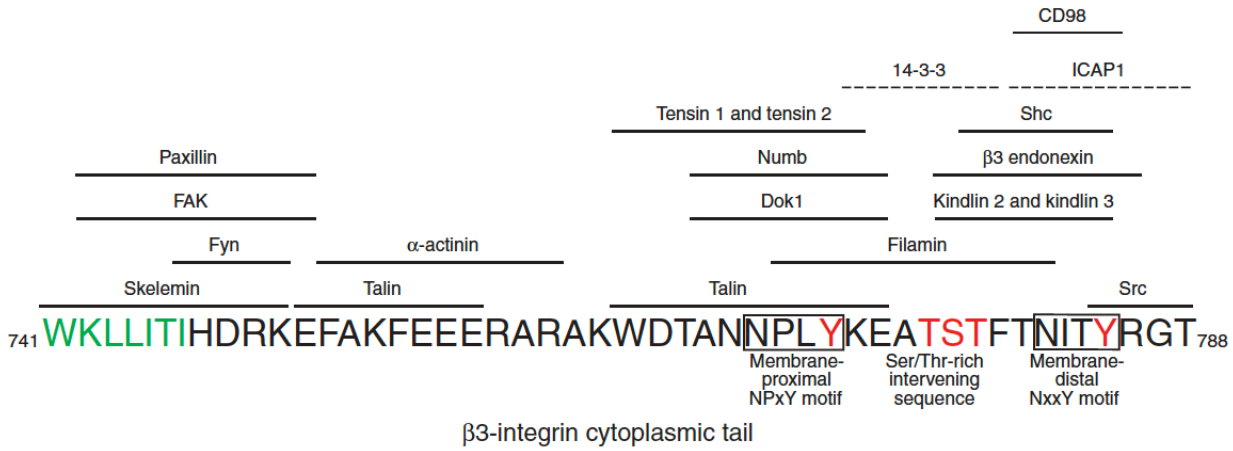

Figure 24: Adaptor-binding sites along the β3-integrin tail. The mapped positions of adaptors that have been shown to bind to the β3-integrin cytoplasmic tail are indicated by solid lines. Positions of adaptors that have binding sites on other integrins, but that have not been shown to bind to β3 integrin, are indicated by broken lines. Residues that are normally buried in the membrane are colored green. Residues that can be phosphorylated by various kinases are highlighted in red. The integrin β3 cytoplasmic tail is shown as a paradigm but results can be generalized to include integrin β1. The figure and legend were reproduced and adapted with permission (166); permission conveyed through the Copyright Clearance Center.

Protein Name	Gene Names						
CD98**	SLC3A2	SLC7A5					
14-3-3	YWHAB	YWHAE	YWHAG	YWHAH	YWHAQ	YWHAZ	YWHAS
ICAP1	ITGB1BP1						
Tensin1	TNS1						
Tensin2	TNS2						
Other Tensins*	TNS3	TNS4					
Shc	SHCA	SHCB	SHCC	SHCD			
Numb	NUMB						
B3 endonexin^	ITGB3BP						
Dok1	DOK1						
Kindlin1*	FERMT1						
Kindlin2	FERMT2						
Kindlin3	FERMT3						
Filamin	FLNA	FLNB	FLNC				
Talin	TLN1	TLN2					
Src	CSK						

Table 2: Proposed CRISPR screen targeting integrin binding proteins that can bind the cytoplasmic tail of integrin β1. List based on review of literature, including work by Legate and Fassler (166). **CD98 is a heterodimer composed of the two genes listed. Other tensins* and kindlins* are included in the screen even though they are less well characterized as integrin β1 binding partners. ^β3 endonexin is not known to have a phosphotyrosine binding domain and thus may bind the cytoplasmic tail via phosphotyrosine-independent mechanisms.

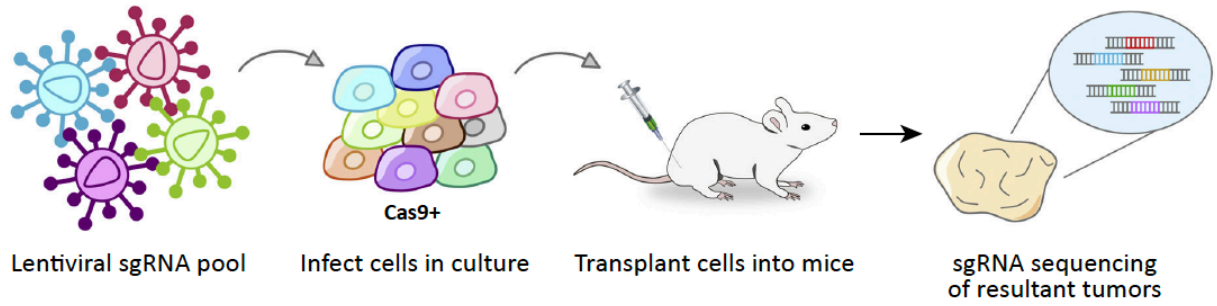

Figure 25: CRISPR screen workflow. To perform an *in vitro* CRISPR screen, the desired single-guide RNA (sgRNA) library must first be cloned into expression vectors. Lentiviral vectors are commonly used, as they can stably integrate into the host genome. After a selection

