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ELUCIDATING THE ROLE OF THE TYROSINE PHOSPHATASE, SHP-2, IN REGULATION OF PD-L1 EXPRESSION IN NON-SMALL LUNG CANCER USING BOTH BIOCHEMICAL ANALYSES AND REAL-WORLD GENOMIC INFORMATION

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy
at the University of Kentucky

By
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Lexington, Kentucky
Director: Dr. Esther P. Black, Professor of Pharmaceutical Sciences
Lexington, Kentucky
2021

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ABSTRACT OF DISSERTATION

ELUCIDATING THE ROLE OF THE TYROSINE PHOSPHATASE, SHP-2, IN REGULATION OF PD-L1 EXPRESSION IN NON-SMALL LUNG CANCER USING BOTH BIOCHEMICAL ANALYSES AND REAL-WORLD GENOMIC INFORMATION

Immune checkpoint inhibitors (ICIs), especially those that target programmed cell death protein 1 (PD-1) and programmed cell death ligand-1 (PD-L1), have been shown to provide substantial clinical benefit in many patients with non-small cell lung cancer (NSCLC). While these therapeutic agents can be highly effective in the correct context, the biological systems that malignant cells draft from normal activities of the cell are poorly characterized. Tumor cell-specific expression of PD-L1 is likely important for clinical benefit from PD-1 and PD-L1 inhibitors. It is known that PD-L1 is inappropriately expressed in many cancers harboring mutations in the RAS family of genes. The KRAS gene is mutated in as many as 30% of NSCLC tumor and drives tumor proliferation. Because there are no FDA-approved KRAS-targeting agents available for NSCLC patients, ICI therapy has been used in patients with tumors harboring mutations in the KRAS gene with clinical success. However, utilization of these therapies will remain hindered until there is a more complete understanding of the mechanisms governing the expression of targets of ICIs, specifically of PD-L1. The work in this dissertation explores the role of the tyrosine phosphatase, SHP-2. SHP-2 has been scrutinized as an important signaling molecule in a variety of cancers that links the activity of several signaling cascades as a regulator of KRAS, resulting in the clinical development of inhibitors of SHP-2. The work encompassed in these studies takes two complementary approaches to explore the role of SHP-2 in control of PD-L1 expression. First, publicly available real-world genomic information was used to establish a connection between the activity and/or expression of SHP-2 and PD-L1 in tumors and how expression relates to response to ICI therapy. Second, this work further sought to elucidate the molecular mechanism by which SHP-2 impacts the expression of PD-L1 in an NSCLC cell line model system. From these investigations, this work established that SHP-2 and PD-L1 have an expression relationship in clinical samples that may impact response to ICI therapies and experimentally identified a possible mechanism by which SHP-2 impacts PD-L1 expression in NSCLC.

KEYWORDS: Non-small Cell Lung Cancer, Immunotherapy, SHP-2, Bioinformatics, Experimental Therapeutics, Phosphatases and kinases

Keller J. Toral

August 11th 2021

Date

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CHAPTER 1.

1.1 INTRODUCTION

Cancer, at its most fundamental level, is a disease characterized by abnormal cells that divide without control and can invade nearby tissues (1). While correct and concise, this deceptively simple definition only scratches the surface of the answer to the question ‘What is cancer?’ Within the past 100 years, the burden of cancer has evolved in a manner that mirrors the pathology of the disease itself. In 1900, the leading causes of death in the USA were primarily due to both bacterial and viral infections, with cancer accounting for 64 deaths per 100,000 people. Between 1900 and 2010 overall mortality from all causes declined by 54%, yet the rates of death due to cancer increased roughly 300%, resulting in cancer as one of the leading causes of death, second only to heart disease by 2019 (2). The decline in deaths due to infectious disease and subsequent increases in lifespan may largely be attributed to the development of antibiotics and vaccines (3). As a result, the physiological landscape upon which human disease occurs was ever altered, facilitating the emergence of illnesses that arise not from extrinsic factors such as pathogens, but rather from malfunctions of our own biology that become more apparent as lifespan increases. This shift in mortality reveals much about the nature of this ever-changing affliction, and so one may think of cancer as a pathology not of the human body, but of the human evolution.

The evolution of an organism requires three core criteria to occur: reproduction, variation, and selective pressure. The increase in life expectancy over the last century and how it relates to cancer reflects one of these core evolutionary principles: **reproduction**. It is thought that, among many factors, the escalation in cancer mortality over the last century can primarily be attributed to a large increase in life expectancy. In 1900 the average life expectancy at birth was 47 years, compared to 79 years by 2010 (2). As an individual grows past the point

in their life at which they may bear offspring, their continued existence becomes evolutionarily disadvantageous to their species. That is to say that, from a purely evolutionary standpoint and from the perspective of a low population, newly developing species, there are diminishing returns on the usefulness of an individual that can no longer reproduce. Once older individuals can no longer serve a caretaker role and must instead be taken care of, in times when there is great pressure to survive, older individuals take more time, energy and resources than they provide. As such, there is minimal evolutionary incentive for the development of biochemical mechanisms which prevent the onset of cancer later in life, at least as is the case in homo sapiens. However, there do exist numerous biochemical mechanisms aimed at restraining **genetic variance** throughout the ages of reproductive capability and onward. Whether it be by detecting and eliminating sources of genetic lesions, preventing the unregulated replication of individual cells, or carefully curating the integrity of the genome itself, our bodies every attempt to cull the inception and progression of cancer aims to minimize the probability of potentially detrimental genetic mutations.

The corrupted application of **selective pressure** by cancerous cells, specifically with regard to the development of anti-cancer therapies, has revealed itself as one of the greatest obstacles in the effective treatment and control of disease progression. A longstanding issue in the use of drugs to combat the growth of pathogenic organisms has been their ability to acquire resistance to therapeutic intervention. Bacterial cells rapidly evolve under the selective pressure of drugs that impede their ability to properly divide, and malignant human cells possess the same capabilities but to a substantially more sophisticated extent. Methicillin-resistant Staphylococcus aureus (MRSA) is a relatively simple single cell organism that has imposed major challenges in the development of antibacterial drugs. MRSA's genome contains some 2.8 million base pairs which encode roughly 2,629 coding sequences, about 10-fold fewer coding sequences than the human genome (4). When one compares the complexity of, and challenges posed by prokaryotic mechanisms of drug resistance against the highly adaptive, multiplex, and

intersecting pathways applied by eukaryotic cells to the same end, the task of overcoming tumoral drug resistance appears incredibly difficult. It is important to consider that the generation of random genetic mutations is a naturally occurring process that leads to phenotypic changes which may or may not be advantageous to an organism. When a rapidly and haphazardly reproducing population of heterogenous cells is put under the selective pressure of pharmacological intervention, their ability to exponentially evolve past even the most efficacious of therapies is unveiled as the true obstacle in cancer drug development.

Of all the drugs which enter clinical trials for the treatment of cancer, a dismal 97% of them fail to receive FDA approval, the lowest among all drug indications (5). The consequences of these failures are enormous; by the time a drug reaches late phase clinical trials, hundreds of millions of dollars and years of time have been invested in its development (6). Furthermore, most of these clinical failures stem not from issues in the toxicity or pharmacokinetics of a drug as one might predict. Rather, these failures are the result of inadequacies in drug efficacy, the cause of which is often indiscernible until later phase clinical trials and may be attributed to unforeseen downstream cellular processes that remain poorly understood (7)(8). Despite our best efforts to predict the success of a drug with preclinical modeling, when they finally reach a human subject, the drugs simply do not work. The decision for which anti-cancer compound is chosen to treat a tumor is based upon the mechanism of action that would prove most effective in stopping that tumor's growth. If we do not fully understand the mechanism of a compound, that is to say the drug isn't doing exactly what we 'think' it's doing, it drastically impairs our ability to utilize our treatments to their full potential. Thus, the failure to completely understand and fully characterize all the facets of how a drug, its target, and the downstream effectors of that target function within an actual tumor has created a wildly unsustainable system for cancer drug development.

The ancient Chinese military strategist, Sun Tzu, once wrote in The Art of War, "If you know your enemy as you know yourself, you will fight without fear in

100 battles. If you know yourself but not your enemy, with every victory gained you will also suffer defeat." Though he wrote this regarding military strategy, this proverb lends relevancy to the war against cancer. Despite the ongoing development of highly sophisticated targeted therapies, cancers continue to evolve, adapt, and overcome our best efforts in manners we remain unable predict. Although we create groundbreaking new therapies which drastically improve the lives of those with previously untreatable cancer, we often fail to predict for whom they will work, how they work, and when they do not work- why not. The enemy now standing in opposition of humanity is cancer, and cancer at its most fundamental level, is evolution. To fully understand how human cells can evolve and overcome our efforts to eradicate them, we must understand as completely as possible every component of every biological process by which cancer cells evade destruction and consume their host.

Thus, to fully utilize any cancer treatment, we must focus our efforts into thoroughly defining every complexity of a drug and its mechanism of action. The factors that control the expression, activity, epigenetic regulation, metabolic turnover, parallel and intersecting pathways, downstream and upstream effectors of the target are all important for discerning the right therapy for the right patient/tumor at the right time. The work herein aims to further elucidate the complexities of the regulation of programmed cell death ligand 1 (PD-L1) expression, the target of some of the most successful cancer therapies to date.

1.2 BIOLOGY OF NSCLC

Hallmarks of cancer

At its most basic level, cancer is the uncontrolled and unregulated replication of a cell. However, there are a variety of cellular mechanisms by which this outcome can be achieved. Canonically, these defining biochemical capabilities have been historically separated into ten distinct categories described a decade ago by Hanahan and Weinberg (9). Depending on the type of cancer and the tissue

of origin, the acquisition of several of these capabilities may allow a cell to evolve into cancer, with very late-stage cancers having the potential to encompass all ten, heavily contributing to the innate heterogeneity of the disease. These ten hallmarks of cancer can be broadly grouped by the mechanism by which they promote unregulated cell growth, and will be expanded on in the forthcoming sections; 1) the recruitment or corruption of extrinsic tissues to assist in tumor development 2) the disruption of cellular checks and balances designed to prevent the occurrence and progression of cancer 3) the exploitation of intrinsic signaling or genetic pathways related to cellular growth or 4) strategies to evade and/or manipulate the immune system.

Recruitment or corruption of extrinsic tissues

In order for a tumor to continue growing beyond the normal limitations of its tissue of origin, it must have a proper supply of oxygen and nutrients and be able to survive in an irregular growth environment. The two major mechanisms by which cancer will coerce neighboring tissues to assist in its progression are by inducing angiogenesis, or by invading the nearby tissues directly through the process of metastasis. The notion that a critical part of tumor progression was the recruitment of blood vessels to supply the growing tumor mass with nutrients was initially met with great resistance. Judah Folkman, the so-called ‘father of angiogenesis’ in 1971 defined angiogenesis as “a cascade of processes emanating from microvascular endothelial cells in response to soluble factors” hypothesizing that developing tumors were being limited in their growth by an inability to acquire enough oxygen and mitogenic factors, and that only by recruiting blood vessels to fuel their growth could tumors develop into later, deadlier stages of disease (10). Over time, further insight was gained to confirm this hypothesis and led to the discovery of a number of growth factors known to induce angiogenesis, namely proteins such as vascular endothelial growth factor (VEGF) and hypoxia-inducible factor alpha (HIF-a). As such, drugs which interfere with these pathways reduce the recruitment of blood vessels and subsequent blood flow to tumors thus serving

as viable therapeutic options for a variety of tumor types, though not many are used in the clinic today.

Perhaps the most deleterious characteristic of advanced cancer is its ability to invade or spread to other tissues in a process known as metastasis. Though one of the deadliest properties of the disease, metastasis is a highly inefficient process that requires numerous specific biological conditions to be met then resulting in the development of heterogenous cell populations capable of surviving outside of their tissue of origin (11). This ultimately leads to the development of secondary tumors, typically to highly vascularized tissues such as the liver, brain, and lungs. Chemotherapy, a branch of therapy which damages and hinders actively proliferating cells non-discriminately throughout the entire body, has served as an effective treatment strategy to prevent cells which have been shed from the primary tumor from forming a colony and replicate in secondary tissues.

Disruption of cellular checks and balances

For a cell to transition into malignancy, a loss of function in mechanisms that restrict unregulated cell growth must occur in addition to oncogenic mutations that drive proliferation. The enabling of replicative immortality, ability to resist programmed mechanisms of cell death, and evasion of growth suppressors are all methods cancer applies to subvert such preventative measures. When describing the ability of a cell to become ‘immortal’ it is important to note that this refers not to any single cell being able to resist death, but rather to the loss of genetic limitations designed to limit the number of times any individual cell may replicate. The ‘Hayflick phenomenon’ describes the limited number of times a primary cell population in culture will divide until cellular division stops and the cell enters a state in which it will no longer continue to undergo mitosis known as senescence (12). Cellular senescence is thought to be a natural process that occurs in most somatic tissue and in general can be thought of as “cellular aging”. It has been hypothesized that the principle of senescence is an evolutionary mechanism

specifically intended to reduce the likelihood of cancer occurring by restricting the total number of times a cell can replicate. Each time a cell divides there-in lies the potential for the occurrence of errors while creating copies of genetic code. By imposing an upper limit to the number of times which a cell may divide, the maximum potential for mutations during mitosis is reduced, thus diminishing the likelihood of a cell becoming cancerous. Importantly, the number of times a cell is programmed to divide is also governed by what type of cell it is and what functions that cell serves. For example, a neuron almost never divides, whereas enterocytes may divide indefinitely.

The biochemical mechanism that exerts this self-imposed senescence manifests in the form of GC rich DNA sequences located at the ends of linear eukaryotic chromosomes known as telomeres. Telomeres function as protective ‘caps’ for the ends of chromosomes and are created by the enzyme telomerase, a specialized reverse transcriptase that adds back telomeric DNA to the ends of chromosomes. Over time, somatic tissues will gradually lose expression of telomerase, resulting in the shortening of telomeric DNA (13). After enough telomeric DNA has been lost, the shortened telomeres are recognized by the cell as double-strand breaks, which will then lead to large amounts of genomic instability and chromosomal aberrations triggering p53 modulated senescence or apoptosis. To subvert the natural process of cellular senescence, cancer cells have been shown to inappropriately express telomerase, thus preventing the loss of telomeric DNA (14). This revelation has prompted the development of telomerase inhibitors currently undergoing clinical trials.

A characteristic shared by nearly every subtype of cancer is the ability to deter or otherwise evade cellular signaling events that result in self-mediated cell death processes such as apoptosis, anoikis, or autophagy. Unlike cellular necrosis which happens in a fast, non-programmed fashion, these processes involve a variety of finely coordinated cellular signaling cascades that result in the controlled breakdown of organelles, proteins, and genetic material. Proteins such as Bax/Bcl-

2 and the caspase family of proteins are heavily involved in transmitting and regulating signal cascades that induce apoptosis to control DNA damage resulting from unregulated cellular growth and division. It is well understood that the loss or dysfunction of these proteins has been strongly correlated to the progression of cancer.

In addition to the evasion of both intracellular and extracellular stimuli that initiate programmed-cell death, another hallmark of cancer is the inactivation of tumor suppressor genes. Tumor suppressor genes contribute to the normal development of healthy cells by controlling DNA damage and cell cycle progression. These genes have gained the title of “tumor suppressors” by influencing processes that stifle the growth and survival of cells that would otherwise progress to malignancy (15). They do this in a number of ways: inhibiting cell growth and division, promoting apoptosis, preventing or hindering genetic change, inhibiting angiogenesis, and inhibiting metastasis. The gene TP53 encodes the tumor suppressing protein p53, one of the most well characterized tumor suppressors. A transcriptional activator, p53 functions to regulate the expression of a multitude of genes that influence growth, DNA damage and repair, and apoptosis (16).

Sustained cell signaling

Cancer results from the uncontrolled replication of a cell population. To maintain unregulated proliferative signaling, cancer cells may utilize any number of signal transduction pathways related to proliferation and survival. Depending on cancer subtype, the number of unique signaling pathways it co-opts may vary greatly, highlighting not only the inherent variance within a single cancer subtype, but also between cancer subtypes. For example, it is not uncommon for leukemias to only require a single oncogenic driver mutation to maintain sustained cellular signaling and progress to malignancy. The tyrosine-protein kinase ABL1 is frequently mutated in leukemias, often through a chromosomal translocation at

t(9;22) that results in the fusion of breakpoint cluster region protein (BCR) with ABL1, distorting the regulatory domains of ABL1 resulting in a constitutively active protein kinase that continuously initiates or sustains cell signaling cascades related to cell division, adhesion, and resistance to apoptosis (17).

In addition to the BCR-ABL fusion protein, many additional oncogenic driver proteins are also kinases responsible for activating signal transduction pathways. Epidermal growth factor receptor (EGFR/Her-2) is a receptor tyrosine kinase commonly mutated in NSCLC and breast cancer, with mutations that result in constant phosphorylation of substrate proteins (18). Phosphoinsolitol-3-kinase (PI3K) is responsible for phosphorylating lipids that transmit signals within the AKT/mTOR pathway resulting in the constant activation of this cellular growth pathway. Janus kinase (JAK) is responsible for activating cell signaling cascades involving the signal-transducer and activator of transcription (STAT) family of proteins. Given the heavy involvement of protein phosphorylation within these growth and survival pathways, protein phosphatases such as Src-homology containing protein 2 (SHP-2) and phosphatase and tensin homolog (PTEN) have been shown to function as negative regulators of these pathways, and a loss in their activity or expression has been linked to tumor progression (19).

Inflammation and the avoidance of immune detection

Outside of the intracellular processes occurring within the tumor itself, there are extrinsic biological systems that influence tumor development, not the least of which is the immune system. It has long been understood that there is a great deal of interplay between the immune system and the tumor itself, however until recently, the importance of the immune response on tumor development had not been thoroughly researched. One of the primary functions of the immune system is to identify and eliminate cancer through the recognition of mutant proteins that may result in tumorigenesis, a process known as immune surveillance. The manner in which the immune system does this is highly complex and involves

many different immune cell types. CD4+ helper T-cells identify cancerous neoepitopes and mobilize natural killer (NK) cells and cytotoxic CD8+ T-cells to eliminate them. Additionally, immune cells secrete immunomodulatory or inflammatory molecules such as cytokines or antibodies which are produced by B-cells. This persistent activity of the immune system against an identified tumor often results in prolonged chronic inflammation (20).

Eventually, a tumor can accumulate mutations to evolve to the final 'escape phase' in which it applies several mechanisms such as antigenic modulation, tumor induced privileged sites, and tumor-induced immune suppression to completely elude immune detection. However, therapies which disrupt these mechanisms of immune evasion and 'unmask' the tumor to the immune system have demonstrated immense clinical benefit in recent years. Immunotherapy first began in the early 20th century when Dr. William B. Coley would inject mixtures of live and attenuated streptococcus into patient tumors to induce a localized immune response (21). In recent years, monoclonal antibodies developed against immunosuppressive proteins such as programmed death protein 1 (PD-1) and its ligand (PD-L1) have revolutionized the field of cancer therapy and will be discussed in greater detail in later chapters.

Biology and epidemiology of NSCLC

Of all cancer types, lung cancer, non-small cell lung cancer (NSCLC) specifically, may be the most burdensome on the American healthcare system. It is the leading cause of cancer deaths each year both world-wide and within the US, accounting for nearly 22% of all deaths due to cancer (22). Furthermore, the Commonwealth of Kentucky suffers the highest rates of death due to lung cancer in the entire country, likely attributable to the high rates of smoking (22).

Lung cancer can be divided into two groups, the more prevalent and slower growing NSCLC, and the less common but more aggressive small cell lung cancer

(SCLC). NSCLC can be further divided into two major histological groups, lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD). LUSC is a slow growing form of the diseases and originates from squamous cells; a type of epithelial cell that lines the inside airways of the lungs. LUAD arises from glandular cells that are found within the smaller airways and alveoli and has the tendency to progress less quickly than other lung cancers. Although all types of lung cancer are associated with smoking, the association is strongest with LUSC when compared to LUAD. LUAD is the most common lung cancer histology amongst non-smokers (23). The most prominent risk factor for NSCLC is tobacco use, contributing to greater than 80 percent of lung cancer deaths. Additional risk factors include pre-existing conditions such as COPD or previous aerodigestive malignancies, genetic predispositions, or exposure to carcinogenic compounds such as asbestos, radon, and arsenic (24).

Although many cancers can be divided into discrete subgroups based upon their histology, tissue, and cell type of origin, the heterogeneity of the disease from a molecular standpoint cannot be overstated. There have been numerous studies which have established high intratumor heterogeneity as a negative prognostic indicator, characterized by factors such as tumor mutational burden (TMB), chromosome instability (CIN), and copy number variation (CNV) (25)(26)(27)(28). NSCLC, specifically, is well understood to be a highly heterogenous disease which likely contributes to the challenges posed in identifying effective therapies that provide durable and long-lasting clinical benefit (29). There are likely many reasons for the genetic diversity of NSCLC; however, the consistent DNA damage induced by tobacco use over a long period of time has been hypothesized to be a major contributing factor (30). Importantly, as we continue to develop our understanding of cancer and its ever-increasing complexity, this heterogeneity highlights the value of mechanistic studies to elucidate the molecular workings of the disease. Though not always immediately apparent, the eventual clinical translatability of such work may function to attenuate previous failures in attempts

to develop efficacious treatments that eventually become FDA-approved therapies (31)(32).

In LUAD, mutations in the genes encoding KRAS and EGFR are extremely common and detected in 32% and 27% of LUAD tumors, respectively. In addition to these well-characterized oncogenes, activating mutations in other genes such as BRAF, ALK, MET, and ROS play an important role in the development of LUAD. Mutations in tumor suppressor genes such as TP53, STK11, and PTEN are all common in LUAD, with nearly 50% of LUAD tumors harboring inactivating mutations in the TP53 gene (33).

Biology of KRAS

The KRAS protein (Kirsten rat sarcoma 2 viral oncogene homolog), encoded by the KRAS gene, is a small, membrane-associated GTPase that functions to transmit external cellular signaling events to the nucleus. It belongs to a large superfamily of genes that encode small GTPases, and more specifically the subfamily known as the Ras family of genes. The RAS proteins include the three highly homologous proteins HRAS, NRAS, and KRAS. The KRAS protein contains a GTP binding pocket that serves to switch between its active GTP-bound state and its inactive GDP-bound state. Furthermore, this GTP-binding pocket is found within its G-domain, a region of the protein where downstream effectors may bind and intimate cellular signaling cascades. The balance of these two states is controlled by guanine nucleotide exchange factors (GEFs) which catalyze the transition from GDP-bound KRAS to a GTP-bound state, and GTPase-activating proteins (GAPs) which bind to activated G proteins, stimulate their activity or expedite GTP hydrolysis. When bound to GTP, KRAS can bind to cellular effector molecules such as son of sevenless (SOS) complexed with adaptor proteins like growth factor receptor-bound protein 2 (Grb2) to transmit signals from the cell membrane to the nucleus. These signaling cascades are known to influence cellular processes such as cell differentiation, proliferation, and apoptosis.

Importantly, the activation of KRAS is not just a single-step process but requires correct interactions with effector and scaffolding proteins such as SHP-2 for proper activation (34).

Figure 1.1. The KRAS signaling cascade. Upon ligation of RTKs with soluble growth factors or cytokines, the RTKs will trans-autophosphorylate allowing for the recruitment of protein complexes (Grb2/SOS/SHP-2) to the cell membrane which may then recruit additional proteins (KRAS) to allow for the initiation of cell signaling cascades that control cell growth and survival.
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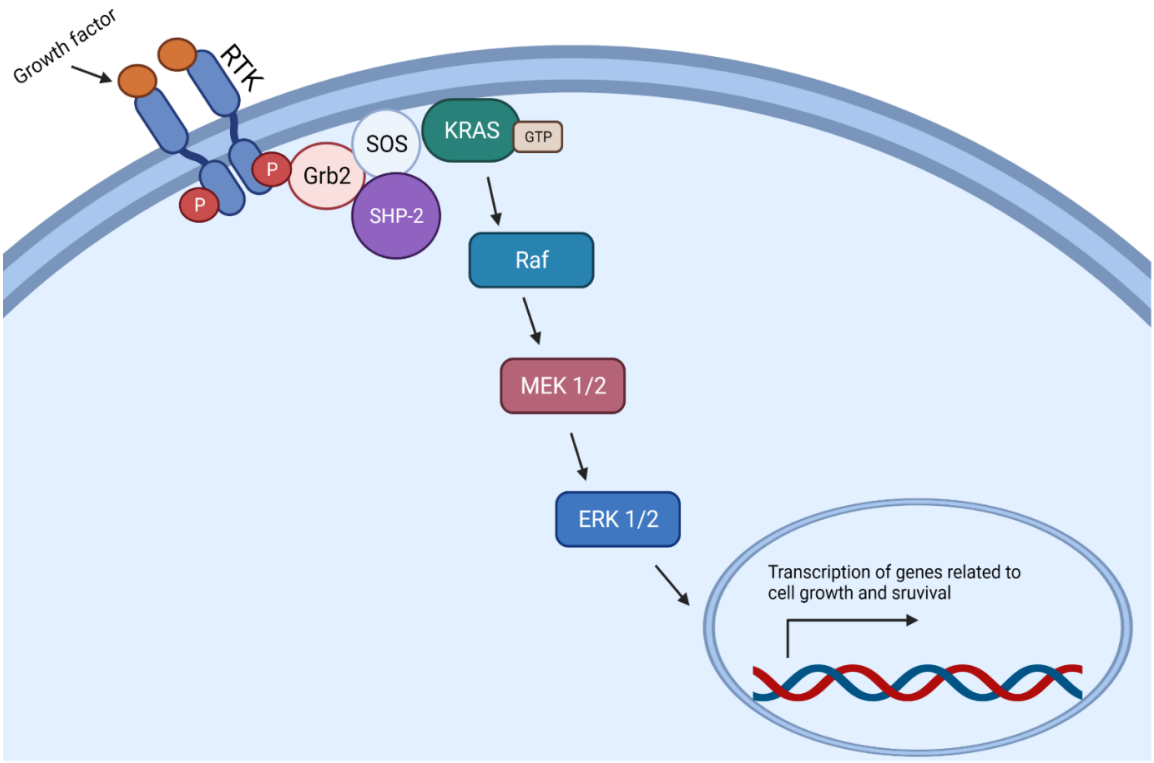


Upstream of Ras is a wide variety of membrane bound RTKs including but not limited to EGFR, Her2, platelet derived growth factor receptor (PDGFR), ROS proto-oncogene 1 (ROS1), ALK, VEGFR and others (35)(36)(37). Activation of RTKs upstream of Ras occurs upon binding an appropriate ligand which are most commonly growth factors like EGF or cytokines. Upon ligation, monomeric RTKs will then dimerize and auto, or trans-autophosphorylate, tyrosine residues on their cytoplasmic domains. The phosphorylated tyrosine residues then function as binding sites for adaptor proteins such as SHP-2 and Grb2 which contain Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. The adaptor proteins may recruit additional scaffold or adaptor proteins such as the GEF SOS to form complexes. The complexes can associate with membrane-bound KRAS and catalyzes its transition to an active GTP-bound state. Once active, KRAS can then bind to and activate additional downstream effector molecules to initiate signal transduction pathways that control proliferation (Raf/MEK/ERK), apoptosis (PI3K/Akt/mTOR), cytoskeletal reorganization (TIAM1/Rac/Rho), and cell cycle progression (PLC/PKC) (37).

