Induction of a Tumor-associated Activating Mutation in Protein Tyrosine Phosphatase \( \text{Ptpn11 (Shp2)} \) Enhances Mitochondrial Metabolism, Leading to Oxidative Stress and Senescence*

Hong Zheng\(^1\), Shanhui Li\(^1\), Peter Hsu, and Cheng-Kui Qu\(^2\)

From the Department of Medicine, Division of Hematology and Oncology, Case Comprehensive Cancer Center, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106

**Background:** The pathogenic effects of tumor-associated activating mutations of \( \text{Ptpn11 (Shp2)} \) have not been well characterized. Here, we report that induction of the \( \text{Ptpn11}^{E76K/\#} \) mutation, the most common and active \( \text{Ptpn11 (Shp2)} \) mutation found in leukemias and solid tumors, in primary mouse embryonic fibroblasts resulted in proliferative arrest and premature senescence. As a result, apoptosis was markedly increased. These cellular responses were accompanied and mediated by up-regulation of p53 and p21. Moreover, intracellular levels of reactive oxygen species (ROS), byproducts of mitochondrial oxidative phosphorylation, were elevated in cells. These analyses revealed that oxygen consumption of \( \text{Ptpn11}^{E76K/\#} \) cells and the respiratory function of \( \text{Ptpn11}^{E76K/\#} \) mitochondria were significantly increased. Furthermore, we found that phosphorylation of mitochondrial Stat3, one of the substrates of Shp2 phosphatase, was greatly decreased in the mutant cells with the activating mutation \( \text{Ptpn11}^{E76K/\#} \). This study provides novel insights into the initial effects of tumor-associated \( \text{Ptpn11 (Shp2)} \) mutations.

**Results:** \( \text{Ptpn11}^{E76K/\#} \) mouse embryonic fibroblasts display increased ROS levels, cellular senescence, and enhanced mitochondrial respiration upon induction of the mutation.

**Conclusion:** Induction of the \( \text{Ptpn11}^{E76K/\#} \) mutation results in oxidative stress by enhancing mitochondrial metabolism.

**Significance:** This study provides novel insights into the initial effects of \( \text{Ptpn11 (Shp2)} \)-activating mutations.

*This work was supported, in whole or in part, by the National Institutes of Health Grants HL068212 and HD070716 (to C. K. Q.).\(^1\)

\(^1\) Both authors contributed equally to this work.

\(^2\) To whom correspondence should be addressed: Department of Medicine, Division of Hematology and Oncology, Case Comprehensive Cancer Center, Case Western Reserve University, 10900 Euclid Ave., Cleveland, Ohio 44106. Tel.: 216-368-3361; Fax: 216-368-1166; E-mail: cxq6@case.edu.

Shp2, encoded by \( \text{Ptpn11} \), is an ubiquitously expressed protein tyrosine phosphatase (PTP)\(^3\) that contains two tandem Src homology 2 (SH2) domains, a PTP domain, and a C-terminal tail (1–3). It has been implicated in multiple cell signaling pathways that are activated by growth factors, cytokines, and hormones, regulating cell survival/growth and differentiation (4–6). Under basal conditions, this phosphatase is held in an inactive conformation by the N-terminal SH2 (N-SH2) domain, which occludes the active site in the PTP domain (7, 8). Shp2 is activated upon binding of the SH2 domains (primarily the N-SH2 domain) to phosphotyrosine residues in signaling partners due to the disruption of the inhibitory intramolecular interaction (7–9). Intriguingly, although dephosphorylating substrate proteins, Shp2 plays an overall positive role in the signal transduction of receptor and cytokolic kinases, particularly the Ras-Erk pathway (4–6). The underlying mechanism, however, remains elusive. Importantly, mutations in \( \text{Ptpn11} \) (heterozygous) have been identified in pediatric hematologic malignancies (10–12) and solid tumors (13). These tumor-associated mutations affect N-SH2 or PTP domain residues involved in the inhibitory domain contacts, resulting in “activated mutants” of Shp2. Recent studies have demonstrated that single \( \text{Ptpn11} \)-activating mutations are sufficient to induce hematologic malignancies in mice (14–17), suggesting a causal role of \( \text{Ptpn11} \) mutations in these diseases.

The pathogenesis of \( \text{Ptpn11} \)-associated malignancies has not been well characterized as the biochemical basis for the positive role that Shp2 plays in cell signaling and other cellular processes is not fully understood. Shp2 functions in growth factor and cytokine signaling in both catalytically dependent and independent manners (18–20). Tumor-associated \( \text{Ptpn11} \) mutations result in hyperactivation of Shp2 catalytic activity (12, 21). In addition, these mutations enhance the binding of mutant Shp2 to signaling partners (14, 17, 22, 23). Our previous studies suggested that both elevated catalytic activity and enhanced binding to signaling partners contributed to the pathogenic
Effects of Oncogenic Ptpn11 on Mitochondrial Metabolism

effects of activating mutations of Ptpn11 on cytokine signaling (17, 24). In addition to the cytosolic localization, emerging evidence has indicated that Shp2 is also distributed to the mitochondria; specifically, the mitochondrial intercristae/intermembrane space (25, 26). Mitochondria are highly dynamic cellular organelles that play multiple roles in cells, including energy production, intermediate metabolism, apoptosis, and maintenance of cytoplasmic calcium homeostasis (27, 28). However, the role of Shp2 in the mitochondria is not clear. Understanding of novel functions of Shp2 in these organelles may help elucidate the molecular mechanisms of Shp2-associated tumorigenesis.

