Gain-of-function mutations of *Ptpn11* (Shp2) cause aberrant mitosis and increase susceptibility to DNA damage-induced malignancies

Xia Liu[a,1], Hong Zheng[a,b,1], Xiaobo Li[a,b,1], Siying Wang[a,2], Howard J. Meyerson[c], Wentian Yang[d], Benjamin G. Neel[e], and Cheng-Kui Qu[a,b,3]

[a]Division of Hematology/Oncology, Department of Medicine, Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, OH 44106; [b]Division of Hematology/Oncology, Department of Pediatrics, Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta, Emory University, Atlanta, GA 30322; [c]Department of Pathology, Case Western Reserve University, Cleveland, OH 44106; [d]Department of Orthopaedics, Brown University Alpert Medical School, Providence, RI 02912; and [e]Princess Margaret Cancer Center, Department of Medical Biophysics, University of Toronto, Toronto, ON M5G 2M9, Canada

Edited by Joseph Schlessinger, Yale University School of Medicine, New Haven, CT, and approved December 9, 2015 (received for review May 4, 2015)

Gain-of-function (GOF) mutations of protein tyrosine phosphatase nonreceptor type 11 *Ptpn11* (Shp2), a protein tyrosine phosphatase implicated in multiple cell signaling pathways, are associated with childhood leukemias and solid tumors. The underlying mechanisms are not fully understood. Here, we report that *Ptpn11* GOF mutations disturb mitosis and cytokinesis, causing chromosomal instability and greatly increased susceptibility to DNA damage-induced malignancies. We find that Shp2 is distributed to the kinetochore, centrosome, spindle midzone, and midbody, all of which are known to play critical roles in chromosome segregation and cytokinesis. Mouse embryonic fibroblasts with *Ptpn11* GOF mutations show a compromised mitotic checkpoint. Centrosome amplification and aberrant mitosis with misaligned or lagging chromosomes are significantly increased in *Ptpn11*-mutated mouse and patient cells. Abnormal cytokinesis is also markedly increased in these cells. Further mechanistic analyses reveal that GOF mutant Shp2 hyperactivates the Polo-like kinase 1 (Plk1) kinase by enhancing c-Src kinase-mediated tyrosine phosphorylation of Plk1. This study provides novel insights into the tumorigenesis associated with *Ptpn11* GOF mutations and cautions that DNA-damaging treatments in Noonan syndrome patients with germ-line *Ptpn11* GOF mutations could increase the risk of therapy-induced malignancies.

Ptpn11 | Shp2 | protein tyrosine phosphatase | mitosis | chromosomal instability

Src homology 2 domain-containing protein tyrosine phosphatase 2 (Shp2), a ubiquitously expressed protein tyrosine phosphatase (PTP), plays multiple roles in cellular processes. This phosphatase is best known to be involved in growth factors, cytokines, and other extracellular protein-induced signal transduction (1, 2). It is normally self-inhibited by hydrogen bonding of the backside of the N-terminal SH2 (*N*-SH2) domain to the deep pocket of the PTP domain. Ligands with phosphorylated tyrosine (pY) residues activate Shp2 by binding the SH2 domains (primarily *N*-SH2) and disrupting the inhibitory interaction between *N*-SH2 and PTP domains. Shp2 is involved in multiple cell signaling pathways and plays an overall positive role in transducing signals initiated from receptor and cytosolic kinases (1, 2). The underlying mechanisms are not completely understood. Shp2 interacts with a number of cell signaling intermediates. Of these signaling partners, some are the targets of its enzymatic activity. However, Shp2 can also function as an adaptor protein independent of its catalytic activity (3, 4). In addition to growth factor/cytokine-induced signaling, Shp2 regulates genotoxic stress-triggered signaling and cellular responses (5, 6). More recently, Shp2 has been found to be required for optimal activation of Polo-like kinase 1 (Plk1) and maintenance of chromosomal stability, although the detailed signaling mechanisms remain unclear (7).

The critical role of Shp2 in cell signaling and other activities is further underscored by its direct association with human diseases. Germ-line or somatic heterozygous mutations in *Ptpn11* (encoding Shp2) have been identified in the developmental disorder Noonan syndrome (8), juvenile myelomonocytic leukemia (JMML) (9, 10), acute leukemias (11, 12), and sporadic solid tumors (13). These mutations cause amino acid changes at the interphase formed between *N*-SH2 and PTP domains, disrupting the inhibitory intramolecular interaction and leading to hyperactivation of Shp2 catalytic activity (8, 9). In addition, disease-associated *Ptpn11* mutations enhance the binding of mutant Shp2 to signaling partners (14–16). Recent studies have demonstrated that these gain-of-function (GOF) mutations of *Ptpn11* are sufficient to drive the development of Noonan syndrome and leukemias in mice (15, 17, 18). Nevertheless, as the biochemical basis for the role that Shp2 plays in cell signaling is not well understood, the mechanisms of the tumorigenesis induced by *Ptpn11* GOF mutations remain poorly defined.

Significance

Genetic mutations of protein tyrosine phosphatase nonreceptor type 11 *Ptpn11* (Shp2) are associated with childhood leukemias and solid tumors. The mechanisms by which *Ptpn11* mutations induce malignancies are not fully understood. In this study, we demonstrate that *Ptpn11* disease mutations disturb mitosis and cytokinesis, causing chromosomal instability and greatly increased susceptibility to DNA damage-induced malignancies. This finding reveals a novel mechanism by which deregulation of Shp2 induces malignancies and has important clinical implications. Given that *Ptpn11* mutations enhance DNA damage-induced tumorigenesis, caution should be taken when considering DNA-damaging preconditioning for stem cell transplantation therapy or DNA-damaging chemotherapy for *Ptpn11*-associated malignancies, especially in Noonan syndrome patients with germ-line *Ptpn11* mutations, as the risk of therapy-induced malignancies in these patients may be increased.

