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Synthetic lethality of TNK2 inhibition in PTPN11-mutant leukemia

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Abstract

The protein tyrosine phosphatase PTPN11 is implicated in the pathogenesis of juvenile myelomonocytic leukemia (JMML), acute myeloid leukemia (AML), and other malignancies. Activating mutations in PTPN11 increase downstream proliferative signaling and cell survival. We investigated the signaling upstream of PTPN11 in JMML and AML cells and found that PTPN11 was activated by the nonreceptor tyrosine/serine/threonine kinase TNK2 and that PTPN11-mutant JMML and AML cells were sensitive to TNK2 inhibition. In cultured human cell-based assays,

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Data and materials availability: The full publically available data set from fig. S4 can be found at HMS LINCS (Harvard Medical School NIH Library of Integrated Network-based Cellular Signatures Program) Project Dasatinib KINOMEScan—Dataset (ID: 20196) (https://lincs.hms.harvard.edu/db/datasets/20196/smallmolecules?sort=facility_salt). The full RNA-seq and whole-exome sequencing data sets, of which a small portion is shown in the current study, will be made publicly available at Genomic Data Commons upon publication of a manuscript describing this full functional genomic cohort; these data are available upon request from B.H.C. or J.W.T. All other data that support the findings of this study are provided in the main text or the Supplementary Materials.

SUPPLEMENTARY MATERIALS

<http://www.sciencesignaling.org/cgi/content/full/11/539/eaao5617/DC1>

PTPN11 and TNK2 interacted directly, enabling TNK2 to phosphorylate PTPN11, which subsequently dephosphorylated TNK2 in a negative feedback loop. Mutations in PTPN11 did not affect this physical interaction but increased the basal activity of PTPN11 such that TNK2-mediated activation was additive. Consequently, coexpression of TNK2 and mutant PTPN11 synergistically increased mitogen-activated protein kinase (MAPK) signaling and enhanced colony formation in bone marrow cells from mice. Chemical inhibition of TNK2 blocked MAPK signaling and colony formation in vitro and decreased disease burden in a patient with PTPN11-mutant JMML who was treated with the multikinase (including TNK2) inhibitor dasatinib. Together, these data suggest that TNK2 is a promising therapeutic target for PTPN11-mutant leukemias.

INTRODUCTION

PTPN11 (also known as SRC homology 2 domain-containing phosphatase 2 or SHP2) is a ubiquitous protein tyrosine phosphatase that dephosphorylates targets in many signaling pathways. Unlike most phosphatases, PTPN11 promotes cellular proliferation (1). PTPN11 is autoinhibited in its basal state in which its N-terminal SH2 domain blocks its catalytic PTP (protein tyrosine phosphatase) domain. PTPN11 is activated by the coincident phosphorylation of its C-terminal tyrosine residues (Tyr⁵⁴² and Tyr⁵⁸⁰) and the binding of its N-terminal SH2 domain to phosphorylated tyrosyl residues in scaffold proteins, such as GAB1 or GAB2 [growth factor receptor-bound protein 2 (GRB2)-associated binding protein 1 or 2], thereby relieving autoinhibition (2). Binding of the adaptor molecule GRB2 to the phosphorylated Tyr⁵⁴² of PTPN11 leads to further activation of downstream pathways. In particular, PTPN11 activates the mitogen-activated protein kinase (MAPK) pathway. Disease-associated mutations in PTPN11 release its autoinhibition, resulting in increased phosphatase activity (2).

Gain-of-function PTPN11 mutations are currently the most common driver of juvenile myelomonocytic leukemia (JMML), found in 35% of cases (3, 4). Mutations in *PTPN11* and other genes encoding MAPK pathway-associated proteins, namely *NFI*, *NRAS*, *KRAS*, and *CBL*, collectively occur in ~85% of JMML patients, for whom prognosis is poorer as the number of RAS-MAPK pathway mutations increase (4). Similar mutations in *PTPN11* are present in ~5 to 10% of acute myeloid leukemia (AML) cases and have been associated with breast and lung tumors (3–7). Gain-of-function, germline *PTPN11* mutations, often less activating than those that cause JMML, occur in 50% of cases with Noonan syndrome (NS), which imparts a susceptibility to JMML (2). Curative treatment for JMML patients is limited to allogeneic hematopoietic stem cell transplantation, but disease reemerges in 50% of transplant recipients within 2 years of treatment (8). Standard care for AML patients is cytotoxic chemotherapy, but that offers a low chance of 5-year survival, at only 24% (9). These current clinical outcomes make development of new therapies imperative.

Research has suggested a link between mutations in PTPN11 and sensitivity to the multikinase inhibitor dasatinib. In a mouse model of PTPN11-mutant lung cancer, dasatinib reduced the interaction of PTPN11 with GAB1 (5,10), suggesting that dasatinib may inhibit the activity of proteins upstream of PTPN11. Another study showed that pretreating

MCF-10a human mammary epithelial cells with dasatinib reduced the phosphorylation of PTPN11 and GAB1 after stimulation of the epidermal growth factor receptor (EGFR), suggesting that PTPN11 activation downstream of EGFR—a receptor tyrosine kinase that activates RAS-MAPK pathway signaling—depends on the activity of one or more of dasatinib's targets (10). Yet another study showed that dasatinib also abrogated both RAS signaling and the heightened sensitivity to granulocyte-macrophage colony-stimulating factor (GM-CSF) in primary JMML cells, which the authors concluded may be mediated through dasatinib's inhibition of SRC family kinases (11). Furthermore, results from a mouse model of NS demonstrated the efficacy of low-dose dasatinib in selectively improving cardiac dysfunction associated with the disease but did not identify the direct target of dasatinib that mediated this effect (12).

Tyrosine kinase nonreceptor 2 [TNK2; also called activated cell division control protein 42 homolog (CDC42)-associated kinase or ACK1] is a cytoplasmic kinase that is overexpressed in many solid tumors (13). Recent work by Wu *et al.* (14) suggests that high expression of TNK2 in triple-negative breast cancer correlates with poorer patient outcome and increased invasive phenotype in cell line and xenograft models. Various reports have identified point mutations, gene amplifications, or ligand-dependent signaling roles for TNK2 in solid tumors of the prostate (15–18), breast (14, 19), gastrointestinal tract (20), kidney (21), lung (22, 23), and others (24). We and others have recently characterized the importance of TNK2 signaling in certain hematologic malignancies (chronic neutrophilic leukemia and atypical chronic myeloid leukemia) and solid tumors, namely in its roles regulating signaling downstream of pathways mediated by EGFR (25), SRC (26), and CSF3 receptor (CSF3R) (27). Here, we investigated the mechanism of action for dasatinib in PTPN11-mutant leukemia cells.