We previously generated a line of conditional knock-in mice with the most common and active Ptpn11 mutation (E76K) found in leukemias and tumors (12, 29–31). Global Ptpn11E76K/+ mutation results in early embryonic lethality. Induced knock-in of this mutation in pan hematopoietic cells leads to myeloproliferative disease followed by malignant evolution into various acute leukemias (32). During the course of examining the role of the Shp2 E76K mutant on growth factor signaling in the cells isolated from this line of mice, we accidently found that induction of the Ptpn11E76K/+ mutation in mouse embryonic fibroblasts (MEFs) caused growth arrest. We therefore further characterized the pathogenic effects of this mutation in MEFs.

MATERIALS AND METHODS

Antibodies and Reagents—Antibodies against phospho-Erk, Erk, phospho-Stat3, Stat3, Shp2, and p21 were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Antibodies against phosphor-Akt and Akt were obtained from Cell Signaling Technology (Danvers, MA). p53 antibody was from Oncogene Science (Cambridge, MA). The lactate assay kit was provided by Biovision (Golden, CO). MTS ([3-(4,5-dimethylthiazol-2-yl)]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was obtained from Promega Life Science (Madison, WI).

Generation of Inducible Ptpn11E76K Knock-in Mouse Embryonic Fibroblasts (MEFs)—Conditional Ptpn11E76K knock-in (Ptpn11E76K Neo+) mice were used to cross estrogen receptor promoter driven Cre transgenic (ER-Cre+) mice at the Animal Resources Center, Case Western Reserve University. All animal procedures complied with the NIH Guideline for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. For generation of inducible Ptpn11E76K knock-in MEFs, embryonic day 15.5–17.5 embryos were dissected from timed pregnant females. Following removal of the heads and internal organs, embryos were minced and tissue masses were plated and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin. Three days later, embryonic fibroblasts were harvested and designated as passage 1. Ptpn11+/+ /ER-Cre+ and Ptpn11E76K Neo+/ER-Cre+ MEFs were treated with 4-hydroxytamoxifen (4-OHT, 0.5 µM) to induce Cre expression, deletion of the Neo cassette, and expression of Shp2 E76K from the targeted allele in Ptpn11E76K+/+ /ER-Cre+ MEFs. To measure cell growth rate, cells were plated in 96-well plates at a density of 1 × 10³ cells and incubated overnight. MTS was added, and incubation was continued at 37 °C for 1 h. Viable cells were determined using a microplate reader at an absorbance of 492 nm.

Senescence-associated β-Galactosidase Activity Assay, Cell Cycle, and Apoptosis Analyses—MEFs were seeded in 35-mm dishes at a density of 4 × 10⁴ cells per dish. The day after, cells were fixed and stained for β-galactosidase activity as previously described (33). Positive cells were counted in 400 cells. For apoptosis analysis, cells were harvested by trypsinization and fixed in ice-cold 75% ethanol at −20 °C overnight. Fixed cells were treated with RNase A (20 µg/ml) at 37 °C for 30 min, washed with PBS, and then stained with propidium iodide (PI) (50 µg/ml in PBS). Apoptotic cells with Sub-G1 DNA content were quantified by standard FACS procedures.

Determination of Mitochondrial Mass, Mitochondrial Membrane Potential, and Reactive Oxygen Species (ROS)—To measure mitochondrial mass, cells were incubated for 15 min with 20 nM MitoTracker Green (Molecular Probes, Grand Island, NY) and then washed with PBS. The intensity of labeling was measured by FACS. To measure mitochondrial membrane potential, cells were incubated with 20 nM MitoFluor Red (Molecular Probes, Grand Island, NY) for 20 min and then washed with PBS. The intensity of labeling was measured by standard FACS. To measure cellular ROS levels, cells were loaded with 2′,7′-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Grand Island, NY) (5 µM) following the instructions provided by the manufacturer. Fluorescence probes were detected and mean fluorescence intensities (MFI) were quantified by FACS.

Oxygen Consumption, Extracellular Flux, and ATP Measurement—Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured at 37 °C using an XF24 extracellular analyzer (Seahorse Bioscience, North Billerica, MA). MEFs were seeded in 24-well plates for 12 h. Medium was changed to un-buffered DMEM (DMEM supplemented with 25 mM glucose, 1 mM sodium pyruvate, 31 mM NaCl, 2 mM GlutaMax, pH 7.4) and incubated at 37 °C in a non-CO₂ incubator for 1 h. Viable cells were determined using a microplate reader at an absorbance of 492 nm. Every point represents an average of 3–5 different wells. After the assays, plates were saved, and protein concentration in each well was determined using a BCA assay (Thermo Fisher Scientific, Waltham, MA). Total cellular ATP levels were assessed using adenosine 5′-triphosphate bioluminescent assay kit (Biovision, Golden, CO) following the instructions provided by the manufacturer.

