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Mechanisms and Molecular Biology of Major Tumor Suppressors

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by

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DEDICATION:

I would like to dedicate this dissertation to my family and friends. I wouldn't have made it through this process without you. Thank you for keeping me focused on my goals and for reminding me how proud you are even when I had trouble seeing my own progress.

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ABSTRACT:

This dissertation is devoted to the study of the molecular biology of major tumor suppressors, defined as those that prevent the cellular processes identified as the hallmarks of cancer. Specifically, the major tumor suppressors pRb and STK11 are explored in the context of osteosarcoma and lung cancer, respectively.

RB1 was the first tumor suppressor gene discovered. Over four decades of work have revealed that the Rb protein (pRb) is a master regulator of biological pathways influencing virtually every aspect of intrinsic cell fate including cell growth, cell-cycle checkpoints, differentiation, senescence, self-renewal, replication, genomic stability and apoptosis. While these many processes may account for a significant portion of *RB1*'s potency as a tumor suppressor, a small, but growing stream of evidence suggests that *RB1* also significantly influences how a cell interacts with its environment, including cell-to-cell and cell-to-extracellular matrix interactions. Chapter 2 highlights pRb's role in the control of cell adhesion and how alterations in the adhesive properties of tumor cells may drive the deadly process of metastasis.

Chapter 3 defines a role for pRb as a suppressor of the progression to metastasis by upregulating integrin α10. Transcription of this integrin subunit is herein found to be pRb-dependent in mouse osteoblasts. Classic pRb partners in cell cycle control, E2F1 and E2F3, do not repress transcription of integrin α10 and phosphorylation of pRb is not necessary for

activation of the integrin α10 promoter. Promoter deletion revealed a pRb responsive region between -108bp to -55bp upstream of the start of the site of transcription. pRb activation of transcription also leads to increased levels of integrin α10 protein and a greater concentration of the integrin α10 protein at the cell membrane of mouse osteoblasts. These higher levels of integrin α10 correspond to increased binding to collagen substrate. Consistent with our findings in mouse osteoblasts, we found that integrin α10 is significantly underexpressed in multiple solid tumors that have frequent inactivation of the pRb pathway. Bioinformatically, we identified data consistent with an 'integrin switch' that occurs in multiple solid tumors consisting of underexpression of integrins α7, α8, and α10 with concurrent overexpression of integrin β4. pRb promotes cell adhesion by inducing expression of integrins necessary for cell adhesion to a substrate. We propose that pRb loss in solid tumors exacerbates aggressiveness by debilitating cellular adhesion, which in turn facilitates tumor cell detachment and metastasis.

Lung cancer is the leading cause of cancer-related death in the U.S. and additional targeted therapies are desperately needed to treat these patients. *STK11* is the third most frequently mutated gene in lung adenocarcinoma following only *KRAS* and *TP53*, yet its mutational status is not currently clinically evaluated and no therapies have been approved to specifically target its pathway. A deep understanding of the complex pathways controlled by STK11 and their alterations in cancer are required to develop effective therapies for patients with loss-of-function mutations. In Chapter 4 we present the current understanding of STK11, focusing on its molecular biology and therapeutic implications, including a compilation of studies evaluating *STK11* somatic mutations in human lung cancer tissue and how the frequency of these mutations varies across histological subtypes and patient populations. Finally, we review the strategies being used to target STK11-deficient cancers at the clinical trial, pre-

clinical, and basic science levels as well as proposing potential new therapies that might benefit this patient population.

STK11 is a tumor-suppressor commonly mutated in lung adenocarcinoma (LuAd). There are a number of agents that may selectively target the deregulated pathways in *STK11* mutated tumors, and thus, identifying the subset of adenocarcinomas that harbor these mutations could have significant clinical benefit. In Chapter 5, we characterized a cohort of 442 adenocarcinoma patients with respect to *STK11* mutation status and subset of this cohort using immunochemistry, gene expression, and western blotting. We found that measuring *STK11* mutation status is complicated by the fact that many *STK11* mutations lead to expression of a stable protein that is indistinguishable from wild type (WT) via immunohistochemistry. To circumvent this, we used published cell line mutation and gene expression data to derive a signature correlating with *STK11* mutation status. This signature was validated in the cohort of 442 lung adenocarcinomas and strongly correlates with mutation status (ROC curve AUC = 85.29). These data suggest that *STK11* mutation status may be best assessed by measuring the downstream targets included in our signature.

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CHAPTER ONE:

Introduction to Major Tumor Suppressors

MAJOR TUMOR SUPPRESSORS AND THE HALLMARKS OF CANCER

Cancer is caused by catastrophic failure of numerous cellular processes. The progression from normal cell to cancer cell is a multi-step process involving functional alterations in a series of key regulatory proteins which can occur due to genetic mutations, alterations in gene expression or due to the activity of infectious agents (e.g., human papilloma virus). Genes that encode proteins that activate cell proliferation are often up-regulated in cancer via over-expression or by mutations that cause the resulting protein to be constitutively active. These cancer promoting genes are called oncogenes. Their negative regulatory counterparts, genes that encode proteins that prevent cancer from forming, are called tumor suppressors. Cancers grow unchecked once these tumor suppressors are lost by mutation, hyper-phosphorylation, or promoter methylation.

There have been more than 70 tumor suppressors identified to date[1] of varying importance. Some are lost late in tumorigenesis and are considered to be 'passenger mutations,' those that confer little or no survival advantage to an existing cancer. Other tumor suppressors are lost at the incipient stages of tumor neogenesis, this loss being crucial for the evolution from normal cell to cancer cell. These 'driver mutations' occur in tumor suppressors that act as

sentinels, each protecting the integrity of cellular processes crucial to maintaining a normally functioning cell. These are the major tumor suppressors.

The processes that must occur in order for cancer to develop have been defined as a set of eight 'hallmarks of cancer.' These processes include: sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enable replicative immortality, inducing angiogenesis, resisting cell death, and the two newest hallmarks: reprogramming energy metabolism, and evading immune destruction.[2] Each of these processes is protected by one or more major tumor suppressors as outlined below.

'Sustaining proliferative signaling' is quintessentially what makes cancer such a deadly disease. All of the other hallmarks support this unrestricted cellular proliferation in some way. While this is a hallmark generally driven by oncogenes, a tumor suppressor that acts as a guardian against uncontrolled growth is the phosphatase and tensin homolog (PTEN). PTEN acts as a negative regulator of the AKT pathway serving to dephosphorylate PIP_3 into PIP_2, thereby repressing AKT, signaling to the cell to stop dividing. Befitting of its status as a major tumor suppressor, PTEN is also involved in regulation of apoptosis, migration, adhesion, and genetic stability. It is commonly lost in many human cancers including 30-70% of prostate cancer.[3,4]

'Evading growth suppressors' is driven by the loss of anti-proliferative signals. This is a process guarded by the first tumor suppressor ever identified, pRb. pRb serves as a regulator of the G_1/S transition of cell cycle acting as a transcriptional repressor by binding E2F family members at the promoter site of E2F-regulated genes. Mitogenic signaling activates the formation of Cyclin D / CDK4/6 complexes which phosphorylate, and thus inactivate, pRb

allowing for cell cycle progression. pRb has also been linked to numerous other cellular processes and is lost or inactivated in the majority of human cancers.

'Activating invasion and metastasis' is the multi-step process by which epithelial cells acquire the ability to invade adjacent tissues and eventually disseminate to distal sites setting up distant metastases. This process begins with a series of steps known as the epithelial-to-mesenchymal transition (EMT), controlled by the next major tumor suppressor, transforming growth factor β (TGFβ). TGFβ acts as a tumor suppressor in the early stages of tumorigenesis, serving to maintain a favorable cytokine and chemokine profile in the tumor microenvironment.[5-7] However, as the disease progresses, oncogenes can serve to convert TGFβ function to induce EMT leading to increased invasion and migration. As such, TGFβ acts as both a major tumor suppressor, and at advanced stages of disease, an oncogene.

'Enabling replicative immortality' occurs when cells are no longer limited to a finite number of cell division cycles. In healthy cells, the progressive shortening of telomere repeat sequences with each subsequent cell division causes cells to lose the ability to protect the ends of the chromosomal DNA from forming end-to-end fusions. The sentinel of genomic integrity is p53, which serves to assess DNA damage and activate DNA repair pathways as necessary, or if the damage is beyond repair, induce apoptosis. *TP53*, which encodes for the p53 protein, is the most frequently mutated gene in human tumors with over 25,000 mutations reported to date[I].

'Inducing angiogenesis' is the process by which tumors stimulate the growth of new blood vessel vasculature in order to meet the increased metabolic demands of tumor tissues. The major tumor suppressor that prevents this from happening is thrombospondin-1 (TSP-1). This inhibitor of angiogenesis serves to limit vessel density by inhibiting chemotaxis toward pro-angiogenic signals and inducing receptor-mediated apoptosis in activated endothelial cells.[8] It does this by

[I] International Agency for Research on Cancer TP53 Database; p53.iarc.fr

binding transmembrane receptors displayed by endothelial cells and evoking suppressive signals that counteract the proangiogenic stimuli.[9]

'Resisting cell death' allows cancer cells to evade all of the aforementioned processes whereby tumor suppressors activate apoptosis. Bax, Bak, and Bok are some of the pro-apoptotic proteins responsible for triggering cell death through disrupting the mitochondrial membrane resulting in release of cytochrome C. Apoptosis is a process activated and controlled by many of the major tumor suppressors. Bax and Bak are transcriptional targets of p53, and Bok is controlled by E2F, a member of the pRb pathway, in addition to acting as tumor suppressors in their own right. These pro-apoptotic proteins are also suppressed in cancer when outnumbered by the anti-apoptotic members of the Bcl-2 family (e.g., Mcl1) which directly bind and inactivate their pro-apoptotic counterparts resulting in immortal cancer cells. Several studies have measured these anti- and pro-apoptotic genes across a variety of cancers and found Bok to be consistently deleted and Mcl1 to be amplified.[10]

'Reprogramming energy metabolism' involves the counterintuitive switch in cancer cells from oxidative phosphorylation to glycolysis even in the presence of oxygen. This phenomenon, called the "Warburg effect," causes cancer cells to compensate for the reduced ATP production of glycolysis in order to fuel their unrestricted growth. The major tumor suppressor that serves to prevent dysregulated cellular energetics is STK11. It accomplishes this by suppressing cell growth, angiogenesis, and bioenergetics under conditions of nutrient or oxygen stress[11] through its downstream effectors AMPK and mTOR. This is one of the two 'emerging hallmarks' and as such, STK11 is one of the least characterized major tumor suppressors.

'Evading immune destruction' is the final hallmark and the second of the 'emerging hallmarks.' It can be argued that the entire immune system acts as one of the body's most effect

tumor suppressors serving to seek and destroy incipient neoplasias before they ever fully develop. Constant immune surveillance routinely eliminates the vast majority of nascent tumors that are highly immunogenic leaving behind weakly immunogenic clones to grow out and develop into tumors. Evading detection by the immune system involves the immunosuppressive branches of the immune system including regulatory T cells and myeloid-derived suppressor cells and efforts to restore the immune response using cytokines, vaccines and antibody immunotherapies (e.g., anti-CTLA4, anti-PD-1) have shown promise in certain patient subsets.[12]

The remainder of this dissertation will focus on the major tumor suppressors pRb and STK11 in the context of osteosarcoma and lung cancer, respectively.

OSTEOSARCOMA

Osteosarcoma is a type of cancer that develops in bone tissue. It primarily affects children and young adults, likely linked to ages of rapid bone growth, but risk rises again in adults over the age of 60, and is a disease that can occur at any age. There are thirteen types of osteosarcoma that are divided into high-, intermediate-, and low-grade subtypes of varying frequencies. When diagnosed at a localized stage, the 5-year survival rate is 60-80%[II]. However, this drops to a survival rate of 15-30% when diagnosed with detectable metastases, increasing to approximately 40% if the metastases are exclusively in the lungs or if the tumor, including metastases, is completely resectable[II]. Osteosarcoma is more common in males than females and slightly more common in African Americans than in whites. Children who have hereditary retinoblastoma, marked by loss of the major tumor suppressor pRb, have an increased risk of osteosarcoma later in life as it is also marked by a high frequency of pRb loss. Similarly,

[II] www.cancer.org

patients with Li-Fraumeni syndrome, characterized by the loss of the major tumor suppressor p53, are also at an increased risk of developing osteosarcoma. Currently, the options to treat osteosarcoma include surgery, chemotherapy, and in certain cases, radiation. First-line chemotherapies include cisplatin, doxorubicin, high-dose methotrexate, ifosfamide, epirubicin, or some combination of the above while second-line therapies for refractory or metastatic disease include docetaxel, gemcitabine, cyclophosphamide, etoposide, topotecan, sorafenib, or some combination thereof[III]. Luckily, osteosarcoma is a relatively rare disease with only 5.5 boys and 4.5 girls out of every 1,000,000 aged 0-19 diagnosed in the U.S. between 2006-2010[IV]. Unfortunately, this rarity also works against the patients with the disease as research funds are channeled to more common diseases. It is our hope that our work in osteosarcoma will help lead to more targeted and effective treatments in the future.

LUNG CANCER

In contrast to osteosarcoma, lung cancer is the second most diagnosed cancer in both men and women following only prostate cancer and breast cancer, respectively and is the leading cause of cancer-related death among both men and women, but similar to osteosarcoma, this disease also suffers from a dearth of research funds. It is a disease inextricably linked to smoking with risk increasing due to both quantity and duration of smoking. This perception of lung cancer as a lifestyle disease has negatively impacted the research funds being directed toward finding a cure for this deadly disease and ignores many of the other causes including exposure to asbestos, radon, pollution, second-hand smoke, as well as other occupational

[III] National Comprehensive Cancer Network; www.nccn.org

[IV] www.cancer.org

hazards. Our work is focused on understanding the most predominant subtype, non-small cell lung cancer (NSCLC) in order to provide benefit to the greatest number of people. NSCLC accounts for approximately 85% of lung cancer diagnoses, with the remainder classified as small cell lung cancer. NSCLC is further subdivided into histological subtypes: adenocarcinoma, which accounts for approximately 35-40% all lung cancers, squamous cell carcinoma, which accounts for 25-30% of all lung cancers, and large cell carcinoma which accounts 10-15% of all lung cancers[V]. Most lung cancers are diagnosed in patients between 50-70 years old and is slightly higher in males compared to females, and slightly higher in African-American men compared to white men. The estimated 5-year survival for lung cancers diagnosed at a local stage is 54%, however only 15% of lung cancers fall into this category. For all stages at diagnosis combined, the 5-year survival rate is only 17%[VI]. Treatment decisions for lung cancer have traditionally been based on histological subtype and stage of disease, however more recently with the advent of targeted therapies, treatment decisions are being made based on a patient's individual spectrum of mutations. One of the most common mutations in lung adenocarcinoma is in the major tumor suppressor, STK11 and this relationship will be discussed in detail later in the dissertation.

OVERVIEW OF THE DISSERTATION

In the following chapters I will describe my work on two major tumor suppressors: pRb and STK11. **Chapter 2** describes emerging work linking the tumor suppressor pRb to control of cell adhesion and how alterations in this pathway may drive the process of metastasis.

[V] emedicine.medscape.com

[VI] www.cancer.org

Chapter 3 presents the novel finding that the adhesion protein, integrin α10 is transcriptionally activated by the tumor suppressor pRb using osteoblasts as a model system. It further elucidates a signature consisting of changes in expression of four integrin subunits that occurs across multiple solid tumor types. **Chapter 4** presents an overview of what is known about the STK11 tumor suppressor, specifically focusing on its molecular biology and strategies to therapeutically target STK11-deficient tumors. **Chapter 5** describes a novel gene expression signature that can be used to determine the mutational status of *STK11* in lung adenocarcinoma patients and clarifies why we believe this is the best option for a clinical diagnostic test. My conclusions and future directions for research are outlined in **Chapter 6**, as well as describing the current methods being explored to restore tumor suppressor function and my thoughts on the future of cancer treatment.

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CHAPTER TWO:

The Retinoblastoma Protein: A Master Tumor Suppressor Acts as a Link Between Cell Cycle and Cell Adhesion[VII]

ABSTRACT

RB1 was the first tumor suppressor gene discovered. Over four decades of work have revealed that the Rb protein (pRb) is a master regulator of biological pathways influencing virtually every aspect of intrinsic cell fate including cell growth, cell-cycle checkpoints, differentiation, senescence, self-renewal, replication, genomic stability and apoptosis. While these many processes may account for a significant portion of *RB1*'s potency as a tumor suppressor, a small, but growing stream of evidence suggests that *RB1* also significantly influences how a cell interacts with its environment, including cell-to-cell and cell-to-extracellular matrix interactions. This review will highlight pRb's role in the control of cell adhesion and how alterations in the adhesive properties of tumor cells may drive the deadly process of metastasis.

[VII] This chapter will be submitted for publication. See Appendix A for details.

THE RETINOBLASTOMA PROTEIN (pRb): THE CLASSIC PARADIGM

Existence of the *RB1* gene was predicted in 1971 from epidemiological evidence from retinoblastoma families[1] and the *RB1* gene was identified over 15 years later.[2] The initial characterization of pRb function was guided by studies of DNA tumor viruses[3,4] which pointed to pRb's role as a regulator of the G_1/S transition.[5] It is now known that both the G_1/S and G_2/M phases of the mammalian cell cycle are controlled by a complex and redundant molecular pathway that involves members of the E2 promoter binding factor (E2F)[6], dimerization partner (DP)[7], pRb[3], cyclin-dependent kinase (CDKs), Cyclins[8], and CDK inhibitor(CDKN)[9] families. This pathway is disrupted in most, if not all, solid tumors.[10]

While initial work on the function of pRb in cell cycle highlighted its role in the G_1/S transition, work over the last three decades demonstrates that pRb controls most cellular processes related to cell fate and DNA metabolism including cell-cycle checkpoints, tissue differentiation and morphogenesis, senescence, self-renewal, replication, tissue-specific gene expression, mitotic fidelity, genomic stability and apoptosis.[11-16]. In this review we will refer to these various cell intrinsic processes collectively as cell cycle. There are a number of outstanding articles[17-21] that review pRb's role in the cell cycle processes and they will not be repeated in detail here.

pRb's activity is regulated by post-translational modifications, phosphorylation being the most predominant.[22] pRb phosphorylation by CDK4-Cyclin D and CDK2-Cyclin E induces S-phase entry.[23,24] pRb is phosphorylated on at least 13 different serine/threonine residues suggesting that specific patterns of pRb phosphorylation may represent a 'pRb code' in which different pRb conformational variants mediate distinct protein-protein interactions. In non-

cancerous cells, anti-proliferative signals activate pRb by promoting its dephosphorylation by serine and threonine type I phosphoprotein phosphatases and by inhibiting the Cyclin-CDK complexes that phosphorylate pRb.[25-27] This activation allows pRb to block progression to S-phase, promoting entry to G_0 instead. A recent review has addressed the complexity of these pRb kinases.[22]

pRb's strong tumor suppressive nature is evident in the fact that pRb function is lost in most human cancers[26,28,29], and also by the fact that oncogenic insults, such as Ras activation, trigger a strong anti-oncogenic senescence program that depends on pRb.[11,30] Every component of the pRb pathway that represses cell cycle is subject to mutational inactivation in some human cancers and every component that induces cell cycle is subject to oncogenic up-regulation, providing genetic evidence that the pathway as a whole is essential in tumor development. CDKN proteins are subjected to inactivating mutations and epigenetic silencing. Cyclins, CDKs, and rarely, E2Fs themselves are upregulated by translocations and gene amplifications.[31-34] The rate of *RB1* gene mutation varies significantly among different tumor types, but is highest in retinoblastoma, osteosarcoma, and small cell lung cancer (SCLC).[28,35] Mutations targeting the *RB1* gene directly affect pRb function by either completely abrogating its expression or by producing a non-functional protein.[36] Other cancer types bearing wild type *RB1* alleles still have impaired pRb function due to alterations in genes coding for upstream pRb regulators. These alterations range from inactivating mutations, deletions or epigenetic silencing of the $p16^{INK4A}$ locus (a CDKN family member), to alterations leading to Cyclin D or CDK4 overexpression.[32] The latter scenario results in pRb inactivation by chronic hyperphosphorylation. Therefore, oncogenesis usually entails either a complete loss of pRb expression or its inactivation by hyperphosphorylation.

DEREGULATION OF ADHESION PROTEINS IN CANCER

Cadherins are calcium-dependent cell adhesion proteins that mediate cell-to-cell adhesion. They are named for the tissue that they were first identified in (eg, E-cadherin, N-cadherin, and OB-cadherin were discovered in epithelial, neural, and osteoblast tissues, respectively), but are not restricted exclusively to those tissues. Cadherins, together with catenins, are the main components of adherens junctions, which are membrane protein complexes that are stabilized by association with actin filaments densely packed under the cell membrane.[37] Their disruption is part of EMT during oncogenic progression and contributes to metastasis by facilitating detachment of cancer cells from the primary tumor.[38] This disruption consists of a 'cadherin switch' whereby expression of E-cadherin is repressed and N-cadherin is upregulated.

Alterations in integrin expression have also been noted in cancers. Similar to the 'cadherin switch' occurring during EMT, an 'integrin switch' has been observed in multiple solid tumors consisting of overexpression of integrin β4, and underexpression of integrins α7, α8, and α10.[39] Integrins are a family of 26 cell-to-extracellular matrix (ECM) adhesion receptor subunits. Each functional integrin heterodimer consists of two type-1 (single membrane-spanning domain with the C-terminus located cytoplasmically) transmembrane subunits, one α- and one β-subunit. Integrins bind to multi-adhesive ECM components, organizing the cytoskeleton and activating intracellular signaling pathways. They have been shown to affect cell shape, polarization, cytoskeletal organization, cell motility, proliferation, survival, and differentiation. Integrins are unusual among transmembrane receptors in that they signal bidirectionally, carrying both mechanical and chemical signals. "Inside-out" signaling, known as "priming," is responsible for

a conformational change in the integrin heterodimer which extends outward and induces adhesiveness to the ECM. Adhesion is further strengthened by the lateral reorganization of integrins into clusters, which may progress to dot-like focal complexes that mature into larger focal adhesions and finally into streak-like fibrillar adhesions.[40] The "integrin adhesome" is comprised of 156 signaling, structural, and adaptor molecules that contribute to cytoskeletal reorganization and catalytic activity as integrin tails have no catalytic activity of their own.[40] Integrin signaling, and the associated cross-talk with adjacent receptor tyrosine kinases, has been linked to many pathways important in human cancer including the Ras/Raf/MEK/MAPK, PI3K/ PIP_3/AKT, NF-κB, and pRb/E2F pathways.

NEW ROLES FOR pRb IN CELL ADHESION: REGULATION OF CADHERIN- AND INTEGRIN-MEDIATED ADHESION

Cancer types showing high frequencies of mutational inactivation of the *RB1* gene are very aggressive relative to tumors with chronically hyperphosphorylated wild-type pRb. An example is osteosarcoma, which at the time of diagnosis is consistently high grade and poorly differentiated. These patients have a poor prognosis with 20%[VIII] of diagnosed cases already having detectable metastases[41] and only 10% achieving long-term disease free intervals.[42] Osteosarcoma incidence is increased 1000 fold in patients who inherit *RB1* mutations relative to the general population,[43] implicating pRb loss in osteosarcoma formation. pRb loss occurs in over 70% of sporadic osteosarcomas, and loss of *RB1* heterozygosity is present in 60-70% of osteosarcomas and is indicative of a poor prognosis.[44]

[VIII] http://www.cancer.org/

Like osteosarcoma, SCLC is characterized by a high rate (~90%) of mutational inactivation of the *RB1* locus.[45] Patients with SCLC have a five-year survival rate of only 6%[I], which can increase to 54% if detected at a localized stage. Unfortunately, only 15% of SCLC are detected at the localized stage. This extreme aggressiveness is in stark contrast to the five-year survival rates of tumors with lower rates of *RB1* mutations such as breast, prostate, and colorectal cancers when detected at a localized stage, which are 99%, 100%, and 90%, respectively, according to the American Cancer Society Cancer Facts and Figures, 2014[I]. Interestingly, lung cancer survival rate triples to 18%[I] in the non-small cell lung cancer (NSCLC) subtype, which usually bears wild type *RB1* alleles but instead exhibits preferential loss of the $p16^{INK4A}$ locus with consequent pRb hyperphosphorylation.[46] The differences in survival rates between SCLC and NSCLC suggest that increased aggressiveness may be associated more with direct alterations of the *RB1* locus than with chronic pRb hyperphosphorylation resulting from alterations in other loci.

