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## Inhibition of the Gab2/PI3K/mTOR signaling ameliorates myeloid malignancy caused by *Ptpn11* (Shp2) gain-of-function mutations

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### Abstract

Activating mutations, such as E76K and D61Y, in *PTPN11* (SHP2), a protein tyrosine phosphatase implicated in multiple cell signaling processes, are associated with 35% of patients with juvenile myelomonocytic leukemia (JMML), an aggressive childhood myeloproliferative neoplasm (MPN). Effective therapeutic interventions for this malignancy are still lacking. Here we show that the interaction between leukemia-associated mutant Shp2 and Gab2, a scaffolding protein important for cytokine-induced PI3K/Akt signaling, was enhanced, and that the mTOR pathway was elevated in *Ptpn11*<sup>E76K/+</sup> leukemic cells. Importantly, MPN induced by the *Ptpn11*<sup>E76K/+</sup> mutation was markedly attenuated in *Ptpn11*<sup>E76K/+</sup>/*Gab2*<sup>-/-</sup> double mutant mice — Overproduction of myeloid cells was alleviated, splenomegaly was diminished, and myeloid cell infiltration in non-hematopoietic organs was decreased in these double mutants. Excessive myeloid differentiation of stem cells was also normalized by depletion of Gab2. Acute leukemia progression of MPN was reduced in the double mutant mice, and as such, their survival was much prolonged. Furthermore, treatment of *Ptpn11*<sup>E76K/+</sup> mice with Rapamycin, a specific and potent mTOR inhibitor, mitigated MPN phenotypes. Collectively, this study reveals an important role of the Gab2/PI3K/mTOR

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#### Authorship contributions

W.L., W.M.Y., and J.Z., conducted the research and summarized the data. R.J.C., M.L.L., Z.Z., and K.D.B. provided critical reagents, discussed the work, and edited the manuscript. C.K.Q. designed the experiments and provided technical training to the first three authors. W.L. and C.K.Q. wrote the manuscript.

#### Conflict-of-interest disclosure

The authors declare no competing financial interests.

pathway in mediating the pathogenic signaling of the *PTPN11* gain-of-function mutations, and a therapeutic potential of Rapamycin for *PTPN11* mutation-associated JMML.

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## INTRODUCTION

Juvenile myelomonocytic leukemia (JMML), an aggressive myeloproliferative neoplasm (MPN) of early childhood, is characterized by hypersensitivity of myeloid progenitors to cytokines, such as granulocyte-macrophage colony stimulating factor (GM-CSF)<sup>1, 2</sup>, and overproduction of myeloid cells and monocytes. JMML cells retain capabilities to differentiate, but can infiltrate into non-hematopoietic organs, disrupting their functions. This disease can progress to acute myeloid leukemia. While the current standard of care for patients with JMML relies on allogeneic hematopoietic stem cell (HSC) transplant, relapse is the most frequent cause of treatment failure<sup>3-5</sup>. New therapeutic interventions are still needed for this fatal disease.

Significant progress in understanding the pathogenesis of JMML has been achieved by deciphering the genetic lesions that occur in patients. The genetic mutations identified in JMML occur in the signaling proteins involved in the RAS/ERK pathway<sup>3-5</sup>, providing new opportunities for both diagnosis and therapy. Thirty-five percent of patients with JMML have mutations, such as E76K and D61Y, in the protein tyrosine phosphatase (PTP) *PTPN11* (Shp2), a positive regulator of the Ras pathway (see below), while activating mutations in *RAS* (*K-RAS* or *N-RAS*), homozygous inactivation of *NFI*, a GTPase activating protein that negatively regulates Ras output, and loss-of-function mutations in the E3 ubiquitin ligase *c-CBL* account for 20%, 15%, and 10–15% of JMML cases, respectively<sup>6-9</sup>. *PTPN11*, *RAS*, *NFI*, and *c-CBL* mutations are usually mutually exclusive in patients. When modeled using murine systems, gain-of-function mutations in *Ptpn11*, *K-Ras*, and *N-Ras*, or loss-of-function in *Nfi* and *Cbl*, are sufficient to induce cytokine hypersensitivity in myeloid progenitors and to produce a JMML-like MPN in mice<sup>10-18</sup>.

*Ptpn11* (Shp2), a ubiquitously expressed PTP, is implicated in multiple cell signaling processes, such as the Ras/Erk, PI3K/Akt, Jak/STAT, and NF- $\kappa$ B pathways that are activated by diverse growth factors, cytokines, insulin, growth hormones, and cell adhesion molecules<sup>19-21</sup>. Shp2 is normally self-inhibited by hydrogen bonding of the backside loop of the N-terminal SH2 (N-SH2) domain with the deep pocket of the PTP domain<sup>22, 23</sup>, and gets activated upon binding to signaling partners with phospho-tyrosine (pY) residues. Intriguingly, this phosphatase plays an overall positive role in transducing signals initiated from receptor kinases, particularly in the Ras pathway<sup>19-21</sup>. The underlying signaling mechanisms are still not yet well understood. *PTPN11* mutations found in JMML are concentrated in the N-SH2 domain and result in amino acid changes at the interphase between N-SH2 and PTP domains, disrupting the inhibitory intramolecular interaction, thus leading to increased Shp2 catalytic activity<sup>7, 24</sup>. Although Ras hyperactivation is central in the pathogenesis of JMML, Ras effector or parallel pathways, such as PI3K/Akt signaling, have also been shown to contribute to the disease<sup>25, 26</sup>. While activated Ras is able to activate PI3K by a direct interaction with the p110 catalytic subunit<sup>27</sup>, cytokines, such as GM-CSF, can also activate PI3K in a Ras-independent manner via a Shp2-containing protein

complex including Gab2, Grb2, and PI3K regulatory subunit p85 $\alpha$  <sup>17, 28</sup>. In the present study, we sought to determine the role of the hematopoietic cell-specific scaffolding protein, Gab2, in gain-of-function Shp2-induced hyperactivation of PI3K/Akt and pathogenesis of MPN *in vivo*.