RESULTS

A primary specimen from a patient with PTPN11-mutant JMML demonstrates dasatinib sensitivity and TNK2 dependence

To identify functional targets in a primary patient sample, peripheral mononuclear white blood cells from a patient with recurrent JMML carrying a PTPN11 G60R mutation (g180c transversion; Fig. 1A) were assayed *ex vivo* against panels of small-molecule kinase inhibitors or small interfering RNA (siRNAs) (9, 28). The sample showed significantly reduced viability in the presence of siRNA targeting TNK2, suggesting that these JMML cells exhibited dependence on TNK2 for cell survival (Fig. 1B and table S1). Treatment of this patient's cells in culture with a panel of small-molecule inhibitors revealed sensitivity to the multitarget kinase inhibitor, dasatinib, with an IC₅₀ (median inhibitory concentration) of 36 nM (table S2). The median IC₅₀ for dasatinib from a diverse cohort of 151 primary leukemia patient specimens was 401 nM. Therefore, this patient's JMML cells were 10-fold more sensitive to dasatinib than the average leukemia sample from this previously published patient cohort (Fig. 1C) (29). Because TNK2 is reportedly a target of dasatinib (30), this suggests that leukemic cells with PTPN11 mutations might be sensitive to drugs that target TNK2, such as dasatinib.

Next, we examined the capacity of the PTPN11 G60R mutant to activate the RAS/MAPK pathway. The most common PTPN11 mutation in JMML, E76K, has been identified as having the highest phosphatase activity (3, 31). We transfected ectopic expression constructs containing human wild-type (WT) PTPN11, PTPN11 G60R, or PTPN11 E76K into human endothelial kidney 293T17 cells, a highly transfectable derivative of the human embryonic kidney (HEK) 293 cell line. Immunoblots of 293T17 cells expressing either G60R or E76K PTPN11 showed significantly increased amounts of p44/42 MAPK phosphorylation [ERK1/2 (extracellular signal-regulated kinase 1/2), MAPK1/3] compared with cells expressing WT PTPN11 (Fig. 1D). To test GM-CSF-induced colony formation, a hallmark of PTPN11 mutation and JMML (32), mouse bone marrow cells were transduced with the constructs expressing mutant or WT PTPN11. Consistently, mouse bone marrow methylcellulose colony formation assays showed GM-CSF-induced colony formation in the context of cells expressing both E76K and G60R PTPN11 (Fig. 1E). These results suggest that G60R and E76K mutations in PTPN11 confer a similar gain-of-function phenotype, and because the E76K mutation is the most commonly occurring PTPN11 variant seen in JMML, we conducted further experiments here using the PTPN11 E76K mutant.

TNK2 enhances signaling of mutant PTPN11 through RAS/MAPK

To examine the potential impact of TNK2 on signaling of PTPN11 mutants, 293T17 cells were cotransfected with constructs expressing mutant or WT PTPN11 with or without TNK2 coexpression. Signaling activity was then assessed by immunoblot (Fig. 2A). Coexpression of PTPN11 E76K and TNK2 resulted in significantly increased abundance of phosphorylated p44/42 MAPK when compared to cells expressing PTPN11 E76K or TNK2 alone (Fig. 2B). We also observed that coexpression of TNK2 with PTPN11 results in increased phosphorylation of PTPN11 at residues Tyr⁵⁴² and Tyr⁵⁸⁰ (Fig. 2, A and B). Notably, phosphorylation of TNK2 at its primary activating tyrosine residue, Tyr²⁸⁴, was reduced when coexpressed with PTPN11, and this reduction of TNK2 phosphorylation was especially pronounced with mutant PTPN11 compared with WT (Fig. 2, B and C). The Tyr²⁸⁴ residue of TNK2 has been shown to be important for TNK2 activation, with the kinase SRC implicated as a possible kinase that phosphorylates this site in TNK2 (13). This decrease in phospho- TNK2 is also observed, although not statistically significant, when WT PTPN11 was cotransfected with TNK2 (Fig. 2B).

Because TNK2 is dephosphorylated while enhancing activation of p44/42 MAPK downstream of mutant PTPN11, we next wanted to test the impact of mutations that activate or deactivate the TNK2 kinase domain. We generated a mutation at the TNK2 “gatekeeper” residue, Thr²⁰⁵, mutated to Ile (T205I), which has increased kinase activity compared with WT TNK2 and has been shown previously to block its sensitivity to kinase inhibitors, as described with gatekeeper mutants of other tyrosine kinases, such as BCR-ABL (the fusion product of the breakpoint cluster region and abelson tyrosine kinase genes) (33, 34). We also generated a kinase-inactive version of TNK2 with a mutation at the critical tyrosine residue in the kinase domain, Tyr²⁸⁴, mutated to Phe (Y284F). We observed that the phosphorylation of p44/42 MAPK by TNK2 and mutant PTPN11 coexpression was further enhanced when coexpressed with the activated TNK2 T205I and mutant PTPN11 and abrogated by

coexpression of mutant PTPN11 and the kinase-inactive TNK2 Y284F (fig. S1A). These data suggest that kinase activity of TNK2 is required to enhance mutant PTPN11 signaling.

To determine whether phosphorylation of C-terminal PTPN11 residues is necessary for activated mutant PTPN11 activation in the presence of TNK2, PTPN11 expression constructs with mutation at two tyrosine residues (Tyr⁵⁴² and Tyr⁵⁸⁰) were generated and 293T17 cells were transiently transfected to express mutant PTPN11, TNK2, or empty vector control (fig. S1). We again observed a significant increase in phosphorylated p44/42 MAPK with the coexpression of PTPN11 E76K and TNK2 (fig. S1). Mutation of either Y542 or Y580 resulted in reduction of phospho-p44/42 MAPK to baseline levels with similar decrease to baseline levels observed with the double Y542/Y580 mutant (fig. S1, E and F). Notably, these data suggest that PTPN11 with the activating E76K mutant, which is thought to allow PTPN11 to constitutively adopt the open, active conformation, requires phosphorylation of both Tyr⁵⁴² and Tyr⁵⁸⁰ for full activation of downstream signaling (fig. S1, E and F). In agreement with the observation that activated mutant PTPN11 E76K greatly reduced phospho-TNK2 abundance, reduced PTPN11 activity correlated with higher phospho-TNK2 abundance (fig. S1G), suggesting that TNK2 dephosphorylation is PTPN11-dependent.

