Gene fusions are known to drive many human cancers. Therefore, the functional characterization of newly discovered fusions is critical to understanding the oncobiology of these tumors and to enable therapeutic development. NPM1–TYK2 is a novel fusion identified in CD30+ lymphoproliferative disorders, and here we present the functional evaluation of this fusion gene as an oncogene. The chimeric protein consists of the amino-terminus of nucleophosmin 1 (NPM1) and the carboxyl-terminus of tyrosine kinase 2 (TYK2), including the kinase domain. Using in vitro lymphoid cell transformation assays and in vivo tumorigenic xenograft models we present direct evidence that the fusion gene is an oncogene. NPM1 fusion partner provides the critical homodimerization needed for the fusion kinase constitutive activation and downstream signaling that are responsible for cell transformation. As a result, our studies identify NPM1–TYK2 as a novel fusion oncogene and suggest that inhibition of fusion homodimerization could be a precision therapeutic approach in cutaneous T-cell lymphoma patients expressing this chimera.
cells to measure NPM1–TYK2 fusion transcript by real-time quantitative polymerase chain reaction (RT-qPCR). NPM1–TYK2 mRNA transcript levels in Ba/F3 cells by qRT-PCR. Myla and SU-DHL-1 cell lines were used as positive and negative controls, respectively. Columns represent the mean of three independent experiments; bars represent the SEM. B Subcellular localization of NPM1–TYK2. Ba/F3-Vector and transformed Ba/F3 (NPM1–TYK2) cells were cytospun onto glass slides, fixed, permeabilized, and stained for FLAG antibody and DAPI. Images were acquired with a fluorescent microscope using a 60x oil immersion lens. C Transformation of Ba/F3 cells to IL-3 independent growth. Ba/F3-Vector and transformed Ba/F3 (NPM1–TYK2) cells were grown in RPMI medium in the absence of IL-3. Cell viability from each condition was determined daily by trypan blue exclusion assay. Points represent the mean of three independent experiments; bars represent SEM. **P < 0.0005 was considered as statistically extremely significant. D The clonogenic potential of transformed Ba/F3 (NPM1–TYK2) cells. Ba/F3-Vector and transformed Ba/F3 NPM1–TYK2 cells were mixed with MethoCult medium and plated. After 7 days of incubation, the number of colonies was counted. Columns represent the mean of three independent experiments; bars represent the SEM.

In vitro transformation potential of the NPM1–TYK2 fusion gene. A Detection of overexpressed NPM1–TYK2 mRNA transcript levels in transformed Ba/F3 cells by qRT-PCR. Myla and SU-DHL-1 cell lines were used as positive and negative controls, respectively. Columns represent the mean of three independent experiments; bars represent the SEM. B Subcellular localization of NPM1–TYK2. Ba/F3-Vector and transformed Ba/F3 (NPM1–TYK2) cells were cytospun onto glass slides, fixed, permeabilized, and stained for FLAG antibody and DAPI. Images were acquired with a fluorescent microscope using a 60x oil immersion lens. C Transformation of Ba/F3 cells to IL-3 independent growth. Ba/F3-Vector and transformed Ba/F3 (NPM1–TYK2) cells were grown in RPMI medium in the absence of IL-3. Cell viability from each condition was determined daily by trypan blue exclusion assay. Points represent the mean of three independent experiments; bars represent SEM. **P < 0.0005 was considered as statistically extremely significant. D The clonogenic potential of transformed Ba/F3 (NPM1–TYK2) cells. Ba/F3-Vector and transformed Ba/F3 NPM1–TYK2 cells were mixed with MethoCult medium and plated. After 7 days of incubation, the number of colonies was counted. Columns represent the mean of three independent experiments; bars represent the SEM.