## MATERIALS AND METHODS

### Mice

Conditional knock-in mice (*Ptpn1*<sup>E76K neo/+</sup>) bearing the most common *PTPN11* mutation found in JMML were generated in a previous study <sup>16</sup>. *Gab2*<sup>+/-</sup> mice <sup>29</sup> were obtained from Dr. Toshio Hirano (Osaka University, Japan). *Mx1-Cre*<sup>+</sup> transgenic mice were purchased from the Jackson Laboratory. *Ptpn1*<sup>E76K neo/+</sup> mice were used to cross *Mx1-Cre*<sup>+</sup> and *Gab2*<sup>+/-</sup> mice to generate *Ptpn1*<sup>E76K neo/+</sup>/*Mx1-Cre*<sup>+</sup>/*Gab2*<sup>+/-</sup> mice. These mice were intercrossed to produce various types of mice for subsequent experiments. Mice of each genotype at the same age were numbered and randomly grouped for subsequent analyses. All mice were kept under specific pathogen-free conditions in the Division of Animal Resources at Emory University. All animal procedures complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

### Retroviral vectors and retroviral transduction

Shp2 E76K and Shp2 D61Y cDNAs were subcloned into the murine stem-cell virus (MSCV)-based bicistronic retroviral vector, pMIEG3, in tandem with enhanced green fluorescent protein cDNA (EGFP). Ecotropic retroviral supernatants (pMIEG3, pMIEG3-WT, pMIEG3-E76K, pMIEG3-D61Y) were prepared using Eco-Phoenix packaging cells by the Indiana University Vector Production Facility, as previously described <sup>14</sup>.

### Generation of BM-derived macrophages

BM cells were cultured in DMEM supplemented with 10% FBS and 20% L cell conditional medium (as a source of mouse colony-stimulating factor-1). Forty-eight hours later, nonadherent cells were collected and seeded into new tissue culture plates. After 5 to 7 days of culture, cells were confirmed to be macrophages as more than 90% of these cells were positive for Mac-1 and F4/80.

### Flow cytometric analysis and cell sorting

Multiparameter FACS analyses were performed to determine populations of HSCs (Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>Flk2<sup>-</sup>CD48<sup>-</sup>CD150<sup>+</sup>), multipotent progenitor sub-population 1 (MPP1, Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>Flk2<sup>-</sup>CD48<sup>-</sup>CD150<sup>-</sup>), MPP2 (Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>Flk2<sup>-</sup>CD48<sup>+</sup>CD150<sup>+</sup>), and MPP3 (Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>Flk2<sup>-</sup>CD48<sup>+</sup>CD150<sup>-</sup>). Antibodies were purchased from eBiosciences, San Diego, CA, unless otherwise noted. BM cells freshly harvested from femurs and tibias were stained with biotin-labeled antibodies against mouse hematopoietic lineage markers: Mac-1 (M1/70), Gr-1 (RB6-8C5), Ter119 (TER-119), CD3e (145-2C11), B220 (RA3-6B2), and subsequently stained with antibodies conjugated with various fluorochromes: streptavidin eFluor® 450, Sca-1-PE-Cy7 (D7, BD Biosciences, San Jose, CA), c-Kit-APC-eFluor780 (2B8), CD150-Alexa Fluor® 647

(TC15-12FF12.2, BD Biosciences), CD48-FITC (HM48-1), Flk2-PE (A2F10.1, BD Biosciences) for HSCs or MPPs. Specific cell populations were gated based on immune phenotypes for quantification or cell sorting. Fluorescence Minus One (FMO) was used for setting the gating on control samples. Flow cytometric analyses were performed on BD LSR II (BD Biosciences, San Jose). Cell sorting was conducted using BD FACSAria. Data were analyzed using FlowJo software (TreeStar, Ashland).

### Apoptosis analysis

Fresh BM cells were stained with biotin-labeled antibodies against lineage markers (Gr-1, Mac-1, Ter119, CD3e, and B220) as described above, followed by staining with: streptavidin eFluor® 450, Sca-1-PE-Cy7 (D7), c-Kit-APC-eFluor780 (2B8). Cells were then incubated with Annexin V-FITC and 7-amino-actinomycin D (7-AAD) using Annexin V-FITC apoptosis detection kit I (BD Biosciences, San Jose, CA). Apoptotic (Annexin V<sup>+</sup>) cells in the gated Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup> (LSK) cell population were quantified by FACS.

### Colony-forming unit assay

For the myeloid progenitor assay, freshly harvested mouse BM nucleated cells ( $2 \times 10^4$  cells/mL) or patient biopsy marrow cells ( $1 \times 10^4$  cells/mL) were assayed for colony forming units (CFUs) in 0.9% methylcellulose IMDM medium containing 30% FBS, glutamine ( $10^{-4}$  M),  $\beta$ -mercaptoethanol ( $3.3 \times 10^{-5}$  M), and IL-3 or GM-CSF at the indicated concentrations. After 7 days (mouse cells) or 14 days (human cells) of culture at 37 °C in a humidified 5% CO<sub>2</sub> incubator, myeloid colonies (CFU-GM and CFU-M) were counted under an inverted microscope.