Author contributions: C.-K.Q. designed research; X. Liu, H.Z., X. Li, S.W., and H.J.M. performed research; W.Y. and B.G.N. contributed new reagents/analytic tools; X. Liu, H.Z., X. Li, S.W., H.J.M., and C.-K.Q. analyzed data; and X. Liu and C.-K.Q. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]X. Liu, H.Z., and X. Li contributed equally to this work.

[2]Present address: Department of Pathophysiology, Anhui Medical University, Hefei 230032, China.

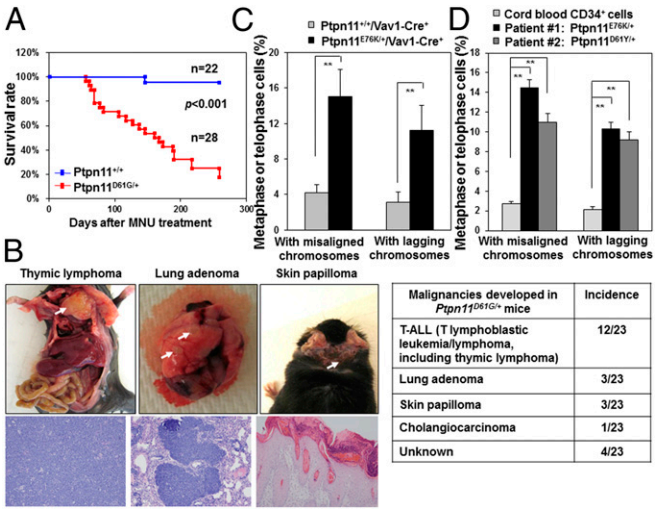
[3]To whom correspondence should be addressed. Email: cheng-kui.qu@emory.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1508535113/-/DCSupplemental.

Results

***Ptpn11*^{D61G} Mutation Induces Chromosomal Instability and Increases Susceptibility to DNA Damage-Induced Malignancies.** During the course of bone marrow transplantation experiments for global *Ptpn11* GOF mutation D61G knock-in (*Ptpn11*$^{D61G/+}$) mice, which develop myeloproliferative disorder (15, 19), we noticed that these animals were susceptible to tumorigenesis following whole-body γ-irradiation (11 Gy) that was used for preconditioning. To further assess responses of *Ptpn11*$^{D61G/+}$ mice to irradiation, we irradiated the mice at a lower dose (6 Gy). During 9 mo of follow-up, 10 of 15 irradiated *Ptpn11*$^{D61G/+}$ mice developed hematological malignancies, mainly T lymphoblastic leukemia/lymphoma, whereas none of the wild-type (WT, *Ptpn11*$^{+/+}$) animals did (Fig. S1*A*). Similarly, 23 of 28 *Ptpn11*$^{D61G/+}$ mice treated with a DNA-damaging agent Methylnitrosourea (MNU) died within 8 mo (Fig. 1*A*), whereas only 1 of 22 WT mice died of thymic lymphoma. In addition to leukemia/lymphoma, lung adenomas and skin papillomas were found in MNU-treated *Ptpn11*$^{D61G/+}$ mice (Fig. 1*B*). Consistent with these results, conditional knock-in mice bearing another *Ptpn11* GOF mutation (E76K) (18), which is stronger than the D61G mutation in activating the catalytic activity of Shp2 (9, 20), showed much accelerated evolution into acute leukemias in response to 6 Gy irradiation (Fig. S1*B*), suggesting that the effects of Shp2 mutants on sensitizing DNA damage-induced malignancies correlate with their enhanced catalytic activities.

Tumorigenesis is highly associated with genomic instability. Genomic instability predisposes cells to malignant transformation and sensitizes the cells to DNA damage-induced malignancies (21, 22). To determine whether the *Ptpn11*^{D61G} mutation might enhance tumorigenesis through disturbing genomic stability, we generated primary mouse embryonic fibroblasts (MEFs) from *Ptpn11*^{D61G} knock-in mice and monitored their chromosomal stability.



Malignancies developed in *Ptpn11*$^{D61G/+}$ mice	Incidence
T-ALL (T lymphoblastic leukemia/lymphoma, including thymic lymphoma)	12/23
Lung adenoma	3/23
Skin papilloma	3/23
Cholangiocarcinoma	1/23
Unknown	4/23

Fig. 1. *Ptpn11* GOF mutations induce chromosomal instability and increase susceptibility to DNA damage-induced malignancy. Eight to 10-wk-old *Ptpn11*$^{D61G/+}$ mice and *Ptpn11*$^{+/+}$ littermates were treated with a single dose of MNU (50 mg/kg body weight) by i.p. injection. (*A*) Kaplan–Meier survival curves of the mice were determined. (*B*) Incidence of tumors in MNU-treated *Ptpn11*$^{D61G/+}$ mice and representative photographs/histopathological images of the indicated tumors. (*C*) Bone marrow cells isolated from *Ptpn11*$^{E76K/+}$/*Vav1-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$/*Vav1-Cre*$^{+}$ littermates were immunostained with anti–β-tubulin antibody and counterstained with DAPI. Metaphase cells with chromosomal misalignment or anaphase/telophase cells with lagging chromosomes were counted. More than 80 mitotic cells for each cell type were examined. (*D*) Leukemic cells from two JMML patients with the indicated *PTPN11* GOF mutations and cord blood CD34^{+} cells were examined as above. More than 70 mitotic cells for each sample were examined.

