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Novel Roles of the Protein Tyrosine Phosphatase SHP2 in Non-small Cell Lung Cancer

Valentina Schneeberger
University of South Florida, vschneeb@mail.usf.edu

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Novel Roles of the Protein Tyrosine Phosphatase

SHP2 in Non-small Cell Lung Cancer

by

Valentina E. Schneeberger

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Cell Biology, Microbiology, and Molecular Biology
College of Arts and Science
University of South Florida

Major Professor: Jie Wu, Ph.D.
Srikumar Chellappan, Ph.D.
Keiran Smalley, Ph.D.
Amer Beg, Ph.D.

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Dedication

I dedicate this dissertation to my parents and my two sisters, Cris and Caro. Words cannot describe what you mean to me. Everything I have is thanks to you and without your love and support, I could never have gotten to where I am today. I love you so incredibly much.
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List of Abbreviations

ATP  Adenosine triphosphate
BASCs Bronchioloalveolar stem cells
Bp   Base pair
Cbp  CSK binding protein
CCSP Clara cell secretory protein
cHS4 Chicken beta globin insulator
COSMIC Catalog of somatic mutations in cancer
CSK c-terminal Src kinase
Dox  Doxycycline
DPC  Day post-coitus
EGFR EGF receptor
Erk1/2 Extracellular signal regulated protein kinase 1 or 2
Gab1 Grb2 associated binder protein 1
Gab2 Grb2 associated binder protein 2
GOF  Gain of function
HSCs Hematopoietic stem cells
IFN  Interferon
Jak  Janus kinase
MAPK Mitogen activated kinase
NSCLC Non-small cell lung cancer
P   Phosphorylated
PI3K Phosphatidylinositol-4, 5-bisphosphate 3-kinase
PTK Protein tyrosine kinase
PTP  Phosphatase
qRT-PCR Quantitative reverse transcription polymerase chain reaction
RMCE Recombinase mediated cassette exchange
RT-PCT Reverse transcription polymerase chain reaction
RTK Receptor tyrosine kinase
rTA Reverse tetracycline transactivator
SFKs Src family of kinases
SHC Src homology and collagen
SHP2 Src homology phosphotyrosine phosphatase 2
SPRED Sprouty-related, EVH1 domain-containing protein
STAT Signal Transducers and Activators of Transcription
SP Side population
tetO tet operon
TKIs Tyrosine kinase inhibitors
Y   Tyrosine
Abstract

The gene *PTPN11* was identified in the early 1990s, and encodes the non-transmembrane protein tyrosine phosphatase SHP2. SHP2 is expressed ubiquitously in cells, and plays an important role in cancer. Unlike most phosphatases, SHP2 positively regulates several signaling pathways including the Ras/MAPK and Src signaling pathways and acts as a proto-oncogene. SHP2 is also a cancer essential gene in certain types of carcinomas, and promotes growth, survival, and epithelial to mesenchymal transformation. Gain of function (GOF) SHP2 mutations are known leukemic oncogenes, and have been identified to a smaller extent in solid tumors as well. Currently, the roles of SHP2 in lung carcinoma are not fully understood. While GOF SHP2 mutations have been detected in lung cancer, their contributions to cellular transformation had not been established. In addition, SHP2 is known to promote EGF growth factor receptor (EGFR) signaling. Since GOF EGFR mutations induce transformation of lung epithelial cells, it is possible that SHP2 plays a role in promoting GOF EGFR mutant driven tumorigenesis. The objective of this dissertation is to determine whether SHP2 can act as an oncogene in lung epithelial cells and whether SHP2 inhibition can affect GOF EGFR mutant induced lung cancer. To achieve these aims, we generated two novel doxycycline (Dox) inducible transgenic mouse models which express either the GOF SHP2$^{E76K}$ leukemic oncogene or the dominant negative SHP2$^{CSDA}$ mutant under the control of the Clara cell secretory protein (CCSP) promoter to regulate transgene expression to type II pneumocytes.
To determine whether SHP2 plays a role in promoting GOF EGFR mutant signaling, we started by disrupting SHP2 function in vitro. Two non-small lung cancer cell lines were used for this project: HCC827 carries the LREA deletion in exon 19, and H1975 co-expresses the EGFR<sup>L858R</sup> point mutation and the EGFR<sup>T790M</sup> gatekeeper mutation. After SHP2 PTP inhibition or knock-down by shRNA and siRNA, both cell lines exhibited decreased cell proliferation and reduced levels of pErk1/2 and c-Myc. Based on these results, we acquired a transgenic mouse line which expresses the EGFR<sup>L858R</sup> mutant under the control of the tet-O promoter and generated bitransgenic CCSP-rtTA/tetO-EGFR<sup>L858R</sup> and tritransgenic CCSP-rtTA/tetO-EGFR<sup>L858R</sup>/tetO-SHP2<sup>CSDA</sup> mice to study the effects of the dominant negative SHP2<sup>CSDA</sup> mutant on EGFR<sup>L858R</sup> mediated carcinogenesis in vivo. After 4, 6, and 8 weeks of Dox induction, pErk1/2 and pSrc levels were increased in the lungs of bitransgenic mice compared to wild type controls. Both kinases were suppressed by SHP2<sup>CSDA</sup> expression in tritransgenic mice. In addition, SHP2<sup>CSDA</sup> expression delayed tumor onset and prevented progression to a more aggressive phenotype. Tritransgenic mice also developed a smaller tumor burden compared to bitransgenic animals. These results suggest that SHP2 is critical for GOF EGFR mutant mediated lung tumorigenesis and describe a new role of SHP2 as a potential therapeutic target for the development of novel NSCLC drugs.

Once we generated our CCSP-rtTA/tetO-SHP2<sup>E76K</sup> transgenic mouse model, we administered Dox for one month and found that SHP2<sup>E76K</sup> expression upregulates pErk1/2, pSrc, pGab1, c-Myc and Mdm2 levels in the lungs of bitransgenic mice compared to controls. After six to nine months of Dox induction, SHP2<sup>E76K</sup> expression caused formation of lung adenomas and adenocarcinoma. We then took advantage of
the reversible feature of our mouse model to test whether lung tumors are dependent on sustained SHP2$^{E76K}$ expression for survival. MRI analysis of lung adenocarcinomas showed full regression of the lung tumors after Dox withdrawal. Histological evaluation of lung tissues revealed residual hyperplastic lesions as well as evidence of necrosis, while biochemical analysis showed that pGab1, pErk1/2, pSrc and c-Myc returned to basal levels. These results demonstrate that sustained SHP2$^{E76K}$ expression is required for lung tumor maintenance. Moreover, this data describe a novel function of SHP2$^{E76K}$ as an oncogene in lung carcinoma.
1.1 Protein tyrosine phosphatases and protein tyrosine kinases

Tyrosine phosphorylation is an essential step to induce cell signaling and was first reported in 1978 with the discovery of the Src tyrosine kinase [1, 2]. Extensive research has identified a multitude of protein tyrosine kinases (PTKs) and elucidated their roles in normal cellular function and cancer. Since for every action, there must be an equal and opposite reaction, the concept of a protein tyrosine phosphatase (PTP) which dephosphorylates tyrosine sites (Y) phosphorylated by PTKs was proposed. This hypothesis was confirmed a decade after the discovery of PTKs with the cloning of the protein tyrosine phosphatase PTP1B [3, 4]. Since then, the human “PTPome” has been extensively characterized with the identification of 107 PTPs grouped into four families. The majority of PTPs are included in the class I cysteine based group, and include the classical receptor PTPs, the non receptor PTPs and the dual specificity PTPs [5]. Class II includes the low molecular weight PTP and class III contains 3 Cdc25 proteins. Both class II and III are cysteine based. The fourth class includes Asp based PTPs [5].

The common assumption at the time was that since PTPs perform the reverse function of PTKs, they should play a negative regulatory role in cell signaling and cancer. This idea was reinforced by the discovery and characterization of PTPs such as PTEN, DEP1, and TCPTP which decrease or terminate cell signaling and block activation of downstream targets of receptor tyrosine kinases (RTKs) [6-9]. Moreover,
these PTPs are often inactivated or lost through mutation, which potentiates cellular transformation leading to cancer development [10, 11]. These studies cemented the role of PTPs as negative regulators of cell signaling.

Further research revealed that tyrosine phosphorylation by RTK does not always activate the target protein [12]. Indeed, multiple proteins are negatively regulated by tyrosine phosphorylation, which would indicate that dephosphorylation by a PTP would have a positive effect on cellular signaling [13-16]. This hypothesis was confirmed in 2003 with the identification of GOF SHP2 mutations in patients afflicted with juvenile myelomonocytic leukemia [17]. In this disease, SHP2 acts as a proto-oncogene and drives leukemogenesis [18, 19]. Following this discovery, additional PTPs were found to act as proto-oncogenes, including RPTPα, PTPH1, and more [5, 20, 21]. These PTPs promote cell signaling, cellular transformation, survival, proliferation, and invasion [5]. Taken together these studies describe complex and opposing roles for the members of the PTPome in normal cellular function, development and cancer. PTPs are an important component of signal transduction, and should not be solely considered erasers of PTKs function.

1.2 Discovery of the protein tyrosine phosphatase SHP2

The gene *PTPN11* was discovered in the early 1990s, and encodes the non-transmembrane protein tyrosine phosphatase Src homology 2 domain tyrosine phosphatase (SHP2), previously called Syp, PTP1D, SH-PTP2, or PTP2C [22-25]. SHP2 contains a C-terminal tail with two tyrosyl phosphorylation sites (Y542 and Y580), a central PTP catalytic domain, and two N-terminal Src homology 2 (SH2) binding
domains (N-SH2 and C-SH2) (Fig. 1). SHP2 is regulated by an auto-inhibitory mechanism. In its inactive conformation, the N-SH2 binding domain folds over the PTP domain and restricts substrate access to the catalytic site (Fig. 1). Upon growth factor or cytokine stimulation, the SH2 binding domains bind to phosphorylated tyrosine sites on growth factor receptors or docking proteins causing SHP2 to unfold, exposing the PTP catalytic domain [26]. SHP2 is expressed ubiquitously, and plays an important role in development, normal cellular function, and cancer [27, 28]. Unlike most phosphatases, SHP2 positively regulates cell signaling and acts as a proto-oncogene [29].

Figure 1. Crystal structure of SHP2. The crystal structure of un-liganded SHP2 was resolved in 1998 by the Shoelson group [30]. This structure is missing the c-terminal tail but otherwise contains the C-SH2 binding domain (yellow), the N-SH2 binding domain (red) and the PTP catalytic domain (green). The structure is in its inactive conformation, whereas the N-SH2 binding domain folds over the PTP domain and blocks substrate access to the catalytic cleft (magenta).
1.3 SHP2 is a positive regulator of cell signaling

SHP2 is activated by several growth factor receptors including the platelet derived growth factor receptor (PDGFR), the epidermal growth factor receptor (EGFR), the fibroblast growth factor receptor (FGFR), and the insulin-like growth factor 1 receptor (IGF-1R) [31, 32]. As a result, SHP2 is involved in multiple cell signaling pathways and performs different roles depending on the activating growth factor receptor, though the main function of SHP2 is to promote the Ras/MAPK and Src signaling pathways through multiple incompletely defined mechanisms.

1.3.1 The role of SHP2 in the Ras/MAPK signaling pathway

An important mechanism for the promotion of Ras/MAPK signaling by SHP2 is its association with the docking protein Grb2 associated binder protein 1 (Gab1) [33, 34]. Phosphorylation of Gab1 on its SHP2 binding sites (Y627, Y659) by growth factor receptors recruits SHP2 to the cell membrane and relieves the auto-inhibitory mechanism, exposing the PTP catalytic domain (Fig. 2) [35]. Once activated, SHP2 positively regulates Ras/MAPK signaling through both direct and indirect approaches. For example, PDGFR binds SHP2 which in turn directly recruits the growth factor receptor bound protein 2 (Grb2)/Son of Sevenless (SOS) complex to the cell membrane (Fig. 2) [31, 36]. SOS is a guanine nucleotide exchange factor that catalyzes the conversion of inactive GDP-bound Ras to active GTP-bound Ras, leading to activation of the Ras/MAPK signaling chain [37].
SHP2 can also indirectly prolong Ras/MAPK activity by interfering with the negative regulators of this signaling pathway such as p120RasGAP and the Sprouty-related, EVH1 domain-containing protein (SPRED) and Sprouty protein families. The p120RasGAP protein catalyzes hydrolyzation of the GTP-bound Ras to the inactive GDP-bound state [38]. To perform this function, p120RasGAP must bind to specific tyrosine sites on growth factor receptors or scaffolding protein such as Gab1. SHP2 dephosphorylates these sites, which disrupts p120RasGAP function by blocking its recruitment to the cell membrane (Fig. 2) [36]. Sprouty is another negative regulator of Ras. This protein functions by binding Grb2 and sequestering the Grb2/SOS complex in the cytoplasm to prevent SOS mediated Ras activation [39]. SHP2 dephosphorylates the Grb2 binding site on Sprouty, enabling relocation of the Grb2/SOS complex to the cell membrane (Fig. 2). Finally, the SPRED family of proteins contains a Sprouty related domain that can bind Ras. The SPRED/Ras complex recruits Raf to the cell membrane, whereas Raf activation through phosphorylation of its Serine338 site is blocked by SPRED. This results in termination of Ras/MAPK signaling [40]. SPRED is a substrate of SHP2 which dephosphorylates the SPRED activating tyrosine sites to restore Ras/MAPK signaling pathway activity (Fig. 2) [41].

1.3.2 The role of SHP2 in the Src family kinase signaling pathway

SHP2 interaction with the members of the Src family of kinases (SFKs) is complex and sometimes tissue specific. For example, after recruitment by the α6β4 integrin, SHP2 binds Fyn and dephosphorylates its negative regulatory tyrosine site. Active Fyn promotes integrin signaling leading to cell growth, migration, and survival [42]. In
hematopoietic cells, the Grb2 associated binder protein 2 (Gab2) recruits both SHP2 and Lyn after G-CSF stimulation. SHP2 then activates Lyn, which in turn induces cell proliferation [43]. Once the SFKs are activated, they can initiate a negative feedback loop by phosphorylating the CSK binding protein (Cbp) SH2 binding sites. Cbp then binds the Src kinase and recruits the C-Src tyrosine kinase (CSK). CSK is a negative regulator of the SFKs, and normally resides in the cytoplasm [44]. Cbp complex formation with SFKs and CSK results in phosphorylation of the SFKs c-terminal inhibitory tyrosine site by CSK, thus terminating Src signaling [44]. SHP2 can interfere with this negative regulatory mechanism by dephosphorylating Cbp to block relocation of CSK to the cell membrane (Fig. 3) [45].

Figure 2. Function of SHP2 in Ras/MAPK kinase signaling pathway. SHP2 is recruited to the cell membrane by an active RTK and binds the Grb2/SOS complex to initiate Ras/MAPK signaling. SHP2 also dephosphorylates the Sprouty, SPRED, and p120RasGAP binding sites to prevent their recruitment to the cell membrane and termination of Ras/MAPK signaling.
1.3.3 The role of SHP2 in the PI3K and Jak/STAT1 signaling pathway

SHP2 functions differently in the Phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K) signaling pathway depending on the activating growth factor and tissue type. After stimulation with IGF, PDGF, or Thrombopoietin, SHP2 associates with the PI3K subunit p85 and induces Akt activation in mouse fibroblasts, glioblastoma cells and megakaryocytes, promoting cell proliferation and survival [46-48]. On the other hand, EGF stimulation causes the opposite effect. In this case, SHP2 dephosphorylates the p85 binding sites on Gab1, leading to decreased p85/SHP2/Gab1 association and Akt phosphorylation [49, 50]. These opposing effects on PI3K signaling further add to the complexity of SHP2 function in signal transduction.

The Janus kinase (Jak)/Signal Transducers and Activators of Transcription 1 (STAT1) signaling pathway activation by type I and II interferons (IFN) elicits a strong anti-proliferative response which is negatively regulated by SHP2 [51]. Indeed, SHP2
dephosphorylates STAT1 after Jak1/2 phosphorylation, disrupting the formation of STAT1 dimers and translocation to the nucleus [52]. This inhibitory mechanism is particularly deleterious in post surgery melanoma patients treated with IFNα adjuvant therapy. Previous work in our lab has shown that SHP2 can restore Ras/MAPK signaling pathway activity post IFN-α2b treatment which significantly curtails the anti-proliferative effects of IFNα therapy [53]. In addition, SHP2 can also regulate STAT1 in a phosphatase independent manner by binding and sequestering non-phosphorylated STAT1 in the cytoplasm [54].