Mitochondria Respiration Assay—Measurement of mitochondrial respiration was performed using a platinum electrode (Dissolved Oxygen Package, Qubit systems, Canada) at 30 °C. Mitochondria were isolated from liver homogenates of Ptpn11E76K+/+ /Alb-Cre+ mice and Ptpn11E76K+/+ /Alb-Cre+ control mice following the protocol previously described (34). The mitochondria (0.5 mg of protein) pellets were resuspended in 0.5 ml of respiration buffer (120 mM KCl, 3 mM Hepes, 1 mM EGTA, 5 mM KH₂PO₄, 1% BSA, pH 7.4) and assayed for respiration using pyruvate (5 mM)/malate (2.5 mM) or palmitoylCoA (10 mM)/carnitine (2.5 µM)/malate (2.5 µM) as substrates for
mitochondria respiration. ADP was added in limiting amounts, and State 3 was measured. Following depletion of ADP, State 4 was measured. Rates of O$_2$ consumption were expressed in nmol O$_2$/mg mitochondrial protein/minute. The ADP:O ratio was calculated as the amount of added ADP expressed in nmol divided by the amount of oxygen consumed during State 3 respiration.

**Immune Complex Phosphatase Assay**—Cells or tissues were lysed. Whole cell lysates (500 μg) were immunoprecipitated with 2 μg of anti-Shp2 antibody. Immune complexes were washed twice with a washing buffer (20 mM HEPES pH 7.5, 50 mM NaCl, 2.5 mM MgCl$_2$, 0.1 mM EDTA, 0.05% Triton X-100), once with a buffer containing 25 mM HEPES pH 7.2, 50 mM dithiothreitol, 2.5 mM EDTA, p-nitrophenyl phosphate (pNPP) (1.5 mg/ml pNPP in tyrosine phosphatase assay buffer containing 20 mM HEPES pH 7.4, 50 mM NaCl, 2.5 mM EDTA, 5 mM DTT, and 100 μg/ml BSA) was then added to each sample and incubated at 30 °C for 1 h. Supernatants were transferred to 96-well plates and absorbance at 405 nm was measured.

**Statistical Analysis**—Data are presented as mean ± S.D. Statistical significance was determined using unpaired two-tailed Student’s t test. p values <0.05 were considered to be significant.

**RESULTS**

**Induction of the Ptpn11$^{E76K/+}$ Mutation Caused Growth Arrest and Apoptosis**—We previously generated a line of conditional knock-in mice with the Ptpn11$^{E76K}$ mutation (32), the most frequent and active Ptpn11 mutation found in leukemias and tumors (12, 29–31). In these knock-in mice, the inserted Neo cassette with a stop codon prevents expression of the targeted allele (Ptpn11$^{E76K Neo/+}$). However, after the deletion of Neo by Cre DNA recombinase, the Neo-deleted mutant allele (Ptpn11$^{E76K}$) is reactivated, producing mutant Shp2 E76K at...
FIGURE 2. Induction of Ptpn11<sup>E76K</sup> mutation in primary MEFs causes cell cycle arrest. A, Ptpn11<sup>+/+</sup>/ER-Cre<sup>+</sup> and Ptpn11<sup>E76K Neo<sup>-</sup>/+</sup>/ER-Cre<sup>+</sup> MEFs were treated with 4-OHT. Forty-eight hours later, cells were starved in serum-free medium for 40 h and then stimulated with EGF (100 ng/ml) for the indicated periods of time. Whole cell lysates were prepared and examined for Akt and Erk activities by immunoblotting with anti-phospho-Erk and anti-phospho-Akt antibodies. Blots were stripped and reprobed with anti-pan-Erk and anti-pan-Akt antibodies to check for protein loadings. Results shown are representative from three independent experiments. The relative ratios of the phosphorylated proteins normalized against the total proteins were analyzed using a PhosphoImager and shown as mean ± S.D. of three independent experiments.

B, total RNA was extracted from 4-OHT or DMSO treated Ptpn11<sup>+/+</sup>/ER-Cre<sup>+</sup> and Ptpn11<sup>E76K Neo<sup>-</sup>/+</sup>/ER-Cre<sup>+</sup> MEFs. p53 and p21 mRNA levels in these cells were determined by real time quantitative PCR. Experiments were repeated three times. Representative results from one experiment are shown.

C, whole cell lysates were prepared from 4-OHT or DMSO treated Ptpn11<sup>+/+</sup>/ER-Cre<sup>+</sup> and Ptpn11<sup>E76K Neo<sup>-</sup>/+</sup>/ER-Cre<sup>+</sup> MEFs. Protein levels of p53, p21, and Shp2 in these cells were determined by immunoblotting. Blots were stripped and reprobed with anti-Shp2 and anti-β-actin antibodies to check for Shp2 expression and protein loading. The relative ratios of p53, p21, and Shp2 normalized against the loading control protein were analyzed using a PhosphoImager and shown as mean ± S.D. of three independent experiments.