Ptpn11$^{D61G/D61G}$ and *Ptpn11*$^{D61G/+}$ cells displayed significantly increased chromosomal instability as opposed to WT cells. Aneuploidy, a state of having abnormal numbers of chromosomes, in *Ptpn11*$^{D61G/D61G}$ and *Ptpn11*$^{D61G/+}$ cells was readily detected at passage 3. At passage 5, the majority of these cells were aneuploid, whereas 50% of WT cells were still diploid (Fig. S1*C*). At passage 18, the percentage of tetraploid cells with 8n DNA content was increased substantially in *Ptpn11*$^{D61G/D61G}$ and *Ptpn11*$^{D61G/+}$ populations (Fig. S1*D*), and this effect of the *Ptpn11*$^{D61G/}$ mutation is clearly gene dosage-dependent. Spectral karyotyping (SKY) analyses validated that *Ptpn11*$^{D61G/D61G}$ cells contained prominent numerical (gain or loss of chromosomes) and structural aberrations (chromosome deletions and translocations) in chromosomes (Fig. S1*E*).

***Ptpn11* GOF Mutations Result in a Defective Mitotic Checkpoint and Aberrant Mitosis/Cytokinesis.** Various cellular surveillance mechanisms function to maintain the integrity of the genome. The mitotic checkpoint, also known as the spindle assembly checkpoint, is a cellular surveillance mechanism that functions to ensure faithful chromosome segregation during mitosis (23, 24). Loss of this highly conserved checkpoint function results in premature mitosis and missegregation of chromosomes, leading to aneuploidy and proneness to cancer. *Ptpn11*$^{D61G/D61G}$ and *Ptpn11*$^{D61G/+}$ cells exhibited aneuploidy; we therefore wondered whether the mitotic checkpoint in these cells was defective. To address this question, *Ptpn11*$^{D61G/D61G}$, *Ptpn11*$^{D61G/+}$, and *Ptpn11*$^{+/+}$ MEFs (at passage 3–5) were treated with nocodazole, a microtubule depolymerizing agent that triggers activation of the mitotic checkpoint and arrests cells at mitosis—in particular, prometaphase. Mitotic checkpoint function was then determined by assessing the percentage of mitotic cells identified by p-histone H3 immunostaining. As shown in Fig. S2*A*, 16 h following nocodazole treatment, the percentage of *Ptpn11*$^{D61G/D61G}$ and *Ptpn11*$^{D61G/+}$ cells arrested at mitosis was decreased compared with that of *Ptpn11*$^{+/+}$ cells. These data demonstrate that the *Ptpn11*^{D61G} mutation compromises mitotic checkpoint function.

To further determine the detrimental effects of the *Ptpn11*^{D61G} mutation on mitosis, we surveyed the morphology of unperturbed mitotic cells (without nocodazole treatment) by p-histone H3 and 4′,6-diamidino-2-phenylindole (DAPI) double staining. The frequency of abnormal mitotic cells with aberrant chromatid morphology (chromosome misalignment at metaphase and chromosome missegregation at anaphase/telophase) was increased in *Ptpn11*$^{D61G/+}$ primary cells, and this difference between WT and *Ptpn11* mutant cells was exacerbated following γ-irradiation (Fig. S2*B*). Furthermore, centrosome amplification was observed in *Ptpn11*$^{D61G/+}$ cells. The percentage of the cells with ≥3 centrosomes, as identified by γ-tubulin immunostaining, was increased in untreated and irradiated *Ptpn11*$^{D61G/+}$ cells (Fig. S2*C*). Consistent with these results, mitotic arrest induced by nocodazole was also decreased in *Ptpn11*$^{E76K/+}$ MEFs (Fig. S3*A*). Moreover, the frequency of abnormal mitotic cells with chromosome misalignment at metaphase or lagging chromosomes at anaphase/telophase in *Ptpn11*$^{E76K/+}$ bone marrow cells (Fig. 1*C*) or MEFs (Fig. S3*B*) and in JMML patient leukemic cells with Ptpn11 GOF mutations (Fig. 1*D*) was greatly increased. Similar to *Ptpn11*$^{D61G/+}$ cells, centrosome amplification in *Ptpn11*$^{E76K/+}$ MEFs was also much more frequent than that in control cells (Fig. S3*C*).

To directly visualize the impact of the *Ptpn11*^{D61G} mutation on mitosis, cell division was monitored in living *Ptpn11*$^{+/+}$ and *Ptpn11*$^{D61G/D61G}$ MEFs with a time-lapse video microscope. The duration of mitosis in *Ptpn11*$^{D61G/D61G}$ cells was prolonged (Fig. 2*A* and representative photomicrographs shown in Fig. S4) likely because mutant cells with chromosome misalignment needed a longer time to satisfy the mitotic checkpoint and because of amplification of centrosomes (25). *Ptpn11*-mutated cells still entered into anaphase with misaligned chromosomes due to the

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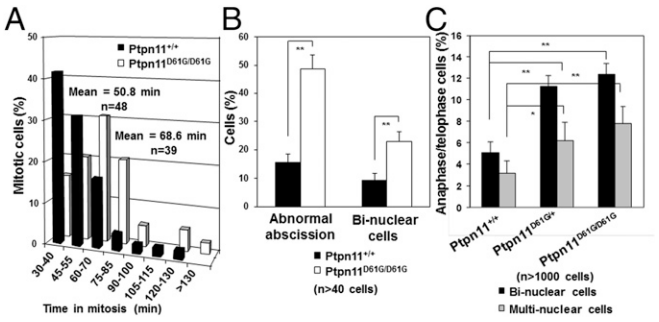