### Rapamycin treatment

Rapamycin (LC Laboratories, Woburn) was dissolved in 100% ethanol at a concentration of 10 mg/mL, diluted in 5% Tween-80 and 5% PEG-400, and administered by intraperitoneal injections at 10 mg/kg body weight, once daily for 12–14 weeks starting on the following day after *Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup> mice were administered pI-pC (to induce the *Ptpn11*<sup>E76K/+</sup> mutation).

### Statistical analysis

All experiments were repeated two to three times with the indicated numbers of mice. Data are presented as mean  $\pm$  S.D. Statistical significance was determined using unpaired two-tailed Student's *t* test. *P* values < 0.05 were considered to be statistically significant.

## RESULTS

### Interaction of Shp2 with Gab2 is enhanced by leukemia-associated *Ptpn11* mutations

The hallmark of JMML is Ras hyperactivation and the hypersensitivity of myeloid progenitors to cytokines<sup>1, 2</sup>. However, signaling partners that mediate the effects of *Ptpn11* mutations in this disease have not been well characterized. We hypothesized that the hematopoietic specific scaffolding protein, Gab2, contributes to *Ptpn11* mutation-induced hyperactivation of Ras and PI3K/Akt signaling. To investigate this hypothesis, we

transduced BM low density mononuclear cells with wildtype (WT) Shp2 or leukemia-associated mutants Shp2 E76K and Shp2 D61Y. As shown in Figure 1A, in response to GM-CSF stimulation, tyrosine phosphorylation of Gab2 was significantly increased in mutant Shp2-expressing cells as compared to WT Shp2-expressing cells. Moreover, interactions between mutant Shp2 and Gab2 were enhanced by Shp2 mutations. We also generated BM-derived macrophages from *Ptpn11*<sup>E76K</sup> conditional knock-in mice (*Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup>), which developed JMML-like MPN following induction of the *Ptpn11*<sup>E76K</sup> mutation<sup>16</sup>, and determined the impact of the *Ptpn11*<sup>E76K</sup> mutation on GM-CSF-induced signaling. In response to GM-CSF stimulation, activation of Erk, Jak2, and Stat5 was increased in *Ptpn11*<sup>E76K/+</sup> mutant cells (Figure 1B). Previously studies have shown that Gab2 plays an important role in the PI3K/Akt pathway by interacting with the p85 subunit of PI3K<sup>28</sup>; we therefore took a closer look at this pathway. As shown in Figure 1C, activation of Akt was increased in *Ptpn11*<sup>E76K/+</sup> cells. Furthermore, activities of the downstream mTOR pathway were also enhanced as evidenced by elevated phosphorylation levels of S6 and 4E-BP1. These observations together indicate that increased interaction of mutated Shp2 with Gab2 might mediate the detrimental effects of *Ptpn11* mutations on cytokine signaling.

### Important role of Gab2 in mediating the pathogenic effects of the *Ptpn11*<sup>E76K</sup> mutation

To further test the role of Gab2 in the pathogenesis and maintenance of MPN induced by *Ptpn11* mutations, we generated *Ptpn11*<sup>E76K/+</sup>/*Gab2*<sup>-/-</sup> double mutant mice from *Ptpn11*<sup>E76K</sup> knock-in<sup>16</sup> and *Gab2* knockout (*Gab2*<sup>+/-</sup>)<sup>29</sup> mice, and monitored disease development and progression in these double mutants. This test is especially interesting given that steady state hematopoiesis in *Gab2* deficient mice is not significantly changed although *Gab2*-depleted stem cells showed decreased repopulation capabilities in competitive repopulation assays<sup>30</sup>. Compared to *Ptpn11*<sup>E76K/+</sup> single mutant mice, gross health signs of *Ptpn11*<sup>E76K/+</sup>/*Gab2*<sup>-/-</sup> double mutants were significantly improved. MPN phenotypes, such as elevated white blood cell (WBC) counts (Supplemental Table 1) and splenomegaly were attenuated in double mutants (Figure 2A). Overproduction of mature myeloid cells (Mac-1<sup>+</sup>/Gr-1<sup>+</sup>) and immature myeloid cells (Mac-1<sup>+</sup>/Gr-1<sup>-</sup>) was largely corrected (Figure 2B). In addition, myeloid cell infiltration in the liver (Figure 2C), lung, and kidney (data not shown) was diminished in double mutant mice. Histopathological examination confirmed that excessive proliferation of myeloid cells in the BM and spleen was reduced and myeloid cell infiltration in non-hematopoietic organs (liver, lung, and kidney) was significantly decreased in these double mutant mice (Supplemental Figure 1). More importantly, only 11% of *Ptpn11*<sup>E76K/+</sup>/*Gab2*<sup>-/-</sup> mice progressed into acute leukemias as opposed to 63% in *Ptpn11*<sup>E76K/+</sup> single mutant mice during 12 month follow-up (Figure 2D), and the overall survival of double mutants was prolonged (Figure 2E). These observations suggest that *Gab2* plays an important role in mediating the pathogenic effects of the *Ptpn11*<sup>E76K</sup> mutation in the development and progression of MPN.

