**RESEARCH BRIEF**

**TNIK Is a Therapeutic Target in Lung Squamous Cell Carcinoma and Regulates FAK Activation through Merlin**

Pedro Torres-Ayuso1, Elvira An1, Katherine M. Nysswaner1, Ryan C. Bensen1, Daniel A. Ritt1, Suzanne I. Specht1, Sudipto Das2, Thorkell Andresson2, Raul E. Cachau3, Roger J. Liang1, Amy L. Ries4, Christina M. Robinson4, Simone Difilippantonio5, Brad Gouker5, Laura Bassel5, Baktiar O. Karim5, Chad J. Miller6, Benjamin E. Turk6, Deborah K. Morrison1, and John Brognard1

**ABSTRACT**

Lung squamous cell carcinoma (LSCC) is the second most prevalent type of lung cancer. Despite extensive genomic characterization, no targeted therapies are approved for the treatment of LSCC. Distal amplification of the 3q chromosome is the most frequent genomic alteration in LSCC, and there is an urgent need to identify efficacious druggable targets within this amplicon. We identify the protein kinase TNIK as a therapeutic target in LSCC. TNIK is amplified in approximately 50% of LSCC cases. TNIK genetic depletion or pharmacologic inhibition reduces the growth of LSCC cells in vitro and in vivo. In addition, TNIK inhibition showed antitumor activity and increased apoptosis in established LSCC patient-derived xenografts. Mechanistically, we identified the tumor suppressor Merlin/NF2 as a novel TNIK substrate and showed that TNIK and Merlin are required for the activation of focal adhesion kinase. In conclusion, our data identify targeting TNIK as a potential therapeutic strategy in LSCC.

**SIGNIFICANCE:** Targeted therapies have not yet been approved for the treatment of LSCC, due to lack of identification of actionable cancer drivers. We define TNIK catalytic activity as essential for maintaining LSCC viability and validate the antitumor efficacy of TNIK inhibition in preclinical models of LSCC.

**INTRODUCTION**

Lung squamous cell carcinoma (LSCC) accounts for one third of all lung cancer cases. Despite extensive genomic sequencing, the identification of oncogenic drivers in LSCC has remained challenging, and actionable alterations are unknown in the majority of patients with LSCC (1, 2). As a result, no targeted therapies have been approved to treat LSCC, and treatment still relies on chemotherapy or radiotherapy. Genomic characterization of LSCC tumors shows that distal chromosome 3q amplification (3q26–29) is the most prevalent genomic alteration in LSCC (3). This region

---

1Laboratory of Cell and Developmental Signaling, Center for Cancer Research, NCI, Frederick, Maryland. 2Protein Characterization Laboratory, Leidos Biomedical Research, Frederick National Laboratory for Cancer Research, Frederick, Maryland. 3Advanced Biomedical Computational Science, Biomedical Informatics and Data Science, Frederick National Laboratory for Cancer Research, Frederick, Maryland. 4Laboratory Animal Sciences Program, Leidos Biomedical Research, Frederick National Laboratory for Cancer Research, Frederick, Maryland. 5Laboratory Animal Sciences Program, Leidos Biomedical Research, Frederick National Laboratory for Cancer Research, Frederick, Maryland. 6Molecular Pathology Laboratory, Leidos Biomedical Research, Frederick National Laboratory for Cancer Research, Frederick, Maryland. 7Department of Pharmacology, Yale School of Medicine, New Haven, Connecticut.

**Note:** Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

**Current address for C.J. Miller:** Institute for Protein Design, University of Washington, Seattle, WA.

**Corresponding Authors:** John Brognard, Laboratory of Cell and Developmental Signaling, Center for Cancer Research, NCI, Frederick, MD 21702. Phone: 301-846-1163 Fax: 301-228-4863 E-mail: john.brognard@nih.gov; and Pedro Torres-Ayuso, Laboratory of Cell and Developmental Signaling, Center for Cancer Research, NCI, Frederick, MD 21702. Phone: 301-846-1914; E-mail: torresayusop2@nih.gov

Cancer Discov 2021;11:1-13

doi: 10.1158/2159-8290.CD-20-0797

©2021 American Association for Cancer Research.

Downloaded from cancerdiscovery.aacrjournals.org on June 5, 2021. © 2021 American Association for Cancer Research.
contains very well-characterized oncogenes, including the transcription factors SOX2 and TP63 and the kinase genes PIK3CA and PRKCI (4). Although the former are not druggable to date, results in head and neck squamous cell carcinoma (HNSCC) suggest that PIK3CA amplification does not predict sensitivity to PI3K inhibitors, and PI3K inhibitors have not shown significant clinical benefit as monotherapies in HNSCC (5, 6). Evaluation of PKC\(\alpha\) is still in pre- or early-phase clinical testing (7).