Fig. 2. Aberrant mitosis/cytokinesis in mutant cells bearing *Ptpn11* GOF mutations. Mitosis and cell division in living $Ptpn11^{+/+}$ and $Ptpn11^{D61G/D61G}$ MEFs at passage 3–5 were monitored by time-lapse video microscopy. (*A*) Average times of mitosis (from nuclear membrane breakdown to completion of cell division) for $Ptpn11^{+/+}$ and $Ptpn11^{D61G/D61G}$ MEFs. (*B*) The percentages of the cells with abnormal abscission and the percentages of the daughter cells with two nuclei due to failed cytokinesis were quantified. (*C*) Mitotic $Ptpn11^{+/+}$ and $Ptpn11^{D61G/D61G}$ MEFs (passage 5) arrested by nocodazole were collected by shake-off and then released to regular culture medium. Eighty minutes later, cells were fixed and immunostained with anti–β-tubulin antibody and counterstained with DAPI. The percentages of the cells with binuclei or multinuclei in anaphase and telophase were quantified.

mitotic checkpoint defect, leading to increased chromosome missegregation. In addition, cytokinesis, the last stage of mitosis, was abnormal in $Ptpn11^{D61G/D61G}$ cells (Fig. S4). The percentage of dividing $Ptpn11^{D61G/D61G}$ cells with abnormal abscission was markedly increased (Fig. 2*B*). Some $Ptpn11^{D61G/D61G}$ cells could not be successfully separated, and a long bridge between two nascent daughter cells was frequently observed (Fig. S4, *Upper* panel of the $Ptpn11^{D61G/D61G}$ group), resulting in binucleate cells (Fig. S4, *Lower* panel of the $Ptpn11^{D61G/D61G}$ group). The percentage of binucleate cells in $Ptpn11^{D61G/D61G}$ MEFs was much higher than that in $Ptpn11^{+/+}$ cells (Fig. 2*B*). To further confirm these results, we collected nocodazole-arrested mitotic cells and then released them to fresh regular culture medium. After 80 min, most of the $Ptpn11^{+/+}$ cells reached cytokinesis and completely separated to two daughter cells, and only less than 10% of cells failed to complete this process. However, the percentage of $Ptpn11^{D61G/D61G}$ cells with binuclear or multinuclear cells resulting from failed cytokinesis was doubled (Fig. 2*C*), providing further evidence for the detrimental effect of the $Ptpn11^{D61G}$ mutation on cytokinesis.

Shp2 Is Activated and Distributed to the Kinetochore, Centrosome, Spindle Midzone, and Midbody in Mitotic Cells. To elucidate the mechanisms by which the $Ptpn11^{D61G}$ mutation disturbs mitosis, we first examined cell signaling activities specifically in mitotic cells, as Shp2 is well established for its important role in cell signaling. Interestingly, no significant changes in Erk, Akt, JNK, and p38 kinase activities in mitotic or synchronized G_1 $Ptpn11^{D61G/D61G}$ and $Ptpn11^{D61G/+}$ MEFs were observed (Fig. S5), making it unlikely that aberrant mitosis and cytokinesis in $Ptpn11^{D61G}$ mutant cells are caused by changes in growth factor or stress signaling. We reasoned that GOF mutations of Shp2 might impact mitosis through other mechanisms. Compared with that in synchronized G_1 cells, tyrosine phosphorylation of Shp2, indicative of its involvement in signaling activities, in "shake-off" mitotic cells was increased, and the catalytic activity of Shp2 was doubled (Fig. 3*A*), suggesting that Shp2 is more active when cells enter into mitosis. Next, we examined the subcellular distribution of Shp2, specifically in mitotic cells. Surprisingly, Shp2 was found to be distributed at the spindle midzone in telophase and concentrated at the midbody in cytokinesis, as evidenced by colocalization with β-tubulin at these mitotic apparatuses (Fig. 3*B*, *Top* panel). This dynamic localization pattern of Shp2 is remarkably similar to those of Aurora B and Plk1, two serine/threonine kinases that play critical roles in mitosis and cytokinesis (26, 27). Shp2 colocalized with Aurora B (Fig. 3*B*, *Middle* panel) and partially colocalized with Plk1 (Fig. 3*B*, *Lower* panel) at the midbody during cleavage and abscission of daughter cells. In addition, Shp2 colocalized with γ-tubulin (Fig. 3*C*), a centrosome-specific protein, indicating its localization at centrosomes. More importantly, Shp2 staining overlapped with CREST (Calcinosis, Raynaud phenomenon, Esophageal dysmotility, Sclerodactyly, and Telangiectasia) staining (Fig. 3*D*), which is restricted to the inner core of the kinetochore, the protein structure on centromeres where the spindle fibers attach, suggesting that Shp2 also is distributed to the kinetochore. Kinetochores, centrosomes, and midbodies play crucial roles in chromosomal segregation and subsequent cytokinesis (28, 29); the distribution of Shp2 to these structures implied that Shp2 might directly participate in the regulation of mitosis. In *Ptpn11*-mutated mouse bone marrow cells (Fig. 3*E*) and JMML patient leukemic cells (Fig. 3*F*), both of which showed greatly increased chromosome misalignment and missegregation in mitosis (Fig. 1 *C* and *D*), Shp2 was also detected in the centrosome. Interestingly, it was only detected in some but not all amplified centrosomes, supporting the hypothesis that centrosome amplification and genomic instability in these leukemic cells are associated at least in part with *Ptpn11* GOF mutations in mitotic structures.