1.4 SHP2 and cancer

SHP2 is a cancer essential gene in certain types of carcinomas. For example, SHP2 is required for survival of tumor initiating cells, tumor growth, and metastasis in Her2 positive and triple negative breast cancers [55, 56]. SHP2 is also required to potentiate EGFRvIII mediated cellular transformation in glioblastoma and SHP2 knock-down in these cells hinders cell-cycle progression and EGFRvIII mediated cell signaling [57]. Moreover, SHP2 is a proto-oncogene. Activating SHP2 mutations in the N-SH2 binding domain or the PTP domain disrupt the auto-inhibitory mechanism, conferring constitutive activity to the PTP catalytic domain (Fig. 4). These GOF SHP2 mutants can induce myeloproliferative disorders and act as leukemic oncogenes. They have been identified in 35% of juvenile myelomonocytic leukemia, 10% of childhood myelodysplastic syndromes, 7% of B-cell acute lymphoblastic leukemia, 4% of acute myelogenous leukemia, and a small percentage of carcinomas [58, 59].
Figure 4. *GOF SHP2 mutations*. The majority of the GOF function SHP2 mutations are located in the N-SH2 or PTP binding domain and disrupt the auto-inhibitory mechanism, conferring constitutive activity to the PTP catalytic domain.

Since SHP2 is involved in multiple signaling pathways, the acquisition of an activating mutation results in a myriad of phenotypic changes. Indeed, GOF SHP2 mutations partly induce cellular transformation by upregulating the Ras/MAPK and Src signaling pathway, leading to increased cell proliferation and survival [60, 61]. In myeloid progenitor cells, GOF SHP2 mutants promote cytokine independent survival while causing hypersensitivity to GM-CSF and IL-3 [62]. Activating SHP2 mutants also induce centrosome amplification which disrupts cell division and causes genomic instability [63]. In addition, mutated SHP2 causes an expansion of the myeloid progenitor cell compartment by blocking the differentiation of early stage hematopoietic progenitors. This process also causes anemia by disrupting normal erythropoiesis [64]. Activating SHP2 mutations have been identified at low frequencies in several cancer types such as lung (1.81%), colon (5.98%), and endometrium (4.27%) according to the
COSMIC database as of April 2014, but might play an important role nevertheless. For example, in a study performed by Sturla et al, GOF SHP2 mutations were identified in 2% of glioblastoma patient samples, and protected tumor cells against senescence [65]. The potential role of SHP2 as an oncogene in carcinoma is still unknown, and further study is required to understand the contributions of GOF SHP2 mutations to epithelial tumorigenesis.

1.5 Lung cancer

Lung cancer is the most prevalent type of cancer in the United States. According to the American cancer society, there will be about 224,210 new cases in 2014, leading to approximately 159,260 deaths (about 27% of all cancer deaths). Lung cancer is divided in two classes, small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC). About 80% of cases fall under the NSCLC category, which is further subdivided in three groups: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma [66]. Each group presents unique histological features and arises in different areas of the lung. Adenocarcinoma is the most commonly indentified subtype in patients. Multiple driver oncogenes have been identified in adenocarcinoma (Fig. 5), and have been targeted for drug development [67]. Unfortunately, the majority of patients develops drug resistance and relapses [68-71]. Moreover, in about 40% of cases the transforming factor is unknown, highlighting an urgent need for alternative therapies. The identification and targeting of the cell of origin of lung cancer could provide a new avenue for therapeutic treatment.
Figure 5. *Common driver oncogenes in adenocarcinoma.* Adenocarcinoma is a subtype of non small cell lung cancer. Several driver oncogenes have been identified and provide valuable targets for drug development.

The concept of a lung cancer cell of origin has generated a lot of interest, and multiple research teams have tried to identify this cell population. Previous studies using lung tissue have established a novel cell type located at the bronchioloalveolar ductal junction with positive expression of both the Clara cell antigen 10 (CC10) and the alveolar type II marker Surfactant protein C (SPC) [72, 73]. These cells are called bronchioloalveolar stem cells (BASCs), and while normally quiescent, become activated following injury and give rise to type I and II pneumocytes. Further work using mouse models supported that BASCs are the cell type from which adenocarcinomas could arise [72, 73]. In squamous cell carcinoma, progenitor tracheal basal cells are the tentative candidate for cell of origin due to similar patterns of gene expression and histological features, but further work is required to confirm this hypothesis [72].
The SCLC cell of origin has been difficult to identify due to tumors often exhibiting mixed cell types and cell surface markers. In general, SCLC is thought to arise from neuroendocrine progenitor cells based on shared patterns of neuroendocrine markers and gene expression [72, 74]. This hypothesis was supported by data from the Kim lab using a mouse model where deletion of the p53 and Rb genes in neuroendocrine cells located in the lung epithelium resulted in hyperproliferative lesions [74].

Aside from BASCs and neuroendocrine progenitor cells, cancer stem cells have also been proposed as the cell of origin for lung cancer. Previous studies have identified a novel lung cell type termed side population (SP) which displays characteristics similar to stem cells described in other tumor types [75]. Lung SP cells efflux Hoechst 33342 dye and express stem cell markers such as CD133, ALDH, and CD 44 [75, 76]. Lung SP cells are usually in a quiescent state, are resistant to chemotherapy, and express genes involved in self-renewal such as Sox2, Nanog, and Oct 4 [77]. Interestingly, this side population is enriched with tumor initiating cells and demonstrates self renewal, tumor repopulating capacity, and have been identified in lung patient tumor samples [78, 79]. Based on this evidence, a strong case can be made that lung cancer is initiated by the deregulation and uncontrolled division of lung cancer stem cells and that lung SP cells could provide a valid therapeutic target for lung cancer treatment [75].

The identification of the lung cancer cell of origin can provide valuable insight into lung tumorigenesis and could open up new avenues for drug development. Extensive research has been done to characterize these cells, but at this stage further study is required to better understand and fully determine their roles in lung carcinogenesis in order to target them for therapeutic treatment.
1.6 The epidermal growth factor receptor

The EGF receptor is a member of the ErbB tyrosine kinase family, and is composed of a cytoplasmic protein tyrosine kinase domain, a single membrane spanning region, and an extracellular ligand binding domain (Fig. 6). Upon ligand binding by a member of the EGF family of ligands, the EGF receptor homo or hetero-dimerizes with a member of the ErbB family resulting in intrinsic kinase trans-activation [80]. The auto-phosphorylation of the EGFR c-terminal tyrosine docking sites recruits a variety of adapter proteins, kinases, and phosphatases, which induce the activation of several signaling pathways involved in cell growth, differentiation, survival and more [81].

1.6.1 EGFR activation induces cell signaling

1.6.1.1 EGFR and the Ras/MAPK signaling pathway

EGFR activation upon ligand binding and dimerization induces the Ras/MAPK cell signaling pathway by recruiting the Grb2/SOS complex to the cell membrane [82]. SOS is a guanine nucleotide exchange factor that catalyzes the conversion of inactive GDP-bound Ras to active GTP-bound Ras. Ras binds multiple targets, but its main function is to activate Raf by inducing a conformational change that disrupts Raf association with the inhibitory protein 14-3-3. This triggers activation of the Raf/Mek/MAPK signaling chain, leading to MAPK translocation to the nucleus and activation of transcription factors that promote cellular growth, proliferation, differentiation and survival [83-85].
1.6.1.2 EGFR and the Src family kinase pathway

Previous studies have described several mechanisms of EGFR mediated SFK signaling pathway activation [86]. EGF ligand binding induces translocation of the Src/SHC complex to the cell membrane whereas SHC binds active EGFR, leading to Src activation [87]. SHC is an adapter protein which associates with multiple partners upon phosphorylation by RTKs [88]. EGF stimulation can also recruit SHP2 to the cell membrane, where it binds to protein complexes composed of Gab1, Paxillin, CSK, or Src. SHP2 dephosphorylates the CSK binding sites on Paxillin, leading to Src activation (Fig. 3) [34]. Src activation by EGFR also triggers a positive feedback loop whereas active Src can in turn phosphorylate and promote EGFR activity, resulting in increased cell proliferation, migration, and anchorage-independent growth [86, 89].

1.6.2 EGFR and cancer

EGFR over expression or amplification in several types of cancer including breast, glioblastoma, and gastrointestinal carcinoma is a negative prognosis marker of survival in patients [90-92]. Moreover, GOF EGFR mutations are oncogenic and induce cellular transformation. They have been identified predominantly in lung cancer, but are present in additional cancer types including malignancies of the central nervous system (4.77%), prostate (3.94%), intestine (4.46%), and more according to the COSMIC database as of April 2014.
Lung cancer is divided in two histological subtypes, non-small cell lung cancer and small cell lung cancer. Non small cell lung cancers make up about 80% of cases, and are further sub-divided into three groups, squamous cell carcinoma, large cell carcinoma, and adenocarcinoma [93]. GOF EGFR mutations are present in about 27.54% of lung adenocarcinoma cases according to the COSMIC database. Interestingly, patients with GOF EGFR mutations are usually never smokers or females of Asian descent. The mechanisms underlying this apparent sensitivity have not been fully elucidated [94-96].

1.6.2.1 Gain of function EGFR mutations

GOF EGFR mutations are classified in two main groups: extracellular domain mutations or tyrosine kinase mutations. The prevalence of these two classes of mutations differs based on cancer type.

*Extracellular domain mutations.*

Previous studies have identified three types of EGFR mutations with extracellular domain deletion. They are classified based on the extent of the extracellular domain deletion. The first type, called EGFRvI, exhibits a complete deletion of the extracellular domain while the second type, EGFRvII, contains an 83 amino acid deletion in domain IV [97]. This mutation does not appear to contribute to tumorigenesis [97]. The third and most commonly identified type, EGFRvIII, lacks domain I and II of the extracellular ligand binding domain (Fig. 6), and is often amplified. This mutation confers constitutive kinase activity and is common in glioblastoma (30-50% of cases) and has been reported in about 5% of squamous cell carcinoma patients [98-100].
Tyrosine kinase domain mutations

About 90% of tyrosine kinase domain mutations are sub-divided in two groups. The first group contains a small deletion, usually amino acids 746-750, in exon 19. The second group contains a missense mutation on exon 21 substituting leucine for arginine at codon 858 [97]. Multiple variants of these two mutations have been described, and include larger deletions, point mutations and insertions, notably in exon 20 (Fig. 6) [101]. Interestingly, tyrosine kinase domain mutations are usually identified in lung cancer, and tend to be mutually exclusive with other oncogenes such as KRAS [97].

Figure 6. Common GOF EGFR mutations in glioblastoma and lung cancer. Extracellular domain mutations are often identified in glioblastoma and exhibit partial to entire deletion of the extracellular domain. Kinase domain mutations are usually identified in lung cancer, and can be found in multiple exons of the tyrosine kinase domain.

1.6.2.2 EGFR as a therapeutic target in NSCLC

The establishment of GOF EGFR mutations as a valid therapeutic target in NSCLC led to the development of multiple specific tyrosine kinase inhibitors (TKIs). Gefitinib (Iressa) was the first of its kind and showed promising anti-tumor potential in vitro and in
vivo. This small molecule inhibitor blocks receptor autophosphorylation by competing with ATP for binding to the intracellular ATP binding pocket of mutant EGFR [102]. Gefitinib treatment abrogates GOF EGFR mutant signal transduction leading to decreased cell growth, proliferation and survival [103]. These effects are mediated through multiple mechanisms. For example, Gefitinib promotes G1 phase arrest through upregulation of p27 and suppresses angiogenesis by decreasing VEGF levels [104, 105]. Gefitinib also decreases cell survival by upregulating BAD, a pro-apoptotic member of the Bcl-2 family of apoptosis regulators [106].

Phase I clinical trials reported Gefitinib is well tolerated in patients, with most side effects falling within Common Toxicity Criteria grade 1 or 2 [107]. Several phase II trials proved the efficacy of Gefitinib in patients with NSCLC [107]. Of these trials, the Iressa dose evaluation in advanced lung cancer 1 (IDEAL1) trial demonstrated a response rate of 18.4% with a median progression free survival of 2.7 months and an overall survival rate of 7.6 months, establishing Iressa as a valid therapeutic agent in the treatment of advanced lung cancer [108]. Based on these results, two phase III clinical trials termed Iressa NSCLC Trial Assessing Combination Treatment (INTACT) 1 and 2 were undertaken to compare a combination of Cisplatin and Gemcitabine with Gefitinib or a combination of Carboplatin and Paclitaxel with Gefitinib to chemotherapy alone. Unfortunately, Gefitinib treatment did not improve overall survival in patients compared to placebo controls [70, 71]. Despite these results, Iressa was approved as a third line therapy after failure of Docetaxel and platinum based chemotherapies by the FDA on May 3rd 2003 [109]. An important point is that these clinical trials were performed without checking the mutation status of the EGF receptor [110]. Subsequent studies
demonstrated that lung cancer patients carrying a GOF EGFR mutation have a significantly higher response rate and time to disease progression compared to patients with wild type EGFR [111, 112]. The Iressa pan-Asia study (IPASS) further validated these results by describing a 71.2% objective response rate in patients carrying a GOF EGFR mutation. Based on this data, Gefitinib is administered as a first line therapy to East Asian lung cancer patients with GOF EGFR mutations [113]. The success of Gefitinib paved the way for the development of a host of novel agents ranging from small molecule tyrosine kinase inhibitors to monoclonal EGFR antibodies [114, 115].

1.6.2.3 Resistance and relapse to EGFR tyrosine kinase inhibitors

Despite the initial efficacy of EGFR tyrosine kinase inhibitors, several studies have shown that the majority of patients stop responding to treatment and relapse. These reports prompted a search to determine the mechanisms of acquired resistance to EGFR tyrosine kinase inhibitors. Pao et al was one of the first groups to describe the T790M mutation in patients resistant to Gefitinib treatment [116]. This mutation is present concurrently with Gefitinib sensitive GOF EGFR mutations, and induces a conformational change that interferes with Gefitinib binding and significantly increases receptor affinity for ATP [117-119]. Acquisition of the T790M mutation occurs in about 50% of lung cancer patients treated with EGFR tyrosine kinase inhibitors and is the main cause of TKIs drug resistance [120]. Additional mechanisms of resistance have been described, including activation of alternative tyrosine kinases (IGF-1R and PI3K) or c-Met amplifications [121-123]. While multiple approaches aiming to overcome these mechanisms of resistance are currently being studied, there is an urgent need for the
identification of novel therapeutic targets to develop treatments for patients who have failed both chemotherapy and EGFR targeted therapy [67, 124].

1.7 Mouse models

Mouse models are an invaluable tool to study the effects of targeted gene alterations on development and disease. Since the generation of the first genetically modified animals in the early 70s, mouse models have become the de facto option to study gene modification in vivo [125, 126]. There are currently multiple approaches available to generate mouse models.

1.7.1 Techniques for mouse model generation

The knock-out system causes partial to total deletion of the gene of interest, resulting in the expression of a non-functional truncated protein or no protein at all [127]. On the other hand, the knock-in system makes use of homologous recombination to insert a mutated gene in its original locus, replacing the wild type gene in the process [128]. These two approaches have many advantages. Targeted insertion/deletion of the gene of interest in its original locus ensures gene expression is comparable to wild type and also prevents disruption of critical genes, a common issue when using random DNA integration. The main drawback with these approaches is that the gene modification is irreversible, so dependency of the observed phenotype on sustained gene expression/deletion cannot be determined. This issue was corrected with the development of inducible transgenic mouse models [128]. One of the main approaches
uses the Dox-inducible reverse tetracycline transcription regulation system. In the presence of Dox, the reverse tetracycline transactivator (rtTA) binds the tet operon promoter (tetO) and induces transcription of the transgene [129]. Transgene expression can be restricted to the organ or cell type of interest by expressing rtTA under the control of a tissue specific promoter [130-132]. Since we are interested in lung cancer, we acquired a mouse strain developed by the Whitsett group, which uses the Clara cell secretory promoter (CCSP) to restricts transgene expression to type II pneumocytes in bitransgenic animals [131].

1.7.1.1 Cre recombinase mediated cassette exchange system

The generation of transgenic, knock-in or knock-out models is a time consuming and costly process. Each new founder line needs to be fully characterized before use, and the random integration and expression levels of DNA in transgenic mouse models makes comparison of transgene expression between two different mouse models unreliable. To address these issues, the Wahl group designed the Cre-recombinase mediated cassette exchange system (RMCE) [133]. They started by designing a novel LoxP site that differs from the original LoxP site by three nucleotides to avoid recombination between the two sites. They then generated a cassette where the hygromycin phosphotransferase-thymidine kinase (HyTK) was flanked by the original and novel LoxP sites (termed L2 and L3). The cassette was inserted in the DHFR locus by homologous recombination or at random sites in the genome through single copy integration using a retrovirus [133]. After confirming the presence and expression of the transgene, RMCE was performed using a donor LoxP target. RMCE efficiently induced
site specific recombination and incoming transgene showed expression levels comparable to original transgene (Fig. 7) [133]. These results establish RMCE as an excellent tool to quickly and efficiently generate novel transgenic mouse models with comparable gene expression.