Collectively, the increased signaling observed with coexpression of TNK2 and PTPN11, as well as the reciprocal increase in PTPN11 phosphorylation and decrease in TNK2 phosphorylation, suggested that PTPN11 and TNK2 might be directly interacting with PTPN11 as a target of TNK2 kinase activity. To test this hypothesis, we performed coimmunoprecipitation experiments. FLAG-tagged WT and mutant (G60R) PTPN11 pulled down with TNK2 immunoprecipitates from lysates of 293T17 cells cotransfected with TNK2 (Fig. 2D), suggesting that these proteins interact. Likewise, endogenous PTPN11 pulled down with TNK2 immunoprecipitates from empty vector- or TNK2-transfected cells resulted in coimmunoprecipitation of endogenous phosphorylated PTPN11 (Fig. 2D), supporting the input immunoblot data showing phosphorylation of endogenous PTPN11 in cells expressing TNK2 (see also Fig. 2D). We observed a similar coimmunoprecipitation of TNK2 with PTPN11 E76K constructs (fig. S1B) and also endogenous interaction between TNK2 and PTPN11 in the human erythroleukemia cell line HEL, which expresses WT PTPN11 and TNK2 (fig. S1C).

Inhibition of TNK2 reduces signaling through PTPN11/RAS/MAPK

Given that our data suggested that TNK2 positively regulates PTPN11 signaling, we next sought to determine the impact of TNK2 inhibition on mutant PTPN11 signaling. To test this, we coexpressed mutant or WT PTPN11 with WT or gatekeeper mutant TNK2 (T205I). The gatekeeper mutant is resistant to dasatinib as well as the TNK2-selective inhibitor, AIM-100 (4-amino-5,6-biaryl-furo[2,3-d]pyrimidine) (33, 35). 293T17 cells expressing these constructs were treated with either dasatinib or AIM-100 for 2 hours, and signaling pathway activity was assessed by immunoblot (Fig. 3 and fig. S2A). Inhibition of TNK2 by either dasatinib or AIM-100 resulted in significantly reduced phospho-p44/42 MAPK levels in cells coexpressing mutant PTPN11 and WT TNK2 (Fig. 3, A to C). In contrast, cells cotransfected with PTPN11 E76K constructs and the inhibitor-resistant gatekeeper TNK2

T205I mutant showed no significant reduction in MAPK signaling (Fig. 3, D to F). In addition, the abundances of phospho-Tyr⁵⁴² PTPN11 and phospho-Tyr⁵⁸⁰ PTPN11 were both reduced in the presence of TNK2 inhibition (Fig. 3G), although only when expressing WT TNK2. Phosphorylated PTPN11 abundance was unaffected by inhibitors in the context of the drug-resistant, gatekeeper TNK2 T205I mutant (Fig. 3H). These results were similar to those comparing dasatinib to the previously characterized TNK2-specific inhibitor XMD8-87 (fig. S2B) (33). Finally, an in vitro kinase assay demonstrated PTPN11 phosphorylation by TNK2, which was abrogated by dasatinib treatment (fig. S1D). Together, these data suggest that TNK2 is an upstream activator of PTPN11 and that targeting of TNK2 abrogates PTPN11 signaling, affecting the capacity of PTPN11 to activate the downstream MAPK pathway.

Given that small-molecule TNK2 inhibitors appeared to block the TNK2-mediated phosphorylation of PTPN11, we next determined whether inhibition of PTPN11 could block the reciprocal dephosphorylation of TNK2. To test this, we used a newly developed allosteric inhibitor of PTPN11, SHP099 (Fig. 3I and fig. S3) (36, 37). Inhibition of PTPN11 with SHP099 in cells transfected with PTPN11 and TNK2 expression constructs resulted in decreased phosphorylation of p44/42 MAPK at concentrations similar to those published (37), suggesting that the increase in MAPK signaling induced by the addition of TNK2 is dependent on PTPN11. Inhibition of PTPN11 also resulted in increased phosphorylation of TNK2 in a dose-dependent manner, consistent with our hypothesis that PTPN11 activity is responsible for the reduction in TNK2 phosphorylation. The amount of phospho-PTPN11 increased concomitantly with the increases in phospho-TNK2, consistent with the model that activated TNK2 phosphorylates PTPN11. Notably, SHP099 is an allosteric inhibitor that binds to and traps the closed, inactive conformation of PTPN11. Because mutant PTPN11 highly favors the open conformation, perhaps even more so in the context of TNK2 coexpression, WT PTPN11 was used for these studies. This mode of inhibition is consistent with our observations that PTPN11 phosphorylation increases with increasing doses of SHP099, yet there is not a concomitant increase in phospho-p44/42 MAPK due to continued trapping of PTPN11 in the closed conformation. Collectively, these data support a feedback mechanism in which TNK2 phosphorylates and activates PTPN11 and is, in turn, deactivated through a PTPN11-dependent dephosphorylation event.