GOF Mutant Shp2 Enhances Plk1 and Aurora B Activities in Mitosis. To further determine the mechanisms responsible for the pronounced effects of *Ptpn11* (Shp2) GOF mutations on mitosis, we tested physical and functional interactions between Shp2 and Plk1, as our previous study demonstrated that WT Shp2 was required for optimal activation of Plk1 in mitosis (7). Shp2 and Plk1 were detected in the same immunocomplex in mitotic cells (Fig. 4*A*). More interestingly, D61G and E76K mutations enhanced the association between mutant Shp2 and Plk1 (Fig. 4*A*), likely due to the open conformation of mutant Shp2 caused by the mutations. Cotransfection of GFP-Plk1 and various Flag-tagged truncated Shp2 followed by Shp2/Plk1 interaction assays showed that full-length Shp2 (FL), Shp2 C (PTP and C-tail), and Shp2 W3 (*N*-SH2, C-SH2, and PTP), but not Shp2 N (*N*-SH2 and C-SH2), bound to GFP-Plk1, indicating that a motif in the PTP domain is required for the binding to Plk1 (Fig. S6*A*). Further in vitro binding assays verified that GST full-length Shp2 and Shp2 W3 but not Shp2 N pulled down Plk1 from the lysates of mitotic cells (Fig. S6*B*). We also transfected Flag-tagged Shp2 FL, Shp2 C, Shp2 W3, and Shp2 N into HeLa cells and examined their localization to the kinetochore. Shp2 N detected in the kinetochore was greatly decreased compared with Shp2 FL, Shp2 C, and Shp2 W3 (Fig. S6*C*), suggesting that the interaction of Shp2 with Plk1 is essential for Shp2 to localize to this mitotic apparatus. Notably, Plk1 kinase activity, determined by phosphorylation of Thr^{210} (p-Thr^{210}), in $Ptpn11^{D61G/D61G}$ and $Ptpn11^{E76K/+}$ mitotic cells was elevated (Fig. 4*B*). In addition, activity of Aurora B, determined by p-Thr^{232}, was also significantly increased (Fig. 4*C*), although no physical interaction between Shp2 and Aurora B was detected. The increase in Aurora B activity might be caused by the enhanced activation of Plk1, owing to the intricate functional interplay between Plk1 and Aurora B kinases (26, 27). Furthermore, we treated $Ptpn11^{E76K/+}$ mutant MEFs with the Plk1 inhibitor BI6727 at a low concentration (5 nM) and assessed its effect on reversing chromosomal instability. Indeed, BI6727 significantly reduced the percentages of metaphase cells with chromosome misalignment and anaphase/telophase cells with lagging chromosomes in these cells (Fig. S7), verifying that aberrant activation of Plk1 is responsible for the chromosomal instability induced by the $Ptpn11^{E76K/+}$ mutation.

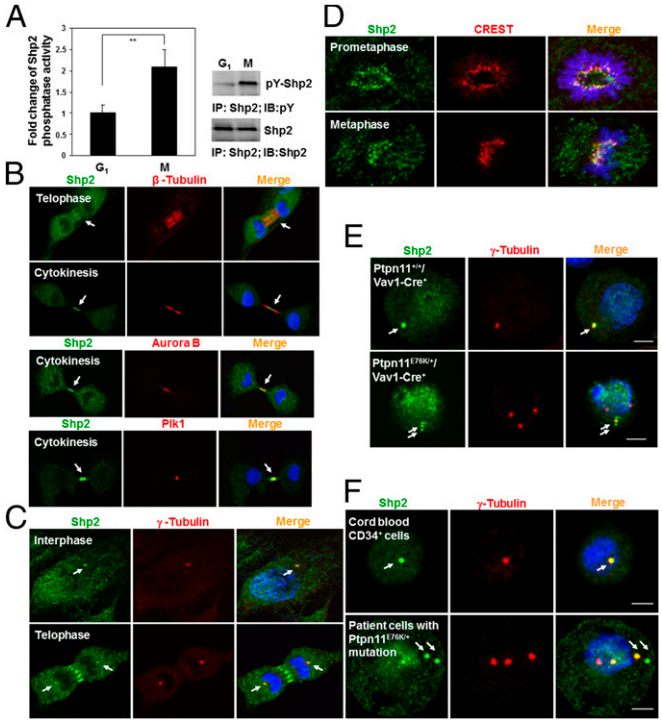

Fig. 3. Shp2 is activated and localized to the kinetochore, centrosome, spindle midzone, and midbody in mitotic cells. (*A*) Synchronized G_1 and mitotic HeLa cells were lysed, and Shp2 catalytic activity in the lysates was assessed by the immunocomplex phosphatase assay. The cell lysates were also examined by immunoprecipitation and immunoblotting as indicated. (*B–D*) NIH 3T3 cells were immunostained with the indicated antibodies. Nuclei were counterstained with DAPI. (*E*) Bone marrow cells isolated from *Ptpn11*$^{E76K/+}$/*Vav1-Cre*$^{+}$ mice and *Ptpn11*$^{+/+}$/*Vav1-Cre*$^{+}$ littermates were immunostained with the indicated antibodies. (*F*) Leukemic cells from two JMML patients with the indicated *PTPN11* mutations and cord blood CD34^{+} cells were immunostained as above.

Plk1 Is Tyrosine Phosphorylated by the c-Src Kinase in Mitosis. During the experiments analyzing the Shp2/Plk1 interaction, we unexpectedly observed tyrosine phosphorylation of Plk1 in mitotic cells (Fig. 5*A*). Phosphoproteomics analyses identified Tyr^{166} and Tyr^{445} as the major phosphorylation sites. To identify the tyrosine kinases that are responsible for Plk1 phosphorylation, we tested a number of tyrosine kinase inhibitors and found that the c-Src kinase inhibitor SU6656 significantly decreased tyrosine phosphorylation of Plk1 in mitosis (Fig. S8*A*). The same results were obtained from c-Src knockdown cells (Fig. 5*B*). Intriguingly, Plk1 kinase activity (p-Thr^{210}) was also suppressed in the knockdown or inhibitor-treated cells (Fig. 5*B* and Fig. S8*A*), suggesting that tyrosine phosphorylation is required for optimal activation of Plk1. Coimmunoprecipitation analyses demonstrated that Plk1 and c-Src were present in the same immunocomplex (Fig. 5*C*). To further test whether the c-Src kinase can directly phosphorylate Plk1, we incubated the active c-Src kinase with purified Plk1 in kinase assay buffer. Plk1 was indeed tyrosine phosphorylated by c-Src (Fig. 5*D*), confirming that tyrosine phosphorylation of Plk1 is most likely carried out by the c-Src kinase.