This *RB1* effect is also observed in epithelial cancers. Although *RB1* mutations are rare in prostate cancer, a recent analysis of 50 castration-resistant prostate cancer patients[47] demonstrated that patients with inactivating *RB1* mutations have a 35 month reduction in median overall survival relative to patients with WT *RB1* ($p=0.025$). Specifically, the 16 patients with *RB1* mutations had a median overall survival of 70 months, versus 105 months in 34 patients with WT *RB1*.

The data discussed above highlight the association between direct mutational targeting of the *RB1* gene and high mortality as demonstrated by the low 5-year survival rates of pRb-null cancers. Given that metastases indicate aggressiveness and cause over 90% of cancer deaths,[48] we propose that pRb deficiency leads to a proclivity for early metastasis, that is, for early

detachment of tumor cells from the primary tumor and invasion of adjacent and distal tissues. If so, blocking cellular events associated with metastasis (eg, loss of cell polarity, epithelial-mesenchymal transition (EMT), loss of cell adhesion) may be part of pRb´s tumor suppressive arsenal. The first hints of a relation between pRb and metastasis came from studies published over a decade ago implicating pRb in the stabilization of adherens junctions. Disruption of these structures is part of EMT and contributes to metastases by facilitating detachment of cancer cells from the primary tumor mass.[38] Early studies showed that retinoblastomas, osteosarcomas, and SCLC, known for their high frequencies of *RB1* mutations, are composed of cells that lack stable adherens junctions. In retinoblastoma, adherens junctions fail to anchor to the cortical actin cytoskeleton.[49] In osteosarcoma and SCLC, adherens junction proteins are downregulated and aberrantly localized in the cytoplasm rather than at the cell membrane.[50,51] Furthermore, a strong correlation was found in retinoblastoma and osteosarcoma between abnormal adherens junctions and invasive capacity,[49,50] underscoring the notion that disruption of these structures is related to invasion, and for the first time, implicating pRb loss in invasive behavior.

These early studies linking pRb to adherens junction integrity remained largely ignored for years following their publication, possibly since they were mostly correlative and did not establish a causal relationship between pRb loss and cell adhesion perturbations. An exception was a study showing that pRb inactivation by SV40 large T antigen in MDCK epithelial cells resulted in a mesenchymal conversion associated with invasiveness that could be reversed by pRb re-activation.[52] This study also offered the first mechanistic explanation of pRb's involvement in cell adhesion by showing that pRb, together with the AP-2 transcription factor, activated transcription of the E-cadherin promoter in epithelial cells.[52] It took approximately a decade for the next reports confirming the link between pRb and cell adhesion. These studies

showed that pRb depletion disrupted cellular adhesion and induced a mesenchymal-like phenotype. They further established that transcriptional regulation of E-cadherin expression by pRb is a molecular link between pRb and cell adhesion.[53,54] Further implicating pRb loss in EMT, pRb depletion results in up-regulation of several EMT-related transcriptional factors including Slug and Zeb-1, which are known E-cadherin transcriptional repressors.[53,51]

The studies described above strongly implicate pRb loss as a promoter of metastasis of carcinomas, or epithelial tumors, specifically via the loss of epithelial markers such as E-cadherin and the acquisition of mesenchymal and migratory phenotypes. Additionally, it has been shown that OB-cadherin, the predominant osteoblast cadherin, is also transcriptionally regulated by pRb,[55] implicating pRb loss in the molecular etiology of non-epithelial tumors such as osteosarcomas. Conditional deletion of pRb in osteoblasts produces a 'cadherin switch' in which OB-cadherin is replaced by N-cadherin,[55] suggesting that pRb promotes the expression of adhesion molecules characteristic of the fully differentiated state, regardless of cell type, while repressing the expression of cell adhesion genes related to an undifferentiated phenotype. The global nature of pRb's influence on cell adhesion was revealed by microarray analyses comparing pRb-proficient versus pRb-deficient osteoblasts, which found that pRb affects the expression of a variety of cell adhesion genes beyond cadherins and that cellular processes related to cell adhesion are strongly affected by pRb.[55] Integrins were also found among the cell adhesion genes whose expression is strongly affected by pRb, and cellular pathways involved in integrin-mediated cell-to-ECM adhesion were also found to be under pRb control.[55] In a follow-up study, it was shown that pRb induces transcription of integrin α10 in osteoblasts, regardless of pRb phosphorylation status, with a corresponding increase in osteoblast binding to a collagen substrate.[39] This suggests that pRb mediates not only cadherin-dependent cell-to-cell adhesion,

but also promotes integrin-dependent cell-to-ECM adhesion. Taken together, the studies summarized above indicate a strong influence by pRb on cell adhesion that can either be activating or repressive depending on the genes involved, up-regulating adhesion genes in differentiated cells (eg, integrin α10) while down-regulating expression of adhesion genes associated with invasiveness and metastasis (eg, N-cadherin).

pRb appears to regulate the formation of functional cell adhesive structures beyond transcriptional regulation of cell adhesion genes, including facilitating the assembly of cell adhesion gene products at the cell membrane. In the absence of pRb, the Rho GTPase Rac1 and its effector the p21-activated protein kinase (Pak1) become up-regulated with consequent phosphorylation of the Merlin tumor suppressor at Serine 518 by Pak1, which in turn causes Merlin to detach from the cell membrane.[55] Therefore, pRb seems to promote adherens junction assembly at the cell membrane by blocking the inactivating phosphorylation of Merlin by Pak1. Merlin is a membrane-bound tumor suppressor and cytoskeleton adapter that stabilizes adherens junctions by anchoring them to the cortical actin cytoskeleton under the plasma membrane.[56,57] Merlin loss, which is frequent in the human cancer syndrome Neurofibromatosis type 2 (NF2), results in adherens junction disruption with consequent inactivation of contact-dependent growth arrest.[56] In summary, studies demonstrate that in the absence of functional pRb, transcription of adherens junction components as well as their assembly at the cell membrane are both compromised. This explains the observation that in pRb-deficient tumors, such as retinoblastoma and osteosarcoma, not only do adherens junction proteins show diminished expression, but they also fail to anchor to the cell membrane instead showing aberrant cytoplasmic localization.[49-51]

Invasiveness and metastases arise from a combination of loss of cell adhesion, onset of migration facilitated by cytoskeletal reorganization and loss of cell polarity, and the capacity to degrade basal laminae in order to escape the primary tumor site and penetrate adjacent tissues. The data summarized above link pRb loss predominantly to perturbations in cell adhesion, but pRb loss could exacerbate invasiveness by affecting other aspects of metastasis. For example, pRb loss has been linked to increased expression of the matrix metalloproteinases (MMPs) that remodel the ECM during cell invasion and metastasis.[58,59] MMP genes such as *MMP9*, *MMP14*, and *MMP15*, which are usually over-expressed in NSCLC, have been shown to be regulated by pRb.[59] pRb reactivation was sufficient to inhibit *MMP* transcription, to reduce the invasion and migration of cancer cells *in vitro*, and to reduce metastatic foci development in a tail vein lung metastasis model in mice.[59] pRb depletion also exacerbates the invasiveness of ErbB2-positive breast cancer, suggesting that pRb loss may play a predominant role in the progression of *in situ* breast ductal cell carcinoma to the invasive stages of the disease.[60]

The data implicating pRb control of cell adhesion in cultured cells are abundant and provide mechanistic insights that were lacking in early correlative studies. There are also data providing insights into the consequences of pRb loss for *in vivo* tissue morphogenesis. When a mouse model of osteosarcoma was generated by conditionally knocking out *RB1* in osteoblasts,[68] structural defects indicative of impaired osteoblast adhesion were observed in the calvaria of pRb knockout mice. Specifically, pRb knockout mice lacked properly organized osteoblast layers and showed osteoblasts that had migrated away from their proper position in the calvaria and invaded the adjacent cartilage.[68] pRb-deficient osteoblasts also expressed elevated levels of Ezrin, a membrane-cytoskeleton linker and osteosarcoma metastasis marker.[55,61,62] Other mouse models of osteosarcoma based on abrogation of pRb function have resulted in mice that develop

fully penetrant, highly metastatic early onset osteosarcomas.[63] Given the importance of cell-to-cell adhesion for osteoblast differentiation, pRb loss can be predicted to alter osteoblast differentiation and lead to the formation of osteosarcoma. Osteoblasts originate from pluripotent mesenchymal stem cells that differentiate into stroma, adipocytes, myoblasts, chrondroblasts, fibroblasts, or osteoblasts.[64,65] Stem cells committed to osteoblastic differentiation are sorted from the rest of the mesenchymal precursors and align with, and adhere to, each other. Homotypic, cadherin-based cell-to-cell interactions play a major role in sorting the pluripotent stem cells into distinct lineages. Consistently, osteoprogenitor cells express a spatio-temporally regulated repertoire of cadherins that provide cues for their alignment into a distinct subpopulation within the bone marrow that will later differentiate into mature osteoblasts.[66,67] Adherens junction loss in pRb-null osteoblasts is accompanied by abnormal expression patterns of the predominant osteoblast-specific cadherins OB- and N-cadherins, suggesting that the timing of cadherin expression during osteoblast differentiation can be altered by pRb loss.[55] This in turn suggests that pRb is required to ensure that expression of specific cadherins proceeds with the right timing during differentiation, and that pRb loss could hamper proper homotypical intercellular contacts, resulting in defective osteoblast differentiation with consequent disruption of bone integrity and/or formation of bone tumors. Based on *in vivo* observations, it is plausible that pRb is instrumental in the orchestration of cell proliferation and cell adhesion as part of differentiation and bone morphogenesis. Disruption of which may be central to the molecular etiology of osteosarcomas, which are characterized by poor differentiation and high frequencies of *RB1* mutations.

A LINK BETWEEN CELL CYCLE CONTROL AND CELL ADHESION

In metastatic cancer cells, adhesion is aberrantly regulated by a variety of pathways resulting in loss of cell-to-cell and cell-to-ECM contact and in dissemination of cancer cells throughout the body. While more work is needed to elucidate those pathways, in many instances this loss of adhesion has been tied to cell-cycle regulators, including members of the pRb-E2F pathway.

Signaling from integrins through their downstream pathways occurs cooperatively through crosstalk with growth factor receptors and has been linked to a variety of pathways involved with cell cycle progression. Integrin-mediated cell-to-ECM adhesion acts as a checkpoint for cell cycle entry. For example, in early work using pRb positive LNCaP and pRb negative RU145 prostate epithelial cell lines, loss of β1 integrin contact to ECM inhibited G_1 CDK activity leading to an accumulation of hypophosphorylated pRb and subsequent Bcl-2 mediated apoptosis.[68] More recently, Wang et al.[69] found that overexpression of integrin α5 and knockdown of integrin α6 decreased pulmonary metastasis of the highly invasive breast cancer cell line 4T1 by inhibiting entry to S-phase through p27 upregulation, resulting in downregulation of Cyclin E/CDK2 complexes. They also found that this modulation of integrin expression upregulated E2F, which may then induce expression of Chk1 to regulate cdc25A/Cyclin E/CDK2/pRb in a feedback loop. These findings implicate integrin α5 as a metastasis suppressor and α6 as a metastasis promoter in breast cancer. For a comprehensive review of how integrins control downstream entry to cell cycle progression see the review by Moreno-Layseca and Streuli.[70]

Expression of E2Fs1-3 was shown to indirectly increase integrin β4 mRNA, protein, and cell surface expression.[71] These E2Fs were found to be downstream of active H-Ras in SUM-159 breast carcinoma cells. Integrin α6β4 has been previously shown to enhance carcinoma invasion, so the mechanism proposed by Yoon et al.[71] links active H-Ras, active E2Fs and integrin α6β4 in a single pathway to promote invasion.

Long-term treatment of three NSCLC cell lines with recombinant CCN1 (Cysteine-rich 61), a secreted matrix-associated molecule, led to permanent cell cycle arrest in G_1. Addition of CCN1 increased abundance of hypophosphorylated pRb and p53 and p21 accumulation. A CCN1 mutant defective for binding integrin α6β1 and co-receptor heparan sulfate proteoglycans was incapable of inducing senescence.[72]

The finding that pRb's effect on integrin expression is unaltered by its phosphorylation state[39] is particularly informative of the mechanisms linking pRb to cell adhesion and of the coupling between cell cycle and cell adhesion. As discussed above, phosphorylation is a mechanism of regulation of pRb function that abrogates pRb's capacity to bind and block E2F transcription factors. The integrin α10 findings[39] suggest that regulation of cell cycle progression and cell adhesion by pRb may be mechanistically uncoupled since while pRb hyperphosphorylation abrogates pRb's capacity to bind E2F and repress the cell cycle, it leaves intact the capacity to induce integrin-mediated cell-to-ECM adhesion. This could shed some light into the aggressive behavior of pRb-deficient tumors. The tendency of pRb-deficient tumors to metastasize early in their development could be explained by the loss of both cell cycle control and cell adhesion resulting from pRb loss. The residual pRb activity retained by tumors with chronically hyperphosphorylated pRb, while not enough to halt initial tumor growth, may

result in a less aggressive tumor and in deterring metastasis by helping to anchor the tumor structure.

The data discussed above expand the paradigm of pRb function beyond cell cycle to include roles in cell adhesion, and therefore implicate pRb loss in later stages of tumor metastasis. **Figure 2.1** shows a model depicting how pRb can integrate cell cycle control and cell adhesion. These dual roles of pRb mechanistically explain how impairment of pRb function contributes to the aggressive nature of some tumor types, expands pRb's arsenal of tumor suppressive abilities, and explains the potency of this preeminent tumor suppressor more adequately than the notion that pRb acts predominantly as a cell cycle repressor.

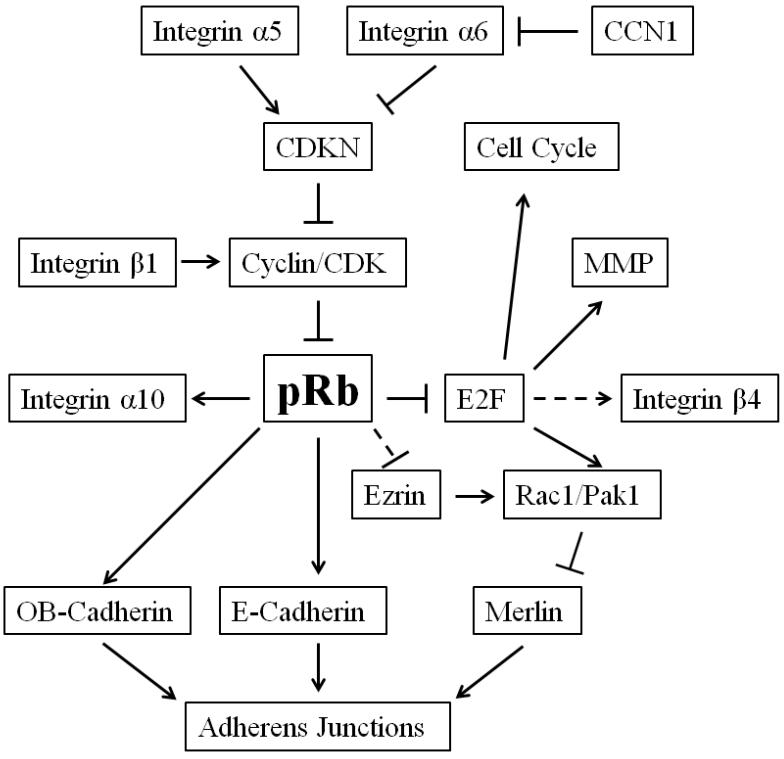

Figure 2.1: Model illustrating the function of pRb in cell cycle control and cell adhesion. Pointed arrows indicate a stimulatory interaction. Blunt arrows indicate pathway repression. Dotted lines represent indirect interaction or interactions via a mechanism that has not been fully elucidated.

TARGETING pRb LOSS AND ABERRANT ADHESION

The activity of the pRb kinases, the CDKs, is central to pRb pathway. For these reasons, small molecule CDK inhibitors are being developed and examined in clinical trials for a number of malignancies.[73]

Aberrant adhesion has been determinant of potential treatment options in several cancers. For example, in erlotinib-resistant lung cancer cells harboring activating *EGFR* mutations, there was increased expression of Src, integrins β1, α2, and α5 along with increased adhesion. Silencing of integrin β1 restored erlotinib sensitivity. There was also increased expression of integrins β1, α2, and/or α5 in refractory tumor samples from patients treated with erlotinib and/or gefitinib.[74]

Furanodiene, a natural terpenoid derived from Rhizoma Curcumae, was found to have anti-proliferative activity in 95-D human lung cancer cells when combined with paclitaxel. These effects included down-regulation of protein levels of Cyclins D1 and B1, CDK6, and c-Myc, as well as down-regulation of expression of integrin β4, focal adhesion kinase, and paxillin.[75] Previous studies had shown that combining furanodiene and paclitaxel had synergistic anti-proliferative effects in NCI-H1299 and 95-D human lung cancer cell lines,[76] and that furanodiene decreased integrin β1 expression in breast cancer cells in a concentration-dependent manner.[77]

Unsurprisingly, aberrant integrin signaling has been implicated in several human cancers and specific therapies are being developed to target the integrin pathway including development of anti-integrin α4 antibodies (eg, Natalizumab currently being evaluated in over 80 clinical trials; http://clinicaltrials.gov), focal adhesion kinase (FAK) inhibitors (eg, GSK2256098

currently being evaluated in three clinical trials; http://clinicaltrials.gov), integrin-linked kinase (ILK) inhibitors, and RGD peptides (competitive inhibitors for the fibronectin-binding consensus sequence such as eptifibatide and tirofiban). Unfortunately, integrins are also known mediators of cell adhesion-mediated drug resistance (CAM-DR). Specifically, melanoma cells expressing $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins are resistant to doxorubicin and melphalan once bound to their fibronectin ligands.[78] This resistance is the result of cell cycle arrest in G_1 and is associated with increased levels of the CDKN p27 and its inhibition of Cyclins A and E.[79]

"Ligand-induced" adhesion, an integrin-mediated Rap-1-independent pathway that allows unstimulated leukocytes to adhere to and migrate through exposed endothelial matrix or high-density ligand, is CDK4-mediated, but pRb-independent. CDK inhibitors were able to block this leukocyte adhesion and migration[80].

The treatment options listed above are all designed to target either the pRb pathway or the process of adhesion. With the new work being pioneered on the link between these two pathways, it is our hope that either combining these drug classes, or developing new drugs to specifically target this newly discovered link that treatment options will be more tailored to individual cancers and increasingly effective in the future.

CONCLUSIONS: IMPLICATIONS FOR pRb AS A METASTASIS SUPPRESSOR

Recent work linking pRb to cell adhesion should reinvigorate the pRb field by challenging the classic paradigm of pRb acting predominantly as a cell cycle regulator. New information about pRb, as well as other oncogenes and tumor suppressors discovered decades ago, continues to uncover novel effects and potentialities beyond cell cycle control.

In the currently accepted model of tumor evolution, a step-wise accumulation of mutations results in the progressive acquisition of aberrant cellular behaviors, each behavior elicited by a particular mutation or sets of mutations. Mutations that inactivate tumor suppressors like *RB1* or that activate proto-oncogenes like *KRAS* would contribute to early stages of tumor evolution by conferring a proliferative advantage to incipient tumor cells. These mutations target cell cycle control by rendering cells capable of bypassing proliferative arrest, contributing to unchecked tumor growth. However, a paradigm in which pRb acts predominantly as a cell cycle repressor does not explain how pRb inactivation in early tumorigenesis would contribute to later stages of metastasis, particularly to the detachment of tumor cells from their original site and dispersion to distant tissues. The current model thus mandates the acquisition of additional secondary mutations that confer metastasis potential at later stages of tumor evolution. This multi-step model has been challenged, however, and deemed conceptually inconsistent since the additional genetic hits that confer metastatic capabilities at later stages of tumorigenesis do not necessarily exacerbate the proliferative advantage conferred by the initial hits that disrupt cell cycle control.[81] In a tumor history that is essentially a micro-evolutionary process, if secondary metastasis-related mutations do not further enhance the previously acquired replicative advantage, the cells that acquired them will remain rare within the tumor mass, outcompeted by more proliferative counterparts. Rather, it has been suggested that mutations acquired by incipient tumor cells early in tumorigenesis confer not only the replicative advantage that allows the initial tumor growth, but also later in tumorigenesis, the proclivity to metastasize.[81] Thus, the tendency to metastasize could be determined by mutant alleles acquired early in tumor history.[81] This revised model predicts that fewer mutations are required for a full-blown malignant phenotype if they target multifunctional genes such as *RB1*.

Several lines of evidence support this. First, primary human breast cancers can shed malignant cells into the bone marrow even when tumors are small and well-localized; second, DNA microarray analyses reveal that metastatic tumor cells show gene expression profiles remarkably similar to the cells contained in the primary tumor from which they were derived; third, certain early gene-expression profiles in primary breast cancer tumors strongly predict metastasis and can be detected before metastasis actually occurs.[81-86] A dual role for pRb in cell cycle and cell adhesion is fully consistent with a model of metastases arising from fewer mutations. Thus, pRb inactivation enhances proliferative capacity and growth of tumor mass during early carcinogenesis, and also contributes to later stages of metastasis by promoting cell detachment from the primary tumor. Further characterization of pRb's role in cell adhesion could contribute to what has been described as "the hope to achieve an understanding of the complex process of neoplastic transformation at the cellular level in terms of a small number of genetic changes."[87]

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CHAPTER THREE:

Expression of Integrin alpha 10 is Transcriptionally Activated by pRb in Mouse Osteoblasts and is Downregulated in Multiple Solid Tumors[IX]

ABSTRACT

pRb is known as a classic cell cycle regulator whose inactivation is an important initiator of tumorigenesis. However, more recently it has also been linked to tumor progression. This study defines a role for pRb as a suppressor of the progression to metastasis by upregulating integrin α10. Transcription of this integrin subunit is herein found to be pRb-dependent in mouse osteoblasts. Classic pRb partners in cell cycle control, E2F1 and E2F3, do not repress transcription of integrin α10 and phosphorylation of pRb is not necessary for activation of the integrin α10 promoter. Promoter deletion revealed a pRb responsive region between -108bp to -55bp upstream of the start of the site of transcription. pRb activation of transcription also leads to increased levels of integrin α10 protein and a greater concentration of the integrin α10 protein at the cell membrane of mouse osteoblasts. These higher levels of integrin α10 correspond to increased binding to collagen substrate. Consistent with our findings in mouse osteoblasts, we found that integrin α10 is significantly underexpressed in multiple solid tumors that have frequent inactivation of the pRb pathway. Bioinformatically, we identified data consistent with

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an 'integrin switch' that occurs in multiple solid tumors consisting of underexpression of integrins α7, α8, and α10 with concurrent overexpression of integrin β4. pRb promotes cell adhesion by inducing expression of integrins necessary for cell adhesion to a substrate. We propose that pRb loss in solid tumors exacerbates aggressiveness by debilitating cellular adhesion, which in turn facilitates tumor cell detachment and metastasis.

INTRODUCTION

The classic pRb pathway comprises pRb, the E2F family of transcription factors, cyclins (primarily D type), cyclin-dependent kinases (CDKs; primarily 4 and 6), and two families of CDK inhibitors (including p16Ink4a). In this classic view, pRb acts as a transcriptional repressor, binding E2F family members at the promoter site of E2F-regulated genes and maintaining transcriptional repression by blocking the transactivation function of E2F and by recruiting additional factors that actively repress transcription.[1] Mitogenic signaling activates the formation of cyclin/CDK complexes, which are responsible for phosphorylating, and thus inactivating, pRb. Once hyperphosphorylated, pRb dissociates from E2F, which is now free to promote the transcription of E2F-regulated genes.