![Diagram of Cre recombinase mediated cassette exchange system](image)

Figure 7. *Cre recombinase mediated cassette exchange system*. The gene is flanked by oppositely oriented hetero-specific LoxP sites and inserted in the genome. In the presence of Cre recombinase, the corresponding LoxP sites from the original cassette recombine with the LoxP site on the replacement cassette replacing the original gene with the DNA from the donor plasmid.

1.7.2 SHP2 mouse models

To determine the role of SHP2 in normal cellular development and cancer, multiple mouse models have been generated. One of the first models used a knock-out approach to delete part of the N-SH2 binding domain (SHP2$^{Δ46-110}$). Homozygous SHP2$^{Δ46-110}$ embryos did not survive past E11.5. At an earlier time point, homozygous SHP2$^{Δ46-110}$ embryos were present but displayed severe developmental abnormalities and growth retardation compared to wild type littermates [134]. Further study revealed that the morphological abnormalities seen in SHP2$^{Δ46-110}$ embryos were most likely caused by the impaired ability of the mutant SHP2 to activate the Ras/MAPK pathway.
downstream of activated PDGFR and FGF [134]. To determine the effect of pan-SHP2 knock-out in adult mice, Bauler et al developed a tamoxifen inducible estrogen receptor-2 cre transgenic mouse model with homozygous floxed ptpn11 (ptpn11fl/fl ert2-cre) [135]. Adult mice displayed impaired hematopoiesis and osteoclastogenesis, cartilage abnormalities, as well as pronounced kyphosis and scoliosis [135]. Targeted SHP2 knock-out in type II alveolar epithelial cells using the SPC-rtTA/tetO-Cre/SHP2fl/fl mouse model caused spontaneous interstitial fibrosis induced by impaired surfactant protein metabolism [136]. These mouse modeling studies established SHP2 as an essential player in embryonic development and survival. Moreover, SHP2 also plays a key role in adult animals, and is required for proper bone and lung epithelial cell homeostasis as well as hematopoiesis.

Aside from its role in development and normal cellular function, SHP2 is also an important factor in cancer. Since SHP2 is a known leukemic oncogene, multiple mouse models were developed to study the role of SHP2 in leukemogenesis [17, 137]. The Qu and Neel groups both generated a knock-in GOF SHP2 mutant mouse model (Ptpn11D61G+/+) to determine the mechanism of leukemogenesis and study Noonan syndrome in vivo. Several Ptpn11D61G+ organs displayed elevated pErk1/2 and pAkt levels and hematopoietic stem cells (HSCs) showed hypersensitivity to GM-CSF and IL-3 [61, 138]. Ptpn11D61G+ mice also showed activation of quiescent HSCs, development of myeloproliferative disease (MPD) and splenomegaly [61]. Interestingly, Ptpn11D61G mediated MPD was dependent on the presence of Gab2 [61]. A knock-in mouse model using the GOF Ptpn11E76K mutant (Ptpn11E76Knexd+/Mx1-Cre+) exhibited a similar phenotype to Ptpn11D61G+ mice with the development of MPD. In addition, these mice
progressed to leukemia and displayed severe impairment of hematopoietic differentiation and GM-CSF/IL-3 hypersensitivity [63]. These mouse modeling studies determined that SHP2 co-localizes with centrosomes, and that HSCs from Ptpn11$^{E76K}$ mice display altered numbers of centrosomes, leading to abnormal cell division and genomic instability [63]. These studies bring an important insight into the mechanism of GOF SHP2 mutant driven leukemogenesis and further our understanding of these diseases.

While the contribution of GOF SHP2 mutations in mouse models of leukemia has been studied, no GOF SHP2 mutant mouse model of carcinoma asides from the one described in this project are currently available. GOF SHP2 mutations are present in a small percentage of solid cancers, and merit further investigation to determine whether SHP2 contributes to carcinogenesis and acts as a proto-oncogene in carcinoma.

1.7.3 GOF EGFR mutant lung cancer mouse models

The prevalence of GOF EGFR mutations in patients with NSCLC led to a strong push to develop transgenic mouse models to study this disease. In 2006, four mouse models were described by the Wong and Varmus lab, and expressed either the exon 19 LREA deletion Del 747-753, the exon 19 deletion Del 747-752, or the exon 21 EGFR$^{L858R}$ point mutation [139, 140]. All four models used the reverse tetracycline transactivation system to direct transgene expression. In this system, bitransgenic animals carry a construct which contains the CCSP promoter and the gene for the reverse tetracycline transactivator and a construct that contains the gene of interest under the control of the tetO promoter. When doxycycline is administered to the mice
either through feed or water, Dox binds to rtTA which can then in turn bind the tetO promoter and induce expression of the transgene [131]. This system is highly versatile, and allows for reversible targeted expression of the transgene in a variety of tissue types depending on the promoter.

Dox induction of bitransgenic CCSP-rtTA/tetO-EGFR<sup>Del 747-753</sup>, CCSP-rtTA/tetO-EGFR<sup>Del 747-752</sup>, CCSP-rtTA/tetO-EGFR<sup>L858R</sup> mice led to formation of hyperplastic lesions with progression to adenocarcinoma at later time points. Tumor burden could be cleared by Dox withdrawal, treatment with Erlotinib or with Cetuximab, demonstrating that tumor cells are dependent on sustained GOF EGFR mutant signaling for survival [139, 140]. These transgenic mouse models provide an excellent tool to better understand the mechanisms of GOF EGFR mutant driven lung carcinogenesis and to identify novel therapeutic targets and mechanisms of drug resistance [141-143].

1.8 Introduction conclusions

The functions of the protein tyrosine phosphatase SHP2 and its role in cancer have been extensively studied since its discovery. SHP2 is a well established leukemic oncogene and GOF SHP2 mutations cause activation of the Ras/MAPK and Src cell signaling pathway, which can have a transforming effect in hematopoietic progenitor cells. The function of SHP2 as a mediator of EGFR cell signaling has also been researched, and it has become clear that the EGF receptor relies on SHP2 for signal transduction. GOF EGFR mutations act as oncogenes in lung epithelial cells and are commonly found in non-smoker and Asian women afflicted with NSCLC. While several tyrosine kinase inhibitors
targeting EGFR have been developed to treat patients, the majority acquire drug resistance and relapse, highlighting an urgent need for the identification of additional therapeutic targets. Since wild type EGFR relies on SHP2 to promote cell signaling, it begs the question of whether GOF mutant EGFR also requires SHP2 to induce and sustain lung tumorigenesis. If GOF EGFR mutants were to actively rely on SHP2 for cell signaling, targeting SHP2 could provide a useful alternative to EGFR tyrosine kinase inhibitors and could provide a potential target for drug development. Several studies have supported this idea, and shown that SHP2 inhibition in a drug resistant GOF EGFR mutant dependent lung cancer cell line can significantly decrease cell proliferation [144]. Further work is required though to confirm these results and validate SHP2 as a therapeutic target in GOF EGFR mutant dependent lung cancer.

As mentioned earlier, the role of SHP2 as an oncogene in hematopoietic malignancies has been thoroughly described, but the contributions of GOF SHP2 mutations to carcinogenesis are largely unknown. GOF SHP2 mutations have been identified in a small percentage of multiple types of carcinoma, notably lung cancer. GOF SHP2 mutations have been found in every lung cancer subtype. Given the function of SHP2 as a promoter of cell signaling, it is possible that the GOF SHP2 mutations found in lung cancer patients are actually contributing to carcinogenesis, and could provide a novel therapeutic target for drug development. At this stage, little research has been done to investigate this hypothesis, so the question remains to be answered.
Chapter 2: Inhibition of SHP2 impairs oncogenic activity of EGF receptor mutants in non-small cell lung cancer

2.1 Abstract

Gain of function EGFR mutations have been identified in 15-25% of lung adenocarcinomas and are targets for therapeutic treatment. EGFR tyrosine kinase inhibitors elicit a strong initial response, but eventually the majority of patients stop responding to treatment and relapse. Multiple approaches have been evaluated to overcome drug resistance, but there is still an urgent need for the identification of novel therapeutic targets to treat lung cancer. SHP2 is an important mediator of EGFR signal transduction, and positively regulates the Ras/MAPK and Src signaling pathway. In this study, we determined whether SHP2 also plays a role in GOF EGFR mutant signaling. SHP2 inactivation using the SHP2 PTP inhibitor SPI-112me, a dominant negative SHP2 mutant, or SHP2 knock-down suppressed cell proliferation and Ras/MAPK signaling in GOF EGFR mutant dependent HCC827 and H1975 lung cancer cells. To assess the role of SHP2 in a mouse model of EGFR driven lung cancer, we generated a Dox inducible transgenic mouse model which expresses the dominant negative PTP-defective SHP2\textsuperscript{CSDA} mutant in type II pneumocytes under the control of the Clara cell secretory protein promoter. Dox induced bitransgenic CCSP-rtTA/tetO-EGFR\textsuperscript{L858R} mice exhibited higher levels of pSrc and pErk1/2 compared to wild type controls. SHP2\textsuperscript{CSDA} expression in tritransgenic CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA} mice suppressed activation
of both kinases. Importantly, SHP2<sup>CSDA</sup> expression significantly delayed EGFR<sup>L858R</sup> induced tumor formation and progression. SHP2<sup>CSDA</sup> expression also reduced pGab1 levels and caused a dissociation of the Gab1/SHP2 complex. This can explain the decreased phosphorylation of pErk1/2 and Src seen in tritransgenic mice, and could be the mechanism of action of SHP2<sup>CSDA</sup>. These results indicate that SHP2 activity is critical for GOF EGFR mutant mediated tumorigenesis, and establish SHP2 as a potential therapeutic target in NSCLC.

2.2 Introduction

Lung cancer is the most prevalent type of cancer in the United States. The American Cancer Society estimates there will be about 224,210 new cases in 2014, leading to approximately 159,260 deaths (about 27% of all cancer deaths). Despite extensive research to develop novel therapeutic treatments and to improve understanding of the biology of this disease, the mortality rate for lung cancer remains high. This is partly caused by diagnosis at an advanced stage which limits treatment options and by patient relapse after therapeutic intervention [145, 146]. Lung cancer is divided in several subtypes, with NSCLC making up about 80% of all cases. NSCLC are further classified into three groups, adenocarcinoma, large cell carcinoma, and squamous cell carcinoma. Adenocarcinoma is often initiated by the activation of a single oncogene. Kras, Alk, and EGFR are the most commonly identified drivers of adenocarcinoma (Fig. 5) [147, 148]. In this study, we focused on GOF EGFR mutant dependent lung cancer. According to the COSMIC database about 27.54% of lung cancers carry GOF EGFR mutations. Although patients initially respond to EGFR tyrosine kinase inhibitor treatment, most of
them relapse and develop drug resistance through multiple mechanisms, including acquisition of the “gatekeeper” T790M mutation in about 50% of cases [149]. This mutation alters the conformation of the ATP binding pocket and interferes with Gefitinib and Erlotinib binding while significantly increasing the affinity of EGFR for ATP. Despite the development of second and third generation tyrosine kinase inhibitors that overcome the effects of the T790M gatekeeper mutation, disease progression still occurs through acquisition of additional drug resistance mechanisms [124]. These observations highlight an urgent need for the identification of new therapeutic targets to treat GOF EGFR mutant dependent lung cancer patients.

SHP2 is a cancer essential gene in multiple cancer types and is required to promote cell transformation, survival, growth and metastasis [55, 57]. SHP2 is an important component of the EGFR signaling pathway as well. Previous work has shown that deletion of the N-SH2 binding domain blocks SHP2 relocation to the cell membrane leading to decreased activity of the Raf and Mek kinases following EGF stimulation [150]. Moreover, SHP2 knock-down in lung cancer cell lines expressing wild type EGFR also decreases basal levels of pErk1/2 [151]. The importance of SHP2 in EGFR mediated signal transduction led us to investigate whether SHP2 activity is also required to promote GOF EGFR mutant signaling and if targeting SHP2 could negatively impact cell growth and survival. To answer these questions, we used an in vitro approach to evaluate the effects of SHP2 knock-down or inhibition on cell signaling and proliferation in GOF EGFR mutant dependent lung cancer cell lines. We also took advantage of the existing EGFR<sup>L858R</sup> transgenic mouse model to test the effects of a dominant negative PTP-inactive mutant of SHP2 (SHP2<sup>CSDA</sup>) on tumor onset and progression in vivo [140].
2.3 Material and Methods

2.3.1 Transgenic mice

2.3.1.1 Construction of L3/L2-tetO vector

The L3/L2-tetO vector was constructed by Dr Noreen Luetteke at the Moffitt Cancer Center Transgenic Animal Core using the transgenic vector tet-op-mp1 as a backbone. This vector was kindly provided by Dr. Katerina Politi and contains the tetO promoter, the mouse protamine1 intron and the polyadenylation sequences [140]. Two tandem repeats of the 1.2 Kb chicken beta globin insulator (cHS4) were subcloned into the Xhol site upstream of the tetO response element to limit the risk of leaky transgene expression [152]. The heterotypic loxP sites were synthesized as oligonucleotides based on published sequences and annealed to produce double-stranded adaptors with compatible overhangs to permit subcloning into restriction sites and facilitate screening [133]. The L3 mutant loxP adaptor contains 5’ Xhol and 3’ SalI sticky ends and was subcloned into the Xhol site upstream of the cHS4 insulators. The L2 wild-type loxP adaptor contains 5’ NotI and 3’ EagI sticky ends and was subcloned in reverse orientation into the NotI site of the tet-op-mp1 vector downstream of the polyA signal. The integrity and orientation of the loxP sites in the final vector (L3/L2-tetO) were confirmed by DNA sequencing.

2.3.1.2 Transgene microinjection

The DNA fragment containing the human SHP2<sup>CSDA</sup> mutant was previously described and excised from a pCDNA3.1 vector and subcloned into the EcoRV site between the tetO and polyA sequences of L3/L2-tetO plasmid [34]. The completed
SHP2\textsuperscript{CSDA} transgene was excised from the vector by digestion with BssHII and isolated by agarose gel electrophoresis followed by EluTrap electroelution and EluTip purification. Ethanol precipitated DNA was resuspended in sterile microinjection buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and microinjected at 3 ng/µl into 0.5 dpc fertilized FVB/N zygotes per standard methods. Zygotes were then surgically implanted into the oviducts of 0.5 dpc pseudopregnant CD-1 females. Offspring were tail biopsied at weaning and genotyped by PCR and slot blot hybridization to identify transgenic lines. This work was performed by Dr. Liwei Chen and Jie Wu from the Wu laboratory and the Moffitt Cancer Center Transgenic Core.

2.3.1.3 Genotyping of tetO-SHP2\textsuperscript{CSDA} transgenic mice

TetO-SHP2\textsuperscript{CSDA} mice were genotyped by PCR using the GoTaq® Hot Start Green Master Mix (Promega) and the following primers: SHP2T1, 5’-AGACGCCATCCACGCTGTTTTGAC-3’ and SHP2T2, 5’-TCTCTTTTAATTGCCCGTGATGTT-3’. PCR reaction was performed in a total volume of 25 µL using the following conditions: 4 min denaturation at 94°C, 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec with a final extension step of 72°C for 4 min yielding a 430-bp PCR fragment.

2.3.1.4 CCSP-rtTA and EGFR\textsuperscript{L858R} transgenic mice

CCSP-rtTA transgenic mice (in inbred FVB/N background) were provided by Dr. Jeffrey A. Whitsett. tetO-EGFR\textsuperscript{L858R} mice (in Bl6 background) were obtained from the NCI/NIH mouse repository. Animals were maintained in pathogen-free housing.
conditions. Rodent chow containing 200 mg/kg Dox (Dox diet, Bio-Serv) was used to activate the reverse tetracycline transactivator. Animal studies and care were approved by the Institutional Animal Care and Use Committee of the University of South Florida and followed institutional and national guidelines.

2.3.2 RT-PCR analysis

Tissue samples were snap frozen in liquid nitrogen. RNA was extracted using Trizol reagent (Life Technologies) according to manufacturer protocol. Samples were treated with DNase I (Life Technologies) to avoid DNA contamination. RT-PCR was performed using the SuperScript One-Step RT-PCR Platinum Taq system (Life Technologies) with the following primers: SHP2F1: 5’-GGTTGGACAAGGGAATCGG-3’ and SHP2R2: 5’-AGGGCTCTGATCTCCACTCG-3’. RT-PCR reaction was performed in a total volume of 50 µL using the following conditions: 30 min cDNA synthesis at 55°C, 4 min denaturation at 94°C then 35 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec with a final extension step of 72°C for 4 min yielding a 462-bp fragment.