As stated above, in NSCLC mutations in the KRAS gene are common and function to impair the ability of KRAS to revert to an inactive GDP-bound state. As many as 30% of all NSCLC tumors harbor mutations in the KRAS gene and are generally mutually exclusive from tumors driven by EGFR mutations. The distribution of mutations in the Ras gene vary by cancer type and Ras isoform. However, a vast majority of the mutations in the KRAS gene occur at the 12th codon (83%) which encodes a glycine residue, followed by the 13th codon (14%) which also encodes a glycine, and lastly the 61st codon (2%) which encodes glutamine (38). Mutations at G12/G13 substantially reduce the GTPase activity of KRAS which prevents its reversion to an inactive GDP-bound state. This then results in the constitute activation of downstream pathways that drive cell growth and survival. In NSCLC, the most common substitutions are $KRAS^{G12C}$, $KRAS^{G12V}$ (found most often in smokers) and $KRAS^{G12D}$ all of which have been shown to have varying effects on the activation levels of different downstream growth and survival

pathways (39). Substitutions in codon 61 that change the polar uncharged amino acid glutamine to charged residues such as histidine are thought to impair the coordination and stabilization of GTP hydrolysis which catalyzes the return of KRAS to an inactive GDP-bound state (40).

Therapeutic interventions for NSCLC

The treatment of NSCLC depends on the stage of the disease upon diagnosis. For early-stage NSCLC (Ia to resectable IIIb) the primary treatment is localized radiation therapy to reduce tumor mass followed by with surgery to resect the tumor. As tumor stage progresses past Ia, chemotherapeutic agents such as cisplatin, docetaxel, etoposide, and vinorelbine are used as neoadjuvant therapies to increase the chances of a complete resection. For tumors that progress to non-resectable stage IIIb/IV, next-generation sequencing (NGS) is often applied to determine the mutational profile of the tumor and direct the application of targeted therapies. In the absence of actionable mutations, chemotherapy is often used as palliative therapy (41).

In LUAD, targeted therapies are available for tumors which harbor mutations in EGFR, ALK, or ROS1. First generation small molecule inhibitors of EGFR such as erlotinib and gefitinib (also known as tyrosine kinase inhibitors, or TKIs) bind to the intracellular tyrosine kinase domain of EGFR and prevent it from phosphorylating substrate proteins. Additionally, monoclonal antibodies like cetuximab have been used to bind to and inhibit EGFR signaling in colorectal and head and neck cancers (42). Although first generation TKIs which bind to and disrupt oncogenic EGFR signaling have demonstrated great clinical success, acquired resistance to these therapies through mutations in the domains to which the drugs bind has presented significant obstacles in their optimal application (43)(44). Mutations such the T790M substitution, which arises from mutations in exon 20 of the EGFR gene, alters the structure of the intracellular tyrosine kinase domain of the protein and is able to overcome competitive small molecule inhibition

by drastically increasing its affinity for ATP binding (45). This has prompted the development of second and third line EGFR inhibitors such as the third generation EGFR TKI Osimertinib, which has a higher affinity specifically towards the T790M EGFR mutant (45).

While there exist targeted therapies against major LUAD oncogenes, direct inhibition of the KRAS protein with small molecules has proven exceptionally difficult, despite decades of research (46). Very recently, a small molecule inhibitor of the KRAS G12C variant, AMG510, has shown promising results in early phase clinical trials. Regardless, there remain no FDA-approved targeted therapies for patients harboring KRAS mutations and thus therapies are limited to cytotoxic chemotherapy. As a result of this unmet need, efforts have been made to find new targets downstream of KRAS signaling that may serve as more suitable drug targets to varying levels of success. Additionally, the use of ICIs in KRAS active cancer offers a new targeted therapeutic avenue for patients with this type of mutation.

1.3 IMMUNE CHECKPOINT INHIBTORS AND PD-L1

Monoclonal antibodies and the advent of Immune checkpoint inhibitors

Within the last decade, the emergence of a class of drugs known as immune checkpoint inhibitors (ICIs) has had an extraordinary impact on the field of cancer therapy. In 2011, the FDA approved the use of the first ICI, ipilimumab, for the treatment of metastatic melanoma (47)(48). It did not take long for the potential efficacy of this newly emerging branch of immunotherapy to be fully recognized (49)(50). Within several years of this drugs approval, the development of monoclonal antibodies was rapidly accelerated, amounting to the FDA approval of the first drug ever given a ‘breakthrough therapy’ designation, obinutuzumab. The impact of ICIs, especially for patients that until recently had no other treatment

options, was so immense that the new ‘breakthrough therapy’ designation was created to get ICIs to the bedsides of patients as quickly as possible.

While there are many proteins for which monoclonal antibody therapies have been developed, by far the most effective are antibodies which target the immune checkpoint proteins cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death protein 1 (PD-1), and its ligand programmed death ligand 1 (PD-L1). These proteins serve a highly important biological function; to modulate the activity and activation of T cells.

The revolutionary discovery of the CTLA-4 immune checkpoint pathway occurred in the early 90s in the lab of James P. Allison when he observed that CTLA-4 had the ability to reduce the action of activated T-cells. He soon developed an antibody against CTLA-4 that could block its function by pharmacologically inhibiting the endogenous inhibitor of immune cell activation. Pre-clinical animal studies quickly confirmed his hypothesis with impressive results, though it wasn’t until roughly a decade later in 2007 that phase 1 clinical trials of ipilimumab began for the treatment of various kinds of treatment resistant cancer (51).

Around the same time as the discovery of CTLA-4, Tasuku Honjo made the discovery of PD-1 and came to understand that it possessed immunomodulatory capabilities like that of CTLA-4. PD-1 is another receptor expressed on the surface of activated T-cells, and upon ligation to PD-L1, is internalized and through a separate mechanism to CTLA-4 deactivates the T-cell. In 2014, the first monoclonal antibody against PD-1, pembrolizumab, was approved by the FDA for the treatment of metastatic melanoma. Soon after in 2015, it was approved for use in patients with treatment resistant metastatic NSCLC, and whose tumors expressed PD-L1.

While activation of both the CTLA-4 and PD-1/PD-L1 pathways results in the deactivation of cytotoxic T-cells, there are some key differences in the

mechanism by which they do so (51). PD-L1 is normally expressed on the surface macrophages, T-cells, B-cells, and healthy epithelial cells in response to an immune reaction and primarily functions as a mechanism for the immune system to differentiate “self” from “non-self”. Upon ligation of PD-L1 to its receptor PD-1, expressed on the surface of activated T-cells, the PD-1 receptor is internalized and initiates a signaling cascade that results in the deactivation of the T-cell. What makes this pathway so pivotally important in the context of cancer is the ability of a tumor to aberrantly express PD-L1 on the cell surface, thus co-opting the immunoregulatory function of PD-1 ligation and inappropriately deactivating cytotoxic T-cells, often times to such an extent it leads to T-cell exhaustion.

CTLA-4 is a receptor expressed on the surface of activated T-cells that competes with the CD28 receptor to bind to its ligand B7 which is expressed on the surface of antigen presenting cells (APCs) (52). The relative expression levels of CTLA-4 and CD28 are based on the activation level of the T-cell receptor (TCR) via major histocompatibility complex (MHC) binding. A weak TCR stimulus causes CD28:B7 binding to overcome CTLA-4:B7 binding and results in activation of signaling cascades that promote T-cell proliferation, survival, and cytokine production (53). When a strong TCR stimulus occurs, the inverse happens, and there is a net negative effect on T-cell activation. The primary function of the CTLA-4 pathway is to reduce T-cell activity in the interest of protecting healthy cells from an overactive immune response, thus functioning as an “immune checkpoint” that restrains immune cell activation. Importantly, the CTLA-4 pathway impacts the earlier phases of immune activation by influencing immune cell proliferative potential (54).

In contrast to the CTLA-4 pathway which produces its effects in the early phases (i.e. priming phase) of immune activation, the PD-1/PD-L1 interaction occurs in the later stages (i.e. effector phase) of T-cell activation by suppressing activated, cytotoxic T-cells. Furthermore, CTLA-4 is primarily expressed on regulatory T-cells, whereas PD-1 is expressed on other types of immune cells such

as B-cells or myeloid cells. These two immune checkpoint pathways also differ in the expression of their ligands, B7 and PD-L1. B7 is expressed by APCs which are typically found within lymphatic tissues, as opposed to PD-L1 which can be expressed upon a wide variety of peripheral and immune cells, and more importantly, on many different types of tumors.

Monoclonal antibodies against PD-1 and PD-L1 have indeed demonstrated clinical success across a variety of cancer types, specifically in advanced melanoma and NSCLC. In 2015, results were published from the KEYNOTE-001 trial, a multicohort phase 1 study of pembrolizumab (brand name Keytruda) in patients with metastatic carcinoma, melanoma, or NSCLC carcinoma (55). Pembrolizumab demonstrated tolerable antitumor activity in NSCLC patients regardless of prior treatment status, and in 2017, the investigators reported a median overall survival (OS) time of 22.1 months (56). Very importantly, these patients all had a PD-L1 tumor proportion score (TPS) of at least 50% (determined by immunohistochemistry (IHC) staining), suggesting that tumors expressing the highest levels of PD-L1 may respond better to PD-1/PD-L1 inhibition than tumors with lower PD-L1 expression (56). In 2019, 5-year results were reported that showed OS rates of 23.2% for treatment-naïve patients and 15.5% for previously treated patients (57). The significance of these improvements to long term survival cannot be overstated when compared to the historic 5-year OS rate of 5.5% (56). Additional KEYNOTE trials have shown repeatable clinical value of PD-1/PD-L1 inhibition in NSCLC and the relevance of a PD-L1 TPS ≥ 50% (58)(59)(60). Importantly, KEYNOTE trials 42 and 189, demonstrated the value of these targeted therapies in patient tumors not harboring mutations in EGFR or ALK (61)(62). Given the mutually exclusive nature of EGFR, KRAS, and ALK mutations, these findings support the notion that patients with KRAS-mutant tumors (for whom there exist no targeted therapies) are poised to greatly benefit from PD-1/PD-L1 blockade (63)(64).

Although the results of these clinical trials provide the foundation for a promising future for drugs which inhibit PD-1 or PD-L1, there remain significant limitations to these therapies that curtail their optimal application. One such limitation is the occurrence of significant adverse reactions that can negatively impact nearly every major organ system (65). These treatments also face a major challenge in feasibility; the price of therapy often exceeds $100,000 over the course of treatment, thus greatly limiting their use only to patients able to afford it. Further compounding these issues is the low rate of response to PD-1/PD-L1 blockade (20-30% overall response rate (ORR) in solid tumors) (66). The objective response rate to PD-1/PD-L1 blockade is also known to vary by tumor type, with melanoma typically demonstrating the most consistent response (30-45%), trailed by NSCLC (15-20%) (67)(68). As demonstrated by the KEYNOTE trials, NSCLC patients with a high PD-L1 TPS (≥50%) responded better to therapy than those with a TPS below 50%. However, a high PD-L1 TPS does not necessitate response to therapy; clinical studies have shown that even in NSCLC patients with high PD-L1 expression, as many as half still did not respond to the PD-1 inhibitor pembrolizumab (69). Conversely, the lack of PD-L1 expression does not mean a patient will not response to therapy. In certain cases, the response rates between those with and without tumoral PD-L1 expression remain relatively similar (70). The lack of understanding as to why some patients without tumoral PD-L1 expression still respond to PD-1/PD-L1 inhibition warrants further investigation into the mechanisms malignant cells apply to aberrantly express PD-L1 and evade immune system detection.

It is apparent that the expression of PD-L1 on a patient tumor is of clinical significance, though it has shown to be insufficient as a sole indicator of response to PD-L1 blockade. This suggests that a more sophisticated metric than the mere quantity of PD-L1 expressing cells is a necessary prerequisite to the complete utilization of PD-L1 expression as a prognostic indicator. As such, many attempts have been made to identify biomarkers of response to PD-1/PD-L1 blockade to replace or supplement tumoral PD-L1 expression. For example, tumor mutational

burden (TMB) is a measure of the quantity of somatic non-synonymous coding mutations in a tumor. Tumors with a high TMB (20 or more mutations per megabase) express a greater number of mutated proteins resulting in heightened immunogenicity through the increased likelihood of neoantigens being recognized by the immune system. Another proposed biomarker, microsatellite instability (MSI), reflects changes in the number of nucleotides in DNA elements composed of repeated motifs (71). MSI quantifies the number of times DNA mismatch repair systems fail to correct errors and is indicative of the levels of genetic instability within a tumor. Though it is true that this instability is often detrimental to an individual cells growth and survival, when this instability is extrapolated to a larger population of cells rapidly replicating, this instability eventually results in advantageous mutations that allow for continued or expedited tumor growth. Both TMB and MSI have been able to provide prognostic value in differing cancer subtypes, but they are inferior in their accuracy compared with PD-L1 TPS. The most effective prognostic indicator response to PD-1/PD-L1 blockade may be a combination of these independent biomarkers (72).

Given the importance of PD-L1 expression (especially when a tumors TPS is ≥50%) in the application of ICIs in NSCLC, a comprehensive understanding of the mechanisms governing PD-L1 expression is vital. PD-L1 expression can vary greatly not only between individual NSCLC tumors, but also within different regions of the tumor itself. This heterogeneity has been hypothesized to be a major factor that cripples the prognostic clinical value of PD-L1 TPS. The factors regulating PD-L1 expression are not yet fully understood, however mechanisms which have been investigated thus far can be grouped by transcriptional and post-transcriptional control.

Intrinsic factors which regulate genetic and epigenetic alterations have been demonstrated to impact the expression of PD-L1. Methylation of the CD274 promoter has been shown to reduce PD-L1 transcriptional activity in NSCLC, and the expression of histone deacetylase has been correlated with PD-L1 expression

as well (73). Further transcriptional regulation of PD-L1 expression is augmented by the activity of signal transduction pathways such as RAS/Raf/ERK, EGFR, PI3K/Akt/mTOR, and tumor suppressing pathways involving TP53 or STK11 all of which may influence PD-L1 expression to some extent (74)(75)(76). Extrinsic factors such as cytokines, specifically molecules such as IFN- γ and IL-6, are known to induce the expression of PD-L1 in many different types of cancers and immune cells alike (77). Growth factors that stimulate the above-mentioned pathways can also induce PD-L1 transcriptional activity (78). DNA damaging agents and angiogenesis/hypoxia may also influence the transcriptional activation of PD-L1 (79). Once transcribed into mRNA, a variety of different miRNAs can target the CD274 transcript to fine tune its translation into protein (80)(81). The stability of CD274 mRNA can also be impacted by alterations to the 3'-UTR of the transcript, and oncogenic Ras signaling has also been shown to influence CD274 mRNA stability (82)(83). Additionally, post translational modifications to the PD-L1 protein such as, glycosylation, ubiquitination, and serine/threonine phosphorylation delicately articulate PD-L1 expression and metabolism (84)(85)(86).

1.4 PHOSPHATASES, KINASES, AND SHP-2

Protein Phosphorylation

Protein phosphorylation is a major modality by which protein function and activity is controlled. Phosphorylation is a reversible post-translational modification (PTM) of eukaryotic proteins carried out by kinase enzymes. Furthermore, protein phosphorylation is an essential component of various signal transduction pathways that relate to the development and progression of cancer. The human genome contains at least 518 protein kinases which may phosphorylate as many as 70% of all known human proteins (87). The possibility that proteins may become post-translationally phosphorylated was discovered in the early 1900s; however, it wasn't until half a century later that protein phosphorylation was described as an

enzymatic process. This discovery stirred further interest into researching the biological relevance of kinases and their importance to cellular signaling pathways. In 1992, Edmon Fischer and Edwin Krebs were awarded the Nobel prize for their discoveries concerning reversible protein phosphorylation (88). Since then, many research groups have devoted their efforts to begin completely characterizing the human kinome (89).

In eukaryotes, protein phosphorylation is carried out by protein kinases which attach a negatively charged hydrophilic phosphate (PO_4^-) group from adenosine triphosphate (ATP) to either serine, threonine, or tyrosine residues. The addition of a phosphate group to a protein likely changes its structure, function, localization, or ability to interact with other proteins. The phosphorylation of a protein can result in a vast array of changes to its function, and the extent of phosphorylation to any specific protein has a unique impact on the cellular processes in which it is involved. For example, the tumor suppressor protein p53 can be phosphorylated on serine residues following DNA damage which influences its ability to bind to DNA and function as a transcription factor. In the case of PD-L1, serine/threonine phosphorylation at T180 or S184 increases its binding affinity to E3 ligases and results in its degradation (16).

Kinases

While there are only three (major) amino acids that become phosphorylated, the kinases responsible for such modifications are specific to either Ser/Thr or Tyr, and also highly specific for their unique protein substrates. The phosphorylation of serine and threonine residues is typically carried out by dual-specificity kinases that have the ability to add a phosphate group to either amino acid (90). Phosphorylation of tyrosine residues is carried out by tyrosine kinases, and a very small family of non-canonical kinases can phosphorylate both Ser/Thr or Tyr residues. Of all known phosphorylation events in the human kinome, a vast majority (95%) are the phosphorylation of serine residues, followed by 3-4% are

that of threonine, and less than 1% tyrosine (90). Importantly, protein phosphorylation plays a considerable role in essential signal transduction pathways that regulate processes such as growth, survival, and cell division. Thus, in cancer, kinase activity is often deregulated resulting in the constitutive activation of pathways that regulate cell growth and survival. Given the wide array of known kinases and the multifaceted functions they carry out, the dysregulation of kinase activity within a tumor does not necessarily mean that all kinases are oncogenes, and that it must be an increase in kinase activity that results in a tumor promoting phenotype (91).

Despite tyrosine phosphorylation events being rare relative to that of serine and threonine, their relevance to pathways often deregulated in cancer is substantial. Receptor tyrosine kinases (RTKs) are transmembrane bound enzymes that selectively phosphorylate tyrosine residues upon ligation to extracellular signal molecules such as growth factors or cytokines. Upon ligation, monomeric RTKs will dimerize and trans-autophosphorylate their cytoplasmic kinase domains resulting in the recruitment of protein complexes which initiate a wide array of signal transduction pathways (34)(35). The largest number of known oncogenes belongs to the family of tyrosine kinases (9). As such, therapeutic strategies which impede deregulated tyrosine phosphorylation are highly pertinent to the treatment of cancer. Tyrosine kinase inhibitors (TKIs) have become some of the most effective targeted therapies for many different cancer types, but especially NSCLC. There have been multiple generations of TKIs that inhibit EGFR activity, greatly stunting the progression of cancers which are dependent on EGFR for growth (92). The clinical success of TKIs provides a logical foundation for the development of drugs which target tyrosine phosphatases that function as negative regulators of these same pathways.

Phosphatases

Protein phosphatases function not only to help maintain and negatively regulate the careful balance of protein phosphorylation within cell signaling cascades, but to also participate in articulating the precise and structured sequence of such phosphorylation events (93). It is important to note that compared to the >500 known kinases (and 90 known tyrosine kinases) encoded within the human genome there are only roughly 200 phosphatases, 38 of which are tyrosine specific phosphatases (PTPs) (94). In the early 21st century, scientific interest was heavily focused upon protein kinases. However, in the past decade, the importance of phosphatases as more than just non-specific housekeeping enzymes has gained appreciation.

Given the well characterized importance of tyrosine kinases in oncogenic cell signaling, it is logical that negative regulators of the same processes should be of equal importance. Although, generally, the phosphorylation of tyrosine resides results in increased protein activity, this is not always the case, and so maintaining the balance of Tyr phosphorylation can have varying effects on protein activity. Understanding the general principle that phosphorylation of a protein increases its activity, this would lead one to believe that PTPs should function as tumor suppressors in an inverse manner to tyrosine kinases. However, though many PTPs do in fact negatively regulate cell growth, migration, and invasion, a number of PTPs have demonstrated tumor promoting properties which often differ based upon the unique PTP as well as the type of cancer in which it is expressed (95)(96). For example, Receptor-type tyrosine-protein phosphatase gamma (PTPRG) has been shown to possess tumor suppressive properties in ovarian and breast cancer (96). Several phosphatases of regenerating liver (PRL) have been reported to have tumor promoting properties, and their overexpression, as is such in the case of PRL3 which is overexpressed in metastatic colorectal cancer, but not in normal or early-stage cancer tissue (97). It is also thought to contribute to the development of lung and liver metastasis (98). Given the wide range of PTP activity on tumor initiation and progression, further interrogation into the role of PTPs in cancer may result in substantial clinical benefit.

Despite recent revelations that PTPs may serve as suitable drug targets in cancer, there have been challenges in developing small molecule catalytic inhibitors against PTPs as a result of their promiscuous nature with regard to protein substrates (99). However, the development of allosteric inhibitors that possess higher specificity for phosphatases relevant to oncogenesis and tumor progression are beginning to show clinical promise, thus warranting further investigation into the biology that underpins PTP function with tumor cells (100). One such phosphatase that has been identified as a critical regulator of oncogenic signaling pathways (such as the KRAS pathway) is the tyrosine phosphatase SHP-2.

SHP-2

The non-receptor tyrosine phosphatase, Src homology region 2 domain-containing, phosphatase-2 (SHP-2) encoded by the PTPN11 gene, is ubiquitously expressed in a variety of mammalian cells and has gained attention as a therapeutic target in recent years. SHP-2 is expressed in both NSCLC patient tumors and cell lines. SHP-2 has three major structural domains; a catalytic protein tyrosine phosphatase (PTP) domain on the C-terminal region and two neighboring Src homology region 2 (SH2) domains on the N-terminal region (101). The SH2 domains function to bind to phosphorylated tyrosine residues on protein substrates, but also possess unique functions that modulate the various activities of SHP-2 itself. In its basal inactive state, the N-SH2 domain binds to and inhibits PTP activity or binds to phosphoprotein substrates, whereas the C-SH2 domain contributes to binding energy and substrate specificity (102). The activity of SHP-2 is controlled through autoinhibitory interactions between the catalytic PTP domain and the N-SH2 domain. The regulation of this inhibitory state is governed through conformational changes induced from binding with protein substrates, mediated through the phosphorylation of two tyrosine residues (Y542 and Y580)

located on its c-terminal domain (CTD) which influence its affinity to protein substrates.

Figure 1.2. Structural conformations of SHP-2. When in an inactive state, the N-terminal SH2 domain will fold back on the catalytic phosphatase domain, preventing phosphatase activity (top). Once activated (through Tyr phosphorylation on its C-terminal domain at Y542 or Y580, or by binding to protein substrates) the N-SH2 domain releases from the PTP domain, allowing phosphatase activity (bottom). Made with Biorender.com

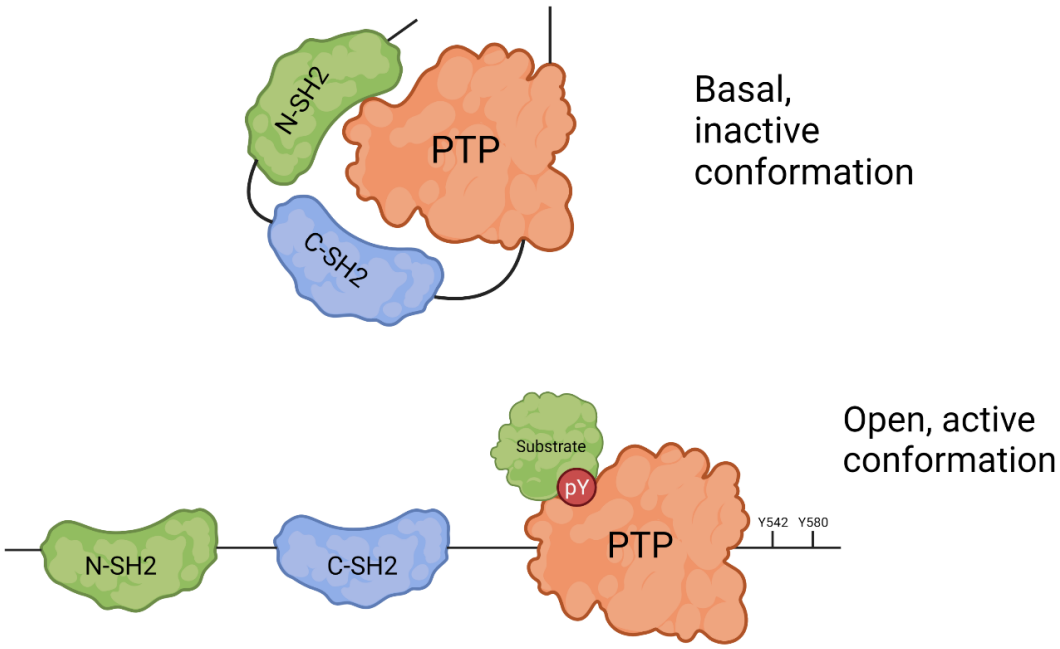
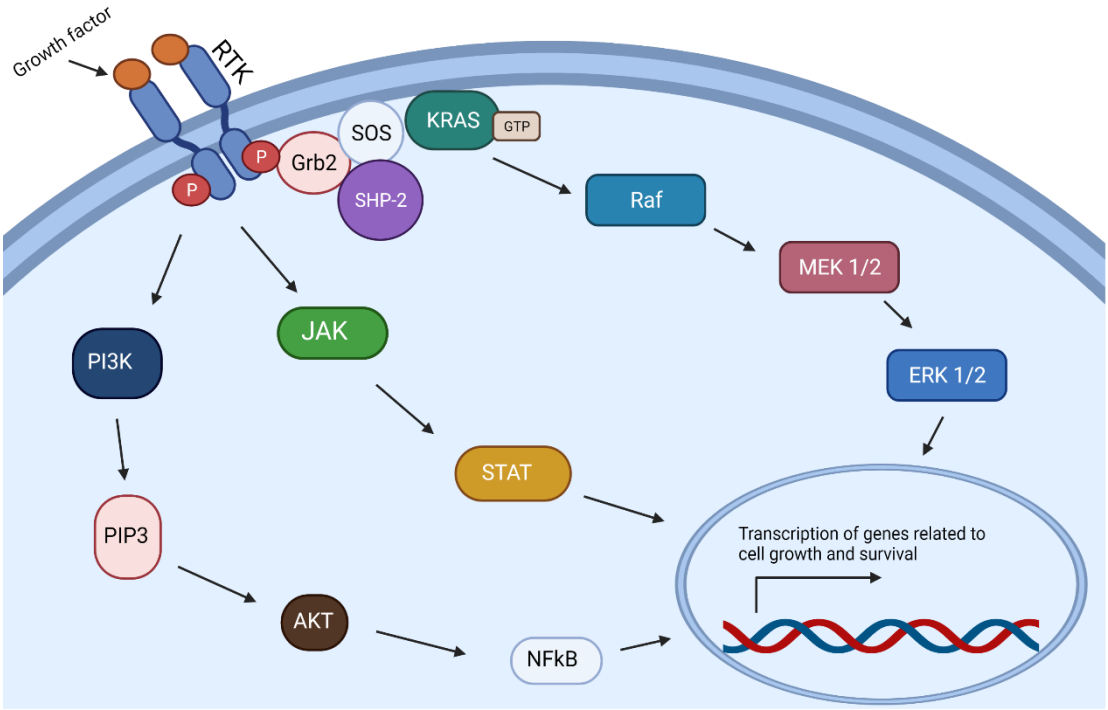


Figure 1.3 The activities of SHP-2. Expanded diagram of SHP-2's involvement within the KRAS signaling cascade, as well as the JAK/STAT and PI3K/Akt pathways. SHP-2 has been shown to both positively and negatively regulate STAT signaling through its catalytic phosphatase activities. Additionally, SHP-2 has been shown to be involved in anti-apoptotic signaling by suppressing caspase-3 mediated apoptosis (104).



SHP-2 has been implicated in numerous pathways relevant to cancer, and the precise nature in which it impacts these pathways is both highly complex and context specific. It has been shown to be a negative regulator of the Jak/STAT pathway by dephosphorylating Tyr701 of STAT1 in response to interferon stimulation. Conversely, SHP-2 can also positively regulate Jak/STAT signaling by dephosphorylating Tyr1007 of Jak2, reducing its ability to form a degradation inducing complex with Socs1, thus preventing Jak2 turnover (103). Additionally, SHP-2 catalytic activity is required for proper regulation of the PI3K/Akt survival pathway (104). However, it is not only the catalytic PTP activity of SHP-2 which is responsible for its signal transducing capabilities. In immune cells, ligation of the PD-1 receptor results in SHP-2 mediated receptor dimerization and internalization which occurs irrespective of SHP-2s PTP activity (105). Indeed, SHP-2 serves a variety of functions in oncogenic signaling pathways, though not to be overshadowed is its critical role in mediating KRAS signaling events. Several groups have demonstrated the importance of SHP-2 within the RAS/Raf/ERK signaling cascade, even going so far as to insinuate SHP-2 as a critical regulator of KRAS-signaling in NSCLC (106). Furthermore, it has been shown that KRAS-mutant NSCLC depends on SHP-2 for carcinogenesis, though ablation of SHP-2 activity or expression was only sufficient to delay tumor progression, not reverse it (107).