In contrast to its negative regulatory control over E2F-mediated transcription, pRb has also been found to positively regulate the expression of a number of genes. One such gene that was discovered to be positively regulated by pRb is E-cadherin, a cell surface adhesion protein that is a marker for epithelial cells. Both pRb and c-Myc activate transcription of E-cadherin in epithelial cells in an AP-2 mediated manner.[2] Similarly, pRb can bind members of the AP-1 family of transcription factors, including c-Jun, at its AP-1 consensus sequence resulting in

stimulation of its transcriptional activity.[3] Other genes known to be positively regulated by pRb include the anti-apoptotic gene, Bcl-2, which is activated in an Ap-2 dependent manner[4] and the CDK inhibitor, p21, which is activated in an Sp1/Sp3 dependent manner[5]. The transcription factor Sp1 has also been found to be important in the upregulation of integrin α5 through interaction with, and activation by, the transcription factor ZEB2[6].

pRb is inactivated either directly, through mutation of the *Rb* gene, or indirectly in the majority of human cancers. Cancer types that have a greater than 90% frequency of pRb gene mutation (e.g. retinoblastoma, osteosarcoma, small cell lung cancer) are also characterized by disrupted cell-to-cell adhesion as mediated by adherens junctions.[7-9] Previous work has shown that pRb-deficient osteoblasts do not undergo contact-dependent growth arrest, lack adherens junctions, and exhibit altered cadherin expression.[10] This same work, via microarray, identified a number of genes involved in cell-to-extracellular matrix (ECM) adhesion that may also be pRb regulated. One such gene that was identified was integrin α10.

Integrins are a family of heterodimeric proteins made up of an α and a β subunit. They mediate adhesion of cells to ECM ligands and are unique among transmembrane receptors in that they have the ability to signal bidirectionally, carrying both mechanical and chemical signals.[11] Integrin α10 interacts exclusively with the β1 subunit to form α10β1 integrin. This is one of four collagen-binding integrins and preferentially binds collagen type IV, the primary collagen type of the basal lamina, but also binds collagens type VI and II, the primary collagen types of the ECM of skeletal muscle and cartilage, respectively.[12] Integrin α10 is found primarily in chondrocytes,[13,14] but has also been found in chondrogenic mesenchymal stem cells, as well as the endosteum (cell lining between bone marrow and bone) and periosteum (cell lining outside the bone)[15], areas rich in osteoblasts. In these regions, osteoblasts are responsible for bone

development and produce osteoid, a matrix composed mainly of collagen type I. A constitutive deletion of integrin α10 in mice resulted in a growth retardation of the long bones while maintaining a normal lifespan and fertility.[16]

We hypothesized that not only was integrin α10 present in osteoblasts, but that it was being regulated by pRb. In this article we demonstrate that pRb transcriptionally activates integrin α10 and that the frequent loss of pRb in multiple solid tumors results in a dramatic downregulation of integrin α10. pRb exerts its tumor suppressive effect primarily through repressing cell proliferation and inducing a post-mitotic state as well as driving differentiation. We would like to add 'maintaining cellular adhesion to the ECM' as a key tumor suppressive function of pRb as we suspect that the subsequent downregulation of integrin α10 is part of a greater 'integrin switch' that may have a vital role in the development of cancer metastasis. Our study builds upon the growing literature that points to the loss of pRb as a key mediator of the progression to metastasis.

MATERIALS AND METHODS

Cell lines and drug treatment

Cell lines and the mice they were derived from were previously described by Sosa-Garcia et al.[10] Briefly, primary osteoblasts were isolated and 3T3-immortalized from *Rb1* conditional knockout embryonic mice and their Rb wild-type littermates to produce pRb null and pRb wild-type MC3T3 cell lines, respectively. They were grown in Minimum Essential Medium Alpha (MEM-α) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Approximately 2 x 10^6 MC3T3 Rb wild-type cells were cultured in p60 plates and received

either a control treatment (complete media) or a drug treatment consisting of complete media supplemented with either 200 nM or 500 nM of the CDK 4/6 specific inhibitor, PD0332991 (ChemiTek). Cells were harvested 48 h after treatment and underwent RNA extraction followed by qRT-PCR as described below.

Plasmid vectors

The pGL3-p27[17], Rb wt LP, Rb 7 LP[18], pEGFPc2 (GenBank Accession #U57606), pcDNA3-E2F1[19], pcDNA3-E2F3[20], CMV-Sp1(Plasmid 12097 purchased from http://www.addgene.org), and pGL3-CIITA-Δ195 (Gift from Ken Wright, Moffitt Cancer Center; Ghosh *et al.*[21]) have all been previously characterized. The novel promoter plasmids were generated by PCR using the following primers: Δ590 forward (5'-GAGAGGTACCTGTTGGGGGAAAGGTGCGGA-3'), Δ463 forward (5'-GAGAGGTACCACAGGCAGTGACTCCCCAAAAGC-3'), Δ397 forward (5'-GAGAGGTACCAGGTCACACAGTAGGACTGCCC-3'), Δ275 forward (5'-GAGAGGTACCCCTACTTTCTGTTCCAAACTGGAGG-3'), Δ232 forward (5'-GAGAGGTACCACCGTGCATAAAAGTAGCCTCAGAA-3'), Δ163 forward (5'-GAGAGGTACCAGGGGGCAGCACCAAGGTAGAG-3'), Δ108 forward (5'-GAGAGGTACCGGGCTCCCCACAGCTCCCTTC-3'), Δ55 forward (5'-GAGAGGTACCTTAGCTGCCAGTGGGAGGGGG-3'), Reverse primer for all of the aforementioned (5'-GAGAAGATCTAGACTCCATGGGCGCTTGTCC-3'). The products were cleaved with KpnI and BglII and cloned into those sites of the pGL3-basic vector (Promega, Chicago, IL, USA). Site-directed mutants were created using the Δ590 plasmid altered with the following internal mutational primers and their reverse complements as reverse primers: Δ590-

YY1 forward (5'-GTTATTTTGCATATCAACGGTTAAGATTAATAAG-3'), Δ590-HBP1(1) forward (5'-TGGAGGAAATTATTGGGCGAATAAACCGTGCATA-3'), Δ590-PAX6 forward (5'-TATTGAATAAATAAAATACGCATAAAAGTAGCCT-3'), Δ590-HBP1(2) forward (5'-TTCCACCACCACTCCACGCCCATCCAACTTTATT-3'), Δ590-SP1 forward (5'-GCTGCCAGTGGGAGGTTTAAGGATAGGAGGGAAA-3')

Luciferase assays

Approximately 100,000 cells per well of MC3T3 Rb null cells were cultured in 24-well plates and transfected with a mixture containing 500 ng promoter construct, 50 ng Renilla luciferase reporter (pRL-TK, Promega), and 2.5 μg of either Rb expression plasmid or empty control vector (pEGFPc2) for an equal amount of DNA diluted in 100 μL serum free MEM-α for each transfection. One microliter X-tremeGENE HP DNA transfection reagent (Roche Disgnostics, Indianapolis, IN, USA) was added to the DNA mixture and allowed to incubate for 20 min before being added to the MC3T3 Rb null cells to a total volume of 600 μL (500 μL serum free medium plus 100 μL transfection mixture). Cells were incubated with the transfection mixture for 4 h before being returned to complete media (MEM-α plus 10% FBS, 1% penicillin/streptomycin). Cells were harvested 48 hours after transfection and luciferase assays were performed using the dual-luciferase reporter assay system (Promega) following the manufacturer's protocol and read using a $20/20^{n}$ Luminometer (Turner Biosystems, Sunnyvale, CA, USA) with standard promega protocol DLR-O-INJ. Experiments were done in triplicate. To control for transfection efficiency, firefly luciferase values were normalized to the values for Renilla luciferase.

Quantitative real-time PCR

Total cell RNA was harvested using the RNeasy Mini Kit (Qiagen, Carol Stream, IL, USA) following the manufacturer's instructions. Reverse transcription reactions were carried out using iScript cDNA Synthesis Kit (Bio-Rad, Chicago, IL, USA). Real-time PCR was performed using Bio-Rad iQ SYBR Green Supermix on a *CFX96*™ real-time PCR detection system (Bio-Rad). The following primers were used: Itgα10 forward (5'-GGCTCCAACAGTATCTATCC-3'), Itgα10 reverse (5'-TGCTCTCACAACTTCTTCC-3'), GAPDH forward (5'-AACGACCCCTTCATTGAC-3'), GAPDH reverse (5'-CTCCACGACATACTCAGCAC-3')

Western blotting

Western blots were performed as previously described[22]. Briefly, cell lysates were normalized for total protein content (35 μg) and subjected to SDS-PAGE. Detection of proteins was accomplished using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL; Amersham Biosciences, GE Life Sciences, Pittsburgh, PA, USA). Antibodies used include a mouse monoclonal antibody specific for endogenous pRb residues 701-928 (9309; Cell Signaling), a goat polyclonal antibody corresponding to amino acids 528-546 of Sp1 (sc-59-G; Santa Cruz Biotechnology), and a mouse monoclonal β-actin antibody (A5441; Sigma).

Immunofluorescence

Immunofluorescence was performed as previously described[23] without permeabilization. Briefly, cells were seeded in a Lab-Tak eight-chamber slide (Thermo Scientific, Waltham, MA, USA) 1 day before experimentation. They were rinsed with PBS and fixed in 4%

paraformaldehyde followed by neutralization with glycine. Cells were then sequentially incubated in 10% bovine serum albumin (BSA), either anti-integrin α10 (AB6030; Millipore) or anti-β-tubulin (2128S; Cell Signaling), followed by secondary AlexaFluor 488-conjugated goat anti-rabbit antibody (Invitrogen, Life Technologies, Grand Island, NY, USA) with added DAPI. This was followed by washing three times in PBS and covering the wells with Vectashield mounting media (Vector Labs, Burlingame, CA, USA) and coverslips. Cells were imaged by the Moffitt Cancer Center Microscopy Core with a Leica SP5, Chicago, IL, USA AOBS tandem scanning inverted confocal microscope.

Functional adhesion assays

Functional adhesion assays were performed as previously described[24]. Briefly, 96-well Immunosorp (Nunc, Sigma Aldrich, St Louis, MO, USA) plates were coated with either 50 μL (40 μg/mL) of soluble Cultrex mouse collagen IV (BD Biosciences, San Jose, CA, USA) or BSA and allowed to evaporate overnight at room temperature. Cells were washed once in serum-free MEM-α and resuspended at a density of 1×10^6 cells/mL with 1×10^5 cells added to each well. After 2 h of adhesion, unattached cells were removed by three washes with MEM-α, and adherent cells were fixed with 70% methanol for 10 min, dried, and subsequently stained with a solution of 0.02% crystal violet at 0.2% ethanol. The stained cells were solubilized in 100 μL Sorenson solution and absorbance was read at 540 nm with an automated 96-well plate reader (VERSAmax, Sunnyvale, CA, USA). Mean and SE values were calculated from the results in four independent wells. Experiments were repeated three times and results of representative experiments are shown.

Mining public databases

Microarray studies conducted on cancers included in the National Cancer Institute's list of the 10 most common solid tumors (bladder, breast, colon and rectal, endometrial, kidney (renal cell), lung, melanoma, pancreatic, prostate, thyroid; http://www.cancer.gov/cancertopics/types/commoncancers) were analyzed using Oncomine (http://www.oncomine.org, Compendia Bioscience, Ann Arbor, MI, USA). Data sets were ordered by under- or overexpression: P-value of integrin analyzed. All known integrins with corresponding probesets were analyzed including integrins α1-11, α2B, αL, αM, αX, αV, αE, β1-8, and βL1. The only integrins found to be significantly under- or overexpressed in at least five of the seven data sets were integrins α7, α8, α10, and β4. These four integrins were further analyzed using seven previously published microarray studies[25-31] to evaluate their mRNA expression. Six studies used Affymetrix human genome arrays with the probe set 216331_at for integrin α7, 214265_at for integrin α8, 206766_at for integrin α10, and 204990_s for integrin β4 with the exception of the Kaiser study which used probe set 211905_at for integrin β4. The seventh study by Haqq et al. used a microarray of 20,862 cDNA targets representing 19,740 unique genes (Research Genetics, Huntsville, AL, USA) with the probe set H44722 for integrin α10, and R87964 for integrin α8. The study by Dyrskjot et al.[32] included nine samples of normal bladder and five samples of normal bladder mucosa as well as 28 samples of superficial bladder cancer (superficial transitional cell carcinoma) comprising 15 tumor biopsies without surrounding carcinoma *in situ* and 13 tumor biopsies with surrounding carcinoma *in situ*. The study by Haqq et al.[26] included three samples of normal skin and six samples of melanoma. The study by Hou et al.[27] included 65 samples of normal lung and 27 samples of squamous cell lung carcinoma. The study by Jones et al.[28] included 23 samples of normal kidney and eight samples

of renal pelvis urothelial carcinoma (transitional cell cancers of the renal pelvis). The study by Kaiser et al.[29] included five samples of normal colon and 13 samples of colon mucinous adenocarcinoma. The study by Landi et al.[30] included 49 samples of normal lung and 58 samples of lung adenocarcinoma. The study by Richardson et al.[31] included seven samples of normal breast and 40 samples of ductal breast carcinoma.

Statistical methods

A data set of non-small cell lung cancer (NSCLC) tumor and adjacent normal samples (GSE19188), was used to identify genes differentially expressed between tumor and adjacent normal. Sample GSM475805 was identified as a corrupt CEL file and excluded from all analyses. Data were normalized with the RMA algorithm using the libaffy software[33], and principle component analysis (PCA) was performed on the remaining 155 samples, using the Evince software (UmBio AB, Umeå, Sweden), to identify outliers. Three tumor samples (GSM475677, GSM475706, GSM475780) clustered in the middle of the adjacent normal samples, and one adjacent normal sample (GSM475666) fell within the tumor distribution. These samples were discarded for all further tumor *versus* adjacent normal analysis. An additional six outlier adjacent normal samples (GSM475752, GSM475755, GSM475766, GSM475781, GSM475807, GSM475811) were identified as falling outside the otherwise tight adjacent normal distribution, lying between the adjacent normal and tumor clusters. Subsequent analyses (data not shown) confirmed that these samples exhibit a more tumor-like gene expression profile than the other adjacent normals, and were thus discarded from further tumor *versus* adjacent normal analysis. The final tumor plus adjacent normal data set, after discarding outliers, consisted of 58 adjacent normal and 87 tumor samples. Samples were then ranked by

their respective scores from the first principle component of a partial least squares discriminate analysis (PLS-DA) model, trained on tumor *versus* adjacent normal, to order them from most normal-like to most tumor-like global gene expression.

Statistical significance of data was calculated using a two-tailed Student's *t*-test in Microsoft Excel.

RESULTS

Integrin α10 expression is pRb-dependent in mouse osteoblasts

In previous work we examined the effects of pRb deficiency in mouse osteoblasts and identified a role for pRb as a regulator of cell adhesion[10]. The microarray screen conducted as part of that work identified integrin α10 as a potential pRb-activated gene. Integrin α10 expression in osteoblasts has not been previously measured, but is known to be high in chondrocytes. Both osteoblasts and chondrocytes are derived from a common progenitor cell so as a first step, we measured the endogenous expression levels of integrin α10 mRNA in mouse osteoblast MC3T3 cells, both wild-type and pRb null, at two different levels of confluency to verify that these cells are integrin α10 positive and that integrin α10 is indeed a pRb target (**Figure 3.1**). We found that integrin α10 expression is much higher in pRb wild-type cells as compared with their pRb null counterparts. Expression levels were also found to be highest at 100% confluency likely due to increased ECM availability at higher cell densities. The high levels of endogenous integrin α10 and the obvious changes in wild-type *versus* pRb null cells make MC3T3 mouse osteoblast cells an ideal model system to study changes in integrin function and expression engendered by pRb.

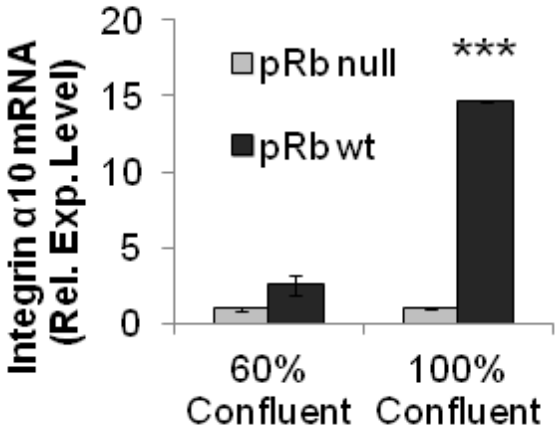


Figure 3.1 Expression of endogenous integrin α10 mRNA in MC3T3 wild-type and MC3T3 pRb null cells. RNA extraction was performed at two different levels of cell confluency followed by qRT-PCR with integrin α10 specific primers to determine expression levels relative to GAPDH. The MC3T3 pRb null expression levels were set as one for each RNA extraction. Asterisk represents significant p-value: *=p<0.05.

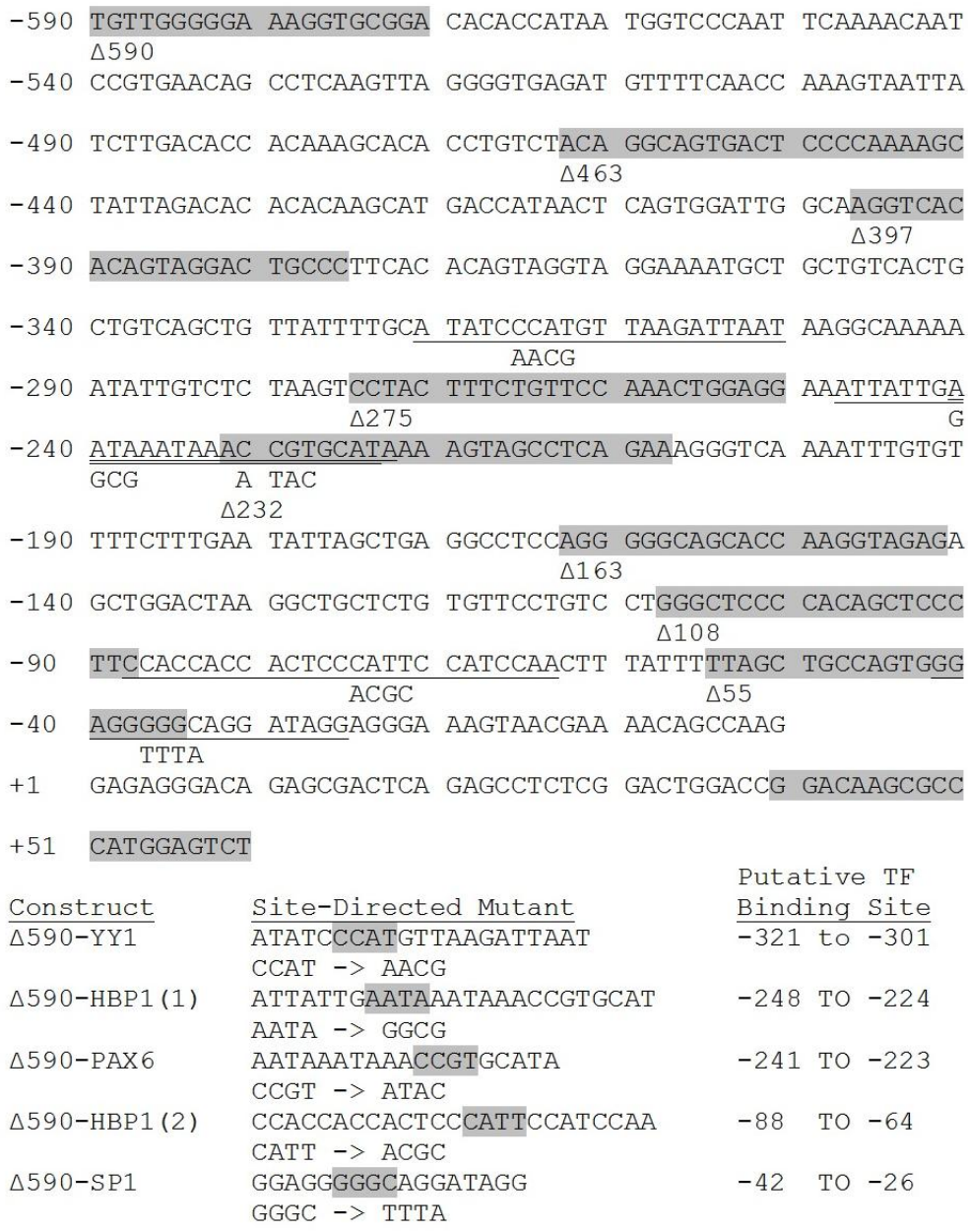
Phosphorylation of pRb is not necessary for activation of integrin α10 promoter

We hypothesized that pRb was regulating integrin α10 at the level of transcription. In order to test this, we defined a putative integrin α10 promoter as the 590 base pairs of DNA immediately upstream of the start of the site of transcription using the fully sequenced chromosome 3 from the Mouse Genome Project (NCBI Reference Sequence: NC_000069.6). A variety of constructs were made of this integrin α10 putative promoter (**Figure 3.2**) and were determined to be active. When acting as a regulator of cell cycle progression, the phosphorylation status of pRb determines whether or not cells proceed through the G1 checkpoint. Upon phosphorylation by cyclin D/CDK4/6 complexes, pRb becomes inactivated and allows progression to S-phase. To determine if phosphorylation status altered the role of pRb in regulating expression of integrin α10, and to narrow down the minimal responsive region of the integrin α10 promoter, three deletion constructs of the integrin α10 promoter were

transfected into MC3T3 cells along with the wild-type pRb large pocket or the non-phosphorylatable pRb large pocket (**Figure 3.3A**). Phosphorylation was not necessary for the activation of the integrin α10 promoter. In order to confirm that differences in activation were due to intrinsic properties of the pRb constructs and not due to increased transfection efficiency of either one, a western blot was conducted and the constructs expressed comparable levels of protein (**Figure 3.3B**). As a final confirmation that the phosphorylation of pRb is not necessary for upregulation of integrin α10 MC3T3 pRb wild-type cells were treated with two doses of PD0332991, a drug specific for CDKs 4 and 6, the two CDKs responsible for phosphorylating pRb (**Figure 3.3C**). Endogenous levels of integrin α10 mRNA were measured 48 h after application of the drug. Cells treated with the drug did not exhibit any inhibition of integrin α10 mRNA expression as compared with the untreated control cells.

E2F1 and E2F3 do not repress transcription of integrin α10

One of the best characterized roles of pRb is as a regulator of transcription involving its interaction with the E2F family of transcription factors. E2F involvement would likely be the result of the E2F transcription factor repressing transcription of the integrin α10 promoter. Addition of pRb would activate the integrin α10 promoter through alleviating this E2F-mediated repression. Previous studies have identified E2F1 as a repressor of the Mcl-1 promoter[19]. We explored the potential of E2F1 and E2F3 to repress the integrin α10 promoter in the absence of pRb. The addition of E2F1 and E2F3 expression plasmids did not result in repressed transcription (**Figure 3.4**) suggesting that pRb is acting through a non-E2F mediated pathway.



Construct	Site-Directed Mutant	Putative TF Binding Site
Δ590-YY1	ATATCCCATGTTAAGATTAAT CCAT -> AACG	-321 to -301
Δ590-HBP1(1)	ATTATTGAATAAATAAACCGTGCAT AATA -> GGCG	-248 TO -224
Δ590-PAX6	AATAAATAAACCGTGCATA CCGT -> ATAC	-241 TO -223
Δ590-HBP1(2)	CCACCACCACTCCCATTCCATCCAA CATT -> ACGC	-88 TO -64
Δ590-SP1	GGAGGGGGCAGGATAGG GGGC -> TTTA	-42 TO -26

Figure 3.2 Schematic of the integrin α10 promoter. (A) Schematic shows the eight 5'-nested deletion constructs characterized (Δ590, Δ463, Δ397, Δ275, Δ232, Δ163, Δ108, Δ55), the five site-directed mutants of the Δ590 construct (Δ590-YY1, Δ590-HBP1(1), Δ590-PAX6, Δ590-HBP1(2), Δ590-SP1) with their 4 base pair substitutions, the putative transcription factor binding sites (underlined), and the primers used for cloning (highlighted in gray). All constructs begin at the base pair by which they are named (upstream relative to the start of the site of transcription) and end at +60.