2.3.3 Cell cultures and reagents

HCC827 and H1975 cell lines were provided by Dr. Eric Haura and cultured in complete RPMI medium1640 (10% fetal bovine serum and1% pen strep) purchased from Life Technologies (Carlsbad, CA) at 37°C in 0.5% CO₂. 293T cells were provided by Dr. Said Sebti and cultured in complete Dulbecco’s modified eagle medium (DMEM) (10% fetal bovine serum and1% pen strep) purchased from Life Technologies (Carlsbad, CA) at 37°C in 0.5% CO₂. The human SHP2^{CSDA} GFP tagged fragment or
the empty vector control GFP tagged fragment were previously generated in our lab and excised from the pcDNA3.1 vector then ligated in the pWP1 transfer vector using Pmel digestion according to established protocols [34]. The SHP2<sub>CSDA</sub> pWP1 transfer vector, the packaging plasmid psPAX2 and the enveloped plasmid pMD2.G were transfected in 293T cells plated in a 6cm plate and grown to near confluence for 6 hours in .5 mL Optimem (Life Technologies) and 2 µL Lipofectamine 2000 (Invitrogen) to obtain lentivirus. Optimem was removed after transfection and replaced with pen strep free complete medium. After 48 hours, viral media was collected and filtered using a .45 µm sterile syringe filter (Corning). HCC827 cells were plated in a 6 well plate and grown to near confluence. Lentiviral infection was performed in 250 µL pen strep free complete medium with 750 µL viral media and 40 µg polybrene for 6 hours. Cells were then washed with PBS (Life Technologies) and grown for 2 weeks in complete medium. The BD Biosciences FACSVantage DIVA digital cell sorter was used to perform three rounds of cell sorting two weeks apart from each other to isolate GFP positive HCC827 cells. Percentage of GFP positive HCC827 cells was calculated using the DIVA software. EGF was purchased from Invitrogen, doxycycline and polybrene from Sigma-Aldrich, Erlotinib from LC Chemicals, WZ4002 from Selleck, and SPI-112me was synthesized in-house at the Moffitt Cancer Center.

2.3.4 RNA interference studies

2.3.4.1 siRNA

Cells were plated in 12 well plates (20,000 cells/well) and grown in complete medium for 48 hours. Cells were transfected with 25 nM PTPN11 On target plus Smart
Pool siRNA or 25 nM On target plus control pool siRNA (Thermo Scientific Dharmacon) for 6 hours in 700 µL Optimem with 2 µL Lipofectamine 2000. Optimem was removed after transfection and cells were grown for 72 hours in complete medium then serum starved for 16 hours prior to protein extraction.

2.3.4.2 shRNA

The TRIPZ Dox inducible lentiviral human PTPN11 shRNA 1049 and 3469 were purchased from Open Biosystems. Lentivirus was obtained using 293T cells according to protocol. HCC827 and H1975 cells were infected with either the 1049 or 3469 lentiviruses and stable clones selected by puromycin treatment. HCC827/1049 and HCC827/3469 were plated in 6 well plates (100,000 cells/well) and grown in complete medium for 24 hours. ShRNA expression was induced using 2 µg/mL doxycycline for 96 hours. Cells were then serum starved for 16 hours prior to protein extraction.

2.3.5 Cell proliferation assay

Cell proliferation was measured using the CellTiter-Glo luminescent cell viability assay (Promega). Cells were plated in 96 well black bottom plates (1000 cells/well) and grown for 24 hours. HCC827/SHP2CSDA cells and HCC827 SPI-112me treated cells were grown for 6 days in 200 µL complete medium. SHP2 knock-down using siRNA or shRNA was adjusted to the 96 well plate format and performed as described earlier in 200 µL complete medium. Cell viability was assayed using 50 µL CellTiter-Glo reagent and luminescence was measured using a Wallac 1420 Victor plate reader.
2.3.6 Soft agar colony formation assay

To perform the soft agar colony formation assay, a 1 mL layer of .8% agar (Fisher Scientific) in complete medium was poured in each well (6 well plate) and left to solidify at room temperature for 20 minutes. Next, a 1 mL layer of 0.4% agar in complete medium containing 10'000 cells was gently poured on top of the bottom layer. All agar solutions were warmed to 48°C. After solidifying, 500 µL of complete medium containing 2 µg/mL doxycycline was added on top of the agar layer. Cells were grown for 21 days and medium was replaced every four days. The number of colonies was assayed by MTT staining. 10 mg/mL MTT was added to each well and plate was incubated for 15 minutes at 37°C. A picture of each well was taken using an alpha imager and the number of colonies was counted manually.

2.3.7 Immunoblotting, immunoprecipitation and mass spectrometry analysis

Antibodies to SHP2, Erk1/2, phospho-Erk1/2 (pErk1/2), Gab1, Akt, c-Myc, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Flag (rabbit), pGab1 (Y627), Phospho-Akt (pAkt), and phospho-Src (pSrc) antibodies were from Cell Signaling Technology (Danvers, MA). Src antibody was from Calbiochem (Billerica, MA) and M2 Flag antibody was from Sigma (St. Louis, MO).

Frozen tissues were crushed and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM Na₂P₂O₇, 1 mM dithiothreitol, 1 mM Na₃VO₄, 100 µg/ml of phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1% Triton X-100). Equal amounts of proteins from cleared tissue lysates were separated by 10% SDS-polyacrylamide gels and transferred
to nitrocellulose filters for immunoblotting. Flag-tagged SHP2 was immunoprecipitated from cleared tissue lysate by using the Flag M2 antibody and Protein-G agarose. Total EGFR was immunoprecipitated from cleared tissue lysate supernatants by using the EGFR antibody and Protein-A agarose. Immunoblotting was performed as described previously [62, 153].

Cells were cultured and cell lysates were prepared for immunoblotting or immunoprecipitation analyses similar to that described previously [60]. Protein identification by mass spectrometry was performed by the Moffitt Center Center Proteomics Core using standard procedure. Essentially, tryptic peptides from gel slices were analyzed with a nanoflow liquid chromatograph coupled to an electrospray ion trap mass spectrometer for tandem mass spectrometry peptide sequencing. Five tandem mass spectra were collected in a data-dependent manner following each survey scan. Sequences were assigned using Mascot (www.matrixscience.com) searches against mouse or human (for SHP2<sup>CSDA</sup>) entries. Results from Mascot were compiled in Scaffold.

2.3.8 Histology and immunohistochemistry

Following euthanasia, mouse lungs were flushed twice with 10 ml PBS and insufflated with 10% buffered formalin. After fixation overnight in 10% buffered formalin solution at room temperature, paraffin blocks were prepared by standard procedure by the Moffitt Cancer Center Tissue Core. Sections (4 µm thick) were stained with hematoxylin and eosin (H&E) for histological examination.
2.3.9 Microscopy analysis

Four micron sections of mouse lung tissues were stained with hematoxylin and eosin and covered in #1.5 cover glass. These slides were used to acquire whole slide images using the Aperio™ (Vista, CA) ScanScope XT with a 20x/0.8NA objective lens at a rate of 5 minutes per slide via Basler tri-linear-array. Digital images were stored in the Moffitt Network Operations Center and accessible to study participants via the password protected Spectrum (Aperio) web-based interface.

Genie® v1 histology pattern recognition software (Aperio) was used to segment tumor from other lung tissues and background. This was completed with a training set of 400 iterations and careful pathologist quality control. The resultant tumor region area was calculated as a percentage of all tissue. This was completed for the isolated tumor regions using the Aperio Nuclear® v9.1 algorithm with optimized thresholds (Averaging radius = 1 µm; Segmentation Type = 2 Cytoplasmic Rejection; Threshold Type = 1-Edge Threshold with weighted Trimming; Min/ Max Nuc Size = 25 µm²/1000000 µm²; RGB stain = 0/ 0.64307/ 0.31756; Positive RGB OD = 0.244583/ 0.509334/ 0.825081).

2.4 Results

2.4.1 SHP2 inhibition decreases cell proliferation and pErk1/2 levels

We selected two cell lines to study the role of SHP2 in GOF EGFR mutant dependent lung cancer. HCC827 cells carry the LREA deletion in exon 19 and are sensitive to EGFR tyrosine kinase inhibitors. On the other hand, H1975 cells express both the EGFR\textsuperscript{L858R} point mutation on exon 21 and the gatekeeper T790M mutation which confers resistance to Gefitinib and Erlotinib but is sensitive to the irreversible
EGFR inhibitor WZ4002. These two cell lines enabled us to study the role of SHP2 in both a drug sensitive and resistant context.

We treated both cell lines with WZ4002 to determine the signaling changes caused by EGFR inhibition. Since SHP2 lies downstream of EGFR and promotes its signaling activity, SHP2 inhibition is predicted to show similar changes to those caused by EGFR inhibition. After 24 hours treatment we detected an increase in p27 and a significant decrease in pErk1/2, pSrc, pAkt, and c-Myc levels by immunoblot (Fig. 8A). WZ4002 activity was confirmed by checking the phosphorylation levels of EGFR. Treatment resulted in complete dephosphorylation of the activating Y1080 site of EGFR (Fig. 8B).

We previously described the novel SHP2 inhibitor SPI-112me. This compound binds the PTP catalytic domain leading to decreased Ras/MAPK signaling and enhanced STAT1 phosphorylation upon IFNγ stimulation [154]. To determine the effect of SHP2 inhibition on cell viability, we treated HCC827 and H1975 cells with SPI-112me for 6 days. We observed decreased proliferation in both cell lines (Fig. 9). SPI-112me treatment for 24 hours reduced pErk1/2 levels in both cell lines and pSrc levels in HCC827 cells, though no major changes were detected in pAkt and pSrc levels (Fig. 10). Since SHP2 inhibition may increase EGFR mediated PI3K pathway activation, we did not expect to see a decrease in Akt activity in our cells [49, 50]. On the other hand, while SHP2 is involved in EGFR mediated Src activation; it is possible that in H1975 cells GOF EGFR mutant can activate Src through an alternative pathway, thus SHP2 inhibition would not result in lowered pSrc levels.
Figure 8. *WZ4002 treatment decreases cell signaling and pEGFR levels.* HCC827 and H1975 cells were plated in 6 well plates and grown to confluence in complete medium then serum starved for 16 hours. Cells were treated with indicated concentrations of WZ4002 in complete medium for 24 hours followed by protein extraction. (A) Immunoblot was performed with indicated antibodies after 10% SDS-polyacrylamide gel separation. (B) 1mg protein from cleared lysate was immuno-precipitated using EGFR antibody and Protein-A agarose beads. Immunoblot was performed with indicated antibodies.
Figure 9. **SPI-112me treatment decreases cell proliferation.** HCC827 and H1975 cells were plated in 96 well plates (1000 cells/well), rested overnight, then treated with indicated SPI-112me concentrations for 6 days in complete medium. Cell proliferation was assayed using the CellTiter-Glo reagent. Graph values represent an average of four independent experiments.

![Graph showing cell viability decrease with SPI-112me concentrations.](image)

Figure 10. **SPI-112me treatment decreases pErk1/2 levels.** HCC827 and H1975 cells were plated in 6 well plates (200,000 cells/well) and rested overnight in complete medium. After 16 hours serum starvation, cells were treated with indicated SPI-112me concentrations in complete medium for 24 hours followed by protein extraction.

![Western blot images showing protein levels.](image)
Immunoblot was performed with indicated antibodies following 10% SDS-polyacrylamide gel separation.

2.4.2 SHP2 knock-down by shRNA decreases cell proliferation and signaling

To confirm that SHP2 inhibition can decrease GOF EGFR mutant mediated cell proliferation and signaling, we generated two HCC827 cell lines which stably express either the SHP2 shRNA construct 1049 or 3469 upon Dox induction. SHP2 knock-down in HCC827/1049 and HCC827/3469 cells resulted in a significant decrease in cell viability and soft agar colony formation compared to uninduced controls (Fig 11A, B).

To determine whether SHP2 can specifically inhibit EGF induced cell growth, we serum starved HCC827/1049 cells for 72 hours then stimulated cells with EGF for an additional 72 hours. Cell proliferation was significantly lower than controls (Fig. 11C) indicating SHP2 knock-down can specifically decrease EGFR mediated cell proliferation upon EGF stimulation. The decrease in cell viability seen in HCC827/1049 and HCC827/3469 cells is correlated with reduced levels of pErk1/2 and c-Myc caused by SHP2 knock-down (Fig. 11D). Similar results were not obtained in H1975 cells because the efficiency of SHP2 knock-down was poor, and no signaling changes or decrease in cell proliferation were detectable following Dox induction (data not included).

2.4.3 SHP2 knock-down by siRNA decreases cell proliferation and signaling

To confirm the results obtained in the previous section using HCC827/1049 and HCC827/3469 cells and to check whether SHP2 knock-down can overcome GOF EGFR mutant signaling in the drug resistant H1975 cell line we used a siRNA approach. SHP2 targeted siRNA transfection efficiently knocked-down SHP2 levels in both cell lines, and
led to a significant decrease in cell proliferation and levels of pErk1/2 and c-Myc.

Transfection of both cell lines using a non-silencing control siRNA did not cause any visible changes. (Fig. 12A, B).

Figure 11. SHP2 knock-down by shRNA decreases cell proliferation and signaling. (A) HCC827/1049 and HCC827/3469 cells were plated in 96 well plates and grown for 6 days in complete medium with or without 2µg/mL doxycycline. Cell proliferation was assayed using the CellTiter-Glo reagent. Graph values represent an average of four independent experiments. (B) After plating in 0.4% agar in six well plates, HCC827/1049 and HCC827/3469 cells were grown for 21 days with or without 2µg/mL doxycycline. Colonies were stained with MTT and counted manually. (C) HCC827/1049 cells were plated in 96 well plates and grown for 3 days in complete medium with or without 2µg/mL doxycycline. 10ng or 20ng EGF was added at day 3 in serum free medium. Cell proliferation was assayed 3 days later using the CellTiter-Glo reagent. (D)
HCC827/1049 and HCC827/3469 cells were plated in 6 well plates (50,000 cells/well) and grown for 6 days in complete medium with or without 2µg/mL doxycycline followed by protein extraction. Immunoblot was performed with indicated antibodies following 10% SDS-polyacrylamide gel separation. (* = p<.0001 as measured using Mann-Whitney test)

Figure 12. SHP2 knock-down by siRNA decreases cell proliferation and signaling. (A) HCC827 and H1975 cells were plated in 96 well plates (1000 cells/well) and rested overnight. Cells were transfected with 25nM non-silencing or SHP2 siRNA in 100µL Optimem and .5 µL Lipofectamine 2000 for 6 hours. Cells were then grown for 72 hours in complete medium. Cell proliferation was assayed using the CellTiter-Glo reagent. Graph values represent an average of two independent experiments (B) HCC827 and H1975 cells were plated in 12 well plates (20,000 cells/well) and rested overnight. Cells were transfected with 25nM non-silencing or SHP2 siRNA for 6 hours in 700µL Optimem with 2µL Lipofectamine 2000. Optimem was removed after transfection and cells were grown for 72 hours in complete medium then serum starved for 16 hours prior to protein extraction. Immunoblot was performed with indicated antibodies after
10% SDS-polyacrylamide gel separation. (P - parental, NS - non-silencing) (* = p<.0001 as measured using Mann-Whitney test)

2.4.4 Expression of a dominant negative SHP2 mutant decreases cell proliferation and signaling

Previous work has shown that different domains of SHP2 play distinct regulatory functions [155]. The previous siRNA and shRNA knock-down experiments describe an important role for SHP2 in GOF EGFR mutant mediated signal transduction, but the drawback of this approach is the removal of the entire SHP2 protein. In that case, it is unclear whether the changes seen are caused by absence of the PTP catalytic function or by a secondary function of SHP2. To address this issue, we generated HCC827 cells that stably express the dominant negative SHP2 mutant SHP2\textsuperscript{CSDA}. The PTP domain of this mutant is catalytically inactive while the rest of the protein is intact. When expressed in cells, SHP2\textsuperscript{CSDA} competes with endogenous wild type SHP2 for substrate binding and decreases the positive regulatory effects of SHP2 [156]. SHP2\textsuperscript{CSDA} expression in HCC827 cells caused a small but significant decrease in cell proliferation and levels of pErk1/2 (Fig. 13A, B). The mild effect of SHP2\textsuperscript{CSDA} on cell viability is most likely caused by failure of the SHP2 mutant to fully out-compete endogenous SHP2 for substrate binding. Indeed, despite three rounds of cell sorting HCC827 cells still only expressed small amounts of the dominant negative SHP2\textsuperscript{CSDA} mutant (Fig. 13B). These results do confirm though that intact PTP catalytic function is required to promote GOF EGFR mutant signal transduction and that the changes in cell signaling are most likely not caused by a secondary function of SHP2.
Figure 13. *Expression of a dominant negative SHP2 mutant decreases cell proliferation and signaling.* (A) HCC827 cells were plated in 96 well plates (1000 cells/well) and grown in complete medium for 6 days. Cell proliferation was assayed using the CellTiter-Glo reagent. Graph values represent an average of four independent experiments. (B) HCC827 cells were plated in 6 well plates (200,000 cells/well) and grown in complete medium for 48 hours before protein extraction. Immunoblot was performed with indicated antibodies following 10% SDS-polyacrylamide gel separation. (P - Parental, EV - empty vector, CSDA - SHP2<sup>CSDA</sup>) (* = p<.002 as measured using Mann-Whitney test)

2.4.5 Characterization of the Dox inducible CCSP-rTA/tetO-EGFR<sup>L858R</sup>/tetO-SHP2<sup>CSDA</sup> transgenic mouse model

After concluding our *in vitro* studies, it became evident that SHP2 activity is indeed required for cell survival and growth of GOF EGFR mutant dependent lung cancer cell lines, regardless of T790M mutation presence. SHP2 inactivation by knock-down, inhibition, or dominant negative SHP2 mutant expression significantly reduced cell proliferation and activation of the Ras/MAPK pathway. To verify these results and test whether targeting SHP2 is relevant to GOF EGFR mutant driven lung cancer *in vivo*, we
46

generated a novel tetO-SHP2\textsuperscript{CSDA} Dox inducible transgenic mouse model which expresses the SHP2\textsuperscript{CSDA} mutant under the control of the tetO promoter (Fig. 14A).