TNK2 is a functional target for PTPN11-mediated transformation

Increased hypersensitivity of cells to the cytokine GM-CSF in colony formation assays is a hallmark of JMML pathogenesis (1, 32). Having established that TNK2 coexpression can enhance the signaling activity of mutant PTPN11, we next wanted to determine whether TNK2 can also enhance the transformation potential of mutant PTPN11 in a functional assay and whether targeting of TNK2 can mitigate PTPN11-mediated colony formation. Accordingly, mouse bone marrow cells were transduced with mutant PTPN11 with or without TNK2 coexpression. We observed significantly increased colony formation when mutant PTPN11 and TNK2 were coexpressed compared to mutant PTPN11 alone, TNK2 alone, or WT PTPN11 coexpressed with TNK2 (Fig. 4A). To test whether PTPN11-mediated colony formation can be successfully targeted with TNK2 inhibitors, we performed mouse bone marrow colony formation assays expressing two different PTPN11

mutant constructs (E76K and G60R) with endogenous levels of TNK2 and treated cells with graded concentrations of dasatinib in the presence or absence of GM-CSF. Dasatinib reduced colony formation in a dose-dependent fashion (Fig. 4B). We investigated the role of TNK2 inhibition in colony assays performed with cells transduced with activated PTPN11 and WT or gatekeeper T205I mutant TNK2 (Fig. 4C and fig. S4). We observed that the TNK2 gatekeeper mutant enhanced GM-CSF-induced colony formation when compared to WT TNK2 in the context of activated mutant PTPN11 (Fig. 4C). In addition, we observed significant sensitivity to TNK2 inhibition by dasatinib in cells transduced with WT TNK2, which was reduced in cells transduced with gatekeeper TNK2 T205I (Fig. 4C). We also examined colony reduction by TNK2- selective inhibitors in bone marrow cells cotransduced with PTPN11 E76K and TNK2, and we observed reduced colony formation in the presence of both dasatinib and the TNK2-specific inhibitors AIM-100 and XMD8-87, suggesting a reliance on TNK2 for PTPN11 transformation potential (Fig. 4D and fig. S4).

PTPN11 mutations confer dasatinib sensitivity in AML

Because a significant percentage of AML cases also harbor gain-of-function mutations in PTPN11, we examined dasatinib sensitivity profiles in primary AML patient samples that have mutant or WT PTPN11. One hundred twenty-eight samples from patients diagnosed with AML were assessed for ex vivo dasatinib sensitivity, and IC₅₀ values were calculated, representing drug sensitivity. A comparison of the average IC₅₀ value for samples with PTPN11 mutations versus samples with WT PTPN11 reveals that the PTPN11 mutant samples are significantly more sensitive to dasatinib than the WT samples (Fig. 5A). To ensure that this relationship is specific, we also assessed the dasatinib sensitivity profiles of KRAS- and NRAS-mutant relative to WT samples in this same cohort. There was no difference in average dasatinib sensitivity of NRAS-mutant versus NRAS-WT specimens, suggesting that TNK2 dependence is specific for PTPN11 mutant samples and does not occur with mutations that occur further downstream in the RAS/MAPK pathway. None of these samples had activating mutations in targets of dasatinib, and none were positive for BCR-ABL (table S3). To further investigate whether other targets of dasatinib might be responsible for this observed sensitivity, we analyzed RNA sequencing (RNA-seq) expression data from a cohort of 356 primary AML patient samples, 10 of which contained PTPN11 mutations (table S4 and fig. S6). PTPN11 mutant samples showed no significant increase in mRNA transcripts for any of the 35 top dasatinib target proteins when compared to PTPN11 WT samples. Although mRNA levels are not a strict indicator of protein activity, the lack of mutation or overexpression of these targets supports TNK2 as the key effector of dasatinib sensitivity in these patient samples.

We identified one AML-specific PTPN11 mutation (S502P) in multiple dasatinib-sensitive patient samples and confirmed its presence by Sanger sequencing (Fig. 5B). To investigate the transformation potential of this variant, PTPN11 S502P was introduced into 293T17 cells or mouse bone marrow. Cell lysates from 293T17 cells expressing PTPN11 S502P showed significant increases in MAPK signaling as well as a reduction of phospho-TNK2 compared with WT (Fig. 5C), and the activity of MAPK signaling was reduced after dasatinib exposure (fig. S5, A to C). In mouse bone marrow colony formation assays, we observed that the PTPN11 S502P mutation increases GM-CSF-induced colony formation

(Fig. 5D). Collectively, these data suggest that the S502P variant of PTPN11 is phenotypically similar to the variants seen in JMML, consistent with the data from primary samples harboring the S502P mutation.

Dasatinib extended survival in a patient with JMML

The leukemia sample used for initial siRNA and drug sensitivity studies shown in Fig. 1 was obtained from a 6-year-old Caucasian male with JMML with mutant PTPN11 diagnosed at age 5 who had failed to respond to two matched sibling donor bone marrow transplants using myeloablative conditioning. Because his leukemia cells were sensitive to TNK2 silencing and dasatinib in vitro (Fig. 1), the family elected to initiate dasatinib therapy at 60 mg/m² per day as an alternative to palliative care under an investigational drug exemption. The patient had a rapid decrease in white blood cell counts to within normal range (Fig. 5E). One month after starting dasatinib, the patient presented with fevers and leukocytosis and was therefore started on mercaptopurine, a chemotherapeutic agent. A repeat marrow at the time showed no overt evidence of disease, but a blood culture at that time grew *Klebsiella* bacteria. After identification of the bacteria and marrow results, mercaptopurine was discontinued. Once treatment for bacteremia was initiated, this patient had a slow resolution of his leukocytosis, and his thrombocytopenia improved after mercaptopurine was discontinued. He achieved a sustained hematologic remission in response to dasatinib therapy and was then eligible to receive a third allogeneic stem cell transplant using an unrelated cord blood donor, which afforded an additional year of life. He eventually died from relapsed disease at age of 7 years. This case study, along with the ex vivo data from PTPN11-mutant AML patients, provided the rationale for further investigations into the efficacy of dasatinib or other TNK2 inhibitors in PTPN11-mutant leukemias.

DISCUSSION

Our study characterized a previously unknown interaction between PTPN11 and the kinase TNK2. These data suggest that TNK2 directly interacts with PTPN11 in what seems to be a closely regulated feedback loop. TNK2 expression results in phosphorylated and activated PTPN11, as evidenced by experiments using the dasatinib-resistant gatekeeper mutant TNK2 T205I. Gain-of-function mutant PTPN11 is uniquely activated when expressed with TNK2. There are two components that seem to regulate the activation of MAPK pathway signaling downstream of PTPN11: an open conformation of the phosphatase and phosphorylation of Tyr⁵⁴²/Tyr⁵⁸⁰ of PTPN11 to facilitate recruitment of adaptor proteins. In the setting of coexpression of WT PTPN11 and TNK2, only one of these two criteria is accomplished (phosphorylated PTPN11 Y542/Y580 but not open conformation of the phosphatase); thus, we do not see the downstream MAPK pathway activation. In the setting of mutant PTPN11 coexpressed with TNK2, both criteria are accomplished (mutation causes open conformation, and TNK2 phosphorylates PTPN11 Y542/Y580), resulting in robust MAPK pathway activation (Fig. 6).