D, 4-OHT or DMSO treated Ptpn11<sup>+/+</sup>/ER-Cre<sup>+</sup> and Ptpn11<sup>E76K Neo<sup>-</sup>/+</sup>/ER-Cre<sup>+</sup> MEFs were loaded with 5-bromodeoxyuridine (BrdU, 10 μM) for 30 min, harvested, and fixed with cold 70% ethanol overnight. The cells were then washed with PBS, treated with HCl (2 N), stained with anti-BrdU antibody, and finally resuspended in PBS containing 50 μg/ml PI. Cell cycle distribution was determined by FACS analyses according to BrdU and PI staining profiles. Representative results of three independent experiments are shown.
the physiological level (32). Although the mechanism underlying the inactivation of the targeted allele is unclear, this conditional allele allows expression of mutant Shp2 E76K under the endogenous Ptpn11 promoter in an inducible manner. To study the pathogenic effects of the Ptpn11E76K mutation on cell signaling and cellular function, we generated conditional Ptpn11E76K mutation knock-in cell models. Ptpn11E76K Neo/+/ER-Cre+ mice were used to cross estrogen receptor promoter-driven Cre transgenic (ER-Cre+) mice. E15.5-E17.5 embryos derived were dissected to generate Ptpn11E76K Neo+/+ /ER-Cre+ and Ptpn11E76K Neo+/+ /ER-Cre+ MEFs following standard procedures. The insertion of Neo inactivated the targeted allele (Ptpn11Neo/Neom) in Ptpn11E76K Neo+/+ /ER-Cre+ cells. However, when these cells were treated with 4-hydroxytamoxifen (4-OHT), Cre expression was induced, leading to the deletion of the Neo cassette (data not shown), and thus the reactivation of the mutant allele (Ptpn11E76K) and restoration of Shp2 E76K expression to the physiological level (Fig. 1, A and B). Shp2 phosphatase activity in 4-OHT-treated Ptpn11E76K+/-/ER-Cre+ MEFs was increased by ~4-fold (Fig. 1C), in agreement with previous observations that the E76K mutation hyper-activates Shp2 (12, 21). Surprisingly, cell growth assessment showed that Neo depletion and Shp2 E76K expression resulted in a substantially decreased cell growth in Ptpn11E76K+/- MEFs while the growth of Ptpn11E76K+/- MEFs was not altered by 4-OHT treatment (Fig. 1D). To characterize the growth arrest induced by the Ptpn11E76K+/- mutation, we determined cell apoptosis. This analysis revealed a markedly increased sub-G1 population in 4-OHT-treated mutant cells compared with that in control cells (Fig. 1E), suggesting that the Ptpn11E76K+/- mutation caused apoptosis and that the apoptotic effect was related to the induction of the oncogene. The apoptosis results were also confirmed by the Annexin V assay (data not shown). Consistent with these observations, mitochondrial membrane potential associated with apoptosis was decreased in Ptpn11E76K+/- mutant cells (Fig. 1F).

**Induction of the Ptpn11E76K+/- Mutation Resulted in Cell Cycle Arrest and Senescence**—We then asked how the Ptpn11E76K+/- mutation caused decreased cell growth and increased apoptosis. Shp2 is well known for its role in growth factor and cytokine-induced signal transduction. We examined the impact of the Ptpn11E76K+/- mutation on growth factor signaling. As shown in Fig. 2A, in response to EGF stimulation, activation of Erk and Akt kinases was elevated in Ptpn11E76K+/- mutant cells, consistent with the positive role of Shp2 in the Ras and Akt pathways (4–6). The moderate increase in growth factor signaling, however, does not seem to suggest that the Ptpn11E76K+/- mutation induces senescence by altering growth factor signaling.

Since Ptpn11E76K+/- mutant cells showed significantly decreased growth, we checked negative regulatory proteins of the cell cycle and found that p53 and its downstream effector p21 were increased in Ptpn11E76K+/- MEFs at both the mRNA (Fig. 2B) and protein levels (Fig. 2C). In agreement with these data, cell cycle analyses showed that upon the induction of the Ptpn11E76K+/- mutation, mutant cells were increased in the G1 phase while the percentage of S phase cells was decreased (Fig. 2D), suggesting a G1/S arrest in these cells. Furthermore, Ptpn11E76K+/- fibroblasts displayed distinctive markers of cellular senescence, such as senescence-associated β-galactosidase (SA-β-gal) activity. The percentage of SA-β-gal positive Ptpn11E76K+/- cells was much higher at the third passage compared with only 5% of control cells being SA-β-gal positive (Fig. 3A). Reactive oxygen species (ROS) are thought to

**FIGURE 3. Ptpn11E76K mutation in primary MEFs induces senescence accompanied by elevated ROS levels.** A, Ptpn11E76K Neo+/+ /ER-Cre+ and Ptpn11E76K Neo+/+ /ER-Cre+ MEFs were treated with 4-OHT. Forty-eight hours later, cells were stained for senescence-associated β-galactosidase (SA-β-gal) activity. Arrows indicate cells with positive SA-β-Gal staining. Experiments were performed with three independent cell lines for each group. Results shown are mean ± S.D. from one experiment. B, ROS levels in 4-OHT or DMSO-treated Ptpn11E76K Neo+/+ /ER-Cre+ and Ptpn11E76K Neo+−/ER-Cre+ MEFs were determined as described in “Materials and Methods.” Experiments were performed three times with three different cell lines for each group. Results shown are mean ± S.D.
play an important role in the aging and/or senescence process (35, 36). Therefore, ROS levels were determined. Ptpn11E76K/+/H11001 mutant cells showed increased cellular ROS compared with control cells (Fig. 3B). These results suggest that oxidative stress might be responsible for cellular senescence caused by the Ptpn11E76K/+ mutation.