MPN is known to be a clonal stem cell disorder; to further determine whether the rescue effects of *Gab2* deletion occurred at the stem cell level, we analyzed HSCs in double mutants and compared to those in *Ptpn11*<sup>E76K/+</sup> single mutants. The frequency and absolute number of HSCs in the BM of *Ptpn11*<sup>E76K/+</sup> mice were reduced due to the hyperactivation

and exhaustion of the reserve capacity, consistent with previous findings<sup>16</sup>. The frequency and absolute number (Supplemental Figure 2A–2C) of HSCs at steady state in *Ptpn11<sup>E76K/+</sup>/Gab2<sup>-/-</sup>* mice were not significantly changed as compared to *Ptpn11<sup>E76K/+</sup>* mice. However, apoptosis of hematopoietic stem/progenitor cells (Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>, LSK cells) in *Ptpn11<sup>E76K/+</sup>/Gab2<sup>-/-</sup>* double mutant mice was nearly restored to the physiological level (Supplemental Figure 2D and 2E) as opposed to significantly reduced apoptosis in *Ptpn11<sup>E76K/+</sup>* single mutant mice.

The myeloid malignancy induced by *Ptpn11* mutations is characterized by cytokine hypersensitivity of myeloid progenitors<sup>1,2</sup>; we therefore assessed the sensitivity of myeloid progenitors to GM-CSF and IL-3 in *Ptpn11<sup>E76K/+</sup>/Gab2<sup>-/-</sup>* double and *Ptpn11<sup>E76K/+</sup>* single mutant mice. As demonstrated in Figure 3A and Supplemental Figure 3, *Gab2* depletion in *Ptpn11<sup>E76K/+</sup>* progenitors significantly decreased the hypersensitivity to GM-CSF and IL-3. Moreover, cytokine-independent baseline colony formation of myeloid progenitors was abolished in *Ptpn11<sup>E76K/+</sup>/Gab2<sup>-/-</sup>* double mutants. To further verify these results, we sorted stem cell-enriched LSK cells and assessed their responses to cytokine-induced differentiation. Compared to *Ptpn11<sup>E76K/+</sup>* cells, myeloid differentiation/expansion (Figure 3B and Supplemental Figure 4 and Figure 3C) of *Ptpn11<sup>E76K/+</sup>/Gab2<sup>-/-</sup>* cells in the presence of IL-3 was significantly decreased. These data together suggest that *Gab2* depletion attenuates *Ptpn11<sup>E76K/+</sup>* mutation-induced myeloid malignancy mainly by decreasing the cytokine sensitivity of myeloid progenitors.

### **Gab2 depletion attenuates the detrimental effects of the *Ptpn11<sup>E76K</sup>* mutation on cytokine signaling**

The signaling mechanisms underlying the effects of *Gab2* deletion on the pathogenesis of *Ptpn11<sup>E76K</sup>* mutation-induced MPN were determined. *Gab2* is an important scaffolding protein in cytokine signaling pathways<sup>28</sup>. We generated BM-derived macrophages from *Ptpn11<sup>E76K/+</sup>* single and *Ptpn11<sup>E76K/+</sup>/Gab2<sup>-/-</sup>* double mutant mice and analyzed GM-CSF signaling in these cells. As shown in Figure 4A, in response to GM-CSF stimulation, activation of Erk and Stat5 was not significantly changed in the double mutant cells. However, Akt activity determined by phosphorylation of Serine<sup>473</sup> and Threonine<sup>308</sup> was decreased in the double mutant cells as compared to that in *Ptpn11<sup>E76K/+</sup>* single mutant cells, suggesting that the rescue effects of *Gab2* deficiency on *Ptpn11<sup>E76K</sup>* mutation-associated aberrant hematopoietic cell development might be attributable to the reduced Akt pathway. To verify this notion, we analyzed the downstream mTOR signaling in primary MPN cells isolated from diseased *Ptpn11<sup>E76K/+</sup>* mice and *Ptpn11<sup>E76K/+</sup>/Gab2<sup>-/-</sup>* double mutants. As shown in Figure 4B, phosphorylation of S6 and 4E-BP1 in the MPN cells of *Ptpn11/Gab2* double mutants was reduced relative to that in *Ptpn11<sup>E76K/+</sup>* single mutant mice.

### **Therapeutic effects of Rapamycin for the MPN induced by the *Ptpn11<sup>E76K</sup>* mutation**

Given that MPN was ameliorated in *Ptpn11<sup>E76K/+</sup>/Gab2<sup>-/-</sup>* double mutant mice and that the mTOR signaling activities were decreased in double mutant cells, we reasoned that targeting the mTOR pathway might be effective in controlling *Ptpn11* activating mutation-induced myeloid malignancy. To test this hypothesis, we treated *Ptpn11<sup>E76K</sup>* knock-in mice with

Rapamycin, a potent and selective inhibitor of mTOR, and monitored disease phenotypes in these mice. As shown in Figure 5A, treatment of Rapamycin at an optimal dosage significantly decreased WBC counts in diseased *Ptpn11*<sup>E76K/+</sup> mice. Importantly, control mice did not show overt hematopoietic suppression, highlighting a potential therapeutic index. Splenomegaly was also reduced in treated *Ptpn11*<sup>E76K/+</sup> mice (Figure 5B). Furthermore, cytokine hypersensitivity of myeloid progenitors was abolished in Rapamycin-treated *Ptpn11*<sup>E76K/+</sup> mice (Figure 5C and Supplemental Figure 5), and the colonies derived in colony assays were much smaller (Figure 5D). Additionally, we analyzed the mTOR signaling pathway in the BM cells isolated from Rapamycin-treated *Ptpn11*<sup>E76K/+</sup> and control mice, and found that phosphorylation of S6 and 4E-BP1 was barely detectable (Figure 5E). These results collectively demonstrate therapeutic effects of Rapamycin in the mouse model of *Ptpn11* mutation-associated myeloid malignancy.