PTPN11 activity correlates with dephosphorylation of TNK2. Experiments evaluating overactive PTPN11 mutants and PTPN11 inhibition experiments support this feedback model. This relationship has functional implications; coexpression of mutant PTPN11 and

TNK2 results in significantly more RAS/MAPK signaling and GM-CSF hypersensitivity, both hallmarks of JMML (32). These increases in MAPK signaling and colony formation are blocked by pharmacological inhibition of TNK2. Data from primary JMML and AML patient samples identify PTPN11 mutations as a genetic marker for sensitivity to TNK2 inhibition.

The PTPN11-dependent dephosphorylation of TNK2 has yet to be fully elucidated. Coimmunoprecipitation of PTPN11 and TNK2 suggests that PTPN11 and TNK2 interact, but whether there is a phosphate removed from the kinase domain tyrosine residue of TNK2 or a reduction of active phosphorylation of that tyrosine remains unknown.

SRC has been reported to be both upstream of TNK2 and downstream/ concurrent with PTPN11 with regard to p44/42 MAPK signaling, as well as a target of dasatinib (26, 38–40). However, the role of SRC kinase in the interplay between PTPN11 and TNK2 and the degree of its involvement is not currently known. The efficacy of TNK2-specific inhibitors in our in vitro and functional assays suggests that SRC is not the sole activator of TNK2, but SRC's role in TNK2 activation with regard to mutant PTPN11 is still under investigation. Although SRC inhibition may occur upstream of TNK2 and contribute to dasatinib-mediated TNK2 inactivation, TNK2 phosphorylation levels were not significantly affected by dasatinib treatment. It is likely that SRC is targeted by dasatinib as a downstream effector of RAS/MAPK signaling, but the activity of mutant PTPN11 as an activator of multiple proliferative pathways might render this inhibition event to be less effective.

The identification of TNK2 as a functional target originated in an ex vivo screen of a primary JMML patient sample. As we move toward personalized cancer therapy, ex vivo functional screening is a promising avenue to identify drug targets that are not directly mutated. Although PTPN11 is known to be necessary for sustaining signaling through RAS/MAPK, its involvement as a regulator at several levels of the signaling cascade has made it challenging to fully determine its exact mechanisms of action. PTPN11 has become an attractive therapeutic target, and an exciting recent development in this area has been the identification of allosteric PTPN11 inhibitors, such as SHP099 (37). However, the potential clinical efficacy of these molecules may be limited, as SHP099 has limited activity against activated PTPN11 because it binds only the closed conformation of PTPN11 (36, 37, 41). Inhibition of proteins in the RAS/MAPK pathway downstream of PTPN11 is an option that is being explored by other groups (42, 43), but the ability of mutant PTPN11 to activate alternate pathways like Janus kinase (JAK)/signal transducer and activator of transcription (STAT) or phosphatidylinositol 3-kinase (PI3K)/Akt signaling makes resistance to these drugs a distinct possibility.

Mutations in PTPN11 drive the pathogenesis of JMML, sometimes even as germline events. Thus, inhibition of TNK2 could be useful for maintenance of remission. Implications for NS patients from work by Yi *et al.* (12) suggests that dasatinib may have a therapeutic future in patients with NS beyond JMML.

Although we have not touched on the contributions of mutant PTPN11 in the stromal compartment of patients with germline mutations, work from other groups suggests that

these cells contribute to leukemogenesis through CCL3 signaling (44). Mice bearing activating PTPN11 mutations in mesenchymal stem/progenitor cells and osteoprogenitors promoted a myeloproliferative phenotype in normal transplanted cells. This effect was reversible with the administration of CCL3 receptor agonists. Perhaps dasatinib could be used in combination with a CCL3 receptor agonist to abrogate the effects of PTPN11-mutant stromal cells in patients with NS or LEOPARD (lentiginos, electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth, deafness, multiple lentiginos syndrome) syndrome.

Efficacy of TNK2 inhibition would depend upon a tumor's dependence on PTPN11 for transformation and survival. In AML, for instance, PTPN11 mutations may occur later in the evolution of the disease, tempering the probability of success of a single-agent therapy. TNK2 inhibitors may prove to be an important component of an eventual molecularly targeted, combinatorial therapeutic strategy, a promising approach recently showing efficacy in AML patient samples (45).

MATERIALS AND METHODS

Patient samples

All clinical specimens were collected with informed consent on a protocol approved by the Oregon Health & Science University (OHSU) Institutional Review Board.

siRNA and kinase inhibitor assays

The siRNA and small-molecule inhibitor screening of patient samples was conducted as previously described (29, 46): Blood or bone marrow from patients was separated on a Ficoll gradient, and mononuclear cells were treated with ammonium-chloride-potassium (ACK) lysis buffer. Cells were cultured in R10 [RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), L-glutamine, penicillin/streptomycin (Invitrogen), and fungizone (Invitrogen)] supplemented with 10^{-4} M 2-mercaptoethanol (Sigma-Aldrich).

Kinase inhibitors were stored at 10 to 100 mM in DMSO (stock concentration was 1000 times the final concentration of the highest tested dose). For creation of replicate plates of the library, each drug concentration was diluted to twice the final concentration, and 50 μ l was plated into 96-well plates using a Hydra 96-channel automated pipettor (Matrix Technologies). Plates were sealed with adhesive lids (microseal B, Bio-Rad), wrapped in aluminum foil, and stored at -20°C until use. Upon receipt of a patient sample, plates were thawed at 37°C , 5% CO_2 for 1 hour and centrifuged at 800g before removal of adhesive lids. Subsequently, patient samples were suspended into culture media at a concentration of 1,000,000 cells/ml, such that the addition of 50 μ l to each well would deliver 50,000 cells to that well (this also dilutes the drugs to their final, desired concentration). Cells were incubated for 3 days at 37°C , 5% CO_2 and subjected to a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega). Each plate contained seven wells without any drug. The average absorbance value of these seven wells was used for data normalization, and the kill curve of each drug gradient was assessed relative to this average no-drug point.