Intracellular signaling that governs lymphoid cell transformation

Cancer cells acquire transformation potential through upregulation of survival signaling pathways resulting from oncogenic genetic alterations. Once we confirmed the oncogenic potential of NPM1–TYK2, we investigated the critical signaling pathway responsible for fusion kinase-driven oncogenicity. In several cancers, TYK2 gene mutations result in constitutive activation of TYK2 kinase leading to upregulation of downstream STAT signaling. Therefore, we examined Ba/F3–NPM1–TYK2 cells for fusion kinase activation and downstream STAT signaling. Our Western blot analysis data clearly indicate the upregulation of phosphorylated NPM1–TYK2 in transformed Ba/F3–NPM1–TYK2 cells. Activation of NPM1–TYK2 fusion kinase resulted in phosphorylation of downstream signaling molecules STAT1, STAT3, and STAT5. Collectively, our NPM1–TYK2 fusion kinase signaling results indicate that Ba/F3–NPM1–TYK2 cells acquire transformation potential through constitutive activation of fusion kinase and downstream STAT signaling.
NPM1–TYK2 fusion kinase activation is an oncogenic driver in lymphoid cell transformation

To further validate the fusion kinase-driven transformation of Ba/F3 cells, we developed a stable Ba/F3–NPM1–TYK2–K462R kinase-dead mutant cell line. Cell lysates were made from Ba/F3-vector, Ba/F3–NPM1–TYK2, Ba/F3–NPM1–TYK2–K462R cell lines, and Western blot analysis was performed for phospho-NPM1–TYK2, phospho-STAT1/3/5, and respective total proteins. Ba/F3-NPM1–TYK2 and Ba/F3-NPM1–TYK2–K462R cells showed ectopic overexpression of FLAG (NPM1–TYK2) protein, whereas no expression was observed in Ba/F3-vector cells (Fig. 3A). Furthermore, wild-type fusion kinase expressing Ba/F3–NPM1–TYK2 cells showed activated fusion kinase and upregulation of downstream STAT1/3/5, whereas no activation of kinase was observed in Ba/F3-vector and Ba/F3–NPM1–TYK2–K462R cells (Fig. 3A). We also examined the IL-3 independent growth and transformation potential of the NPM1–TYK2 kinase-dead mutant compared to wt-NPM1–TYK2 and vector control cells. Ba/F3 vector and NPM1–TYK2 kinase-dead mutant cells were unable to survive without IL-3 in the medium, however, Ba/F3 cells expressing wt-NPM1–TYK2 cells showed IL-3-independent growth (Fig. 3B). The lack of signaling and cell viability in the absence of IL3 of fusion kinase-dead mutant cells clearly demonstrates that the NPM1–TYK2 fusion kinase activity is the oncogenic driver responsible for Ba/F3 cell transformation.

In vivo tumorigenic potential of the NPM1–TYK2 fusion gene

Based on the in vitro transformation potential of the NPM1–TYK2 fusion gene, we further investigated the fusion gene’s tumorigenic potential in an in vivo xenograft model. To assess NPM1–TYK2 tumorigenicity, we subcutaneously injected Ba/F3-Vector or transformed Ba/F3–NPM1–TYK2 cells into the flanks of 5- to 7-week-old female Hsd:Athymic Nude-Foxn1nu mice (Envigo, Indianapolis, IN). The control mice (six per group) injected with Ba/F3-Vector cells showed no evidence of tumor formation, while the mice (six per group) injected with transformed Ba/F3–NPM1–TYK2 cells developed palpable tumors in 3 days (Fig. 4A, B).
progression was rapid and reached approximately 1400 mm³ in size within 15 days (Fig. 4D). The size and weight of lymphoma tumors excised from NPM1–TYK2 mice are shown in Fig. 4C. In addition to tumor growth, Ba/F3–NPM1–TYK2 mice also developed splenomegaly and hepatomegaly, while control mice did not (Fig. 4E, F).