### Rapamycin corrects the hypersensitive growth pattern of JMML patient cells with the *PTPN11*<sup>E76K</sup> mutation

BM cells from patients with JMML display a characteristic hypersensitive growth pattern, resulting in greater production of granulocyte-macrophage colonies in response to GM-CSF<sup>1,2</sup>. We next determined whether Rapamycin could inhibit colony formation and proliferation of JMML patient cells with *PTPN11* activating mutations at an optimal dosage that does not inhibit normal marrow progenitors. As shown in Figure 6A, GM-CSF-induced colony formation of myeloid blasts from JMML patients with *PTPN11* activating mutations was more sensitive to Rapamycin than healthy donor progenitors. Consistent with this data, *PTPN11* mutation-positive JMML cells cultured in GM-CSF-containing liquid medium was also more sensitive than healthy donor BM cells to the inhibition by Rapamycin at 2.5 nM (Figure 6B). The finding that Rapamycin is able to overcome the dominant effects of oncogenic mutant SHP2 in primary human leukemia cells support for a potential therapeutic benefit of this drug in *PTPN11* mutation-associated JMML.

## DISCUSSION

Hyperactivation of the Ras/Erk signaling pathway is known to underlie the pathogenesis of JMML<sup>3-5</sup>. Interestingly, the PI3K/Akt signaling pathway is also dysregulated in this disease, and this can be dependent and independent of the Ras pathway<sup>25</sup>. *PTPN11* gain-of-function mutations are associated with 35% of JMML. However, it has not been well characterized how *PTPN11* mutations lead to Ras-dependent and/or independent hyperactivation of the PI3K/Akt signaling. In this study, we demonstrate that the interaction between JMML-associated mutant Shp2 E76K and Gab2, a scaffolding protein important for cytokine-induced PI3K/Akt signaling<sup>28</sup>, was enhanced, and that the mTOR pathway was highly activated in *Ptpn11*<sup>E76K/+</sup> leukemic cells. Importantly, MPN induced by the *Ptpn11*<sup>E76K/+</sup> mutation was markedly attenuated in *Ptpn11*<sup>E76K/+</sup>/*Gab2*<sup>-/-</sup> double mutant mice. These observations indicate that Gab2 may play a key role downstream of mutated Shp2 in mediating the leukemogenic effects of *Ptpn11* mutations. *Ptpn11* gain-of-function mutations appear to disturb cytokine signaling through multiple mechanisms. These mutations result in increased catalytic activity of Shp2<sup>7,24</sup> and also enhanced binding to signaling partners<sup>13,31</sup> due to the disruption of the inhibitory intramolecular interaction and



the conformational change of the Shp2 protein. The gain-of-function mutations of Shp2 enhance Ras activity likely through elevated catalytic activity of Shp2 since Shp2 catalytic activity plays a positive role in the Ras pathway although the detailed mechanism is still elusive<sup>19–21</sup>. Hyperactivation of Ras leads to increased PI3K/Akt signaling due to the well-established crosstalk between the two pathways<sup>27</sup>. In addition, mutated Shp2 may directly induce increased PI3K/Akt signaling through enhanced binding with the scaffolding protein Gab2 that couples the p85 subunit of PI3K to the cytokine receptor<sup>28</sup> and the plasma membrane. In this signaling complex, mutant Shp2 may serve as an adaptor protein independent of its catalytic activity. Indeed, this phosphatase can function in cytokine signaling in both catalytically-dependent and –independent manners<sup>32</sup>.

Another important finding in this study is the pre-clinical therapeutic efficacy of Rapamycin in the mouse model of *Ptpn11* mutation-induced MPN. We showed that administration of Rapamycin at a tolerable dose significantly alleviated the MPN burden in *Ptpn11*<sup>E76K/+</sup> mice, and also that this drug at a low dose selectively suppressed *PTPN11* mutation-positive JMML patient cells over healthy donor cells *in vitro*. Given that Rapamycin is a clinically-used immunosuppressant and anti-cancer drug for adult patients, this drug may be further developed, used alone or in combination with MEK inhibitors that target the hyperactivated Ras/Erk pathway, as a novel therapeutic strategy for *PTPN11* mutation-associated JMML. This notion is also supported by the previous observation that PTEN expression, a negative regulator of PI3K/Akt/mTOR signaling, frequently was reduced in primary JMML patient samples<sup>33</sup> although the gene mutation status of the JMML specimens was not determined. The elevated Ras-independent activation of PI3/Akt/mTOR signaling may be specific for *Ptpn11* mutation-induced MPN since this is caused by enhanced interaction of mutated Shp2 with Gab2. It remains to be determined whether the PI3/Akt/mTOR pathway may also be an effective target for the treatment of other subtypes of JMML associated with the RAS, NF1, or CBL mutations.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