The protein kinase TNIK has been identified as a potential genetic dependency in tumors with distal amplification of the 3q chromosome (8); however, an in-depth evaluation of its functional role in LSCC has never been performed. Here we tested the potential of TNIK as a therapeutic target in LSCC. Our results suggest that targeting amplified TNIK could serve as a new intervention strategy for a subset of patients with LSCC.

**RESULTS**

To investigate the consequences of targeting TNIK in LSCC, we queried LSCC data sets in The Cancer Genome Atlas (TCGA) to determine the frequency of TNIK amplification in LSCC samples. We observed that TNIK is amplified (>3 gene copies) in 35% to 44% of cases, with an additional 46% to 54% of cases displaying copy-number gains (Fig. 1A; Supplementary Fig. S1A). In contrast, TNIK was amplified in 3% to 4% of lung adenocarcinoma cases (Fig. 1A; Supplementary Fig. S1A). Consistent with TCGA data, we observed increased expression of TNIK mRNA in a panel of LSCC cell lines compared with nontransformed primary lung cells (Fig. 1B). Although TNIK expression was high in LSCC cell lines with 3q amplification, some cell lines lacking TNIK amplification (SW900 and Calu-1) also showed enhanced TNIK mRNA levels. Analogous expression patterns were observed when TNIK levels were assayed by immunoblot (Fig. 1C).

To assess if LSCC cells require TNIK to maintain cancer cell viability, we depleted TNIK with doxycycline (dox)-inducible TNIK-targeting shRNAs, which led to a significant reduction (60%–80%) in the clonogenic growth of LSCC cells with high TNIK expression (LK2, NCI-H520, and SW900 cells; Fig. 1D and E). No significant effect on clonogenic growth was observed in low-expressing TNIK LSCC cells when TNIK expression was evaluated by immunoblot. The blot is representative of three independent experiments (LK2, NCI-H520, and SW900 cells) and two independent experiments (NCI-H157). Tubulin or GAPDH were used as loading controls.

**Figure 1.** Identification of the protein kinase TNIK as a genetic dependency in LSCC. A, TNIK amplification in LSCC in comparison with lung adenocarcinoma (LUAD) cases from the TCGA data set. Graph from cBioPortal [34]. B, Analysis of TNIK mRNA expression by RT-qPCR in a panel of LSCC cell lines and two primary lung cells (NHBE and SAEC). C, Analysis of TNIK expression by immunoblot. The blot is representative of three independent replicates. D, Doxycycline (dox) induction (1 \(\mu\)g/mL, 72 hours) of TNIK shRNA reduces TNIK expression in LK2, NCI-H520, SW900, and NCI-H157 cells. The blot is representative of three independent experiments (LK2, NCI-H520, and SW900 cells) and two independent experiments (NCI-H157). Tubulin or GAPDH were used as loading controls. E, Colony formation assay (14 days) following dox-inducible (1 \(\mu\)g/mL, replaced every 48 hours) TNIK knockdown. The image is representative of at least three independent experiments. Data, mean value ± SD; one-way ANOVA, Tukey multiple comparisons posttest. F, Validation of a dox-inducible (1 \(\mu\)g/mL, 72 hours) TNIK shRNA rescue system in LK2 cells. pLenti6.3-CN5406R linked with TNIK V31W (kinase dead) containing lentiviruses were transduced in corresponding LK2 cells, and rescue of TNIK expression was evaluated by immunoblot. The blot is representative of three independent experiments. GAPDH was used as a loading control. TNIK levels relative to GAPDH were normalized to untreated pLKO-shRNA-negative/GFP LK2 cells (top) or untreated pLKO-shRNA NCI-H520 (bottom). G, Rescue of clonogenic growth (14 days; doxycycline 1 \(\mu\)g/mL, replaced every 48 hours) in LK2 cells was evaluated in 3-D Matrigel cultures. Data, mean value ± SD; one-way ANOVA, Tukey multiple comparisons posttest. n.s., not significant. Immunoblot band intensity was quantified with ImageJ.
TNIK Inhibition as a Therapeutic Approach for Patients with LSCC