Patient blood or bone marrow was prepared as above, and 2.25×10^7 cells were resuspended in 4.2 ml of siPORT buffer (Ambion). Cells were aliquoted at 42 μ l per well onto a 96-well electroporator (Ambion), and 2 μ l of siRNA at 20 μ M was added to each well [tyrosine kinase library (Dharmacon) with single and pooled nonspecific siRNA]. Cells were electroporated at 1110 V (equivalent of 150 V per well), 200 μ s, and two pulses, and 50,000 cells per well were replated into triplicate plates containing 100 μ l per well of culture media. For determination of cell viability, cells were subjected to the CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) (Promega). All values were normalized to the median plate value.

Sequencing of patient samples

Peripheral blood and bone marrow samples from patients with JMML or AML were processed by Ficoll gradient centrifugation followed by red blood cell lysis. Cell pellets for subsequent genomic analysis were snap-frozen in liquid nitrogen. DNA was extracted using Qiagen DNeasy kits performed according to the manufacturer's protocols. For exome sequencing, Illumina Nextera capture probes and protocol with libraries run on a HiSeq 2500 using paired-end 100 cycle protocols were used. Initial data processing and alignments were performed using in-house workflows. Briefly, for each flow cell and each sample, the FASTQ files were aggregated into single files for reads 1 and 2. BWA MEM (Burrows-Wheeler Aligner MEM algorithm) version 0.7.10-r789 (47) was used to align the read pairs for each sample-lane FASTQ file. As part of this process, the flow cell and lane information were kept as part of the read group of the resulting SAM (Sequence Alignment Map) file. The Genome Analysis Toolkit (v3.3) and the bundled Picard (v1.120.1579) were used (48) for alignment after processing. The files contained within the Broad Institute's bundle 2.8 were used including their version of the build 37 human genome. The following steps were performed per sample-lane SAM file: (i) sorting and conversion to BAM via SortSam; (ii) MarkDuplicates was run, marking both lane-level standard and optical duplicates; (iii) read realignment around indels from the reads (RealignerTargetCreator/ IndelRealigner); and (iv) Base Quality Score Recalibration. The resulting BAM files were then aggregated by sample, and an additional round of MarkDuplicates was carried out at the sample level. For genotyping, each AML sample BAM was realigned at the sample level and then genotyped for single-nucleotide variations using MuTect v1.1.7 (49) and VarScan 2 v2.4.1 (50). In addition, indels were produced using VarScan 2. Each VCF (variant call format) file was annotated using the Variant Effect Predictor v83 against GRCh37 (51). The resulting VCF files were filtered to include only those annotated to a gene and converted to MAF (mutation annotation format) using the vcf2maf v1.6.6 tool (52) and converted to MAF. Data were filtered to include patient samples for which dasatinib IC₅₀ and PTPN11 mutation status were known. The full results of this exome sequencing cohort are being prepared in a separate manuscript.

For RNA-seq, libraries were constructed using the SureSelect stranded RNA-seq protocol (Agilent) on the Bravo robot (Agilent). Briefly, poly(A)⁺ RNA was chemically fragmented. Double-stranded complementary DNAs (cDNAs) were synthesized using random hexamer priming with 3' ends of the cDNA adenylated, and then indexed adaptors were ligated. Library amplification was performed using three-primer polymerase chain reaction using a

uracil DNA glycosylase addition for strandedness. Libraries were validated with the Bioanalyzer (Agilent) and combined to run four samples per lane, with a targeted yield of 200 million clusters. Combined libraries were denatured, clustered with the cBot (Illumina), and sequenced on the HiSeq 2500 using a 100-cycle paired-end protocol. For each sample, FASTQ files were aggregated into single files for reads 1 and 2. Alignment was performed using Subjunc aligner (1.4.6) (53). SAM files obtained from Subjunc were used as inputs into featureCounts (1.4.6) (54), and read summarization were performed.

Plasmid construction

TNK2 transcript variant 1 in pDONR was purchased from GeneCopoeia (GC-Y4392). Gene mutations were made using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), and primers were designed using the Agilent Technologies primer design tool. WT and mutated TNK2 were transferred into a Gateway-converted version of pMXs-IRES-Puro (Cell Biolabs Inc.) or MSCV-IRES-GFP using a Gateway LR Clonase kit (Invitrogen). Plasmid sequencing was confirmed via Sanger Sequencing (Eurofins Genomics).

PTPN11 transcript variant 1 in pDONR was purchased from GeneCopoeia (A0360). Gene mutations were made using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies), and primers were designed using the Agilent Technologies primer design tool. WT and mutated PTPN11 were transferred into a Gateway-converted version of p3X-CMV-FLAG14, MSCV-IRES-GFP, or pLenti CMV/TO GFP-Zeo DEST (719-1) (Addgene #17431), or pcDNA 3.2 (Invitrogen) using a Gateway LR Clonase kit (Invitrogen). Plasmid sequencing was confirmed via Sanger Sequencing (Eurofins).

Cell culture and transfection

The HEK 293T17 cell line [American Type Culture Collection (ATCC)] was grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 10% FBS (Atlanta Biologicals), L-glutamine, penicillin/streptomycin (Invitrogen), and amphotericin B (HyClone). Murine retrovirus was created by cotransfecting TNK2 or PTPN11 plasmids and the EcoPac plasmid (provided by R. Van Etten) into 293T17 using FuGENE 6 (Promega). Supernatants were harvested 72 hours later. Human lentivirus was created by cotransfecting PTPN11 plasmids and the psPAX2 (Addgene #12260) and pCMV-VSV-G (Addgene #8454) plasmids into 293T17 using FuGENE 6 (Promega). Supernatants were harvested 72 hours later.

Inhibitor assays

293T17 cells were transfected using FuGENE 6 (Promega) and cultured for 48 hours. The inhibitors dasatinib (SelleckChem), AIM-100 [Calbiochem; (55)], and SHP099 (DC Chemicals) were diluted in DMEM to attain a final concentration in a six-well plate at 1:2000 dilution. After the treatment period, media were aspirated, and lysates were collected as for immunoblot assays. Lysates were subjected to immunoblot.

The human erythroleukemia cell line HEL 92.1.7 (ATCC TIB-180) was grown in RPMI 1640 (Invitrogen) with 10% FBS (Atlanta Biologicals), L-glutamine, penicillin/streptomycin

(Invitrogen), and amphotericin B (HyClone). Cells were cytokine-starved overnight by washing twice with RPMI 1640 and resuspending in RPMI 1640 with 1% bovine serum albumin (BSA).

