SHP2\textsuperscript{E76K} mutant promotes lung tumorigenesis in transgenic mice

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Lung cancer is a major disease carrying heterogeneous molecular lesions and many of them remain to be analyzed functionally in \textit{vivo}. Gain-of-function (GOF) SHP2 (PTPN11) mutations have been found in various types of human cancer, including lung cancer. However, the role of activating SHP2 mutants in lung cancer has not been established. We generated transgenic mice containing a doxycycline (Dox)-inducible activating SHP2 mutant (tetO-SHP2\textsuperscript{E76K}) and analyzed the role of SHP2\textsuperscript{E76K} in lung tumorigenesis in the Clara cell secretory protein (CCSP)\textsuperscript{-}reverse tetracycline transactivator (rtTA)/tetO-SHP2\textsuperscript{E76K} bitransgenic mice. SHP2\textsuperscript{E76K} activated Erk1/Erk2 (Erk1/2) and Src, and upregulated c-Myc and Mdm2 in the lungs of bitransgenic mice. Atypical adenomatous hyperplasia and small adenomas were observed in CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} bitransgenic mice induced with Dox for 2–6 months and progressed to larger adenoma and adenocarcinoma by 9 months. Dox withdrawal from bitransgenic mice bearing genetic resonance imaging-detectable lung tumors resulted in tumor regression. These results show that the activating SHP2 mutant promotes lung tumorigenesis and that the SHP2 mutant is required for tumor maintenance in this mouse model of non-small cell lung cancer. SHP2\textsuperscript{E76K} was associated with Gab1 in the lung of transgenic mice. Elevated pGab1 was observed in the lung of Dox-induced CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice and in cell lines expressing SHP2\textsuperscript{E76K}, indicating that the activating SHP2 mutant autoregulates tyrosine phosphorylation of its own docking protein. Gab1 tyrosine phosphorylation is sensitive to inhibition by the Src inhibitor dasatinib in GOF SHP2-mutant-expressing cells, suggesting that Src family kinases are involved in SHP2 mutant-induced Gab1 tyrosine phosphorylation.

Introduction

Because protein tyrosine phosphatases (PTPs) counter the biochemical reaction of protein tyrosine kinases (PTKs) and many PTKs have oncopgenic activity, PTPs were mostly perceived as tumor suppressors. However, increasing evidence suggests that in some cases PTPs cooperate with PTKs to promote cell signaling and oncogenesis ([1,2]). It is known that certain tyrosine phosphorylation sites exert an inhibitory effect on enzyme activity or trigger a signaling termination response ([3–5]). For instance, phosphorylation of CDK1 Tyr(Y)-15 or SRC Y530 residue inhibits these kinases ([4,5]). In response to the epidermal growth factor receptor (EGF)-induced tyrosine phosphorylation events, p120RasGAP and the E3 ubiquitin ligase c-CBL are recruited to specific tyrosine phosphorylated sites to terminate EGFR signaling ([6–8]). Therefore, dephosphorylation of CDK1 Y15 and SRC Y530 activates these kinases, whereas dephosphorylation of p120GAP and c-CBL SH2 domain docking sites prolongs the PTK-induced signaling pathway activation. This notion of positive cooperation between PTKs and PTPs is exemplified by SHP2.

SHP2 is a classical, non-receptor PTP encoded by the PTPN11 gene ([9]). SHP2 consists of two SH2 domains, a PTP domain and a C-terminal region ([9]). In the wild-type SHP2, the backside of the N-SH2 domain binds to the PTP domain, resulting in autoinhibition of the PTP catalytic activity ([10]). Binding of the SHP2 SH2 domains to specific phosphotyrosine docking sites such as Gab1 in response to tyrosine kinase activation induces a conformational change that leads to SHP2 PTP activation ([11]). Activated SHP2 mediates RAS-ERK1/2 and SRC activation ([9,12,13]). SHP2 was shown to be required for transformation of NIH3T3 cells by oncogenic FGFR3 mutant ([14]). Experiments with short hairpin RNAs and a dominant-negative, PTP-inactive SHP2 mutant demonstrated that SHP2 is required for tumor growth of H292 and DU145 carcinoma cells ([15]).