Despite the known importance of SHP-2 in this oncogenic signaling pathway, whether SHP-2 is a tumor suppressing or promoting protein remains unclear and may depend on the context in which it is expressed. SHP-2 has displayed tumor suppressing activities in hepatocellular carcinoma and has been shown to suppress oncogenesis by dephosphorylating Ras in glioblastoma (108). Outside the realm of cancer, mutations that impact the catalytic activity of SHP-2 are linked to diseases such as Noonan syndrome (NS) and Noonan syndrome with multiple lentigines (NSML). These diseases can cause a wide variety of developmental defects that vary from one individual to another and can change over the course of an affected individual’s lifespan (109). This demonstrates that the impact of

SHP-2 expression bears great relevance to the molecular context in which it is expressed. As it stands, further understanding of the impact of SHP-2 expression within the context of a lung tumor and how that relates to the regulation of oncogenic lies the potential for the development of new therapies that diminish SHP-2 activity or expression. However, until we are able to better understand the complex nature of phosphatases (and SHP-2 specifically) involvement in oncogenic signaling pathways, we remain limited in our ability to create efficacious therapies that synergize with currently available treatments, such as TKIs and immunotherapies.

1.5 PROJECT OVERVIEW

The aim of the work described herein was to combine the application of *in vitro* biochemical experimentation with analysis of real-world, publicly available clinicogenomic information to uncover a mechanism by which SHP-2 controls the regulation of PD-L1 in KRAS active non-small cell lung cancer (NSCLC). It has been a long-standing goal of this laboratory to harness the power of pharmacogenomic data to improve the personalization of therapy for NSCLC patients. While the scope of the presented work resides primarily within the realm of foundational biology, its reach expands into the domain of clinical practice by explicating the underlying mechanisms that influence the efficacy and application of existing and emerging cancer therapies.

As the use of immune checkpoint inhibitors (ICIs) rapidly transitioned from bench to bedside within the past decade, the accumulation of clinical information has begun to reveal the flaws preventing optimal utilization of these therapies. To date, the ability to predict which patients will elicit a response to immune checkpoint inhibition remains strikingly poor and has been estimated to lead to responses in less than 13% of patients with cancer in the US (110). Importantly, the benefit of ICI treatment in responding NSCLC patients exceeds that of other cancers, further demanding improved methods to predict responsive patients in this type of cancer (110). Among the methods that have been approved to predict which patients will

respond to immune checkpoint inhibition based upon the stage of the disease, the most impactful has been tumoral expression of PD-L1, specifically tumors with >50% total PD-L1 expression. It is therefore important to gain a complete understanding of the factors which govern PD-L1 expression in NSCLC. The mechanisms governing the expression of PD-L1 in tumor cells are diverse and continue to grow in number adding complexity to the combined impact of their actions. Resulting from this complexity is the discovery of drug targets which may synergize with PD-L1 inhibition. These combinations may have considerable clinical value, especially in patients harboring activating KRAS mutations who have few targeted therapeutic options.

I hypothesized that the tyrosine phosphatase, SHP-2, is capable of regulating PD-L1 expression, specifically in KRAS-active NSCLC. To address this hypothesis, **I analyzed publicly available protein and gene expression data collected from NSCLC patients to investigate correlations in both the expression and activity of SHP-2 and the expression of PD-L1. I then acquired data sets from two additional studies to further interrogate the relationship between SHP-2 and PD-L1 expression and to determine whether SHP-2 expression correlates with response to immune checkpoint inhibition (Chapter 2). Second, I conducted *in vitro* biochemical analyses to examine the expression and activity of SHP-2 in KRAS active NSCLC cell lines and measure the impact on PD-L1 expression (Chapter 3). Finally, I utilized biochemical and proteomic techniques to uncover novel protein-protein interactions using SHP-2 as bait to define the partners with whom SHP-2 interacts as a means to discover the mechanism(s) by which SHP-2 governs PD-L1 expression in KRAS active NSCLC.**

CHAPTER 2.

2.1 Overview

The identification of novel therapies, new strategies for combination of therapies, and repurposing of drugs approved for other indications are all important for continued progress in the fight against lung cancers (22). Perhaps the most significant advance in therapy for many cancer types was the entry of immune checkpoint inhibitors (ICI) as a standard of care therapy for melanomas in 2014 (111). For non-small cell lung cancers (NSCLC), specifically those without targetable mutations in epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK), ICIs that target programmed cell death 1 (PD-1) or programmed death ligand 1 (PD-L1), have revolutionized cancer therapy even though response rates are relatively low (112).

Both pembrolizumab and atezolizumab, PD-1 and PD-L1 inhibitors respectively, are approved ICI for frontline lung adenocarcinoma therapy for patients with high levels of PD-L1 expression on tumor cells (113). Durvalumab, an anti-PD-1 agent, is approved as maintenance therapy (114). Decisions to implement ICI therapy are often dependent on the PD-L1 tumor proportion score (TPS) using evidence from the KEYNOTE-024 and -042 trials (115) (116). Importantly, PD-L1 expression may not be the optimal biomarker of response as suggested in pivotal clinical studies (e.g. KEYNOTE and OAK trials), but it is clear that patients with high levels of tumoral PD-L1 are likely to experience a robust response to checkpoint inhibition (117). While many research groups have searched for improved biomarkers of response for checkpoint inhibitors, others have focused on identification of therapies that might be combined with ICI to improve patient outcomes (118).

I found that inhibition of the tyrosine phosphatase, SHP-2, increased gene and cell surface protein expression of PD-L1 in KRAS-active NSCLC cell lines.

PD-L1 is normally expressed on the surface of antigen presenting cells while PD-1 is expressed on T cells. It is the abnormal expression of PD-L1 on tumor cells, and the subsequent engagement with PD-1 on T cells, that causes tumors to be masked from an immune response (119). Inhibiting this interaction with antibodies against either PD-1 or PD-L1 can release a potent immune response toward the tumor. I hypothesized that because SHP-2 provides some level of control of expression of PD-L1 on NSCLC cells that inhibition of SHP-2 would increase PD-L1 expression and synergize with ICI therapy. Supportive of my hypothesis is recently published data by Chen and colleagues who showed in a NSCLC model system that combined SHP-2 and PD-L1 inhibition, with accompanying radiation, can overcome resistance to PD-1 inhibitors (120). Other groups have suggested that SHP-2 activity maybe more important in T cells, that infiltrate the tumor, to carry out signaling events downstream of PD-1 stimulation (121). Uncovering the precise mechanism of SHP-2 action on PD-L1 expression consumes many research groups, the model systems are expensive, and experimental time is long to get a drug to the clinic.

With these obstacles in mind, I chose to go to real world data to determine whether SHP-2 activity is related to PD-L1 expression and thereby focus my research efforts. I took advantage of three publicly available data sets to assess whether wet lab experimentation to determine if exploring the combination of ICI and SHP-2 inhibition would likely have clinical impact. First, The Cancer Genome Atlas (TCGA), now known as the NCI Genetic Data Portal (NCI-GDC), holds well-annotated expression and functional proteomic data (The Cancer Proteome Atlas (TCPA)) for patient tumors. However, most samples were collected prior to FDA approvals for ICI therapy, so no response data for ICI treatment is available (https://portal.gdc.cancer.gov/projects/TCGA-LUAD). Unfortunately, larger, industry-sponsored trials evaluating the clinical space for PD-1 and PD-L1 inhibitors are still open (e.g. KEYNOTE and OAK), and full genomic and patient response datasets are not yet published. Therefore, in order to link expression of SHP-2 and PD-L1 with response to ICI, I uncovered two small studies: one in

NSCLC and one in melanoma patients (122) (123). Using real world data from the three studies identified, the hypothesis that inhibition of SHP-2 activity is likely to improve response to PD-L1/PD-1 inhibitors justifies wet-lab characterization of the mechanism(s) of activity.

2.2 Methods

TCPA and GDC analysis of PTPN11 and CD274 expression

TCPA (https://tcpaportal.org/tcpa/index.html), a functional proteomics database which contains reverse phase protein array (RPPA) data from a wide variety of clinical tumor samples was used to identify a lung adenocarcinoma (TCGA-LUAD-L4) dataset containing RPPA data from 362 individual patient samples. These data contain quantitative protein expression levels of 237 unique proteins for each subject. From the TCPA data, SHP-2_pY542, the phosphorylated and active form of SHP-2 and PD-L1 were compared from 362 patient tumors for relative protein expression levels using a two-tailed, non-parametric Spearman correlation analysis with 95% confidence intervals. Corresponding RNA-sequencing data was acquired from TCGA, now NCI-GDC (https://portal.gdc.cancer.gov), for the genes PTPN11 (SHP-2) and CD274 (PD-L1). RNA sequencing data that matched the previously-queried RPPA data for the 362 patient using identifiers linking the TCPA database and the corresponding RNA-sequencing data in GDC. I utilized fragments per kilobase-upper quartile (FPKM-UQ) values. The FKPM-UQ values for the genes PTPN11 and CD274 for each tumor were analyzed by Spearman correlation analysis as previously described.

I conducted the same correlation analysis between PTPN11 and CD274 on the FPKM-UQ values for the full TCGA-LUAD dataset of 585 tumor samples. The tumors were sub-grouped using information on KRAS variants (KRAS-mutants n=99, KRAS-WT n=486) known to be active and performed the same analysis of PTPN11 and CD274 mRNA levels.

I then identified another data warehouse (cBioPortal: cbioportal.org) that that contains gene expression data from clinical cancer studies. Specifically, we located a study sought to characterize the genomic landscape of lung adenocarcinomas in East Asians (197). This study contains RNAseq data for 169 patients, from which I conducted a two-tailed, non-parametric Spearman correlation analysis with 95% confidence intervals between PTPN11 and CD274 mRNA levels (normalization method: z-score).

Last, I selected 10 proteins which I hypothesized to be involved in the regulation of PD-L1 expression from the mass spectrometry analysis of proteins that co-immunoprecipitated with SHP-2 in Chapter 4. For each of the 10 proteins, FPKM-UQ values from the full TCGA-LUAD dataset (n=585) were subjected to the same correlation analysis as previous described between each of the 10 genes and CD274 and PTPN11.

PTPN11 and CD274 expression compared with resistance to ICI in melanoma tumors

Single-cell RNA-sequencing (scRNA-seq) data from 31 melanoma tumors that were 1) not treated with ICIs or 2) became resistant to ICIs following treatment was acquired from the Gene Expression Omnibus (GEO). The R-studio Bioconductor GEOquery package was used to capture raw scRNA-seq transcript per mission values, cell counts, and annotations from this study (GSE115978). TPM values were calculated and annotated by the authors as described in Jerby-Amon L., et al (122) and then imported into Graphpad Prism for statistical analyses.

I established that scRNA-seq reads were available for several cell types, including immune cell types and malignant cells in tumors that were not treated with ICI (n=15). From these data, only single cells that were determined to be malignant melanoma cells by flow cytometry were selected for study. Of the 15 untreated tumors, the analysis was arbitrarily narrowed to include patient tumors

that had scRNA-seq data for at least 30 unique malignant cells (n=6). To identify proportion of single cells in an individual tumor that expressed PTPN11, the percentage of cells with non-zero TPM scores for PTPN11 for each tumor was calculated. The mean TPM and standard deviation values for PTPN11 and CD274 for all single malignant cells in these six tumors were then calculated.

Similarly, tumors (n=15) that had acquired resistance to ICI therapy were processed to include only tumors (n=6) with at least 30 unique malignant cells. I applied the methods used above to calculate the proportion of single cells expressing PTPN11 for each tumor, and the mean TPM values with standard deviation for PTPN11 and CD274 when the single cells of all six tumors were evaluated together. These values were then used to assess any correlations between PTPN11 and CD274 expression and resistance to ICI therapy.

PTPN11 and CD274 expression compare with response to ICI in NSCLC tumors

A study was identified for which expression data was evaluable that analyzed immune signatures predictive of response to anti-PD-1 inhibitors in NSCLC (123). The dataset contains RNA-sequencing and clinical response data for 21 NSCLC patients treated with single agent anti-PD-1 therapies. The R-studio Bioconductor GEOquery package was used to capture raw RNA-sequencing TPM values from this study (GSE136961). Patients who demonstrated progression of disease or stable disease that lasted less than 24 weeks were deemed by the authors to have no durable clinical benefit (DCB) to anti-PD-1 therapy. Patients showing partial or complete response by Response Evaluation Criteria in Solid Tumor (RECIST) v1.1 or stable disease for more than 24 weeks were defined as receiving DCB.

Of the 21 NSCLC patients in this study, nine demonstrated a DCB to ICI therapy and twelve showed no DCB. I binned the data by DCB status and then averaged all TPM values for PTPN11 and CD274 for each patient tumor to

generate the mean TPM score and standard deviation for each group. An outlier analysis was conducted on the PTPN11 TPM data for both DCB and no-DCB groups. Outliers were determined by the 1.5 interquartile range (IQR) method which adds 1.5 times the IQR to the third quartile and excludes data points that fall above that value and subtracts 1.5 times the IQR from the first quartile and excludes data points that fall below that value.

2.3 Results

Phosphorylated SHP-2 correlates with the loss of PD-L1 expression in lung adenocarcinomas

First, protein array data from TCPA LUAD-L4 dataset (n = 362) was analyzed to assess correlations between the active, phosphorylated form of SHP-2 and PD-L1. The analysis revealed that levels of SHP-2_pY542 show a significant negative correlation with PD-L1 expression (r = -0.157, p-value = 0.0028) in these subjects, suggesting that inhibition of SHP-2 activity may increase PD-L1 protein expression (Figure 2.1A and Figure 2.2A).

Next, to better understand the relationship between expression of SHP-2 (PTPN11) and PD-L1 (CD274) mRNA in these patient tumors, I analyzed corresponding RNA-sequencing data (n=362) from the NCI-GDC. In this database, the full TCGA LUAD dataset contained 585 tumor samples, but the TCPA data does not contain complete coverage of the samples. Interestingly, analysis of the matched data did not reveal a significant correlation (p-value = 0.3488) between PTPN11 and CD274 mRNA expression levels (Figure 2.1B). Following this observation, I wanted to know if any relationship between PTPN11 and CD274 expression was found using the entire TCGA-LUAD RNA-sequencing dataset. I found a modest but significant positive correlation existed (r = 0.095, p-value = 0.0211) between PTPN11 and CD274 mRNA levels (Figure 1C). Tumors from this dataset were then sub-grouped by KRAS variants known

to be either constitutively active or KRAS-wildtype, I uncovered no significant relationships between PTPN11 and CD274 mRNA levels regardless of KRAS status.

Extending my observations from the aforementioned data that suggest a relationship between SHP-2 activity and PD-L1 expression, I identified another data warehouse (cBioPortal: cbioportal.org) that contains gene expression data from clinical cancer studies. The analysis revealed a positive (r = 0.267) and significant (p-value = 0.0005***) correlation between PTPN11 and CD274 mRNA, again suggesting that SHP-2 and PD-L1 protein are co-expressed in LUAD tumors (Figure 2.1D). Together, these TCPA and RNA seq data suggest that SHP-2 and PD-L1 protein are co-expressed in LUAD tumor tissue and that activation of SHP-2, not simply expression, may control levels of PD-L1. However, without knowing the expression levels of inactive SHP-2, we cannot state with certainty that SHP-2 activity is the primary role by which SHP-2 regulates PD-L1 expression.

PTPN11 mRNA expression weakly associates with reduced CD274 mRNA expression in melanoma tumors

Having established a correlation between tumoral SHP-2 activity and PD-L1 expression, but not corresponding gene expression levels in lung adenocarcinomas, I sought to understand whether PTPN11 and CD274 expression levels associate with response of patient tumors treated with ICIs. A study was identified (Jerby-Amon L., et al. 2018) that analyzed scRNA-seq data from melanoma tumors that were either treatment naive, or that had become resistant to ICIs following treatment, and the authors of the study were interested in characteristics of the melanoma cells that lead to immune evasion (122). Using the expression data from this study, I sought to answer two main questions: 1) does PTPN11 mRNA expression correlate with CD274 mRNA levels and 2) does PTPN11 expression correlate with poor response to PD-1 inhibition? The workflow scheme for the analysis is found in Figure 2.2B.

To address the first question, the tumors were identified that were not treated with ICI (n=15). The analysis focused on only melanoma tumors (n=6) which had at least 30 malignant cell TPM reads resulting in a mean of 108 (s.d. 91-487) single cells per tumor. The proportion of cells which had non-zero TPM values for PTPN11 was then calculated, revealing six untreated tumors (Mel71, Mel79, Mel103, Mel80, Mel81, Mel89) that demonstrated ≥50% of single malignant cells (mean= 69; range= 50-83%) that expressed PTPN11. Similar to the TCPA/NCI-GDC analysis, I observed that elevated expression of PTPN11 associated with lower expression of CD274 in treatment naïve tumors.

Finally, I wanted to understand the relationship of PTPN11 and CD274 expression and response to ICI therapy. Patient tumors with acquired resistance to therapy (n=15) were used to determine whether the relative levels of PTPN11 and CD274 were different from the treatment-naïve tumors. Again, these data were processed to include only tumors with at least 30 malignant cells (n=6) and resulted in a mean of 79 single cells (range 96-169) per tumor. The six ICI resistant tumors (Mel78, Mel88, Mel98, Mel102, Mel110, Mel94) showed ≥50% single malignant cells (mean=67; s.d.=57-82%) expressed PTPN11, with mean/standard deviation TPM values for PTPN11 (mean=1.29; s.d.=0.12) and CD274 (mean=0.09; s.d.=0.06). Here, similar expression patterns of PTPN11 and CD274 were observed compared with treatment-naive tumors, and again CD274 levels remain low when PTPN11 is expressed. Importantly, I did not observe any relationship between PTPN11 or CD274 expression and acquired resistance to ICI in this dataset.

CD274 mRNA expression associated with response to ICI in NSCLC tumors

Using the data from the next study (Hwang, et al. 2020), I asked whether expression of PTPN11 and CD274 mRNA associates with response to ICI therapy in NSCLC (123). The investigators in this report aimed to find immune signatures

predictive of response to anti-PD-1 inhibitors in NSCLC. I utilized the gene expression data to sort the 21 patient tumors to two groups based upon their DCB status, resulting in nine patients who demonstrated DCB and twelve patients who did not.

After calculating average TPM values for PTPN11 and CD274 and excluding outlying tumors, the analysis revealed no significant difference in the expression of PTPN11 mRNA between subjects with DCB from those that did not respond to anti-PD-1 therapy. Specifically, the mean PTPN11 TPM score for DCB was 576.15 with s.d.=281.78 and for no DCB (mean=487.73 with s.d.=361.24) (Figure 2.3A). Importantly, the mean expression of CD274 mRNA was nearly 3-fold higher in patients who responded to therapy (mean=151.16, s.d.=198.33) compared to those who did not (mean=61.96, s.d.=64.54) (Figure 2.3B). Together, these data showed that PTPN11 gene expression does not associate with CD274 expression or response therapy in NSCLC patients. These findings are consistent with the results from the first study that suggested that SHP-2 activity, not expression, correlates with PD-L1 expression. In contrast, these data suggest a positive relationship between PD-L1 expression and response to ICI which was not observed in the melanoma study.

Proteins that interact in vivo with SHP-2 significantly correlate with both PTPN11 and CD274 mRNA expression in lung adenocarcinomas

In Chapter 4, mass spectrometry analysis was performed to determine the identity of proteins that co-precipitated with SHP-2 to investigate the protein-protein interactions in which SHP-2 is involved. I hypothesized that these proteins may assist SHP-2 with maintenance of PD-L1 expression. To understand whether these proteins are co-expressed with SHP-2 in patient tumors and not just in cell culture, I applied the same analysis of the TCPA and GDC data sets to further inform my wet-lab research.

First, I searched for RPPA data from TCPA for the top 10 scoring proteins from that analysis, presented in Table 2.1A. Unfortunately, no protein array data were available for any of my 10 proteins of interest. I then evaluated the RNA-seq data in GDC to identify gene expression data for these proteins in order to correlate expression with that of PTPN11 mRNA. Of the expression of 10 genes analyzed against PTPN11 in the full TCGA-LUAD dataset (n=585), I found significant positive correlations (p-value ≤ 0.001) for 5 genes (DDX3X, HSP7C, CPSF5, IF2G, SF3A1) and significant negative correlations for 3 genes (PUF60, EIF1A1, RSMB) (Table 2.1B and Figure 2.3A-2.3H). I then aimed to find whether any correlation existed between these genes and CD274 mRNA. This analysis revealed 2 genes with weak positive correlations (DDX3X, HSP7C) and four genes with moderate negative correlations (PUF60, GRP78, RSMB, IF2G) (Table 2.1C, and Figure 2.4A-2.4F).

2.4 Discussion

In this chapter, information obtained from publicly-available protein and gene expression datasets was used to gain real-world insight into my overarching research question: does SHP-2 activity or expression influence PD-L1 mRNA and protein levels and subsequent response to anti-PD-1 or PD-L1 therapies in NSCLC? I used this approach because I believe that the utilization of patient tumor datasets can inform and direct wet-lab experimentation. The design and execution of pre-clinical and clinical studies is expensive, time-consuming, and labor-intensive. Here, a simple and efficient process is presented that, when combined with bench-side experimentation, can offer substantial insight into the clinical translatability of commonly-used, highly-controlled model systems designed for drug discovery applications. Through the analysis of two major cancer data repositories and two smaller clinical studies, I was able to take

further steps towards establishing a connection between the activity of SHP-2 and PD-L1 expression in human tumors without carrying out a study *de novo*.

Of the datasets chosen for this study, the most statistically-powerful and revealing information arose from the composite analyses of the TCPA and GDC data repositories. Using genomic and protein information from a large cohort of NSCLC patients, my most important observation was the significant negative correlation between the active, tyrosyl-phosphorylated form of SHP-2 and PD-L1 protein expression (Figure 2.1A). A limitation of these data was that the RPPA data did not include expression levels of the unphosphorylated and inactive form of SHP-2 which would have been a useful control as informed by my wet-lab studies. The conformational changes induced by phosphorylation of SHP-2 could alter protein-protein interactions and intracellular signaling cascades that impact PD-L1 expression (124). Following from that concern, I observed no statistically significant correlation between the levels of SHP-2 and PD-L1 mRNA in the patients in the NCI-GDC dataset that were initially studied in the TCPA dataset, again highlighting the potential importance of molecular interactions of SHP-2 dependent on its activated state. Interestingly, when I conducted the same analysis on the entire cohort of LUAD patients in the NCI-GDC repository, a weak, but inverse correlation between PTPN11 and CD274 mRNA was observed, enforcing that the activity of SHP-2 is likely of greater importance in PD-L1 regulation than mRNA expression. It is important to note that activity of SHP-2 is not necessarily impacted by the level of expression.

When I embarked on these studies, I most desired to understand how SHP-2 influences response to ICI therapy in KRAS-active tumors to direct my drug discovery efforts in a wet lab setting. A limitation of the data deposited in the NCI-GDC is that the clinical data are often incomplete and lacking details on drug treatment and associated response or perhaps pre-date a particular therapy, like ICIs in this case. However, I was able to address the expression of SHP-2 and PD-L1 in KRAS active LUAD (~26% of the tumors). KRAS status did not change

the outcome of the analysis. I identified other studies in which RNA-seq data was collected from tumors treated with ICIs, one in melanoma and one in NSCLC (122) (123). While the focus of this study is on NSCLC, treatment of melanoma using ICIs was approved several years prior to use in NSCLC, and thus the data available in this cancer with respect to ICI treatment is more mature. It should be noted that melanomas rarely harbor KRAS mutations and more often HRAS mutations. Additionally, Raf mutations are more likely the oncogenic drivers of cell division in melanoma tumors. Neither of the two small studies made the mutation status of Ras available.

The melanoma study was embarked by Regev and colleagues (122) to identify a gene expression profile that is associated with immune evasion that might predict response to ICI treatment. They conducted scRNA-seq on melanoma tumors that were either treatment naïve at that time or had acquired resistance to ICI therapy. These data allowed me to test whether PTPN11 and CD274 gene expression associated with response to therapy. The authors of the study were more interested with defining signatures of resistance that could be used to screen patients prior ICI therapy, so the experimental design was not ideal for my hypothesis and the sample size was small. As observed in the TCPA and NCI-GDC analysis, melanoma tumors also demonstrate that higher PTPN11 expression was associated with less CD274 mRNA expression. Importantly, PTPN11 mRNA levels were roughly equivalent between the treatment naïve and ICI resistant tumors. While this analysis provides some insight into the landscape of SHP-2 and PD-L1 co-expression, it is important to acknowledge that these tumors are not lung tumors, have different oncogenic mutations, and study sample sizes were relatively low.

Data from the NSCLC study carried out by Hwang and colleagues in which they sought to identify immune gene signatures that may predict clinical response to anti-PD-1 therapy were re-purposed for another question (123). Expression of PTPN11 did not associate with DCB, but the tumors from patients

who experienced DCB displayed increased expression of CD274 mRNA, consistent with other studies (68)(125)(126) (Figure 2.3A-2.3B). Taken together, SHP-2 activity, not expression, may be important for PD-L1 expression in NSCLC and subsequent response to anti-PD-1 and -PD-L1 therapy.

Finally, using data from wet lab experimentation, bioinformatic techniques were used to further investigate the mechanism by which SHP-2 regulates PD-L1 expression. RNA-seq data from the NCI-GDC were used to search for correlations in mRNA expression of proteins that co-precipitate with SHP-2, as identified by mass spectrometry. From that mass spectrometry screen for SHP-2 interactors, ten proteins were selected for exploration, eight are known to be involved in mRNA processing, and two are involved in protein transport and maintenance (Table 2.1A). The primary objective from this analysis was to inform and direct subsequent wet-lab experimentation by determining if the protein-protein interactions observed from *in vitro* cell culture could be corroborated by the co-expression of the mRNA which encodes these proteins in NSCLC patient tumors. As such, the findings presented in this chapter will be discussed later and in greater detail in chapter 4.

From these analyses, the most striking observation was the strong correlation between mRNA expression of the RNA helicase DDX3X and SHP-2 ($r = 0.4841$, p-value < 0.0001) (Figure 2.4A). DDX3X was one of the highest scoring proteins across multiple repetitions of the mass spectrometry screen, and therefore, this finding supports the hypothesis that SHP-2 and DDX3X interact within the context of a NSCLC tumor. Importantly, DDX3X contains a tyrosine phosphorylation site at Y104, making it a potential substate for SHP-2. In addition to DDX3X, four other proteins (Table 2.1B, Figure 2.4B-2.4E) had positive correlations with SHP-2 mRNA expression, three of which (CPSF5, IF2G, SF3A1) are mRNA processing proteins and one (HSP7C) a protein chaperone. However, there exists no experimental evidence that any proteins other than DDX3X are phosphorylated on tyrosine residues. This finding suggests that SHP-

2 may be involved in the processing and regulation of mRNA within LUAD tumors, a novel function of SHP-2. However, as stated above, the activity of SHP-2 may be of greater importance than its expression regarding PD-L1 expression, though unfortunately there does not exist protein expression data for DDX3X on TCPA. Although there is a strong positive correlation in the mRNA expression of SHP-2 and these five proteins, these data provide no information on the activation status of SHP-2 or if it directly interacts with these proteins *in vivo.*

The same analysis was then repeated, this time compared with the mRNA expression of CD274. HSP7C, which positively correlated with CD274 mRNA, is a molecular chaperone, and PD-L1 is expressed on the cell surface. Therefore, it is reasonable to suggest that these two proteins would be co-expressed, if indeed PD-L1 is a substrate for trafficking by HSP7C. Interestingly, DDX3X mRNA also correlated with that of PD-L1, though the statistical confidence (p-value = 0.0013) is relatively weak for this type of analysis (Table 2.1C, Figure 2.5A).