![Diagram A]

Figure 14. PCR analysis to identify tetO-SHP2\textsuperscript{CSDA} founder lines. (A) The tetO-SHP2\textsuperscript{CSDA} construct contains 2 chicken beta globin insulators upstream of the tetO promoter and a mouse protamine 1 poly-adenylation tail. The tetO-EGFR\textsuperscript{L858R} construct expresses the GOF EGFR\textsuperscript{L858R} mutant under the control of the tetO promoter. The CCSP-rtTA construct directs expression of the reverse tetracycline transactivator in type II pneumocytes under the control of the Clara cell secretory protein promoter. (B) PCR analysis using DNA extracted from tail snips to identify positive founder lines.

Transgenic mice were obtained by microinjecting the DNA fragment containing the tetO-SHP2\textsuperscript{CSDA} cassette into zygotes from FVB/N mice and growing the embryos in pseudo-pregnant CD-1 mice. After two rounds of DNA microinjection, we obtained nine
founder lines (Line 61, 65, 669, 674, 676, 374, 382, 389, and 390) exhibiting germline transmission of the transgene (Fig. 14B). Unfortunately, line 61, 65, 674, 374, 382, and 390 displayed leaky transgene expression at the mRNA and protein levels in lung tissue (Fig. 15). Based on these results, we selected line 669 and line 65 for further study.

![Figure 15. Founder line leaky expression check. Lungs were collected from wild type and monotransgenic tetO-SHP2<sup>CSDA</sup> mice and snap frozen in liquid nitrogen. Total lung tissue was split in two. Half was used for RNA extraction, half for protein extraction. RT-PCR was performed according to protocol. SHP2<sup>CSDA</sup> was immunoprecipitated using Flag tag antibody and Protein-G agarose beads. Immunoblot was performed using Flag antibody following 10% SDS-polyacrylamide gel separation.](image)

We bred bitransgenic CCSP-rtTA/tetO-SHP2<sup>CSDA</sup> mice to direct expression of the transgene to lung epithelial cells (abbreviated C/65 and C/669). After one month of Dox induction, SHP2<sup>CSDA</sup> expression was almost tripled in the lungs of C/65 mice and was also detectable in the lungs of C/669 mice (Fig. 16). We decided to include line 65
despite its leakiness (Fig. 17A) because of its high expression of the \( \text{SHP2}^{\text{CSDA}} \) transgene (Fig. 17B). Line 669 does not exhibit leaky expression (Fig. 17A) but transgene expression is low (Fig. 17B), so it might have a more moderate effect on GOF EGFR mutant signaling.

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**RT-PCR**

**Flag IP-IB**

**tetO-SHP2\({}^{\text{CSDA}}\)/CCSP-rtTA (65)**

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**RT-PCR**

**Flag IP-IB**

**tetO-SHP2\({}^{\text{CSDA}}\)/CCSP-rtTA (669)**

Figure 16. *Dox induction triggers \( \text{SHP2}^{\text{CSDA}} \) expression in the lungs of bitransgenic CCSP-rtTA/tetO-SHP2\({}^{\text{CSDA}}\) mice*. Lungs were collected from Dox induced/uninduced bitransgenic and wild type mice and snap frozen in liquid nitrogen. Total lung tissue was split in two. Half was used for RNA extraction, half for protein extraction. RT-PCR was performed according to protocol. \( \text{SHP2}^{\text{CSDA}} \) was immunoprecipitated using Flag tag antibody and Protein-G agarose beads. Immunoblot was performed using Flag antibody following 10% SDS-polyacrylamide gel separation. (WT - wild type, B - bitransgenic CCSP-rtTA/tetO-SHP2\({}^{\text{CSDA}}\))
Figure 17. **Comparison of SHP2\(^{\text{CSDA}}\) transgene levels and leaky expression between founder line 669 and 65.** (A) Multiple organs were collected from monotransgenic tetO-SHP2\(^{\text{CSDA}}\) mice from line 65 or 669. RNA was extracted using Trizol and RT-PCR performed according to protocol. (B) SHP2\(^{\text{CSDA}}\) transgene levels of founder line 65 and 669 were checked at the DNA level using slot-blot hybridization. Founder lines lacking the SHP2\(^{\text{CSDA}}\) transgene were used as negative controls. (Br - Brain, Lu - Lung, Li - Liver, Sp - Spleen, Ki - Kidney, In - Intestine, Co - Colon)

We also obtained an additional transgenic mouse line which expresses the EGFR\(^{L858R}\) mutant under the control of the tetO promoter (Fig. 14A). When Dox is administered to CCSP-rtTA/tetO-EGFR\(^{L858R}\) bitransgenic mice, the GOF EGFR mutant is expressed in type II lung pneumocytes and induces epithelial cell transformation. Within a few weeks of Dox induction, the mice develop diffuse hyperproliferative lesions
which progress to a more aggressive phenotype with the formation of multifocal adenomas. Dox withdrawal results in tumor regression, highlighting a dependence on sustained \( \text{EGFR}^{L858R} \) expression for tumor survival [140]. We bred our bitransgenic mice to the \( \text{EGFR}^{L858R} \) mice to generate tritransgenic \( \text{CCSP-rtTA/tetO-EGFR}^{L858R}/\text{tetO-SHP2}_{\text{CSDA}} \) mice (abbreviated C/EGFR/65 for founder line 65 and C/EGFR/669 for founder line 669). Dox induction triggered expression of both transgenes in the lungs of tritransgenic mice (Fig. 18).

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**Figure 18.** Dox induction triggers \( \text{SHP2}_{\text{CSDA}} \) and \( \text{EGFR}^{L858R} \) expression in the lungs of tritransgenic \( \text{CCSP-rtTA/tetO-EGFR}^{L858R}/\text{tetO-SHP2}_{\text{CSDA}} \) mice. Lungs were collected from tritransgenic mice after 0, 2 and 4 weeks of Dox induction. Total lung tissue was split in two. Half was used for RNA extraction, half for protein extraction. RT-PCR was performed according to protocol. \( \text{SHP2}_{\text{CSDA}} \) was immunoprecipitated using Flag tag antibody and Protein-G agarose beads. Immunoblot was performed using indicated antibodies following 10% SDS-polyacrylamide gel separation.
2.4.6 SHP2\textsuperscript{CSDA} expression counters EGFR\textsuperscript{L858R} mediated kinase activation

After characterization of our transgenic mouse models, we fed CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA}, CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}, and wild type mice Dox diet for 4, 6, and 8 weeks. EGFR\textsuperscript{L858R} expression increased Erk1/2 and Src activity as indicated by higher levels of pErk1/2 T202/Y204 and pSrc Y416 in the lungs of bitransgenic mice compared to wild type controls (Fig. 19). As expected, SHP2\textsuperscript{CSDA} expression countered the EGFR mutant induced pErk1/2 increase. Interestingly, the SHP2 mutant also reduced pSrc levels in both tritransgenic line and pAkt levels in C/EGFR/669 mice (Fig 19). It is possible that expression of the SHP2\textsuperscript{CSDA} mutant is higher in the lungs of tritransgenic mice than in our HCC827/SHP2\textsuperscript{CSDA} cell culture model, and can better out-compete endogenous SHP2 for substrate binding resulting in a stronger impact on EGFR\textsuperscript{L858R} mediated signal transduction.

2.4.7 SHP2\textsuperscript{CSDA} expression decreases EGFR\textsuperscript{L858R} mediated tumor onset, progression and burden

As described earlier, bitransgenic CCSP-rtTA/tetO-EGFR\textsuperscript{L858R} mice develop a diffuse form of adenocarcinoma within four weeks of Dox induction. At later time points, the disease progresses to a more aggressive phenotype and mice develop multifocal adenomas. Dox induced SHP2\textsuperscript{CSDA} expression in tritransgenic CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA} mice still triggered cellular transformation, but tumor areas were smaller and more restricted compared to bitransgenic controls (Fig. 20A). Moreover, SHP2\textsuperscript{CSDA} expression seemed to prevent progression of the disease to a more aggressive phenotype since few adenomas were detected (Fig. 20A).
Figure 19. Signaling changes caused by \( \text{SHP2}^{\text{CSDA}} \) expression in CCSP-rtTA/tetO-\( \text{EGFR}^{L858R} \)/tetO-\( \text{SHP2}^{\text{CSDA}} \) mice. Lungs were collected and snap frozen in liquid nitrogen from wild type, bitransgenic CCSP-rtTA/tetO-\( \text{EGFR}^{L858R} \), and tritransgenic CCSP-rtTA/tetO-\( \text{EGFR}^{L858R} \)/tetO-\( \text{SHP2}^{\text{CSDA}} \) mice after 4, 6, and 8 weeks of Dox induction. Immunoblot was performed using indicated antibodies following 10% SDS-polyacrylamide gel separation.
To compare tumor burden in bitransgenic versus tritransgenic animals, we collaborated with the Moffitt Cancer Center Microscopy Core to design a computer algorithm that objectively quantifies the percentage of healthy epithelial cells and tumors cells in lungs using H&E slides. The software was trained to differentiate between different cell types to calculate the percentage of tumor cells in the mouse lungs. The results of the analysis are an average of three H&E slides per animal representing about half of the total lung volume. SHP2<sup>CSDA</sup> expression significantly decreased tumor burden in C/EGFR/65 and C/EGFR/669 mice starting at week 4 of Dox induction compared to C/EGFR mice (Fig. 20B, Table 1). These results confirm that SHP2 also plays an important role in mediating the tumorigenic activity of the GOF EGFR<sup>L858R</sup> mutant <i>in vivo</i>, and reinforce the validity of SHP2 as a potential therapeutic target in GOF EGFR mutant dependent lung cancer.

Table 1. <i>P-values for tumor burden of tritransgenic mice compared to bitransgenic mice</i>

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<th>n</th>
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<td>C/EGFR/669</td>
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P-values were calculated using an unpaired parametric t-test with Welch’s correction comparing results of either C/EGFR/65 mice to C/EGFR mice or C/EGFR/669 mice to C/EGFR mice for each time point.
Figure 20. SHP2<sup>CSDA</sup> expression decreases EGFR<sup>L858R</sup> mediated tumorigenesis. (A) Lungs were collected from wild type, CCSP-rtTA/tetO-EGFR<sup>L858R</sup>, and CCSP-rtTA/tetO-EGFR<sup>L858R</sup>/tetO-SHP2<sup>CSDA</sup> mice, flushed with PBS then insufflated and fixed overnight in 10% buffered formalin. Lungs were processed by the Moffitt Cancer Center Histology.
2.4.8 GOF EGFR mutations induce Gab1 phosphorylation and association with SHP2

After establishing the importance of SHP2 in GOF EGFR mutant dependent lung cancer, we next investigated how SHP2\textsuperscript{CSDA} decreases tumor burden and signaling in CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA} mice. We performed a mass spectrometry analysis to determine the binding partners of SHP2\textsuperscript{CSDA} in the lungs of tritransgenic mice (Fig. 21, Table 2). One of the top candidates was Gab1, which is a well known binding partner of SHP2 [34, 35].

![Gel Slice Image]

**Figure 21.** Mass Spectrometry analysis of SHP2\textsuperscript{CSDA} binding partners in the lungs of CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA} mice. Protein was extracted from the lungs of three CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA} mice and combined in one sample. 10mg protein was immunoprecipitated using Flag antibody and Protein-G agarose beads. Immunoblot was performed using PY20 antibody following 10% SDS-polyacrylamide gel separation. Gel slices corresponding to phosphorylated bands on immunoblot were cut out and analyzed by mass spectrometry.
Table 2. **SHP2\textsuperscript{CSDA} bound proteins identified by mass spectrometry.**

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Table contains top candidates with a minimum of 3 matching peptides. Protein identification by mass spectrometry was performed by the Moffitt Cancer Center Proteomics Core. Tryptic peptides from gel slides were analyzed with a nanoflow liquid chromatograph coupled to an electrospray ion trap mass spectrometer for tandem mass spectrometry peptide sequencing. Five tandem mass spectra were collected in a data-dependent manner following each survey scan. Sequences were assigned using Mascot (www.matrixscience.com) searches against mouse entries. (M.W.-Molecular weight is an approximation, not exact value)

To assess the role of Gab1 in GOF EGFR mutant dependent lung cancer, we started by checking its phosphorylation status in our HCC827 and H1975 cells and found that Gab1 is indeed phosphorylated in these cell lines (Fig. 22A). We then treated both HCC827 and H1975 cells with Erlotinib. In HCC827 cells, EGFR inhibition resulted in decreased levels of pGab1, concurrent with a dissociation of the Gab1/SHP2 complex (Fig. 22A). These results indicate that SHP2/Gab1 association is directly promoted by GOF EGFR mutant signaling. Since H1975 cells express the drug resistant
T790M mutation, Erlotinib treatment should have no effect on these cells. As expected, Erlotinib treatment of H1975 cells did not affect Gab1/SHP2 association and only slightly decreased Gab1 phosphorylation at the highest concentration (Fig. 22A).

Figure 22. **GOF EGFR mutant mediated Gab1 phosphorylation and Gab1/SHP2 association is disrupted by EGFR inhibition in EGFR dependent lung cancer cell lines.** (A) HCC827 and H1975 cells were plated in 10cm plates in complete medium and grown to near confluence. Cells were treated with indicated concentration of Erlotinib for 24 hours before protein extraction. Immunoprecipitation was done using 1mg protein, Gab1 antibody and Protein-G agarose beads. Immunoblot was performed using indicated antibodies following 10% SDS-polyacrylamide gel separation. IP-IB and western blot data was obtained from the same cell lysate per sample. (B) The same procedure described in (A) was used, except cells were treated with WZ4002 instead.
To further confirm our results, we then treated both cell lines with the EGFR inhibitor WZ4002 which overcomes the drug resistant T790M mutation. In this case, both cell lines showed a significant decrease in pGab1 levels and association of Gab1 and SHP2 (Fig. 22B). These results indicate GOF EGFR mutant phosphorylates Gab1 then recruits and activated SHP2 which in turn positively regulates EGFR signal transduction.

Based on our 

in vitro results we wondered whether a similar pattern of SHP2 activation takes place in our bitransgenic CCSP-rtTA/tetO-EGFR

L858R mice. We detected increased levels of pGab1 and Gab1/SHP2 association in C/EGFR mice compared to wild type controls (Fig 23). Interestingly, SHP2

CSDA expression in C/EGFR/65 and C/EGFR/669 mice significantly decreased pGab1 levels leading to dissociation of the Gab1/SHP2 complex (Fig 23), potentially revealing the mechanism by which SHP2

CSDA decreases GOF EGFR mutant mediated Ras/MAPK and Src pathway activation.

Figure 23. EGFR

L858R mediated Gab1 phosphorylation and Gab1/SHP2 association is disrupted by SHP2

CSDA expression in the lungs of tritransgenic mice. Protein was extracted from mouse lungs. Immunoprecipitation was performed using 1mg protein,
Gab1 antibody and Protein-G agarose beads. Immunoblot was performed using indicated antibodies following 10% SDS-polyacrylamide gel separation. IP-IB and western blot data was obtained using the same cell lysate per sample. (WT - wild type, B - bitransgenic CCSP-rtTA/tetO-EGFR<sup>L858R</sup>, 669 - C/EGFR/669, 65 - C/EGFR/65)

2.5 Discussion

The EGFR oncogene is a well described driver of lung tumorigenesis and an established therapeutic target in a molecularly defined subset of NSCLC [157]. Multiple successful approaches have been developed to target GOF EGFR mutants including small tyrosine kinase inhibitors and monoclonal antibodies [158, 159]. Unfortunately, the majority of patients relapse, and develop tumors that no longer respond to EGFR tyrosine kinase inhibition [160]. Drug resistance is achieved by lung cancer cells through multiple mechanisms including the acquisition of secondary EGFR mutations, c-Met amplifications, and others [120-123]. While second and third generation tyrosine kinase inhibitors are being developed, it is obvious that solely targeting EGFR is not sufficient to treat patients with recurrent tumors [67, 124]. These observations highlight an urgent need for the identification of additional therapeutic targets for the treatment of GOF EGFR mutant NSCLC patients.