Besides activation via binding of its SH2 domains to phosphotyrosine-based docking sites, activating SHP2 mutations that disrupt the autoinhibitory mechanism have been found in human cancer ([16–18]). SHP2 mutations are most frequently observed in hematologic malignancies, including 35% of juvenile myelomonocytic leukemia, 5–10% of childhood myelodysplastic syndrome, 7% of B-cell precursor acute lymphoblastic leukemia, and some cases of pediatric and adult acute myelogenous leukemia. In addition to hematologic diseases, SHP2 mutations also occur in solid tumors such as lung, colon and prostate carcinomas ([19–21]). The SHP2 mutation rate in lung cancer (1.81%) ranks in third after colon (5.98%) and endometrial cancer (4.27%) among carcinomas in the Catalogue of Somatic Mutations in Cancer (COSMIC) database ([www.sanger.ac.uk]). Although the SHP2 mutation rates in carcinomas are lower than those in hematologic malignancies, it is nevertheless not insignificant.

Lung cancer is a heterogeneous disease comprising many molecular subtypes. Besides the major non-small cell lung cancer (NSCLC) driver ongenes \textit{Kras}, \textit{Egfr} and \textit{Alk} that are mutated in >5% of NSCLC, several of the known or potential lung cancer ongenes are mutated at <5% rates. For example, the recently identified RET fusion genes occur in 1–2% of NSCLC ([22–24]). However, because lung cancer is a major lethal disease, a small percentage of mutation could represent a large number of affected patients and thus should not be ignored, especially for mutations that are actionable for developing new targeted therapies ([25]).

Cancer-associated SHP2 mutations are prevalent in the interface between the N-SH2 domain and the PTP domain ([19]). In particular, E76 located in the N-SH2 domain is the most frequently mutated residue in human cancer. Previous studies have shown that retroviral expression of cancer-associated SHP2 mutants E76K, D61Y and D61V in mouse bone marrow cells or human cytokine-dependent myeloid cells induced their transformation ([26–29]). Conditional expression of SHP2 D61Y or E76K mutant in hematopoietic cells of knock-in mice caused fatal myeloproliferative disorder ([30,31]). These studies have established mutant SHP2 as a driver oncogene in hematologic malignancies.

Although gain-of-function (GOF) SHP2 mutations have been detected in several types of carcinoma, very little is known about the oncogenic activity of SHP2 mutants in carcinoma. It was reported that a SHP2 T507K mutation identified in liver cancer could transform NIH3T3 cells ([32]), which may be due in part to a change in
substance specificity (33). SHP2 mutations, including E76 mutations, have been identified in lung cancer. However, it is unclear if any of these SHP2 mutants has oncogenic activity in lung carcinoma. In this study, we created doxycycline (Dox)–inducible SHP2E76K transgenic mice to generate a mouse model to study the role of the activating SHP2 mutant in the lung adenocarcinoma.

Clara cell secretory protein (CCSP)-reverse tetracycline transactivator (rtTA) transgenic mice contain a rat CCSP promoter-driven rtTA to regulate tetO activity in type II lung epithelial cells (34). CCSP-rtTA mice have previously been used in mouse models of NSCLC (35–37). To assess if SHP2E76K induces lung tumor development, we crossed tetO-SHP2E76K mice with CCSP-rtTA mice and analyzed lung tumorigenesis in Dox-induced CCSP-rtTA/tetO-SHP2E76K bitransgenic mice. Our results demonstrate that the SHP2E76K mutant induces lung adenomas and adenocarcinomas and that these lung tumors are dependent on continued expression of this oncogene to maintain tumor growth.

Materials and methods

Generation of transgenic mice

Construction of the L3/L2-tetO vector is described in Supplementary Materials and Methods, available at Carcinogenesis Online. The DNA fragment containing a human SHP2E76K mutant was excised from a pCDNA3 vector (29) by Pmel/EcoRI and subcloned into the EcoRV site between the tetO and polyA sequences of L3/L2-tetO plasmid. The transgene was excised from the vector by digestion with BssHIII 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 ethylenediaminetetraacetic acid, pH 7.5) and microinjected at 3 ng/µl into 0.5 dpc fertilized FVB/N zygotes. Zygotes were surgically implanted into the oviducts of 0.5 dpc pseudopregnant CD-1 females for development. Offspring were tail biopsied at weaning and genomic DNA screened by PCR (see Supplementary Materials and Methods, available at Carcinogenesis Online) to identify transgenic lines.