There are limitations to these analyses that warrant consideration. First, these data only describe the expression of mRNA of pairs of genes and not of protein. While it is well understood that when the mRNA of a gene is expressed, often the protein is as well, however this is not always the case as there are many mechanisms that influence the translation of mRNA into protein such as miRNA involvement and mRNA degradation/metabolism (127)(128). Second, RNA-seq data requires special considerations when evaluating the statistical significance of the findings. When comparing the mRNA expression of 20,000 unique genes, the likelihood of false discoveries is much higher than in smaller data sets, and so correlations that do not have very high confidence (p-values < 0.001) of statical significance should be observed with caution (129)(130). Contrary to the retrospective analyses of massive genetic datasets collected under conditions I was unable to control, the genes I chose to investigate were

not random findings of a fishing expedition, but rather are supported by and identified from controlled biochemical assays. It has been suggested that pure *in silico* interrogations of differential gene expression may encompass considerable pitfalls when applied to large datasets, such as those contained within the GDC. Thus, the decision to reinforce my informatic interrogations with reproducible *in vitro* experimental data further underpins the scientific rigor and reliability of conclusions drawn from these analyses. Furthermore, the primary intent of these exploratory analyses was to inform and direct further bench-top molecular interrogation and not serve as the sole information upon which final conclusions are to be drawn. Going forward, these hypotheses will be tested using in vitro cell based assays altering SHP-2 activity and expression to help ratify the inferences from this chapter.

2.5 Conclusions

The value of simple, but grounded, methods to use real-world data and apply the information gained from this study to direct new wet lab experimentation should not be overlooked. Each previously published study I used had limitations for my purposes, but I operate within those limitations. Additionally, the strength and versatility of these bioinformatics analyses are fortified when combined with benchtop molecular analyses. Together they serve as powerful tools when elucidating biological mechanisms. These data convince me that continued exploration into the role of SHP-2 on both PD-L1 expression and response to certain immune checkpoint inhibitors has value. Ultimately there is likely value in combining the use of molecules that inhibit the activity of SHP-2, specifically in lung tumors, and immune checkpoint inhibitors that depend on expression of PD-L1 on tumor cell surfaces.

Figure 2.1. SHP-2 activity and expression correlates with expression of PD-L1 in NSCLC adenocarcinomas. A. Two-tailed non-parametric Spearman correlation analysis of RPPA protein expression data for Y542 phosphorylated SHP-2 and PD-L1 from 362 adenocarcinomas taken from The Cancer Proteome Atlas (TCPA: https://gdc.cancer.gov/about-data/publications/pancanatlas) LUAD-L4 dataset. **B.** Two-tailed non-parametric Spearman correlation analysis of bulk RNA-seq FPKM-UQ values taken from TCGA (GDC) for the 362 patients that had corresponding RPPA protein expression data from TCPA. **C.** Two-tailed non-parametric Spearman correlation analysis of bulk RNA-seq FPKM-UQ values taken from TCGA (GDC) for all 585 patients in the TCGA-LUAD dataset. **D.** Two-tailed non-parametric Spearman correlation analysis of mRNA z-scores taken from cBioPortal (PMID:32015526) for 169 lung adenocarcinoma tumors**.** The red line in each panel represents a linear regression line of best fit.

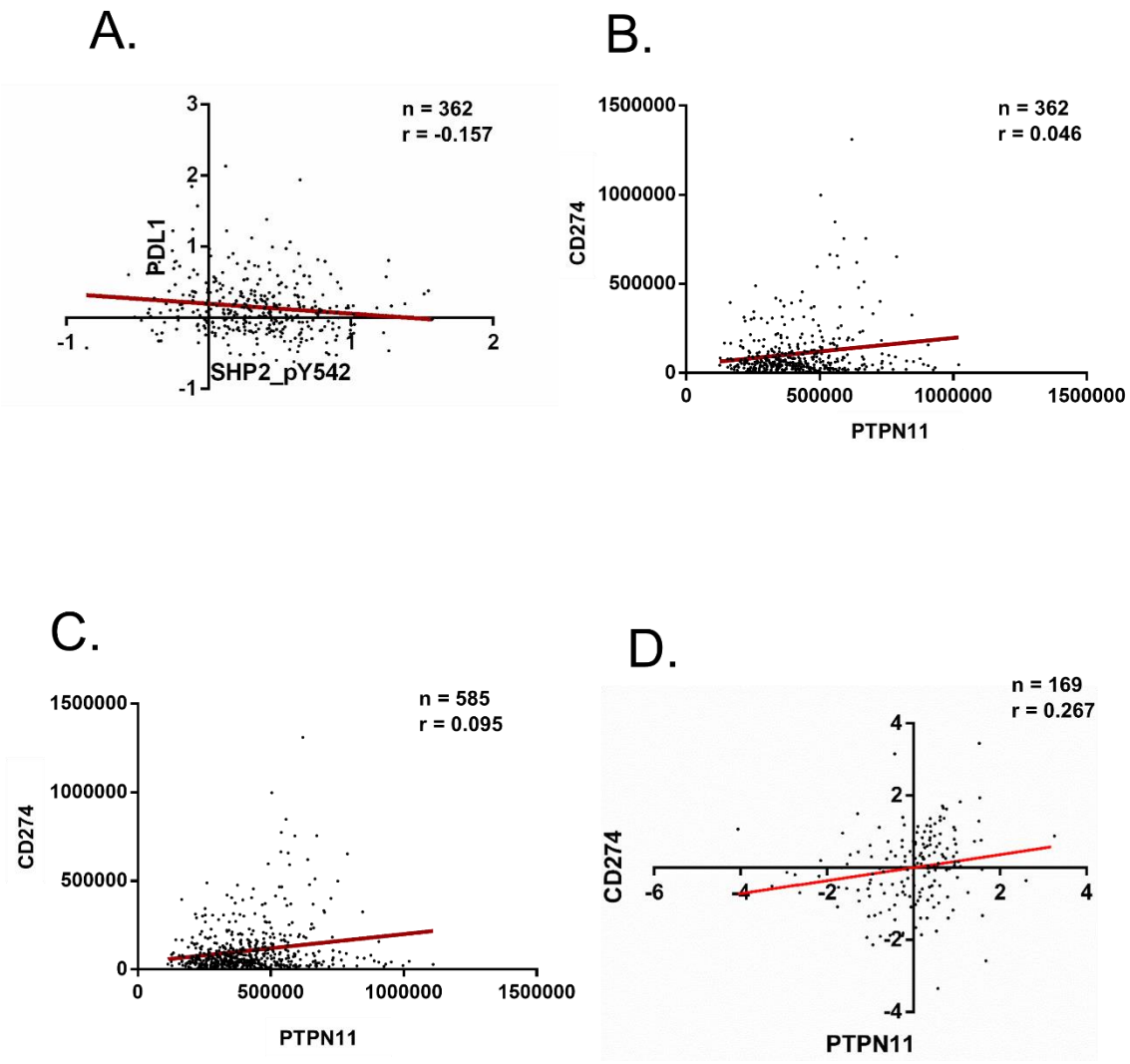


Figure 2.2. Workflow scheme for evaluation of SHP2 and PD-L1 relationships. Reverse-phase protein array (RPPA) was collected from the TCPA data repository (https://gdc.cancer.gov/about-data/publications/pancanatlas) for 362 total patients labeled as the TCPA-LUAD-L4 data set. RNAseq data was collected from GDC for the full TCGA-LUAD dataset (n=585). These data were parsed to include only patients for which there was matching RPPA data on TCPA (n=362). Single-cell RNAseq reads for 31 melanoma tumors were collected and separated into two groups based on ICI treatment status. Only single-cell reads for 'malignant melanoma cells' were retained for analysis. Tumors which had ≥ 30 unique malignant cells with non-zero PTPN11 values were included in the analysis. NSCLC tumors (n=21) with sequence data were first separated into two groups based on response to ICI treatment. Average TPM values were calculated for PTPN11 and CD274, and tumors that had PTPN11 TPM value >2 standard deviations from the mean were excluded from the analysis.

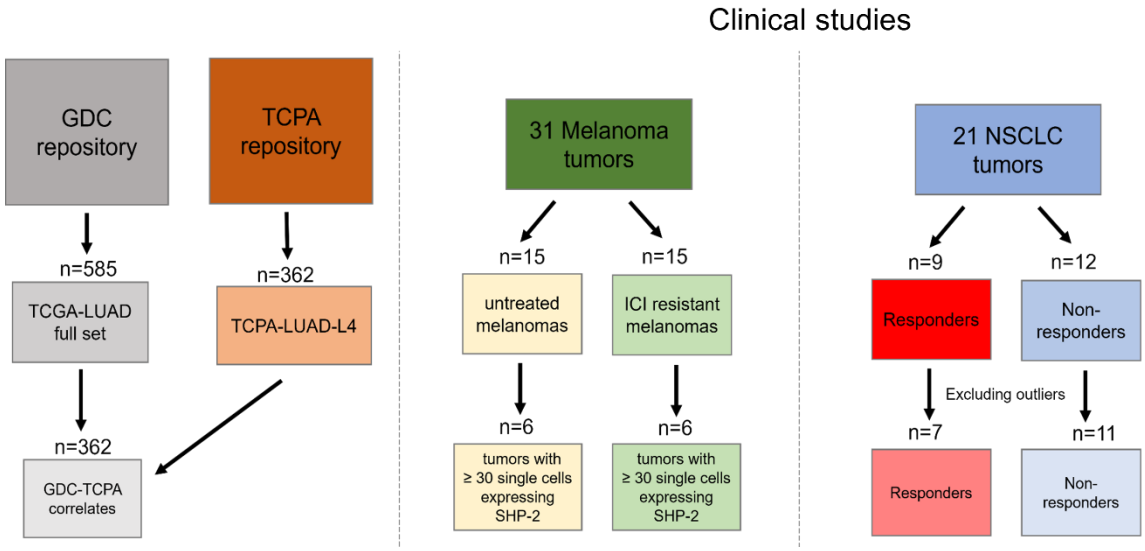


Figure 2.3. CD274, but not PTPN11, mRNA expression is associated with response to ICI in NSCLC tumors. A. PTPN11 TPM values for patients who did or did not demonstrate a durable clinical benefit (DCB) from ICI therapy, as determined by RECIST criteria (123). There was no significant difference between groups, as measured by a student's t-test. **B.** TPM values for CD274 in patients who did or did not demonstrate a durable clinical benefit (DCB) from ICI therapy, as determined by RECIST criteria. There was no significant difference between groups, as measured by a

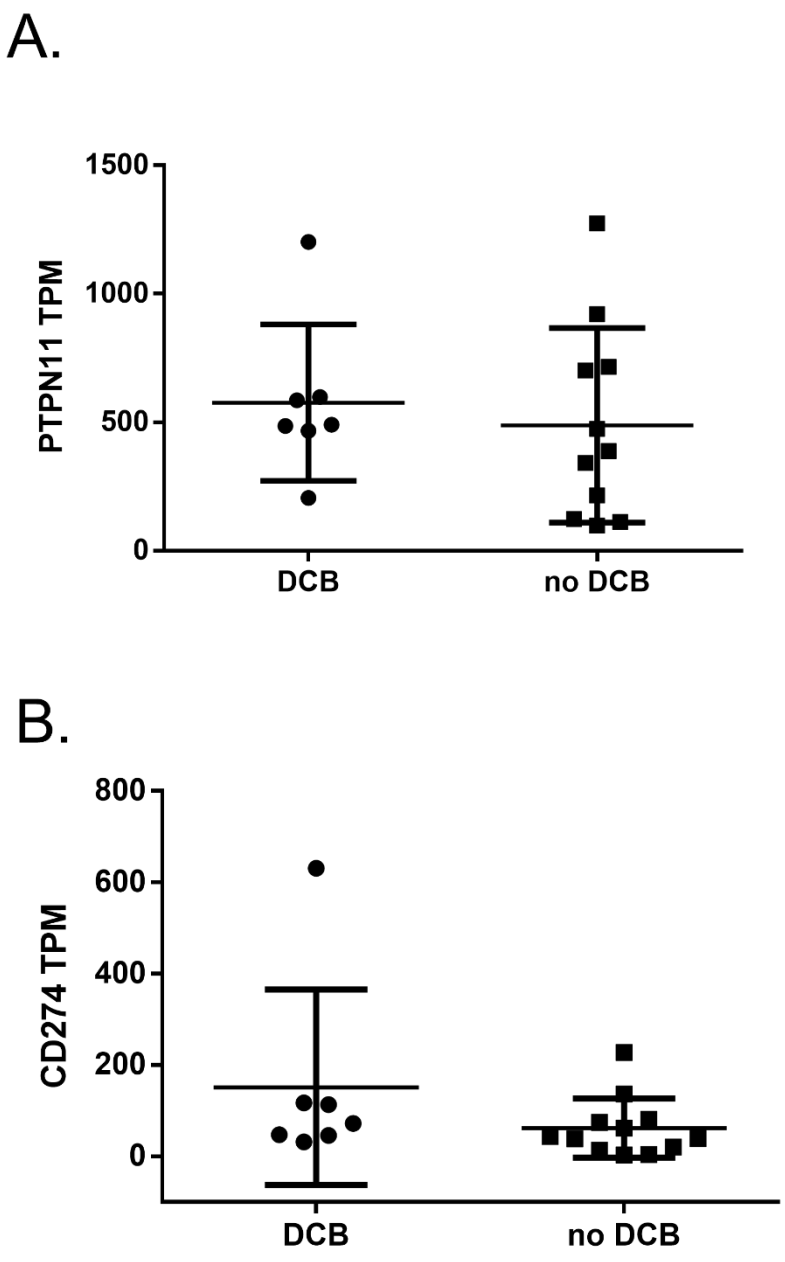
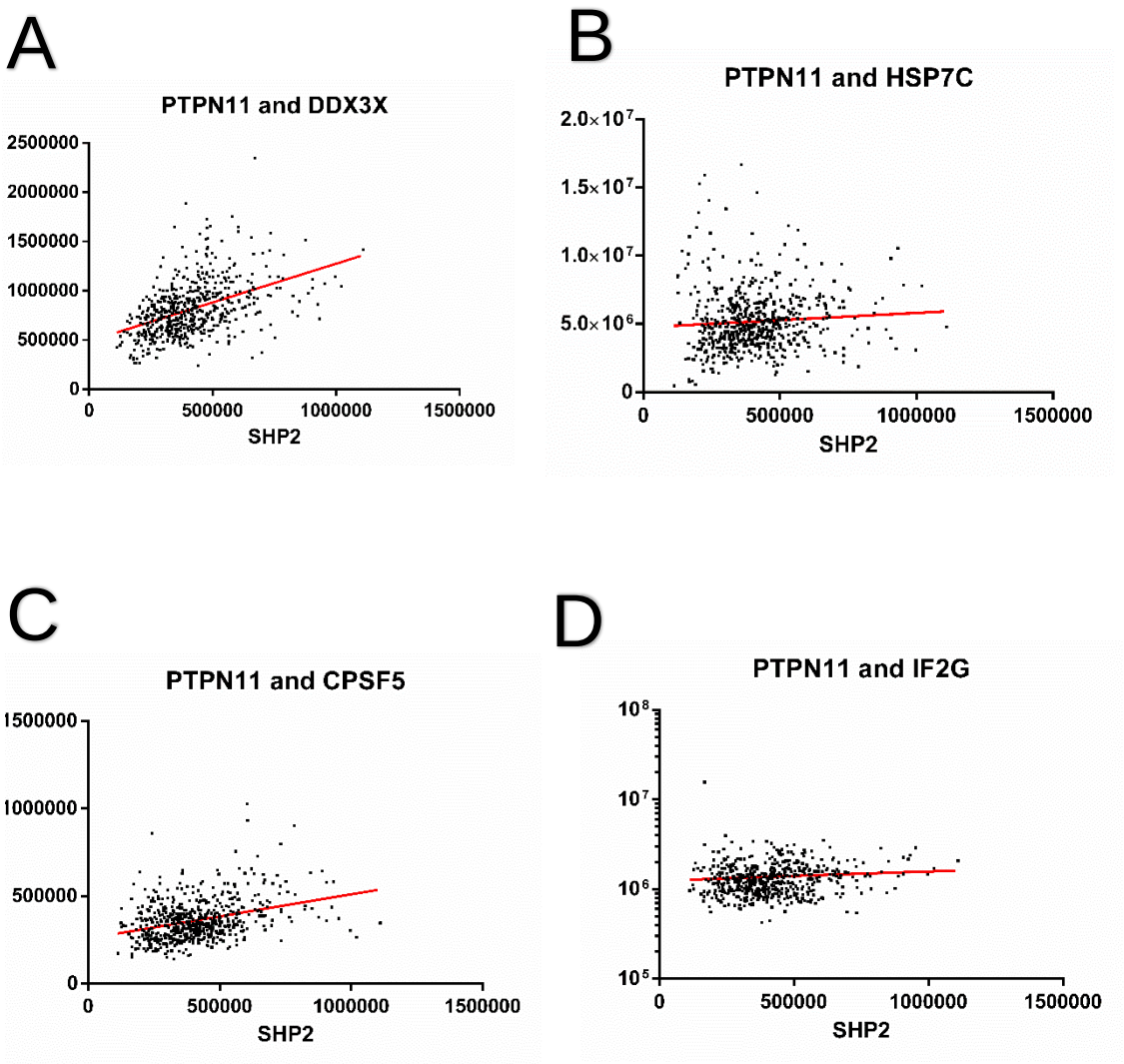


Figure 2.4. Genes identified in mass spec screening correlate with PTPN11 expression in NSCLC adenocarcinomas. Two-tailed non-parametric Spearman correlation analysis of of bulk RNA-seq FPKM-UQ values taken from TCGA (GDC) for all 585 patients in the TCGA-LUAD dataset for PTPN11 and **A.** DDX3X **B.** HSP7C **C.** GPSF5 **D.** IF2G **E.** SF3A1 **F.** PUF60 **G.** EF1A1 **H.** RSMB. The red line represents a non-linear regression line of best fit.



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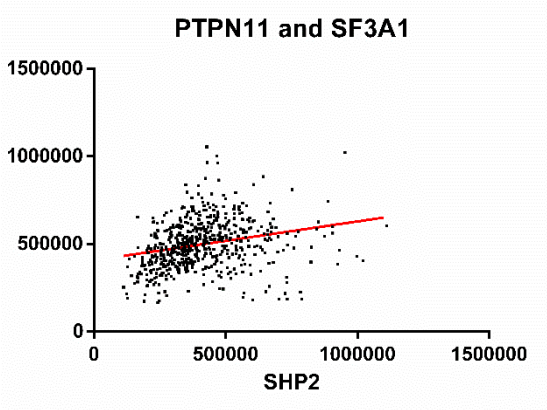
PTPN11 and SF3A1
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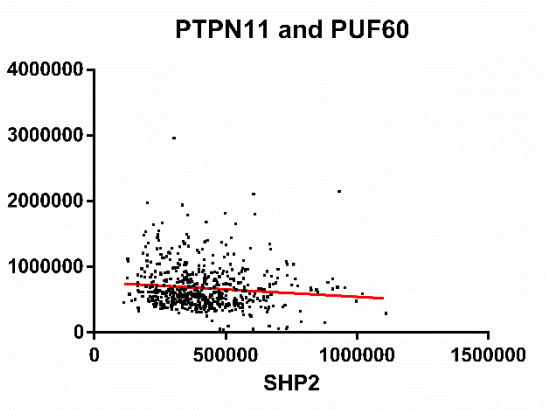
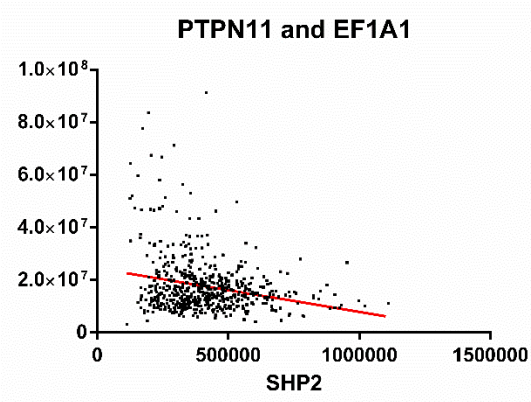
SHP2

F


PTPN11 and PUF60
4000000
3000000
2000000
1000000
0
0
500000
1000000
1500000
SHP2

G


PTPN11 and EF1A1
1.0×10⁸
8.0×10⁷
6.0×10⁷
4.0×10⁷
2.0×10⁷
0
0
500000
1000000
1500000
SHP2

H

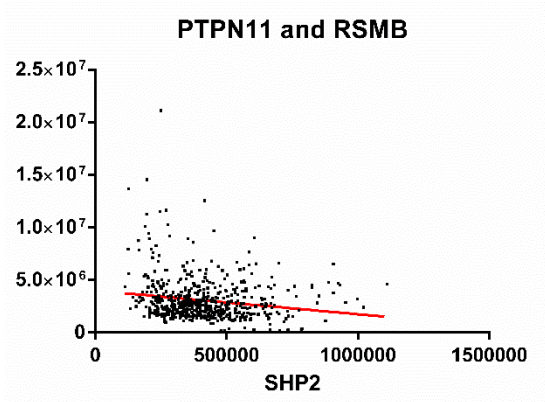
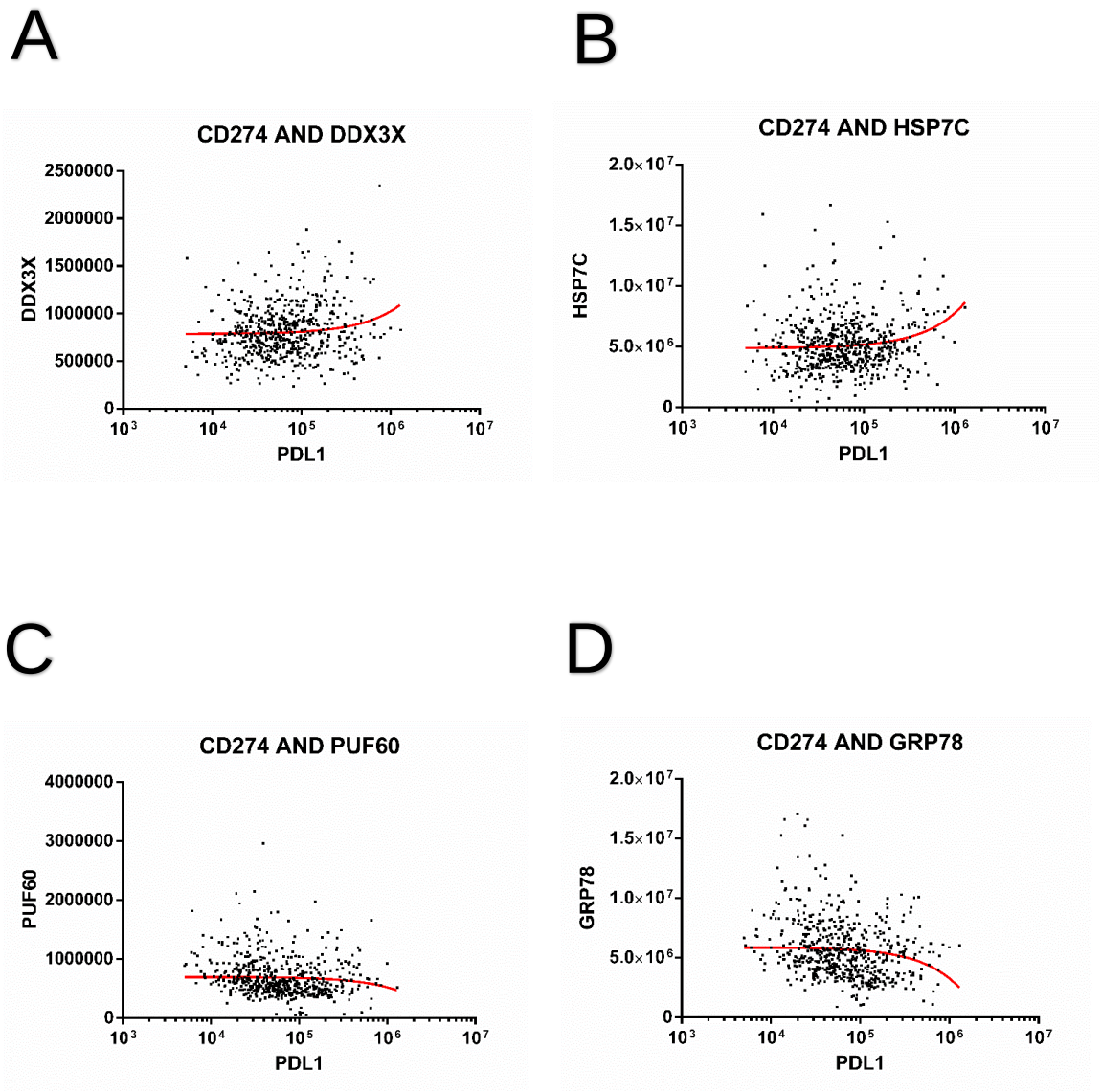
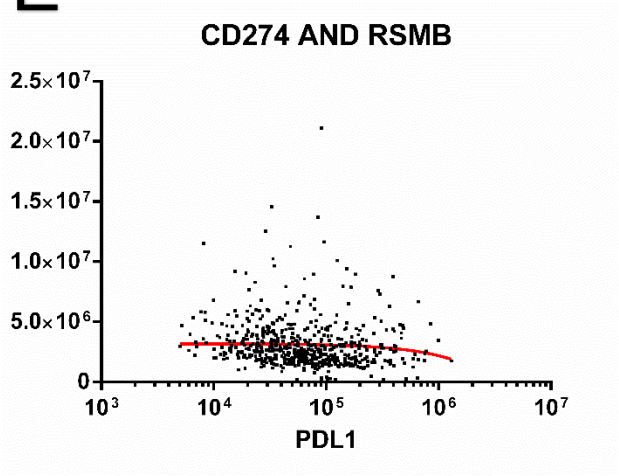

PTPN11 and RSMB
2.5×10⁷
2.0×10⁷
1.5×10⁷
1.0×10⁷
5.0×10⁶
0
0
500000
1000000
1500000
SHP2

Figure 2.5. Genes identified in mass spec screening correlate with CD274 expression in NSCLC adenocarcinomas. Two-tailed non-parametric spearman correlation analysis of bulk RNA-seq FPKM-UQ values taken from TCGA (GDC) for all 585 patients in the TCGA-LUAD dataset for CD274 and **A.** DDX3X **B.** HSP7C **C.** PUF60 **D.** GRP78 **E.** RSMB **F.** IF2G. The red line represents a non-linear regression line of best fit.



E


CD274 AND RSMB
2.5×10^7
2.0×10^7
1.5×10^7
1.0×10^7
5.0×10^6
0
10^3
10^4
10^5
10^6
10^7
PDL1

F

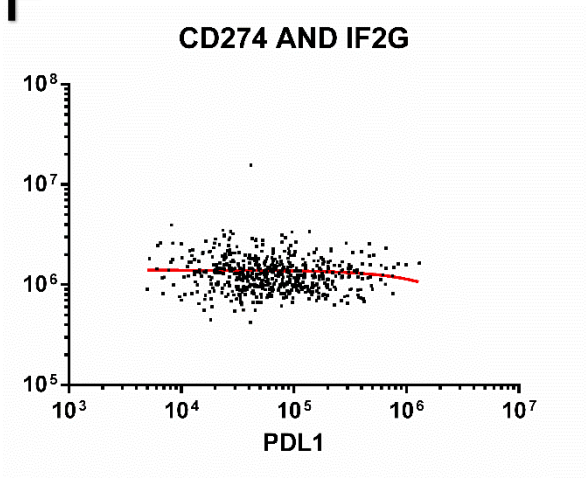

CD274 AND IF2G
10^8
10^7
10^6
10^5
10^3
10^4
10^5
10^6
10^7
PDL1

Table 2.1. Proteins that co-precipitate with SHP-2 identified by mass spectrometry correlate to PTPN11 and CD274 mRNA expression in NSCLC adenocarcinomas. A. Table of the top 10 scoring proteins that co-immunoprecipitated with SHP-2 as identified by mass spectrometry that includes their score value as well as their biological function. **B.** Table depicting the values of a two-tailed non-parametric spearman correlation analysis of bulk RNA-seq FPKM-UQ values taken from TCGA (GDC) for all 585 patients in the TCGA-LUAD dataset for proteins that co-precipitated with SHP-2 analyzed against PTPN11 or **C.** CD274.