phase to enrich for a desired phenotype, the sgRNA cassettes are amplified from genomic DNA and sequenced to identify the top candidate genes. Rather than mice, *in vitro* workflows can be utilized as well utilizing soft agar. The figure and legend were reproduced and adapted with permission (176); permission conveyed through the Copyright Clearance Center.

Cellular Localization of Integrin Cytoplasmic Tail Signaling

As discussed previously, the necessary cellular localization of integrin β1 cytoplasmic tail signaling remains undefined. Does the cytoplasmic tail of integrin β1 need to be localized to the plasma membrane, perhaps enabling interaction with the oncogenic drivers that are also tethered to the plasma membrane such as activated Ras proteins? One could test this hypothesis by expressing a free cytosolic integrin β1 tail. If expression of free cytosolic integrin β1 tail failed to restore tumor formation in integrin β1-KO cells, then this experiment would suggest that this signaling must occur at the plasma membrane. Such experiments may provide clues regarding the nature of integrin β1 signaling in *KRAS*-mutated lung adenocarcinoma.

Integrin Cytoplasmic Tail Signaling Outside the Context of KRAS-Mutated Tumors

In the current study, we made a deliberate decision to limit the models to *KRAS*-mutated lung adenocarcinoma models. The exclusive use of *KRAS*-mutated lung adenocarcinoma models was an attempt to maintain consistency across all experiments rather than an assumption that integrin β1 reliance is unique to *KRAS*-mutated tumors. The more likely scenario is that a reliance on integrin β1 signaling in lung adenocarcinoma is shared amongst several molecular

subtypes. For example, in normal epithelial cells, integrin β1 is sufficient to partially activate EGFR and is required for full activation of EGFR in response to EGF (177-179). Furthermore, in lung cancer cells integrin β1 regulates EGFR signaling and tumorigenic properties (16). Even in *EGFR*-mutated lung adenocarcinoma cells, integrin β1 is required for propagation of oncogenic signaling, including PI3K/AKT pathway activation (180). Thus, it seems likely that integrin β1 is required for tumorigenesis of multiple oncogene-driven lung adenocarcinomas, specifically *KRAS*-mutated and *EGFR*-mutated subtypes. A more exhaustive approach is possible, where one catalogs the net effect of integrin β1 expression on tumor formation in the context of several other lung adenocarcinoma-relevant oncogenes though this experiment may be a relatively lower priority.

Can Cancer Cells Lose their Reliance on Integrin β1?

Thus far, integrin β1 has been shown to be necessary for lung adenocarcinoma development and growth. However, is this integrin β1 requirement absolute or can it be overcome as tumors continue to evolve? Indeed, years of treating cancer patients with targeted therapies has demonstrated that eventual resistance is extremely common (181). Understanding how cancer cells can adapt to circumvent integrin β1 deletion may be beneficial for several reasons. First, such knowledge may provide clues regarding the mechanism whereby integrin β1 deletion inhibits tumor formation. Second, it may educate investigators regarding patient selection for integrin β1-targeted therapies. For example, if *TP53* mutations correlate with primary resistance to integrin β1 deletion, investigators may choose to avoid this population in clinical trials utilizing integrin β1-targeted therapies. Finally, such knowledge may allow

clinicians to predict mechanisms of acquired resistance in patients treated with integrin β1-targeted therapies. To explore this biology, we have proposed a CRISPR-based screen (**Table 3**). A CRISPR library has bene developed containing 4 guide gRNAs targeting each of 45 tumor suppressors common among human tumors, including *CDKN2A, TP53, PTEN* and others. Controls include 10 non-targeted gRNA.