Different analytical assays confirmed the fusion gene expression at mRNA and protein levels in tumor tissues. Our immunocytochemistry results confirm FLAG (NPM1–TYK2) expression in tumor cells (Fig. 5A). We further analyzed NPM1–TYK2 expression using RT-qPCR to measure fusion gene mRNA transcript levels in tumor tissue (Fig. 5B). Since tumor growth was associated with hepatosplenomegaly, we examined spleen and liver tissue samples of Ba/F3-Vector and Ba/F3–NPM1–TYK2 mice for lymphoma cell infiltration by H&E staining. Immunohistochemistry on the spleen and liver tissue from Ba/F3–NPM1–TYK2 injected mice demonstrated the infiltration of lymphoma cells, but not on tissue from Ba/F3-Vector mice (Fig. 5C).
It is essential to study each fusion partner on oncogenic signaling to better understand disease pathogenesis, ultimately leading to cell death. We performed Western blot analysis to understand the molecular pathways responsible for the fusion kinase and downstream STAT signaling activation. Tissue lysates were prepared from each fusion partner-deleted cell line and Western blot analysis was performed to understand the activation of the fusion kinase and downstream STAT signaling. We observed a clear downregulation of STAT1, STAT3, and STAT5 signaling in fusion-deleted cells (lane 3, Fig. 7A). After studying the role of NPM1 fusion partner in HEK293T cells, we found that deletion of NPM1 resulted in downregulation of STAT1/3/5 signaling as observed in Fig. 2 fusion signaling data. In contrast, deletion of the NPM1 portion from the fusion protein inhibited fusion kinase activity and downstream STAT signaling (lane 3, Fig. 7A). Together, our results in both HEK293T and Ba/F3 cells exemplify the essential role of the NPM1 fusion partner in NPM1–TYK2 oncogenicity.

Loss of NPM1 partner abrogates NPM1–TYK2 transformation potential

Next, we tested the loss of the NPM1 partner's influence on the viability of Ba/F3 cells. We observed a clear downregulation of STAT1, STAT3, and STAT5 expression in fusion-deleted cells (lane 3, Fig. 7A). As observed in HEK293T cells, NPM1 fusion partner deletion from the full-length fusion kinase inhibited NPM1–TYK2 activity and downstream signaling in Ba/F3 cells (Fig. 7B). Together, our results in both HEK293T and Ba/F3 cells exemplify the essential role of the NPM1 fusion partner in NPM1–TYK2 oncogenicity.

DISCUSSION

Existing cancer genomics data has shown that gene fusions derived from chromosomal translocations are frequent oncogenic drivers of many hematologic and solid cancers. Targeted therapies are raising hope as effective treatment options for fusion gene-driven malignancies. There has been limited progress in the development of targeted therapies for CTCL due to the lack of effective treatments. The development of potential targeted therapeutic interventions is crucial for the treatment of CTCL.
of understanding of novel genetic alterations and related oncogenic signaling pathways involved in their pathogenesis. An earlier study identified NPM1–TYK2 in CD30+ LPDs, but the oncogenic potential of NPM1–TYK2 overexpression on lymphoid cell transformation or tumorigenicity in biological assays had not yet been explored. Therefore, our current studies focused on functional characterization of fusion gene oncogenicity utilizing in vitro and in vivo biological assays.

Understanding the protein subcellular localization is critical due to its influence on diverse cellular processes. In general, wild-type (wt) proteins of NPM1 and TYK2 are localized in the nucleolus and cytoplasm, respectively. Our immunofluorescence data with

Fig. 7 Fusion partner NPM1 is essential for NPM1–TYK2 mediated oncogenic signaling. A, B Deletion of NPM1 partner in NPM1–TYK2 fusion gene inhibits activation of fusion kinase NPM1–TYK2 mediated signaling in HEK293T and Ba/F3 cells. Western blot analysis of NPM1–TYK2 (FLAG), phospho-NPM1–TYK2, phospho-STAT1/3/5, and respective total proteins. β-Actin served as an internal control.