GOF Mutant Shp2 Enhances Plk1 Tyrosine Phosphorylation by Promoting Activation of the c-Src Kinase. We next examined tyrosine phosphorylation of Plk1 in *Ptpn11* GOF mutant mitotic cells, which displayed increased Plk1 activity (Fig. 4*B*). Plk1 tyrosine phosphorylation in *Ptpn11*$^{E76K/+}$ mitotic cells was significantly increased (Fig. 5*E*). Coimmunoprecipitation assays showed that Shp2, c-Src, and Plk1 were present in the same protein complex and that the associations between the Shp2 E76K mutant and c-Src or Plk1 were enhanced compared with those in control cells (Fig. 5*F*). Previous studies suggest that Shp2 promotes c-Src activation by directly or indirectly controlling phosphorylation levels of C-terminal Tyr^{527} (30, 31), which negatively regulates its kinase activity (32). We thus determined tyrosine phosphorylation of c-Src in the Shp2 immunocomplex. Autophosphorylation (Tyr^{416}) of Src, indicative of kinase activity, in the Shp2 immunocomplex was clearly increased in mitotic cells compared with G_1 cells, whereas the inhibitory phosphorylation at Tyr^{527}, the target of Shp2, was decreased in mitotic cells (Fig. 5*G*). Furthermore, phosphorylation levels of Tyr^{416} and Tyr^{527} of Src were increased and decreased, respectively, in *Ptpn11*$^{E76K/+}$ cells relative to those in control cells (Fig. 5*H*), indicating that the activating mutation E76K of Shp2 increases Plk1 tyrosine phosphorylation by directly or indirectly enhancing dephosphorylation of the inhibitory Tyr^{527} of the Src kinase. Consistent with this notion, overexpression of the catalytically inactive Shp2 E76K C459S double mutant greatly decreased tyrosine phosphorylation as well as activity (p-Thr^{210}) of Plk1 in mitotic cells (Fig. S8*B*). These data strongly suggest that the elevated catalytic activity is required for GOF mutant Shp2 to enhance tyrosine phosphorylation and activation of Plk1.

Finally, to verify the role of phosphorylation at Tyr^{166} and Tyr^{445} of Plk1 in mediating the detrimental effects of *Ptpn11* GOF mutations on mitosis, we knocked down Plk1 from Shp2 E76K-expressing HeLa cells with shRNA, transfected the cells with WT Plk1 or Plk1 mutants with Y166F and Y445F mutations, and then assessed their rescue effects on the mitotic checkpoint in Plk1 knockdown cells. Shp2 E76K-expressing cells displayed a reduced mitotic checkpoint, as evidenced by

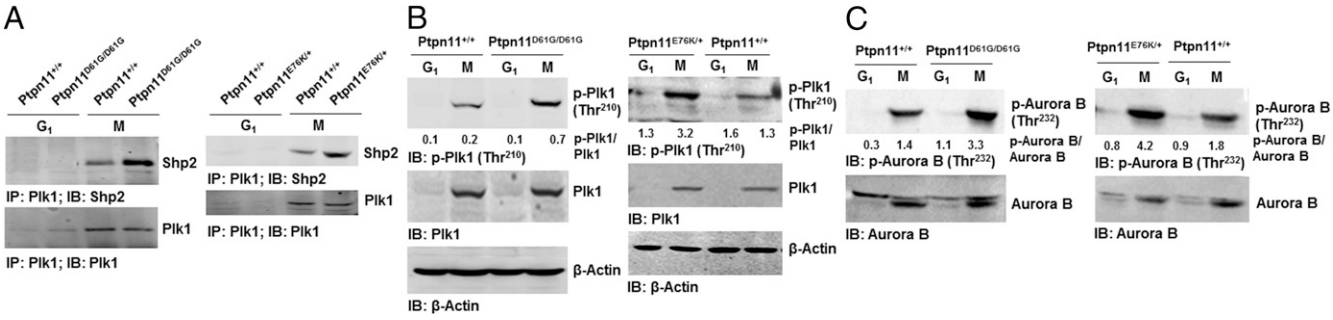

Fig. 4. GOF mutant Shp2 enhances Plk1 activation in mitosis. (*A*) Whole-cell lysates prepared from synchronized G_1 and mitotic *Ptpn11*$^{+/+}$ and *Ptpn11*$^{D61G/D61G}$ MEFs (*Left*) or *Ptpn11*$^{+/+}$ and *Ptpn11*$^{E76K/+}$ MEFs (*Right*) were examined by immunoprecipitation and immunoblotting as indicated. Numbers shown in *B* and *C* indicate arbitrary units of p-Plk1/Plk1 or p-Aurora B/Aurora B from densitometric analyses.