Colony formation assays

All murine studies were performed in accordance with OHSU Institutional Animal Care and Use Committee protocol IS00002358. Bone marrow was harvested from mice by flushing the femur and tibia with medium. Cells were cultured at 5×10^5 per ml with viral supernatant, polybrene (AmericanBio), and Hepes buffer by centrifugation at 2500 rpm at 30°C for 90 min and then placed back at 37°C. After 24 hours, the medium was replaced with fresh retroviral “cocktail,” and cells were centrifuged as above and then placed back at 37°C for additional 24 hours. Next, cells were analyzed for GFP expression by fluorescence-activated cell sorting.

GFP⁺ cells were then sorted and plated in triplicate L into M3234 methylcellulose (STEMCELL Technologies) supplemented with mSCF (murine stem cell factor; 50 ng/ml), mIL-3 [murine interleukin-3 (IL-3); 10 ng/ml], and mIL-6 (PeproTech). Cells were enumerated at day 7 or 14 using the STEMvision colony counter (STEMCELL Technologies) with blinded manual counting to ensure accuracy.

Coimmunoprecipitation assays

Proteins were immunoprecipitated from cell lysates by incubation with rabbit anti-ACK1 09-142 (Millipore) or anti-immunoglobulin G (IgG) isotype control overnight at 4°C on a rotator. Lysates were then incubated with protein G agarose beads (Millipore) for 1 hour at 4°C on a rotator. FLAG-tagged proteins were incubated with anti-FLAG M2 affinity gel (Sigma-Aldrich) overnight at 4°C on a rotator. Beads or affinity gel were washed three times using cell lysis buffer (Cell Signaling Technology) with added cOmplete mini protease inhibitor tablets (Roche), phenylmethylsulfonyl fluoride, and phosphatase inhibitor cocktail 2 (Sigma-Aldrich). Proteins were dissociated from beads using an SDS loading buffer for 5 min at room temperature and then subjected to immunoblotting analysis.

Immunoblotting

Transfected 293T17 lysates were spun at 8000 rpm for 10 min at 4°C to pellet cell debris, mixed 2:1 with 3× glycine SDS sample buffer [75 mM tris (pH 6.8), 3% SDS, 15% glycerol, 8% β-mercaptoethanol, and 0.1% bromophenol blue] and heated at 95°C for 5 min. Lysates were run on criterion 4 to 15% tris-HCl gradient gels (Bio-Rad), transferred to polyvinylidene difluoride membrane, and blocked for 30 min in TBS-T (tris-buffered saline with Tween 20) with 5% BSA. Blots were probed overnight at 4°C with the following antibodies: rabbit anti-ACK1 (A-11) (Santa Cruz Biotechnology), rabbit anti-SHP2 (CST 3572), rabbit anti-phospho-ACK1 (Millipore), rabbit anti-p44/42 MAPK (ERK1/2) (CST 9102), rabbit phospho-p44/42 MAPK (ERK1/2) (Thr²⁰²/Tyr²⁰⁴) (CST 9101), mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 6C5 (AM4300, Ambion), rabbit anti-MEK1/2 (CST 9122), rabbit anti-phospho-MEK Ser^{217/221} (CST 9121), rabbit anti-phospho-STAT5 (Y694) (CST 9351), and mouse anti-STAT5 (BD 610192). Primary antibody was followed by anti-rabbit or anti-mouse IgG horseradish peroxidase-conjugated

secondary antibodies. Blots were developed using Clarity chemiluminescent substrate (Bio-Rad) and imaged using a Bio-Rad ChemiDoc MP imaging system.

Quantification of relative protein values was calculated as follows: Total and phosphoprotein levels were analyzed using Bio-Rad ImageLab Software. Each of these values was normalized to GAPDH protein levels for each membrane to control for loading effects. We then normalized each normalized phosphoprotein value to the normalized total protein value to get a relative phosphoprotein value.

Statistical analysis

Means $\pm$ SEM are shown unless otherwise stated. One-way ANOVA or two-tailed Student's *t* test were used for comparisons. Welch's *t* test was used in cases where population variances were not equal as described in each figure legend. Statistical analysis was conducted using GraphPad Prism version 6.0. Sample sizes are consistent with those reported in similar studies and provide sufficient power to detect changes with the appropriate statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. A primary patient sample containing a PTPN11 mutation demonstrates dasatinib sensitivity and over-reliance on TNK2.

(A) Sanger sequencing confirming that the PTPN11 mutation G60R was first identified in a patient with recurrent JMML by whole-exome sequencing. (B) Peripheral blood mononuclear cells from this patient were incubated with an siRNA library, and viability was assessed by MTS assay. Each bar represents cell viability after silencing of an individual kinase (table S2). (C) Peripheral blood mononuclear cells from the same JMML patient were incubated with graded concentrations of each of 66 small-molecule kinase inhibitors for 3 days. Cell viability was determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay, and the IC_{50} for each drug was calculated with respect to cells incubated in the absence of drug. These IC_{50} values were compared to the median IC_{50} for each drug across 151 patient samples. Each bar represents the percentage of median IC_{50} for an individual kinase inhibitor (table S1). (D) Mouse bone marrow cells were transduced to express PTPN11, PTPN11 G60R, or PTPN11 E76K and plated in a methylcellulose GM-CSF sensitivity colony formation assay. Colonies were counted at 14 days [GM-CSF] = 0.05 nM (0.71 ng/ml). ** $P < 0.005$ and *** $P < 0.0005$. (E) 293T17 cells were transiently transfected with expression constructs containing PTPN11, PTPN11 G60R, PTPN11 E76K, or empty vector, and lysates were subjected to immunoblot. Blots are representative of four biological replicates. Data (B to D) are means $\pm$ SEM of four experiments.

Fig. 2. TNK2 increases signaling through PTPN11/RAS/MAPK in cells overexpressing mutant PTPN11.