Previous work has shown that SHP2 is a key downstream effector of EGFR signal transduction [34, 57]. EGFR activation recruits SHP2 to the cell membrane where it becomes activated and positively regulates the Ras/MAPK and Src signaling pathways through multiple mechanisms [36, 42, 44]. The importance of SHP2 in EGFR signal transduction led us to inquire whether SHP2 is also required by GOF EGFR mutants and could potentially serve as a therapeutic target in GOF EGFR mutant dependent NSCLC.
To determine whether targeting SHP2 impacts cell proliferation and survival in both a drug sensitive or resistant context, we selected the HCC827 and H1975 lung cancer cell lines for our experiments. HCC827 cells respond to EGFR inhibition while H1975 cells carry the gatekeeper mutation T790M and are resistant to Erlotinib. SHP2 inhibition using the SPI-112me inhibitor decreased cell proliferation and pErk1/2 levels. Similarly, SHP2 knock-down using shRNA or siRNA also decreased cell viability, colony formation, and Ras/MAPK signaling in both GOF EGFR mutant dependent lung cancer cell lines. These results demonstrate that SHP2 function is required to mediate cell signaling and demonstrate a key role for SHP2 in cell growth of both drug resistant and sensitive lung cancer cells. To check whether intact SHP2 PTP catalytic activity is necessary to mediate GOF EGFR mutant signal transduction, I expressed the dominant negative SHP2<sup>CSDA</sup> mutant in HCC827 cells and observed a decrease in cell proliferation and Erk1/2 activation. This data indicate that intact PTP catalytic function is indeed required to mediate GOF EGFR mutant signaling.

In the next step of our study, we decided to test our hypothesis in vivo. We generated a novel Dox inducible tetO-SHP2<sup>CSDA</sup> transgenic mouse model which expresses the dominant negative SHP2<sup>CSDA</sup> mutant under the control of the tetO promoter in lung epithelial cells. We also obtained a second transgenic mouse model that expresses the EGFR<sup>L858R</sup> mutant under the control of the tetO promoter. We then bred bitransgenic CCSP-rtTA/tetO-EGFR<sup>L858R</sup> and tritransgenic CCSP-rtTA/tetO-EGFR<sup>L858R</sup>/tetO-SHP2<sup>CSDA</sup> mice to study the effects of the SHP2<sup>CSDA</sup> mutant in an EGFR<sup>L858R</sup> driven mouse model of lung carcinogenesis. Dox induction of bitransgenic mice increased pErk1/2 and pSrc levels compared to wild-type controls. SHP2<sup>CSDA</sup>
expression countered EGFR\textsuperscript{L858R} mediated kinase activation and significantly reduced tumor formation and burden in the lungs of tritransgenic mice. The dominant negative SHP2\textsuperscript{CSDA} mutant also conferred a protective effect by blocking progression of the disease to a more aggressive phenotype. These results demonstrate that targeting SHP2 can significantly impact GOF EGFR mutant dependent lung tumorigenesis and support the validity of SHP2 as a therapeutic target for GOF EGFR mutant dependent NSCLC.

We performed a mass spectrometry analysis to identify the binding partners of SHP2\textsuperscript{CSDA} in the lungs of tritransgenic CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA} mice and determine how SHP2\textsuperscript{CSDA} can decrease cell signaling and tumor growth. The results of the analysis identified Gab1 as a top candidate. Gab1 is the main docking protein for SHP2, and association between the two is required for proper SHP2 activation and function [35]. Using our lung cancer cell lines, we determined that EGFR inhibition decreases Gab1 phosphorylation and disrupts Gab1/SHP2 association. In our bitransgenic CCSP-rtTA/tetO-EGFR\textsuperscript{L858R} mouse model, we found that EGFR\textsuperscript{L858R} increases pGab1 levels and Gab1/SHP2 complex formation in the lungs. SHP2\textsuperscript{CSDA} expression significantly decreases Gab1 phosphorylation and Gab1/SHP2 association. A possible explanation for this observation is that SHP2 can regulate the activity of its own docking protein [35, 60]. When SHP2\textsuperscript{CSDA} binds to pGab1, it prevents the activation of a positive feedback loop and blocks the phosphorylation of additional Gab1 proteins, thus disrupting Gab1/SHP2\textsuperscript{WT} complex formation and causing the decreases in cell signaling seen in tritransgenic CCSP-rtTA/tetO-EGFR\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA} mice (Fig. 24).
In conclusion, we have described an important function of SHP2 as a key mediator of GOF EGFR mutant driven lung tumorigenesis and established SHP2 as a potential therapeutic target for the treatment of GOF EGFR mutant dependent NSCLC.
Chapter 3: Active SHP2 mutant induces lung hyperproliferative lesions and adenoma in transgenic mice

3.1 Abstract

SHP2 plays an important role in carcinogenesis. Gain of function SHP2 mutants are known leukemic oncogenes and have been identified in multiple cancer types, including lung cancer. However, the oncogenic potential of activating SHP2 mutants in lung tumorigenesis has not been established. To address this question we generated Dox inducible bitransgenic CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice that express the GOF SHP2\textsuperscript{E76K} mutant in lung epithelial cells under the control of a rat Clara cell secretory protein promoter fragment. SHP2\textsuperscript{E76K} expression increased pErk1/2, pSrc, c-Myc and Mdm2 levels in the lungs of bitransgenic mice. After 6 to 9 months of Dox induction, bitransgenic CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice developed atypical adenomatous hyperplasia that progressed to adenoma and adenocarcinoma in the lungs. These results demonstrate a new role for this GOF SHP2 mutant as a lung cancer oncogene. To determine whether tumor survival is dependent on continued SHP2\textsuperscript{E76K} expression, we analyzed tumor-bearing mice after 1 month of Dox withdrawal by Magnetic Resonance Imaging (MRI). The results of the MRI analysis indicate that SHP2\textsuperscript{E76K} expression is required for tumor maintenance in this mouse model of lung adenocarcinoma. Further study revealed that SHP2\textsuperscript{E76K} expression increased Gab1 phosphorylation and promoted Gab1/SHP2 complex formation in the lungs of
bitransgenic mice and cancer cell lines. This data indicate that GOF SHP2 mutants auto-regulate the tyrosine phosphorylation of their own docking protein. Since inhibition of Src family kinases (SFKs) decreases pGab1 levels in cell lines expressing an activating SHP2 mutant, and SHP2$^{E76K}$ expression increases Src phosphorylation, it seems likely that the SFKs are an important mediator of this positive feedback loop mechanism.

3.2 Introduction

Protein tyrosine kinases have traditionally been identified as inducers of cell signaling and act as oncogenes when acquiring gain of function mutations [161]. Since protein tyrosine phosphatases usually counter the biochemical reaction of PTKs, they have generally been considered tumor suppressors. However, increasing evidence suggests that some PTPs collaborate with PTKs to promote cell signaling and transformation [162, 163]. Indeed, tyrosine phosphorylation does not always positively regulate the target protein. For example, phosphorylation of tyrosine sites such as Src Y530 or CDK1 Y15 negatively regulates enzyme activity of these kinases [14-16].

On the other hand, phosphorylation of the p120RasGap and c-CBL tyrosine binding sites upon EGFR activation recruits these negative regulators to the cell membrane and terminates EGFR signaling [164-166]. In these circumstances, dephosphorylation of the p120RasGap and c-CBL docking sites and the inhibitory Src Y530 and CDK1 Y15 sites promotes PTK signal pathway activity. This concept of positive collaboration between PTKs and PTPs is exemplified by SHP2.
Indeed, growth factor receptors recruit and activate SHP2 by inducing a conformational change that relieves its auto-inhibitory mechanism. Active SHP2 promotes Ras/MAPK and Src signaling pathway activation [26, 34, 45]. Because of its positive regulatory effects on cell signaling, SHP2 is a cancer essential gene in certain types of carcinoma and is required to maintain cell growth and survival [55, 57]. Moreover GOF SHP2 mutations are known leukemic oncogenes and have been identified in approximately 35% of juvenile myelomonocytic leukemia, 10% of childhood myelodysplastic syndromes, 7% of B-cell acute lymphoblastic leukemia, and 4% of acute myelogenous leukemia [17, 167]. The majority of activating SHP2 mutations are located in the N-SH2/PTP interface and disrupt the auto-inhibitory mechanism, conferring constitutive activity to the PTP catalytic domain (Fig. 4) [58]. Activating SHP2 mutations have also been identified in carcinoma at lower rates than in hematologic malignancies. According to the COSMIC database, as of 4-14-2014, about 1.81% of lung cancer cases carry GOF SHP2 mutations. Even though this percentage may seem small, it still represents a large number of people due to the high prevalence of lung cancer in the population.

The American Cancer Society estimates there will be about 224,210 new cases of lung cancer in 2014, causing approximately 159,260 deaths (about 27% of all cancer deaths). Lung cancer is usually initiated by activation of a single oncogene. Kras, EGFR, and Alk are the most common drivers of lung tumorigenesis [147, 148]. Additional oncogenes such as RIT1 and RET fusions have been identified in a smaller percentages of cases [168-170]. These genes provide interesting targets for the development of new drugs that can broaden treatment opportunities for patients. Since
GOF SHP2 mutations are present in lung cancer, the contribution of SHP2 to tumorigenesis merits further investigation.

To achieve this goal, we generated a novel Dox inducible mouse model that carries the GOF SHP2\textsuperscript{E76K} mutant (tetO-SHP2\textsuperscript{E76K}). We bred these mice to a second strain of transgenic mice which express rtTA under the control of the Clara cell secretory protein to direct expression of SHP2\textsuperscript{E76K} in type II pneumocytes [131]. The CCSP-rtTA mouse model has previously been validated as a reliable model of NSCLC [131].

### 3.3 Material and methods

#### 3.3.1 Transgenic mice

##### 3.3.1.1 Transgene microinjection

The L3/L2-tetO vector was constructed as described in the previous section. The DNA fragment containing the human SHP2\textsuperscript{E76K} mutant was excised from a pCDNA3.1 vector and subcloned into the EcoRV site between the tetO and polyA sequences of L3/L2-tetO plasmid [62]. The completed SHP2\textsuperscript{E76K} transgene was excised from the vector by digestion with BssHII and isolated by agarose gel electrophoresis followed by EluTrap electroelution and EluTip purification. Ethanol precipitated DNA was resuspended in sterile microinjection buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) and microinjected at 3 ng/μl into 0.5 dpc fertilized FVB/N zygotes per standard methods. Zygotes were then surgically implanted into the oviducts of 0.5 dpc pseudopregnant CD-1 females. Offspring were tail biopsied at weaning and genotyped by PCR and slot blot hybridization to identify transgenic lines. This work was performed
by Dr. Liwei Chen and Dr. Wu from the Wu laboratory and the Moffitt Cancer Center Transgenic Mouse Model Core.

3.3.1.2 Genotyping of tetO-SHP2$^{E76K}$ transgenic mice

TetO-SHP2$^{E76K}$ mice were genotyped by PCR using the GoTaq® Hot Start Green Master Mix (Promega) and the following primers: SHP2T1, 5’-AGACGCCATCCACGCTGTTTTGAC-3’ and SHP2T2, 5’-TCTCTTTTAATTGCCCGTGATGTT-3’. PCR reaction was performed in a total volume of 25 µL using the following conditions: 4 min denaturation at 94°C, 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec with a final extension step of 72°C for 4 min yielding a 430-bp PCR fragment.

3.3.1.3 CCSP-rtTA transgenic mice

CCSP-rtTA transgenic mice (in inbred FVB/N background) were provided by Dr. Jeffrey A. Whitsett. Animals were maintained in pathogen-free housing conditions. Rodent chow containing 200 mg/kg Dox (Dox diet, Bio-Serv) was used to activate the reverse tetracycline transactivator. Animal studies and care were approved by the Institutional Animal Care and Use Committee of the University of South Florida and followed institutional and national guidelines.

3.3.2 RT-PCR analysis

Tissue samples were snap frozen in liquid nitrogen. RNA was extracted using Trizol reagent (Life Technologies) according to manufacturer protocol. Samples were treated
with DNase I (Life Technologies) to avoid DNA contamination. RT-PCR was performed using the SuperScript One-Step RT-PCR Platinum Taq system (Life Technologies) with the following primers: SHP2F1: 5’-GGTTGGACAAGGGAATACGG-3’ and SHP2R2: 5’-AGGGCTCTGATCTCCACTCG-3’. RT-PCR reaction was performed in a total volume of 50 µL using the following conditions: 30 min cDNA synthesis at 55°C, 4 min denaturation at 94°C then 35 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec with a final extension step of 72°C for 4 min yielding a 462-bp fragment.

3.3.3 Immunoblotting, immunoprecipitation, and mass spectrometry analysis

Antibodies to SHP2, Erk1/2, phospho-Erk1/2, Gab1, Akt, c-Myc, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Flag (rabbit), pGab1 (Y627), Phospho-Akt, and phospho-Src antibodies were from Cell Signaling Technology (Danvers, MA). Src antibody was from Calbiochem (Billerica, MA) and M2 Flag antibody was from Sigma (St. Louis, MO). Antibodies to MDM2 (clone 2A9) and MDMX (clone 8C6) were obtained from Dr. Jiandong Chen and generated as described previously [171, 172]. The p53 antibody was purchased from IMGENEX (San Diego, CA).

Frozen tissues were crushed and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5 mM Na₄P₂O₇, 1 mM dithiothreitol, 1 mM Na₃VO₄, 100 µg/ml of phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml aprotinin, and 1% Triton X-100). Equal amounts of proteins from cleared tissue lysate were separated by 10% SDS-polyacrylamide gels and transferred to nitrocellulose filters for immunoblotting. Flag-tagged SHP2 was immunoprecipitated
from cleared tissue lysate by using the Flag M2 antibody and Protein-G agarose. Immunoblotting was performed as described previously [62, 153]. Hel cells were provided by Dr. Ken Zuckerman and cultured and cell lysates prepared for immunoblotting or immunoprecipitation analyses similar to that described previously [62, 153]. Tyrosine kinase inhibitors were from LC Laboratories (Woburn, MA).

Protein identification by mass spectrometry was performed by the Moffitt Cancer Center Proteomics Core using standard procedure. Essentially, tryptic peptides from gel slides were analyzed with a nanoflow liquid chromatograph coupled to an electrospray ion trap mass spectrometer for tandem mass spectrometry peptide sequencing. Five tandem mass spectra were collected in a data-dependent manner following each survey scan. Sequences were assigned using Mascot (www.matrixscience.com) searches against mouse or human entries. Results from Mascot were compiled in Scaffold.

3.3.4 qRT-PCR

qRT-PCR was performed using Power SYBR Green reagents (Applied Biosystems) and proprietary primers for 18s rRNA or Mdm2 exon 1-3 from IDT (San Jose, CA). Samples were assayed in triplicates whereas standards, no amplification controls, and no DNA controls were performed in duplicates. The ABI PRISM 7900HT Sequence Detection System from Applied Biosystems was used to run qPCR. Data was normalized using 18s rRNA as the internal control and analyzed using the SDS software version 2.3.
3.3.5 Histology and immunohistochemistry

Mice were euthanized by CO₂ asphyxiation followed by cervical dislocation. Lungs were flushed twice with 10 ml PBS and insufflated with 10% buffered formalin. After overnight fixation in 10% buffered formalin solution at room temperature, paraffin blocks were prepared by standard procedure by the Moffitt Cancer Center Tissue Core. Sections (4µm thick) were stained with hematoxylin and eosin (H&E) for histological examination.

For IHC analysis of pErk1/2, slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson). Slides were deparaffinized with EZ Prep solution (Ventana). Heat-induced antigen retrieval method was used in Cell Conditioning 1 (Ventana). A rabbit pErk1/2 antibody (#4376, Cell Signaling, Danvers, MA) was used at a 1:200 dilution in PSS diluent (Ventana) and incubated for 32 min. Anti-rabbit secondary antibody (Ventana) was used for 20 min. The detection system used was the Ventana OmniMap kit and slides were counterstained with Hematoxylin. All slides were scanned and uploaded as digital images using the Aperio™ (Vista, CA) ScanScope XT with a 20x/0.8NA objective lens at a rate of 5 minutes per slide via Basler tri-linear-array. Digital images were stored in the Moffitt Network Operations Center and accessible to study participants via the password protected Spectrum (Aperio) web-based interface.