A

Protein	MS score	Function
HSP7C	540.88	Protein folding and transport, protein complex formation, proteome stress protection
DDX3X	511.12	RNA-Helicase; binds rG4s structures, including in 5'-UTS of NRAS
PUF60	482.65	DNA/RNA binding protein; RNA splicing, apoptosis, and transcription regulation
CPSF5	387.32	Cleavage factor complex; activator of pre-mRNA-3' end cleavage. mRNA maturation.
EIF1A1	353.86	Elongation initiation factor complex subunit. Th1 specific TF, binds to IFNγ promoter
GRP78	321.38	ER chaperone, protein folding, degrades misfolded proteins
RSMB	244.19	pre-mRNA splicing; SMN-Sm complex, component of snRNPs
KPYM	234.93	Pyruvate kinase, stimulates POU5F1-mediated transcriptional activation. Assists in caspase cell death in tumor cells
IF2G	184.97	Eukaryotic initiation factor (e-IF2) subunit
SF3A1	119.2	pre-mRNA splicing; SF3A complex

B

	PTPN11 + correlation	
Protein	Correlation	p-value
DDX3X	0.4841	< 0.0001
HSP7C	0.1396	0.0006
CPSF5	0.3152	< 0.0001
IF2G	0.1604	< 0.0001
SF3A1	0.3226	< 0.0001
	PTPN11 - correlation	
Protein	Correlation	p-value
PUF60	-0.1473	0.0003
EIF1A1	-0.1518	0.0002
RSMB	-0.1929	< 0.0001

C

	CD274 + correlation	
Protein	Correlation	p-value
DDX3X	0.1316	0.0013
HSP7C	0.1149	0.0051
	CD274 - correlation	
	Correlation	p-value
PUF60	-0.2527	< 0.0001
GRP78	-0.2754	< 0.0001
RSMB	-0.2269	< 0.0001
IF2G	-0.1206	0.0032

Table 2.2. PTPN11 mRNA expression weakly associated with reduced CD274 mRNA expression in melanoma tumors regardless of ICI exposure. A. Six tumors had ≥ 30 unique malignant cells that had non-zero PTPN11 and are ICI therapy naïve. Shown are the percentage of single cells that expressed PTPN11, the combined TPM values for all the single cells within each individual tumor, and the average TPM values for all 6 untreated tumors combined**. B.** The same details as **(A)** but for tumors that did not respond to ICI therapy. There was no significant difference in average PTPN11 and CD274 TPM values between treatment naïve and treatment resistant groups.

A.

Treatment	Tumor	% cells expressing PTPN11	Tumoral PTPN11 TPM	Tumoral CD274 TPM	Average PTPN11 TPM	Average CD274 TPM
none	Mel80	80.4	1.35	0.07		
none	Mel81	81.7	1.57	0.02		
none	Mel89	83.0	1.78	0.18	1.40	0.14
none	Mel71	56.5	1.24	0.07		
none	Mel79	58.8	1.40	0.18		
none	Mel103	50.4	1.08	0.12		

B.

Treatment	Tumor	% cells expressing PTPN11	Tumoral PTPN11 TPM	Tumoral CD274 TPM	Average PTPN11 TPM	Average CD274 TPM
Ipilimumab+ nivolumab	Mel78	75.8	1.49	0.15		
Ipilimumab+ pembrolizumab	Mel110	82.1	1.39	0.05		
Tremlimumab	Mel88	66.9	1.16	0.17	1.29	0.09
Ipilimumab	Mel98	61.8	1.19	0.14		
Ipilimumab+ nivolumab	Mel102	56.8	1.20	0.03		
Ipilimumab+ pembrolizumab+ nivolumab	Mel194	59.4	1.29	0.03		

CHAPTER 3.

3.1 Overview

Non-small cell lung cancer (NSCLC) remains one of the most onerous forms of cancer. In 2020, it was estimated that roughly one quarter of all cancer-related deaths will be due to cancer of the lung and bronchus (22). The severity of the disease can be attributed to late-stage diagnoses and the rapid resistance of NSCLC to both cytotoxic chemotherapy and targeted therapies, resulting in a poor 5-year survival rate of 20% (131). In NSCLC, as many as one third of tumors harbor mutations in RAS genes, especially KRAS, a protein which until recently has been exceptionally difficult to target with small molecule inhibitors (132)(133)(134). The RAS protein is a GTPase which, in response to growth factor stimulation, is activated, providing a trigger for initiation of intersecting signaling cascades that drive proliferation and cell survival.

Historically, direct inhibition of the KRAS protein or downstream effector proteins (such as farnesyltransferases) with small molecules has proven exceptionally difficult in providing control of tumor growth (135). Thus, treatments for patients with tumors harboring KRAS mutations remain limited to cytotoxic chemotherapies or immune checkpoint inhibitors (136). Monoclonal antibodies that interfere with PD-1/PD-L1 checkpoints between tumors and cytotoxic/effector T-cells have shown substantial clinical benefit for a subset of patients without EGFR, ALK, or ROS actionable mutations (137). Importantly, evidence is mounting that suggests that many KRAS-active tumors possess the unique characteristic of aberrant expression of PD-L1. In a 2018 study examining 219 lung adenocarcinomas (83), Falk et al. (2018) observed that KRAS-active tumors maintained significantly higher PD-L1 expression than KRAS-wildtype tumors, and that increased PD-L1 expression was associated with improved overall survival (138). In a meta-analysis of over 23 studies, Liu et al. (2020) observed that KRAS-active NSCLC is more likely to be PD-L1 positive compared with wild-type tumors

(137). Furthermore, they observed that patients harboring KRAS-active tumors displayed a significantly higher overall response rate to PD-L1 blockade. Together, these studies suggest that KRAS-active NSCLC may be particularly susceptible to interruption of the PD-L1/PD-1 interaction.

The ability of the immune system to identify and target tumors is a natural process that occurs irrespective of pharmacological intervention. In the 1960s and 1970s, immune cells were further classified as cytotoxic T-cells and dendritic cells with specific functions, for example, and in 1975 natural killer cells were found to specifically target leukemia cells in mice (139)(140)(141). Within the past several decades, proteins involved in modulating the immunological identification and targeting of tumors have received a great deal of scientific attention. One such protein, PD-L1, has been shown to play a crucial role in modulating immune response and T-cell activity. PD-L1 is normally expressed on the surface of cells of the immune system, usually antigen presenting cells, as a mechanism by which the immune system distinguishes self from non-self (142). Specifically, ligation of PD-L1 to the receptor PD-1, which is found on the surface of activated T-cells, results in T cell deactivation and protection of self. When PD-L1 is expressed on tumor cells and engages PD-1 on effector T cells the tumor is hidden from cytotoxic T cells. Once the PD-1/PD-L1 interaction is interrupted, the tumor can now be targeted by the immune response. After Honjo et al. (1992) uncovered the biology of this interaction, antibodies that bind either PD-1 and PD-L1 were developed to unmask the tumor from the immune system and have shown substantial clinical benefit for many solid tumors (142)(143)(144).

Although several studies have demonstrated that KRAS-active cancers often have increased levels of PD-L1 expression, the precise mechanism(s) by which PD-L1 is aberrantly expressed in KRAS-active cancers remains unclear. It is likely that multiple signaling pathways, such as PI3K/AKT/mTOR and EGFR, govern PD-L1 expression (145)(146)(147)(148)(149). One mechanism reported by Coelho et al. (2017) showed that oncogenic RAS signaling leads to dysregulation of the RNA-

binding protein, TTP, that can upregulate PD-L1 expression by stabilizing PD-L1 mRNA (83). Other studies demonstrate that cytokine-activated pathways, such as JAK/STAT, also impact the normal expression of PD-L1 in immune cells (and also may be the case in some cancer subpopulations), specifically through interferon gamma-induced activation of the transcription factor, signal transducer and activator of transcription-3 (STAT3) (150)(151)(152). Additionally, post-translational modifications to PD-L1 protein can influence its metabolism and localization within a cell. Glycosylation of PD-L1 has been shown to impact PD-L1 stability, thus influencing its ability to impede T-cell activation in cancer cells (153). Phosphorylation of PD-L1 on Ser/Thr residues by GSK3β alters the interactions of PD-L1 with E3 ligases and thus the extent of PD-L1 ubiquitination, further influencing protein turnover and activity (154).

In prior work from this laboratory, mRNA and microRNA gene expression profiles that differentiated mRNA expression levels in KRAS-active and EGFR-mutant lung cancer cell lines were mined using novel bioinformatic approaches to examine co-incident changes in mRNA and microRNA expression to identify potential therapeutic targets for KRAS-active NSCLC (155). One of the potential targets identified that warranted further exploration was the non-receptor tyrosine phosphatase, Src homology region 2 containing protein phosphatase (SHP-2). SHP-2 has been implicated in regulating the RAS/Raf/ERK pathway as well as integrating the activities of the JAK/STAT and PI3K/Akt/mTOR survival pathways (124)(156)(157). Along with other laboratories, we initially hypothesized that SHP-2 might be a direct therapeutic target for KRAS-active NSCLC in the absence of efficacious KRAS inhibitors (106)(107)(158). The preponderance of evidence from our lab and others indicates that inhibition of SHP-2 is unlikely to cause cell death and forced us to consider other explanations for our observations.

SHP-2 is encoded by the PTPN11 gene and contains two SH-2 domains that are used to dock phosphorylated tyrosine residues of substrate proteins, and a catalytic protein tyrosine phosphatase (PTP) domain that can act upon those

substrates. Following receptor tyrosine kinase (RTK) activation, SHP-2 is recruited to the cell membrane and may be phosphorylated at two Tyr residues (Y542 and Y580) on the C-terminal domain, though the kinases responsible for such phosphorylation events remain understudied. Once in an active conformation, SHP-2 may interact with other proteins involved in RAS signal transduction such as Grb2 and SOS, resulting in subsequent ERK activation, tumor proliferation, growth, and survival (159)(160). It is thought that both the ability of SHP-2 to enucleate this protein complex, as well as catalytic phosphatase activity both play a role in these signal transduction events (108). SHP-2 loses phosphatase activity when the N-SH2 domain folds on the phosphatase domain leading to auto-inhibition (161). However, activating mutations can occur in the N-SH2 or PTP domains of the PTPN11 gene and lead to a protein that has disrupted auto-inhibitory activity associated with unfolding of the protein resulting in a constitutively-active phosphatase. The phosphatase domain of SHP-2 is responsible for dephosphorylation and deactivation of p120-RASGAP, a negative regulator of ERK activation, as well as a collection of other proteins (159)(160). Though the catalytic phosphatase activity of SHP-2 contributes to its signal transduction capabilities, it is also capable of serving as a scaffold that enucleates protein complexes, specifically through interactions of its SH2 domains with p-Tyr residues on protein substrates (162). Dissecting the relative influence of SHP-2 PTP activity from scaffolding functionality is of critical importance as it relates to PD-L1 expression.

My initial experiments aligned with the published work of others that pharmacological inhibition of SHP-2 activity does not lead to cell death of NSCLC cell lines (106). However, I found that ablation of SHP-2, and to a lesser extent inhibition of phosphatase activity, did alter the expression of PD-L1. I postulated that SHP-2 engages in a multi-protein complex to control PD-L1 mRNA expression in KRAS-active cells that does not require phosphatase activity. The future implications of this work are that inhibition of SHP-2 may synergize with ICI therapy in NSCLC by promoting the expression of PD-L1.

3.2 Methods

Cell lines and treatment conditions

KRAS-active NSCLC cell lines NCI-H460 and A549 were acquired from ATCC (STR authenticated) and UKY-29 cells were developed by, and a gift from, John Yannelli, Ph.D. at the University of Kentucky, College of Medicine (163). Cells were seeded at $1x10^5$ cells/well on a 6-well plate for flow cytometry, or $2x10^4$ on a 24-well plate for qRT-PCR and western analysis. Cells were allowed to adhere and grow for 24 hours in RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and maintained at 37°C, 5% CO2. For assays involving treatment with SHP099, RMC-4550, SHP-2 siRNA or CRISPR, media was then removed and replaced with RPMI 1640 containing 1% FBS for 24 hours. Media was again replaced with RPMI 1640 containing 1% FBS with either a final concentration of [10µM] of the SHP-2 inhibitor SHP099 (Novartis), [20nM] of RMC-4550 (SelleckChem), or vehicle (4% DMSO). For genetic ablation of SHP-2, media was removed and replaced with RPMI 1640 containing 1% FBS and no antibiotics. Cells were then transfected with Lipofectamine RNAiMAX (ThermoFisher) and SHP-2 siRNA [30nM] (Cell Signaling Technologies) or SignalSilence Control siRNA (Cell Signaling Technologies, #6568). A Cas9-guide RNA complex (New England Biolabs) or a non-targeting CRISPR/Cas9 guide RNA was transfected using Lipofectamine RNAiMAX. Following all treatments, cells were incubated for another 24 hours prior to harvest for western analysis in 100µl protein sample buffer (62.5mM Tris-HCL pH 6.8, 2% SDS, 10% Glycerol, 5% β-mercaptoethanol, bromophenol blue) or for qRT-PCR in 350µl buffer RLT (QIAgen). Cells plated on 6-well plates designated for flow cytometry were washed with ice cold Ca+/Mg+ free PBS prior to trypsinization and flow cytometric analysis.

CRISPR/Cas9 SHP-2 knockout

Guide RNA (gRNA) targeting exon 3 of PTPN11 (SHP-2) was generated using the CHOPCHOP online web tool (https://chopchop.cbu.uib.no/), resulting in

a gRNA sequence of GATTACTATGACCTGTATGG. The DNA oligonucleotide was synthesized by IDT technologies and used to generate gRNA with the EnGen sgRNA Synthesis Kit (New England BioLabs). Cells were seeded at $1x10^5$ cells/well on a 6-well plate or $2x10^4$ cells/well on a 24-well plate for 24 hours in RPMI 1640 containing 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and maintained at 37°C in 5% CO2. Media was then replaced with RPMI 1640 containing 1% FBS for another 24 hours. Media was removed and replaced with RPMI 1640 containing 1% FBS and no antibiotics prior to CRISPR treatment. gRNA was complexed with EnGen Spy Cas9 NLS (New England BioLabs) and added to wells for 48 hours for western analysis or 24 hours for flow cytometry. Cells were then lysed in 100µl protein lysis buffer for western blotting or 350µl buffer RLT for RNA extraction and RT-qPCR.

Flow Cytometry

Following treatment, cells were washed with cold Ca2+/Mg2+-free PBS (VWR) and detached using trypsin-EDTA 0.05% (ThermoFisher). After a 5-minute centrifugation at 300xg and 4°C, supernatant was removed and cells were resuspended in cold FACS buffer (pH 7.0: 1X PBS, 1mM EDTA, 25 mM HEPES, 1% heat-inactivated FBS) and transferred to a 96-well round bottom plate (Nunc) at $2x10^5$ cells/well for staining. Cells were stained with fluorescent-conjugated antibodies specific for PD-L1 (PE) and EGFR (Alexa Fluor 647) (BD Biosciences) and fluorescence was measured using (Sony SY3200). UltraComp eBeads (Thermo Fisher) were used for fluorescence compensation. Analysis was performed using FlowJo v. 10 (Tree Star) and incorporated live and singlet gates prior to gating on individual markers. Flow cytometry was performed by the University of Kentucky flow cytometry core facility.

Western blot analysis

Following treatment and harvest, cells were lysed with protein sample buffer, subjected to a freeze/thaw cycle, sonicated, then heated to 95°C for 5 minutes prior to protein separation using 10% SDS-polyacrylamide gel electrophoresis (PAGE) at 135V for 80 minutes. Proteins were transferred to a nitrocellulose membrane and blocked in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% bovine serum albumin for 30 minutes prior to overnight incubation at 4°C with primary antibody: SHP-2 (#3397), PD-L1 (#13684), or GAPDH (#2118) (Cell Signaling Technologies). After overnight incubation, antibody was removed, and blots were washed with TBS containing 0.1% Tween 20. Secondary HRP-labeled antibodies were applied to the blot in 5% non-fat, dry milk in 1X TBS containing 0.1% Tween 20 for one hour. The blot was washed three times for 5 minutes each in 1X TBS containing 0.1% Tween prior to developing and detection of HRP signal. Signal was quantified using ImageJ software (NIH) and values for SHP-2 and PD-L1 were normalized to GAPDH.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from lysed cells using the RNeasy kit (Qiagen). cDNA was then generated using the iScript cDNA synthesis kit (BioRad). qRT-PCR was performed using human TaqMan primer probe sets (Thermo- Fisher) for PD-L1 (Assay ID: Hs00204257_m1), SHP-2 (Hs06636344_g1), GAPDH (Hs02786624_g1) and *β*-Actin (Hs99999903_m1). qRT-PCR data were analyzed by calculating ΔCt values for PD-L1 by standardizing PD-L1 Ct values against those for the averaged Ct values of housekeeping genes GAPDH and *β*-Actin.

PD-L1 ΔCt = (PD-L1 Ct) – (GAPDH Ct + *β*-Actin Ct / 2)

Statistical analysis

For flow cytometry, the raw cell counts for cells expressing PD-L1 for three separate experiments were averaged and a student's t-test was applied to determine statistical significance. For qPCR analysis, ΔCt values for three separate experiments were then averaged and a student's t-test was applied to determine significance.

3.3 Results

PD-L1 mRNA abundance is increased in SHP-2 ablated cells

To determine whether pharmacological inhibition or genetic ablation of SHP-2 altered expression of PD-L1 mRNA, total RNA was prepared from A549, H460, and UKY-29 cells that were treated with SHP-099, RMC-4550, SHP-2 siRNA, or CRISPR. qRT-PCR was used to quantify levels of PD-L1 and GAPDH mRNA following treatments. The abundance of PD-L1 mRNA was not significantly changed in any cell line with SHP-2 pharmacological inhibitors or siRNA treatment (Figure 3.1). Specifically, in H460 and A549 cells, treatment with siRNA or RMC-4550 caused slight, but not statistically significant, increases in PD-L1 mRNA as indicated by a reduction in dCt values. CRISPR/Cas9-mediated knockout of SHP-2 resulted in significant increases in PD-L1 mRNA in all three cell lines (Figure 3.1). I used CRISPR/Cas9-mediated ablation to confirm that the findings from siRNA-mediated ablation. CRISPR/Cas9 ablation is more reliable, but transfection of the gRNA complexes tends to be more toxic to these cells.

PD-L1 protein expression is increased in SHP-2 ablated cells

Following from the mRNA analysis, I then asked whether PD-L1 protein levels changed consistently with the changes in mRNA expression. Western blot analysis revealed that PD-L1 protein expression increased in all three cells treated

with CRISPR (Figure 3.2A-3.2F). Specifically, in CRISPR-treated H460 cells, an 83% reduction in SHP-2 resulted in a five-fold increase in PD-L1 relative to control (Figure 3.2A, 3.2B); in A549 cells, a 92% reduction of SHP-2 resulted in a greater than ten-fold increase in PD-L1 relative to control (Figure 3.2C, 3.2D); and in UKY-29 cells, a 78% reduction of SHP-2 resulted in an eight-fold increase (Figure 3.2E, 3.2F) relative to control. Although siRNA ablation is not as efficient as CRISPR in all cells, H460 cells treated with siRNA also showed an increase in PD-L1 expression consistent with CRISPR ablation. In both UKY-29 and H460 cells, treatment with allosteric SHP-2 inhibitor RMC-4550 led to 1.5-fold and 3.5-fold increases in PD-L1 expression, respectively, relative to untreated cells. SHP099 treatment did not lead to increased PD-L1 expression over that of the vehicle controls. Additionally, the negative control cell transfections with either SignalSilence Control siRNA or non-targeting CRISPR/Cas9 guide RNA are shown in Figure 3.5.

Expression of PD-L1 on the cell membrane increased after SHP-2 ablation

Because cell surface expression of PD-L1 is required for therapeutic antibody binding and is often measured prior to initiation of ICI therapy for frontline ICI therapy decisions, I investigated whether cell surface expression of PD-L1 also changed in response to pharmacological inhibition or genetic ablation of SHP-2. Following the same conditions used to assess mRNA and protein levels, I measured cell surface expression of PD-L1 and EGFR as a control using flow cytometry. An increase in PD-L1 cell surface expression was observed in A549 (Figure 3.3, 3.4B), H460 (Figure 3.4A), and UKY29 (Figure 3.4C) cell lines following treatment in CRISPR/Cas9-mediated SHP-2 knockouts and consistent with, but to a lesser extent than, siRNA treatment. Specifically, H460 cells demonstrated a 33.7% increase in PD-L1 surface expression; A549 cells showed 62.9% increase in PD-L1; and UKY-29 cells demonstrated a 27% increase in PD-L1 surface expression following CRISPR knockout of SHP-2 (Figure 3.4B-3.4D). EGFR surface expression was measured as a control surface protein and was

unaltered by any treatments. These results follow from total protein analyses that loss of SHP-2 increases both total and PD-L1 expressed on the cell membrane.

3.4 Discussion

In this chapter, I present my efforts to understand the impact of the tyrosine phosphatase SHP-2 on the tumor expression of PD-L1. Currently, tumoral expression of PD-L1 is a guideline measure to initiate anti-PD-1 and PD-L1 therapies in some lung cancer settings. Specifically, for newly diagnosed NSCLC patients, treatment with a PD-1 antibody (pembrolizumab) is dependent on tumoral expression of PD-L1 that must be greater than 50% to initiate therapy and for second-line therapy, greater than 1% (164). Importantly, PD-L1 expression levels in tumor biopsies are impacted by many variables: heterogeneity of cell types within the tumor, region of the tumor tested, and the individual antibody/platform combination utilized which is often unique for an individual therapeutic entity (164). It is also reasonable to expect that control of PD-L1 expression differs within and among tumors. Given the clinical implications of the variability of PD-L1 expression, a comprehensive understanding of the regulatory mechanisms controlling when and where PD-L1 is expressed on tumor cells is vital.

Prior studies found that PD-L1 expression is aberrantly upregulated in KRAS-active NSCLC. Importantly, Coehlo et al. (2017) implicated the kinase and phosphatase pair of ERK and PP2A in control of the RNA-binding protein, TTP, that preserves PD-L1 mRNA stability (83). I found that the absence of SHP-2 activity via pharmacological inhibition of phosphatase activity contributed to increased PD-L1 expression in some KRAS active cells. I hypothesized that SHP-2 exerts control over PD-L1 expression downstream of growth factor signaling/KRAS signaling by impacting transcription initiation. Dissection of this process is of primary importance wherein pharmacological inhibition of SHP-2 may have therapeutic value in combination with PD-L1/PD-1 antibodies (145)(165). Notably, the proposed mechanisms of PD-L1 regulation in cancerous cells are

unlikely to mirror the normal regulation of PD-L1 expression in healthy cells. Therein lies the possibility that in malignant cells, PD-L1 regulatory mechanisms may be altered by the accumulation of multiple mutations that impact aberrant PD-L1 expression.

While my initial observation that loss of SHP-2 function resulted in increased PD-L1 levels, the level of expression of PD-L1 differs among cell lines. As other laboratories have observed, PD-L1 expression is highest in KRAS active cells (83)(137). Treatment with small molecule inhibitors of SHP-2 had minimal impact on PD-L1 mRNA levels, suggesting that the phosphatase activity associated with SHP-2 is not important at this level of control (Figure 3.1). Importantly, ablation of SHP-2 with siRNA or CRISPR resulted in consistent increases in PD-L1 mRNA, unlike treatment with small molecule inhibitors, also in Figure 3.1. These findings hinted toward another role of SHP-2 in control of PD-L1 expression as it may participate in a regulatory protein complex that controls PD-L1 mRNA or protein half-life much like ERK/PP2A control of TTP (83)(166). Thus, allosteric inhibition of SHP-2, with either SHP-099 or RMC-4550, may not interfere with the ability of SHP-2 to interact with other proteins. Bivona (2019) suggests that a GRB/SOS/GAB/SHP-2 complex transmits information from receptor tyrosine kinases to the nucleus to regulate expression of a panel of genes necessary for proliferation (167). It is unclear whether these interactions exclusively control the flow of phosphates as secondary signals among proteins or other complex interactions of proteins provide the control of expression of PD-L1. Finally, flow cytometry analysis revealed that cell surface expression of PD-L1 increased in cells with SHP-2 ablation consistent with the findings of Liu et al. (2017) (168). It is however unclear whether these changes in surface expression are the result of increases in total protein, or if there is some mechanism by which SHP-2 is involved in trafficking PD-L1 to the cell surface. Further experimentation, such as ****come back to this****

3.5 Conclusions

My results provide novel insight into additional regulatory mechanisms governing the aberrant expression of PD-L1 in KRAS-mutant NSCLC. Ablation of SHP-2 significantly increased PD-L1 mRNA and surface expression, while pharmacological inhibition of phosphatase activity resulted in minimal changes in PD-L1 levels. Furthermore, I hypothesized that the formation of multi-protein complexes is controlled by SHP-2 and provide another layer of regulatory control of PD-L1 expression. These observations hold both clinical and basic biological implications. Ablation of SHP-2 may synergize with PD-L1 blockade in some KRAS-active NSCLC and should be predicted by the expression levels of PD-L1. Thus, dissection of the unreported activities of SHP-2 protein complexes in the regulation of mRNA processing and trafficking of PD-L1 may offer additional insights for both biomarkers and therapy.

Figure 3.1. PD-L1 mRNA expression depends on SHP-2. PD-L1 mRNA levels were measured by qRT-PCR in H460, A549, and UKY29 cell lines treated with SHP099 [10μM], RMC-4550 [20nM], SHP-2 siRNA [30nM] or CRISPR/Cas9 mediated SHP-2 knockout. PD-L1 dCt values for three independent experiments were generated using GAPDH as a reference gene and then averaged. Error bars represent standard deviation. Significance was determined using the students t-test ($P<0.05$).

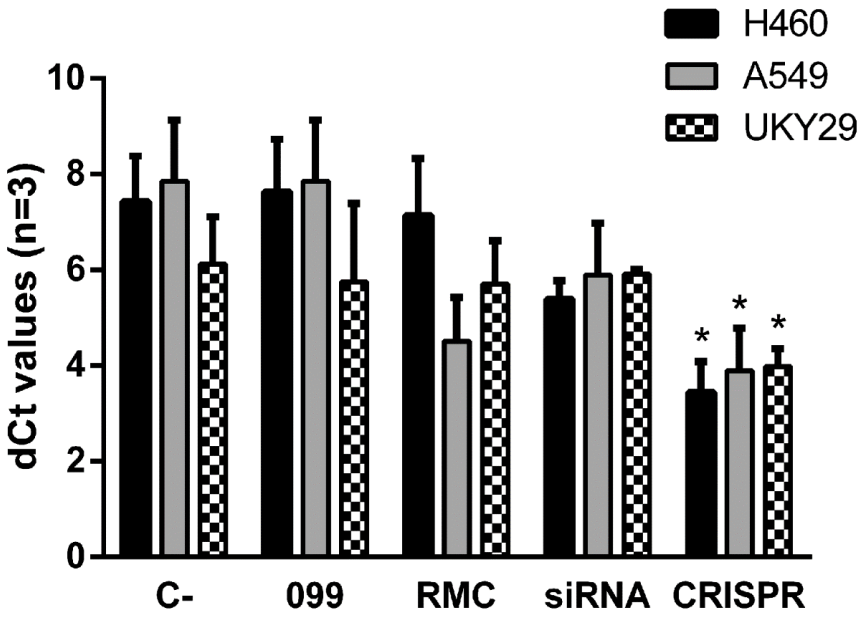


Figure 3.2. PD-L1 protein levels increase in the absence of SHP-2. Representative western blots of **A.** H460 cell lysates, **C.** A549 cell lysates, and **E.** UKY-29 cell lysates that were untreated or treated with vehicle [4% DMSO], allosteric inhibitors SHP099 [10μM], RMC-4550 [20nM], and SHP-2 siRNA [30nM] or CRISPR/Cas9-mediated SHP-2 knockout**.** Densiometric quantification of SHP-2 and PD-L1 signal normalized to GAPDH signal from **B.** H460 cell lysates (A), **D.** A549 cell lysates (C), and **F.** UKY-29 cell lysates (E).