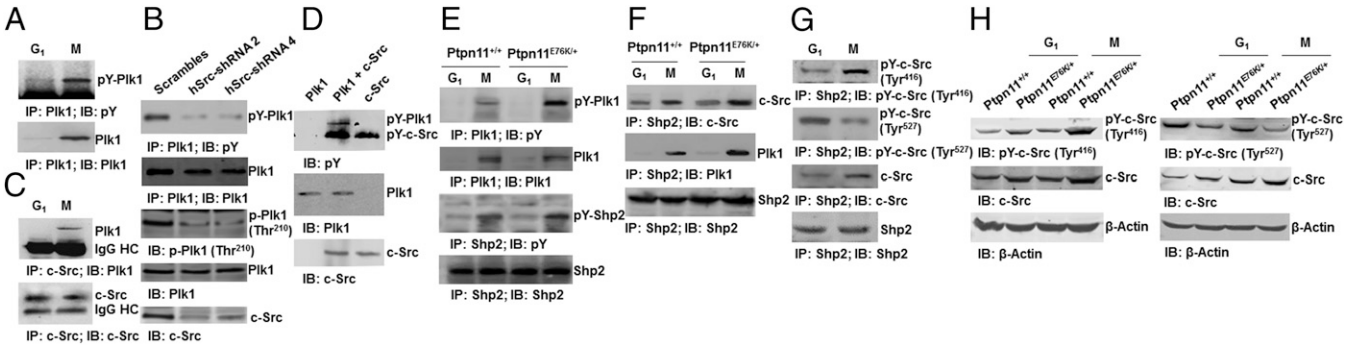

Fig. 5. Plk1 is tyrosine phosphorylated by the c-Src kinase in mitosis, and GOF mutant Shp2 enhances Plk1 tyrosine phosphorylation by increasing c-Src activity. (*A*) Synchronized G_1 and mitotic HeLa cells were collected, and cell lysates were examined by immunoprecipitation and immunoblotting as indicated. (*B*) HeLa cells were transfected with human c-Src shRNA. Cell lysates prepared from mitotic cells were examined by immunoprecipitation and immunoblotting as indicated. (*C*) Whole-cell lysates prepared from synchronized G_1 and mitotic HeLa cells were examined by immunoprecipitation and immunoblotting as indicated. (*D*) Purified c-Src kinase (5 units) and Plk1 (0.1 μg) were incubated at 37 °C for 30 min in tyrosine kinase assay buffer. The entire reaction system was analyzed by immunoblotting with the indicated antibodies. (*E* and *F*) Whole-cell lysates prepared from synchronized G_1 and mitotic *Ptpn11*$^{+/+}$ and *Ptpn11*$^{E76K/+}$ MEFs were examined by immunoprecipitation and immunoblotting as indicated. (*G* and *H*) Synchronized G_1 and mitotic HeLa cells or synchronized G_1 and mitotic *Ptpn11*$^{+/+}$ and *Ptpn11*$^{E76K/+}$ MEFs were examined by immunoprecipitation and immunoblotting as indicated.

the decreased mitotic arrest response to nocodazole (Fig. 6*A*) and increased abnormal mitosis with misaligned and lagging chromosomes (Fig. 6*B* and Fig. S9), similar to *Ptpn11*$^{E76K/+}$ MEFs (Fig. S3*B*). Silencing Plk1 in the Shp2 E76K-expressing cells partially rescued mitotic checkpoint function (Fig. 6*A*) and decreased the percentages of metaphase cells with misaligned chromosomes and anaphase/telophase cells with lagging chromosomes (Fig. 6*B* and Fig. S9) to similar levels in WT Shp2-expressing cells. Importantly, reintroduction of WT Plk1 into the Plk1 knockdown cells restored the Shp2 E76K effect on the mitotic checkpoint (Fig. 6*A*) and chromosome instability (Fig. 6*B* and Fig. S9). However, neither Plk1^{Y166F} nor Plk1^{Y445F} mutants showed rescue capabilities in both assays (Fig. 6 *A* and *B* and Fig. S9), reaffirming the role of the enhanced tyrosine phosphorylation of Plk1 in mediating the detrimental effect of the *Ptpn11* GOF mutation on genomic stability. Indeed, compared with WT Plk1, Plk1^{Y166F} and Plk1^{Y445F} mutants had greatly decreased activities, as determined by p-Thr^{210} levels (Fig. 6*C*).

Discussion

In this report, we present several findings that shed light on the tumorigenesis associated with *Ptpn11* (Shp2) GOF mutations. Although the overall positive role of Shp2 in growth factor/cytokine signaling has been well established (1, 2), *Ptpn11* GOF mutations appear to induce tumorigenesis not only by promoting cell proliferation but also by being permissive for cancer-forming genetic alterations due to the disruption of the mitotic machinery. The role of Shp2 in cell signaling processes represents the function of Shp2 mostly in interphase cells. By specifically focusing on cells in mitosis, a very short yet critical phase in the cell life, our study now demonstrates that Shp2 GOF mutations have a significant impact on genomic stability in both mouse models and patient cells. This impact appears to be independent of their effects on growth factor signal transduction because cell signaling activities during this particular time were not significantly changed by a GOF mutation of Shp2 (Fig. S5). Rather, the increased catalytic activity (8, 9) and enhanced binding of GOF mutant Shp2 to its interacting proteins (14–16) directly disturb mitosis and cytokinesis, evoking chromosomal instability and other genetic alterations that cooperatively drive tumor cell formation. The notion that Shp2 GOF mutations induce chromosomal instability is also supported by clinical observations, in which *Ptpn11* GOF mutations were frequently found in childhood leukemias with hyperdiploid DNA content (11, 33), karyotypic changes were often observed in JMML patients with *Ptpn11* mutations (34, 35), and Noonan syndrome patients with *Ptpn11* mutations were predisposed to hematological malignancies, relative to *Ptpn11* mutation-negative cases (36).