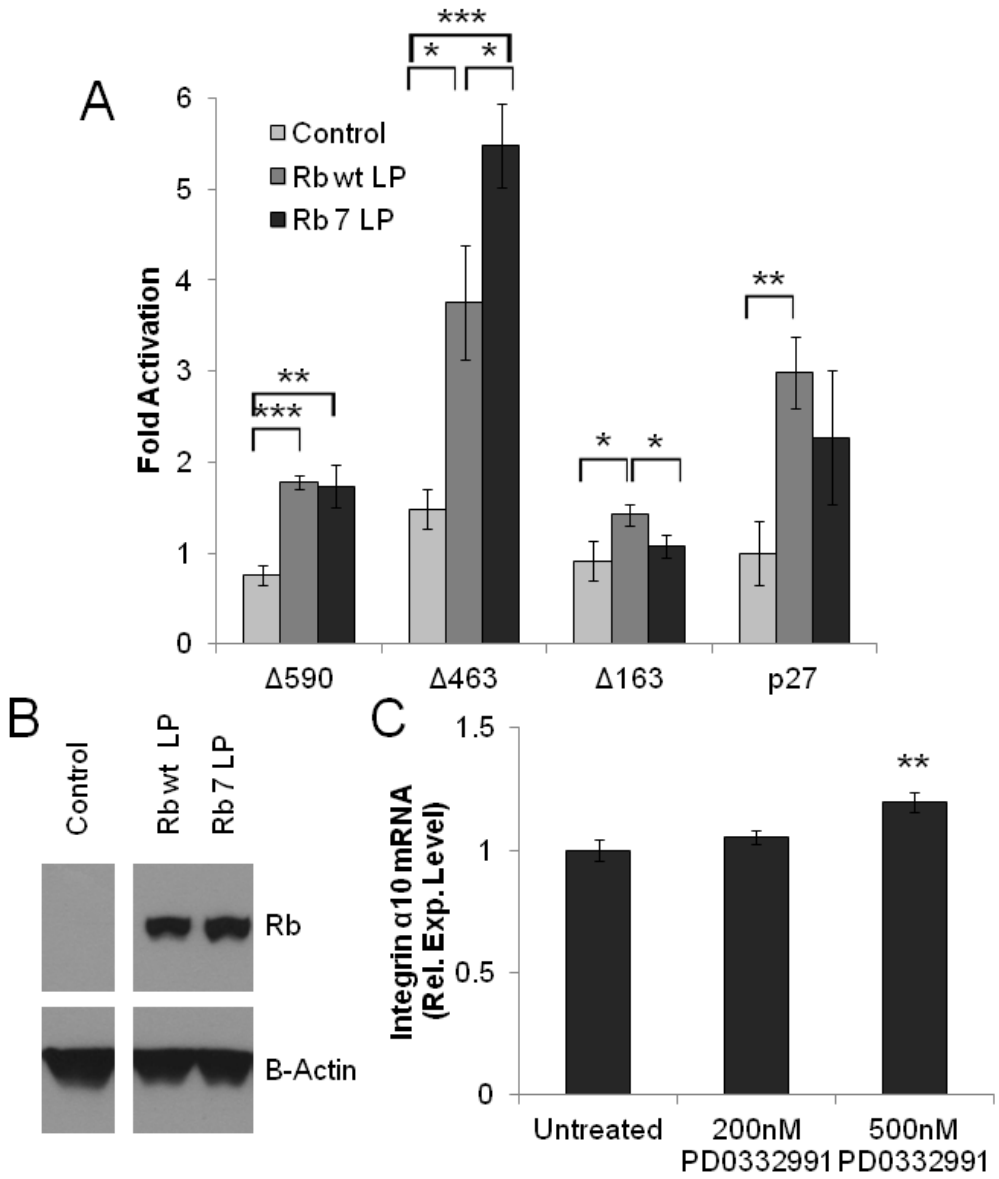


Figure 3.3 Effect of pRb phosphorylation status on integrin α10 transcription. (A) Three integrin α10 promoter deletion constructs (Δ590, Δ463, Δ163) and a p27 promoter construct control were co-transfected separately with either pEGFPc2 (control), pRb wild-type large pocket (Rb wt LP), or pRb non-phosphorylatable large pocket (Rb 7 LP) into MC3T3 pRb null cells. Promoter activation was measured by luciferase activity. p27 control activity is set to one. (B) Western blot of control (pEGFPc2), pRb wild-type large pocket (Rb wt LP), and pRb non-phosphorylatable large pocket (Rb 7 LP) transfected into MC3T3 pRb null cells. Blot was probed for pRb (9309; Cell Signaling) and β-Actin (A5441; Sigma). (C) Expression of endogenous integrin α10 mRNA in MC3T3 wild-type cells treated with the CDK 4/6 specific drug, PD0332991 at two different doses (200nM and 500nM). RNA extraction was performed 48 hours post drug treatment followed by qRT-PCR with integrin α10 specific primers to determine expression levels relative to GAPDH. The untreated MC3T3 pRb wild-type expression levels were set as one. Asterisks represent significant p-values as follows: *=$p<0.05$, **=$p<0.01$, and ***=$p<0.001$.

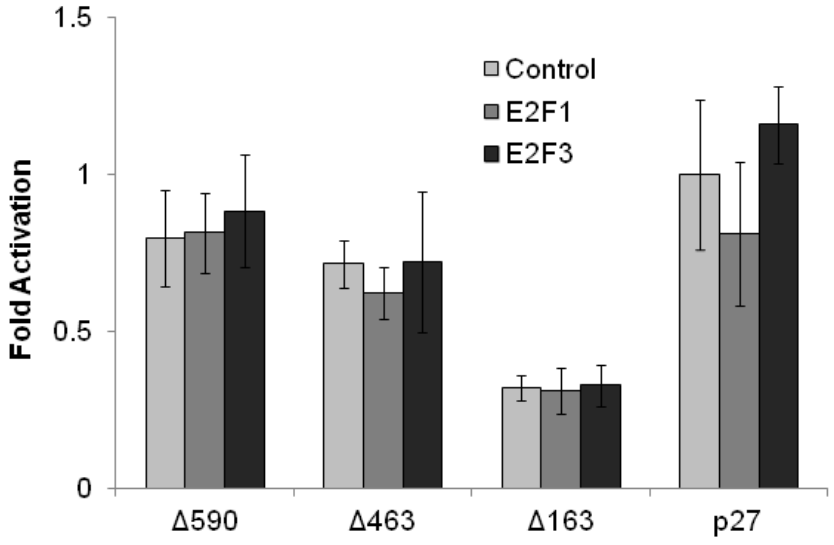


Figure 3.4 Effect of E2F1 and E2F3 on Integrin α10 promoter activation. Three integrin α10 promoter deletion constructs (Δ590, Δ463, Δ163) and a p27 promoter construct control were co-transfected separately with either pEGFPc2 (control), E2F1pcDNA3 (E2F1), or E2F3pcDNA3 (E2F3) into MC3T3 pRb null cells. Promoter activation was measured by luciferase activity. p27 control activity is set to one. No changes were statistically significant.

Exploring the possible involvement of other transcription factors

According to the Biological General Repository for Interaction Data sets (BioGRID; http://www.thebiogrid.org),[34] human pRb associates with 145 unique interactors. Bioinformatic analysis of the integrin α10 promoter was conducted to look for putative binding sites for these 145 interactors using the Genomatix program MatInspector (Genomatix Software GmbH, Munich; http://www.genomatix.de; Cartharius *et al.*[35] and Quandt *et al.*[36]). This analysis revealed putative transcription factor binding sites for known pRb interacting transcription factors YY1, yin-yang 1, from -321 to -301bp, HBP1, high-mobility group box transcription factor 1, from -248 to -224bp (HBP1(1)), PAX6, paired box homeotic gene-6, from -241 to -223, a second HBP1 site from -88 to -64bp (HBP1(2)), and Sp1, specificity protein 1, from -42 to -26bp (**Figure 3.2**). We decided to further analyze the potential involvement of Sp1 as it is

not only known to interact with pRb, but has previously been shown to have a role in the upregulation of integrin α5 along with ZEB2[6]. Additionally, this site identified on the mouse promoter was also conserved on the human promoter with a putative binding site of base pairs -17 to -1 with respect to the start of the site of transcription (data not shown). We explored the possibility that Sp1 could act with pRb as a co-activator of transcription of the integrin α10 gene. Sp1 was added in the presence and absence of pRb to three integrin α10 promoter constructs in MC3T3 pRb null cells looking for a synergistic activation when both Sp1 and pRb were added, however no activation was observed to correspond with the addition of Sp1 (data not shown).

As there was no evidence of Sp1 having a role in the activation of integrin α10, we decided to look at the other putative pRb interacting transcription factor binding sites on the integrin α10 promoter. Site-directed mutants were made in which the four base pairs most critical for the binding of the transcription factor to the promoter (the 'canonical binding site' as determined by Genomatix) were mutated (Δ590-YY1, Δ590-HBP1(1), Δ590-PAX6, Δ590-HPB1(2), and Δ590-Sp1). In addition to the site-directed mutants, a series of progressive deletion mutants were made in which each successive deletion removed one of the putative transcription factor binding sites (Δ397, Δ275, Δ232, Δ108, and Δ55). The site-directed mutation of individual binding domains did not reveal a single motif responsible for pRb activation of the integrin α10 promoter (**Figure 3.5B**) pointing to either a lack of individual importance of these transcription factors in the activation of the integrin α10 promoter, or continued binding despite the mutated base pairs suggesting that they were not, in fact, the canonical binding site. Interestingly, the progressive deletions of the integrin α10 promoter resulted in progressively diminished transcriptional activation, all the while not losing the

activating effect of pRb up until the Δ55 construct suggesting a pRb responsive region between -108bp to -55bp upstream of the start of the site of transcription (**Figure 3.5C**).

As all of the constructs and controls are activated by the addition of pRb, a control experiment was conducted using the promoter of CIITA, the class II major histocompatibility complex transactivator, a gene not known to be pRb regulated. As expected, the addition of pRb did not cause any significant change in CIITA transcription (**Figure 3.5A**).

We used a bioinfomatic approach to create the list of transcription factors that were potentially involved in activating integrin α10, but relying on the computer algorithm and exploring only the region directly upstream of the start of the site of transcription may have excluded a key transcription factor. A different starting point could have been a literature search, or including a greater region of the upstream sequence to search. With this list in mind, additional experiments that could have been conducted to determine the involvement of other transcription factors include using siRNA to deplete the transcription factors to see if any significant decrease in integrin α10 activation occurred. Conversely, only Sp1 DNA was added into the luciferase experiments to see if it activated the expression of integrin α10, but other transcription factor DNAs could also have been added. An EMSA could have been used to determine if any of these transcription factors bind to the promoter sequence of integrin α10. Although not shown in any figures, multiple ChIP experiments were conducted to observe pRb binding along the integrin α10 promoter. These same samples could have immunoprecipitated using antibodies directed against the putative transcription factors involved to show their binding to the integrin α10 promoter. This binding could be in complex with pRb, a possibility that could be explored using co-immunoprecipitation to determine if the transcription factors were

directly bound to pRb. Additional mutagenesis experiments could have been used to confirm the specific promoter regions necessary for activation by these transcription factors.

pRb wild-type osteoblasts exhibit higher protein levels and adhesion of Integrin α10 compared with matched pRb null cells

pRb-activated expression of integrin α10 mRNA is effectively translated into higher levels of integrin α10 protein as visualized by immunofluorescence (**Figure 3.6A**). Integrin α10 protein in pRb wild-type osteoblasts is located in high concentrations at the cell membrane forming a well-defined cellular margin. The matched pRb-null osteoblasts are characterized by lower levels of membranous integrin α10 protein with irregular signal at cell margins. For both cell types nuclear staining with the integrin α10 antibody is likely nonspecific. No differences in β-tubulin staining levels and patterns were observed between the two cell lines.

Next, we decided to test the functionality of the integrin α10 protein in both the pRb wild-type and pRb null osteoblasts. As expected, the higher levels of integrin α10 protein in pRb wild-type osteoblasts directly corresponded to a statistically significant change of approximately 47% greater binding to collagen IV as compared with matched pRb null cells (**Figure 3.6B**). This indicates that not only is more integrin α10 protein present in pRb wild-type osteoblasts, but this protein is functional and maintains cellular adhesion to ECM substrate that is lost when pRb is no longer present.

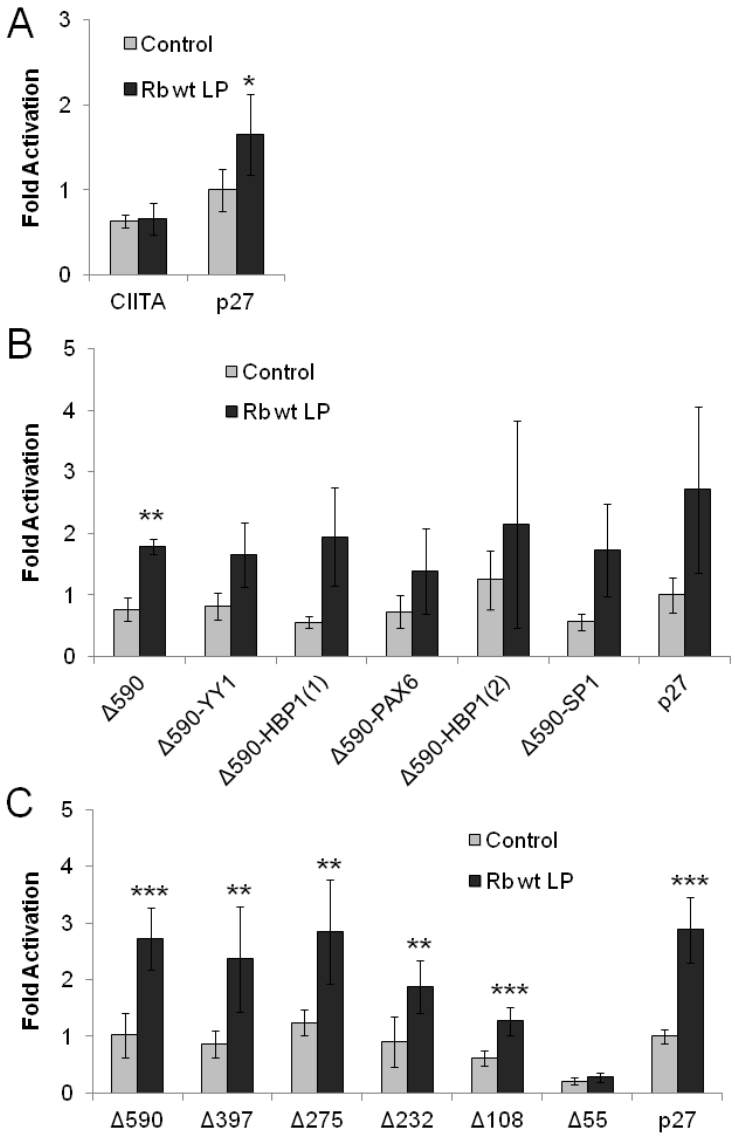


Figure 3.5 Effect of pRb on activation of site-directed and deletion mutants of the integrin α10 promoter. (A) A CIITA promoter construct and a p27 promoter construct control were co-transfected separately with either pEGFPc2 (control) or pRb wild-type large pocket (Rb wt LP) into MC3T3 pRb null cells. Promoter activation was measured by luciferase activity. p27 control activity is set to one. (B) An integrin α10 promoter construct (Δ590), five site-directed mutants of that construct (Δ590-YY1, Δ590-HBP1(1), Δ590-PAX6, Δ590-HBP1(2), Δ590-SP1) and a p27 promoter construct control were co-transfected separately with either pEGFPc2 (control) or pRb wild-type large pocket (Rb wt LP) into MC3T3 pRb null cells. Promoter activation was measured by luciferase activity. p27 control activity is set to one. (C) Six integrin α10 promoter deletion constructs (Δ590, Δ397, Δ275, Δ232, Δ163, Δ55) and a p27 promoter construct control were co-transfected separately with either pEGFPc2 (control) or pRb wild-type large pocket (Rb wt LP) into MC3T3 pRb null cells. Promoter activation was measured by luciferase activity. p27 control activity is set to one. Asterisks represent significant p-values as follows: *=$p<0.05$, **=$p<0.01$, and ***=$p<0.001$.

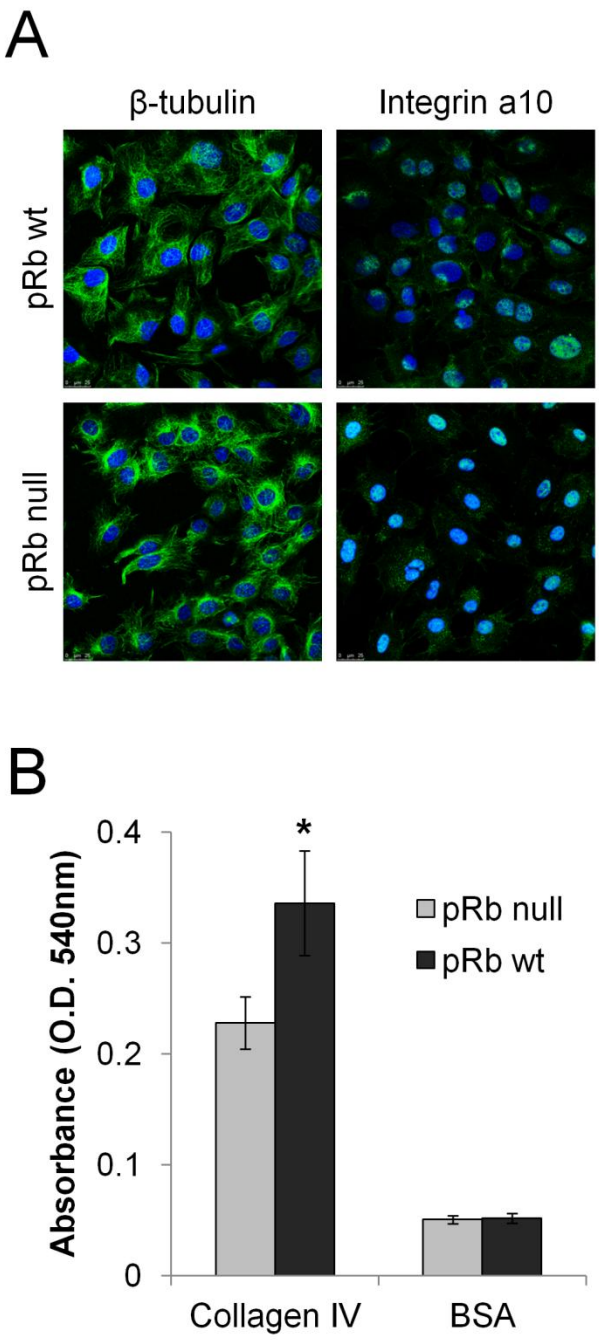


Figure 3.6 Effect of pRb on Integrin α10 protein levels and function. (A) MC3T3 pRb wild-type and pRb null cells were probed with antibodies against β-tubulin (2128S; Cell Signaling) and integrin α10 (AB6030; Millipore) (green) and stained with DAPI (blue; nuclei). They were then examined using confocal immunofluorescence microscopy. Representative images are shown. (B) MC3T3 pRb wild-type and pRb null cells were incubated with either collagen IV or BSA substrate and allowed to adhere. Attached cells were permeabilized and stained with crystal violet. Absorbance at 540nm was measured. Asterisk represents significant p-value: *=$p<0.05$.

Identification of an 'integrin switch' that occurs in multiple solid tumors

Analysis of integrin expression in public databases of the most common solid tumors led to the identification of a four integrin signature that appears as consistently significant changes in mRNA expression levels between tumors and their matched normal tissues. Specifically, the signature consists of overexpression of integrin β4 and underexpression of integrins α7, α8, and α10 (**Table 3.1, Figure 3.7A-D)**. Increased expression of integrin β4 has also previously been linked to metastasis in human osteosarcoma cells.[37]

Due to the relative rarity of osteosarcoma and the propensity to treat the disease before tumor resection, no data on integrin α10 expression in tumor and matched normal tissue could be obtained. Instead, seven solid tumor types from the National Cancer Institute's list of the ten most common solid tumors were examined, including two types of lung cancer, and all exhibited significantly underexpressed integrin α10 as compared to their matched normal control tissues (**Table 3.1, Figure 3.7E-H**). Specifically, ductal breast carcinoma, the most common type of breast cancer, had the largest fold-change[31] (**Figure 3.7G**) followed by melanoma[26] (**Figure 3.7H**). Squamous cell lung carcinoma, a subtype of non-small cell lung cancer (85% of all lung cancers) which accounts for 25-30% of all non-small cell lung cancers, had the third highest fold-change[27] (**Figure 3.7F**). Next was superficial bladder cancer, which accounts for 80% of bladder cancers[32]. Renal pelvis urothelial carcinoma was next[28]; this study also showed downregulation or no change in renal pelvis urothelial cancer in all of the adhesion genes analyzed (e.g. ADAM12, ADAMTS5, ADAM9, TNFAIP6, GNRH2, CD47, CD36, ICAM1, CD96, CD99, SCARB1, CDW52). Sixth was lung adenocarcinoma, which accounts for roughly 50% of all non-small cell lung cancers[30] **(Figure 3.7E)**. Finally, colon mucinous adenocarcinoma was seventh.[29]

Table 3.1 Integrin expression in seven common solid tumor types.

	Integrin α7		Integrin α8		Integrin α10		Integrin β4	
Database	**Fold Change**	**p-value**	**Fold Change**	**p-value**	**Fold Change**	**p-value**	**Fold Change**	**p-value**
Dyrskjot Bladder 3	-1.587	1.63E-05	-1.475	2.25E-04	-1.604	3.99E-07	2.313	1.82E-05
Haqq Melanoma	NC	2.68E-01	-1.504	3.00E-03	-3.471	2.04E-04	N/A	N/A
Hou Lung	-1.734	1.64E-14	-5.742	1.91E-15	-1.828	2.88E-18	3.591	8.92E-10
Jones Renal	-1.523	2.45E-05	-3.993	7.00E-17	-1.499	5.91E-10	2.500	2.00E-03
Kaiser Colon	NC	9.40E-02	-1.494	1.00E-03	-1.222	6.09E-06	2.852	5.81E-05
Landi Lung	-1.239	1.01E-07	-2.375	4.58E-17	-1.357	9.02E-14	1.588	3.36E-07
Richardson Breast	-5.362	3.41E-06	NC	1.70E-02	-3.491	2.00E-06	-2.735*	1.86E-08

Data comes from OncomineTM (www.oncomine.org, Compendia Bioscience, Ann Arbor, MI). The fold change value is determined by comparing the means of the two classes in an analysis on a log2 scale and then converting that difference to a linear scale (www.oncomine.org). NC = no significant difference in expression between normal and tumor tissue. N/A = this study did not have a probe for the gene of interest. Only one significant change occurred in the opposite direction of the other six datasets and is denoted with an asterisk.

CONCLUSIONS

In this study we have demonstrated that integrin α10 is expressed in mouse osteoblasts and that the expression of this gene is activated at a transcriptional level by pRb. This activation of expression directly leads to increased integrin α10 protein levels and greater adhesion to a collagen substrate. Our analysis of publically available databases revealed that integrin α10 is significantly downregulated in tumor tissue compared with normal in multiple solid tumors. These findings point to an important role for changes in integrin α10 expression during disease progression.