		Renal Cell Carcinoma				Lung Adenocarcinoma		
Gene	**Function**	**Renca^^**	**A498**	**A704**	**786-O**	**A549**	**H358**	**LLC^^**
FLCN^	Birt-Hogg-Dube							
FAT1	Adhesion			x			x	
FAT3	Adhesion						x	
FAT4**	Adhesion	x			x	x	x	x
CDKN2A	Cell cycle							
CDKN2B	Cell cycle			x				
RB1	Cell cycle							
ARID1A	Chromatin							
ARID1B	Chromatin			x				
BAP1	Chromatin							
KDM5C	Chromatin							
KMT2A	Chromatin	x	x		x			
KMT2C/MLL3	Chromatin		x	x	x	x	x	
KMT2D	Chromatin				x			
MEN1	Chromatin							
PBRM1	Chromatin			x				
SETD2	Chromatin		x	x				
SMARCA4	Chromatin		x			x		x
SMARCB1^	Chromatin							
ATM	DNA damage	X			x			
BRCA1*	DNA damage	X						
BRCA2	DNA damage							
FANCF	DNA damage							
TP53	DNA damage	X		x	x			
LATS1*	Hippo							
LATS2	Hippo		x					
NF2	Hippo							
VHL	Hypoxia		x	x	x			
FH^	Krebs					x		
SDHA	Krebs							
SDHB	Krebs							
SDHC	Krebs							
SDHD	Krebs							
IDH1	Krebs							
IDH2	Krebs							
PTEN	PI3K				x			
TSC1	PI3K							
TSC2*	PI3K				x		x	
NF1	Neurofibromatosis							
NOTCH1	NOTCH							
NFE2L2	NRF2							
TERT	Telomeres							
SMAD4	TGFbeta							
APC	WNT		x			x		
CTNNB1	WNT						x	

Table 3: Proposed CRISPR screen targeting known tumor suppressors. Shown are tumor suppressors whose function is commonly lost in human cancers. Also shown are several human and mouse^^ cell lines to which we have access to in the lab (many of which have already had

integrin β1 deleted). "X" signifies that the parent cell line has a known, preexisting mutation in that gene described in the literature. ^Some genes included because they are well-described in smaller subtypes of renal cell carcinoma. *Others are included for purposes of library redundancy despite the fact they are less frequently found to be mutated in human tumors. This library has already been prepared and includes four guide RNAs per gene as well as 10 non-targeting guide RNA included as a control.

Integrins as Tumor Promoters versus Tumor Suppressors

While we have speculated that most carcinomas require some integrin signaling, there is rich literature suggesting some integrins in some contexts function as tumor suppressors (17). If investigators inhibit an integrin functioning as a tumor suppressor, the outcomes could be highly detrimental for the patient. Understanding the biology that determines how an individual integrin subunit and/or heterodimer affects tumor behavior is critical so that investigators can predict the impact of targeted integrin inhibition. In addition, a deeper biological understanding of these factors influencing integrin behavior in tumors may reveal new approaches to targeting integrin biology in cancer. For example, if certain integrin binding proteins preferentially bind the cytoplasmic tail of integrins functioning as tumor promoters, clearly these integrin binding proteins would be high priority targets during drug development. Conversely, if other integrin binding proteins preferentially bind the cytoplasmic tail of integrins functioning as tumor suppressors, then inhibition of these proteins should be avoided. Thus, understanding the biological cues governing how integrins impact tumor behavior is critical.

In terms of mouse models, most studies investigating the role of integrin β1 in cancer have been performed in the context of powerful, activated oncogenes. For example, deletion of integrin α1 (resulting in abrogation of integrin α1β1) (61) or deletion of all β1 integrins (Chapter 3) resulted in decreased tumor formation within the context of an activated *Kras* allele. Similarly, there was decreased tumor formation in a polyomavirus middle T (PyMT)-driven breast cancer model with inducible knock out (KO) of integrin β1 in breast epithelium (15). This model is driven by expression of the PyMT antigen that activates the nonreceptor kinase c-Src (182) and closely mimics the oncogenic signaling of receptor tyrosine kinases which are commonly activated in many human malignancies (183). Interestingly, the only mouse model suggesting integrin β1 can function as a tumor suppressor is the TRAMP prostate adenocarcinoma mouse model (126), where tumorigenesis is driven by prostate-specific expression of SV40 early T/t antigen genes which results in inhibition of the tumor suppressors TP53 and Rb. Thus, in the context of a tumor suppressor-driven cancer lacking a dominant oncogene, deletion of integrin β1 produces a different effect on tumor growth than in tumors driven by a dominant oncogene (i.e., *KRAS*-mutated lung adenocarcinoma). Some of the effects of integrin β1 on tumor development is likely tissue dependent. Thus, to advance this line of investigation further, investigators likely need to examine the effect of integrin β1 expression (and/or other integrins) in the same tissue/cell type in the context of various oncogenes and tumor suppressors to clarify whether distinct drivers of cancer influence the net effect of integrin expression on the cancer phenotype.