CCSP-rtTA transgenic mice (in inbred FVB/N background) (34) were provided by Dr Jeffrey A. Whitsett. Animals were maintained in specific pathogen-free housing conditions. To activate the transactivating function of the rtTA protein, mice were fed with rodent chow containing 200 ppm Dox (Dox diet, Bio-Serv). 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.

Reverse transcription–PCR analysis of SHP2E76K messenger RNA expression

Tissue samples were snap frozen in liquid nitrogen. RNA was extracted using Trizol reagent (Life Technologies). Samples were treated with DNase I (Life Technologies) to avoid DNA contamination and reverse transcription–PCR (RT–PCR) was performed using the SuperScript One-Step RT–PCR Platinum Bq system (Life Technologies) with the following primers: SHP2F1: 5′-GGTTGCAAGAACGATTCAAG-3′ and SHP2R2: 5′-AGGCTCTCGATCCACTCG-3′. The protocol for a 50 µl RT–PCR reaction was as follows: 30 min complementary DNA synthesis at 55°C, 4 min denaturation at 94°C then 35 cycles of 94°C for 30 s, 57°C for 30 s, then 72°C for 30 s with a final extension step of 72°C for 4 min, which yields a 462 bp fragment.

Histological and immunohistochemical examination

After euthanasia, the mouse lungs were flushed twice with 10 ml phosphate-buffered saline and insufflated with 10% buffered formalin. After fixation overnight at 4°C, the 10% buffered formalin solution was decanted, and formalin blocks were prepared by standard procedure by the Histology Service of the Tissue Core of the Moffitt Cancer Center. Sections (4 µm thick) were stained with hematoxylin and eosin (H&E) for histological examination. For immunohistochemical analysis of pErk1/2, slides were stained using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ). Slides were deparaffinized with EZ Prep solution (Ventana). Heat-induced antigen retrieval method was used in Cell Conditioning 1 (Ventana) and 2. Antibody anti-pErk1/2 (#4376, Cell Signaling, Danvers, MA) was used at a 1:200 dilution in PSS diluent (Ventana) and incubated for 30 min. Anti-rabbit secondary antibody (Ventana) was used for 20 min. The detection system used was the Ventana Omniplex kit and slides were counterstained with hematoxylin.

Immunoblotting, immunoprecipitation, kinase assay and mass spectrometry

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), phosho-Akt (pAkt) and phospho-Src (pSrc) antibodies were from Cell Signaling Technology. Anti-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 described as (38,39). The anti-p53 antibody was from IMMGENEX (San Diágo, CA).

Frozen tissues were crushed and lysed with lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM ethyleneglycol-bis(aminomethyl)-tetraacetic acid, 25 mM NaF, 5 mM Na3PO4, 1 mM diithiothreitol, 1 mM Na2VO4, 100 µg/ml phenylmethylsulfonyl fluoride, 2 µg/ml leupeptin, 2 µg/ml apronin and 1% Triton X-100). Equal amounts of proteins from cleared tissue lysate supernatants were separated by 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose filters for immunoblotting. Flag-tagged SHP2 was immunoprecipitated from cleared tissue lysate supernatants by using the anti-Flag M2 antibody and Protein-G agarose. Immunoblotting was performed as described previously (15,29).

Cells were cultured and cell lysates were prepared for immunoblotting or immunoprecipitation analyses similar to that described previously (15,29). Methylcellose colony formation assay was performed as described (29). Nutlin-3 was from ENZO Life Sciences (Farmingdale, NY). Tyrosine kinase inhibitors were from LC Laboratories (Woburn, MA). LYN kinase activity in TF-1 cells was measured by an immune complex kinase assay similar to that described (12). For knockdown experiments, 3 × 105 cells in six-well plates were transfected with 100 pmol of small interfering RNAs (siRNAs; On-TARGETplus SMARTpool, Fisher Scientific) using lipofectamine 2000.

