## Supplementary Methods

*Cell lines and culture media composition*

| Cell line | Medium |
|---|---|
| NHBE | BEGM (Lonza, cat. CC-3170) |
| SAEC | SAGM (Lonza, cat. CC-3118) |
| LK2 | RPMI 1640 (Quality Biological, cat. 112-024-101) + 10% fetal bovine serum (FBS, Atlanta Biologicals) + GlutaMAX (Gibco, cat. 35050061) + 1% penicillin-streptomycin (pen-strep; Gibco, cat. 15140122) |
| NCI-H520 | |
| SW900 | |
| LUDLU-1 | |
| HCC15 | |
| HCC95 | |
| NCI-H157 | |
| NCI-1703 | |
| SK-MES-1 | |
| KNS-62 | MEM (Gibco, cat. 11090099) + 20% FBS + GlutaMAX + 1% pen-strep |
| Lc-1-sq | DMEM:F12 (Gibco, cat. 10565018) + 10% FBS + GlutaMAX + 1% pen-strep |
| Calu-1 | DMEM (Gibco, cat. 11995073) + 10% FBS + GlutaMAX + 1% pen-strep |
| HEK 293T | |

*Reagents*

NCB-0846 was purchased from Selleck Chemicals (cat. S8392). Doxycycline was purchased from Sigma-Aldrich (cat. D3072). Staurosporine was purchased from Cell Signaling Technology (cat. #9953). NCB-0846 for *in vivo* experiments was synthesized by Synnovator (Centrewest Business Park, Cary, NC, USA). Lipofectamine 2000 (cat. 11668019) and Lipofectamine RNAiMAX (cat.

13778075) were purchased from Invitrogen. JetPRIME was acquired from Polyplus Transfections (cat. 114-15).

*Generation of doxycycline-inducible knockdown and rescue cell lines*

shRNA sequences (Supplementary Table S5) were cloned into the Tet-pLKO-Puro vector (Addgene #21915; kindly donated by Dr. D. Wiederschain [Novartis]) and verified by Sanger sequencing. For generation of shRNA TNIK rescue constructs, *attB*-containing TNIK was amplified by PCR from pRK5-HA-TNIK (kindly donated by Dr. A. Kiendser [Helmholtz Zentrum München – German Research Center for Environmental Health, Munich, Germany]) using Phusion High-Fidelity Polymerase (Invitrogen, cat. F530S) according to the manufacturer's protocol. PCR products were cloned first into pDONR221 entry vector, in which mutagenesis was conducted using the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent, cat. 200522). Primers used are listed in Supplementary Table S5. Vectors confirmed to harbor the corresponding mutations were then cloned into the pLenti6.3/TO/V5-DEST vector. Entry and destination vectors were generated by cloning using the ViraPower HiPerform T-REx Gateway Expression System (Invitrogen, A11141) per the vendor's instructions. All vectors and mutations were verified by Sanger sequencing.

Lentiviruses were produced in HEK 293T cells according to standard procedures. In brief, Tet-pLKO-Puro shRNA or pLenti6.3/TO/V5-DEST vectors were co-transfected with pMDLg/pRRE (Addgene #12251), pRSV-Rev (Addgene #12253), and pMD2.G (Addgene #12259), all kindly donated by Dr. Trono (Swiss Federal Institute of Technology Lausanne [EPFL]), using Lipofectamine 2000. Viral supernatants were collected at 72 hours post-transfection. Cells were selected with the corresponding antibiotic (puromycin [Gibco, cat. A1113803] or blasticidin [Gibco, cat. R21001]) 48 hours after lentiviral transfection.

*siRNA-mediated transient knockdown*

LK2 or NCI-H520 cells ($2 \times 10^5$ cells/well in six-well plate) were reverse-transfected with corresponding siRNA oligonucleotides (30 nM) using Lipofectamine RNAiMAX according to the manufacturer's protocol. Medium was replaced 16 hours after transfection, and cells were analyzed for protein expression or cell viability (MTS assay) 96 hours post-transfection. siRNA against Merlin ([*NF2*] #1: cat. s194647, #2: cat. s194648, #3: s528738), FAK ([*PTK2*] #1: cat.

s11485, #2: cat. s11484, #3: cat. s11486), YAP ([*YAP1*] #1: cat. s20366, #2: cat. s20367), and TAZ ([*WWTR1*] #1: cat. s27787, #2: cat. s24789) were purchased from Ambion.