3.3.6 MRI analysis

Mice were anesthetized with 2% isoflurane, restrained in a mouse cradle mounted on an insertion device and positioned within the RF coil of the magnet while
continuously receiving isoflurane. Respiratory function and temperature of the animals were monitored and controlled throughout the imaging session using the SAI system (Small Animal Instruments, Inc.). Temperature control of the animals was achieved by a variable temperature gas unit and set to maintain a body temperature of 37 ± 1°C. MRI was performed using a 7-T horizontal magnet (ASR 310, Agilent Technologies) equipped with nested 205/120/HDS gradient insert and a bore size of 310 mm. Temperature control of the imaging gradients was achieved by means of a water chiller (Neslab Waters) and maintained at 12°C for all acquisitions. Using a 35-mm Litz-cage coil (Doty Scientific), coronal $T_2$-weighted fast spin-echo (FSE, 3D) sequences were acquired with TE/TR = 64/1000 ms, Matrix = 256x128x16, field of view (FOV) = 90x45x16 mm and slice thickness of 1 mm over 8.5 minutes. Tumor volumes and dimensions were quantified by manually drawn regions of interest (ROI) using VnmrJ (Agilent Technologies, Inc.). MRI was performed by the Moffitt Cancer Center Animal Imaging Core.

In an initial pilot study, $T_2$-weighted FSEMs sequences were acquired with TR = 954 ms, ESP = 7.5 ms, ETL = 8 (eff. TE = 60 ms), FOV = 40 x 90 mm, Matrix = 128 x 256, 15 slices at 1 mm thickness, 8 averages and fat suppression in 3.75 minutes.

3.4 Results

3.4.1 Generation of Dox inducible tetO-SHP2$^{E76K}$ transgenic mice

We selected the SHP2$^{E76K}$ mutant for our transgenic mouse model because it is a well characterized leukemic oncogene, confers the highest PTP catalytic activity of known GOF SHP2 mutants, and has previously been identified in lung cancer patients
This point mutation is located in the N-SH2 binding domain and disrupts the auto-inhibitory mechanism conferring constitutive catalytic activity to the PTP domain (Fig 25).

Figure 25. Crystal structure of the GOF SHP2\textsuperscript{E76K} mutation (PDB: 2SHP). This structure is missing the c-terminal tail but otherwise contains the C-SH2 binding domain (yellow), the N-SH2 binding domain (red) and the PTP catalytic domain (green). The blue residue highlights the location of the E76K point mutation which disrupts the auto-inhibitory mechanism.

To generate our transgene, we modified the tetracycline inducible tet-op mp1 vector by inserting two chicken beta globin insulators upstream of the tetO promoter and flanking the cassette with a pair of oppositely oriented heterotypic L2 and L3 loxP sites [133, 152]. The L2/L3 transgenic vector system is capable of undergoing Cre-recombinase mediated cassette exchange [133]. We then subcloned a C-terminal Flag
tagged human SHP2\textsuperscript{E76K} coding sequence downstream of the tetO promoter of our L2/L3 vector to obtain the tetO-SHP2\textsuperscript{E76K} transgenic construct (Fig. 26A). Transgenic mice were obtained by microinjecting the DNA fragment containing the tetO-SHP2\textsuperscript{E76K} cassette into zygotes from FVB/N mice and growing the embryos in pseudo-pregnant CD-1 mice. We identified eight founder lines (Line 394, 398, 400, 404, 417, 422, 425, 428) that displayed germline transmission of the transgene (Fig. 26B).

Figure 26. Transgenic constructs and founder line identification. (A) The tetO-SHP2\textsuperscript{E76K} construct contains 2 chicken beta globin insulators upstream of the tetO promoter and a mouse protamine 1 poly-adenylation tail. The CCSP-rTTA construct directs expression of the reverse tetracycline transactivator in type II pneumocytes under the control of the Clara cell secretory protein promoter. (B) PCR analysis using DNA extracted from tail snips to identify positive founder lines.

We selected founder lines 400, 398, 417 and 425 for further investigation. Founder line 400 expressed leaky expression of the SHP2\textsuperscript{E76K} transgene at the mRNA level in all
organs tested (Fig. 27). Based on these results, we selected lines 398, 417, and 425 that displayed no leaky expression for further study (Fig. 27).

![Image]

Figure 27. Founder line leaky expression check. Lungs were collected from monotransgenic tetO-SHP2E76K mice and snap frozen in liquid nitrogen. Trizol was used for RNA extraction and RT-PCR was performed according to protocol. (Lu - lung, Li - liver, Ki - kidney, Co - colon)

We bred bitransgenic CCSP-rtTA/tetO-SHP2E76K mice (abbreviated C/398, C/417, and C/425) and screened for Dox inducible expression of SHP2E76K in the lungs. After one month of Dox induction, SHP2E76K was readily detectable in the lungs of bitransgenic mice at the mRNA and protein level (Fig 28).

3.4.2 SHP2E76K expression induces Ras/MAPK and Src signaling pathway activation

After characterizing our transgenic mouse model, we determined the biochemical changes caused by SHP2E76K expression in the lungs of CCSP-rtTA/tetO-SHP2E76K mice. SHP2E76K expression increased pErk1/2 T202/Y204 and pSrc Y416 levels but did not affect pAkt S473 in the lungs of bitransgenic mice compared to wild type controls.
(Fig. 29). SHP2\textsuperscript{E76K} expression also increased c-Myc levels in the lungs of bitransgenic mice (Fig. 29). C-Myc is an important promoter of cell proliferation and is a driver oncogene of lung cancer [153, 174].

![Image](image.png)

**Figure 28.** *Dox induced SHP2\textsuperscript{E76K} expression is detectable in the lungs of bitransgenic CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice.* Lungs were collected from Dox induced bitransgenic and wild type mice and snap frozen in liquid nitrogen. Total lung tissue was split in two. Half was used for RNA extraction, half for protein extraction. RT-PCR was performed according to protocol. SHP2\textsuperscript{E76K} was immunoprecipitated using Flag tag antibody and Protein-G agarose beads. Immunoblot was performed using Flag antibody following 10% SDS-polyacrylamide gel separation.

Previous work has shown that activation of the Ras/MAPK pathway upregulates Mdm2 which suppresses p53 activity [175]. Experiments performed by Dr. Yuan Ren in the Wu laboratory have consistently shown that SHP2\textsuperscript{E76K} transformed TF-1 cells and increased Mdm2 while reducing p53 but did not have any effect on MDMX [60, 62]. Moreover, Ras/MAPK pathway inactivation by the Mek inhibitor U0126 restored Mdm2 to non-transformed levels, indicating that SHP2\textsuperscript{E76K} upregulates Mdm2 through activation of the Ras/MAPK pathway [60]. To determine whether a similar mechanism of Mdm2 upregulation is present in our bitransgenic CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice, we
measured Mdm2 mRNA level expression by qRT-PCR. Results showed that Mdm2 mRNA levels are significantly higher in the lungs of bitransgenic mice (n=4) compared to wild type controls (n=4) (Fig. 30)

Figure 29. Signaling changes caused by SHP2\textsuperscript{E76K} expression in CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice. Lungs were collected and snap frozen in liquid nitrogen from wild type, monotransgenic tetO-SHP2\textsuperscript{E76K}, and bitransgenic CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice after 1 month of Dox induction. Immunoblot was performed using indicated antibodies following 10% SDS-polyacrylamide gel separation. (WT - wild type, M - monotransgenic, B - bitransgenic)
Figure 30. *qRT-PCR analysis of Mdm2 mRNA levels in the lungs of bitransgenic mice compared to wild type controls.* Lungs were collected from wild type or CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mice and snap frozen in liquid nitrogen. RNA was extracted using Trizol according to manufacturer protocol. qRT-PCR was repeated two times. Lung samples from each animal was assayed in triplicates (n=4). Average Ct values were 27.5 for WT and 25.8 CCSP/E76K. Statistical analysis was performed using the non parametric Mann-Whitney test.

3.4.3 SHP2<sup>E76K</sup> expression induces hyperplasia and adenoma formation in lung epithelial tissue

SHP2<sup>E76K</sup> expression caused significant histological alterations in the lungs of CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mice. After 6 months of Dox induction, the majority of animals displayed extensive atypical adenomatous hyperplasia (n=12) (Fig. 31A). 25% of bitransgenic mice (n=12) developed adenomas as well, including in one case as early as 2 months after Dox induction (Fig. 31A). At the 9 months time point, 86.7% of bitransgenic mice (n=15) developed adenomas that were larger in size and several animals developed adenocarcinomas (defined by a diameter of ≥5mm) (Fig. 31B) [176]. Histological evaluation by a pathologist indicated that tumors were of papillary, solid, or mixed subtypes (Table 3) with features resembling human NSCLC.
In comparison, none of our monotransgenic tetO-SHP2\textsuperscript{E76K} or wild type control mice developed hyperplasia or adenoma after 6 months of Dox induction (WT, n= 6; E76K, n=7). At the 9 month time point, 2 control mice developed tumors with similar histological features to tumors identified in bitransgenic mice (one WT and one E76K), but they were significantly smaller in size (WT, n= 9; E76K, n=4) (Fig. 30B) At 12 months of Dox induction, two control mice displayed tumors (one WT and one E76K), one of them had features similar to adenocarcinoma, the other to squamous cell carcinoma (WT, n= 12; E76K, n=12).

Overall, Dox induced CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice developed lung tumors at a significantly higher rate than monotransgenic tetO-SHP2\textsuperscript{E76K}, CCSP-rtTA, or wild type controls (Fig. 32) indicating that SHP2\textsuperscript{E76K} promotes lung tumorigenesis with NSCLC features in this transgenic mouse model of lung cancer.

3.4.4 Dox withdrawal induces lung tumor regression

For the next step of our study, we took advantage of the reversible feature of our transgenic mouse model and the small animal imaging facilities at the Moffitt Cancer Center to determine whether constant SHP2\textsuperscript{E76K} expression is required for tumor maintenance. We set up a small pilot study to determine whether MRI can be used to detect established tumors in the lung of bitransgenic mice (n=4). Tumor presence was verified by dissection following positive identification by MRI analysis (Fig. 33). The results of our pilot study established MRI as a valid technique to detect lung tumors \textit{in vivo}.  79
Figure 31. Histological evaluation of lung hyperplasia and adenoma formation in CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mice. Lungs were collected from wild type and CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mice, insufflated with 10% neutral buffered formalin, fixed overnight, then processed by the Moffitt Cancer Center Histology Core. Slides were stained with H&E, and histological alterations were evaluated by a pathologist (A) Bitransgenic mice
developed hyperproliferative lesions and small adenomas after 6 months of Dox induction. (B) Bitransgenic mice developed lung adenomas and adenocarcinomas after 9 months of Dox induction. Only two mice out of 24 control animals developed adenomas after 9 months of Dox induction.

Table 3. Histological evaluation of tumor subtype.

<table>
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<th>Genotype (ID)</th>
<th>Time (months)</th>
<th>Histology</th>
</tr>
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<tbody>
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<td>6</td>
<td>Adenoma (papillary)</td>
</tr>
<tr>
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<tr>
<td>C/398 (4235)</td>
<td>9</td>
<td>Adenocarcinoma (mixed subtypes); focal hyperplasia</td>
</tr>
<tr>
<td>C/398 (4233)</td>
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<td>Adenoma (mixed subtypes)</td>
</tr>
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<td>Adenoma (papillary); focal hyperplasia; squamous dysplasia</td>
</tr>
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<td>Adenoma (mixed subtypes); hyperplasia</td>
</tr>
<tr>
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<td>Adenoma (papillary)</td>
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<td>Adenoma (papillary)</td>
</tr>
<tr>
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</tr>
<tr>
<td>C/417 (4246)</td>
<td>9</td>
<td>Not available; evidence of tumor in lung photo</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Adenocarcinoma (solid) with adjacent areas of hyperplasia</td>
</tr>
<tr>
<td>WT (2956)</td>
<td>12</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>WT (2202)</td>
<td>12</td>
<td>Adenoma (papillary)</td>
</tr>
</tbody>
</table>

H&E stained slides from representative lung tumor-carrying CCSP-rtTA/tetO-SHP2E76K and control mice were evaluated by a resident Moffitt Cancer Center pathologist to determine the histological subtype of the lesions.
Biochemical analysis confirmed that SHP2$^{E76K}$ expression was no longer detectable after Dox withdrawal (Fig. 35A). In the absence of the positive regulatory effects of SHP2$^{E76K}$, pErk1/2, pSrc, and c-Myc all returned to levels comparable to wild type controls (Fig. 35B). These results were further supported by IHC staining of lung tissues with a pErk1/2 antibody. Strong pErk1/2 staining is detectable in SHP2$^{E76K}$ induced lung tumors (Fig. 35C). After Dox withdrawal, pErk1/2 staining intensity was comparable to wild type controls (Fig. 35C).
Figure 33. *MRI pilot study*. CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mice were Dox induced for 9 months then imaged by the Moffitt Cancer Center small animals imaging core staff. Animals with MRI detectable lung tumors were euthanized and dissected to confirm the presence of tumors in the area seen in MR images. (n=4)

3.4.5 SHP2<sup>E76K</sup> auto-regulates phosphorylation of its docking protein Gab1

To determine the mechanisms of SHP2<sup>E76K</sup> driven lung tumorigenesis we sought to identify the binding partners of SHP2<sup>E76K</sup> in the lungs of CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mice. SHP2<sup>E76K</sup> immunoprecipitates were obtained from lung tissue of Dox induced bitransgenic mice, separated on a 10% SDS polyacrylamide gel, and immunoblotted with a PY20 antibody (Fig. 36 A). Gel bands corresponding to phosphorylated tyrosines
on immunoblot were analyzed by mass spectrometry. The proteins identified by mass spectrometry are listed in Table 4. One of the top candidates was Gab1. Further analysis using co-immunoprecipitation confirmed that Gab1 and the SHP2\(^{E76K}\) mutant associate in the lungs of bitransgenic CCSP-rtTA/tetO-SHP2\(^{E76K}\) mice (Fig. 36B).

---

**Figure 34.** *Dox withdrawal causes lung tumor regression.* (A) CCSP-rtTA/tetO-SHP2\(^{E76K}\) mice were Dox induced for 9 months. A first round of MR imaging confirmed presence of tumors in the lungs of bitransgenic animals. Mice were then placed on regular chow for a month, and imaged for a second time using similar conditions. (n=6) (B) After the second round of MRI analysis, bitransgenic mice were sacrificed and lung tissue
collected. H&E slides were prepared by the Moffitt Cancer Center Histology Core and evaluated by a pathologist to check for presence of residual lesions.
Figure 35. Dox withdrawal abrogates the positive regulatory effects of SHP2$^{E76K}$ in the lungs of CCSP-rtTA/tetO-SHP2$^{E76K}$ mice. (A) Lung tissue was collected from CCSP-rtTA/tetO-SHP2$^{E76K}$ mice following the second round of MRI after 1 month Dox withdrawal. Tissue was snap frozen in liquid nitrogen and divided in half to extract both RNA and protein. RT-PCR was performed according to manufacturer’s protocol. Flag-tagged SHP2$^{E76K}$ was immunoprecipitated using Flag antibody and Protein-G agarose beads followed by 10% SDS-polyacrylamide gel separation and immunoblot with Flag antibody. (B) Lungs were collected from wild type and pre or post Dox induced CCSP-rtTA/tetO-SHP2$^{E76K}$ mice. Protein extraction was followed by 10% SDS-polyacrylamide gel separation and immunoblot with indicated antibodies. (C) Lung slides from pre and post induction CCSP-rtTA/tetO-SHP2$^{E76K}$ or wild type mice were stained with pErk1/2 antibody. (WT - wild type, B - bitransgenic)

Figure 36. SHP2$^{E76K}$ expression induces Gab1 phosphorylation and Gab1/SHP2 association in the lungs of CCSP-rtTA/tetO-SHP2$^{E76K}$ mice. (A) Protein was extracted from the lungs of CCSP-rtTA/tetO-SHP2$^{E76K}$ mice and immunoprecipitated using Flag antibody and Protein-G agarose beads. Immunoblot was performed using PY20 antibody following 10% SDS-polyacrylamide gel separation. (B) Protein was extracted from the lungs of CCSP-rtTA/tetO-SHP2$^{E76K}$ mice and immunoprecipitated using Flag antibody and Protein-G agarose beads. Immunoblot was performed using indicated antibodies following 10% SDS-polyacrylamide gel separation. (C) Lungs were collected from wild type and pre or post Dox induced CCSP-rtTA/tetO-SHP2$^{E76K}$ mice. Protein extraction was followed by 10% SDS-polyacrylamide gel separation and immunoblot with indicated antibodies. (WT - wild type, B - bitransgenic)
Table 4. *SHP2^{E76K}* bound proteins identified by mass spectrometry.