Fig. 8 Loss of NPM1 partner abrogates NPM1–TYK2 transformation potential. A Deletion of NPM1 portion in NPM1–TYK2 fusion gene abrogates the transformation potential of the NPM1–TYK2 fusion gene in Ba/F3 cells. Ba/F3 cells were transduced with lentiviral particles expressing vector, NPM1–TYK2, and ΔN-1-257-NPM1–TYK2. Cells were grown in RPMI medium without IL-3 growth factor for 10 days. Cell viability from each condition was determined daily by trypan blue exclusion assay. Points represent the mean of three independent experiments; bars represent SEM. ***P < 0.0005 was considered as statistically extremely significant. B The deletion of the NPM1 portion in the NPM1–TYK2 fusion gene inhibits the clonogenic potential. Columns represent the mean of three independent experiments; bars represent the SEM.
transformed Ba/F3–NPM1–TYK2 cells showed fusion protein distribution in both nuclear and cytoplasmic compartments. A possible explanation for the nuclear distribution of NPM1–TYK2 is through its heterodimerization with wt-NPM1, as seen in NPM1–ALK expressing ALCL cells. Malignant cellular transformation is characterized by continuous uncontrolled proliferation. The transformed cells are distinguished from normal counterparts by acquired alterations in growth patterns like anchorage-independent growth potential with no intercellular contact inhibition and xenograft formation.

First, we performed in vitro lymphoid cell transformation assays to determine the transformation potential of NPM1–TYK2 using IL-3-dependent lymphoid Ba/F3 cells. The dependency on cytokine signaling of Ba/F3 cell survival is extensively used in hematologic malignancies to understand the oncogenicity of novel kinases, fusion kinases, and downstream signaling of activated tyrosine kinases. Our in vitro transformation assay results distinctly demonstrate that NPM1–TYK2 overexpression leads to the transformation of Ba/F3 cells with survival and proliferation independent of IL-3. Subsequently, our studies focused on identifying the signaling network that is responsible for this transformation potential. In previous fusion kinase oncogenic characterization studies, Ba/F3 cells acquired IL-3-independence through fusion-driven signaling pathways.

TYK2, the first identified JAK family member, is associated with cytokine and growth factor receptors which activate STAT signaling. TYK2 is activated by various cytokines, including numerous interleukins and interferons. TYK2 point mutations found in T-ALL (T-cell acute lymphoblastic leukemia) cell lines exhibited transformation potential via the STAT1/STAT2 pathway. Several activating TYK2 mutations are known to trigger TYK2 signaling, which is essential to the development of T-ALL. Screening of cancer datasets revealed more than fifty TYK2 chromosomal translocations, mostly in hematologic malignancies. As shown in other TYK2 mutated cancers, we investigated NPM1–TYK2 fusion kinase hyperactivation and downstream STAT signaling by Western blot analysis. Our data displayed constitutive phosphorylation of the TYK2 fusion kinase and upregulated downstream effectors STAT1, 3, and 5 in transformed Ba/F3 cells. Furthermore, our NPM1–TYK2–K462R kinase-dead mutant cells showed abrogated signaling and cell viability, confirming that wt-NPM1–TYK2 mediated constitutive activation of fusion kinase is the oncogenic driver responsible for cell transformation.

In vivo xenograft models play an essential role in understanding molecular mechanisms and pathobiology of the disease. Once our in vitro transformation studies characterized NPM1–TYK2 chimera’s oncogenic potential and survival signaling that governs transformation, we conducted in vivo studies using NPM1–TYK2 transformed xenograft models. In line with in vitro transformation, we demonstrated the in vivo tumorigenic potential of the fusion gene in NPM1–TYK2 transformed xenograft models. These mice developed robust tumor progression with associated hepatosplenomegaly. Further immunohistochemistry results revealed infiltration of lymphoma cells in tumors, spleen, and liver. From the excised tumors, signaling data revealed hyperactivation of fusion kinase and upregulation of STAT signaling.

Several fusion proteins involving receptor tyrosine kinases have demonstrated transformation potential in many solid cancers and hematologic malignancies. The majority of these oncogenic fusion kinases contain the carboxyl-terminus kinase and amino-terminus non-kinase partners. The oligomerization sequences of the amino terminus fusion partner are usually responsible for the constitutive activation of carboxyl-terminus kinase partner. Studying the role of each fusion partner helps improve the understanding of disease pathogenesis and directly promotes the development of potential targeted therapeutic interventions. Here, we explored the amino-terminus NPM1 fusion partner’s functional role in fusion kinase oncogenicity. Native NPM1 protein exists as dimeric and oligomeric forms through the amino-terminal oligomerization domain. In other NPM1-fusion proteins, the amino-terminus NPM1 fusion partner provides a self-association interface for fusion kinase to form homodimers. In our fusion mapping studies, NPM1 deletion from the NPM1–TYK2 fusion gene completely inhibited fusion gene activation and downstream STAT signaling. Further, in vitro transformation results showed that removal of the amino-terminus NPM1 fusion partner abrogated the transformation and

Fig. 9 Visual overview. NPM1–TYK2 fusion mediated oncogenic signaling.
clonogenic potential of the fusion kinase. Our results suggest that the NPM1 fusion partner facilitates a homodimerization interface for NPM1–TYK2 which is necessary for its constitutive activation and transformation potential.