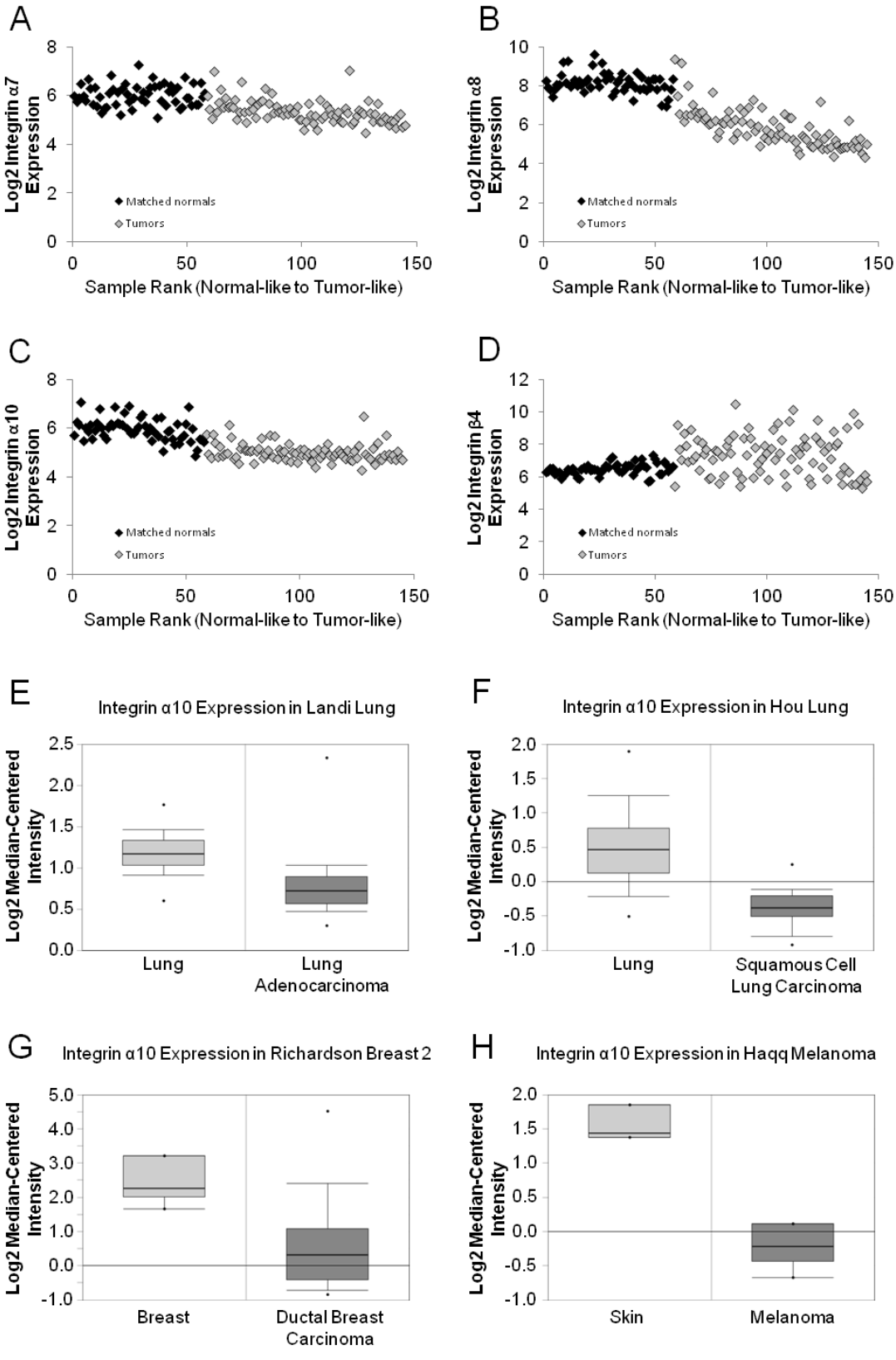

A
Log2 Integrin α7 Expression
Matched normals
Tumors
Sample Rank (Normal-like to Tumor-like)
B
Log2 Integrin α8 Expression
Matched normals
Tumors
Sample Rank (Normal-like to Tumor-like)
C
Log2 Integrin α10 Expression
Matched normals
Tumors
Sample Rank (Normal-like to Tumor-like)
D
Log2 Integrin β4 Expression
Matched normals
Tumors
Sample Rank (Normal-like to Tumor-like)
E
Integrin α10 Expression in Landi Lung
Log2 Median-Centered Intensity
Lung
Lung Adenocarcinoma
F
Integrin α10 Expression in Hou Lung
Log2 Median-Centered Intensity
Lung
Squamous Cell Lung Carcinoma
G
Integrin α10 Expression in Richardson Breast 2
Log2 Median-Centered Intensity
Breast
Ductal Breast Carcinoma
H
Integrin α10 Expression in Haqq Melanoma
Log2 Median-Centered Intensity
Skin
Melanoma

Figure 3.7 Changes in integrin expression occur in multiple solid tumors. (A-D) The GSE19188 dataset of NSCLC tumor and adjacent normal samples was ranked from most normal to most tumor-like. Samples 1-58 are the adjacent normals (black diamonds) while samples 59-145 represent tumor samples (light gray diamonds). Gene expression was determined via microarray for integrin α7 using probe 216331_at (A), α8 using probe 214265_at (B), α10 using probe 206766_at (C), and β4 using probe 204990_s_at (D). (E-H) Oncomine™ (www.oncomine.org, Compendia Bioscience, Ann Arbor, MI) was used for analysis and visualization of integrin α10 expression in four common solid tumor types. Datasets were ordered by under-expression: P-value of Itga10. (E) Analysis of integrin α10 expression in normal lung (n=49) vs. lung adenocarcinoma (n=58) from the Landi Lung database. (F) Analysis of integrin α10 expression in normal lung (n=65) vs. squamous cell lung carcinoma (n=27) from the Hou Lung database. (G) Analysis of integrin α10 expression in normal breast (n=7) vs. ductal breast carcinoma (n=40) from the Richardson Breast 2 database. (H) Analysis of integrin α10 expression in normal skin (n=3) vs. melanoma (n=6) from the Haqq Melanoma database.

The downregulation of integrin α10 following pRb loss may be part of a larger oncogenic event in which cellular differentiation is lost. Differentiation has previously been measured as part of a 'cadherin switch' in which epithelial cells lose their epithelial phenotype and become more mesenchymal in character. We propose that there is a simultaneous 'integrin switch' involving the downregulation of integrins α10, α7, α8, and potentially other positive prognostic integrins with concurrent upregulation of less favorable integrins, including integrin β4, that results in a loss of adhesion to the local extracellular matrix allowing previously anchored cells to metastasize. As a highly tissue specific integrin, loss of α10 may be associated with a loss of differentiation. There is potential to use integrin α10 levels as a prognostic marker. High levels indicate a tumor that has retained differentiation and will likely have better overall survival while decreased expression levels are an indicator of a more advanced disease state in which pRb has been lost or mutated.

This study builds upon our previous work defining a role for pRb as a regulator of mouse osteoblast cell adhesion[10] and adds to the growing literature that links pRb to the metastatic cascade.[39]

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CHAPTER FOUR:

The Molecular Biology and Therapeutic Implications of *STK11*/*LKB1* Mutations in Lung Cancer[x]

ABSTRACT

Lung cancer is the leading cause of cancer-related death in the U.S. and additional targeted therapies are desperately needed to treat these patients. *STK11* is the third most frequently mutated gene in lung adenocarcinoma following only *KRAS* and *TP53*, yet its mutational status is not currently clinically evaluated and no therapies have been approved to specifically target its pathway. A deep understanding of the complex pathways controlled by STK11 and their alterations in cancer are required to develop effective therapies for patients with loss-of-function mutations. In this article we present the current understanding of STK11, focusing on its molecular biology and therapeutic implications, including a compilation of studies evaluating *STK11* somatic mutations in human lung cancer tissue and how the frequency of these mutations varies across histological subtypes and patient populations. Finally, we review the strategies being used to target STK11-deficient cancers at the clinical trial, pre-clinical, and basic science levels as well as proposing potential new therapies that might benefit this patient population.

[x] This chapter will be submitted for publication. See Appendix A for details.

OVERVIEW

In the U.S., lung cancer is the second most diagnosed cancer in both men and women following only prostate cancer and breast cancer, respectively.[1] Although the incidence rate has been declining in men over the past two decades, in women the incidence rate has just recently started to decrease. Lung cancer is the leading cause of cancer-related death among both men and women. Non-small-cell lung cancer (NSCLC) represents more than 80% of lung cancer diagnoses and has an overall 5-year survival rate of approximately 16%, which decreases precipitously among patients diagnosed with late stage disease.[2]

Traditionally, decisions on lung cancer treatment have been based on clinical characteristics such as stage at diagnosis, performance status of the patient, and tumor histology. More recently, however, treatment strategies involve the subdivision of NSCLC into molecular subsets based on gain-of-function mutations in oncogenes and loss-of-function mutations in tumor suppressors. Many of these alterations occur disproportionately across lung tumor histopathologies,[3-5] which likely indicate differences in carcinogenesis and cell type of origin. Mutations in these genes confer an advantage to tumor cells by activating signaling pathways crucial for cancer cell proliferation and survival. Loss-of-function mutations in tumor suppressor genes are more common events than gain-of-function mutations in oncogenes, but have traditionally been much more difficult to treat therapeutically as restoring their function would involve delivery of wild type DNA to tumor cells and no viable delivery systems have been developed to date. Instead, the most effective therapies are small molecule inhibitors that block gain-of-function activity, especially of proteins on the cell membrane. Drugs designed to specifically inhibit these molecular targets have significantly extended survival times for NSCLC

patients whose tumors harbor these gain-of-function mutations.[6] Restoring the function of mutated tumor suppressors is generally achieved through targeting key downstream signaling pathways that exhibit increased activity when the tumor suppressor is lost. Development of therapeutic regimes to replace the activity of tumor suppressors requires a deep understanding of the far-reaching effects of their loss-of-function mutations and studying the molecular biology of these key proteins is central to this strategy. STK11 is one of these key tumor suppressors.

The *STK11* gene encodes a tumor suppressor located on chromosome 19p13.3 that encodes the serine/threonine protein kinase also known as liver kinase β1 (LKB1). The gene spans 23kb and is made up of nine coding exons (exons 1-9) and a final non-coding exon (exon 10). Germline mutations in *STK11* were first identified in patients with Peutz-Jeghers syndrome,[7] a rare autosomal dominant disorder which is associated with an increased risk of gastrointestinal and other malignancies,[8,9] with over 80% of patients developing cancer by the age of 70.[10] Studies have also found that *STK11* somatic mutations are quite common in NSCLC, especially among adenocarcinomas, suggesting an important role for STK11 in lung tumorigenesis.[11-18] In this article we review the current evidence regarding the role of *STK11* mutations in lung cancer focusing on the molecular biology and therapeutic implications.

STUDIES OF *STK11* MUTATIONS IN HUMAN LUNG CANCER

Although rarely mutated in most human cancers, *STK11* is the third most frequently mutated gene in lung adenocarcinoma after *KRAS* and *TP53*.[5,11,15,16,19] **Table 4.1** summarizes studies that have generated *STK11* somatic mutation data from human lung cancer tissue. The collection of these data from primary tumor samples is often complicated by the fact that tumor

suppressor genes such as *STK11* can undergo mutational and deletional events that can be missed due to normal tissue contamination, resulting in marked underrepresentation. With that in mind, the frequency of *STK11* mutations in these studies range from 0.6% to 44.4%. Two studies reported the frequency of *STK11* mutations across adenocarcinoma subtypes,[5,20] some analyzed the overall frequency of *STK11* mutations across multiple histological subtypes,[21-25] while other studies reported *STK11* inactivating mutations in other lung cancer histology subtypes (e.g., 19% of squamous cell carcinomas, 14% of large cell carcinomas, and 25% of adenosquamous carcinomas).[14-16,21,26,27] The spectrum of mutation frequency across histological subtypes is consistent with previously published data from mouse models where *STK11* deficiency altered the resulting spectrum of tumor histology.[12] Specifically, *STK11*-deficient tumors were found in adenocarcinoma, squamous, and large cell carcinoma, whereas the tumors in other genetic models of murine lung cancer were solely adenocarcinoma histology.[12] None of the studies found any associations with overall survival which contrasts what is known about *EGFR* and *KRAS* mutations. *EGFR* mutations are associated with sensitivity to EGFR-tyrosine kinase inhibitors, namely gefitinib and erlotinib,[28] but it is also known that patients with *EGFR* mutant tumors have better overall survival regardless of treatment,[29] while lung cancer patients with *KRAS* mutant tumors are associated with worse overall survival, especially in patients with adenocarcinoma and early stage disease.[30] However, STK11 inactivation has been shown to be a prominent biomarker for poor outcome in cervical cancer, with a median survival of only 13 months for patients with STK11-deficient tumors versus greater than 100 months for wildtype tumors.[26]

Table 4.1: Studies reporting *STK11* somatic mutations in human lung cancer.

REFERENCE	HISTOLOGY	ASSAY	STAGE	GENDER	N= ≥1 *STK11* MUTATIONS (%)	ASSOCIATIONS
Avizienyte et al.[31]	12 Squamous cell, 3 Large cell carcinoma, 1 Small cell carcinoma, 12 Adenocarcinoma	SSCP analysis	NR	NR	1 Adenocar. (12%)	NR
Sanchez-Cespedes et al.[11]	Adenocarcinoma	Manual sequencing	NR	NR	6 (30%)	NR
Matsumoto et al.[15]	106 Adenocarcinoma, 24 Adenocarcinoma, 25 Brain metastases	Manual sequencing	Stage I only, Stage I-III, Mets	NR	1 (0.9%) 3 (12.5%) 3 (12%)	*STK11* mutation only men/smokers; No *EGFR/ STK11* double mutants
Onozato et al.[32]	81 Adenocarcinoma, 14 Squamous cell, 1 Adenosquamous, 2 Large cell carcinoma, 1 Small cell carcinoma, 1 Carcinoid	Manual sequencing	57 Stage I, 13 Stage II, 27 Stage III, 3 Stage IV	59 Males, 41 Females	3 (3%)	All 3 patients with *STK11* mutations were male smokers
Ji et al.[16]	80 Adenocarcinoma, 42 Squamous cell, 5 Adenosquamous, 10 Large cell carcinoma, 6 Unknown	Direct exon sequencing and copy loss by MLPA	NR	NR	37 (26%)	NR
Lee et al.[33]	105 Adenocarcinoma, 54 Squamous cell	Direct sequencing	82 Stage I, 77 Stage II-IV	108 Males, 51 Females	1 (0.6%)	824delC in male smoker with adenocarcinoma
Wilkerson et al.[20]	Adenocarcinoma	Direct sequencing	NR	NR	9 (12.3%)	No association with survival
Gill et al.[25]	68 Adenocarcinoma, 49 Squamous cell	Direct sequencing	78 Stage I, 47 Stage II-IV	79 Males, 45 Females	7/62 (11.4%)*	6 mutants were Adenocarcinoma; 1 was Squamous
Okuda et al.[34]	Adenocarcinoma	Direct sequencing	105 Stage I, 24 Stage II, 38 Stage III, 7 Stage IV	157 Males, 17 Females	5 (2.9%)**	No mutations among light smoker males.
Koivunen et al.[21]	207 Adenocarcinoma, 92 Squamous cell, 9 Adenosquamous	Direct sequencing	188 Stage I, 59 Stage II, 47 Stage III, 8 Stage IV	187 Males, 187 Females	34 (11%)	No association with survival; Caucasians and smokers more mut.
Strazisar et al.[22]	51 Adenocarcinoma, 67 Squamous cell, 11 Large cell carcinoma	DHPLC mutation screening	NR	107 Males, 22 Females	3 (2.3%)	All 3 mutations were in Adenocarcinoma
Tan et al.[24]	206 Adenocarcinoma, 24 Large cell carcinoma	Sequenom LungCarta panel	NR	158 Males, 72 Females	13 (5.7%)	No association with overall survival
Tam et al.[35]	Adenocarcinoma	cDNA sequencing	31 Stage I, 8 Stage II, 5 Stage III, 1 Stage IV	16 Males, 29 Females	20 (44.4%)	NR
An et al.[23]	354 Adenocarcinoma, 144 Squamous cell, 26 Large cell carcinoma	Direct sequencing	143 Stage I, 72 Stage II, 135 Stage III, 174 Stage IV	361 Males, 163 Females	8/101 (7.9%)	No association with overall survival
Suzuki et al.[36]	Adenocarcinoma	Direct sequencing	NR	54 Males, 43 Females	14 (14.4%)	3 *EGFR*/*STK11* double mutants
Ding et al.[5]	Adenocarcinoma	Direct sequencing	75 Stage I, 91 Stage II, 8 Stage III, 10 Stage IV	NR	34 (18%)	Neg. correlation between *EGFR* and *STK11* mutations
Chitale et al.[37]	Adenocarcinoma	Sequenom-based mutation screens	135 Stage I, 27 Stage II, 36 Stage III, 2 Stage IV	78 Males, 121 Females	28 (11%)	Mutations in *EGFR* and *STK11* mutually exclusive

NR=not reported *Sequenced 8 coding exons in 62 of 124 tumors that had loss of heterozygosity and identified eleven tumors (11.3% overall) with *STK11* mutation. **Only exons 1, 6, and 7.

Similar to mutations in *KRAS* and *EGFR*, there is evidence to suggest ethnic/racial differences in *STK11* mutations. Studies in Asian populations including Japanese, Korean, and Chinese have reported much lower *STK11* mutation rates, ranging from 3-7% in lung adenocarcinoma, compared to Whites.[21,32,38,39] This observation is similar to *KRAS* mutations in lung cancer, which frequency co-occur with *STK11* mutations, where it has been noted that lung tumors in Western populations harbor a higher frequency of *KRAS* mutation (15-50%) compared to Asian populations (5-15%). Asian populations have been found to express an *STK11* germline F354L polymorphism at a higher frequency than Western populations which has been reported in 10% of Chinese[38] and 6% of Korean populations compared to 0.2% of a Finnish population.[40] This allele has not been associated with cancer predisposition, and is likely a polymorphism that has no effect in lung cancer, but has been previously reported to affect cell polarity maintenance in an AMPK-dependent manner.[41]

The STK11 kinase domain spans over 60% of the entire length of the protein, encompassing amino acids 49-309 of the total 433. Mutations have been found throughout the entire gene without any well characterized hotspots. In general, recurrent C-terminal mutations located outside of the kinase domain do not impair STK11 kinase activity or interfere with its ability to promote growth arrest. These mutations do, however, impair STK11's regulation of the AMP-activated protein kinase (AMPK) cascade and cell polarity. These mutations include P324L, F354L (described above to have an increased frequency in Asian popluations), and T367M.[41]

The STK11 protein is catalytically active as a heterotrimeric complex with the STE20-related adaptor protein α (STRADα) and mouse protein-25 (MO25) and mutations have been found to interfere with STK11's interaction with these binding partners. An investigation of 34

point mutations in *STK11* revealed that 12 of these mutants failed to interact with these STRADα and MO25.[42] An analysis of the mutation sites led to the discovery of two binding sites on opposite surfaces of MO25 required for assembly of the heterotrimeric complex.[42]

Variations in *STK11* single nucleotide polymorphisms (SNPs) have also been linked to various diseases including gene variants in *STK11* rs8111699 which contribute to differences in insulin sensitivity and metformin efficacy in hyperinsulinemic girls with androgen excess, leading to the conclusion that the girls with the least favorable endocrine-metabolic profile improved the most with metformin therapy.[43] Another study that analyzed 772 patients with surgically resected colorectal adenocarcinoma for the *STK11* rs741765 SNP found that the GG genotype was significantly associated with a worse disease free survival and overall survival.[44]

DOWNSTREAM TARGETS OF STK11

As mentioned above, STK11 is catalytically active as part of a heterotrimeric complex. After translation, the nuclear localized STK11 binds STRADα, which shuttles the complex to the cytoplasm.[45] STRADα is also responsible for stimulating STK11 catalytic activity through an allosteric mechanism involving binding STK11 as a pseudosubstrate.[46] This interaction between STRADα/STK11 is further stabilized by MO25, which interacts with STK11's activation loop.[46]

An additional STK11 complex was discovered in which an STK11 isoform that differs in the C-terminal region, but not one that lacks a portion of the kinase N-terminal lobe domain, was found to interact with the chaperones Hsp90 and Cdc37.[47] This non-canonical STK11/Hsp90/Cdc37 complex is catalytically inactive unlike the STK11/STRADα/MO25 complex. Dissociation of the STK11/Hsp90 complex triggers recruitment of both Hsp/Hsc70

and CHIP proteins which are responsible for activating STK11 degradation. They proposed that these two chaperone complexes with antagonizing activities are responsible for fine tuning the cellular levels of STK11 protein.[47]

STK11 is a multi-functional kinase and has been found to be involved in a broad spectrum of cellular activity including metabolism, polarity, epithelial-mesenchymal transition, cell cycle regulation, apoptosis, and autophagy.[14] Many of these functions are regulated through STK11 activation of the AMPK cascade,[48] which is achieved through STK11 directly phosphorylating T172 on AMPK.[49] Identification of an STK11 splice variant that is lacking S431, but is able to activate AMPK equally as effective as full-length STK11, led to the conclusion that phosphorylation of STK11 at S431 is not required for downstream phosphorylation of AMPK and other kinases, as has been previously suggested.[50] Activation of AMPK and other members of the AMPK family is crucial to STK11's role as a regulator of cellular energy metabolism and cell polarity.[49,51] AMPK in particular has been found to have great importance as it acts as a tumor suppressor, serving to promote p53 acetylation and subsequent apoptosis of hepatocellular carcinoma cells.[52]

Downstream of STK11, AMPK activation negatively regulates the mTOR pathway. AMPK directly phosphorylates the TSC2 tumor suppressor under conditions of energy starvation, which serves to down-regulate mTOR signaling,[53] and STK11 is required for this repression of mTOR under low energy conditions.[54] In addition to inhibiting mTOR through TSC2, AMPK also directly phosphorylates the mTOR binding partner RAPTOR which is required for inhibition of mTORC1 and cell cycle arrest following energy stress.[55] These data together suggest a model for STK11 as a "low-energy-checkpoint tumor suppressor" in that wild

type STK11 acts as a sensor inducing AMPK signaling, which halts ATP-consuming processes in conditions of low cellular energy.[49]

STK11 is important in embryonic organogenesis in a tissue-dependent manner. Some tissue specific examples of the effects of STK11 inhibition can be found in the pancreas where this loss can lead to development of precancerous lesions in an AMPK-independent manner, whereas inhibition of STK11 in the lung leads to a cell-autonomous branching defect, a phenotype that can be rescued by an AMPK activator.[56]

For a representation of all of the downstream targets of STK11 see **Figure 4.1**.

STK11 AND TRANSCRIPTIONAL CONTROL

While regulation of the TSC2/mTOR pathway is clearly a major component of the biology of *STK11* mutations, STK11 also regulates the activity of a number of transcription factors and transcriptional programs via poorly understood mechanisms.[57,58] The best understood of these is the indirect control of CREB-regulated transcriptional cofactor (CRTC) phosphorylation. CRCT proteins are excluded from the nucleus due to phosphorylation events downstream of AMPK and AMPK-regulated salt-inducible kinases. In the absence of STK11, CRTC1 accumulates in the nucleus due to lack of phosphorylation, binds to the CREB transcription factor and activates transcription of CREB-regulated genes including LYPD3, NR4A2,[59] and NEDD9.[60] Two papers have recently examined the transcriptional program alterations associated with *STK11* mutation in large lung adenocarcinoma databases[61,62] and both confirmed that CREB-regulated genes are dramatically affected by STK11 mutations. One of

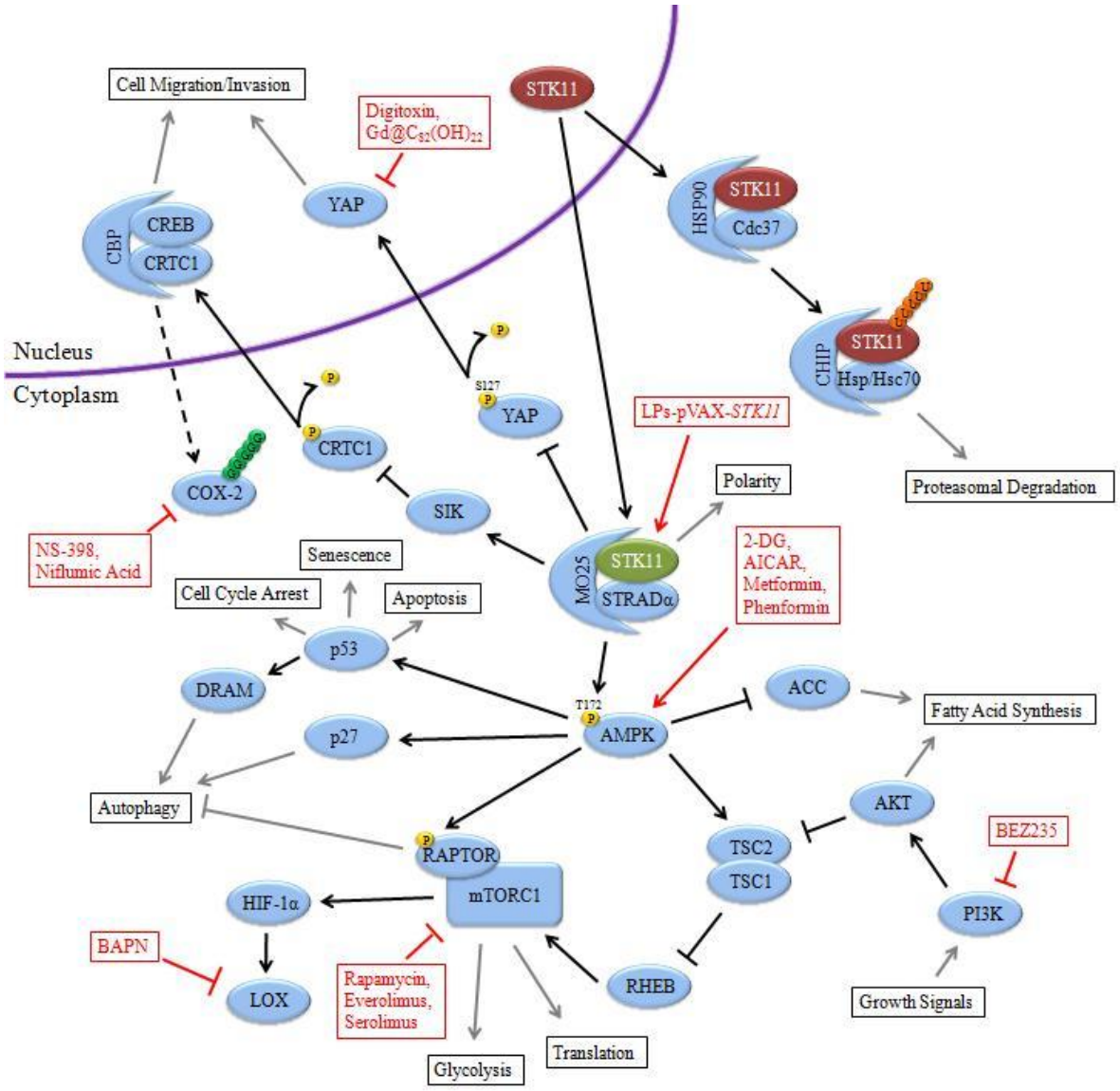

Figure 4.1 Downstream pathways controlled by STK11 and therapeutic targets. Inactive STK11 is shown in red; active STK11 in green. Yellow 'P's represent phosphorylation events; orange 'U's represent ubiquitination. Boxes in black represent downstream cellular processes; boxes in red represent drugs used to inhibit or activate targets.

these two analyses[62] also identified NRF2 activation and attenuation of the PI3K-AKT pathway as elements of STK11 mutations, suggesting the potential for targeted therapies (discussed below). Interestingly, the other paper demonstrated the STK11-CRTC1 circuit regulates COX-2 expression and its activation by glycosylation.[61] Furthermore, this paper used the C-MAP drug

response database to demonstrate that the highest ranking drugs correlating positively with STK11-mutated gene expression signature were activators of CRTC1 including forskolin and numerous PGE-2 analogs. Finally, this work demonstrated that COX-2 inhibitors specifically inhibited growth and motility in STK11-null cell lines and not in STK11-wild type cell lines, suggesting potential clinical application (discussed below).