Protein identification by mass spectrometry was performed by the Proteomics Core of the Moffitt Cancer Center 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 (for SHP2E76K) entries. Results from Mascot were compiled in Scaffold.

Quantitative RT–PCR

Quantitative RT–PCR was performed using Power SYBR Green reagents (Applied Biosystems) and proprietary primers for 18s ribosomal RNA 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 quantitative PCR. Data were normalized using 18s ribosomal RNA as the internal control and analyzed using the SDS software version 2.3.

Magnetic resonance imaging protocol

Magnetic resonance imaging (MRI) protocol is provided in the Supplementary Materials and Methods, available at Carcinogenesis Online.

Statistical analysis

Statistical methods used for data analysis are indicated in the legends of Figures 2 and 3.

Results

Generation of inducible SHP2E76K transgenic mice

We modified the tetracycline-inducible tet-op-mpl transgenic vector (35) that contains seven copies of the tet operator by placing tandem repeats of chicken β-globin insulator sequence (cSH4) (40) upstream of tetO and then flanking the transgenic cassette with a pair of oppositely oriented heterologous L3 and L2 loxP sites (41). This L3/L2-tetO vector (Figure 1A) was designed to be capable of undergoing Cre-recombinase-mediated cassette exchange (RMCE) (41). SHP2E76K is a constitutively active SHP2 mutant (29,42). To generate transgenic mice containing Dox-inducible SHP2E76K, a C-terminal Flag-tagged human SHP2E76K coding sequence was subcloned into L3/L2-tetO to generate the tetO-SHP2E76K transgenic construct (Figure 1B). By design, controlled expression of SHP2E76K in the progenitor cells of NSCLC can be achieved by crossing tetO-SHP2E76K transgenic mice with CCSP-rtTA transgenic mice (34) and feeding the CCSP-rtTA/tetO-SHP2E76K bitransgenic mice with Dox containing chow (Figure 1B).

Transgenic mice were generated by microinjecting the 5.8 kb BssHIII DNA fragment containing the tetO-SHP2E76K transgenic
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Histological examination indicates that these tumors in bitransgenic mice had tumors in the lung (P < 0.05). Dox-induced lung tumors in transgenic mice regress after Dox withdrawal (9). Atypical adenomatous hyperplasia was observed in CCSP-rtTA/tetO-SHP2E76K bitransgenic mice had small lung adenomas (Fig. 3, Supplementary Table 1, available at Carcinogenesis Online). At 9 months after Dox induction, 13 of 15 CCSP-rtTA/tetO-SHP2E76K bitransgenic mice had tumors in the lung (Fig. 3, Supplementary Figure 3 and Supplementary Table 1, available at Carcinogenesis Online). Compared with the 6 months time point, tumors at 9 months were larger in size and some had progressed to adenocarcinomas (defined as tumors ≥5 mm in diameter (46) (Fig. 3B)). Histological examination indicates that these tumors were papillary or mixed subtypes of adenomas and progressed to mixed subtypes and solid adenocarcinomas (Supplementary Table 1, available at Carcinogenesis Online) (47).

In contrast, none of 13 wild-type, tetO-SHP2E76K or CCSP-rtTA monotransgenic mice used as littermate controls of the above bitransgenic mice developed any lung tumor after 6 months of Dox induction. At the 9 months Dox-treatment time point, one wild-type and one tetO-SHP2E76K monotransgenic mice among 13 mice had lung adenomas. Moreover, tumors from these two mice were much smaller than those from CCSP-rtTA/tetO-SHP2E76K bitransgenic mice (Fig. 3B and C). Two mice among 24 wild-type, tetO-SHP2E76K or CCSP-rtTA monotransgenic mice had tumors at 12 months after Dox induction. Both of them occurred in the wild-type mice and one of these tumors was squamous cell carcinoma. Statistical analysis indicated that Dox-induced CCSP-rtTA/tetO-SHP2E76K bitransgenic mice had a statistically significant (P < 0.0001) increase in lung tumorigenesis (Fig. 3C). These data clearly show that SHP2E76K promotes lung tumorigenesis that resembles NSCLC in this mouse model.