The Ptpn11E76K/+ Mutation Enhanced Mitochondrial Aerobic Metabolism—To further define the mechanism by which the Ptpn11E76K/+ mutation increases cellular ROS levels, we determined effects of this mutation on mitochondrial function as ROS are produced as by-products during mitochondrial energy production and the source of a majority of cellular ROS can be traced back to the mitochondria (27, 28). Moreover, Shp2 has been shown to be distributed to the mitochondrial intercristae/intermembrane space (25, 26). We assessed cellular ATP levels in Ptpn11E76K/+/H11001 knock-in MEFs, but steady state total cellular ATP levels were not significantly changed (Fig. 4A). Since mitochondria are the primary cellular respiratory organelles, we decided to determine mitochondrial function of Ptpn11E76K/+ MEFs by real-time measurement of oxygen consumption. The results showed that Ptpn11E76K/+ MEFs had increased basal oxygen consumption (Fig. 4B). As the addition of the mitochondrial inhibitor oligomycin resulted in a similar and nearly complete reduction in oxygen consumption in WT and mutant cells, the oxygen consumptions in both cell types under resting conditions appeared to be derived almost exclusively from the mitochondrial cytochrome chain activity. To measure maximal oxygen consumption, we treated the cells with the mitochondrial uncoupling reagent carbonylcyanide-4-trifluoromethoxyphenylhydrazone (FCCP). Under the maximally uncoupled conditions, the difference in oxygen consumption between WT and Ptpn11E76K/+ cells was even larger (Fig. 4B). Subsequent treatment with respiratory chain inhibitor rotenone abolished oxygen consumption to basal levels in both WT and Ptpn11E76K/+ cells, confirming that the oxygen consumption following FCCP treatment reflected maximal reserve oxygen consumption capacity. We also measured extracellular proton flux and no difference in the basal levels was detected between WT and Ptpn11E76K/+ MEFs during the 30-min observation time. However, under the maximal uncoupling conditions, Ptpn11E76K/+ cells had increased extracellular acidification rates, indicating an potentially enhanced glyco-
lytic capacity in Ptpn11E76K/+ knock-in MEFs (Fig. 4C). This notion was supported by the observation that more lactate was produced by Ptpn11E76K/+ cells relative to control cells (Fig. 4D).

To gain further insights into the pathogenic effects of the Ptpn11E76K/+ mutation in the mitochondria, isolated mitochondria were needed for direct functional analysis. To this end, we generated liver-specific Ptpn11E76K/+ knock-in (Ptpn11E76K/Alb-Cre/) mice based on Ptpn11E76K Neo/H11001 and Alb-Cre/H11001 mice. Mitochondria were then isolated from livers (enriched for mitochondria) and mitochondrial respiration was measured with pyruvate/malate as a substrate. Representative traces for pyruvate-supported respiration are shown in Fig. 5A. State 3 represents the capacity of mitochondria to metabolize oxygen in the presence of ADP, and State 4 represents O2 consumption by leakage of protons through the inner membrane after ADP exhaustion. We found that the oxygen consumption was substantially increased in Ptpn11E76K/+ mitochondria, indicating an elevated capacity of mitochondrial metabolism (Fig. 5B). The ADP:O ratio, which reflects how effectively mitochondrial respiratory chain consumes O2 to phosphorylate ADP to ATP, was decreased in Ptpn11E76K/+ mitochondria compared with control mitochondria (Fig. 5C), suggesting an increased mitochondrial uncoupling in Ptpn11E76K/+ mitochondria. Similar observations were made with fatty acid substrates (palmitoyl-CoA/carnitine/malate) (data now shown).

Together, these data from isolated mitochondria suggest that a gain of function of the Shp2 phosphatase enhances the respiratory activity of mitochondria.

To verify the role of Shp2 in mitochondria, we assessed mitochondrial aerobic metabolism in Ptpn11 knock-out cells. Basal oxygen consumption of Ptpn11 conditional knock-out MEFs was not significantly changed. However, maximal reserve oxygen consumption capacities of Ptpn11 knock-out cells were greatly decreased compared with those of WT cells (Fig. 6A). Also, cytosolic glycolysis in Ptpn11-depleted cells under the maximal uncoupling conditions was decreased (Fig. 6B). Consistent with these results, cellular ATP (Fig. 6C) and ROS levels (Fig. 6D) in Ptpn11 knock-out cells were significantly decreased. These data, together with those from Ptpn11E76K/+ knock-in cells, confirm that Shp2 phosphatase plays an important role in mitochondrial aerobic metabolism. While loss of this protein decreases mitochondrial metabolism, gain-of-function in Shp2 enhances mitochondrial respiration.