*Plasmid transfection*

Plasmid DNA was transfected into cells using JetPrime according to the manufacturer's protocol, and cells were analyzed 48 hours to 72 hours post-transfection.

*MTS cell viability assays*

Cells ($2 \times 10^3$ cells/well) were seeded in triplicate in 96-well plates in a volume of 100 μl and treated with the corresponding compound the following day. Cells were analyzed 72 hours later. For MTS assays, the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, cat. G3580) was used per the manufacturer's protocol. In brief, MTS (10% final concentration) was added and incubated for two hours, and absorbance was measured at 490 nm.

*Crystal violet cell viability assays*

For NCB-0846 rescue with TNIK inhibitor-resistant mutant, $5 \times 10^4$ cells/well were seeded in 24-well plates. Cells were transfected with corresponding plasmids the following day, and treated with NCB-0846 (500 nM, for 48 hours) 24 hours post-transfection. For TNIK knockdown studies, LK2 cells ($1 \times 10^3$ cells/well) or NCI-H520 cells ($2.5 \times 10^3$ cells/well) were seeded in triplicate in 24-well plates. Cells were treated with doxycycline (2 μg/ml) for 72 hours to induce TNIK shRNA expression. The medium was then refreshed, and cells treated with doxycycline and NCB-0846 (500 nM) for an additional 72 hours. After the corresponding treatments, cells were washed with PBS, fixed in ice-cold methanol, and stained with 0.5% crystal violet (Sigma-Aldrich, cat. C0775) in 25% methanol. Colonies were quantified using ImageJ (National Institutes of Health, RRID:SCR_003070) (1).

*Colony formation assays*

Cells ($5 \times 10^2$ cells/well) were seeded in triplicate in 12-well plates and treated with the corresponding compound the following day. Medium was refreshed every 48 hours for 14 days. Colonies were then washed with PBS, fixed in ice-cold methanol, and stained with 0.5% crystal violet (Sigma-Aldrich, cat. C0775) in 25% methanol. Colonies were quantified using ImageJ (National Institutes of Health, RRID:SCR_003070) (1).

*Matrigel 3D assays*

Three-dimensional "on-top" cell culture on Matrigel was performed as described (2). In brief, 150 µl of growth-factor-reduced Matrigel (Corning, cat. 356231) was added to each well of a 12-well chamber (3.5 $cm^2$ surface) and allowed to solidify (20 minutes, 37 ºC). Cells were trypsinized, resuspended in assay medium (normal medium with 2.5% Matrigel), and seeded (0.5–1 × $10^3$ cells/$cm^2$). Cells were treated after 48 hours. The medium was replaced every 48 hours for 14 days. Spheroids were stained with MTT (Sigma-Aldrich, cat. M2128) according to the vendor's protocol and quantified using ImageJ (National Institutes of Health, RRID:SCR_003070).

*RNA extraction*

Genomic DNA was removed and RNA was prepared using the RNeasy kit (Qiagen, cat. 74104) according to the manufacturer's protocol. RNA quantity and quality were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

In vitro *kinase assays*

For kinase assays, immunoprecipitates, obtained as described above, were additionally washed three times with kinase buffer (Cell Signaling Technology, #9802) and incubated with 50 ng of TNIK-kinase domain (Carna Biosciences, cat. 07-438-20N) and 50 µM ATP (Cell Signaling Technology, #9804) in a final volume of 50 µl of kinase buffer for 30 minutes at 30 °C. Kinase assays were started by the addition of ATP. TNIK-kinase domain was not included in TNIK autophosphorylation assays. When inhibitors were used, they were added 15 minutes before starting the kinase assays. Kinase assays were terminated by the addition of Laemmli buffer (2× final concentration), followed by incubation at 100 °C for 15 minutes. Immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by immunoblot.

*Mass spectrometry*

pcDNA3-FLAG-Merlin (Addgene #11623, kindly donated by Professor V. Ramesh [Massachusetts General Hospital]) was transfected alone into LK2 cells, or together with pRK5-HA-TNIK into HEK 293T cells (6 × $10^6$ cells in 15-cm plates) with jetPRIME according to the manufacturer's protocol. Cells were lysed and Merlin was immunoprecipitated (two hours, 4°C) using Pierce™ Anti-DYKDDDDK Magnetic Beads (Thermo Scientific, cat. A36797) 24 hours

after transfection. Immunoprecipitates were washed three times in lysis buffer, washed once with TBS, and then resuspended in 25 mM $NH_4HCO_3$, pH 8.4 (Millipore cat. S2454).