Lung tumors in transgenic mice regress after Dox withdrawal

Recent results indicate that the capacity of MMR detection of lung tumors in small animals. In pilot trials, we dissected mice after MMR analyses and verified the presence of lung tumors corresponding to the MMR-detected tumor masses in the lung (Supplementary Figure 4, available at Carcinogenesis Online). To determine if continued SHP2E76K expression is required for lung tumor maintenance, we identified two CCSP-rtTA/tetO-SHP2E76K mice in which Dox-induced lung tumors were detected by T2-weighted MRI at 7T (Fig. 4A). Dox diet was then replaced with regular chow. When these mice were examined again by MRI 1 month after Dox withdrawal, the lung tumors were no longer detectable (Fig. 4A). After the second MRI, lung tissues were collected for further analysis. Histological examination revealed residual hyperplastic lesions and scar tissue in H&E slides from areas corresponding to where the tumors were detected by MRR prior to Dox withdrawal (Fig. 4B). Thus, both
radiological and histological data demonstrated that the lung tumors regressed after deinduction of SHP2E76K in these bitransgenic mice, suggesting that the lung tumors at this stage remain dependent on continued expression of SHP2E76K.

To assess SHP2E76K expression after Dox withdrawal, we analyzed lung tissues of these two mice for the presence of SHP2E76K mRNA and protein. As shown in Figure 4C, neither SHP2E76K mRNA nor protein was detected in these lung tissues, consistent with data shown in Figure 2 that SHP2E76K expression was Dox-dependent in the CCSP-rtTA/tetO-SHP2E76K bitransgenic mice. In addition, intense pErk1/2 staining was observed in every lung tumor that we have analyzed (n = 4) from Dox-induced CCSP-rtTA/tetO-SHP2E76K mice as represented in Figure 4D. After the Dox withdrawal, the pErk1/2 immunohistochemical stain intensity was similar to that of the wild-type and monteransgenic mice (n = 5; Figure 4D).

In a subsequent experiment, we extended the MRI analysis of lung tumors to four additional CCSP-rtTA/tetO-SHP2E76K bitransgenic mice that were Dox-induced for 7–8 months. All of them showed tumor regression after Dox withdrawal (Supplementary Figure 5, available at Carcinogenesis Online).

**SHP2E76K autoregulates its docking protein Gab1**

We immunoprecipitated SHP2E76K from the lung tissue of a Dox-induced CCSP-rtTA/tetO-SHP2E76K mouse. Immunoprecipitates were separated on a sodium dodecyl sulfate–polyacrylamide gel. Gel slides corresponding to phosphotyrosine bands in the immunoblot were analyzed by mass spectrometry to identify proteins in these bands. Proteins identified in these gel slides that have been observed previously to be tyrosine-phosphorylated proteins are shown in Figure 5A. Gab1, but not other Gab family of docking proteins, were among these proteins. It is known that a constitutively active SHP2 is non-functional if it lacks intact SH2 domains (11,26). This indicates that both an activated PTP as well as SHP2 docking to a specific scaffold protein are necessary for the cellular function of SHP2. Because SHP2 binding to Gab1 or Gab2 has been demonstrated to be essential for SHP2 signaling and transformation activity (11,26), we focused our study here on Gab1.

Immunoprecipitation of Gab1 from the lung of Dox-induced CCSP-rtTA/tetO-SHP2E76K mouse confirmed Gab1 tyrosine phosphorylation and binding to SHP2E76K (Figure 5B). Moreover, pGab1 level was higher in Dox-induced CCSP-rtTA/tetO-SHP2E76K mice.
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than that in the wild-type or bitransgenic mouse after Dox withdrawal (Figure 5C). In TF-1 and H292 cells, SHP2E76K induced Gab1 tyrosine phosphorylation and SFKs were activated (Figure 5D and E). These data indicate that SHP2E76K can autoregulate tyrosine phosphorylation of its own docking protein Gab1.