A. LUAD 4% LSCC 44%

B. Genetic alteration: Amplification Deletion

C. Primary cells LSCC cells

D. IB: TNIK

E. IB: TNIK

F. IB: TNIK

G. IB: TNIK

TNIK Inhibition as a Therapeutic Approach for Patients with LSCC

A. LUAD 4% LSCC 44%

B. Genetic alteration: Amplification Deletion

C. Primary cells LSCC cells

D. IB: TNIK

E. IB: TNIK

F. IB: TNIK

G. IB: TNIK

TNIK Inhibition as a Therapeutic Approach for Patients with LSCC

A. LUAD 4% LSCC 44%

B. Genetic alteration: Amplification Deletion

C. Primary cells LSCC cells

D. IB: TNIK

E. IB: TNIK

F. IB: TNIK

G. IB: TNIK

TNIK Inhibition as a Therapeutic Approach for Patients with LSCC

A. LUAD 4% LSCC 44%

B. Genetic alteration: Amplification Deletion

C. Primary cells LSCC cells

D. IB: TNIK

E. IB: TNIK

F. IB: TNIK

G. IB: TNIK
Figure 2. TNIK inhibition significantly reduces viability of LSCC cells. A, Dose–response curves of a panel of LSCC cell lines to NCB-0846. The cells were treated with increasing concentrations of NCB-0846 (72 hours). The lines represent the fitted curves, where each dot indicates the mean value ± SD of at least three independent experiments each conducted in triplicate. B, Cell viability (MTS assay) of selected LSCC cell lines treated with NCB-0846 (500 nmol/L) for 72 hours. At least three independent experiments with triplicates were conducted for each cell line. Data, mean value ± SD; two-tailed t test with Welch correction. C, Cell viability (crystal violet assay) of NCI-H520 cells treated with NCB-0846 (500 nmol/L, 48 hours) is rescued by expression of a TNIK inhibitor–resistant mutant (TNIKV31W). n = 3 independent experiments with triplicates. WT, wild-type. D, Reduced effect of NCB-0846 treatment (72 hours) on NCI-H520 cell viability after TNIK knockdown (72 hours doxycycline 2 μg/mL induction followed by 72 hours cotreatment with doxycycline and NCB-0846). Data, mean value ± SD; two-way ANOVA, Tukey multiple comparisons posttest. n = 3 independent experiments with triplicates. E, Left, tumor growth curve of LK2 cell–derived xenografts treated with NCB-0846 (100 mg/kg, twice a day (b.i.d.), five days on/two days off). Mean tumor volumes ± SEM are shown. Center, average tumor volume at the end of treatment; mean ± SEM. n = 10 mice in the vehicle–treated group, 8 mice in the NCB-0846–treated group; two-tailed t test. Right, tumor images from the study at the end of treatment. F, Left, tumor growth curve of NCI-H520 cell–derived xenografts treated with NCB-0846 (100 mg/kg, b.i.d., five days on/two days off). Mean tumor volumes ± SEM are shown. Center, average tumor volume at the end of treatment; mean ± SEM. n = 10 mice in the vehicle–treated group, nine mice in the NCB-0846–treated group; two-tailed t test. Right, tumor images from the study at the end of treatment. (continued on following page)
TNIK Inhibition as a Therapeutic Approach for Patients with LSCC

To determine if TNIK was essential for maintaining LSCC growth in vivo, we generated mouse xenograft models derived from the LK2 and NCI-H520 cell lines. Treatment of the mice with 100 mg/kg of NCB-0846 significantly reduced tumor growth in vivo without effects on the overall weight of the mice (Fig. 2E and F; Supplementary Fig. S2C), consistent with our results indicating that TNIK is essential for maintaining LSCC clonogenic growth in culture. Tumor analysis by immunohistochemistry (IHC) showed that NCB-0846 treatment significantly increased cell apoptosis but did not affect cell proliferation (Fig. 2G and H; Supplementary Fig. S2D). We then established LSCC mouse xenograft models derived from two treatment-naive patients with LSCC (PDX), as these models more accurately recapitulate patients’ pharmacologic responses to cancer drugs (12). A similar response to NCB-0846 was observed in these PDx models. In PDx model 1 (PDx-1), TNIK inhibition significantly halted tumor growth from the very onset of the treatment while in the second model (PDx-2), TNIK inhibition effectively stabilized tumor growth (Fig. 3A and B). There was a minor overall decrease in the relative weight of the mice treated with NCB-0846 in the PDx-1 model (5.84% ± 3.92%; Supplementary Fig. S3A). Analysis of tumor samples by IHC demonstrated that NCB-0846 treatment increased cell apoptosis in PDx-2 but did not affect cell proliferation; however, we observed that NCB-0846 suppressed proliferation in PDx-1 (Fig. 3C and D; Supplementary Fig. S3B). These results indicate that TNIK represents a pharmacologic target in LSCC and that TNIK inhibitors could represent new treatment options for patients with LSCC.