Kidney cancer may offer an ideal context in which to further explore integrin β1. Clear cell renal cell carcinoma is a tumor suppressor-driven cancer, typically lacking activation of

classic oncogenes such as the Ras proteins. Interestingly, deletion of integrin β1 in two human clear cell renal cell carcinoma cell lines, A498 and 786-O, resulted in *increased* colony formation relative to integrin β1-wild type cells (**Figure 26**). This is a similar result to that observed in the tumor suppressor-driven adenocarcinoma of the prostate mouse model referenced previously (126). While these early results require validation, it could offer a valuable comparator to our lung adenocarcinoma cell lines, where deletion of integrin β1 results in an opposite phenotype. Should these results be validated, we could proceed with expression of full length integrin β1, Tacβ1 or YYAA constructs in the human kidney cancer integrin β1-KO cells to determine whether the pro-tumorigenic effect of integrin β1 deletion is mediated by the cytoplasmic tail or ectodomain (with follow-up studies mirroring our approach from the lung cancer studies). Other studies could facilitate a more direct comparison of the lung cancer and kidney cancer cell lines. For example, if a functional integrin β1 cytoplasmic tail restores the tumor suppressive effects of integrin β1 in kidney cancer cells, this would suggest that the integrin β1 cytoplasmic tail is mediating opposite effects in lung cancer and kidney cancer cells. A comparison of cytoplasmic tail-binding integrin binding proteins in the lung cancer and kidney cancer cells could then be pursued using a proteomics-based approach to test the hypothesis that distinct integrin binding proteins mediate integrin β1 cytoplasmic tail signaling and tumorigenesis. Another hypothesis is that deletion of integrin β1 in lung cancer and kidney cancer results in opposite cancer phenotypes because of the distinct global integrin repertoire of each cell. Thus, when integrin β1 is deleted from the two cell types, the remaining integrin repertoire is distinct and results in opposite phenotypes. This hypothesis could be readily tested using a flow cytometry-based approach to catalog integrins expressed in both cell lines. Thus,

these cell lines may provide useful comparators in evaluating the effect of integrin β1 expression on tumor biology.

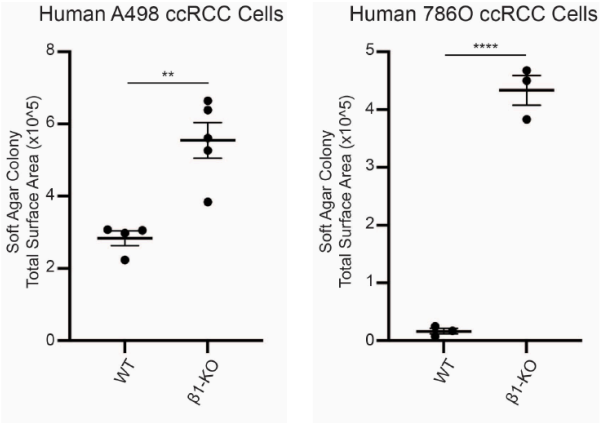

Figure 26: Integrin β1 deletion in human clear cell renal cell carcinoma cells results in decreased soft agar colony formation. Integrin β1 was deleted in two human clear cell renal cell carcinoma cell lines: A498 (left) and 786-O (right). In both experiments, the integrin β1-KO cells formed more colonies in soft agar. These experiments have only been performed once with additional replicates underway. **p<0.01; ****p<0.0001 by unpaired, two-tailed *t* test.

Another system may offer other opportunities to explore the biological factors influencing whether an integrin functions as an oncogene or tumor suppressor. Collaborators have developed a tamoxifen-inducible *Lats1/2*-KO mouse with targeted deletion in renal epithelial tissues (184). Deletion of *Lats1/2* results in activation of Hippo signaling including translocation of the transcription factors Yap and Taz into the nucleus, driving development of tumors with sarcomatoid appearance that spontaneously metastasize to liver and lung, resulting in premature mouse death. The Zent lab has created immortalized cell lines from this renal