To further define the mechanisms underlying the effects of the Ptpn11E76K/+ mutation in the mitochondria. We analyzed the activity of Stat3, one of the known substrates of Shp2 phosphatase (37, 38) that has been shown to play an essential role in the mitochondria (39). We found that phosphorylation levels of tyrosine 705 of Stat3, the target of Shp2 enzymatic activity, were...
substantially decreased in the cytosol and mitochondria of Ptpn11E76K/H11001 cells (Fig. 7). Interestingly, phosphorylation of serine 727, which has been shown to be important for the mitochondrial function of Stat3 (39), was also decreased in Ptpn11E76K/H11001 cells although the mechanism remains unclear. Consistent with these observations, phosphorylation levels of tyrosine 705 and serine 727 of Stat3 in mitochondria isolated from the liver of Ptpn11E76K/H11001/Alb-Cre/H11001 mice were also decreased (data not shown). A recent study has shown that depletion of Stat3 causes increased mitochondrial oxygen consumption and elevated ROS levels (40), similar to the mitochondrial phenotypes demonstrated by Ptpn11E76K/H11001 cells. Thus, the increased inhibition (dephosphorylation) of Stat3 by the Shp2 E76K mutant accounts at least in part for the deleterious effects of this mutation in the mitochondria.

**Ptpn11E76K/+ Mutation-induced Senescence Was Attributed to Elevated ROS Levels**—ROS are highly reactive compounds. They can damage macromolecules such as DNA, proteins, and lipids, a process that is believed to contribute to senescence, aging, and tumorigenesis (35, 36). To test whether Ptpn11E76K/+ mutation-induced senescence is attributed to elevated levels of ROS, we treated the cells with an antioxidant N-acetyl-cysteine (NAC). As shown in Fig. 8A, treatment of Ptpn11E76K/+ mutant MEFs with NAC resulted in decreased ROS levels in these cells. As a result, cell senescence caused by the induction of the Ptpn11E76K/+ mutation was greatly decreased (Fig. 8B), indicating that elevated ROS in Ptpn11E76K/+ mutant cells contributed significantly to the senescence.

**Ptpn11E76K/+ Mutation-induced Senescence Was p53-dependent**—The tumor suppressor p53 is up-regulated in MEFs in response to the induction of the Ptpn11E76K/+ mutation (Fig. 2, B and C). To further understand the role of p53 in mediating the Ptpn11 mutation-associated cell growth arrest, we generated Ptpn11E76K Neo/H11001/ER-Cre/H11001/p53/H11001/H11002 mice and determined senescent cells following the induction of the Ptpn11 mutation by the treatment of 4-OHT. As shown in Fig. 8C, compared with Ptpn11E76K/H11001 single mutant cells, Ptpn11E76K/+p53−/− double mutant cells exhibited significantly decreased SA-β-gal-positive cells. These results suggest that p53 indeed plays an essential role in mediating the senescence response induced by the Ptpn11E76K/+ mutation.

**DISCUSSION**

Activating mutations of the tyrosine phosphatase Ptpn11 (Shp2) are associated with leukemias and solid tumors. However, the pathogenic effects of Ptpn11 mutations have not been well characterized. In this report, we demonstrate that induction of the Ptpn11E76K/+ mutation causes oxidative stress and senescence in primary fibroblasts. As oncogene-induced senescence has been recognized as an important tumor-suppressive barrier (41, 42), senescence and apoptosis activated by the
**Effects of Oncogenic Ptpn11 on Mitochondrial Metabolism**

Ptpn11E76K/+ mutation might be an initial protective mechanism for preventing tumorigenesis.

The Ptpn11E76K/+ mutation appears to induce oxidative stress and senescence by dysregulating mitochondrial function. Previous studies have indicated that the ability of Ras to induce senescence depends on activation of the Raf/Mek/Erk pathway (43, 44). Although the positive role of Shp2 in the Ras pathway has been well established (4–6), Ptpn11E76K/+ mutant cells showed a moderate increase in growth factor-induced signaling (Fig. 2A), which does not seem to contribute significantly to senescence. Rather, altered mitochondrial function is likely responsible for the proliferative arrest. Shp2 is distributed to the mitochondrial intermembrane space in addition to the cytosol (25, 26). Mitochondria play an important role in cell metabolism and energy production, and mitochondrial dysfunction has been linked to a wide array of human diseases (27, 28). There were notable changes in mitochondrial function following the induction of the Ptpn11E76K/+ mutation. For example, oxygen consumption of Ptpn11E76K/+ mutant cells was increased (Fig. 4B) and the respiratory function of isolated Ptpn11E76K/+ mutant mitochondria was enhanced (Fig. 5B). Moreover, cellular ROS levels were elevated (Fig. 3B), which appeared to directly cause cellular senescence as treatment of the mutant cells with the antioxidant NAC significantly decreased senescence (Fig. 8B). This notion is supported by previous studies showing that ROS regulate cell cycle progression (45) and that oxidative stress imposed by elevated ROS can lead to cellular senescence (46).

It appears that the tumor suppressor p53 plays an important role in mediating Ptpn11E76K/+ mutation-induced senescence. p53 has been shown to be essential for cellular senescence induced by DNA damage and oxidative stress (47). It activates cell cycle arrest by inducing cyclin-dependent kinase inhibitors, such as p21, p16, and p19. Previous studies have shown that the ability of oncogenic Ras to induce senescence is accompanied by the up-regulation of several inhibitors of cell proliferation, including p53, p21, and p14/p19 (48, 49). In Ptpn11E76K/+ mutation-induced senescence, p21 was greatly increased (Fig. 2, B and C), but no significant changes of p16 and p19 were detected (data not shown). p53 appears to play an important role in the senescence induced by Ptpn11E76K/+ mutation as senescence in Ptpn11E76K/+p53−/− cells was decreased (Fig. 8C). Instead, these double mutant cells became transformed and immortalized rather quickly. Thus, the p53 pathway needs to be inactivated to bypass Ptpn11E76K/+ mutation-induced senescence and cause malignant transformation, highlighting the tumor suppressor function of p53.