We next investigated the mechanisms through which TNIK could promote LSCC cell viability. In colon cancer cells, TNIK has been reported to contribute to cell viability through regulation of the WNT/β-catenin pathway (13, 14); however, analysis of β-catenin expression and activity did not differ in LSCC cells depleted of TNIK (Supplementary Fig. S4A). To identify alternative mechanisms by which TNIK could modulate LSCC viability, we scanned established oncogene and tumor suppressor gene products for occurrences of the consensus TNIK phosphorylation site motif (15). We found that the tumor suppressor Merlin (moesin–ezrin–radixin-like protein; NF2) harbored a potential TNIK phosphorylation site at T576, a position analogous to known regulatory sites on the related ERM proteins (ezrin, radixin, and moesin). Moreover, Merlin had several additional sites (S13, T23, and T272) conforming to the TNIK consensus sequence. Of note, these phosphorylation sites are evolutionarily conserved in metazoans, suggesting that their posttranslational modification has functional consequences (Supplementary Table S2). Also, structural modeling of Merlin indicates that these sites are accessible to the solvent, and thus exposed to be phosphorylated. These data suggested that Merlin may be a novel substrate of TNIK. We also performed a global reverse-phase protein array analysis in cells depleted of TNIK and identified focal adhesion kinase (FAK) as a downstream effector (Supplementary Fig. S4B). FAK has been reported to be regulated by Merlin (16), providing us with an additional rationale to evaluate Merlin as a downstream substrate of TNIK. Indeed, coexpression of Merlin and TNIK resulted in increased Merlin phosphorylation as assessed with a pan-phospho-threonine antibody; this phosphorylation was sensitive to the addition of the TNIK inhibitor NCB-0846 (Fig. 4A).

Figure 2. (Continued) G, Left, IHC analysis of apoptosis (cleaved caspase-3; n = 10 in vehicle group, n = 8 in the NCB-0846–treated group) and proliferation (Ki-67; n = 9 in the NCB-0846–treated group) and proliferation (Ki-67; n = 4/group) in LK2 cell xenografts. Data are represented as mean ± SD; two-tailed Mann–Whitney test. Right, representative hematoxylin and eosin (H&E) and cleaved caspase-3 and Ki-67 IHC images from each treatment group. H, Left, IHC analysis of apoptosis (cleaved caspase-3; n = 10 in the vehicle-treated group; n = 9 in the NCB-0846–treated group) and proliferation (Ki-67, n = 4/group) in NCI-H520 cell xenografts. Data, mean ± SD; two-tailed Mann–Whitney test. Right, representative H&E and cleaved caspase-3 and Ki-67 IHC images from each treatment group. n.s., not significant. Scale bar, 300 μm.
Figure 3. Preclinical evaluation of TNIK inhibitor NCB-0846 in LSCC PDX. A, Left, tumor growth curve of PDX-1 treated with NCB-0846 (100 mg/kg, b.i.d., five days on/two days off). Mean tumor volumes ± SEM are shown. Center, average tumor volume at the end of treatment; mean ± SEM. n = 8 mice/group; two-tailed t test. Right, tumor images from the study at the end of treatment. B, Left, tumor growth curve of PDX-2 treated with NCB-0846 (100 mg/kg, b.i.d., five days on/two days off). Mean tumor volumes ± SEM are shown. Center, average tumor volume at the end of treatment; mean ± SEM. n = 9 mice/group; two-tailed Mann–Whitney test. Right, tumor images from the study at the end of treatment. C, Left, IHC analysis of apoptosis (cleaved caspase-3; n = 8/group) and proliferation (Ki-67; n = 4/group) in PDX-1 cell xenografts. Data, mean ± SD; two-tailed Mann–Whitney test. Right, representative H&E and cleaved caspase-3 and Ki-67 IHC images from each treatment group. D, IHC analysis of apoptosis (cleaved caspase-3; n = 9/group) and proliferation (Ki-67; n = 4/group) in PDX-2 cell xenografts. Data, mean ± SD; two-tailed Mann–Whitney test. Right, representative H&E and cleaved caspase-3 and Ki-67 IHC images from each treatment group. n.s., not significant. Scale bar, 300 μm.
we observed that TNIK and Merlin coimmunoprecipitated (Fig. 4A). A TNIK kinase-dead mutant (K54R) also interacted with Merlin but did not induce its phosphorylation (Fig. 4A).

We verified that TNIK directly phosphorylated Merlin in vitro in a radiolabel kinase assay, and this phosphorylation was confirmed by phosphopeptide mapping and mass spectrometry analysis on Merlin isolated from cells coexpressing TNIK and Merlin, because they were not detected by phosphopeptide mapping, nor were these sites of phosphorylation increased upon TNIK overexpression (Supplementary Fig. S4C; Supplementary Table S3). The mass spectrometry analysis of Merlin isolated from cells coexpressing TNIK and Merlin also revealed phosphorylation of Merlin at S13, a site that is phosphorylated by PKA and the PAK kinases (17), S566, and S581, which were unlikely to be phosphorylated by TNIK because they were not detected by phosphopeptide mapping, nor were these sites of phosphorylation increased upon TNIK overexpression (Supplementary Fig. S4C; Supplementary Table S3). Coexpression of TNIK and Merlin in HEK 293T cells resulted in increased phosphorylation of Merlin at S13, which did not occur when the TNIK kinase-dead mutant