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Table contains top candidates with a minimum of 3 matching peptides. Protein identification by mass spectrometry was performed by the Moffitt Cancer Center Proteomics Core. Tryptic peptides from gel slides were analyzed with a nanoflow liquid chromatograph coupled to an electrospray ion trap mass spectrometer for tandem mass spectrometry peptide sequencing. Five tandem mass spectra were collected in a data-dependent manner following each survey scan. Sequences were assigned using Mascot (www.matrixscience.com) searches against mouse entries. (M.W. - Molecular weight is an approximation, not exact value)

Previous work has shown that Gab1/SHP2 association is required for SHP2 activation and that GOF SHP2 mutants are non-functional if they lack intact SH2 binding.
domains [33, 34]. These studies indicate that acquisition of a GOF SHP2 mutation is not sufficient to induce cellular transformation; recruitment of the PTP to the cell membrane by docking proteins is a required step in this process. Since Gab1 is the main docking protein of SHP2 and was identified in our mass spec analysis, we decided to focus our attention on Gab1.

Dox induced CCSP-rtTA/tetO-SHP2\(^{E76K}\) mice expressed elevated levels of phosphorylated Gab1 compared to wild type controls or bitransgenic mice after Dox withdrawal (Fig. 36C). To confirm these results, we expressed the SHP2\(^{E76K}\) mutant in TF-1 and H292 cells, and saw an increase in pGab1 as well as activation of the Src family of kinases [60]. These results indicate that the SHP2\(^{E76K}\) mutant can promote its own activation by regulating the phosphorylation of its own docking protein.

To determine which protein tyrosine kinase is responsible for SHP2\(^{E76K}\) mediated Gab1 phosphorylation, we treated H292/SHP2\(^{E76K}\) cells with Ruxolitinib, Dasatinib or Erlotinib inhibitors which target Jak, SFKs, and EGFR respectively. Ruxolitinib had no effect on pGab1 levels, while Dasatinib and Erlotinib significantly reduced Gab1 phosphorylation [60]. To determine whether Src activity is also required for induction of Gab1 phosphorylation by a different GOF SHP2 mutant we treated H661 lung cancer cells which express the SHP2\(^{N58S}\) mutant with Dasatinib. As described earlier, Src inhibition decreased pGab1 levels and caused a dissociation of the Gab1/SHP2 complex [60]. We further confirmed these results using a siRNA approach. Pan Src knock-down using a Src SiRNA also reduced pGab1 levels in H661 cells [60]. To identify specifically which SFK is responsible for Gab1 phosphorylation, we used siRNA targeting Lyn and Lck. Only Lyn knock-down in H292/SHP2\(^{E76K}\) cells decreased pGab1
levels, indicating that Lyn is the mediator of SHP2$^{E76K}$ induced Gab1 phosphorylation [60]. These results indicate that GOF SHP2 mutants potentially induce Gab1 phosphorylation through activation of the SFKs.

To confirm that this mechanism of Gab1 activation is specific to GOF SHP2 mutants, we tested the erythroleukemia cell line Hel as a control. Cell transformation and aberrant tyrosine phosphorylation events in this cell line are mediated by the GOF Jak2$^{V617F}$ mutant. Ruxolitinib but not Dasatinib treatment inhibited Gab1 tyrosine phosphorylation in Hel cells (Fig. 37). To verify the specificity of these two inhibitors, we confirmed that Ruxolitinib treatment decreased Jak2 phosphorylation but not Src phosphorylation, while Dasatinib reduced active Src but not pJak2 in these cells (Fig. 37).
3.5 Discussion

GOF SHP2 mutations are well known leukemic oncogenes but their contribution to carcinogenesis is mostly undefined [177, 178]. SHP2<sup>E76K</sup> is a constitutively active GOF SHP2 mutant found in human cancers, including NSCLC. To determine the role of SHP2<sup>E76K</sup> in lung tumorigenesis, we generated a novel Dox inducible CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mouse model. SHP2<sup>E76K</sup> expression promoted Ras/MAPK and Src pathway cell signaling by activating the Erk1/2 and Src kinases and increasing c-Myc levels.

After 6 months of Dox induction, the majority of CCSP-rtTA/tetO-SHP2<sup>E76K</sup> bitransgenic mice displayed atypical adenomatous hyperplastic lesions and 31% developed adenomas. At the 9 months time point, 87% of Dox-induced CCSP-rtTA/tetO-SHP2<sup>E76K</sup> bitransgenic mice displayed adenomas and adenocarcinomas, whereas only 15% of control mice of the same inbred strain developed lung tumors. Moreover, the tumors found in control mice were notably smaller compared to those found in bitransgenic CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mice, indicating that SHP2<sup>E76K</sup> expression might trigger earlier tumor onset, faster tumor growth, or both. The fact that no control mice developed tumors at the 6 months time point further supports this notion. These results demonstrate that the SHP2<sup>E76K</sup> mutant is a driver of...
carcinogenesis in lung epithelial cells and describe a novel role for SHP2 as a lung cancer oncogene.

The majority of Dox induced CCSP-rtTA/tetO-SHP2^{E76K} bitransgenic mice displayed a tumor latency longer than 6 months. A potential explanation for this phenomenon is that endogenous wild type SHP2 competes with the SHP2^{E76K} mutant for the same docking proteins thus reducing the transforming effect of SHP2^{E76K}. This does not seem to be the case in our mice though, since SHP2^{E76K} expression positively regulated cell signaling in the lungs of our bitransgenic mice. An alternative mechanism is that SHP2^{E76K} expression induces genomic instability leading to the acquisition of one or more secondary mutations that promote the expansion of proliferative lesions. The validity of this multigenic hypothesis is supported by the fact that CCSP-rtTA/tetO-Myc mice display a long tumor latency of 300 days. The lung tumors observed in these mice acquire a secondary Kras mutation. Moreover, tumor development is also accelerated in mice by exposure to a chemical carcinogen or breeding onto a high Mcl1 background [174]. Consistent with our previous finding that SHP2 upregulates c-Myc in lung carcinoma cells in culture, we observed increased c-Myc levels in the lungs of Dox-induced CCSP-rtTA/tetO-SHP2^{E76K} bitransgenic mice. Myc plays an important role in cell proliferation and might significantly contribute to SHP2^{E76K} mediated tumorigenesis [153].

If the multigenic hypothesis of SHP2^{E76K} mediated lung tumorigenesis is correct, then tumor growth and survival should be independent of SHP2^{E76K} expression. We investigated this concept by taking advantage of the reversible feature of our Dox inducible transgenic mouse model. We used an MRI approach to detect lung tumors in
Dox induced bitransgenic mice. We then withdrew doxycycline for a month and performed a second round of MRI. The MR images and follow-up histological evaluation revealed that lung tumors not only stopped growing, but regressed in the absence of SHP2<sup>E76K</sup> expression. These results indicate that tumor maintenance in Dox induced CCSP-rtTA/tetO-SHP2<sup>E76K</sup> mice is dependent on sustained SHP2<sup>E76K</sup> expression and that SHP2<sup>E76K</sup> is the main driver of lung carcinogenesis in this bitransgenic mouse model.

Previous work has shown that augmentation of PTP activity is not sufficient to induce cellular transformation. SHP2 must first be recruited to the cell membrane and associate with docking proteins to function properly [33, 179]. This association is mediated by phosphorylation of the SHP2 binding sites on the docking proteins. An important question is since the GOF SHP2<sup>E76K</sup> mutant is clearly functional in the lungs of our bitransgenic mice, how is phosphorylation of the docking protein maintained in these animals? A potential answer is that GOF SHP2 mutants can positively regulate the phosphorylation of their own docking protein. This notion is supported by previous research in our lab showing that SHP2 knock-down in H292 cells impairs basal and EGF stimulated tyrosine phosphorylation of the Gab1 SHP2 binding sites Y627 and Y659 [153].

Since Gab1 is the main SHP2 docking protein and was identified as a top candidate in our mass spectrometry experiment, we started by evaluating the tyrosine phosphorylation of the SHP2 binding sites. We identified higher levels of pGab1 in the lungs of our bitransgenic mice, TF-1/SHP2<sup>E76K</sup> cells, and H292/SHP2<sup>E76K</sup> cells. These data indicate that SHP2<sup>E76K</sup> does indeed autoregulate the tyrosine phosphorylation of
Gab1 and its binding to this docking protein. Additional experiments using PTK inhibitors revealed that EGFR or SFKs inhibition by Erlotinib or Dasatinib reduces pGab1 levels in H292/SHP2\textsuperscript{E76K} and H661 cells. This effect can be replicated using SFKs siRNAs in these cells. More specifically, Lyn knock-down had the strongest impact on Gab1 phosphorylation in H661 cells. Since SHP2 knock-down has a negative effect on SFKs activation and SHP2\textsuperscript{E76K} expression increases pSrc levels in cell culture models and the lungs of CCSP-rtTA/tetO-SHP\textsuperscript{E76K}, our results suggests that the SHP2\textsuperscript{E76K} mutant promotes its own activation by activating the SFKs and triggering a positive feed-forward loop system [153].

Figure 38. SHP2\textsuperscript{E76K} mechanism of action in CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice. EGFR phosphorylates Gab1 and recruits SHP2\textsuperscript{E76K} which induces activation of the Lyn kinase. Lyn then phosphorylates Gab1, initiating a positive feedback loop.

In conclusion, we have described a novel role for the GOF SHP2\textsuperscript{E76K} mutation as an oncogene in lung carcinoma by generating a Dox inducible transgenic mouse model.
that exhibits a disease phenotype similar to NSCLC patients. We also described a new mechanism of GOF SHP2 mutant activation through a positive feedback loop involving the docking protein Gab1 and the SFKs.
Chapter 4: Concluding remarks

The protein tyrosine phosphatase SHP2 plays an important role in normal cellular function. This protein is expressed ubiquitously and positively regulates multiple signaling pathways, including the Ras/MAPK and Src cell signaling pathways resulting in increased cell proliferation, survival, and motility [34, 36, 47, 180]. Because of its extensive function, SHP2 also plays a prominent role in cancer [58]. GOF SHP2 mutations are known leukemic oncogenes, and SHP2 acts as a cancer essential gene through over-expression or amplification in several types of malignancies [55, 59, 177]. Since the role of SHP2 in lung cancer is incompletely defined, the studies described in this dissertation aim to elucidate the oncogenic potential of a GOF SHP2 mutant \textit{in vitro} and \textit{in vivo} and to figure out whether SHP2 is a synthetic lethal gene in GOF EGFR mutant dependent lung cancer.

4.1 Chapter 2 summary and conclusions

In the second chapter of this dissertation, we aimed to establish SHP2 as a synthetic lethal gene in GOF EGFR mutant dependent lung cancer. To achieve this goal, we performed \textit{in vitro} experiments and generated a novel Dox inducible transgenic mouse model expressing the dominant negative SHP2^{CSDA} mutant under the control of the CCSP promoter to direct expression to lung epithelial cells.
Using two GOF EGFR mutant dependent lung cancer cell lines, we showed that interfering with normal SHP2 function through shRNA knock-down, siRNA knock-down, PTP catalytic domain inhibition, or expression of the dominant negative SHP2\textsuperscript{CSDA} mutant reduced cell proliferation and Ras/MAPK cell signaling pathway activity. These results indicate that targeting SHP2 \textit{in vitro} can have a significant effect on GOF EGFR mediated cell signaling and proliferation.

Based on the previous results, we generated a Dox inducible transgenic mouse model and bred bitransgenic CCSP-\textsc{rtTA/tetO-EGFR}\textsuperscript{L858R} mice and tritransgenic CCSP-\textsc{rtTA/tetO-EGFR}\textsuperscript{L858R}/tetO-SHP2\textsuperscript{CSDA} mice. Expression of the SHP2\textsuperscript{CSDA} mutant in the lungs of tritransgenic mice decreased EGFR\textsuperscript{L858R} mediated Ras/MAPK and Src cell signaling pathway activation, leading to delayed tumor onset and reduced tumor burden.

To elucidate the mechanism of action of the SHP2\textsuperscript{CSDA} mutant in the lungs of tritransgenic mice, we performed a mass spectrometry analysis and identified Gab1 as one of the main binding partners of SHP2\textsuperscript{CSDA}. Further experiments \textit{in vitro} revealed that GOF EGFR mutants phosphorylated Gab1 on its SHP2 binding site, and that GOF EGFR mutant inhibition caused a dissociation of the Gab1/SHP2 complex which might be responsible for the decreased levels of pErk1/2, pSrc, and cell proliferation. These results were further supported by showing that EGFR\textsuperscript{L858R} expression in the lungs of bitransgenic mice increased pGab1 levels and Gab1/SHP2 association, while SHP2\textsuperscript{CSDA} expression in tritransgenic mice countered these effects and fully disrupted Gab1/SHP2 complex formation.
SHP2 is required to promote wild type EGFR mediated cell signaling [34, 150, 151]. In this project we demonstrated that SHP2 is also required to transduce GOF EGFR mutant cell signaling and that targeting SHP2 has significant effects on GOF EGFR mutant mediated lung tumorigenesis. Our work and others describes a novel role for SHP2 as a synthetic lethal gene in GOF EGFR mutant dependent lung cancer [144]. In a time where alternative options to EGFR tyrosine kinase inhibitors are urgently needed, our results establish the potential of SHP2 as a novel therapeutic target for drug development to treat GOF EGFR mutant dependent lung cancer patients.

4.2 Chapter 3 summary and conclusions

In the third chapter of this dissertation we determined whether the GOF SHP2\(^{E76K}\) mutant can induce tumor formation in the lungs of bitransgenic CCSP-rtTA/tetO-SHP2\(^{E76K}\) mice. We generated a novel doxycycline inducible transgenic mouse model using the tet-on approach to induce transgene expression and used a recombinant Clara cell secretory protein promoter to direct expression of the GOF SHP2\(^{E76K}\) mutant to lung epithelial cells.

SHP2\(^{E76K}\) expression in the lungs of bitransgenic mice following one month of Dox induction significantly increased levels of pErk1/2, pSrc, Myc and Mdm2. Myc is a downstream target of SHP2 and an important driver of cell proliferation [153]. Elevated Myc levels combined with the increased Ras/MAPK and Src cell signaling pathways activation are most likely responsible for the appearance of atypical adenomatous hyperplastic lesions and adenomas after 6 months of Dox induction. At the nine months time point, a significant majority of bitransgenic CCSP-rtTA/tetO-SHP2\(^{E76K}\) mice
developed adenomas compared to wild type or monotransgenic controls. These results establish the GOF SHP2\textsuperscript{E76K} mutant as a novel driver oncogene of lung tumorigenesis in our mouse model.

Since our bitransgenic mice displayed such a long latency to tumor development, we suspected that the animals had acquired secondary mutations responsible for tumor formation. Despite the presence of these additional mutations, we hypothesize that SHP2\textsuperscript{E76K} remained the main driver of tumorigenesis and that sustained transgene expression was required for tumor maintenance. To test this hypothesis, we took advantage of the reversible feature of our transgenic mouse model. We used MRI to check for the presence of lung tumors in bitransgenic mice after nine months of Dox induction then withdrew doxycycline for a month. A second round of MRI revealed that the lung tumors had either regressed or completely disappeared indicating that while it is highly likely that our bitransgenic mice acquired secondary mutations, the main driver of tumorigenesis remained the GOF SHP2\textsuperscript{E76K} mutant.

In an effort to determine the mechanism of SHP2\textsuperscript{E76K} driven tumor formation in the lungs of our bitransgenic CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice we performed a mass spectrometry analysis to identify the binding partners of the SHP2\textsuperscript{E76K} mutant. The docking protein Gab1 was identified as one of the top candidates. Previous work has shown that solely enhancing the catalytic activity of SHP2 through acquisition of a GOF mutation is not sufficient to increase cell signaling, the GOF SHP2 mutant must be recruited to the cell membrane to properly function [33, 34]. Gab1 is one of the main docking proteins of SHP2 and association between the two proteins leads to SHP2 relocation to the cell membrane and activation [35]. Interestingly, we demonstrated that
GOF SHP2 mutants can increase the phosphorylation level of Gab1 both *in vitro* and *in vivo*, indicating that mutant SHP2 can auto-regulate the phosphorylation of its own docking protein. Further work revealed that this mechanism is most likely mediated through recruitment of the SFKs and is specific to GOF SHP2 mutants.

GOF SHP2 mutants are well known leukemic oncogenes, but their contribution to carcinogenesis in solid tumors is mostly unknown. According to the COSMIC database, GOF SHP2 mutations, including the SHP2$^{E76K}$ mutation used in our transgenic mouse model, have been identified in about two percent of lung cancer cases, but their potential contribution to tumorigenesis is unclear. Due to the high prevalence of lung cancer, even two percent of cases accounts for a large number of patients. In this project, we describe for the first time that a GOF SHP2 mutant can act as an oncogene in lung epithelial cells, meaning that the GOF SHP2 mutations identified in lung cancer patients could potentially be contributing or even driving carcinogenesis. Our results establish SHP2 as a potential novel lung cancer oncogene and highlight a need to further investigate the role of SHP2 in lung cancer to determine its potential as a therapeutic target for drug development.
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