STK11 AND ADHESION

As a multifunctional tumor suppressor, STK11 has also been linked to not only intra-cellular processes, but also the extra-cellular process of adhesion. The N-terminal domain of STK11 in particular has been found to be necessary for repressing the focal adhesion kinase (FAK) and stabilizing focal adhesions.[63] Loss of STK11 results in increased phosphorylation of FAK and enhanced adhesion to fibronectin.[63] Adhesion of lung cancer cells to fibronectin has previously been found to enhance tumorigenicity and confer cell adhesion mediated drug-resistance.[64] Specifically, fibronectin prevents apoptosis through integrin α5β1 mediated activation of COX2 and inhibition of p21 gene expression, an effect not seen with other matrix components such as collagen type I.[65] Fibronectin has also been found to stimulate NSCLC cell growth through activation of Atk, mTOR, and S6K with concurrent repression of STK11, AMPK, and PTEN.[66] This fibronectin-induced cascade can by blocked using an antibody against integrin α5β1.[66] Additionally, a novel tumor suppressor that binds to the cytoplasmic domain of integrin α5, Nischarin, has also been found to directly interact with STK11.[67] Loss of Nischarin and STK11 was found to increase migration and tumor growth through increased phosphorylation of PAK1 and LIMK1, as well as increased Cyclin D1 levels.[67] Another STK11

pathway found to negatively regulate cell adhesion involves the AMPK family member NUAK1 which is directly phosphorylated and activated by STK11. Inhibition of this STK11-NUAK1 pathway has been found to increase cell adhesion, and this pathway has been shown to be activated under conditions of cell detachment.[68]

Used as our model system for the study in **Chapter 3**, osteoblast differentiation was found to correspond with decreased phosphorylation of AMPK, a phenotype that was found to be inhibited by glucose restriction and metformin stimulation,[69] so there is likely a link between the two pathways described. Another cross-over between the two major tumor suppressors described in this dissertation is through the downstream regulation of the Rac1 pathway. Treatment of endothelial cells with simvastatin resulted in increased phosphorylation of STK11 and AMPK with subsequent AMPK-mediated activation of Rac1. Through its downstream pathway members STK11 has been shown to either activate or repress adhesion, adding to its arsenal of tumor suppressive abilities.

THERPEUTIC IMPLICATIONS AND TARGETING OF *STK11* MUTATIONS

At the molecular level, lung cancer is a complex and heterogeneous disease. Molecular alterations in lung cancer occur at multiple levels (e.g., genetic, epigenetic, and protein expression) and understanding the functional significance of these alterations can yield improvements in diagnosis, prognosis, and treatment.[70] New technologies in the identification of key and potentially targetable genetic alterations have resulted in a greater understanding of the molecular underpinnings of lung cancer. Loss-of-function mutations, while more common, are difficult to take advantage of therapeutically, so greater understanding of the multiple

biochemical pathways and characterization of these molecular alterations is needed to develop new therapeutic treatments and targeted precision medicine. STK11 in particular is an attractive target because it is the major upstream activator of the energy-sensing kinase AMPK and has been linked to a variety of important pathways in cancer. Restoring STK11 activity in mutant tumors is predicted to both sensitize tumors to additional chemotherapies and to increase susceptibility of cancer cells to cell death. **Table 4.2** summarizes all of the clinical trials, pre-clinical studies, and basic science studies that have focused on targeting STK11 deficient tumors using a variety of therapies.

A recent study sought to combine *STK11* gene therapy with low-dose cisplatin-based chemotherapy using cationic liposomes-mediated *STK11* gene, which sensitized lung cancer cells to cisplatin *in vitro* and *in vivo*, resulting in fewer lung metastatic nodules, and prolonged lifespan.[75] They believe this sensitization occurred through up-regulation of p53 and JNK and down-regulation of mTOR and MMPs 2 and 9.

Apart from attempts to replace the down-regulated *STK11* gene itself, strategies to target downstream members of the STK11 pathway are in various stages of development and have shown a range of efficacies. Some examples of this targeting strategy include taking advantage of the fact that cells deficient in STK11 are known to be hypersensitive to apoptosis induced by energy stress[49] as a side effect of the dysregulated AMPK pathway. Using AICAR, an AMP analog capable of stimulating AMPK, it was found that AICAR treatment prevented cell death upon glucose depletion only in *STK11* wild type cells[79] and that multiple disparate types of STK11-deficient cells are sensitized to cell death by AICAR.[49] Another means of targeting the metabolic dysregulation caused by STK11 loss is use of the glucose analog, 2-deoxyglucose (2-DG), which targets tumor cells due to their increased glucose uptake. In STK11-negative cells,

Table 4.2: Therapies targeting the STK11 pathway by stage of development.

REGIMEN	DISEASE	STK11 STATUS	N=	RESULTS	REFERENCE
CLINICAL TRIALS					
Standard of Care	Colorectal cancer	Evaluated *STK11* SNP rs741765	772 patients	The STK11rs741765 SNP GG genotype is prognostic for a worse DFS (p=0.030) and OS (p=0.038).	Lee et al.[71]
Everolimus	PJS Patient with Pancreatic Cancer	LOH analysis of 19p locus using four polymorphic markers	1 patient	Everolimus used to achieve a partial remission in advanced pancreatic cancer in a PJS patient. Progressive disease was noted after 9 mo. of treatment.	Klumpen et al.[72]
Metformin + Paclitaxel / Carboplatin / Bevacizumab	Lung Adeno-carcinoma	Secondary Outcome; Retrospective evaluation of *STK11* WT vs. Mut	Expect 60 patients	Recruiting for Phase 2; Estimated completion April 2015.	NCT01578551
Sirolimus + Metformin	Advanced Solid Tumors	Secondary Outcome; Retrospective evaluation of *STK11* WT vs. Mut	Expect 64 patients	Recruiting for Phase 1; Estimated completion July 2017.	NCT02145559
Metformin + Carbohydrate Restriction + Platinum based chemotherapy	Stage IV NS-NSCLC	Secondary Outcome; Retrospective evaluation of *STK11* WT vs. Mut	Expect 60 patients	Recruiting for Phase 2; Estimated completion June 2018.	NCT02019979
PRE-CLINICAL STUDIES					
Docetaxel +/- Selumetinib	NSCLC	*KRAS*, *p53*, and *STK11* mutant mice were generated using conditional knockout model.	9-28 mice/ therapy	*KRAS*/*p53* and *KRAS*/*STK11* mutant mice were resistant to docetaxel monotherapy. Addition of Selumetinib restored sensitivity in *KRAS*/*p53* mutant tumors, but not *KRAS*/*STK11* mutant.	Chen et al.[73]
BAPN	NSCLC	Generated KRAS; KRAS, $STK11^{L/L}$; KRAS, $p53^{L/L}$ mice.	8 mice/ therapy	BAPN treatment for 4 wk significantly decreased both tumor number and tumor volume in *STK11*-deficient mice, but not *STK11* WT mice.	Gao et al.[74]
LPs-pVAX-*STK11* + cisplatin	NSCLC	Added *STK11* back into mice using gene therapy.	7 mice/ therapy	Combination of conventional ciplatin-based and gene therapy resulted in fewer lung metastatic nodules and prolonged lifespan.	Ou et al.[75]
Phenformin	NSCLC	*KRAS*, *p53*, and *STK11* mutant mice were generated using Cre/Lox system.	5-12 mice/ therapy	*KRAS*/*STK11* mutant NSCLC but not *KRAS*/*p53* mutant mice responded to phenformin as a single agent leading to prolonged survival.	Shackelford et al.[76]
BEZ235 + Selumetinib + Dasatinib	Lung Cancer	*KRAS*/*STK11* mutant tumors were generated in mice.	3-7 mice/ therapy	*KRAS*/*STK11* mutant lung tumors did not respond to Dasatinib or BEZ235/ Selumetinib, the triple combination Dasatinib/ BEZ235/Selumetinib resulted in significant tumor regression.	Carretero et al.[77]
BASIC SCIENCE STUDIES					
CI-1040 or rapamycin	NSCLC	Known *STK11* status cell lines.	10 cell lines	*KRAS*/*STK11* mutant NSCLC cell lines are sensitive to single-agent treatment with CI-1040 or rapamycin whereas *KRAS* or *STK11* mutant alone are not sensitive.	Mahoney et al.[78]
NS-398 or Niflumic Acid	Lung Adeno-carcinoma	Known *STK11* status cell lines; confirmed with Western blot.	7 cell lines	NS-398 or Niflumic acid treatment of *STK11*-null cell lines resulted in growth and cell motility inhibition, but not in *STK11* WT cells.	Cao et al.[61]
AICAR / glucose depletion	Lung Cancer	Known *STK11* status cell lines; confirmed with Western blot.	4 cell lines/ STK11 status	AICAR treatment prevented cell death upon glucose depletion only in STK11 WT cells.	Carretero et al.[79]
2-DG	NSCLC	Known *STK11* status cell lines; confirmed with Western blot.	4 cell lines	2-DG acted as a potent activator of apoptosis and caused a decrease in cell viability in STK11-null cells.	Inge et al.[80]
AICAR	*STK11* WT and null MEFs	$STK11^{-/-}$ MEFs were produced by *in vitro* excision of *STK11* lox allele.	N/A	STK11-null MEFs, but not WT or heterozygous controls, lost downstream phosphorylation of AMPK upon AICAR treatment and underwent rapid apoptosis.	Shaw et al.[81]

2-DG was found to activate apoptosis in response to energetic stress.[80] A third means of exploiting the inability of *STK11*-mutant cells to appropriately respond to metabolic stress is the use of therapeutic biguanides commonly used to treat diabetes, such as metformin and phenformin.

Targeting mTOR has also been shown to be effective in *STK11* mutant tumors. The mTOR inhibitor everolimus was used to achieve a partial remission in a patient with advanced pancreatic cancer induced by Peutz-Jeghers syndrome, although observable progressive disease occurred after nine months of mTOR inhibition likely due to selective pressure for cancer cells with alternative driver mutations, or a hyperactivation of an alternate pathway such as the AKT pathway.[72]

STK11 has also been found to negatively regulate lysyl oxidase (LOX) through mTOR-HIF-1α signaling. LOX mediates lung cancer malignancy progression by triggering extracellular matrix remodeling allowing for increased cell proliferation and invasion and could be a therapeutic target for the treatment of STK11-deficient lung cancer. This is supported by the work done by Gao et al. using the LOX pharmacological inhibitor, BAPN.[18]

Another downstream target found to be upregulated in response to *STK11*-deficiency is the COX-2 pathway (described above). A gene signature developed to determine *STK11* mutational status was used to search the Connectivity-MAP drug response database and returned results that were known or predicted activators of CRTC1, a transcription factor responsible for regulation of COX-2.[61] The COX-2 inhibitors NS-398 and Niflumic acid were shown to result in growth and cell motility inhibition in STK11-null cell lines, but not their wild type counterparts.[61]

Previously, analysis of primary and metastatic de novo lung cancers with integrated genomic and proteomic profiles led to the identification of gene and phosphoprotein signatures associated with STK11 loss and progression to invasive and metastatic lung tumors. They found that SRC is activatied in STK11-deficient primary and metastatic tumors and that combined inhibition of SRC (using dasatinib), PI3K (using BEZ235), and MEK1/2 (using AZD6244) resulted in synergistic tumor regression.[77]

An additional method of STK11-mediated tumor suppression involves STK11 inhibition of the Yes-associated protein (YAP), an oncogene more commonly associated with the Hippo tumor suppressor pathway, which functions as a transcription factor responsible for promoting expression of proliferative genes. STK11 suppresses YAP via indirect, non-canonical regulation,[82] preventing YAP from inducing EMT when constitutively active.[83] The structure of YAP includes several protein domains that are promising drug targets. One such structure is the N-terminal domain where YAP binds the TEAD transcription factor. This YAP-TEAD interaction can be inhibited by verteporfin, which has been shown to prevent YAP-induced liver overgrowth.[84] Another YAP target is its WW domain which was identified *in silico* to be targetable by digitoxin,[85] and the endohedral metallofullerenol $Gd@C_{82}(OH)_{22}$,[86] a molecule that has previously been shown to have anti-neoplastic effects in tumor cells including the *STK11* mutant A549 cell line.[87] To our knowledge, no one has compared the efficacy of these therapies in STK11-proficient and -deficient lung cancer cell lines.

Therapy decisions based solely on *STK11* mutational status may not be enough, however. Evidence in several studies has pointed to the idea that *STK11*/*KRAS* co-mutational patients may represent a genetic and functionally distinct subset of NSCLC.[73,78] *STK11*/*KRAS* mutant NSCLC cell lines were found to be sensitive to the MEK inhibitor CI-1040 and the mTOR inhibitor

rapamycin, whereas cell lines carrying single *STK11* or *KRAS* mutations alone are not.[78] Similarly, phenformin was found to selectively induce apoptosis in STK11-deficient NSCLC cells, and in therapeutic trials using KRAS-dependent mouse models of NSCLC, tumors with *STK11*/*KRAS* , but not those with *KRAS*/*TP53* mutations, responded to phenformin as a single agent leading to prolonged survival.[76] An example where *STK11*/*KRAS* mutations represent a resistant phenotype was in a "co-clinical" trial featuring genetically engineered mouse models concurrently mirroring an ongoing human clinical trial in patients with *KRAS*-mutant lung cancer where it was found that concomitant loss of either p53 or STK11 impaired the response of *KRAS*-mutant tumors to docetaxel monotherapy.[73] Addition of the MEK inhibitor selumetinib (AZD6244) provided substantial benefit for mice with *KRAS* mutations (92% overall response rate) and *KRAS*/*TP53* mutations (61% overall response rate), but mice with *KRAS*/*STK11* mutations were more resistant (33% overall response rate).[73]

Currently, a series of on-going clinical trials are recruiting patients to evaluate metformin combined with either paclitaxel, carboplatin, or bevacizumab in lung adenocarcinoma (trial # NCT01578551), metformin and sirolimus in advanced solid tumors (trial # NCT02145559), and metformin combined with carbohydrate restriction and platinum-based chemotherapy in stage IV non-squamous NSCLC (trial # NCT02019979). These trials will all evaluate *STK11*-mutational status as a secondary outcome retrospectively.

CONCLUSION AND FUTURE DIRECTIONS FOR RESEARCH

Loss-of-function mutations, despite being common events in cancer, are largely ignored when developing and selecting therapies. This is due to the fact that they are difficult to exploit

as restoring function of a gene is not something that we are currently capable of. Despite this, we have a responsibility to gain a complete understanding of the biology of these tumor suppressors and the multitude of changes in key signaling pathways and downstream events caused by their loss. With this information available, we have the best hope for developing the most effective therapeutic regimens possible.

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CHAPTER FIVE:

A Gene Expression Signature Reflecting *STK11* Mutation in Lung Adenocarcinoma[XI]

ABSTRACT

STK11 is a tumor-suppressor commonly mutated in lung adenocarcinoma (LuAd). There are a number of agents that may selectively target the deregulated pathways in *STK11* mutated tumors, and thus, identifying the subset of adenocarcinomas that harbor these mutations could have significant clinical benefit. In the current work, we have characterized a cohort of 442 adenocarcinoma patients with respect to *STK11* mutation status and subset of this cohort using immunochemistry, gene expression, and western blotting. We find that measuring *STK11* mutation status is complicated by the fact that many *STK11* mutations lead to expression of a stable protein that is indistinguishable from wild type (WT) via immunohistochemistry. To circumvent this, we used published cell line mutation and gene expression data to derive a signature correlating with *STK11* mutation status. This signature was validated in the cohort of 442 lung adenocarcinomas and strongly correlates with mutation status (ROC curve AUC = 85.29). These data suggest that *STK11* mutation status may be best assessed by measuring the downstream targets included in our signature.

[XI] This chapter will be submitted for publication. See Appendix A for details.

INTRODUCTION

STK11 is a serine/threonine kinase also known as liver kinase β1 (LKB1). It was first discovered as the gene responsible for Peutz-Jeghers syndrome,[1] a rare heritable disease characterized by hamartomatous polyps of the gastrointestinal tract as well as hyper-pigmented macules on the lips, gums, and inner lining of the mouth. *STK11* has further been characterized as a potent tumor suppressor, explaining the fact that Peutz-Jeghers patients have an approximately 80% risk of developing cancer by age 70,[2] especially of gastrointestinal origin.

The *STK11* gene is located on chromosome 19p13.3 and spans 10 exons, the 10th of which is non-coding. The kinase domain of the 433 amino acid protein that it encodes spans more than half the length of the protein encompassing amino acids 49-309. Mutations have been found throughout the lengthy kinase domain with no obvious hotspots. The STK11 protein is catalytically active as a heterotrimeric complex with the STE20-related adaptor protein α (STRADα) and mouse protein-25 (MO25). STRADα binds STK11 in the nucleus and transports it to the cytoplasm where it is active,[3] and it has been found that binding to STRADα increases STK11's kinase activity more than 10-fold.[4,5] In contrast, MO25 acts as a scaffold protein and serves to stabilize the entire complex. Many mutations in *STK11* have been found to interfere with the capability to bind these partners,[6] rendering the expressed protein inactive.

While the diversity of mutations discovered in the *STK11* gene is large, the range of cancers that these mutations have been found in is comparatively small. The only cancer type found to harbor a high percentage of *STK11* mutations is non-small cell lung cancer (NSCLC). The adenocarcinoma histological subtype of NSCLC in particular has a high frequency of *STK11* mutations[7-14] and is reported to be the third most commonly mutated gene in this cancer subtype

following only *KRAS* and *TP53*,[15] pointing to its importance as a driver mutation in tumorigenesis. Mutations in *STK11* have been found to commonly co-occur with *KRAS* mutations, and to be mutually exclusive with *EGFR* mutations.[11,15,16]

The loss of STK11 can be particularly devastating to a cell as it plays a key role in maintaining glucose homeostasis. STK11 is responsible for directly phosphorylating the AMP-activated kinase (AMPK) at T172, as well as other members of the AMPK family of kinases under conditions of energy stress,[17,18] which in turn suppress mTOR activity through phosphorylation of the tumor suppressor TSC2.[19,20] Without STK11 acting as a glucose sensor, cells are able to continue to grow unchecked in low glucose conditions such as those commonly found in tumors. In addition to its role in metabolism, STK11 has also been linked to cell polarity, epithelial-mesenchymal transition, cell cycle regulation, apoptosis, and autophagy.[10]

Kinases, such as STK11, most commonly act as oncogenes, but as a tumor suppressor STK11 drives tumor progression when it is lost, rendering it impossible to target directly. Consequently, research is focused on targeting downstream targets of STK11. Therapies that reactivate AMPK, such as metformin, phenformin,[21] and AICAR,[18,22] and an AMP mimetic (2-deoxyglucose)[23] have been shown to render tumors more susceptible to chemotherapies. Additionally, drugs that target the downstream proteins that are up-regulated by loss of STK11 such as mTOR inhibitors (e.g. rapamycin[24] and everolimus[25]), cyclooxygenase 2 (COX-2) inhibitors (e.g. NS-398 and Niflumic acid[26]), and lysyl oxidase inhibitors (e.g. BAPN[14]) have shown efficacy in *STK11* mutant cells as compared to their WT counterparts. Studies have also been done to evaluate the differences in chemosensitivity between tumors that harbor *STK11*/*KRAS* double mutations compared to *STK11* alone.[27,28]

Despite the growing body of evidence pointing to the efficacy of targeting this pathway in lung adenocarcinoma, patients are not currently evaluated for *STK11* mutational status as part of standard of care treatment. Our work seeks to develop a clinically applicable test to determine *STK11* status by evaluating downstream markers. With this key piece of information, clinicians will be able to design a more personalized therapy regimen for *STK11* mutant lung adenocarcinoma patients.

MATERIALS AND METHODS

Human subjects protection

This study includes data from 442 lung adenocarcinoma patients that consented to the Moffitt Cancer Center's Total Cancer Care (TCC^{TM}) protocol either at the Moffitt Cancer Center (179 patients) or at one of 18 TCC affiliates (263 patients) between April 2006-August 2010. This multi-institutional protocol has no exclusion or inclusion criteria and is open to all patients willing to permit access to self-reported demographics, clinical data, medical records, and tissue samples. These prospectively enrolled patients are followed for life. All work was approved by the University of South Florida Institutional Review Board (**Appendix B**).

Western blotting

Frozen cell pellets were obtained from Fumi Kinose of the Moffitt SPORE in Lung Cancer Cell Core facility. All lines were authenticated by genotyping and maintained free of *Mycoplasma.* As previously described[29], cell lysates were normalized for protein content (30 μg) and separated using SDS-PAGE. Proteins were visualized using horseradish peroxidase

conjugated secondary antibodies and enhanced chemiluminescence (ECL; Amersham Biosciences, GE Life Sciences, Pittsburgh, PA, USA). Antibodies used include an STK11 mouse monoclonal antibody (sc-32245, Santa Cruz Biotechnology, Santa Cruz, CA, USA), a MO25 rabbit polyclonal antibody (M7195, Sigma), a STRAD goat polyclonal antibody (sc-55052, Santa Cruz Biotechnology), a threonine 172 phospho-AMPKα rabbit monoclonal antibody (2535, Cell Signaling), an AMPKα1 rabbit polyclonal antibody (2795, Cell Signaling), and a β-actin mouse monoclonal antibody (A5441, Sigma).

Immunohistochemistry

A tissue microarray (TMA) was constructed from available diagnostic paraffin blocks from a subset of the Moffitt patients (N = 145) of the cohort described above. Slides from potential donor blocks were stained with hematoxylin-eosin and examined by a board-certified clinical pathologist. Appropriate blocks were released for study and representative tumor areas (and a subset of normal tissue areas) marked. Donor tissue cores with a diameter of 0.6 mm were punched and arrayed into a recipient paraffin block using a tissue arrayer (Beecher Instrument, Silver Spring, MD, USA). The TMA included 145 cores from primary adenocarcinomas, 58 cores of adjacent normal lung tissue, 14 cores from non-lung tissue controls (normal and cancer) and 10 samples of lung cancer cell lines of known STK11 status (which were used to demonstrate the specificity of STK11 staining).

TMA slides were cut into 4 μM sections and stained with a mouse anti-STK11 monoclonal antibody (sc-32245, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:100 dilution (staining details are available upon request). The stained TMA was reviewed by a board-certified clinical pathologist blind to the molecular data. Normal tissue cores were examined to

determine staining criterion. The staining of tumor tissue was scored as either negative or positive with positive values ranging from +2 to +4.