Supplementary Table S3). The mass spectrometry analysis of Merlin isolated from cells coexpressing TNIK and Merlin also revealed phosphorylation of Merlin at S13, a site that is phosphorylated by PKA and the PAK kinases (17), S566, and S581, which were unlikely to be phosphorylated by TNIK because they were not detected by phosphopeptide mapping, nor were these sites of phosphorylation increased upon TNIK overexpression (Supplementary Fig. S4C; Supplementary Table S3). Coexpression of TNIK and Merlin in HEK 293T cells resulted in increased phosphorylation of Merlin at S13, which did not occur when the TNIK kinase-dead mutant

Supplementary Table S3). The mass spectrometry analysis of Merlin isolated from cells coexpressing TNIK and Merlin also revealed phosphorylation of Merlin at S13, a site that is phosphorylated by PKA and the PAK kinases (17), S566, and S581, which were unlikely to be phosphorylated by TNIK because they were not detected by phosphopeptide mapping, nor were these sites of phosphorylation increased upon TNIK overexpression (Supplementary Fig. S4C; Supplementary Table S3). Coexpression of TNIK and Merlin in HEK 293T cells resulted in increased phosphorylation of Merlin at S13, which did not occur when the TNIK kinase-dead mutant
was expressed, indicating that TNIK was directly phosphorylating S13 (Fig. 4C). This phosphorylation was lost when expressing a Merlin mutant where the serine is exchanged for an alanine (S13A; Fig. 4C), confirming its specificity. In addition, pThr-phosphorylated Merlin was largely diminished when the nonphosphorylatable T272A mutant was expressed (Supplementary Fig. S4E); however, expression of the T576A or T581A mutants did not affect global Merlin threonine phosphorylation, indicating that Merlin pT272 is the main substrate recognized by the pan-phospho-threonine antibody (Supplementary Fig. S4F). Phosphorylation of Merlin at S315 has been previously reported (18) but could not be verified by immunoblot due to lack of site-specific antibodies. In agreement with the above observations, molecular dynamics simulations were performed and overall indicate that TNIK-mediated phosphorylation of Merlin at the sites identified (S13, T272, S315, and T576) will affect Merlin structure and activity (Supplementary Fig. S5; Supplementary Table S4).

We next determined if endogenous TNIK could phosphorylate Merlin at these sites in LSCC cells. Pulldown of endogenous Merlin from NCI-H520 cells followed by mass spectrometry analysis of phosphopeptides identified...
phosphorylation of Merlin S13 and S315. Phosphorylation of S13 was not identified in NCB-0846–treated samples, indicating TNIK is directly phosphorylating this site in NCI-H520 cells (Supplementary Fig. S6A and S6B). Similarly, phosphorylation at S315 was reduced after TNIK inhibition (fold change: 1.00 vs. 0.34, DMSO/NCB-0846 samples; Supplementary Fig. S6C), indicating endogenous TNIK is directly phosphorylating S315 in NCI-H520 cells. Because we lack sensitive phospho-site-specific antibodies that recognize endogenous Merlin, we validated phosphorylation of Merlin at S13 by endogenous TNIK in LK2 and NCI-H520 cells by overexpressing Merlin and showed that this phosphorylation was sensitive to treatment with the TNIK inhibitor NCB-0846 (Fig. 4D). However, the pan-pThr antibody was not sensitive enough to detect phosphorylation on Merlin in LK2 or NCI-H520 cells. Combined, these results demonstrate that Merlin is a direct TNIK substrate and indicate that S13 and S315 were the main TNIK-mediated Merlin phosphosites. We were unable to identify phosphorylation of endogenous Merlin T272 in the pulldown mass spectrometry experiments, but cannot exclude that this site may be phosphorylated by endogenous TNIK.

Merlin is a scaffold protein regulated by phosphorylation and can modulate downstream effectors such as FAK (16). We found that FAK activation (as determined by FAK-pY397 levels) was reduced in LK2 and NCI-S20 cells upon TNIK knockdown (Fig. 4E). Importantly, reexpression of catalytically active TNIK, but not a kinase-dead mutant, rescued the effects of TNIK knockdown on FAK activation in LK2 cells (Fig. 4F). In agreement with TNIK activity being required for FAK activation, treatment of LK2 and NCI-H520 cells with NCB-0846 reduced FAK phosphorylation at Y397 (Supplementary Fig. S7A). In addition, Merlin negatively regulates the pro-oncogenic YAP/TAZ transcription factors through activation of effectors of the Hippo pathway (16). Consistent with TNIK-modulating Merlin, we also observed reduced YAP levels following TNIK knockdown in LK2 and NCI-H520 cells (Supplementary Fig. S7B).