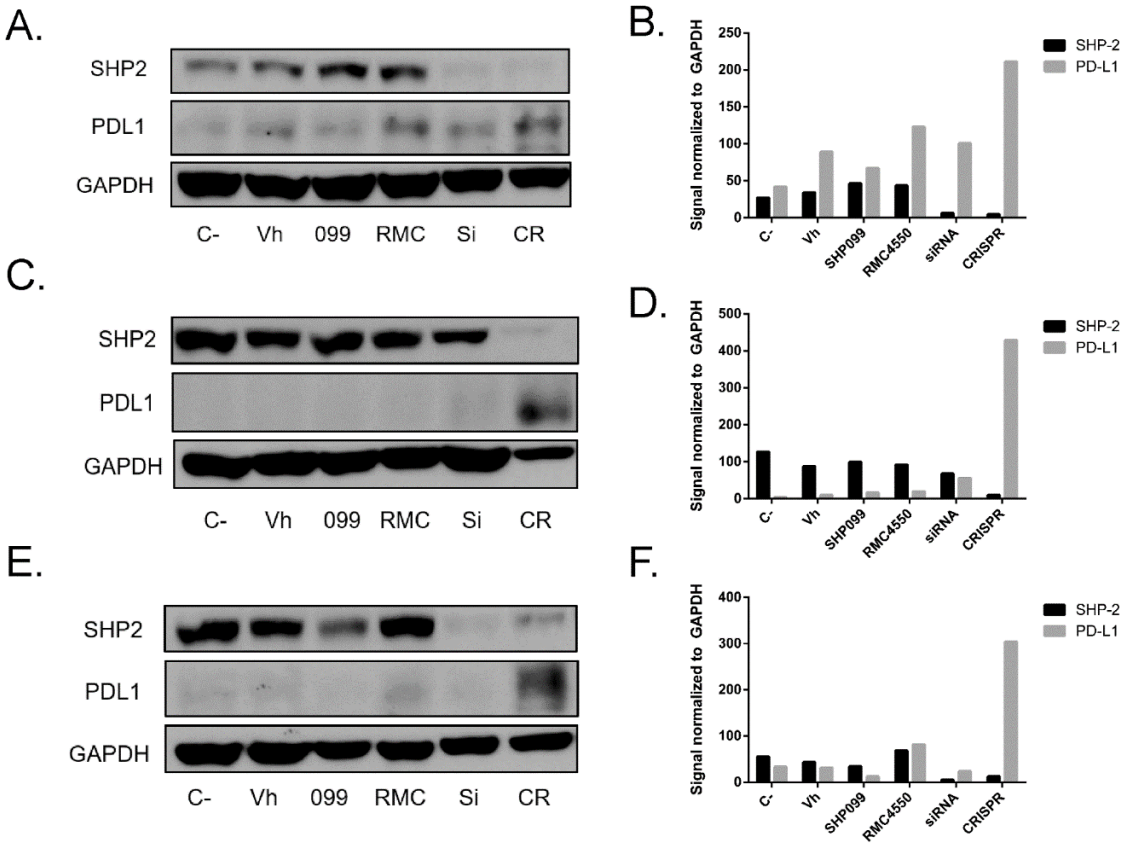


Figure 3.3. PD-L1 surface expression increases in the absence of SHP-2 in A549 cells. Representative pseudo-colored density plots of A549 cells treated with vehicle [4% DMSO], SHP099 [10μM], RMC-4550 [20nM], SHP-2 siRNA [30nM] or CRISPR/Cas9-mediated SHP-2 knockout. The Y-axis represents EGFR-AlexaFluor 647 signal (control) and the X-axis represents PD-L1-PE signal.

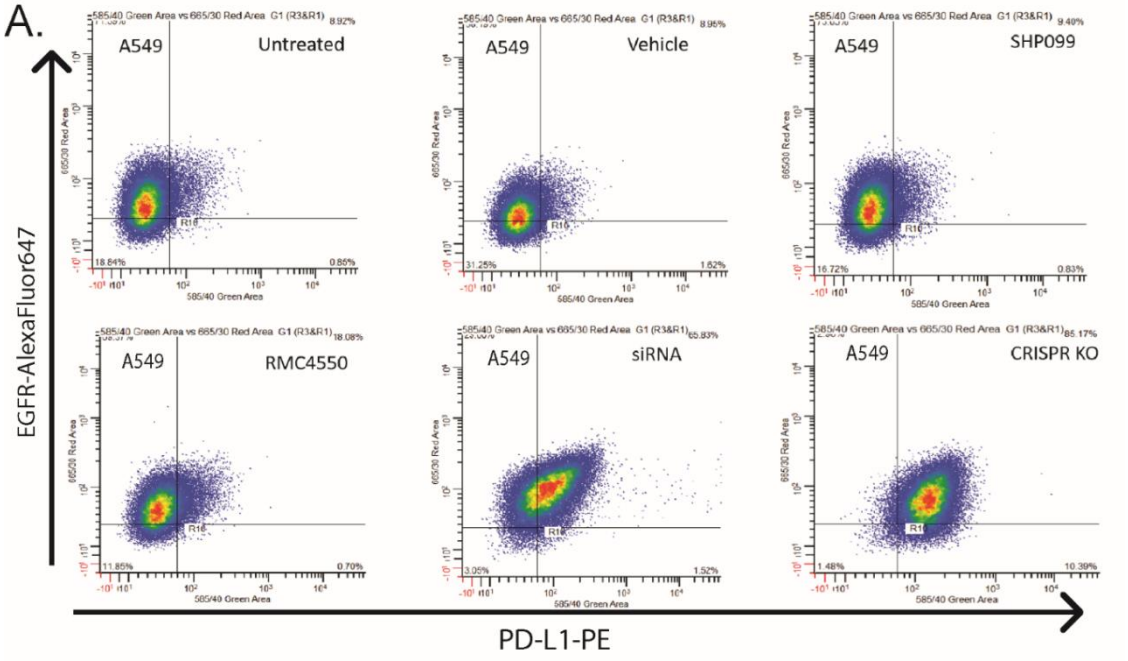


Figure 3.4. PD-L1 surface expression increases in the absence of SHP-2. Flow cytometry quantification of PD-L1 on the surface of **A.** H460 cells, **B.** A549 cells, and **C.** UKY-29 cells expressing PD-L1 on their surface treated as described in figure 3.3. Three independent experiments were averaged, and error bars represent the standard error of the mean. Significance was determined using the students t-test ($P<0.05$).

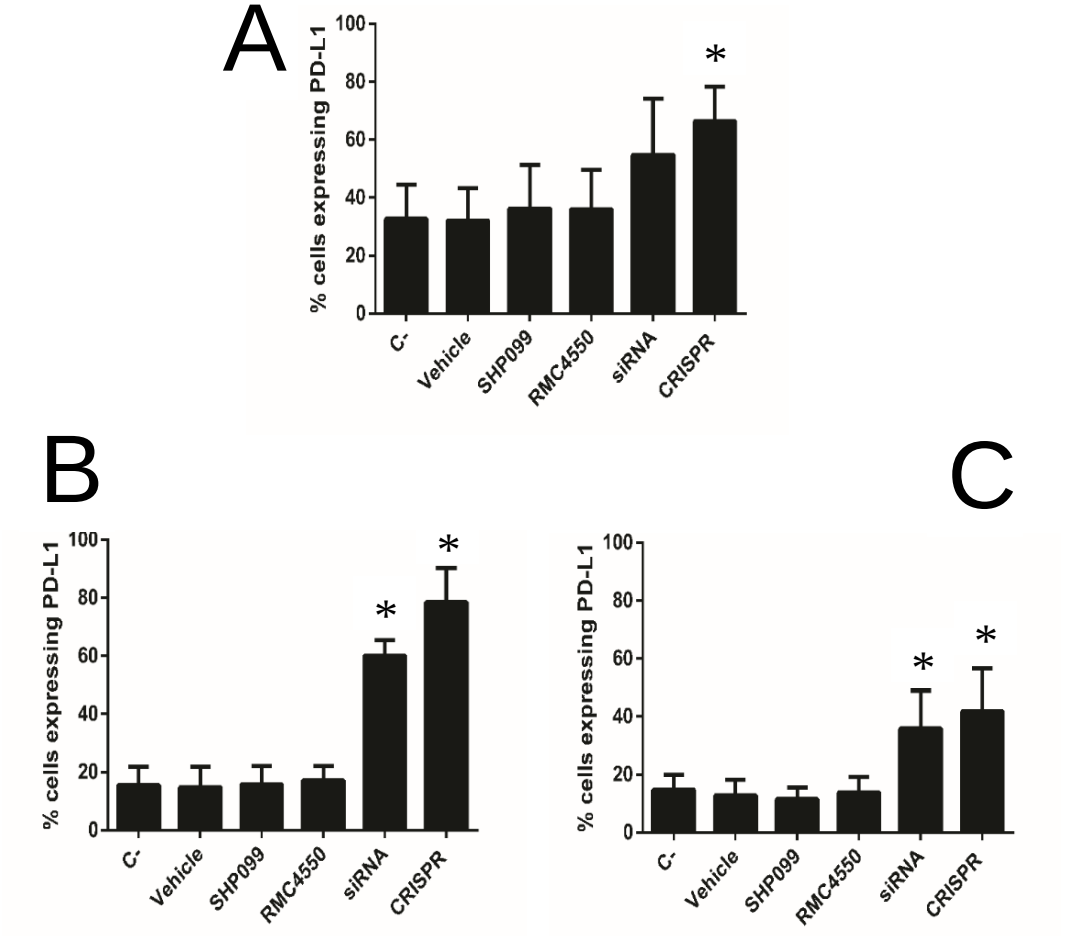
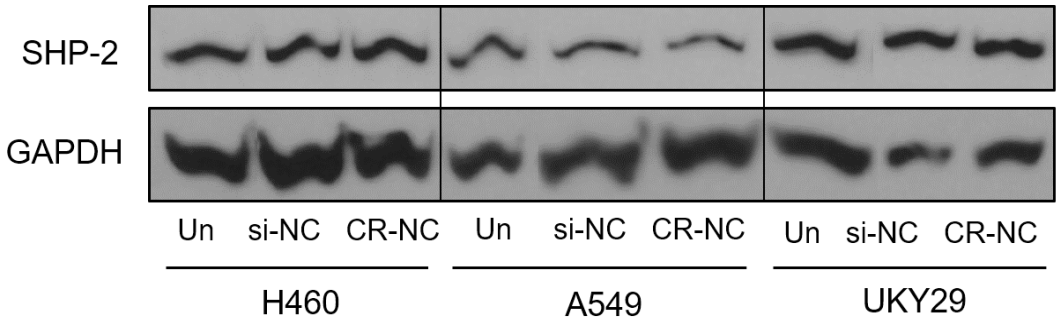


Figure 3.5. Negative control siRNA and CRISPR gRNA do not reduce SHP-2 expression. Representative western blots of H460, A549, and UKY-29 cell lysates that were untreated (Un) or treated with SignalSilence negative control siRNA [30nM] (si-NC) or CRISPR/Cas9 negative control gRNA (CR-NC). Bands for each cell line were all run on the same gel and negative control bands were grouped next to untreated cells for clarity.



CHAPTER 4.

4.1 Overview

Over the past decade there has been growing interest in understanding the multifaceted roles of tyrosine phosphatases as they relate to human cancers. It has long been established that phosphorylation of tyrosine residues by tyrosine kinases plays a critical role in the transmission of cellular signaling cascades that regulate cell growth and survival (169)(170). Considering the gravity of tyrosine phosphorylation events, it is logical to expect that the expression and activity of negative regulators of these pivotal pathways should hold equal importance to abnormal utilization by malignant cells (171). It is also important to note that the relationship between kinases and phosphatases is more complex than the simply positive or negative regulation of pathway activity; both classes of enzymes are capable of more than functioning solely as “on/off” switches, and the addition or subtraction of a phosphate group does not necessarily correspond to greater or lesser activity. To begin dissecting the complex involvement of protein tyrosine phosphatases in cell signaling pathways, this chapter aims to further elucidate the mechanism by which the non-receptor tyrosine phosphatase SHP-2 is involved in regulating the expression of genes downstream of RTK signaling, specifically PD-L1.

SHP-2 has been shown to participate in the transmission of extracellular signaling events mediated by several different RTKs and functions as a focal point of intersecting signaling cascades such as the Ras/Raf/MEK, JAK/STAT, and PI3K/mTOR pathways in cancer cells (124). In NSCLC, SHP-2 plays a critical role in regulating oncogenic Ras signaling by forming a multi-protein complex with SOS and Gab1/Grb2 at cytosolic phosphorylated tyrosine residues located on RTKs and is required for the growth of KRAS-driven NSCLC (106)(107). Additionally, SHP-2 has been demonstrated to interact with the intracellular immunoreceptor tyrosine-based switch motif (ITSM) domain of the PD-1 receptor on activated T-cells and

macrophages. Upon ligation of PD-1 with its ligand, SHP-2 serves as a scaffold to create a PD-1:PD-1 dimer which results in the activation of SHP-2, the internalization the PD-1 receptor, and subsequent T-cell activation (172)(173). Importantly, the mechanism of SHP-2-mediated activation and PD-1 internalization has been shown to occur irrespectively of the catalytic phosphatase activity of SHP-2, suggesting that SHP-2 may engage in signal transduction activities solely through its ability to initiate protein complex formation (172)(174).

SHP-2 contains three major functional domains; two SH-2 domains (N-SH2, C-SH2) that allow SHP-2 to associate with phosphorylated tyrosine residues present on its protein substrates, and a catalytic phosphatase domain (175). On the C-terminal domain (CTD) of SHP-2 reside two tyrosine residues (Y542 and Y580) which may become phosphorylated, releasing SHP-2 from its autoinhibitory conformation and allowing for catalytic PTP activity. Although it is well understood that SHP-2 phosphorylation correlates with its activity, the exact tyrosine kinases responsible for SHP-2 activation remain unknown.

Mutations in the PTPN11 gene which encodes SHP-2 have been linked to several types of human diseases, including cancer (176)(177)(178)(179). Noonan's syndrome (NS) and Noonan syndrome with multiple letigines (NSML), formerly known as LEOPARD syndrome, are diseases in which the regulatory mechanisms for SHP-2 activation are altered (180). Activating mutations of SHP-2, such as E76K, are associated with NS and juvenile myeloid leukemia, and the E76K mutation is the most commonly found SHP-2 mutation in NSCLC tumors, although the incidence of SHP-2 mutations is low in NSCLC (181). Such activating mutations activate SHP-2 by disrupting the auto-inhibitory interactions between the N-SH2 and PTP domains, resulting in constitutively active phosphatase activity. Additionally, these mutations may impact the protein complex formation capability of SHP-2, as demonstrated by Fragale et al. Specifically, SHP-2 mutants with disrupted autoinhibitory activity maintained prolonged binding with adaptor proteins Gab1/Grb2 (182).

As previously described, the involvement of SHP-2 (whether through PTP or scaffolding activity) in numerous immunomodulatory pathways suggests that SHP-2 activity or expression would be capable of influencing PD-L1 levels. The factors governing how and when PD-L1 is aberrantly expressed by malignant cells are only beginning to be understood. However, there are multiple regulatory levels of PD-L1 expression that have been described. For example, the activity of signal transduction pathways such as the RAS/Raf/ERK, Jak/STAT, and PI3K/Akt/mTOR all have been shown to influence transcriptional activity of PD-L1 (183)(184). More specifically, NSCLC patient tumors harboring mutations in the KRAS gene have upregulated levels of PD-L1, as well as improved responses to ICI therapy compared to patients with wildtype KRAS. Coelho et al. demonstrated that oncogenic RAS signaling can upregulate PD-L1 expression by stabilizing PD-L1 mRNA through the mRNA binding protein tristetraprolin (TTP) (83). Finally, post translational modifications to PD-L1 such as glycosylation, ubiquitination, and Ser/Thr phosphorylation have been shown to impact PD-L1 protein turnover and metabolism, as well as the trafficking of PD-L1 to the cell surface (86)(185).

As described earlier in chapters 2 and 3, I observed that PD-L1 protein significantly and negatively correlates with levels of active (p-Y452) SHP-2 in NSCLC patient tumors, and that there also exists a slight positive correlation between PTPN11 and CD274 mRNA levels. From my in vitro experimentation in chapter 3, I observed increases in PD-L1 mRNA, protein, and surface expression following genetic ablation of SHP-2 protein, concurrent with my finding that SHP-2 activity negatively correlates with PD-L1 expression in human tumors. Importantly, I observed that inhibition of SHP-2 phosphatase activity with two small molecule allosteric inhibitors of SHP-2 did not impact levels of PD-L1 expression, and thus the ability of SHP-2 to enucleate protein-protein complexes may be of greater importance than its catalytic PTP activity with regard to PD-L1 expression. To begin unraveling the mechanism by which SHP-2 influences PD-L1 expression, I first sought to pinpoint the layer of expression at which SHP-2 exerts control, as

well as identify any protein binding partners of SHP-2 that may be responsible for the PD-L1 phenotypes observed in earlier chapters.

4.2 Methods

Cell lines and treatments

For cycloheximide and actinomycin D treatments, NCI-H460 cells were seeded at $2x10^4$ cells/well on a 24-well plate in RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO2 for 24 hours. Media was then removed and replaced with RPMI 1640 containing 10% FBS and no antibiotics prior to transfection with SHP-2 siRNA [30nM] (ThermoFisher) and Lipofectamine RNAiMAX (ThermoFisher). After a 48-hour incubation with the lipid complexes, media was removed and replaced with RPMI 1640 containing 10% FBS and either Cycloheximide [10mg/mL] or Actinomycin D [1mg/mL]. Cells were harvested after 0, 2, 4, 6, 8, and 10 hours of exposure to either compound in protein sample buffer for western blot analysis or buffer RLT (QIAgen) for RNA extraction and RT-qPCR. To assess the status of SHP-2 expression in our cell lines of interest NCI-H460, A549, and UKY29 cells were seeded at $2x10^4$ cells/well in a 24-well plate in 1mL of RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO2 for 24 hours. Designated wells then had media removed and replaced with RPMI 1640 containing 1% FBS. After 24 hours, designated wells had FBS added back to a final concentration of 10% and were allowed to incubate for another 24 hours prior to harvest.

For growth factor stimulation assays, NCI-H460, A549, and UKY29 were seeded at $2x10^5$ cells/well in all well of a 6-well plate in 3mL of RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO2 for 24 hours. For five of the wells, media was then replaced with 3mL RPMI 1640 containing 0.1% FBS. After 24 hours, FBS was added back to four of the starved wells and cells were

harvested 1, 2, 4, and 8 hours post addition of FBS. The remaining two wells were harvested 48 hours after being plated.

For carfilzomib treatments, NCI-H460, A549, and UKY29 cells were seeded at $2x10^4$ cells/well on a 24-well plate in 1mL RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO2 for 24 hours. Cells then were allowed to either remain in 10% FBS RPMI 1640 for an additional 48 hours, or media was replaced with 1mL of 1% FBS RPMI 1640 and allowed to incubate another 24 hours. Finally, designated cells had media removed and replaced with 10% FBS RPMI 1640 for 24 hours. Carfilzomib (SelleckChem) and/or FBS to a final concentration of 10% FBS were added 72 hours after initial seeding to designated wells to a final concentration of [50nM] for 8 hours prior to harvest. Cells were harvested in 100µL protein sample buffer prior to protein separation by SDS-page and western blot analysis.

For immunoprecipitations, NCI-H460 cells were seeded on a 60mm plate at a density of $1x10^5$ cells in 5mL of RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin and maintained at 37°C, 5% CO2 for 24 hours. Media was then replaced with 2.5mL of RPMI 1640 containing 1% FBS and no antibiotics. Cells were transfected with 5mg of a plasmid construct encoding wild-type (WT) SHP-2 fused to EGFP (AddGene, plasmid #12283) using the Lipofectamine 3000 (ThermoFisher) transfection reagent and incubated for 48 hours to ensure the expression of SHP-2. Media was then removed, and cells were washed with 5mL of ice-cold 1X phosphate buffered saline before lysing the cells with 1mL ice-cold IP lysis buffer (50mM HEPES pH 7.5, 150mM NaCl, 1% Triton X-100, 10% glycerol, 1.5mM MgCl2, 1mM EDTA) on ice for 5 minutes.

SHP-2 transfection and co-immunoprecipitation

Wildtype (WT)-SHP-2 transfected H460 cells were lysed then transferred into a 1.5mL tube, sonicated three times with a handheld sonicator to disrupt cell

membranes, and centrifuged for 10 minutes (>13,000xg) at 4°C. Half of the supernatant was transferred to a new 1.5mL tube and a monoclonal SHP-2 antibody (Cell Signaling Technologies, #3397) was added at a dilution of 1:150 then rotated overnight at 4°C. The remaining supernatant was handled in the same manner but without the addition of antibody.

The following day, protein A magnetic beads (10µL slurry per 100µL lysate; Cell Signaling Technologies) were pre-washed 3 times with 5 times their volume of IP lysis buffer and captured using a magnetic tube rack before being added to the cell lysate. The slurry was incubated while rotating for 20 minutes at 4°C. Protein-complexed magnetic beads were then collected in a magnetic tube rack, washed 5 times with 5 times their volume of IP lysis buffer, washed three times with 50mM HEPES pH 7.5, then captured and resuspended in 30µL protein sample buffer. The samples were heated to 95°C for 5 minutes. Beads were collected and the supernatant was loaded onto a 10% SDS-PAGE acrylamide gel and separated at 135V for 70 minutes. The gel was then transferred to a clean glass container containing Coomassie blue staining solution (2.5% Coomassie Blue, 10% glacial acetic acid, 50% MeOH, 50% dH2O) and incubated, while rocking, at room temperature for 60 minutes. Coomassie was then removed, and the gel was washed twice with dH2O for 30 minutes before a final overnight de-staining in dH2O. The gel was then transferred to a bio-safety cabinet and protein bands were excised using a clean razor blade and then transferred to 1.5mL tubes. The University of Kentucky Proteomics Core completed mass spectrometry analysis.

CD274 promoter luciferase assay

NCI-H460, A549, and UKY29 cells were seeded at $2x10^4$ cells/well on a 24-well plate in 1mL RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO2 for 24 hours. Media was then replaced with 500µL RPMI 1640 with 1% FBS containing no antibiotics prior to transfection with SHP-2 siRNA (ThermoFisher) with RNAiMAX (ThermoFisher) to a concentration of [30nM]. After a 24-hour

incubation, media was replaced with RPMI 1640 with 10% FBS containing no antibiotics and cells were transfected with plasmid vectors containing Renilla luciferase with a TK promoter (Promega #E2241) and firefly luciferase with the CD274 promoter (Addgene #107003) using Lipofectamine3000 (ThermoFisher). Additionally, one group of transfected cells were also treated with RMC-4550 (SelleckChem) at [20nM]. Media was removed 24 hours post-transfection, and cells were washed with 1x phosphate buffered saline. Cells were then lysed by 15 minutes of incubation with 100µL of passive lysis buffer (Promega #E1910) while rocking at room temperature. To measure luminescence, lysate was warmed to room temperature and then 20µL was plated onto a white-bottom 96-well plate (USA Scientific #5665-5074). The Promega dual-luciferase reporter assay system was then used to measure luminescence with a Synergy H1 multi-mode microplate reader (BioTek). To control for transfection efficiency, firefly luciferase luminescence was standardized to Renilla luminescence by dividing the firefly signal values by the Renilla signal values.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from lysed cells using the RNeasy kit (Qiagen). cDNA was then generated using the iScript cDNA synthesis kit (BioRad). qRT-PCR was performed using human TaqMan primer probe sets (Thermo- Fisher) for PD-L1 (Assay ID: Hs00204257_m1), SHP-2 (Hs06636344_g1), GAPDH (Hs02786624_g1) and β-Actin (Hs99999903_m1). qRT-PCR data were analyzed by calculating ΔCt values for PD-L1 by standardizing PD-L1 Ct values against those for the averaged Ct values of housekeeping genes GAPDH and β-Actin.

PD-L1 ΔCt = (PD-L1 Ct) – (GAPDH Ct + *β*-Actin Ct / 2)

Western blot analysis

Following treatment and harvest, cells were lysed with protein sample buffer, subjected to at least one -20°C freeze/thaw cycle, sonicated, then heated to 95°C for 5 minutes prior to protein separation using 10% SDS-polyacrylamide gel electrophoresis (PAGE) at 135V for 80 minutes. Proteins were transferred to a nitrocellulose membrane and blocked in Tris-buffered saline (TBS) containing 0.1% Tween 20 and 5% bovine serum albumin for 30 minutes prior to overnight incubation at 4°C with primary antibody: SHP-2 (#3397), PD-L1 (#13684), or GAPDH (#2118) (Cell Signaling Technologies). After overnight incubation, antibody was removed, and blots were washed with TBS containing 0.1% Tween 20. Secondary HRP-labeled antibodies were applied to the blot in 5% non-fat, dry milk in 1X TBS containing 0.1% Tween 20 for one hour. The blot was washed three times for 5 minutes each in 1X TBS containing 0.1% Tween prior to developing and detection of HRP signal. Signal was quantified using ImageJ software (NIH) and values for SHP-2 and PD-L1 were normalized to GAPDH.

Mass spectrometry

Each gel slice was treated with dithiothreitol (DTT), iodoacetamide (IAA), and digested with trypsin. The tryptic samples were filtered with 0.22µM PVDF filters and subject to liquid chromatography-mass spectrometry (LC-MS/MS) analysis. MS data sets were searched in MASCOT against a custom database containing a reviewed dataset of Homo sapiens proteins from Uniprot. (https://www.uniprot.org/uniprot/?query=taxonomy:%22Homo%20sapiens%20(Human)%20%5b9606%5d%22&fil=organism%3A%22Homo+sapiens+%28Human%29+%5B9606%5D%22+AND+reviewed%3Ayes). The MASCOT files were filtered with peptide medium confident filter (MCF, target peptide false discovery rate of 5% for ‘medium’ confidence) to filter out low confidence peptides.

4.3 Results

SHP-2 ablation changes PD-L1 mRNA and protein half-life

To dissect the mechanism by which SHP-2 influences PD-L1 expression, I first wanted to determine the role of SHP-2 in either transcription or translation, using actinomycin D and cycloheximide treatments, respectively. Cycling H460 cells with or without ablation of SHP-2 using siRNA were treated with either actinomycin D or cycloheximide for 10 hours. siRNA ablation of SHP-2 was chosen here to reduce toxicity from transfection and allow for sufficient cell numbers for subsequent treatments. mRNA and protein expression of SHP-2, PD-L1 and GAPDH were measured by qRT-PCR and western blot, respectively. We discovered that in cells lacking SHP-2 then subsequently treated with actinomycin D, both PD-L1 protein and mRNA were degraded more rapidly than in cells expressing SHP-2 (Figure 4.1A, 4.1B, 4.3). In cells lacking SHP-2 then treated with cycloheximide, modest changes in PD-L1 protein and mRNA levels were observed when compared to SHP-2 expressing cells (Figure 4.2A, 4.2B, 4.4). Collectively, these data suggest that SHP-2 likely functions in regulating mRNA turnover.

SHP-2 ablation increases CD274 promoter activity

To begin investigation of the impact of SHP-2 expression on PD-L1 mRNA expression, a CD274 promoter-driven luciferase construct was ectopically expressed to measure whether altering SHP-2 expression influences CD274 promoter activity. I discovered that A549 and UKY29 cells treated with either SHP-2 siRNA or RMC-4550 had greater CD274 promoter activity compared to control (Figure 4.5A, 4.5C). Importantly, these changes in CD274 promoter activity varied by cell type, with H460 cells demonstrating no differences in CD274 promoter activity in either treatment.