(A) 293T17 cells were cotransfected with expression constructs containing PTPN11, PTPN11 E76K, TNK2, or empty vector controls. Lysates were collected at 48 hours and subjected to immunoblot. (B) Relative phospho-p44/42 MAPK (phospho-ERK1/2), relative phospho-TNK2 (Y284), and relative phospho-PTPN11 values were calculated with GAPDH as a loading control. Data are means $\pm$ SEM of four experiments. *** P = 0.0005 and ** P < 0.005 by one-way analysis of variance (ANOVA). (C) 293T17 cells were cotransfected with expression constructs containing PTPN11, PTPN11 E76K, TNK2, or empty vector controls. Lysates were collected 48 hours after and subjected to immunoblot. (D) 293T17 cells were

cotransfected with expression constructs containing PTPN11 WT FLAG or PTPN11 G60R FLAG constructs, TNK2, or empty vector controls. Lysates were collected 48 hours after and subjected to TNK2 immunoprecipitation, followed by Western blot. Blots (A, C, and D) are representative of four biological replicates.

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Fig. 3. Inhibition of TNK2 reduces signaling through PTPN11/RAS/MAPK.

(A) Phospho-p44/42 MAPK (phospho-ERK1/2) in 293T17 cells that were cotransfected with expression constructs containing PTPN11, PTPN11 E76K, TNK2, TNK2 T205I, or empty vector controls and treated 48 hours later with dasatinib (100 nM), AIM-100 (500 nM), or 0.05% dimethyl sulfoxide (DMSO) vehicle control for 2 hours. Lysates were subjected to immunoblot. (B and C) Quantification of Western blots represented in (A). Relative phospho-MAPK p44/42 (phospho-MAPK1/2), values were calculated with GAPDH as a loading control. *** $P < 0.0005$ by one way ANOVA. (D) As described in (A), phospho-PTPN11 (Y542) in 293T17 cells treated with TNK2 inhibitors. (E and F) Quantification of Western blots represented in (A). Relative phospho-p44/42 MAPK values were calculated with GAPDH as a loading control. No significance, as determined by one-way ANOVA. (G and H) Quantification of Western blots represented in (D). Relative phospho-PTPN11 values were calculated with GAPDH as a loading control. (I) Inhibition of PTPN11 in cells cotransfected with PTPN11 WT and TNK2 vectors. Cells were treated with SHP099 or vehicle control in increasing doses for 2 and 48 hours after transfection. Lysates were then immunoblotted. Blots (A and I) are representative of four biological replicates. Data (B to G) are means $\pm$ SEM of four experiments.

Fig. 4. Functional assays show increased transformation potential and sensitivity to TNK2 inhibition.

(A) Total colony formation in mouse bone marrow colony formation assay. Mouse bone marrow cells were cotransduced to express PTPN11, PTPN11 E76K, TNK2, or empty vector controls. Cells were selected for GFP⁺ (green fluorescent protein-positive) and puromycin resistance and plated in a methylcellulose GM-CSF sensitivity colony formation assay. Colonies were counted at 14 days [GM-CSF] = 0.05 nM (0.71 ng/ml). **** $P < 0.0001$ by one-way ANOVA. (B) Total colony formation in mouse bone marrow colony formation assay in cells transduced with PTPN11, PTPN11 E76K, or PTPN11 G60R. Cells were sorted for GFP⁺. Cells were plated with increasing concentrations of dasatinib. *** $P < 0.005$ and **** $P < 0.0005$ by one-way ANOVA. (C) Total colony formation and percent total colony formation in mouse bone marrow colony formation assay. Mouse bone marrow cells were cotransduced to express PTPN11 E76K and TNK2 or TNK2 T205I gatekeeper mutant (fig. S4). Cells were selected for GFP⁺ and puromycin resistance and plated in a methylcellulose GM-CSF sensitivity colony formation assay. Colonies were counted at 7 days [GM-CSF] = 0.05 nM (0.71 ng/ml). *** $P < 0.005$ and ** $P < 0.005$ by one-way ANOVA. (D) Total colony formation in mouse bone marrow colony formation assay. Mouse bone marrow cells were cotransduced to express PTPN11 E76K and TNK2 (fig. S4). Cells were selected for GFP⁺ and puromycin resistance and plated in a methylcellulose GM-CSF sensitivity colony formation assay. Colonies were counted at 7 days [GM-CSF] = 0.05 nM (0.71 ng/ml). No significance, as determined by one-way ANOVA. Data (A to D) are means $\pm$ SEM of three experiments.

Fig. 5. PTPN11 mutations in AML confer dasatinib sensitivity.

(A) Mean dasatinib IC_{50} of AML samples from patients, by PTPN11, NRAS, or KRAS mutation status (table S4). If no IC_{50} was reached here, IC_{50} was set to 1 μM . $n = 128$ samples. P values are determined by two-tailed Student's t tests. $*P < 0.05$ or no significance, as determined by one-way ANOVA. (B) Sanger sequencing confirming a PTPN11 S502P mutation, identified by GeneTrail analysis, in a patient's AML sample. Asterisk indicates the mutation. (C) Immunoblotting on lysates from 293T17 cells were transiently cotransfected with expression constructs containing PTPN11, PTPN11 S502P, TNK2, or empty vector controls. Blot is representative of five biological replicates. (D) Total colony formation in mouse bone marrow colony formation assay. Mouse bone marrow cells were cotransduced to express PTPN11, PTPN11 E76K, PTPN11 S502P, TNK2, or empty vector controls and plated in a methylcellulose GM-CSF sensitivity colony formation assay. Colonies were counted at 14 days [GM-CSF] = 0.05 nM (0.71 ng/ml). Data are means $\pm$ SEM of three experiments. (E) Peripheral blood counts for JMML patient at the time of

recurrence after second bone marrow transplant. Dasatinib therapy is shown over a 3-month period. The patient was diagnosed with *Klebsiella* bacteremia (denoted by asterisk), which is resolved with antibiotic therapy.

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Fig. 6. Working model: Synthetic lethality of TNK2 inhibition in PTPN11-mutant leukemia. PTPN11 signaling is necessary for sustaining RAS/MAPK activation, with activating mutations of PTPN11 leading to increased RAS/MAPK signaling and cell proliferation (left). Our findings suggest a new paradigm in which TNK2 activates PTPN11, especially mutant PTPN11, leading to even more RAS/MAPK signaling and leukemogenesis (middle). Inhibition of TNK2 with dasatinib abolishes this RAS/MAPK signaling (right).