In conclusion, our in vitro and in vivo preclinical findings provide functional evidence to identify NPM1–TYK2 as a novel fusion oncogene. Our lymphoid cell transformation and tumorigenicity in xenograft models represent tools necessary to understand the uncovered cellular mechanisms underlying the disease and to assist in future drug screening to develop precision therapies. Importantly, our fusion mapping studies suggest inhibition of NPM1–TYK2 homodimerization by targeting the NPM1 fusion partner could be a potential therapeutic strategy to treat CD30+ LPDs expressing the chimera. Additionally, future preclinical studies focused on inhibition of the TYK2 fusion partner must also be considered in the development of treatment for these fusion-driven lymphomas.

METHODS

Ethics statement

All animal experiments were approved by the University of Kansas Medical Center institutional animal care and use committee and performed in accordance with relevant regulations and guidelines.

Cell culture

Ba/F3, SU-DHL-1 (obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany), HEK293T, and WEHI-3B cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Myla's cell line was kindly provided by Ryan Wilcox, University of Michigan, USA. HEK293T and WEHI-3B cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Myla, SU-DHL-1, and Ba/F3 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Ba/F3 cells were additionally supplemented with a 10% WEHI-3B conditioned medium (as a source of interleukin 3) and all stable clones were maintained with 2 μg/ml of puromycin.

Chemicals and reagents

All reagents and antibodies were purchased from the following: Thermo Scientific, Waltham, MA, USA (RPMI-1640 SH30027; DMEM-SH30081); Corning Life Sciences, USA (Fetal Bovine Serum-MT35010CV, Penicillin/Streptomycin-30-002-CI); InvivoGen, San Diego, CA, USA (Puromycin-58-58-04100); Cell Signaling Technology, Beverly, MA, USA (TYK2/β-Actin-612656, FLAG-F3165, ACTGGCGAGATGGTGGCGGTGCGGGCCCTCAAGCAGACTGCGGC-3′ and RP 5′-GCCGAGCTCTCCTGGCGGACGGCCATCTGGCCACG-3′) and wt-NPM1–TYK2 cDNA as a template (Q5 site-directed mutagenesis kit, New England BioLabs Inc, Ipswich, Massachusetts, USA). The mutant sequence was confirmed by Sanger sequencing. Ba/F3 cells were transfected with FG-NPM1–TYK2–K462R lentiviral particles and stable clones were established using puromycin antibiotic selection. Ectopic kinase-dead fusion protein expression levels were confirmed by Western blot using FLAG antibody.

Western blot analysis

The cells were harvested, washed with phosphate-buffered saline (PBS), added in lysis buffer (25 mM Tris. HCl, 150 mM NaCl, 25 mM NaF, 0.5 mM Na-orthovanadate, 1% Triton-X, 1 mM Benzamidine) with protease and phosphatase inhibitors and incubated on ice for 20 min. Cell lysates were centrifuged at 10,000 RPM for 15 min, and protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL USA). The protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting was performed. The blots were scanned using the Odyssey IR scanner (LI-COR Biosciences, Lincoln, NE, USA). All blots derive from the same experiment and were processed in parallel.