SHP-2 interacts with proteins involved in mRNA processing and cellular chaperoning

Because SHP-2 appears to influence both PD-L1 mRNA and protein half-life, I wanted to determine whether SHP-2 is interacting with other proteins that might assist in understanding the mechanism used to manage PD-L1 expression. I sought to identify protein partners of SHP-2 in cells using co-immunoprecipitation followed by mass spectrometry identification of protein partners. Ectopically-expressed SHP-2 was immunoprecipitated from H460 cells and resolved by SDS-PAGE. LC-MS/MS was carried out on gel regions subjectively chosen to contain prominently stained proteins. We then queried the tryptic protein fragments to determine identification of proteins in that slice. Importantly, the bait protein, SHP-2, was among the proteins discovered in our analysis. We then generated a list of the highest scoring proteins, with scores ranging from 234.93 to 540.88 and amino acid coverage ranging from 18.33% to 51.98%. Of these proteins, two (HSP7C, GRP78) have been shown to be involved in the chaperoning and proper folding of proteins, and the remaining eight (DDX3X, PUF60, CPSF5, EF1A1, EF1A3, DDX3Y, RSMB, RSMN) have been shown to be involved in transcriptional regulation/activation and mRNA processing (Table 4.1). These results are consistent with a role for SHP-2 in either mRNA processing or regulation of turnover.

SHP-2 expression is not targeted by the proteasome

During this experimentation, I observed that SHP-2 is poorly expressed in NSCLC cells that are actively growing, consistent with the observations of other groups (Figure 4.6)(106)(107). Thus, NSCLC cell lines were maintained in low serum for assays designed to evaluate the impact of SHP-2 activity or expression. I sought to understand why SHP-2 expression was not expressed in cycling cells as would be expected of a protein that was crucial for KRAS pathway control. I suspected that loss of SHP-2 expression was linked to increased proteasomal degradation in cycling cells.

To first understand the context of SHP-2 expression as it relates to the cell cycle, I first cultured cells in low serum media (0.1% FBS), added back serum to a concentration of 10% FBS, and harvested cells after 1, 2, 4, and 8 hours. I discovered that in H460 and A549 lines, SHP-2 expression was rapidly and substantially decreased after 1 hour, and this reduction in expression was maintained through the 8-hour time point (Figure 4.7). To test if the loss of SHP-2 was mediated by proteasomal degradation, cells were cultured under varying media conditions for 72 hours followed by treatment with the selective 26S proteasome inhibitor carfilzomib (CFZ). We found that cells maintained in low serum conditions (48 hours of 1% FBS RPMI) had the highest levels of SHP-2 expression, compared with cells in high serum conditions. Importantly, SHP-2 expression was unaffected following CFZ treatment, suggesting that the loss of SHP-2 expression in cycling cells is not due to degradation by the 26S proteasome (Figure 4.8)

4.4 Discussion

The aim of this final data chapter was to elucidate the mechanism by which SHP-2 exerts control over PD-L1 expression. In chapter 3, I observed changes in PD-L1 levels at all layers of expression (mRNA, protein, and surface expression) in response to genetic ablation of SHP-2 protein. These results suggested that SHP-2 had multiple roles on PD-L1 expression, but it was important to understand why. It was discovered that in H460 cells treated with actinomycin D, the half-life of both PD-L1 mRNA and protein was decreased in cells with reduced SHP-2 expression, while in cells treated with cycloheximide demonstrated minimal changes in PD-L1 mRNA and protein half-life. Thus, the impact of SHP-2 on PD-L1 expression likely resides in the regulation of mRNA half-life rather than by influencing the translation or turnover of PD-L1 protein, potentially in a manner similar to that of TTP as described by Coehlo et al. Importantly, these findings suggest a previously unreported function of SHP-2 in direct control of mRNA expression and metabolism. With these observations in hand, we further

hypothesized that SHP-2 serves as a protein scaffold for a multi-protein complex involved in mRNA turnover. To begin dissecting novel SHP-2 protein-protein interactions that would support our hypothesis, we performed immunoprecipitation of SHP-2 followed by mass spectrometry analysis to identify co-immunoprecipitating proteins.

From the bioinformatic perusal of the tryptic peptide sequences from the mass spectrometry analysis, I uncovered proteins previously identified as: 1) components of multi protein complexes, 2) participants in mRNA translation and processing, and 3) chaperones of proteins from the endoplasmic reticulum (ER) to the cell surface. Of the top 10 scoring proteins from the analysis shown in Table 1, two have been shown to serve as chaperones for proteins from the endoplasmic reticulum, and eight are involved in transcriptional regulation, mRNA processing and maturation. The protein with the most independent hits in the analysis, HSP7C, plays significant roles in the transport and folding of newly synthesized polypeptides, the formation and dissociation of protein complexes, and can even function as a repressor of transcriptional activation (186)(187)(188). The second highest scoring protein, DDX3X, is a multifunctional RNA helicase involved in a large variety of cellular processes, specifically with regard to RNA transcription and metabolism. Importantly, DDX3X contains a tyrosine at residue 104 which may be phosphorylated and could serve as a potential interaction site for SHP-2 SH2 domains (189). Additionally, DDX3X has been reported to have both tumor-promoting and tumor-suppressive effects, similar to SHP-2, and has been shown to have deregulated expression in several types of cancer (124)(189)(190).

Having established a connection between SHP-2 expression and PD-L1 mRNA levels in the bioinformatic analyses of tumor data, I wanted to understand if SHP-2 and its potential protein partners were capable of impacting PD-L1 transcriptional activity by influencing activity of the CD274 promoter. I discovered that A549 and UKY29 cells with reduced SHP-2 activity or expression maintained significantly higher levels of CD274 promoter activity than those that express active

SHP-2 (Figure 4.7). This finding further reinforces our hypothesis that SHP-2 exerts transcriptional control of PD-L1, potentially through its involvement in upstream signaling pathways such as the RAS/Raf/ERK and Jak/STAT pathways, both of which have been shown to influence CD274 transcriptional activity (191)(192). Interestingly, the reductions in SHP-2 activity had minimal impact on CD274 promoter activity in H460 cells when compared to A549 and UKY29 cells. Across multiple repetitions, H460 cells demonstrated the lowest levels of CD274 promoter activity of all three cell lines even though they consistently express the highest levels of PD-L1 protein (as measured by western blot) (Figure 4.6). This suggests that in some cell types, there are post-transcriptional regulatory events that modulate the stability of the CD274 mRNA available for translation. It is important to note that H460 cells with ablated SHP-2 expression demonstrated a more rapid degradation of PD-L1 mRNA and protein, however SHP-2 knockdown had no impact on CD274 promoter activity in these cells. Additional repetitions of the actinomycin D/cycloheximide experiments, also expanded to A549 and UKY29 cells, are necessary to help understand these observations and provide additional information on if these trends are impacted by cell type. It is also important to dissect whether there is an impact of SHP-2 at the CD274 promoter that directs transcription, or if SHP-2 serves a protective role for CD274 mRNA by forming a complex with eIF1-a and DDX3X at the cap of newly transcribed mRNA that influences mRNA stability and delivery to the ribosome. Interestingly, treatment with the SHP-2 allosteric inhibitor RMC-4550 similarly increased CD274 promoter activity and so the catalytic PTP activity of SHP-2 may bear relevance to transcriptional control of PD-L1. However, it is still unclear as to what extent the presence of RMC-4550 impacts the proposed SHP-2 protein complex formation.

From our mass spectrometry screen, we identified 8 proteins that are involved in mRNA processing, however only one protein (PUF60) has been shown to directly bind to the promoter region of genes and regulate their transcriptional activity (193). Additionally, our analysis of RNA-seq data from NSCLC patient tumors revealed a significant negative correlation ($r = -0.253$, $p<0.0001$) between

PUF60 and CD274 mRNA. Though there remains the possibility of SHP-2's direct involvement in a complex that binds to the CD274 promoter, further experimentation is required to dissect these processes and uncover the mechanism by which SHP-2 impacts CD274 transcriptional activity.

Finally, the expression of SHP-2 primarily in non-cycling cells made it difficult to understand how SHP-2 regulates PD-L1 in growing tumor cells. Thus, in cycling cells, is SHP-2 expressed only for a brief period to transmit a signal or interact with a protein substrate, followed by prompt SHP-2 degradation or promoter deactivation? Or are there other mechanisms that limit SHP-2 protein expression? I hypothesized that SHP-2 may be a target for degradation by the proteasome in cells that are actively dividing, and that SHP-2 expression in cycling cells could be both ephemeral and contingent upon the activation of pathways in which it serves a necessary function. I discovered that cells rapidly lost expression of SHP-2 after the addition of mitogens to low serum cell media. Within 1 hour SHP-2 expression was greatly diminished and did not return by the 8-hour time point. This suggests that regulation of SHP-2 expression is cell-cycle mediated, and that as cells enter the S-phase of the cell cycle, expression of SHP-2 is lost, and expression does not return until cells either progress past the S-phase or leave the cell cycle completely. To determine whether SHP-2 expression is lost due to proteasomal activity, cells were treated with CFZ after starvation and serum addition. However, I discovered that CFZ treatment for 8 hours did not prevent the degradation of SHP-2, suggesting the 26S proteasome is not responsible for this loss of SHP-2 expression. These observations suggest that SHP-2 expression is differentially modulated by the replicative status of malignant cells, which is an important consideration for pharmacologically inhibiting SHP-2 *in vivo.*

Each of the findings in this chapter are also important to consider within the context of a human tumor *in situ*. As noted in earlier chapters, NSCLC tumors are comprised of not one singular cell type, but of heterogenous sub-populations of cells. As a tumor grows it will recruit blood vessels through the process of

angiogenesis to acquire sufficient amounts of oxygen and mitogenic factors. However, tumor angiogenesis is not an efficient or articulate process, and so it may render parts of the tumor devoid of sustenance, specifically areas like the core of tumor, which may become populated by necrotic tissue (194)(195)(196). My findings suggest that SHP-2 expression, and its influence on PD-L1 expression, may be most prevalent in specific areas of a tumor, such as the core, with slowed growth. Furthermore, it suggests that SHP-2 is of greater importance to cells under conditions in which survival is of greater importance than replication. Further experimentation, such as immunohistochemical interrogation of KRAS-active NSCLC tumor biopsies for the co-occurrence of SHP-2 and PD-L1, is required to understand whether the *in vitro* expression patterns mirror that of an actual tumor.

4.5 Conclusions

The experimentation in this chapter imparts evidence that SHP-2 regulates PD-L1 mRNA half-life. Further, CD274 promoter activity was impacted by a loss of SHP-2 expression, thereby further narrowing the scope of SHP-2 to a transcriptional regulatory role. Although SHP-2 is not expressed as cells enter S-phase, evidence presented here shows that SHP-2 is not degraded by the 26S proteasome in actively cycling NSCLC cells. Additional experimentation should include studies to determine the mechanisms controlling SHP-2 expression in cycling cells. Finally, using the information gleaned from the immunoprecipitation analyses, I predict that multi-protein complexes, controlled by transient SHP-2 expression, are the regulatory agents of PD-L1 control. These observations hold both clinical and basic biological implications. Ablation of SHP-2 may synergize with PD-L1 blockade in NSCLC and may be predicted by the expression levels of PD-L1 from a biopsy as standard of care. Thus, dissection of the unreported activities of SHP-2 protein complexes in the regulation of mRNA processing and trafficking of PD-L1 may offer additional insights for both biomarkers and therapy.

Figure 4.1. SHP-2 expression influences PD-L1 protein half-life in actinomycin D treated cells. A. NCI-H460 cells were seeded at $2x10^4$ cells/well on a 24-well plate in RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO_2 for 24 hours. Media was then removed and replaced with RPMI 1640 containing 10% FBS and no antibiotics prior to transfection with SHP-2 siRNA [30nM] for 48 hours prior to treatment with actinomycin D [1ug/mL] and timepoints were taken every 2 hours for 10 hours. **B.** Densitometric quantification of the representative western blot of PD-L1 signal normalized to GAPDH in actinomycin D treated cells.

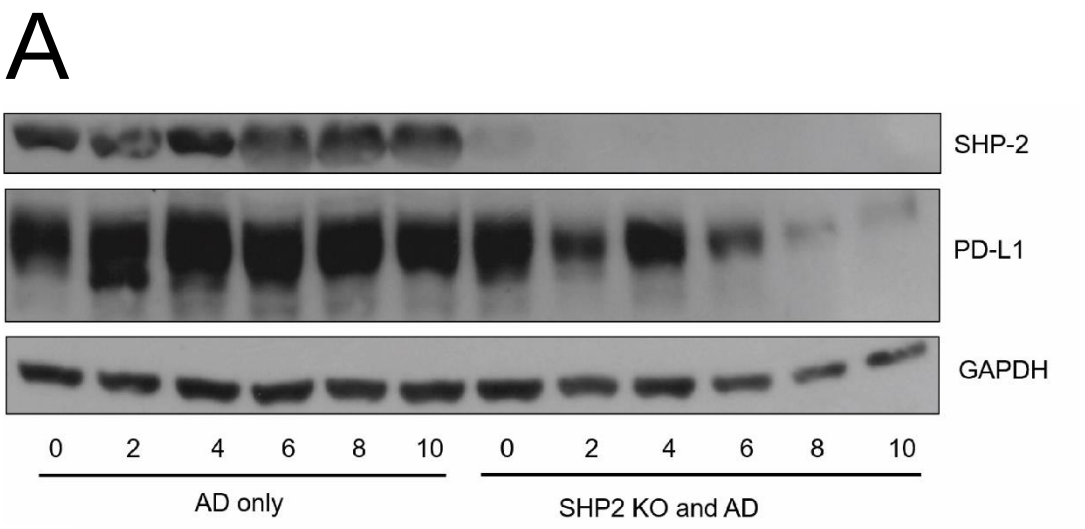
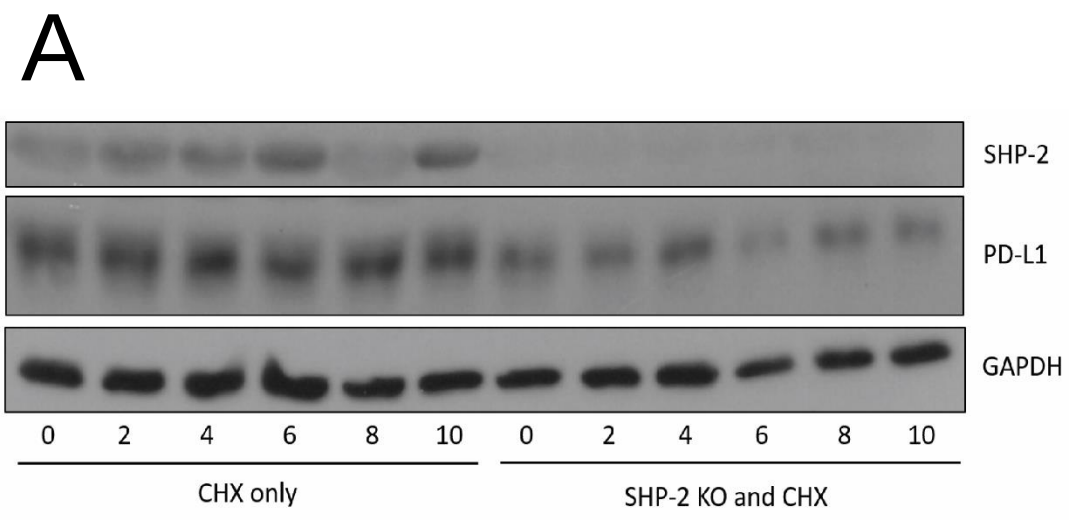


Figure 4.2. SHP-2 expression modestly influences PD-L1 protein half-life in cycloheximide treated cells. A. NCI-H460 cells were seeded at $2x10^4$ cells/well on a 24-well plate in RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO_2 for 24 hours. Media was then removed and replaced with RPMI 1640 containing 10% FBS and no antibiotics prior to transfection with SHP-2 siRNA [30nM] for 48 hours prior to treatment with cycloheximide [10ug/mL] and timepoints were taken every 2 hours for 10 hours. **B.** Densitometric quantification of the representative western blot of PD-L1 signal normalized to GAPDH in cycloheximide treated cells.



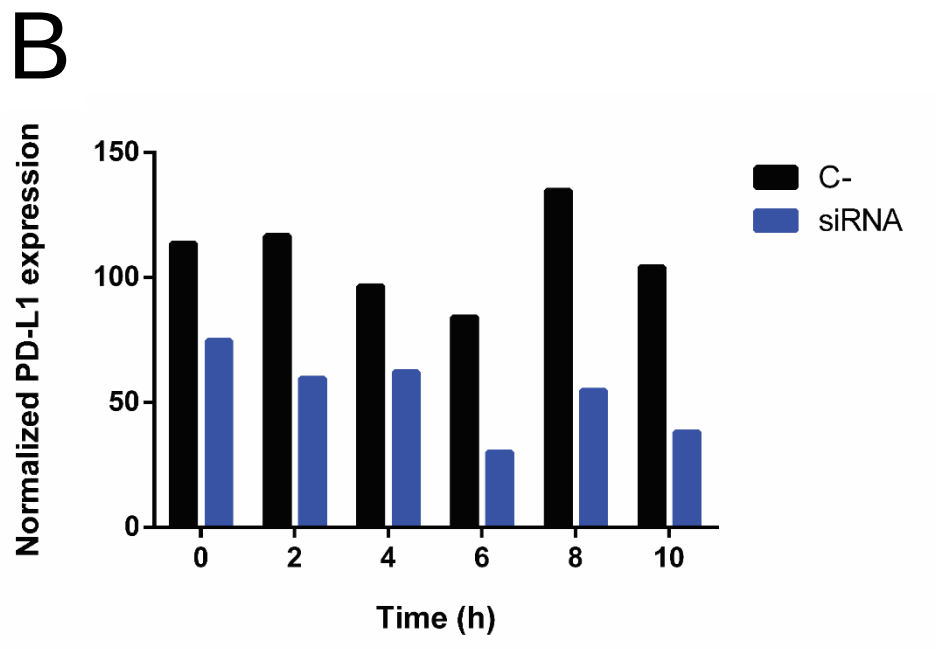


Figure 4.3. SHP-2 expression influences PD-L1 mRNA half-life in actinomycin D treated cells. A Time-course of mRNA expression levels of CD274 (PD-L1) in NCI-H460 cells treated as previously described in Figure 4.1 with actinomycin D.

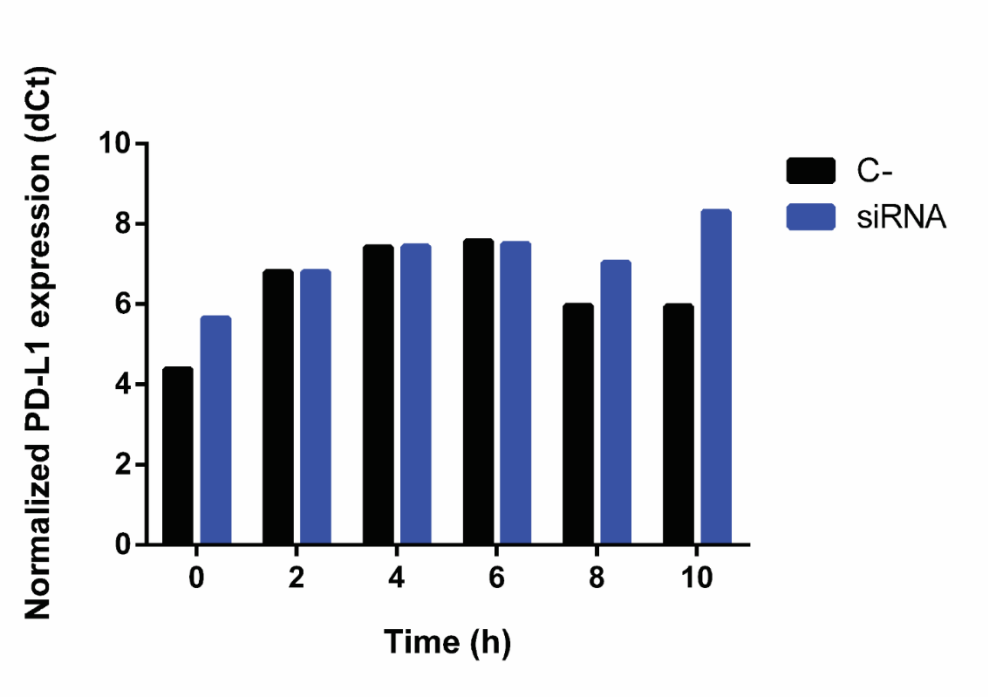


Figure 4.4. SHP-2 expression does not impact PD-L1 mRNA half-life in cycloheximide treated cells. A Time-course of mRNA expression levels of CD274 (PD-L1) in NCI-H460 cells treated as previously described in Figure 4.2 with cycloheximide.

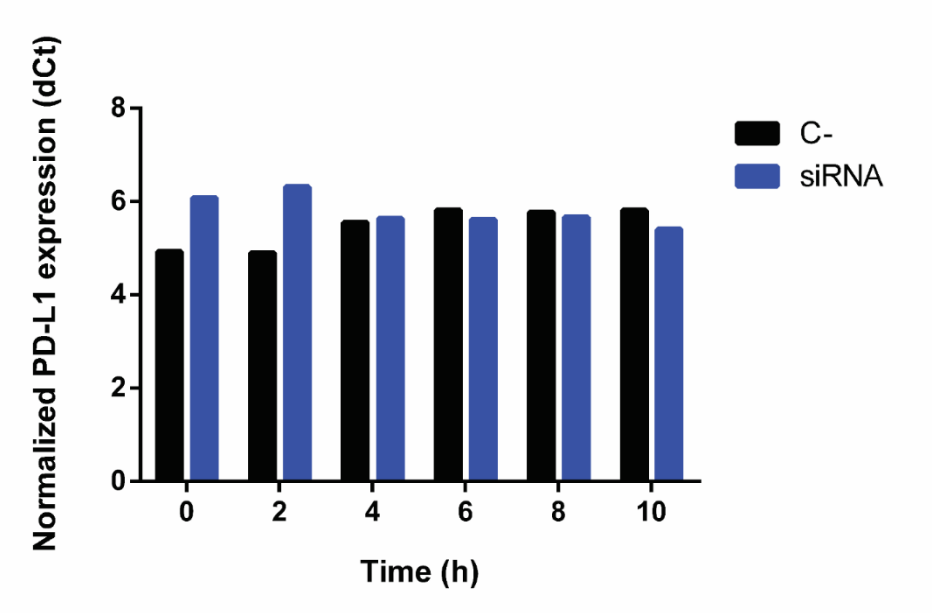
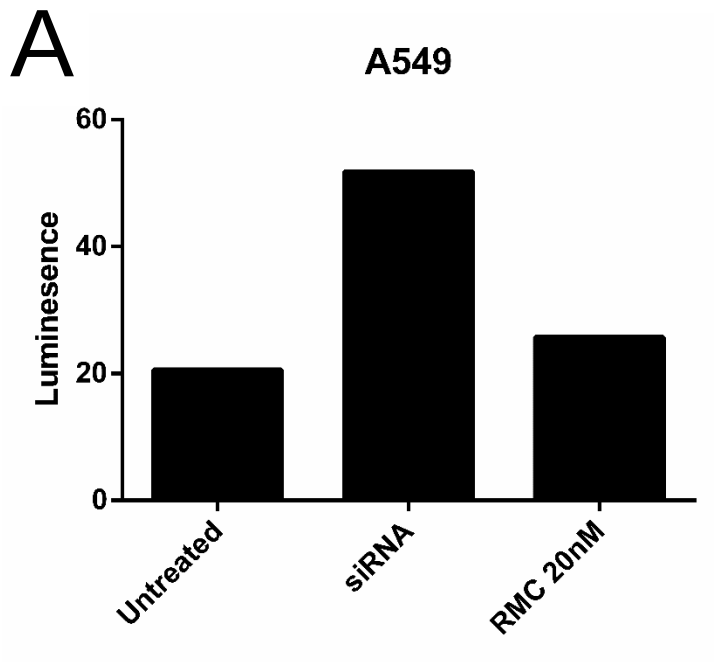
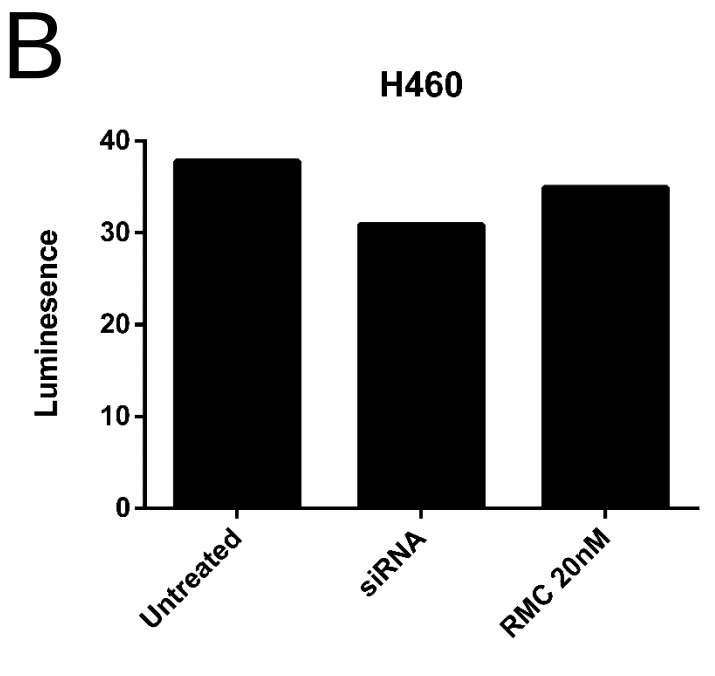


Figure 4.5. CD274 transcriptional activity is increased in cells with ablated SHP-2 activity or expression. A. Ectopic expression of a CD274 promoter driven luciferase cassette (pGL3 2 kb prom. CD274) in A549 cells, **B.** H460 cells, or **C.** UKY29 cells either untreated after transfection or treated with SHP-2 siRNA [30nM] or RMC-4550 [20nM]. Firefly luminescence expression was normalized to Renilla luminescence signal driven by the thymidine kinase promoter.




B
H460
Luminesence
40
30
20
10
0
Untreated
siRNA
RMC 20nM

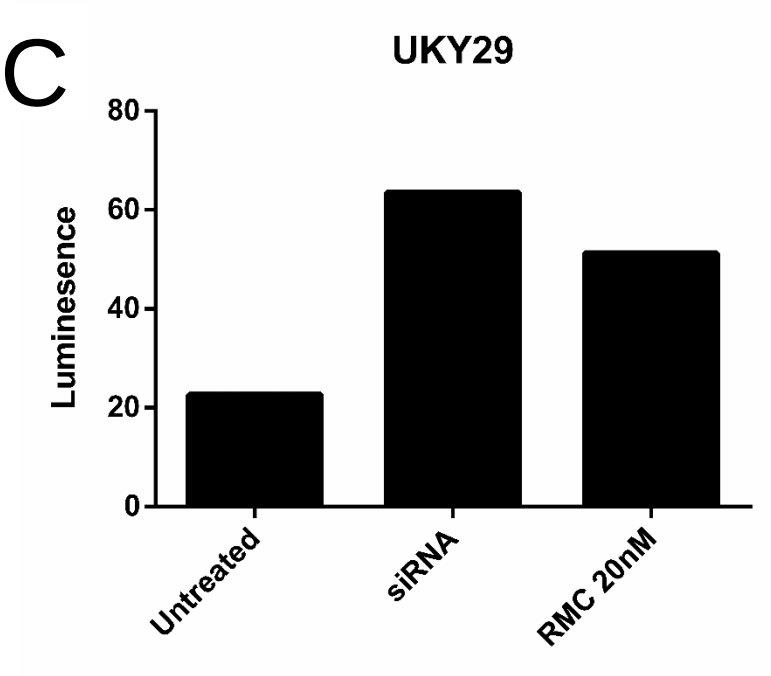

C
UKY29
Luminesence
80
60
40
20
0
Untreated
siRNA
RMC 20nM

Figure 4.6. SHP-2 is not expressed in actively cycling cells. NCI-H460, A549, and UKY29 cells were seeded at $2x10^4$ cells/well in a 24-well plate in 1mL of RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO_2 for 24 hours. Cells labeled 1% and S+ then had media removed and replaced with RPMI 1640 containing 1% FBS. After 24 hours, cells labeled S+ had FBS added back to a final concentration of 10% and were allowed to incubate for another 24 hours prior to harvest. Cells labeled 10% remained in 10% media for the full 72 hours.