To assess which PTK may be involved in GAB1 tyrosine phosphorylation, we treated H292/SHP2E76K cells with various concentrations of the JAK, SFK or EGFR inhibitors ruxolitinib, dasatinib or erlotinib and then analyzed GAB1 tyrosine phosphorylation. ruxolitinib (up to 30 μM) did not affect GAB1 tyrosine phosphorylation, whereas both dasatinib and Erlotinib inhibited GAB1 tyrosine phosphorylation in H292 cells (Figure 5F). The effect of dasatinib on pGAB1 was detectable at the lowest concentration that we tested in H292/SHP2E76K cells (0.2 μM). In the vector control H292 cells (H292/V), the basal pGAB1 level was very low and EGF increased the GAB1 tyrosine phosphorylation. Higher concentrations of dasatinib (≥1 μM) were needed to inhibit EGF-stimulated GAB1 tyrosine phosphorylation (Supplementary Figure 6, available at Carcinogenesis Online).

In another control experiment, we treated HEL cells with dasatinib and ruxolitinib. HEL cells contain a constitutively active JAK2V617F mutant and thus the aberrant tyrosine phosphorylation events in this cell line were mainly attributed to the JAK2V617F activity. ruxolitinib but not dasatinib inhibited GAB1 tyrosine phosphorylation in HEL cells (Supplementary Figure 7, available at Carcinogenesis Online). Consistent with the specificities of these two inhibitors, control immunoblots showed that ruxolitinib reduced active JAK2 but not active SRC in HEL cells, whereas dasatinib reduced active SRC but not JAK2 in these cells.

H661 is a lung cancer cell line harboring a GOF (N58S) mutation in the N-SH2 domain of SHP2. As shown in Figure 5G, GAB1 tyrosine phosphorylation and GAB1-SHP2 association were sensitive to dasatinib in H661 cells, suggesting that SFK is involved in GAB1 tyrosine phosphorylation in H661 cells. Using siRNAs, we successfully knocked down c-SRC in H661 cells (Figure 5H). In agreement with the experiment using the SFK inhibitor dasatinib, knocking down of c-SRC in H661 cells reduced the pGAB1 level. Besides c-SRC, H292 cells express three SFKs (c-SRC, LYN and LCK) at high levels (48). Knockdown of LYN was most effective to reduce pGAB1 level in H292/SHP2E76K cells (Figure 5H).

Discussion

Besides hematologic malignancies, GOF SHP2 mutations are found in human carcinomas such as NSCLC (19,21), but their contribution to carcinogenesis is largely undefined. SHP2E76K is a constitutively activated GOF SHP2 mutant found in human cancers, including NSCLC. In this study, we generated Dox-inducible tetO-SHP2E76K transgenic mice and evaluated the role of the SHP2 mutant in lung tumorigenesis using the CCSP-rTA-driven tetO transgenic mouse model of NSCLC. At the 9 months time point, lung tumor burden was found in 87% of Dox-induced CCSP-rTA/tetO-SHP2E76K bitransgenic mice, whereas only 15% of control mice of the same inbred strain developed lung tumors. Moreover, tumors in the bitransgenic mice were notably larger compared with those in the control mice, suggesting that either the hyperproliferative lesions occurred earlier in time, tumors grew faster or both in the SHP2E76K-expressing

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In support of this notion, 31% of the Dox-induced CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} bitransgenic mice developed lung tumors by 6 months. These data demonstrate that the GOF SHP2 mutant can promote lung tumorigenesis. Most of the Dox-induced CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} bitransgenic mice had a tumor latency of \(>6\) months. One possible explanation is that in our transgenic mouse model, besides the SHP2\textsuperscript{E76K} mutant, the endogenous wild-type SHP2 is present in the same cells that could reduce the effect of SHP2\textsuperscript{E76K} by competing for the same docking proteins. However, this does not appear to be the main reason because we could detect the biochemical signaling effects of SHP2\textsuperscript{E76K} in the lungs of Dox-induced bitransgenic mice (Figure 2). Another possible explanation is that one or more secondary mutational events, such as tumor suppressor gene mutations, collaborate with SHP2\textsuperscript{E76K} expression to allow expansion of the proliferative lesions. Compatible with this multigenic hypothesis of lung tumorigenesis, it was reported that CCSP-rtTA/tetO-Myc mice displayed a long tumor latency of 300 days, Myc-induced lung tumors also acquired kras mutations and tumor development was accelerated in mice exposed to a chemical carcinogen or bred onto a high Mcl1 background (44). Consistent with our previous finding that SHP2 upregulates c-Myc in lung carcinoma cells in culture (15), we observed an increased Myc level in the lungs of Dox-induced CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} bitransgenic mice and the elevated Myc level dropped to normal after Dox withdrawal (Figure 5C).