tissue, resulting in isogenic cell lines with and without expression of *Lats1/2*. We have then used CRISPR to delete integrin β1 in both the WT and Lats1/2-KO cell lines. Interestingly, in the non-transformed WT cells, deletion of integrin β1 results in increased colony formation, suggesting increased proliferation upon deletion of integrin β1. However, in the transformed Lats1/2-KO cells, deletion of integrin β1 results in fewer colonies when cells are grown in soft agar. Thus, the WT non-transformed cells and the transformed Lats1/2-KO cells exhibit opposite soft agar phenotypes in response to deletion of integrin β1 (**Figure 27**). This dichotomy offers a unique opportunity to explore the biology regarding whether an integrin subunit functions as a tumor promoter or tumor suppressor. Why would deletion of Lats1/2 cause such a marked change in the function of integrin β1? Perhaps activation of the Hippo pathway and resultant oncogenic transformation changes the integrin repertoire of the cell, and this changes the cellular response to integrin β1 deletion? Or perhaps deletion of Lats1/2 alters downstream integrin signaling in such a way that cellular response to integrin β1 deletion is profoundly changed. We have developed the tools and assays to explore this biology thoroughly, including flow cytometry panels to measure surface expression of various integrins, mutated and chimeric integrin β1 constructs to dissect out the role of the ectodomain versus cytoplasmic tail, proteomic workflows to catalog integrin binding proteins in both contexts, and finally RNAseq workflows to assess gene expression in these cell lines. Finally, a unique advantage of this system is the availability of the corresponding mouse model that provides an *in vivo* system in which to further evaluate mechanistic details within a physiologic context.

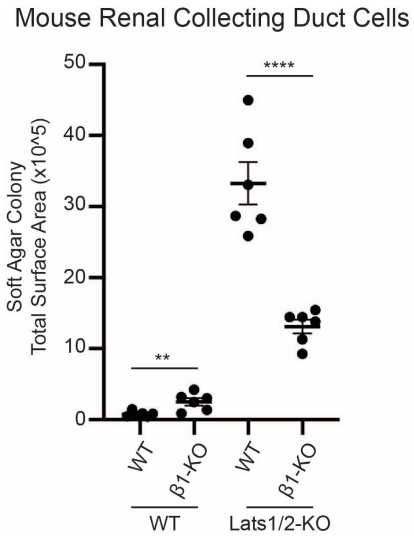

Figure 27: Integrin β1 deletion in mouse renal sarcomatoid carcinoma cells results in decreased soft agar colony formation. Integrin β1 was deleted in WT and *Lats1/2*-KO cells (184). Deletion of integrin β1 in the WT cells results in increased soft agar colony formation whereas deletion of integrin β1 in the transformed, *Lats1/2*-KO cells results in decreased soft agar colony formation. These experiments have only been performed once with additional replicates underway. **p<0.01; ****p<0.0001 by unpaired, two-tailed *t* test.

New Strategies to Target Integrin Signaling in Cancer

Given the nature of constitutively activated integrins in cancer, and integrin signaling that is independent of the ECM-binding extracellular domain, new strategies are needed to target integrin signaling in cancer. The previous strategies, which focused on abrogating ECM:integrin ligation is insufficient in this context. Thus, anti-integrin antibodies that block ECM from binding to the integrin extracellular domain, or ECM-mimetics that bind the ECM-binding site of

integrins, are likely to be insufficient to stop cancer growth if integrin cytoplasmic tail signaling continues unabated. One strategy that has targeted integrin cytoplasmic tail signaling is the use of small molecules that inhibit kinases downstream of integrin activation, namely FAK and SRC tyrosine kinase inhibitors. However, these drugs have been tested extensively in cancer and, while some strategies remain promising, their success in large clinical trials has thus far been modest (185,186). Indeed, these FAK- and SRC-containing signaling pathways are complex and include many inputs aside from integrins, and integrins likewise are likely capable of signaling independent of these kinases. Thus, new strategies are likely needed to target integrin cytoplasmic tail signaling more precisely. These newer strategies will require us to refine our understanding of integrin cytoplasmic tail-binding proteins and require us to define which ones are necessary for ligand-independent integrin signaling in cancer. Such knowledge could nominate new therapeutic targets, and technology is emerging that could facilitate such an approach. For example, emerging targeted protein degradation technologies such as proteolysis-targeted chimeras (PROTACs) may offer new opportunities to precisely target these cytoplasmic proteins and/or integrins (187). It should be noted that any attempt to delete or inhibit integrin function would likely require sophisticated techniques to target such inhibition to cancer cells as integrin function is essential in most cells and tissues.

Summary & Conclusions

The work described in this thesis has made a valuable contribution to the integrin and cancer biology literature. However, it has raised more questions than it has answered. The experiments proposed in Chapter 5 are numerous and expansive. We will continue to work to

validate preliminary data and strategize with the full committee to prioritize which experiments are most essential for eventual R01 level funding applications within this field.

CHAPTER 6

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