A histology slide from a tissue micro array stained for STK11 (sc-32245, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was scanned using the Aperio™ (Vista, CA, USA) ScanScope XT with a 200x/0.8NA objective lens at a rate of 7 min per slide via Basler tri-linear-array detection. Each core was then segmented using the TMA block software associated with Spectrum to be analyzed individually. Image analysis for 227 stained cores was performed using an Aperio Positive Pixel Count® v9.0 algorithm with the following thresholds: [Hue Value =.1; Hue Width =.5; Color Saturation Threshold =0.04; IWP(High) = 220; Iwp(Low)=Ip(High) = 175; Ip(low) =Isp(High) =100 Isp(Low) =0] to segment positive staining of various intensities. The algorithm was applied to the entire digital core image to determine the percentage of positive biomarker staining by applicable area.

Statistical analysis

The mutational data, IHC data, clinical information, and vital status data were merged into a single file for subsequent statistical analyses using Stata/MP 12.1 (StataCorp LP, College Station, TX, USA). Pearson's chi-squared test was used to test for differences in the distributions of mutational status by study population characteristics. The Student's t-test was used to test for differences in the mean IHC values by mutational status. Survival analyses were performed using Cox proportional hazard regression, Kaplan-Meier survival curves, and the log-rank test.

Plasmid vectors

The pcDNA3-FLAG-LKB1 vector was purchased from Addgene (Plasmid #8590; Addgene, Cambridge, MA, USA). The *STK11* gene insert was amplified from this plasmid using the T7 promoter and custom primer (5'-ATACTCGAGCTGCTGCTTGCAGGC-3'), excised using EcoRI and XhoI restriction enzymes, and cloned into those sites of the pNTAPb vector (Agilent Technologies, Santa Clara, CA, USA). *STK11* variants were generated by PCR using the following primers: D194Y forward (5'-ACCCTCAAAATCTCCTACCTGGGCGTGGC-3'), D194Y reverse (5'-GCCACGCCCAGGTAGGAGATTTTGAGGGT-3'), P281fs*6 forward (5'-GACTGTGGCCCCCGCTCTCTGACCTG-3'), P281fs*6 reverse (5'-CAGGTCAGAGAGCGGGGGCCACAGTC-3'), F354L forward (5'-AGGACGAGGACCTCTTGGACATCGAGGATG-3'), F354L reverse (5'-CATCCTCGATGTCCAAGAGGTCCTCGTCCT-3').

***STK11* gene expression**

Patients consented under the TCC^{TM} protocol described above were profiled for expression of ~60,000 distinct transcripts using a custom Affymetrix GeneChip. Tissues were processed and RNA quality assessed according to the TCC^{TM} protocol. The patient cohort was then de-identified, GeneChip data extracted, analyzed for hybridization quality, and processed using Robust Multi-Array Analysis, a model-based method of calculating expression signal. Microarray expression analyses were performed with CRAN, R Bioconductor using the LIMMA package. The gene expression data were normalized using a pin-based Lowess-fit normalization algorithm. Probesets were merged on gene symbols using a signal-to-noise-based weighted approach and features with more than 20% missing values were removed.

Transfection and immunoprecipitation

Cells were cultured in RMPI supplemented with 10% fetal bovine serum plus 1% penicillin/streptomycin. H1299 cells were cultured in 150 mm plates to approximately 70-80% confluency. They were then transfected with 13 μg vector (pNTAPb), Mef2a control, or one of the *STK11* variants, D194Y, P281fs*6, or F354L, plus 41 μg ssDNA mixed with 130 μL Lipofectamine-2000 in a total of 30 mL of serum-free media. This mixture was left on the cells for 4 hours, then changed to complete media. After 48 hours, cells were harvested and immunoprecipitated using streptavidin beads following the manufacturer's protocol (InterPlay Mammalian TAP System, Agilent Technologies, Santa Clara, CA, USA) stopping after the streptavidin elution with the exception that NETN (0.5% v/v Nonidet P-40, 20 mM Tris pH 8.0, 50 mM NaCl, 50 mM NaF, 100 μM Na_3NO_4, 1 mM DTT, and 50μg/mL PMSF) was used in place of both the manufactuer's Lysis buffer and Streptavidin binding buffer. The proteins were eluted in 30 μL 2X Laemmli buffer and the entire elution was run on the SDS-PAGE gel for western blotting.

A549 cells were cultured as above in 100 mm plates to 70-80% confluency. They were then transfected with 1 μg vector (pNTAPb), WT *STK11*, or one of the *STK11* variants, D194Y or F354L, additional plates received co-tranfections of WT *STK11* with each of the *STK11* variants at ratios of 1 μg variant plus 1 μg WT, 1 μg variant plus 5 μg WT, or 1 μg variant plus 10 μg variant. All transfections were brought up to a total of 11 μg DNA using ssDNA mixed with 60 μL Lipofectamine-2000 in a total of 15 mL of serum-free media. This mixture was left on the cells for 4 hours, then changed to complete media. After 48 hours, cells were harvested and western blotted as described above.

Generation of *STK11* mutation signature

Cell line gene expression and metadata was obtained from ArrayExpress[30] accession E-MTAB-783, supplemented with additional data from the Sanger Cell Line Project (http://www.broadinstitute.org/cgi-bin/cancer/datasets.cgi). CEL files were normalized using IRON[31] against the median sample. Histology and site of origin was conformed, and several mis-annotated cell lines, identified as outliers through principle component analysis (PCA), were corrected for histology and site of origin where supported by literature (A4-Fuk, MDA-MB-435, NCI-H1155, NCI-H1299, NCI-H1770, NCI-H810, SK-NEP-1). Large differences in gene expression were observed due to batch effect (Affy_batch in the Sanger/Broad metadata), and corrected for with COMBAT[32] using a conformed combination of site of origin and histology as the covariate.

STK11 mutational status for NSCLC cell lines was curated from the literature (Luc Girard, personal communication) and confirmed in select cases by western blot. Preliminary partial least squares discriminant analysis (PLS-DA), trained to separate mutant vs. WT, indicated that four WT cell lines (H292, H2170, H2342, EKVX) exhibited mutant-like gene expression patterns. These four outlier cell lines were omitted from further signature generation analysis. An *STK11* mutation signature was generated by comparing mutant vs. WT groups using the following criteria for differential expression: at least three samples must have a log2 intensity greater than 5, $|\text{fold-change}| \geq 2$, and p-values from both T-test and Mann-Whitney U-test < 0.01. The signature was further reduced by keeping only those probesets most associated with the first PCA component.

RESULTS

Expression of STK11 protein in lung adenocarcinoma

Forty-two cell lines with known *STK11* mutational status were examined via western blot for STK11 protein expression (**Figure 5.1A**). In all *STK11* mutant cell lines, protein expression was absent. In *STK11* WT cell lines the protein generally appeared as a single band, however some cell lines blotted as a doublet or triplet, although always expressing the band for full-length protein of approximately 52 kD. The exceptions were H2170, which only blotted for lower weight bands, and the EKVX and Calu-3 cell lines, which expressed extremely faint bands of the expected size.

In work described elsewhere, we examined a cohort of 442 adenocarcinoma patients for mutations in *KRAS*, *EGFR*, *TP53*, and *STK11*. This analysis revealed that a high percentage of LuAd possessed mutations and copy number variations in the *STK11* gene. Many of the mutations identified in the previous study were of unknown significance, and thus, we sought to determine whether LuAd samples harboring these *STK11* mutations would express STK11 protein. In contrast to the cell lines, blotting patient samples for STK11 yielded a wide range of protein expression for both WT and mutant tumors (**Figure 5.1B**). The presence of WT normal tissue contaminating the tumor sample may account for some mutant tumors that express high levels of protein, but assigning *STK11* mutational status by the presence or absence of a western blot band would be highly inaccurate.

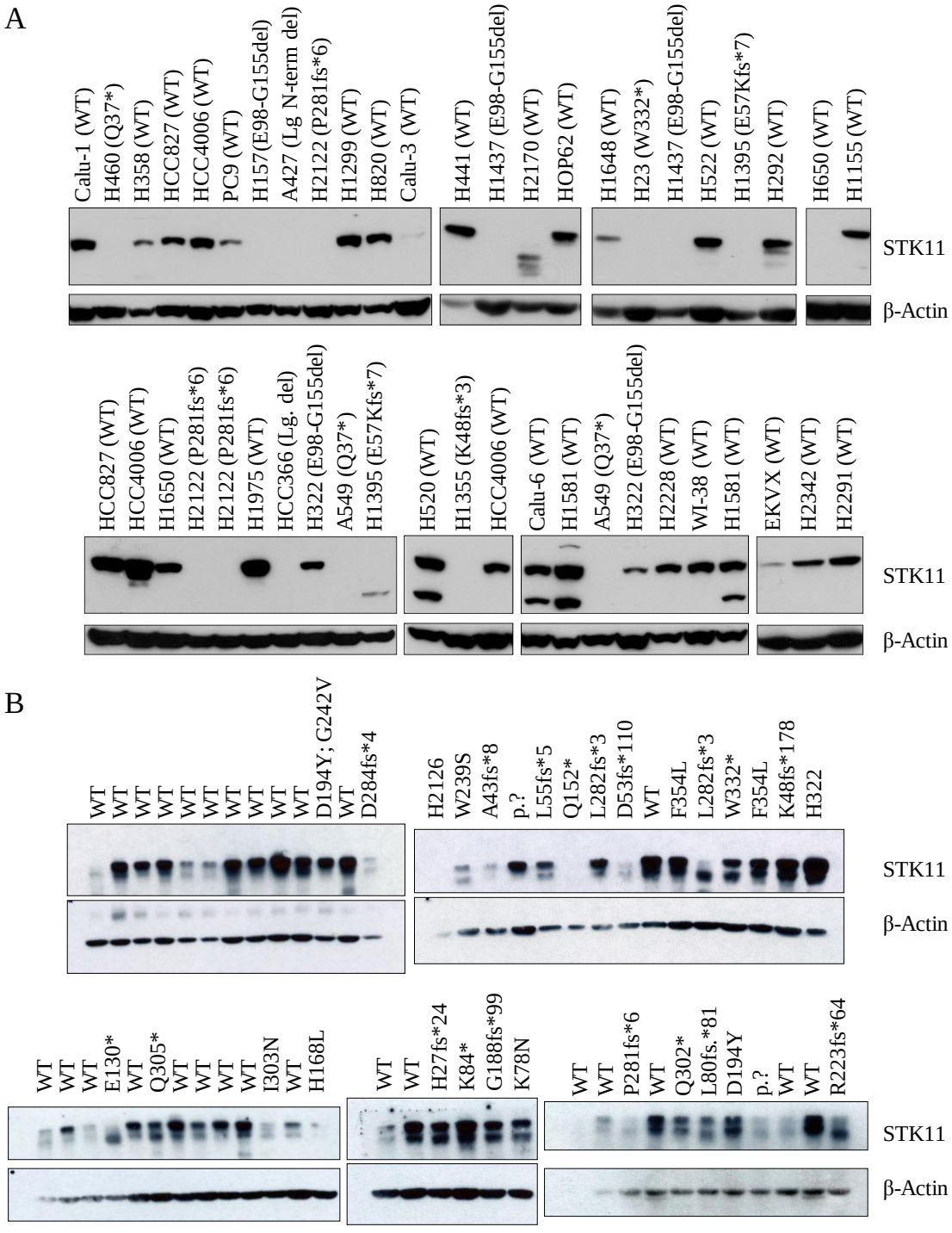

Figure 5.1: STK11 protein expression in cell lines and lung adenocarcinoma tumor samples. (A) Cell lines of known *STK11* mutational status (denoted in parentheses) and (B) patients with tumors sequenced for *STK11* mutational status (listed above each lane) were blotted for STK11 (sc-32245, Santa Cruz Biotechnology) and β-actin (A5441; Sigma). Repeated cell lines were obtained from different laboratory sources.

IHC staining

STK11 staining in normal lung epithelial tissue was generally light, cytoplasmic and diffuse and was set to a +4 value. The staining of tumor tissue ranged from 0 to 4+ (**Figure 5.2A**). Under the staining conditions used, infiltrating lymphocytes stained a very dark brown, but were ignored when assigning a staining score. Upon measuring staining using automated software, *STK11* mutant tumors were found to have statistically significantly lower levels of protein compared to the WT tumors (P=0.001) (**Figure 5.2B**). However, despite this trend, similar to the western blotting results, both *STK11* WT and mutant tumors exhibited a wide range of staining.

When we dichotomized the IHC data at the overall median value, we found that tumors with low STK11 protein levels had improved survival compared to those with high STK11 protein levels (**Figure 5.2C**), however this difference was not statistically significant (P = 0.142). The trend remained when we restricted this analysis to early stage tumors (data not shown), but the data were not statistically significant (P = 0.183).

Characterization of three recurring *STK11* variants

Three *STK11* patient variants were chosen from the list of mutations that were found to reoccur in our previous work and previously published studies from other groups. These variants, D194Y, P281fs*6, and F354L, were examined for mRNA expression levels of the *STK11* gene (**Figure 5.3A**). Due to our small sample size for each mutation, no statistically significant trends were found, but the D194Y mutant had the highest *STK11* expression, almost equivalent to the average expression of WT samples. The F354L polymorphism, which has

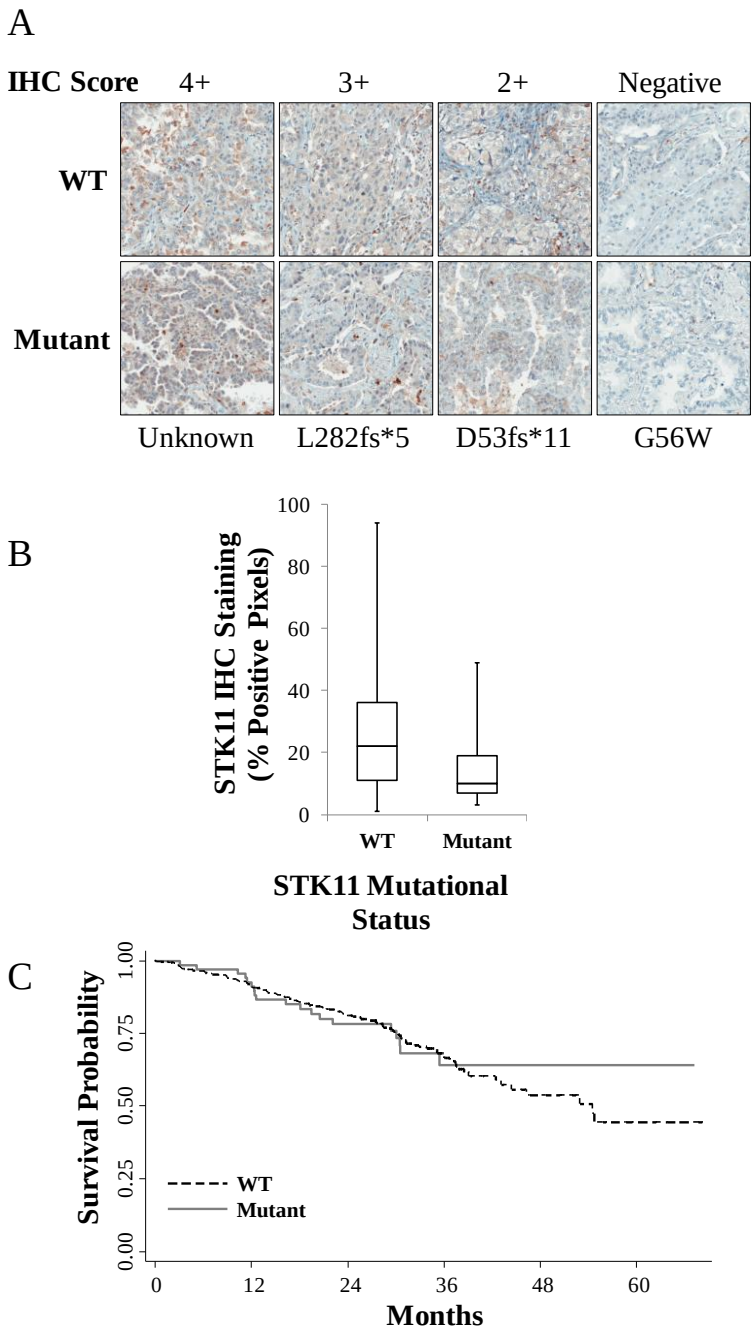

Figure 5.2: STK11 IHC and survival probability. (A) IHC was run on a panel of 145 lung adenocarcinoma tumors of known STK11 mutational status; representative cores were chosen for each staining level for both STK11 WT (on top) and STK11 mutant tumors (on bottom, with mutation listed under picture). For the 4+ staining mutant sample, the DNA mutation occurred two base pairs into the intron splice site with unknown effect on the resulting protein (Unknown). (B) The percent positive pixels staining for STK11 for each core was determined using the Aperio Positive Pixel Count® algorithm, stratified by mutational status of the core, and separated into quartiles. (C) Survival probability for both STK11 mutant and WT tumors was determined using a Kaplan Meier curve. No significant difference was noted.

previously been found in Asian populations at approximately 10% frequency,[33] has been called a polymorphism in lung cancer, having no effect on STK11 catalytic activity. We decided to explore it further, however, because it has also been reported to affect cell polarity through an AMPK-dependent mechanism.[34] This F354L variant expressed *STK11* at levels just greater than the average mutant sample still falling within the range of the second quartile of WT *STK11* expression. The P281fs*6 mutation resulted in the lowest *STK11* expression of the three variants examined, with expression levels just above the first quartile of all *STK11* mutant samples.

We next sought to determine if the three variants could bind the other members of the catalytically active STK11 trimeric complex, MO25 and STRAD. This was done by transfecting WT *STK11* as well as each of the three variants into H1299 cells which contain an intact STK11 pathway. The exogenous STK11 was immunoprecipitated using streptavidin beads and blotted for its binding partners. The WT STK11, as well as the D194Y and F354L variants were able to bind MO25 and STRAD (**Figure 5.3B**). The P281fs*6 mutant, however, showed no binding to either MO25 or STRAD, and was thus dropped from further characterization as STK11 is catalytically inactive without the other members of the complex.

Finally, we examined the remaining two variants for dominant negative activity by transfecting the variant alone, and variant plus WT in 1:1, 1:5, and 1:10 ratios, into A549 cells which lack endogenous STK11 and looking for downstream phosphorylation of AMPK. The D194Y mutant was able to suppress activation of AMPK by the WT STK11 at all ratios examined, keeping AMPK phosphorylation levels close to ambient background, as measured when transfecting in the empty vector alone (**Figure 5.3C**) marking it as a dominant negative form of the STK11 protein. This D194Y mutation has previously been noted in Peutz-Jeghers syndrome, NSCLC, and has been found to be a residue involved in Mg^{2+} binding and catalysis,

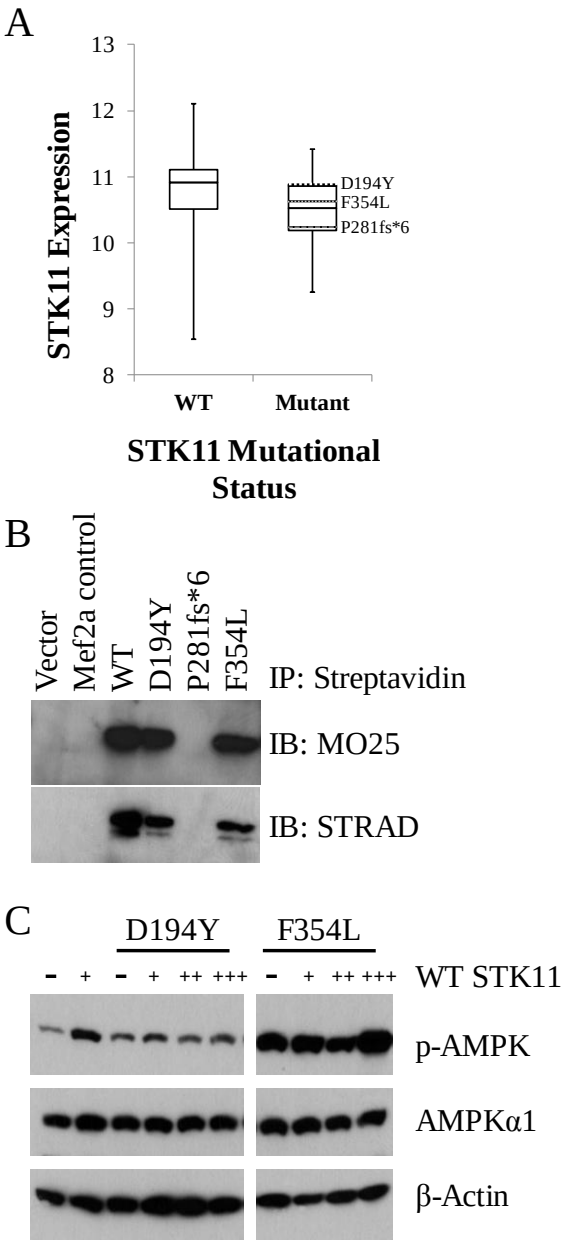

Figure 5.3: Characterization of three recurring *STK11* variants. (A) The panel of 442 lung adenocarcinomas were evaluated via microarray for gene expression of *STK11*, stratified by mutational status, and separated into quartiles. *STK11* expression for the three variants that were chosen, D194Y (N=3), P281fs*6 (N=4), and F354L (N=4), were averaged and plotted against the *STK11* mutant quartiles. (B) Empty vector, a vector control, WT *STK11*, and each of the three variants were transfected into H1299 cells, immunoprecipitated using streptavidin beads, and blotted for binding to MO25 (M7195, Sigma), and STRAD (sc-5502, Santa Cruz). (C) Empty vector, WT *STK11*, and each of the three variants alone, and variant plus WT in 1:1 (denoted with +), 1:5 (denoted with ++), and 1:10 (denoted with +++) ratios, were transfected into A549 cells and blotted for AMPK phosphorylated at T172 (p-AMPK; 2535, Cell Signaling), total AMPK (AMPKα1; 2795, Cell Signaling) and β-actin (A5441; Sigma).

so despite being expressed, the lack of downstream activation of the pathway correlates to the importance of this residue.[35-37] Conversely, the F354L variant phosphorylated AMPK in the presence, or absence, of WT protein, and is likely a polymophism that does not have any real effect on the protein's catalytic activity.

An *STK11* gene expression signature derived from a panel of cell lines of known *STK11* mutational status

Forty-eight cell lines (**Table A1**) with defined *STK11* status and gene expression data from the Connectivity Map (CMAP) of the Broad Institute (http://www.broadinstitute.org/) were used to define a gene signature correlating with *STK11* mutation status. The genes used to make the signature are listed in **Table A2**. **Figure 5.4A** shows the first and second principle components of the signature for all cell lines. The first principle component separates the cell lines into WT and mutant, except for five lines indicated in yellow (H292, H2170, H2342, and two sources of EKVX). In the Western blot analysis of these cell lines (**Figure 5.1A**), H292 appears as a triplet, H2170 is missing the wild type band but has three lower molecular weight bands, H2342 has a band at the expected wild type size, and EKVX has a faint band of the expected wild type size. All of these lines were excluded from further analysis. NR4A2 is the strongest gene in the signature and has previously been found to be downstream of STK11[38,39] with potential to be used as a marker of *STK11* mutation.

The *STK11* gene signature can be used to classify human tumors

We next assessed the STK11 gene signature using the panel of 442 lung adenocarcinoma for which the mutation status and copy number (data not shown) of *STK11* was determined.