An important question is whether the mutant SHP2-induced tumors require SHP2\textsuperscript{E76K} to maintain tumor growth. Unlike the conditional knock-in mice that are irreversible, an advantage of the Dox-inducible transgenic mouse model is that the transgene is readily reversible and can be used to address this important issue. We withdrew Dox diet from lung tumor-bearing CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} bitransgenic mice and examined the lesions again 1 month after deinduction. Our MRI and histological analyses reveal that lung tumors not only stopped growing, but regressed after cessation of SHP2\textsuperscript{E76K} expression. These data indicate that SHP2\textsuperscript{E76K} is required to maintain the lung tumors induced in this bitransgenic mouse model.

Although the PTP activity is essential for SHP2 signaling, it is not sufficient. It is known that a constitutively activated SHP2 without its SH2 domains docking to specific cellular SHP2 binding proteins are non-functional in the cells (11,26). In fact, the first SHP2 knockout mouse was a deletion of the N-SH2 domain (49), resulting in a highly active SHP2 but unable to bind its docking proteins. Most of the GOF SHP2 mutants found in human diseases are located in the interface between the N-SH2 and the PTP domains that do not affect the binding affinity of SHP2 to their phosphotyrosine-based binding sites. Thus, an important question is how do cells harboring these SHP2 mutations, such as SHP2\textsuperscript{E76K}, maintain an elevated tyrosine phosphorylation state on the SHP2 docking sites in order to mediate the biological function of the mutant SHP2?

Fig. 4. Lung tumors in CCSP-rtTA/tetO-SHP2\textsuperscript{E76K} mice regress after Dox withdrawal. (A) 3D FSE datasets (TE/TR = 64/1000 ms) demonstrating coronal sections of tumor-bearing mice before and 1 month after Dox withdrawal, as indicated. The tumor sizes were 27.2 (mouse #1) and 22.3 mm\(^3\) (mouse #2) prior to Dox withdrawal. Arrows in panel indicate the positions of tumors or where tumors were detected prior to Dox withdrawal. (B) H&E sections of lung tissue corresponding to where tumors were detected by MRI. Residual atypical adenomatous hyperplasia and scar tissues are indicated by arrows. (C) Lung tissues from Dox withdrawn mice were analyzed by RT–PCR (left) or immunoprecipitation-immunoblotting (right) to verify the absence of SHP2\textsuperscript{E76K} mRNA or protein following deinduction. (D) Immunohistochemical analysis of pErk1/2 in mouse lung tissues. Slides were processed under identical conditions in the same experiment using a Ventana Discovery XT automated system.
We found previously that knockdown of SHP2 in H292 cells reduced basal and EGF-stimulated GAB1 tyrosine phosphorylation on the SHP2 docking sites (pY627 and pY659) in H292 tumor xenografts and in cultured cells (15). This indicates that SHP2 mediates tyrosine phosphorylation of its own activating sites on GAB1. However, it was unclear if activating SHP2 mutations can induce GAB1 tyrosine phosphorylation. In this study, we have found increased Gab1 tyrosine phosphorylation in the lung tissues of transgenic mice, TF-1 cells and H292 cells that express exogenous SHP2E76K. These data indicate that SHP2E76K can autoregulate tyrosine phosphorylation of Gab1 and its binding to this docking protein. Our experiments using PTK inhibitors showed that Gab1 tyrosine phosphorylation in H292 and H661 cells are sensitive to the SFK inhibitor dasatinib and/or the EGFR inhibitor erlotinib. The effect of dasatinib is phenocopied by SFK siRNAs in these cells. Consistent with the observation that SHP2 knockdown reduces SFK activation (15), our data indicate that SHP2E76K activates SFKs. Previous studies have revealed two mechanisms by which SHP2 regulated SFK activation via regulation of CSK.