These results indicate that TNIK phosphorylates Merlin and led us to hypothesize that phosphorylated Merlin is required to maintain FAK activation. To test this model, we knocked down Merlin, TNIK, and the combination of Merlin and TNIK. Knockdown of Merlin in LK2 and NCI-S20 cells reduced FAK activation, which was further diminished with the combination of Merlin and TNIK knockdown (Fig. 4G; Supplementary Fig. S7A and S7C). Furthermore, we found that expression of a Merlin phosphomimetic mutant (Merlin 4D) restored FAK activation and YAP levels in NCI-H520 cells with TNIK knockdown (Fig. 4H), but a wild-type Merlin or a nonphosphorylatable mutant (Merlin 4A) did not affect FAK activation and YAP levels in cells depleted of TNIK (Fig. 4H). We also assessed if these pathways contributed to maintain cell survival; siRNA-mediated knockdown of Merlin, FAK, and YAP significantly reduced cell viability in LK2 and NCI-H520 cells (Fig. 4I and J; Supplementary Fig. S7D–S7F). In summary, our results suggest that phosphorylated Merlin is required for the maintenance of FAK activation and YAP stabilization and provides one possible mechanism by which TNIK maintains LSCC cell survival (Fig. 4K; Supplementary Movie S1).

**DISCUSSION**

We have identified amplified *TNIK* as a promising therapeutic target in LSCC. Using a variety of functional assays, we determined that targeting TNIK significantly reduced cell viability in LSCC cell lines with high TNIK expression as evaluated by colony formation assays and cell growth on 3-D matrices. Importantly, TNIK depletion had no significant effect on LSCC cells that lack TNIK amplification and have low TNIK expression. Moreover, we have shown that pharmacologic targeting of TNIK with a small-molecule inhibitor, NCB-0846, significantly reduced viability of LSCC cells in vitro and in vivo, including in established LSCC PDXs. Our data indicate that NCB-0846 effects on cell viability are specifically due to catalytic inhibition of TNIK, as cell viability could be rescued by expression of an inhibitor-resistant mutant (TNIK<sup>1101R</sup>) or viability was not affected by NCB-0846 in cells with almost complete TNIK knockdown. Although TNIK was mainly overexpressed in LSCC cell lines with 3q amplification, we found a subset of LSCC cell lines (SW900 and Calu-1) that lacked TNIK amplification but displayed high TNIK levels, suggesting that other mechanisms can drive increased TNIK abundance in these cell lines. Importantly, these cells were also sensitive to TNIK depletion.

TNIK has been previously involved in the promotion of colorectal cancer, triple-negative breast cancer, prostate cancer, and chronic myelogenous leukemia (9, 19–21). Mechanistically, we observed that TNIK did not mediate its effects by regulating the Wnt/β-catenin pathway, contrary to previous reports in other cancer types (14, 19). We found that TNIK’s effects were in part mediated through regulation of Merlin/NF2, although the contribution of additional substrates cannot be ruled out. Merlin is a tumor suppressor protein, closely related to members of the ERM family. The ERM proteins are an evolutionarily conserved group of proteins that act as cross-linkers between membrane receptors and the actin cytoskeleton. ERM proteins are present in two conformations, a closed or inactive conformation, which involves the intramolecular association between the C-terminal and the FERM domain, and an open or active conformation in which this intramolecular association is disrupted (22, 23). Activation of ERM is a multiple-step process that requires association to the plasma membrane through binding of the FERM domain to phosphatidylinositol-4,5-bisphosphate (PIP₂), and subsequent phosphorylation at a conserved C-terminal threonine (T567 in ezrin), that can be mediated by multiple protein kinases (22, 23). Because of the structural conservation between Merlin and ERM proteins, a similar mechanism was thought to operate to regulate Merlin’s function. Indeed, phosphorylation of Merlin at the C-terminal SS18 (equivalent to ezrin T567) has been proposed to modulate Merlin activity, but contrary to ERM proteins, several studies have shown that Merlin is inactivated by phosphorylation (16, 17, 24). Of note, LSCC cells with distal 3q amplification (such as the LK2 and NCI-H520 cell lines) also harbor amplification of the *PAK2* gene, which encodes one of the kinases responsible for the phosphorylation of Merlin at SS18. However, the functional relevance of SS18 phosphorylation has been recently questioned using a CRISPR knock-in approach to generate the respective phosphomimetic and
nonphosphorylatable Merlin S518 mutants (25). Similar to ERM proteins, Merlin binding to phosphoinositides, such as PIP2, is critical for its activity (25).