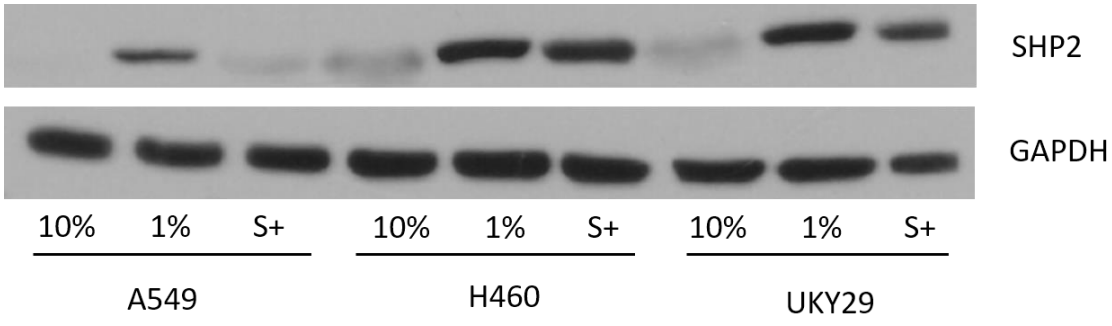


Figure 4.7. SHP-2 expression is lost as cells re-enter the cell cycle. NCI-H460 and A549 cells were seeded at $2x10^5$ cells/well in all well of a 6-well plate in 3mL of RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO_2 for 24 hours. For five of the wells, media was then replaced with 3mL RPMI 1640 containing 0.1% FBS. After 24 hours, FBS was added back to four of the starved wells and cells were harvested 1, 2, 4, and 8 hours post addition of FBS. Lanes labeled 10% remained in 10% media for 48 hours, and well labeled 1% were harvested 24 hours after being changed to 1% media.

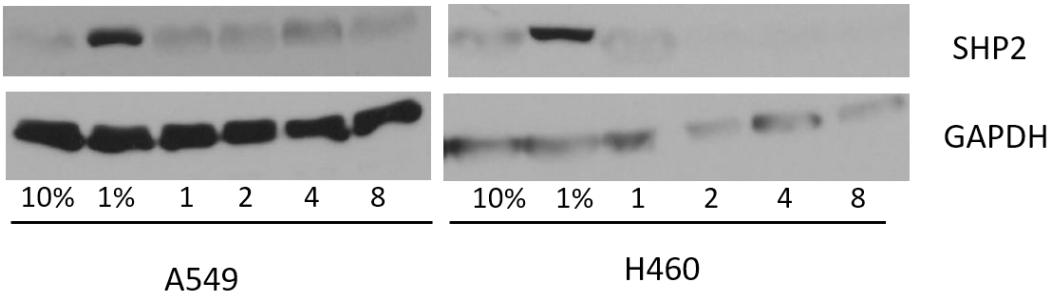


Figure 4.8. Carfilzomib treatment has no impact on SHP-2 degradation. For carfilzomib treatments, NCI-H460, A549, and UKY29 cells were seeded at $2x10^4$ cells/well on a 24-well plate in 1mL RPMI 1640 containing 10% FBS and incubated at 37°C, 5% CO_2 for 24 hours. Cells then were allowed to either remain in 10% FBS RPMI 1640 for an additional 48 hours, or media was replaced with 1mL of 1% FBS RPMI 1640 and allowed to incubate another 24 hours (lanes labeled 1%). Finally, designated cells had media removed and replaced with 10% FBS RPMI 1640 for 24 hours. Carfilzomib and/or FBS to a final concentration of 10% FBS were added 72 hours after initial seeding to designated wells to a final concentration of [50nM] for 8 hours prior to harvest. Wells labeled S+ received FBS for 8 hours in addition to carfilzomib.

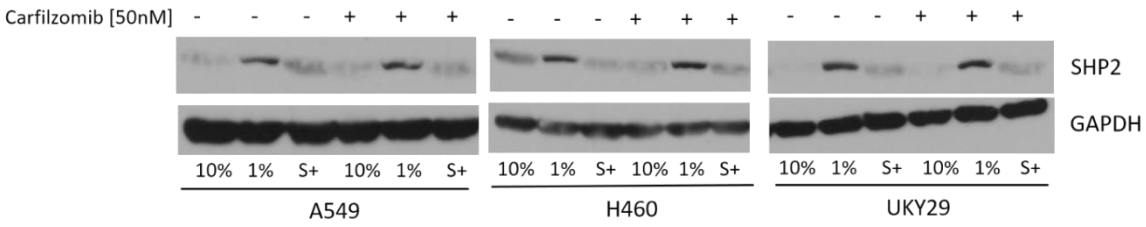


Table 4.1. Top 10 scoring proteins identified in LS-MS/MS analysis. Mass spectrometry analysis of proteins which co-immunoprecipitated with SHP-2, ranked by percent coverage and categorized by function.

Protein	Score	% Coverage	Function
SHP-2	648.58	48.57	Protein tyrosine phosphatase
HSP7C	540.88	35.29	Chaperone, protein folding, proteolysis activation
DDX3X	511.12	33.08	RNA-helicase, binds rG4s structures
PUF60	482.65	30.77	DNA/RNA binding protein, RNA splicing, transcription regulation
CPSF5	387.32	51.98	Cleavage factor complex, activator of pre-mRNA-3' cleavage
EF1A1	353.86	35.06	Promotes tRNA binding during peptide synthesis
EF1A3	353.86	35.06	Promotes tRNA binding during peptide synthesis
DDX3Y	353.09	24.55	RNA-helicase, binds rG4s structures
GRP78	321.38	22.94	ER chaperone, protein folding, degrades misfolded proteins
RSMB	244.19	18.33	pre-mRNA splicing, SMN-Sm complex, component of snRNPs
RSMN	244.19	18.33	pre-mRNA splicing, SMN-Sm complex, component of snRNPs

CHAPTER 5.

5.1 Overview

The work described herein utilizes the immense power of publicly available gene and protein expression data to serve as a quick and effective tool to form a foundation upon which the logical development of a discretely testable hypothesis may be formatted for benchtop laboratory experimentation. Given the importance of SHP-2 to numerous oncogenic pathways, the multifaceted functionality of SHP-2 as both a tyrosine phosphatase and a protein scaffold, and the strong correlation between SHP-2 activity and PD-L1 protein expression in patient tumors, **I hypothesized that SHP-2 regulates PD-L1 expression through the enucleation of a protein complex that influences PD-L1 mRNA expression and metabolism.**

Indeed, the findings described in this work bear clinical and translational significance by suggesting that inhibition or targeted degradation of SHP-2 may synergize with PD-L1 blockade, especially in KRAS-driven NSCLC. An important caveat of this suggestion is the lack of SHP-2 in growing cells, or at least those in S-phase. However, the true value of this work lies not in the expedited application of synergistic drug combinations, but rather in the expansion of knowledge regarding the basic biology behind the anomalous utilization of immune checkpoint proteins by malignant cells. Though arguably the greatest achievement in cancer therapy of this century, the optimal application of immune checkpoint inhibitors remains restricted by a lack of understanding behind the regulatory mechanisms that govern ICI target expression. These studies, through the interrogation of the molecular mechanisms underpinning PD-L1 expression, function to ameliorate the shortcomings of modern ICI therapeutic interventions. Furthermore, the investigation of the biological relationship between SHP-2 and PD-L1 has revealed the potential of previously unreported functions of SHP-2 in the trafficking of

proteins and processing of mRNA. These revelations present the potential for new therapeutic avenues that manipulate these novel mechanisms of PTPs in cancer, further accentuating the value of basic biology research in the field of cancer therapeutics.

Summary of Results

To begin my exploration into the relationship between SHP-2 and PD-L1 expression, I turned first to one of the most powerful cancer informatic databases available to date: The Cancer Proteome Atlas, and the NCI Genetic Data Commons. These databases contain protein and gene expression data with substantial sample sizes of real-world patient tumors across a plethora of cancer subtypes. Additionally, I looked to the Gene Expression Omnibus (GEO), a database that serves as depository for published results. While the data contained in the TCPA/GDC predates the advent of ICIs in the clinic, GEO provides data from smaller studies that, in some cases, collected gene expression data from patients who did or did not respond to ICI therapy. Together, the information contained in these three databases was used to address two research questions: **does there exist a correlation between SHP-2 activity/expression and PD-L1 expression, and does the expression of SHP-2 correlate to response to ICI therapy?**

Upon interrogation of reverse-phase protein array data for lung adenocarcinoma (LUAD) patients (n=362) I uncovered a significant negative correlation between active SHP-2 and PD-L1 protein levels. Interestingly, when I analyzed mRNA expression of SHP-2 and PD-L1 for these same 362 patient tumors, I uncovered no significant correlations in mRNA levels. Upon expanding our analysis to the entire LUAD dataset (n=585), I discovered a slight and significant positive correlation in SHP-2 and PD-L1 mRNA. These findings suggest that these two proteins may be co-expressed in NSCLC tumors, and the activity of SHP-2 is used to more finely tune tumoral expression of PD-L1.

Next, I turned to GEO to find smaller studies that analyzed malignant cells for SHP-2 and PD-L1 expression that also contained data for response to immune checkpoint inhibition, as the GDC/TCPA was lacking ICI response data. I found that, although PTPN11 mRNA expression maintained no significant correlation to ICI response in both melanoma or NSCLC, PTPN11 and CD274 mRNA did weakly associate in melanoma. Furthermore, I found that CD274 mRNA levels were associated with response to ICI in NSCLC tumors, an observation made by many others that supports the current clinical ICI treatment recommendation of ICI usage in patients with tumors with a PD-L1 tumor proportion score ≥50%.

Finally, I conducted a similar correlation analysis of proteins that co-precipitate with SHP-2 in KRAS-active NSCLC cells, detailed in chapter 4. The protein which I had the most interest in, DDX3X, had a strong positive correlation with SHP-2, suggesting that these proteins are indeed co-expressed not only in NSCLC cells *in vitro*, but in real-world patient tumors as well. Additionally, both DDX3X protein and mRNA are expressed in cycling and non-cycling H460, A549 and UKY29 cells

Following the insight gained from our bioinformatic analysis, I used several methods to ablate SHP-2 expression and activity and measured their effects on PD-L1 expression at the levels of mRNA, total protein, and cell surface protein. **I hypothesized that reductions in SHP-2 activity or expression would result in increases to PD-L1 expression in KRAS-active NSCLC cells.** I used two allosteric inhibitors (SHP099 and RMC-4550) to inhibit the phosphatase activity of SHP-2. To diminish SHP-2 expression, I used both siRNA to prevent PTPN11 mRNA translation, and CRISPR/Cas9 mediated knockout to edit the PTPN11 genomic template and prevent PTPN11 mRNA expression altogether.

My experimentation revealed that changes in PD-L1 expression were primarily in response to genetic ablation of SHP-2 protein, rather than pharmacological inhibition. Genetic ablation of SHP-2 removes both its ability to

enucleate protein complexes and its catalytic phosphatase activity. Allosteric inhibition of SHP-2 prevents phosphatase activity, but it is unknown to what extent this type of inhibition influences protein-protein interactions of SHP-2. However, it is important to note that the TCPA dataset did not contain expression data for total SHP-2, only for pY542-SHP-2. At the mRNA and total protein levels we observed a significant increase in PD-L1 expression following SHP-2 CRISPR knockouts, but not with siRNA knockdowns. At the level of cell surface expression, I again observed significant increases in PD-L1 levels in cells with diminished SHP-2 expression. Taken together, our findings provide a novel mechanism for the aberrant expression of PD-L1 in KRAS-active NSCLC.

Having observed increases in PD-L1 at multiple levels of expression following SHP-2 knockout, but not small molecule inhibition**, I next hypothesized that multiprotein complexes enucleated by SHP-2 are the regulatory agents of transcriptional control of PD-L1.** To test this, I began by using actinomycin D and cycloheximide to measure the impact of SHP-2 knockdown on PD-L1 mRNA and protein half-life. I discovered that cells lacking SHP-2 and treated with actinomycin D, both PD-L1 protein and mRNA were more rapidly degraded (and to a lesser extent the same was observed with cycloheximide) suggesting that SHP-2 likely functions in regulating mRNA turnover. I immunoprecipitated SHP-2 coupled with mass spectrometry to identify co-precipitating protein partners. Interestingly, the top ten scoring proteins were involved in either mRNA processing or as ER chaperones, observations concurrent with my discoveries from chapter 3. These findings are highly significant as they suggest previously unreported functions of SHP-2 complexes in the regulation of mRNA turnover and protein trafficking but will require more experimentation to definitively understand.

I followed up on these observations by utilizing a luciferase reporter assay to measure the transcriptional activity of the CD274 promoter in the presence or absence of SHP-2. I discovered that SHP-2 knockdown cells had higher levels of CD274 promoter activity than those expressing SHP-2, though importantly, this

varied by cell type. It is worth noting that H460 cells, the cell line I used to measure mRNA stability, had no changes in promoter activity following SHP-2 ablation or inhibition. This suggests that the function of the proposed SHP-2 complex may not be to directly interact with the CD274 promoter, but rather to post-transcriptionally regulate mRNA. Though all three cell lines harbor activating mutations in the KRAS gene, they also harbor additional mutations in pathways known to regulate PD-L1 transcriptional activity. For example, H460 cells harbor an activating mutation in PIK3CA, the catalytic subunit of the PI3K complex. Constitutive activation of this pathway may be responsible for the high levels of CD274 transcriptional activity in untreated H460 cells and may serve as an explanation for why diminished SHP-2 activity/expression had no impact on CD274 promoter activity in these cells (74).

Finally, I used the selective proteasome inhibitor carfilzomib and stratified media conditions to discern under what conditions cells express SHP-2 and investigate a potential mechanism of SHP-2 turnover. I found that as cells enter S-phase, SHP-2 expression is rapidly diminished, though not through proteasomal degradation. It is possible that ablation of SHP-2 activity in these cell lines impacts CD274 mRNA expression at multiple levels; first, by disrupting the transmission of signaling cascades that positively regulate CD274 mRNA expression (such as the RAS/Raf/ERK cascade) (83)(106)(107), and then also by preventing the formation of SHP-2 protein complexes which negatively regulate CD274 mRNA stability or metabolism, as suggested by my own experimentation.

Indeed, the complex interplay between these proteins and the manner by which they interact and intersect at multiple nodes of molecular operations poses a great challenge in elucidating the mechanism of SHP-2s regulation of PD-L1. Future experimentation which picks apart the involvement of SHP-2 in these signaling cascades from my newly proposed mechanisms of SHP-2 complexes in mRNA processing will be necessary to elucidate the role of SHP-2 in the expression of PD-L1.

Experimental limitations

In these studies, I combined the power of publicly available gene and protein expression data with benchtop biochemical analysis to test my hypothesis that SHP-2 influences PD-L1 expression in KRAS-active NSCLC. Through this analysis, I demonstrated that SHP-2 activity and expression impact PD-L1 levels both in patient NSCLC tumors and in vitro cell culture. Furthermore, I began investigating possible mechanisms by which SHP-2 does this and uncovered previously unreported functions of SHP-2 as well as provide a mechanism for SHP-2 degradation in replicating cells. Though I am confident in both the reliability and reproducibility of these findings, there are important considerations that must not be ignored regarding the experimental design and biological/bioinformatic models applied in these studies.

Through my analysis of protein expression data deposited on TCPA, I uncovered a significant negative correlation between SHP-2 activity and PD-L1 expression. However, the TCPA data did not include expression levels of total SHP-2 protein, only the Y542 activated form. As a result of this, I am unable to dissect the impact of inactive from active SHP-2, particularly as it relates to the catalytic phosphatase activity of the protein verses its protein enucleation capabilities. The TCPA data also did not include protein expression for any of the top 10 identified protein hits from the mass spec screen, limiting the usefulness of the combined bioinformatic/biochemical methods used. Though the sample size of TCPA NSCLC patient samples was still several hundred (n=362) this was still a subset of the full TCGA-LUAD data set (n=585) and thus further limited the power of the analysis. Thankfully, these shortcomings were attenuated by the more complete data contained in the GDC, however this dataset too had its own limitations. One aim of my bioinformatic analyses was to understand how SHP-2 expression correlated to ICI response, but the GDC contained incomplete or missing drug response data. And lastly, the datasets acquired from GEO were

limited by either small sample sizes (n<30), non-NSCLC patient samples, or not entirely complete ICI response data.

Following the results of my informatic analyses, I tested the effects of diminished SHP-2 activity or expression on PD-L1 expression in KRAS-active NSCLC cells. The major limitation of these studies is that all my work was confined to cell-based assays, and so the scope of my observations does not cross into how the expression of these two proteins relate within an actual living organism. I also observed that expression of SHP-2 was greatly reduced in cells that are actively cycling, and so to allow for the expression of the target of my small molecules and genetic knockouts, it was necessary to culture the cells in low serum containing media. The fact that cells were not cycling in these experiments results in alterations in the expression of genes that regulate cell cycle progression, or those that are expressed more strongly in conditions in which survival outweighs proliferation. Though these cells were certainly impacted on a molecular level by the lack of mitogenic factors which disallowed cell cycle progression, this model does indeed reflect the hypoxic, mitogen devoid conditions and non-cycling cell populations that exist within actual tumors. In my experiments that called for ablated SHP-2 expression, the differential efficacy of siRNA versus CRISPR/Cas9 mediated knockdown is not to be ignored. Furthermore, the transfection of these cells with lipid complexes had increased toxicity compared to that of the small molecule inhibitors, and so the less efficacious (and also less toxic) method of siRNA ablation was more appropriate for experiments involving cycloheximide or actinomycin D. Additionally, both the cycloheximide/actinomycin D and co-immunoprecipitation experiments were carried out only in the H460 cell line, not in A549 or UKY29 cells. Lastly, though the clinicogenomic analyses of my top ten mass spec hits provided additional evidence for the co-expression and potential interactions of these proteins and SHP-2, reverse pulldowns of the identified proteins followed by analysis for the presence of SHP-2 is required to reinforce my conclusions.

Conclusions and future directions

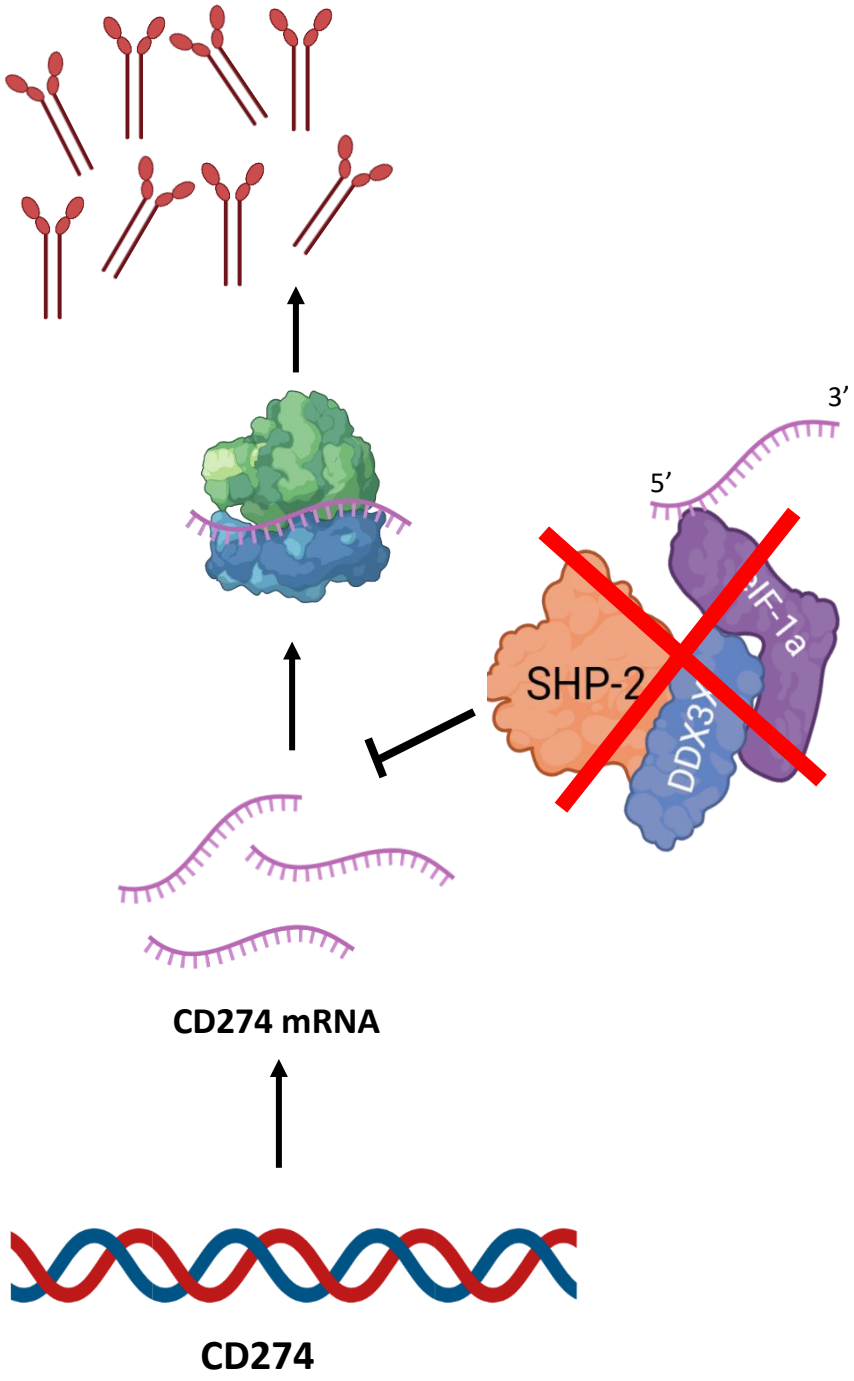
The work described herein successfully identified SHP-2 as a protein that could influence the expression of PD-L1 through the combined application of cancer informatics and biochemical analyses. In doing so, I also uncovered new information about the biological functions of SHP-2 in NSCLC cells. In these studies, I demonstrated the usefulness and power of informatic analyses of publicly available datasets such as TCPA and GDC, which helped to focus the aim of my biochemical experimentation and expedite the interrogation of my hypotheses. My most significant finding is a new mode of regulation for PD-L1 in NSCLC cells through the activity and expression of SHP-2. Furthermore, these observations suggest that the inhibition of SHP-2 would synergize with PD-L1 blockade in NSCLC patients, which bears a great deal of clinical relevance as SHP-2 inhibitors are already on their way through clinical trials. Through my biochemical analyses, I also was able to uncover several previously unreported biological functions of SHP-2. First, of the top ten hits from my mass spec analysis, eight of these proteins are directly involved in the regulation and processing of mRNA, which until now, SHP-2 was not known to interact with. The other two proteins identified were both ER chaperone proteins, again suggest another previously unknown function in the trafficking of proteins to the cell surface.

Based upon my findings, my working hypothesis is that SHP-2 forms a complex with the RNA helicase DDX3X and eIF-1a at the cap of newly transcribed mRNA that functions to negatively regulate PD-L1 expression (Figure 5.1). It may do this by altering mRNA stability or half-life, or by participating in the chaperoning of CD274 mRNA to the ribosome, impacting the translation of CD274 mRNA into protein. At the same time, there also exists the possibility that ablation of SHP-2, specifically in KRAS active cells, diminishes the activity of signaling pathways known to positively influence PD-L1 transcriptional activity, thus simultaneously increasing PD-L1 expression through the removal of the negative regulatory SHP-

2 complex and decreasing PD-L1 expression through the reduced activation of intersecting signaling pathways.

Moving forward, I plan to continue my investigation of GEO datasets which include NSCLC patient tumors treated with ICIs as more mature data for ICI treatment in this tumor subtype becomes available. I have also generated both hyperactive and catalytically inactive mutants of SHP-2 which I plan to transfect into these same cells and observe their impact on PD-L1 expression and which may help to dissect the roles of SHP-2 signal transduction from complex formation as they relate to CD274 mRNA expression. The findings of the mass spec screening provide many potential avenues for further experimentation, however I have chosen the most promising candidate, DDX3X, to be the first protein for which I conduct reverse immunoprecipitation to confirm its interaction with SHP-2. Furthermore, I will knock out DDX3X with CRISPR/Cas9 and conduct a similar analysis to that detailed in chapter 3. Lastly, my finding that SHP-2 is not degraded by the proteasome in actively cycling cells, but through a different unknown mechanism, lays the groundwork for additional experimentation that further details the precise nature of how and why replicating NSCLC cells eliminate SHP-2.

Figure 5.1. Working hypothesis for the mechanism of PD-L1 regulation by SHP2-. SHP-2 forms a complex with the RNA helicase DDX3X and eIF-1a at the cap of newly transcribed mRNA that functions to negatively regulate PD-L1 expression. It may do this by altering mRNA stability or half-life, or by participating in the chaperoning of CD274 mRNA to the ribosome, impacting the translation of CD274 mRNA into protein.



APPENDIX 1. TABLE OF ABBREVIATIONS

Abbreviation	Gene name	Protein name
VEGF	VEGF	Vascular endothelial growth factor
HIF-a	HIF-a	Hypoxia-inducible factor 1-alpha
p53	TP53	Cellular tumor antigen p53
Bax	BAX	Apoptosis regulator BAX
Bcl-2	BCL2	B-cell lymphoma 2
ABL1	ABL1	ABL proto-oncogene 1
BCR	BCR	Breakpoint cluster region protein
EGFR/Her-2	EGFR	Epidermal growth factor receptor
PI3K	PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
Akt	PKB	Protein kinase B
mTOR	MTOR	Mammalian target of rapamycin
JAK	JAK	Janus Kinase
STAT	STAT	Signal transucer and activator of transcription
SHP-2	PTPN11	SH2 containing protein tyrosine phosphatase
PTEN	PTEN	Phosphatase and tensin homolog
CD4	CD4	Cluster of differentiation 4
CD8	CD8	Cluster of differentiation 8
PD-1	PDCD1	Programmed cell death protein receptor 1
PD-L1	CD274	Programmed cell death protein ligand 1
KRAS	KRAS	Kirsten ras oncogene homolog
BRAF	BRAF1	B-Raf Proto-Oncogene, Serine/Threonine Kinase
ALK	ALK	ALK Receptor Tyrosine Kinase
MET	MET	MET proto-oncogene receptor tyrosine kinase
ROS	ROS1	ROS Proto-oncogene 1
STK11	STK11	Serine/threonine kinase 11
SOS	SOS1	Son of Sevenless nucleotide exhange factor
Grb2	GRB2	growth factor receptor bound protein 2
Raf	RAF1	Raf-1 proto-oncogene serine/threonine kinase
MEK	MAP2K	Mitogen-activated protein kinase kinase 1
Erk	MAPK1	Mitogen-activated protein kinase 1
TIAM1	TIAM1	TIAM Rac1 Associated GEF1
Rac	RAC1	Rac family small GTPase 1
Rho	RHO	Rhodopsin
PLC	PLCG1	Phospholipase C Gamma 1
PKC	PRKCA	Protein kinase C
CTLA-4	CD152	Cytotoxic T-lymphocyte-associated protein 4

CD28	CD28	Cluster of differentiation 29
TCR	TRA	T-cell receptor alpha
B7	CD80	B-lymphocyte activation antigen B7
PTPRG	PTPRG	Receptor-type tyrosine protein phosphatase gamma
PRL	PRL	Prolactin

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PUBLICATIONS:

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