The molecular mechanisms by which the Ptpn11E76K/+ mutation enhances mitochondrial aerobic metabolism and causes increased ROS production remain to be further determined. Although Shp2 has been shown to be distributed to the mitochondrial intermembrane space (25, 26), little is known about its signaling mechanisms in these organelles. Recently, Lee et al. proposed that oxidative phosphorylation complexes might be direct or indirect targets of Shp2 (50). We now show that Shp2 is required for optimal mitochondrial metabolism.
and that gain-of-function of Shp2 caused by the tumor-associated mutation substantially enhanced mitochondrial respiration (Figs. 4B and 5B). Since Stat3 activity (phosphorylation) in the mitochondria was greatly decreased in Ptpn11E76K/H11001 mutant cells (Fig. 7), it is likely this mutation disturbs mitochondrial function partly through enhanced dephosphorylation and inhibition of Stat3. Additionally, as total cellular ATP levels were not significantly changed (Fig. 4A) in the mutant cells but the ADP:O ratio in Ptpn11E76K/+ mitochondria was decreased (Fig. 5C), this mutation appeared to cause increased uncoupling activity in the mutant mitochondria. Further studies are required to determine the underlying mechanisms. Identification of novel Shp2 substrates in the mitochondria will shed light not only on molecular mechanisms by which Shp2 regulates mitochondrial function and mitochondria-dependent cellular activities, but also how disease mutations in this phosphatase change mitochondrial metabolism and cellular functions by altering the functional interactions with these substrates.

REFERENCES

1. Ahmad, S., Banville, D., Zhao, Z., Fischer, E. H., and Shen, S. H. (1993) A widely expressed human protein-tyrosine phosphatase containing src homology 2 domains. Proc. Natl. Acad. Sci. U.S.A. 90, 2197–2201

2. Feng, G. S., Hui, C. C., and Pawson, T. (1993) SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. Science 259, 1607–1611
Effects of Oncogenic Ptpn11 on Mitochondrial Metabolism

3. Freeman, R. M., Jr., Plutzky, J., and Neel, B. G. (1992) Identification of a human src homology 2-containing protein-tyrosine phosphatase: a putative homolog of Drosophila corkscrew. Proc. Natl. Acad. Sci. USA. 89, 11239–11243

4. Chan, G., Kalaizidis, D., and Neel, B. G. (2008) The tyrosine phosphatase Shp2 (PTPN11) in cancer. Cancer Metastasis Rev. 27, 179–192

5. Tonks, N. K. (2006) Protein tyrosine phosphatases: from genes, to function, to disease. Nat. Rev. Mol. Cell Biol. 7, 833–846

6. Xu, D., and Qu, C. K. (2008) Protein tyrosine phosphatases in the IAK/STAT pathway. Front Biosci. 13, 4925–4932

7. Eck, M. J., Pluskey, S., Trüb, T., Harrison, S. C., and Shoelson, S. E. (1996) Spatial constraints on the recognition of phosphoproteins by the tandem SH2 domains of the phosphatase SH-PTP2. Nature 379, 277–280

8. Hof, P., Pluskey, S., Dhe-Paganon, S., Eck, M. I., and Shoelson, S. E. (1998) Crystal structure of the tyrosine phosphatase SHP-2. Cell 92, 441–450

9. Barford, D., and Neel, B. G. (1998) Revealing mechanisms for SH2 domain mediated regulation of the protein tyrosine phosphatase SHP-2. Structure 6, 249–256

10. Loh, M. L., Vattikutti, S., Schubbert, S., Reynolds, M. G., Carlson, E., Lieuw, K. H., Cheng, J. W., Lee, C. M., Stokoe, D., Bonifás, J. M., Curtiss, N. P., Gotlib, J., Meshinchi, S., Le Beau, M. M., Emanuel, P. D., and Shannon, K. M. (2004) Mutations in PTPN11 implicate the SH-2 phosphate in leukemogenesis. Blood 103, 2325–2331

11. Tartaglia, M., Martinelli, S., Cazzagna, G., Cordeddu, V., Iavarone, I., Spinelli, M., Palmi, C., Carta, C., Pession, A., Aricò, M., Masera, G., Baso, G., Sorcini, M., Gelb, B. D., and Biondi, A. (2004) Genetic evidence for lineage-related and differentiation stage-related contribution of somatic PTPN11 mutations to leukemogenesis in childhood acute leukemia. Blood 104, 307–313

12. Tartaglia, M., Niemeyer, C. M., Fragale, A., Song, X., Buechner, J., Jung, A., Hähnen, K., Hasle, H., Licht, J. D., and Gelb, B. D. (2003) Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat. Genet. 34, 148–150

13. Bentires-Alj, M., Paez, J. G., David, F. S., Keilhack, H., Halmos, B., Naoki, K., Maris, J. M., Richardson, A., Bardelli, A., Sugarbaker, D. J., Richards, W. G., Du, J., Girard, L., Minna, J. D., Loh, M. L., Fisher, D. E., Velculescu, V. E., Vogelstein, B., Meyerson, M., Sellers, W. R., and Neel, B. G. (2004) Activating mutations of the Noonan syndrome-associated SHP2/PTPN11 gene in human solid tumors and adult acute myelogenous leukemia. Cancer Res. 64, 8816–8820