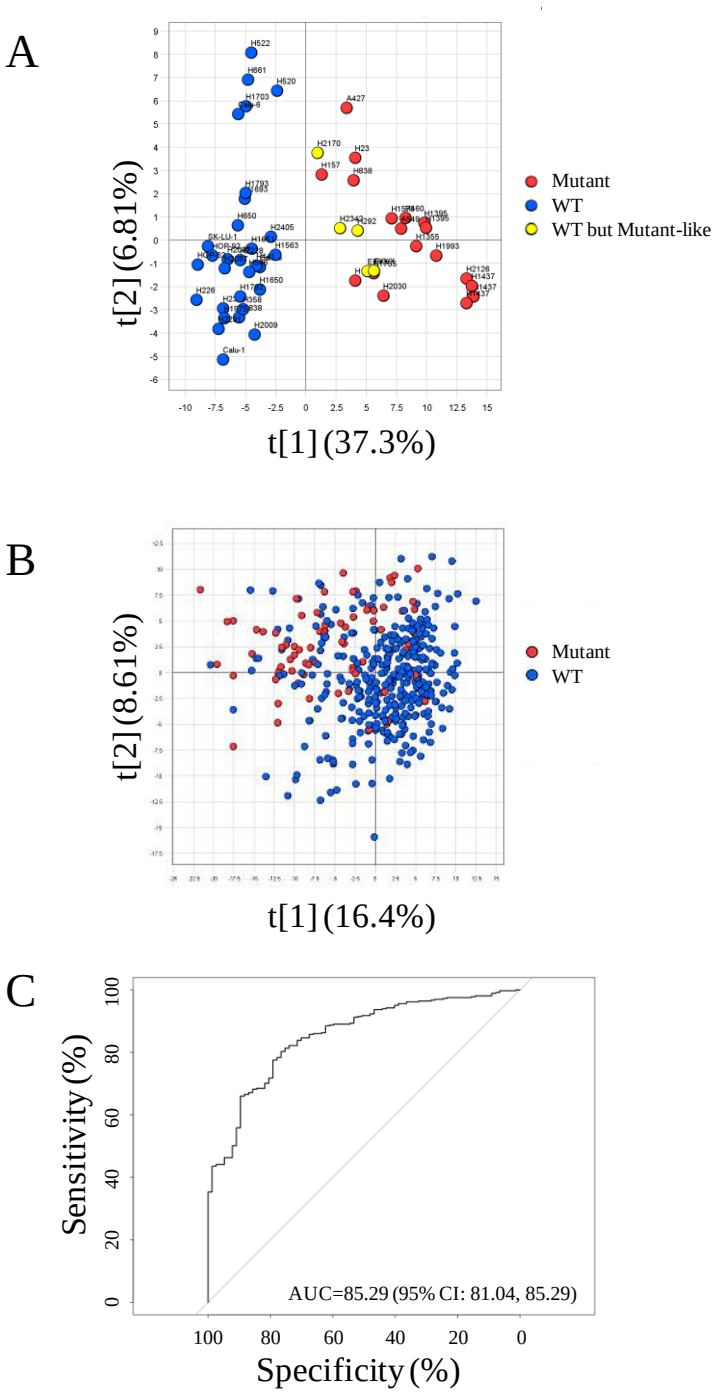

Figure 5.4: *STK11* gene expression signature. (A) First principal component analysis of an *STK11* gene expression signature was developed in cell lines; units are arbitrary. (B) First principal component analysis of this gene expression signature validation in the cohort of 442 patients; units are arbitrary. (C) The performance of the STK11 gene signature, developed in cell lines was tested using a receiver operating characteristic (ROC) curve to determine how well it correlated with known *STK11* mutational status samples in the 442 patient cohort.

Figure 5.4B demonstrates that the signature derived in cell lines is also present in *in vivo* tumors. There are exceptions on both sides. Our gene expression signature was also found to highly correlate with *STK11* mutational status as seen in the ROC curve (AUC = 85.29) (**Figure 5.4C**).

CONCLUSIONS

We have examined *STK11* DNA mutational status and the resulting levels of both STK11 mRNA and protein and found that while a mutation in the DNA sequence results in an inactive protein, it does not necessarily abrogate expression of either mRNA or protein. Conversely, WT *STK11* tumors have been shown to have a wide range of expression of both STK11 mRNA and protein. While DNA sequencing is a viable option to determine a patient's *STK11* mutational status, it is a cumbersome process involving the sequencing of nine individual exons. The majority of mutations revealed through sequencing have not been characterized for downstream activity, so even after sequencing it is unclear whether the STK11 downstream pathway has indeed been inactivated. In order to solve this problem, we have developed an *STK11* gene expression signature comprising genes both related to and downstream of STK11 and the STK11 pathway. This signature can separate both *STK11* WT and mutant cell lines and patient samples.

With the resulting *STK11* mutational status in hand, a clinician will eventually be able to offer patients a more personalized treatment regimen of one of the many drugs in development to target the altered STK11 pathway. In our own work we have found that our *STK11* signature correlates with drug sensitivity to COX-2 inhibitors (data not shown) and have published a study looking at the effects of these COX-2 inhibitors in both *STK11* WT and mutant cell lines.[26]

With the American Cancer Society estimating over 224,000 new incidences of lung cancer in the U.S. in 2014 (Cancer Facts and Figures), and approximately 35-40% of these lung cancers being classified as adenocarcinoma, there is a huge demand for a test like ours and we look forward to helping these patients receive the most efficacious treatment possible.

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CHAPTER SIX:

Conclusions and Future Directions for Research

TARGETING TUMOR SUPPRESSORS

Current strategies to treat cancer rely heavily on reducing the activity of overactive oncogenes, however this approach largely ignores tumor suppressors, as restoring function of these sentinel proteins is a more difficult task. There are several strategies that seek to restore the activity of tumor suppressors, most commonly through targeting downstream pathway members which are amplified upon loss of the tumor suppressor. Other targeting strategies include directly restoring normal gene expression through gene therapy. This involves packaging wild type tumor suppressor DNA in viral hosts and using them to infect tumor tissues, or less commonly by using either DNA plasmids directly, or packaging these plasmids in liposomes or polymers.[1] For tumor suppressors that are being suppressed by DNA methylation or histone deacetylation, activity may be restored through the use of DNA methyltransferase inhibitors (e.g. Decitabine) or histone deacetylase inhibitors (e.g. Vorinostat and Romideosin). It has also been suggested that bypassing restoration of tumor suppressor function may be a viable option through the use of microRNAs. Similar to proteins with oncogenic or tumor suppressive functions, specific microRNAs may fulfill these same roles and restoring faulty microRNAs may be an easier task with more far-reaching effects as individual microRNAs have been shown to

regulate hundreds of genes. A riskier strategy involves actually targeting normally functioning tumor suppressors that are redundant activators of pathways no longer activated by the tumor suppressor lost in cancer cells. The idea behind this strategy is that targeting a redundant protein in healthy tissues will be compensated for by the normally functioning tumor suppressor. In tumor tissues, however, where the tumor suppressor is already lost, targeting the redundant protein will abrogate all activation of the essential pathway leading to cancer cell death. Proof-of-concept studies using this strategy targeted the genes *ENO1* and *ENO2* in glioblastoma.[2] There is still a lot of work that needs to be done to develop strategies to restore activity of each of the major tumor suppressor, but the concepts described above may prove effective in the future.

The two major tumor suppressors discussed in this dissertation can currently be targeted in cancer using several of the strategies mentioned above. Specifically, the pRb pathway can be targeted by inhibiting downstream pathway members that are up-regulated upon loss of pRb using CDK inhibitors and E2F inhibitors. Restoration of pRb activity is also being attempted by re-expressing the CDKN family member, p16, which is itself a tumor suppressor, using adenovirus-mediated gene therapy.[3] Similarly, adenovirus-mediated gene therapy has been used to try to restore the *STK11* gene, while the downstream pathway is being targeted by inhibiting proteins that are up-regulated upon loss of STK11 using mTOR inhibitors, COX-2 inhibitors, and LOX inhibitors. Reactivation of the downstream pathway is being achieved using AMPK activators such as metformin, phenformin, and AICAR. Understanding the molecular biology of these two major tumor suppressors is vital in our quest to develop additional strategies to specifically restore the function of these critical proteins.

CONCLUSIONS FROM THE WORK PRESENTED IN THIS DISSERTATION

Chapters 2 and 3 explored the major tumor suppressor, pRb. This protein, which is lost in the majority of human cancers has been widely characterized as a cell cycle suppressor, but newly emerging work is also linking it to the process of cell-to-cell and cell-to-extracellular matrix adhesion. The status of current work outlining the mechanisms and pathways by which pRb controls these adhesive properties is leading toward a greater understanding of this protein and paving the way for future means of exploiting its loss therapeutically. Specifically, pRb was found to up-regulate expression of the adhesion protein, integrin α10. In addition to changes in this integrin subunit, three others, integrins α7, α8, and β4 were also found to undergo changes in expression in tumor tissue compared to normal tissue in multiple common solid tumor types.

Chapter 4 and 5 discussed the major tumor suppressor STK11 with a focus on its molecular biology and therapeutic implications. Currently there are no approved therapies specifically for patients with *STK11* loss-of-function mutations, however a variety of strategies are being explored at the basic science, pre-clinical, and clinical trial levels. These therapies seek to restore normal pathway activity in patients affected by loss-of-function *STK11* mutations. Once therapies are approved, the next question will be how to identify patients that will benefit from these therapies, as patients are not currently clinically evaluated for their *STK11* mutational status. Our work has identified a gene signature made up of downstream pathway members and other genes affected by *STK11* loss-of-function mutations. This signature can identify patients with an aberrant STK11 pathway with the hope that these patients will soon be eligible to receive therapies specifically targeting their personal mutational spectrum.

FUTURE WORK ON INTEGRINS

The majority of our experimental work on integrins was focused on the transcriptional control of integrin α10 with bioinformatic work that led to the identification of an 'integrin switch' in multiple solid tumors. Further work would be necessary to fully characterize the four integrins involved and confirm the bioinformatically identified 'switch.' Real-time PCR could be used to quantify differences in transcriptional levels of integrin mRNA, while western blotting or immunofluorecence could be used to visualize changes in the levels of these proteins in the predicted solid tumor types. Changes in the mRNA or protein levels of these four integrins could be further analyzed for their expected correlation to the proclivity of tumors to metastasize with high levels of the three protective integrins, α7, α8, and α10 indicating a good prognosis, and high levels of the detrimental integrin, β4, indicating a poor prognosis. This four integrin signature could potentially be developed into a prognostic patient biomarker test. Increased levels of integrin β4 could also be targeted therapeutically as the extracellular domains of integrins could be ideal substrates for small molecule inhibitors, targeted antibodies, or binding-domain peptide mimetics.

Another potential future direction for the integrin project involves the continued elucidation of pRb's control of several other adhesion proteins. Our preliminary work identified multiple integrins and cadherins that appeared to be regulated by pRb including upregulation of cadherins 1, 11, and 26 as well as integrins α1, α8, and β3 and downregulation of cadherins 2 and 6, as well as integrins α6, β7, and βL1, relationships all confirmed by real-time PCR in pRb-proficient and -deficient SAOS-2 osteosarcoma cells. These relationships could all be further explored for the mechanism linking them to pRb.

Ultimately, our work serves to strengthen the link between pRb and adhesion. While there are drugs that target each of these pathways individually, developing therapeutic regimens that combine these drugs, or eventually developing targeted therapies to restore adhesion dysregulated by the loss of pRb may be an extremely beneficial strategy for treating the greater than 50% of human tumors that are characterized by loss of pRb.

FUTURE WORK ON STK11

Additional work on STK11 is currently underway with our gene expression signature being transformed into codesets compatible with the Nanostring platform. This platform is an amplification-free method for detecting small amounts of mRNA from patient samples through hybridization to fluorescently bar-coded probes for predetermined target genes. These results will be compared with our previous microarray-based signature to narrow down our gene list to those that are similarly modified on both platforms helping to create the smallest, most robust signature possible. This newly refined signature will also be tested in outside datasets independent of the 442 patients from the SPORE cohort. These datasets, including the TCGA, may help refine the gene list to narrow it down to the smallest number of maximally informative genes. With the final gene list defined, the ultimate goal for this signature is to develop it into a patient diagnostic test used to clinically identify the mutational status of *STK11* in order for patients to receive treatment specific to their unique mutational landscape. With multiple clinical trials currently underway investigating the response of *STK11* mutant and wild type patients to a variety of therapies, it is our hope that evaluating the status of this gene will be informative for future standard of care therapy options.

This gene expression signature was also tested against CMAP drug-response signatures resulting in a series of hits. These drugs were largely related to prostaglandins and the COX-2 pathway. Some initial work was done on testing a few of these drugs in *STK11*-proficient and -deficient cell lines. Follow-up studies on more of the drugs, including the subset that are not COX-2 inhibitors, may yield viable treatment options not yet explored for *STK11* mutant patients. Lung adenocarcinoma is a very deadly disease and currently no targeted therapies are available for patients with *STK11* mutations. Our work will continue to focus on identifying new therapeutic strategies and the patients that will benefit most from them.

THE FUTURE OF CANCER TREATMENT

In the words of Hippocrates, "It is more important to know what sort of person has a disease than to know what sort of disease a person has." While this concept has been around for several millennia, it is truly the future of cancer treatment. With the widespread use of oncogenomics to analyze each patient's individual mutation spectrum and develop a unique treatment regimen, 'personalized medicine' is truly becoming a reality. With a strong foundation of basic science being translated into the clinic, it is our hope that each piece of information we uncover about the inner workings of these devastating cancers will result in more effective treatments in the future.

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APPENDICES

APPENDIX A: Permission to Use Published Papers and Author Contributions

Chapter 2: " The Retinoblastoma Protein: A Master Tumor Suppressor Acts as a Link Between Cell Cycle and Cell Adhesion" was written as an invited review for the journal *Cell Health and Cytoskeleton* and will be submitted for review in September, 2014. Authors on this paper include Brienne E. Engel[1] (wrote 40% of manuscript), W. Douglas Cress[1] (wrote 20% of manuscript), and Pedro G. Santiago-Cardona[2] (wrote 40% of manuscript)

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Chapter 3: "Expression of Integrin alpha 10 is Transcriptionally Activated by pRb in Mouse Osteoblasts and is Downregulated in Multiple Solid Tumors" appeared in the journal Cell Death and Disease (2013) 4, e938. PMID: 24287699. Authors on this paper included Brienne E. Engel[1] (wrote manuscript, carried out experiments for Figures 3.1-3.7), Eric Welsh[2] (carried out bioinformatics for data to create Figure 3.7A-D), Michael F. Emmons[1] (carried out experiment for Figure 3.6B), Pedro G. Santiago-Cardona[3] (developed concepts and provided comments on manuscript), and W. Douglas Cress[1] (developed concepts and provided comments on manuscript).

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Chapter 4: "The Molecular Biology and Therapeutic Implications of *STK11/LKB1* Mutations in Lung Cancer" was written as an invited review for the journal *Carcinogenesis* and will be submitted for review in September, 2014. Authors on this paper include Brienne E. Engel[1] (wrote 70% of manuscript, made Table 4.2), W. Douglas Cress[1] (wrote 10% of manuscript), Frederic J. Kaye[2] (developed concepts and provided comments on manuscript), and Matthew B. Schabath[3] (wrote 20% of manuscript, made Table 4.1).

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Chapter 5: "A Gene Expression Signature Reflecting *STK11* Mutation in Lung Adenocarcinoma" is a manuscript in preparation for submission by the end of 2014 to a journal to be determined. Authors on this paper include Brienne E. Engel[1] (wrote manuscript, carried out experiments for Figures 5.1A, 5.2A-B, 5.3), Eric A. Welsh[2] (carried out bioinformatics for Figures 5.4A-B), Lu Chen[1] (carried out preliminary molecular biology work), Chunxia Cao[3] (carried out experiment for Figure 5.1B), Stephen G. Brantley[4] (scored IHC cores for Figure 5.2A), Dung-Tsa Chen[2] (made ROC curve for Figure 5.4C), Matthew B. Schabath[5] (provided data for Figures 5.2C, 5.3A), Amer Beg[6] (provided funding for some analyses), Frederic J. Kaye[3] (provided funding/reagents for Figure 5.1B), Eric B. Haura[7] (developed concepts), and W. Douglas Cress[1] (developed concepts, provided funding, provided comments on the manuscript) Departments of [1]Molecular Oncology, [2]Biostatistics and Bioinformatics, [5]Cancer Epidemiology, [6]Immunology, [7]Thoracic Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL, [3]Department of Medicine, University of Florida, Gainesville, FL, USA, and [4]Pathology Services M2Gen®, Tampa, FL

APPENDIX B: Institutional Review Board Approval Letter

USF
UNIVERSITY OF
SOUTH FLORIDA

DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX (813) 974-5618

February 4, 2011

Matthew Schabath, PhD
H Lee Moffitt Cancer Center
12902 Magnolia Drive
MRC-CANCONT
Tampa, Florida 33612

RE: **Expedited Approval** for [Application Type: Initial or Continuing Review]
IRB#: Pro00003347
Title: Defining the Molecular Heterogeneity of KRAS Mutant Lung Adenocarcinoma - MCC# 16487

Dear Dr. Schabath:

On 2/1/2011 the Institutional Review Board (IRB) reviewed and **APPROVED** the above referenced protocol**.** Please note that your approval for this study will expire on 02/01/2012.

Approved Items:
Protocol Document(s):

Protocol (version 2, 01/11/2011)	1/18/2011 3:05 PM	0.01

It was the determination of the IRB that your study qualified for expedited review which includes activities that (1) present no more than minimal risk to human subjects, and (2) involve only procedures listed in one or more of the categories outlined below. The IRB may review research through the expedited review procedure authorized by 45CFR46.110 and 21 CFR 56.110. The research proposed in this study is categorized under the following expedited review category:

(5) Research involving materials (data, documents, records, or specimens) that have been collected, or

will be collected solely for nonresearch purposes (such as medical treatment or diagnosis).

Your study qualifies for a waiver of the requirements for the documentation of informed consent as outlined in the federal regulations at 45CFR46.116 (d) which states that an IRB may approve a consent procedure which does not include, or which alters, some or all of the elements of informed consent, or waive the requirements to obtain informed consent provided the IRB finds and documents that (1) the research involves no more than minimal risk to the subjects; (2) the waiver or alteration will not adversely affect the rights and welfare of the subjects; (3) the research could not practicably be carried out without the waiver or alteration; and (4) whenever appropriate, the subjects will be provided with additional pertinent information after participation.

Your study qualifies for a waiver of the requirement for signed authorization as outlined in the HIPAA Privacy Rule regulations at 45 CFR 164.512(i) which states that an IRB may approve a waiver or alteration of the authorization requirement provided that the following criteria are met (1) the PHI use or disclosure involves no more than a minimal risk to the privacy of individuals; (2) the research could not practicably be conducted without the requested waiver or alteration; and (3) the research could not practicably be conducted without access to and use of the PHI.Waiver of HIPAA Authorization has been approved for you to conduct a secondary analysis of identifiable data that are collected under Moffitt Cancer Center's Total Cancer Care Protocol (IRB Study#104189/MCC# 14690). This study will involve data of those patients with adenocarcinoma without a prior history of chemotherapy and/or radiation-therapy that have been collected under the TCC Protocol.

As the principal investigator of this study, it is your responsibility to conduct this study in accordance with IRB policies and procedures and as approved by the IRB. Any changes to the approved research must be submitted to the IRB for review and approval by an amendment.

We appreciate your dedication to the ethical conduct of human subject research at the University of South Florida and your continued commitment to human research protections. If you have any questions regarding this matter, please call 813-974-9343.

Sincerely,

USF Institutional Review Board

Cc: Vicki Stecher, MA, USF IRB Professional Staff
Diane Martinez, Manager, Moffitt

APPENDIX C: Cell Lines Used to Derive the *STK11* Signature

Table A1: Cell lines used to derive the *STK11* signature

Cell Line	NSCLC Subtype	STK11 Status
H1437**	Adenocarcinoma	E98-G155del
H2126	Adenocarcinoma	Y156-G268del
H1993	Adenocarcinoma	E199*
H1395*	Adenocarcinoma	E57Kfs*7
H1355	Adenocarcinoma	K48fs*3
H460	Large Cell	Q37*
A549	Carcinoma, unspecified	Q37*
H1573	Adenocarcinoma	S216F
H2030	Adenocarcinoma	E317*
EKVX*	Adenocarcinoma	WT
H1755	Adenocarcinoma	P281fs*6
H292	Mucoepidermoid	WT
H23	Adenocarcinoma	W332*
H1666	Adenocarcinoma	A200fs*87
H838	Adenocarcinoma	T212fs*75
A427	Carcinoma, unspecified	Large N-term deletion
H2342	Adenocarcinoma	WT
H157	Squamous Cell Carcinoma	E98-G155del
H2170	Squamous Cell Carcinoma	WT
H520	Squamous Cell Carcinoma	WT
H1563	Adenocarcinoma	WT
H2405	Adenocarcinoma	WT
H1650	Adenocarcinoma	WT
Calu-3	Adenocarcinoma	WT
H441	Adenocarcinoma	WT
H2009	Adenocarcinoma	WT
H1651	Adenocarcinoma	WT
H522	Adenocarcinoma	WT
H596	Adenocarcinoma	WT
H661	Large Cell Carcinoma	WT
H1703	Squamous Cell Carcinoma	WT
H1793	Adenocarcinoma	WT
H1693	Adenocarcinoma	WT
H358	Bronchioalveolar	WT
H1792	Adenocarcinoma	WT
H2228	Adenocarcinoma	WT
H1838	Adenocarcinoma	WT
Calu-6	Adenocarcinoma	WT
H650	Bronchioalveolar	WT
H2087	Adenocarcinoma	WT
H1975	Adenocarcinoma	WT
Calu-1	Epidermoid carcinoma	WT
H2347	Adenocarcinoma	WT
H2291	Adenocarcinoma	WT
HOP-92	Large Cell Carcinoma	WT
SK-LU-1	Adenocarcinoma	WT
HOP-62	Adenocarcinoma	WT
H226	Squamous Cell Carcinoma	WT

NSCLC subtype from ATCC; STK11 status from the Connectivity Map (CMAP) of the Broad Institute (http://www.broadinstitute.org/). Cell lines are listed in the ranked order of their first principle component with most 'mutant-like' listed first and most 'WT-like' listed last. Single asterisks indicate cell lines that had two subtypes that were used to derive the signature. Double asterisks indicate cell line that had three subtypes that were used to derive the signature.

APPENDIX D: Genes in the *STK11* Signature

Table A2: Genes in the *STK11* signature

ABCA1	LHFP
ADRB2	LYST*
ALDH3B1	MALT1
APBB2	MAP7*
APOBEC3G	MECOM
ARHGEF3	MERTK
ATF7IP	MFGE8
AVPI1	MUC5B
BCAT1*	MX2
C21orf96	MYLK
CASP9	NPC2
CDKN1C	NR4A1
CEP170	NR4A2*
CFI	NR4A3
CLIC2	PDE4B*
CLIP4	PDE4D*
CPS1*	PDLIM4
CSGALNACT1	PDP1
CTSB	PELI1
CYP1B1*	PLSCR1
DENND4B	PRKAA2
DLG1	PSIP1
DPM3	PTGES*
DPYD	PTP4A1*
DPYSL3	SEC14L1*
EDNRA	SEC14L1 LOC729799
EFNB3	SETD6
EPB41L2	SH2B3
EPHB2*	SHANK2*
FGA*	SIK1
FGB	SLFN12
FGG	SLIT2
FLJ20935 fis, clone ADSE01534	SMAD2*
FOXN3	SPATS2L
Glutaminase isoform C	SPDEF
GRAMD1B	STAC
GRK5	TACC2*
GUCY1B3	TBC1D30
HAL*	TFF1
HEG1	TRIM2
HERC6	TRIO*
HGD	TYMP*
IFI27	UBE2L6
IFI44	ZCCHC24
IFI44L	ZFP36L1*
IFIT1	ZNF177 ZNF559-ZNF177
IGF2BP2	ZNF415
KCTD14 NDUFC2-KCTD14	ZNF43*
KIAA1598	ZNF85
LGSN	ZNF93*

Genes are listed alphabetically. Asterisks indicate that multiple probesets were used for that gene.

ABOUT THE AUTHOR

Brienne Engel grew up outside Albany, New York in the town of East Greenbush. She was valedictorian of her graduating class from Columbia High School. For her undergraduate education, she attended Brandeis University on a full-tuition Justice Louis Brandeis scholarship. While at Brandeis she double-majored in Biochemistry (B.S.) and Biology (B.S.) and double-minored in Chemistry and Art History. From her sophomore to senior years, she conducted research in the biochemistry department with Dr. Dan Oprian where her projects included the expression of Taxadiene Synthase in mammalian cells and the crystallization of rhodopsin. She spent the spring semester of her junior year abroad in the School for International Training's program, Australia: Natural and Cultural Ecology. Upon graduating, she moved to St. Petersburg, Florida to attend the University of South Florida, College of Marine Science as a USF Presidential Fellow where she worked with Dr. Pam Hallock-Muller and received her Master's Degree in Biological Oceanography in 2010. While at the Moffitt Cancer Center, in addition to performing research, Brienne was a member of the Cancer Biology Student Organization, the Government Relations Taskforce, an intern at the Office of Technology Management and Commercialization, and a volunteer for the Great American Teach-In.