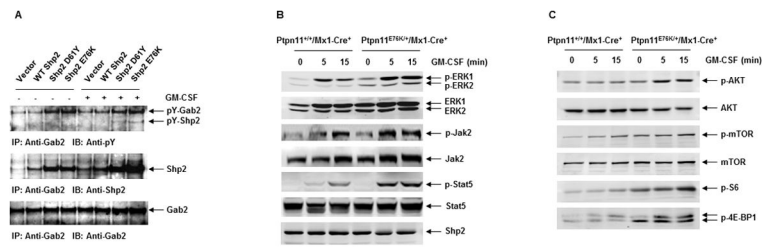
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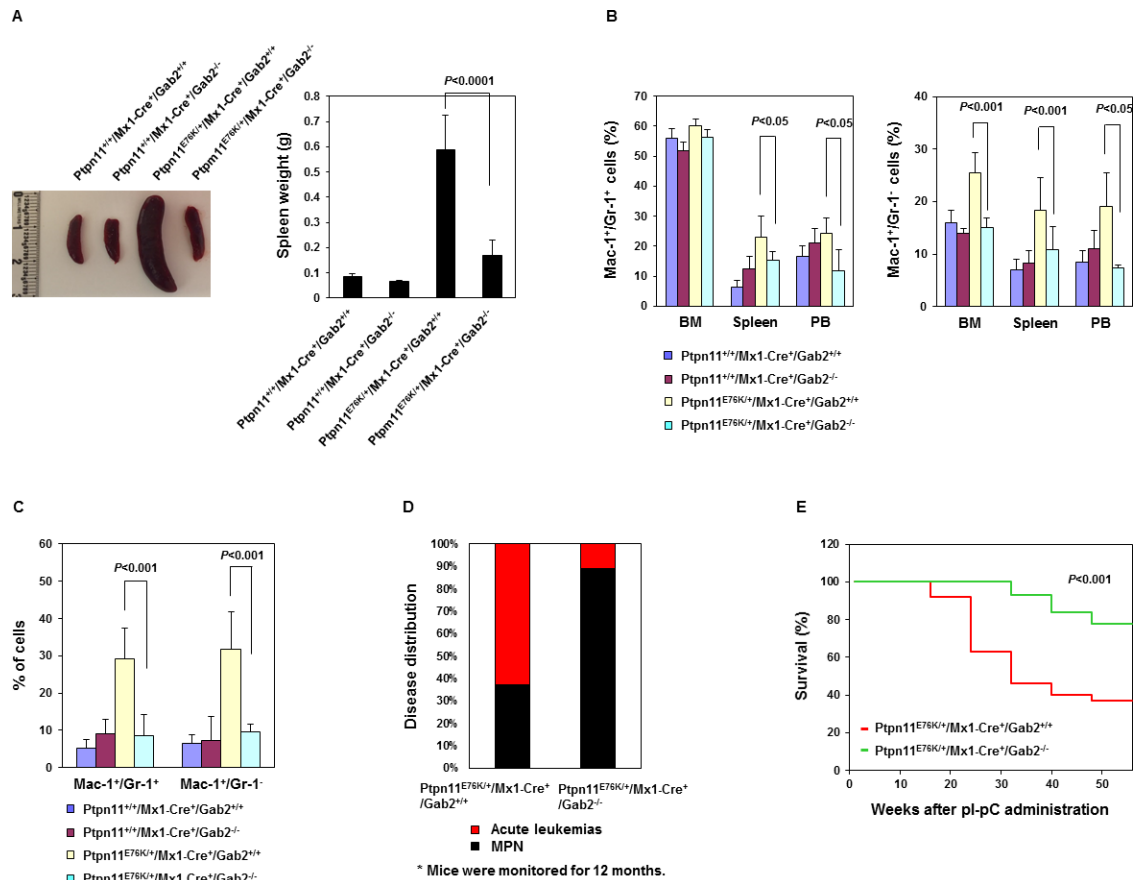
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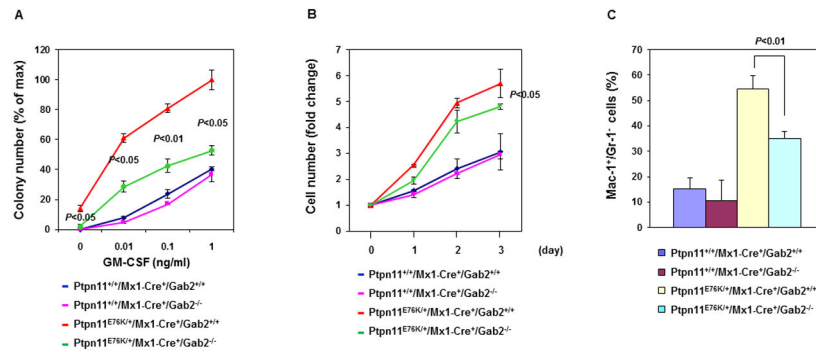


**Figure 1. The *Pttn1*<sup>E76K</sup> mutation enhances cytokine-induced PI3K/Akt/mTOR signaling**  
 (A) BM progenitors transduced with WT Shp2, Shp2 D61Y, Shp2 E76K, or control vector were sorted and differentiated to macrophages, which were starved in serum and cytokine-free medium for 48 hours and then stimulated with GM-CSF (50 ng/mL) for as long as 60 minutes. Protein extracts were prepared and immunoprecipitated with anti-Gab2 antibody followed by anti-pY immunoblotting. Blots were stripped and reprobbed with anti-Shp2 and then anti-Gab2 antibodies. (B) BM-derived macrophages were generated from *Pttn1*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup> and *Pttn1*<sup>+/+</sup>/*Mx1-Cre*<sup>+</sup> mice 6–8 weeks following pI-pC administration. These cells were starved and then stimulated with GM-CSF (50 ng/mL) for the indicated periods of time. Whole cell lysates were prepared. Levels of p-ERK, p-Jak2, and p-Stat5 were determined by immunoblotting analyses. Blots were striped and reprobbed with anti-ERK, anti-Jak2, anti-Stat5, and anti-Shp2 antibodies to check for protein loading. (C) The cell lysates were also examined for levels of p-AKT, p-mTOR, p-S6, and p-4E-BP1 by immunoblotting analyses. Blots were stripped and reprobbed with anti-AKT and anti-mTOR antibodies to check for protein loading. Experiments were repeated three times with similar results obtained in each.