**Fig. 5.** SHP2E76K autoregulates Gab1 tyrosine phosphorylation. (A) Lung tissue from a Dox-induced CCSP-rtTA/tetO-SHP2E76K mouse was immunoprecipitated with an anti-Flag (M2) antibody. Immunoprecipitated proteins were eluted from the Protein-G agarose with a Flag peptide. One-tenth of the eluted immunoprecipitate was used for immunoblotting with an anti-pY antibody. The rest of eluted immunoprecipitate was processed for mass spectrometric identification of proteins from corresponding slides of Coomassie blue-stained gel. Major proteins (excluding keratins) identified in each band were searched against PhosphoSitePlus (www.phosphosite.org) database and those that have been reported as tyrosine-phosphorylated proteins are shown. (B) Lung tissue from a Dox-induced CCSP-rtTA/tetO-SHP2E76K mouse was immunoprecipitated with an anti-Gab1 antibody. The immunoprecipitate was analyzed by immunoblotting with antibodies to pGab1 (Y627) and Flag-tag. After removal of antibodies, the membranes were re-probed with antibodies to Gab1 and SHP2. (C) Immunoblot analyses of lung tissue lysates from the wild-type (W), Dox-induced CCSP-rtTA/tetO-SHP2E76K (P), or after Dox withdrawal of CCSP-rtTA/tetO-SHP2E76K mouse with MRI-detected tumor (A). (D) Left panels, Gab1 was immunoprecipitated from cytokine-starved TF-1 cells containing control vector (V), wild-type SHP2 (W) or SHP2E76K (K). The immunoprecipitates were analyzed by immunoblotting with antibodies to pY or SHP2. Right panels, LYN was immunoprecipitated and its tyrosine kinase activity was assayed using a glutathione S-transferase-GAB1 fusion protein (12) as the substrate. (E) H292 cells expressing a control vector (V), wild-type SHP2 or SHP2E76K (K) were analyzed by immunoblotting with indicated antibodies. Note that the anti-pSRC antibody cross-reacts with other SFKs. (F) H292/SHP2E76K cells were treated with indicated concentrations of ruxolitinib, dasatinib or erlotinib for 24h. Cell lysates were analyzed for pGAB1 by immunoblotting. (G) H661 cells were treated with dasatinib for 24h. Gab1 was immunoprecipitated from cell lysates and the immunoprecipitates were analyzed by immunoblotting with indicated antibodies (upper panels). Cell lysates were analyzed by immunoblotting as indicated (lower panels). (H) H292/SHP2E76K or H661 cells were transfected with non-targeting (NT), LYN or c-SRC (SRC) siRNAs or left untransfected (N). Cell lysates were prepared and analyzed by immunoblotting with indicated antibodies.
(12,13). However, we have not ruled out additional mechanism(s). Nevertheless, because SHP2 activates SFKs and SFKs are involved in the activation of SHP2 via phosphorylation of GAB1, our data suggest that SHP2E76K triggers a positive feedforward loop to regulate cell signaling.

Many transgenic mice produced by the traditional approach, in which transgenes are randomly integrated into the host chromosomes, either exhibit undesirable leaky expression or do not express transgenes in the desired tissues due to positional effects. Thus, new transgenic mice have to undergo costly and time-consuming characterization to identify suitable lines for further study. This is especially difficult for CreO transgenic mice because each line has to be bred to transactivator transgenic mice (expressing tTA or rtTA) to test the inducibility and specificity of transgene expression in the bitransgenic mice. Cre-RMCE can streamline the generation of new transgenic mice by allowing high-efficiency site-specific replacement of already characterized integrated transgenes flanked by hetero-specific loxP in single cell-stage embryos (zygotes) (50). Our tetO-SHP2E76K transgene is flanked by the improved L3/L2 loxP sites placed in opposite orientation to allow efficient Cre-RMCE (41). The multiple lines of inducible tetO-SHP2E76K transgenic mice that we derived and characterized here are a potential resource for generating new transgenic mice by Cre-RMCE as mouse models for studying other genetic lesions identified in human lung cancer.

**Supplementary material**

**Supplementary Materials and Methods, Table 1 and Figures 1–7** can be found at [http://carcin.oxfordjournals.org/](http://carcin.oxfordjournals.org/)

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