We have established that Merlin interacts with TNIK and is a direct TNIK substrate. In this study, we have identified S13 and S315 as the primary TNIK phosphorylation sites after mass spectrometry analysis of endogenous Merlin pulldowns. However, we cannot completely exclude the possibility that TNIK could activate a downstream protein kinase that would phosphorylate Merlin at these residues. Consistent with our observations, phosphorylation of endogenous Merlin at S13 and S315 has been detected in high-throughput proteomics analysis (26, 27), including studies in non–small cell lung cancer (28). Using phosphopeptide mapping from in vitro kinase assays, we identified that Merlin could also be phosphorylated by TNIK at T272 and T357. Phosphorylation of T272 was also detected in mass spectrometry analysis from cells coexpressing TNIK and Merlin. We were unable to detect phosphorylation of endogenous Merlin at T272 in cells that expressed endogenous TNIK levels; however, our data do not exclude the possibility that TNIK T272 can be phosphorylated in cells. One possible explanation for lack of detection of T272 phosphorylation is that these sites might be easily accessible to the action of protein phosphatases and their identification is difficult under more stringent conditions. In addition, these sites fall on short tryptic peptides, which complicate their detection by mass spectrometry. AKT-mediated phosphorylation of Merlin at S315 has been described in previous studies (18), although there are conflicting data on which is the responsible upstream kinase of this site (29). Mechanistically, phosphorylation at this site triggered Merlin’s polyubiquitination and proteasomal degradation (18). However, we did not observe changes in Merlin levels after TNIK knockdown in LSCC cells, suggesting that this regulatory mechanism of Merlin might not be operative in the cell lines used in this study.

Knockdown of TNIK led to reduced FAK activation (measured as FAK Y397 phosphorylation) that was rescued upon expression of shRNA-resistant TNIK, but not of a kinase-dead mutant. Consistently with a requirement for catalytic activity, TNIK inhibition with NCB-0846 also reduced FAK pY397 levels. FAK is a nucleo-cytosolic non–receptor tyrosine kinase that can regulate FAK activity (31). Our results show that knockdown of Merlin also reduces FAK activation in LSCC cells and that this effect is enhanced by TNIK depletion. Moreover, reduced FAK activation in NCI-H520 cells with TNIK knockdown could be rescued after expression of a Merlin phosphomimetic mutant, suggesting that FAK activation depends on expression of a phosphorylated form of the Merlin protein (Fig. 4K). In addition, siRNA-mediated depletion of Merlin or its downstream effectors significantly diminishes cell viability in LK2 and NCI-H520 cells, indicating that this is a relevant pathway in LSCC. Combined, our studies suggest that phosphorylation of Merlin by TNIK induces a conformation of Merlin that is needed to maintain FAK activation. This proposed model is supported by multiple lines of evidence. First, depletion of Merlin suppresses FAK activation (Fig. 4G; Supplementary Fig. S7A and S7C), and second, expression of a phosphomimetic mutant of Merlin (4D) can rescue loss of FAK activation in cells depleted of TNIK (Fig. 4H). Interestingly, wild-type Merlin or a nonphosphorylatable Merlin mutant (4A) were unable to rescue FAK activation in cells depleted of TNIK, highlighting that negative charges at these positions in Merlin are required to maintain FAK in an active conformation. Furthermore, catalytic activity of TNIK was essential for maintaining viability of LK2 and NCI-H520 cells, and TNIK catalytic inhibition led to loss of endogenous Merlin phosphorylation on S13 and S315. Overall, this provides a detailed mechanism whereby TNIK directly phosphorylates endogenous Merlin at S13 and S315 to promote FAK activation. How exactly phosphorylated Merlin sustains FAK activation will require further study.

In summary, we propose a model in which TNIK contributes to LSCC in part by modulating the Merlin–FAK signaling axis (Fig. 4K; Supplementary Movie S1). The lack of effective treatments for LSCC has been a large therapeutic gap. The success of TNIK inhibition in our in vitro models, including established LSCC PDXs, highlights the translational potential of our findings and confirms TNIK as a potential therapeutic target for a subset of patients with LSCC who harbor TNIK copy-number gains.

METHODS

Ethical Approval and Patient Details

LSCC tumor fragments from treatment-naive patients were collected by the Manchester Cancer Center Biobank between January 2015 and December 2016. All patients provided written informed consent for the use of donated samples for this research. All studies were conducted in accordance with the Human Tissue Act 2004 (UK) and regulations by the NIH. All patient information has been anonymized.