Immunofluorescence

To analyze the cellular distribution of NPM1–TYK2, Ba/F3–FG–NPM1–TYK2 cell slides were prepared by cytospin technique. Similarly, Ba/F3–pCDH- vector cells were also processed and used as a control. Both control and FG–NPM1–TYK2 overexpressed cells were fixed with 4% paraformaldehyde and permeabilized with methanol/glacial acetic acid (3:1). After fixation, the cells were incubated with FLAG primary antibody overnight, washed with PBS, and then incubated with secondary antibody conjugated with Dylight-594 for an hour (D1-2594, Vector Laboratories, Burlingame, CA). Coverslips were mounted with Vectashield antifade mountant medium containing DAPI (4′, 6-diamidino-2-phenylindole, Vector laboratories, Burlingame, CA). The images were acquired using an Eclipse E1000 microscope (Nikon).

Immunohistochemistry

Paraffin-embedded tissues were sectioned at 4 μm size and mounted on the glass slide. Sections were dewaxed in xylene overnight and rehydrated through in gradient ethanol series, followed by washing with PBS. Hematoxylin staining was performed using Leica Surgipath SelectTech Hematoxylin. This is followed by brief immersion in Richard-Allan Scientific Eosin-y with Phloxine, then dehydration with reagent alcohol, xylene, and mounted with a coverslip using permanent mounting media. The slides were examined with Nikon Eclipse E1000 microscope under a 40× objective.

RT-qPCR

Reverse transcription. Total RNA was isolated using an RNA isolation kit (Roche Applied Bioscience) and reverse transcribed with High-Capacity cDNA reverse transcription kit (Applied Biosystems) according to the manufacturer's protocol. The resulting cDNA was diluted and used in SYBR green qPCR assay.

SYBR green qPCR assay. qPCR was performed using Power SYBR Green Mastermix (Applied Biosystems, Carlsbad, CA, USA) on an Applied Biosystems StepOne Plus Real-Time PCR System. All oligonucleotide
Clonogenic assay
The clonogenic assays were performed with Ba/F3 cells to determine the oncogenic potential of ΔN–1–257-NPM1–TYK2 in comparison to full-length NPM1–TYK2 along with vector control. Stable Ba/F3 cells were grown in Methocult media without IL-3 in triplicates. The colonies were allowed to grow and form colonies for seven days. The colonies were counted and presented as percentages.

Cell viability
To compare the oncogenic mechanisms of the full-length NPM1–TYK2 and ΔN–1–257-NPM1–TYK2, resulting in the survival of Ba/F3 cells, we used trypsin blue exclusion assays, and cell viability was represented by a percentage. Further, we conducted similar viability experiments with NPM1–TYK2–K62R kinase-dead mutant cells to validate the fusion kinase-driven transformation potential.

Animal experiments
Upon confirming in vitro transformation potential of the NPM1–TYK2 fusion gene, we next aimed to characterize the in vivo tumorigenic potential of a novel fusion gene utilizing an NPM1–TYK2-transformed Ba/F3-xenograft model. We used five to seven-week-old female Hsd:Athymic Nude-Foxn1nu mice (Envigo, Indianapolis, IN) to validate the tumorigenic potential of fusion kinase NPM1–TYK2. We injected 5 million Ba/F3 vector control and Ba/F3-FG–NPM1–TYK2 cells in the flank via a subcutaneous route (six mice in each group). Tumor size was measured by the Vernier caliper and tumor volumes were calculated using the modified ellipsoid formula: \( V = \frac{1}{2}(\text{length} \times \text{width}^2) \). The mice were euthanized when the tumor size reached 2000 mm\(^3\) or the tumors became necrotic. Tumor presented as percentages.

Statistical analysis
Statistical analysis was performed using Microsoft Excel. Significant differences were determined using the Student's t-test.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY
The authors declare that all relevant data for this study are included within the paper. For any additional information regarding the supporting data, please contact the corresponding author with a reasonable request.

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AUTHOR CONTRIBUTIONS
S.K. and R.B. designed the experiments. S.K., C.J.V., D.R.W., and R.B. carried out experiments and generated data. S.K., R.W.B., M.U.M., T.L.L., I.S., C.J.V., A.V., S.A., W.C., D.R.W., R.A.J., Y.S., J.P.M., and R.B. provided resources and acquired funding. S.K. and R.B. wrote the original paper. R.B. supervised the work. All authors reviewed and edited the paper.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
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npj Precision Oncology (2022) 3