14. Araki, T., Mohi, M. G., Ismat, F. A., Bronson, R. T., Williams, I. R., Kutok, J. L., Yang, W., Pao, L. I., Meyerson, H. J., Guo, C., Gerson, S. L., and Qu, C. K. (2011) Non-lineage/stage-restricted effects of a gain-of-function mutation in tyrosine phosphatase Shp2 in malignant transformation of hematopoietic progenitors. J. Exp. Med. 208, 1977–1988

15. Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, E., and Pereira-Smith, O. (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. U.S.A. 92, 9363–9367

16. Frezza, C., Cipolat, S., and Scorrano, L. (2007) Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. Nat. Protoc. 2, 287–295

17. Barja, G. (1999) Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. J. Bioenerg. Biomembr. 31, 347–366

18. Sastre, J., Pallardó, F. V., García de la Asunción, J., and Viña, J. (2000) Activating mutations of the noonan syndrome-associated SHP2/PTPN11 gene in tyrosine phosphatase Ptpn11 on malignant transformation of hematopoietic progenitors. Cancer Cell 7, 179–191

19. Chan, R. J., Leedy, M. B., Munugalavadla, V., Voorhorst, C. S., Li, Y., Yu, M., and Kapur, R. (2005) Human somatic PTPN11 mutations induce hematopoietic-cell hypersensitivity to granulocyte-macrophage colony-stimulating factor. Blood 105, 3737–3742

20. Yu, W. M., Daino, H., Chen, J., Bunting, K. D., and Qu, C. K. (2006) Effects of a leukemia-associated gain-of-function mutation of SHP-2 phosphatase on interleukin-3 signaling. J. Biol. Chem. 281, 5426–5434

21. Bennett, A. M., Tang, T. L., Sugimoto, S., Walsh, C. T., and Neel, B. G. (1994) Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor beta to Ras. Proc. Natl. Acad. Sci. U.S.A. 91, 7335–7339

22. Wegrzyn, J., Potla, R., Chwae, Y. J., Sepuri, N. B., Zhang, Q., Koeck, T., Derecka, M., Szczepanek, K., Szeland, M., Gornicka, A., Moh, A., Moghadas, S., Chen, Q., Bobbili, S., Cichy, J., Dulak, J., Baker, D. P., Wolfman, A., Stuehr, D., Hassan, M. O., Avadhani, N., Drake, J. I., Fawcett, P., Stuehr, D., Hassan, M. O., Fu, X. Y., Avadhani, N., Drake, J. I., Fawcett, P., Lesniewski, E. J., and Larner, A. C. (2009) Function of mitochondrial Stat3 in cellular respiration. Science 323, 793–797
Effects of Oncogenic Ptpn11 on Mitochondrial Metabolism

40. Mantel, C., Messina-Graham, S., Moh, A., Cooper, S., Hangoc, G., Fu, X. Y., and Broxmeyer, H. E. (2012) Mouse hematopoietic cell-targeted STAT3 deletion: stem/progenitor cell defects, mitochondrial dysfunction, ROS overproduction, and a rapid aging-like phenotype. *Blood* **120**, 2589–2599

41. Prieur, A., and Peeper, D. S. (2008) Cellular senescence *in vivo*: a barrier to tumorigenesis. *Curr. Opin. Cell Biol.* **20**, 150–155

42. Larsson, L. G. (2011) Oncogene- and tumor suppressor gene-mediated suppression of cellular senescence. *Semin. Cancer Biol.* **21**, 367–376

43. Zhu, J., Woods, D., McMahon, M., and Bishop, J. M. (1998) Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.* **12**, 2997–3007

44. Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., and Lowe, S. W. (1998) Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes Dev.* **12**, 3008–3019

45. Havens, C. G., Ho, A., Yoshioka, N., and Dowdy, S. F. (2006) Regulation of late G1/S phase transition and APC Cdh1 by reactive oxygen species. *Mol. Cell Biol.* **26**, 4701–4711

46. Dumont, P., Burton, M., Chen, Q. M., Gonos, E. S., Frippiat, C., Mazari, J. B., Eliaers, F., Remacle, J., and Toussaint, O. (2000) Induction of replicative senescence biomarkers by sublethal oxidative stresses in normal human fibroblast. *Free Radic Biol. Med.* **28**, 361–373

47. Wu, C., Miloslavskaya, I., Demontis, S., Maestro, R., and Galaktionov, K. (2004) Regulation of cellular response to oncogenic and oxidative stress by Seladin-1. *Nature* **432**, 640–645

48. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**, 593–602

49. Ferbeyre, G., de Stanchina, E., Lin, A. W., Querido, E., McCurrach, M. E., Hannon, G. J., and Lowe, S. W. (2002) Oncogenic ras and p53 cooperate to induce cellular senescence. *Mol. Cell Biol.* **22**, 3497–3508

50. Lee, I., Pecinova, A., Pecina, P., Neel, B. G., Araki, T., Kucherlapati, R., Roberts, A. E., and Hüttemann, M. (2010) A suggested role for mitochondria in Noonan syndrome. *Biochim. Biophys. Acta* **1802**, 275–283