Cell Lines

Normal human bronchial epithelial cells (NHBE) and small airway epithelial cells (SAEC) were purchased from Lonza in February 2016. Cells were expanded for two passages and frozen. NCI-H520 (RRID:CVCL_1566), SW900 (RRID:CVCL_1731), and HEK 293T (RRID:CVCL_0063) cells were obtained from ATCC. LK2 (RRID:CVCL_1377), KNS-62 (RRID:CVCL_1335), and Lc-1-sq (RRID:CVCL_3008) were obtained from the Japanese Collection of Research Bioresources (JCRB) in February 2015. LUDLU-1 (RRID:CVCL_2582) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) General Cell Collection. Calu-1 (RRID:CVCL_0608) cells were kindly donated by Dr. D.K. Morrison (NCI) in March 2017. All cell lines were maintained at 5% CO2 at 37°C and cultured as recommended by the vendor. In brief, NHBE and SAEC were cultured in BEGM (Lonza, cat. CC-3170) or SAGM (Lonza, cat. CC-3118), respectively. LK2, NCI-H520, SW900, LUDLU-1, HCC15, HCC95, NCI-H157, NCI-H1703, and SK-MES-1 (RRID:CVCL_0630) cells were kindly donated by Dr. D.K. Morrison (NCI) in March 2017. All cell lines were verified by short tandem repeat profiling upon receipt [CRUK Manchester Institute (cell lines obtained between 2015 and 2016) or Frederick National Laboratory for Cancer Research (cell lines obtained in or after 2017)]. Cells were expanded (one to two passages) upon receipt, and several vials were frozen. All cell lines were maintained at 5% CO2 at 37°C and cultured as recommended by the vendor. In brief, NHBE and SAEC were cultured in BEGM (Lonza, cat. CC-3170) or SAGM (Lonza, cat. CC-3118), respectively. LK2, NCI-H520, SW900, LUDLU-1, HCC15, HCC95, NCI-H157, NCI-H1703, and SK-MES-1 were cultured in RPMI-1640 (Quality Biological, cat. 112-024-101) + 10% FBS (Atlanta Biologicals) + GlutaMAX ( Gibco, cat. 35050061) + 1% penicillin–streptomycin.
TNIK Inhibition as a Therapeutic Approach for Patients with LSCC

A. Noailles: Conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.
B. Y. Li: Conceptualization, resources, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.
C. M. Robinson: Investigation and methodology.
D. S. Difilippantonio: Resources, formal analysis, investigation, visualization, methodology, writing–review and editing.
E. A. Ries: Investigation and methodology.
F. P. Torres-Ayuso: Resources, formal analysis, investigation, visualization, methodology, writing–review and editing.
G. B. Gouker: Investigation and methodology.
H. Brognard: Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, validation, visualization, methodology, writing–review and editing.
supervision, funding acquisition, validation, visualization, methodology, writing—original draft, project administration, writing—review and editing. K.M. Nyström: Investigation, methodology, project administration, writing—review and editing. R.C. Bensen: Investigation and methodology. D.A. Ritt: Investigation and methodology. S.I. Specht: Investigation and methodology. S. Das: Data curation, formal analysis, investigation, and methodology. T. Andresson: Data curation, formal analysis, investigation, and methodology. R.E. Cachau: Formal analysis, investigation, and methodology.

Acknowledgments

We thank the patients and their families for providing samples for this research, as well as the Manchester Cancer Research Centre Biobank (Manchester, UK) and Dr. R. Booton (University Hospital of South Manchester, Manchester, UK) for logistic support to provide tissue samples for the generation of LSCC PDX. We thank Dr. E.W. Trotter (CRUK Manchester Institute, Manchester, UK) for lab logistics. We thank members of the Laboratory of Cell and Developmental Signaling for helpful discussions. This research was mainly funded by the NCI, project number 1ZIABC011691-01 (J. Brognard). Additional funding came from a Fundacion Ramon Areces post-doctoral fellowship (P. Torres-Ayuso), the Lung Cancer Research Foundation (J. Brognard and P. Torres-Ayuso), and National Institute for General Medical Sciences R01 GM102262 (B.E. Turk). This project has been funded in part with federal funds from the NCI, NIH, under Contract No. 75N91019D00024. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

Received June 5, 2020; revised November 21, 2020; accepted January 20, 2021; published first January 25, 2021.

REFERENCES

ness in mesothelioma cells and negatively regulates FAK. Oncogene 2006;25:5960–8.
TNIK Is a Therapeutic Target in Lung Squamous Cell Carcinoma and Regulates FAK Activation through Merlin

Pedro Torres-Ayuso, Elvira An, Katherine M. Nyswaner, et al.

Cancer Discov  Published OnlineFirst January 25, 2021.

Updated version  Access the most recent version of this article at: doi:10.1158/2159-8290.CD-20-0797

Supplementary Material  Access the most recent supplemental material at: http://cancerdiscovery.aacrjournals.org/content/suppl/2021/01/23/2159-8290.CD-20-0797.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link http://cancerdiscovery.aacrjournals.org/content/early/2021/05/19/2159-8290.CD-20-0797. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.