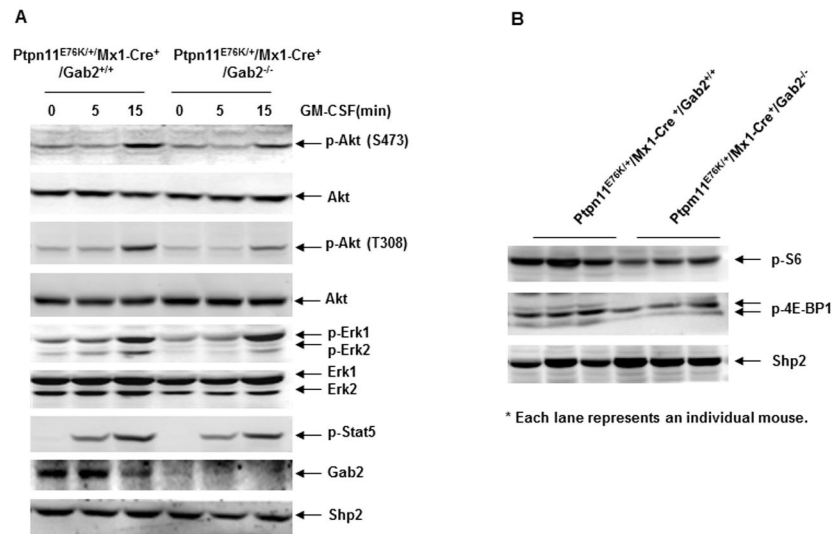
**Figure 2. Development and progression of  $Ptpn11^{E76K/+}$  mutation-induced MPN are attenuated by deletion of Gab2**

$Ptpn11^{+/+}/Mx1-Cre^{+}/Gab2^{+/+}$ ,  $Ptpn11^{+/+}/Mx1-Cre^{+}/Gab2^{-/-}$ ,  $Ptpn11^{E76K/+}/Mx1-Cre^{+}/Gab2^{+/+}$ , and  $Ptpn11^{E76K/+}/Mx1-Cre^{+}/Gab2^{-/-}$  mice were analyzed 10~14 weeks after pi-pC administration. Spleen weights (A) (n=5–10/group), percentages of Mac-1<sup>+</sup>/Gr-1<sup>+</sup> and Mac-1<sup>+</sup>/Gr-1<sup>-</sup> cells in the BM, spleen, peripheral blood (B), and liver (C) (n=4–10/group) were determined. Data are shown as mean±S.D. (D) A cohort of pi-pC-administered  $Ptpn11^{E76K/+}/Mx1-Cre^{+}/Gab2^{+/+}$  (n=27) and  $Ptpn11^{E76K/+}/Mx1-Cre^{+}/Gab2^{-/-}$  (n=18) mice were monitored for acute leukemia progression for 12 months as we previously described<sup>16</sup>. The percentages of the mice that progressed to acute leukemias were documented. (E) Kaplan–Meier survival curves of  $Ptpn11^{E76K/+}/Mx1-Cre^{+}/Gab2^{+/+}$  and  $Ptpn11^{E76K/+}/Mx1-Cre^{+}/Gab2^{-/-}$  mice.



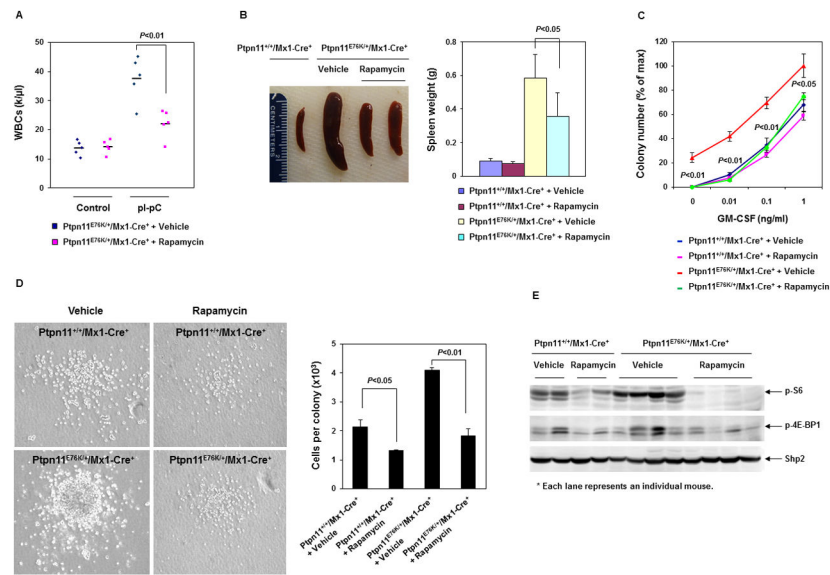
**Figure 3. Depletion of Gab2 corrects the hypersensitive growth pattern of *Ptpn11*<sup>E76K/+</sup> myeloid progenitors**

(A) BM cells ( $2 \times 10^4$  cells/mL) freshly harvested from the mice with the indicated genotypes ( $n=3$ /group) were assessed by CFU assays 8 weeks after pI-pC administration with a range of GM-CSF concentrations. (B) BM-derived macrophages were generated from the mice with the indicated genotypes ( $n=3$ /group) and cultured in DMEM supplemented with 10% FBS and 20% L cell conditional medium for 3 days. Cell growth rates were determined using a One Solution Cell Proliferation Assay kit. (C) LSK (Lineage<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>) cells were sorted from the mice with the indicated genotypes ( $n=6$ /group) and cultured in the presence of IL-3 (2 ng/mL). After 7 days of *in vitro* culture, percentages of myeloid (Mac-1<sup>+</sup>/Gr-1<sup>+</sup>) cells differentiated from LSK cells were assayed by FACS. Three independent experiments were performed with similar results obtained in each. Data shown are mean $\pm$ S.D. from one experiment.