Shp2 GOF mutations appear to induce genomic instability by directly disturbing mitosis. One of the findings in this report is that part of the Shp2 protein is localized to the mitotic apparatus, including the centrosome, spindle midzone, midbody, and the kinetochore (Fig. 3 *B*–*F*), all of which are known to play critical roles in chromosomal segregation and maintaining

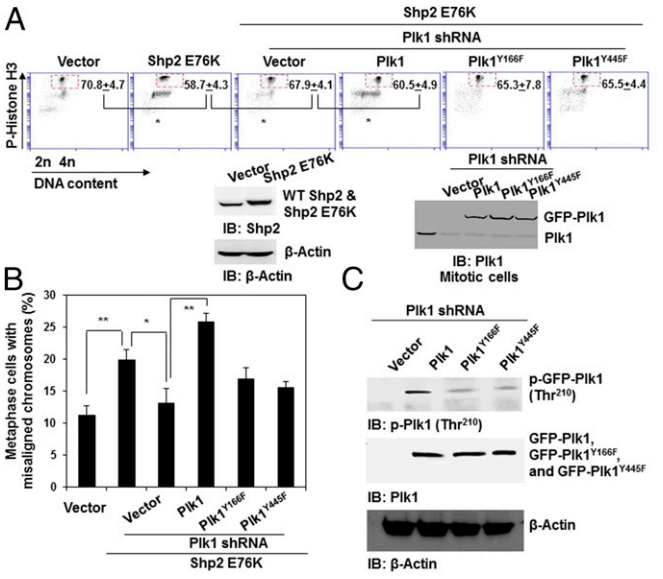

Fig. 6. Important role of phosphorylation of Y^{166} and Y^{445} for the activation of Plk1. Shp2 E76K cDNA and the control vector were transfected into HeLa cells. Shp2 E76K-overexpressing cells were then transfected with Plk1 shRNA targeting 3′UTR. These Plk1 knockdown cells were subsequently transfected with WT GFP-Plk1, GFP-Plk1 mutants with Y166F or Y445F mutations, or the control vector. (*A*) Forty-eight hours later, cells were treated with nocodazole (200 ng/mL) for 16 h, and the percentages of mitotic cells were determined by propidium iodide (PI) and p-histone H3 double staining followed by FACS analyses. (*B*) Metaphase cells with chromosomal misalignment were counted. More than 50 mitotic cells for each group were examined. (*C*) Whole-cell lysates prepared from mitotic cells were examined by immunoblotting as indicated.

genome integrity (23, 28, 29). In addition, Shp2 was detected in the same protein complex with the mitotic kinase Plk1 (Fig. 4*A*), and the Shp2/Plk1 interaction was required for Shp2 to localize to the mitotic apparatus (Fig. S6*C*). More importantly, Plk1 was aberrantly activated in *Ptpn11* GOF mutant cells (Fig. 4*B*). Plk1 plays a pleotropic role in mitotic processes. It drives the G_2/M progression (26, 27). It is also localized to the centrosome, kinetochore, and spindle midzone; participates in the spindle assembly checkpoint by phosphorylating checkpoint proteins; and is required for the formation of stable kinetochore–microtubule attachments (26, 27). Elevated Plk1 activity causes kinetochore–microtubule attachment errors (37), and Plk1 is indeed overexpressed in various human cancers with poor prognosis (38). Because treatment with a Plk1 inhibitor at a low concentration restored chromosome stability in Shp2 GOF mutant cells (Fig. S7), disrupted mitosis and chromosomal missegregation in Shp2 GOF mutant cells appear to be attributed to the enhanced Plk1 activity.

Another interesting finding in this report is that Shp2 GOF mutations hyperactivate Plk1 by enhancing c-Src kinase-mediated tyrosine phosphorylation of Plk1. We discovered that Plk1 was phosphorylated on Tyr^{166} and Tyr^{445} in mitotic cells and that tyrosine phosphorylation of Plk1 correlated with the activation status of Plk1 (Fig. 5*B* and Fig. 6*C*). The mitosis-specific tyrosine phosphorylation of Plk1 was likely carried out by the c-Src kinase because tyrosine-phosphorylated Plk1 was detected in the same protein complex with c-Src (Fig. 5*C*), and the c-Src kinase was indeed able to directly phosphorylate Plk1 in the kinase assay (Fig. 5*D*). Moreover, knockdown or inhibition of c-Src substantially decreased tyrosine phosphorylation as well as activity of Plk1 (Fig. 5*B* and Fig. S8*A*). Plk1 tyrosine phosphorylation (Fig. 5*E*) and activity (Fig. 4*B*) were significantly increased in $Ptpn11^{E76K/+}$ mitotic cells, in which c-Src activity was also increased (Fig. 5*H*). Given that the inhibitory tyrosine phosphorylation of c-Src (Tyr^{527}) was decreased in $Ptpn11^{E76K/+}$ mitotic cells expressing the hyperactive Shp2 E76K mutant (Fig. 5*H*), the increased c-Src activity might be caused by enhanced dephosphorylation of Tyr^{527} or enhanced dephosphorylation of PAG/Cbp, tyrosine phosphorylation of which is required for the Csk kinase to get access to and phosphorylate c-Src (Tyr^{527}) (31). This notion is supported by the observation that inactivation of the catalytic activity of the Shp2 E76K mutant greatly decreased tyrosine phosphorylation and activity of Plk1 (Fig. S8*B*). Further studies, however, are required to determine how WT Shp2 initially gets activated in mitosis to facilitate Plk1 tyrosine phosphorylation and how elevated phosphorylation of Tyr^{166} and Tyr^{445} promotes Plk1 activation. The detailed structural basis for the physical and functional interactions among Shp2, Src, and Plk1 also needs to be determined.

Materials and Methods

To synchronize cells at the G_1 phase, cells were treated with thymidine (2 mM) for 17 h, washed with PBS three times, and then incubated in fresh medium without thymidine. After 9 h, thymidine (2 mM) was added again for another 16 h. To arrest cells in mitosis, cells were treated with nocodazole (200 and 400 ng/mL for HeLa cells and MEFs, respectively) for 16 h. Mitotic cells were then collected by shake-off.

All animal procedures were approved by the Institutional Animal Care and Use Committee at Case Western Reserve University and Emory University. Deidentified patient specimens were utilized in this study. Informed consent was obtained from patients.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health Grants CA181754 and DK092722 (to C.-K.Q.).

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