**Figure 4. Gab2 deletion reverses the enhanced PI3K/Akt/mTOR signaling caused by the *Ptpn11*<sup>E76K/+</sup> mutation**

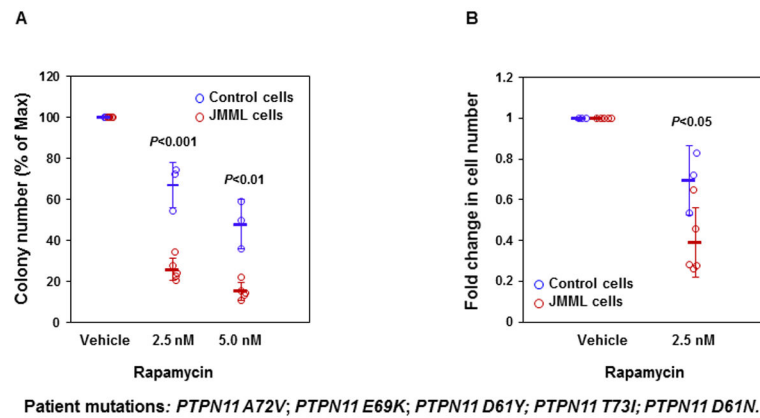
(A) BM-derived macrophages generated from pI-pC administered *Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup>/*Gab2*<sup>+/+</sup> and *Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup>/*Gab2*<sup>-/-</sup> mice were starved in serum and cytokine-free medium for 48 hours and then stimulated with GM-CSF (50 ng/mL) for the indicated periods of time. Whole cell lysates were prepared. Levels of p-Erk, p-Akt, and p-Stat5 were determined by immunoblotting analyses. Blots were striped and reprobed with anti-Erk, anti-Akt, anti-Shp2, and anti-Gab2 antibodies to check for protein loading and Gab2 deletion efficiency. Three independent experiments with three pairs of mice were performed. Similar results were obtained in each. (B) MPN cell lysates prepared from *Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup>/*Gab2*<sup>+/+</sup> and *Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup>/*Gab2*<sup>-/-</sup> mice 10 weeks after pI-pC administration when MPN was fully developed were immunoblotted with p-S6 and p-4E-BP1. Shp2 levels in the tissue lysates were examined by anti-Shp2 immunoblotting. Each lane represents an individual mouse.



### Figure 5. Rapamycin treatment mitigates MPN in *Ptpn11*<sup>E76K/+</sup> mice

(A) WBC counts in the peripheral blood of *Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup> mice were determined before and after 12–14 weeks of treatment with Rapamycin (10 mg/kg body weight, once daily) or the vehicle (n=5/group). (B) Spleen weights were determined after 12–14 weeks of treatment with Rapamycin or vehicle. (C) BM cells were harvested from *Ptpn11*<sup>+/+</sup>/*Mx1-Cre*<sup>+</sup> and *Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup> mice after 12–14 weeks of treatment with Rapamycin or vehicle. These cells were assessed by CFU assays at the indicated GM-CSF concentrations. Experiments were performed three times with similar results obtained in each. Data shown are mean±S.D. from one experiment in triplicates. (D) Representative colonies growing in the medium containing GM-CSF (0.1 ng/mL) are shown. The average number of the cells per colony was determined by dividing the total number of the cells harvested from each plate with the number of the colonies in that plate (n=3/group). Experiments were performed in three pairs of mice with similar results obtained in each. Data shown are mean±S.D. from one experiment in triplicates. (E) Spleen lysates were prepared from *Ptpn11*<sup>+/+</sup>/*Mx1-Cre*<sup>+</sup> and *Ptpn11*<sup>E76K/+</sup>/*Mx1-Cre*<sup>+</sup> mice after 12–14 weeks of treatment with Rapamycin or vehicle. Levels of p-S6, p-4E-BP1, and Shp2 were determined by immunoblotting analyses. Each lane represents an individual mouse.





**Figure 6. Rapamycin abrogates cytokine-induced overgrowth of JMML patient cells with the *PTPN11*<sup>E76K</sup> mutation**

(A) BM cells ( $1 \times 10^4$  cells/mL) from five JMML patients with the indicated *PTPN11* mutations and three normal donors were plated in methylcellulose medium containing GM-CSF (1.0 ng/mL) and Rapamycin at the indicated concentrations. Colonies were enumerated 14 days later and normalized against the number of the colonies derived without Rapamycin treatment. (B) BM cells from five JMML patients with the indicated *PTPN11* mutations and normal donors were cultured in IMDM containing GM-CSF (1.0 ng/mL) and Rapamycin (2.5 nM) or vehicle. Cell numbers were determined using the One Solution Cell Proliferation Assay kit. Data shown are mean  $\pm$  S.D. of all samples tested.