FLAG-Merlin-containing beads were heated at 95 °C for five minutes to denature the proteins. The samples were digested overnight either with 2 μg of trypsin (Promega, cat. V5280) or 2 μg of Arg-C (Promega, cat. V1881) at 37 °C. The supernatant containing the protease digest was obtained after centrifuging the beads. The beads were washed twice with 25 mM $NH_4HCO_3$, pH 8.4, to get maximum yield. The digest was desalted using C18 columns (Thermo Scientific) and lyophilized.

Phosphopeptides were enriched using magnetic $TiO_2$ beads (Thermo Fisher, cat. 88811) according to the manufacturer's protocol. The lyophilized digest was resuspended in 80% acetonitrile/2% formic acid. The solution was incubated with conditioned $TiO_2$ beads for 30 minutes at room temperature. After incubation, phosphopeptide-bound $TiO_2$ beads were collected by magnetic separation. The beads were washed three times with buffers provided in the kit and eluted using elution buffer. The eluate was dried and reconstituted in 0.1% trifluoroacetic acid for mass spectrometry analysis. The flow through, containing all the non-phosphopeptides was also dried and analyzed using mass spectrometry.

The samples were then subjected to nanoflow liquid chromatography (Thermo EASY-nLC 1000, Thermo Scientific) coupled to high-resolution tandem mass spectrometry (Q Exactive HF, Thermo Scientific). Mass spectrometry scans were performed in the Orbitrap analyzer at a resolution of 70,000 with an ion accumulation target set at $3 \times 10^6$ over a mass range of 380–1,580 m/z, followed by tandem mass spectrometry analysis at a resolution of 15,000 with an ion accumulation target set at $2 \times 10^5$. MS2 precursor isolation width was set up at 1.4 m/z, normalized collision energy was 29, and charge state 1 and unassigned charge states were excluded. Acquired tandem mass spectrometry spectra were searched against the human UniProt protein database using a SEQUEST and percolator validator algorithms in the Proteome Discoverer 2.2 software (Thermo Scientific, RRID:SCR_014477). The precursor ion tolerance was set at 10 ppm, and the fragment ion tolerance was set at 0.02 Da, along with methionine oxidation and phosphorylation of serine, threonine, and tyrosine included as dynamic modification. A false discovery of 0.01 and a minimum peptide length of six amino acids were used for peptide identification.

*Mouse experiments*

Female NOD-scid-IL2Rgamma null (NSG) mice used in these experiments were obtained from in-house breeding colonies. All animals were injected at 6–8 weeks of age. For cell-line-derived xenografts, $2 \times 10^6$ cells were injected subcutaneously (s.c.) into the left flank of NSG mice. For generation of LSCC PDX cohorts, tumor fragments were received viably frozen from a protocol approved by the Manchester Cancer Centre Biobank. The original fragments were implanted s.c. at 5 × 5 mm tissue fragments with a drop of Matrigel. When the tumors reached 15 mm in diameter, 2 × 2 mm tissue fragments in a drop of Matrigel were implanted into a new set of animals to produce the cohort used for treatment studies. In all experiments, the tumor dimensions were measured two to three times a week with digital calipers to obtain three diameters of the tumor sphere, from which the tumor volume was determined using the equation length × width × height × pi/6.

When the tumor volumes reached between 95 and 150 $mm^3$, the animals were randomized into treatment groups (n = 8–10 mice/group) based on tumor volume and body weights using the Studylog software (Studylog Systems, RRID:SCR_016682). For NCB-0846 efficacy studies, mice received 100 mg/kg NCB-0846 or vehicle (10% DMSO [Fisher Scientific, cat. BP231-100], 45% polyethylene glycol 400 [Sigma-Aldrich, cat. 1546445], 45% of 30% 2-hydroxypropyl-β-cyclodextrin [Sigma-Aldrich, cat. H107) orally twice a day (AM and PM dose) on a five days on and two days off schedule until the end of the study. All animals in each study were euthanized when the first animal reached tumor endpoint, which was defined as a tumor greater than 2 cm in any direction or a tumor volume of 1,500 $mm^3$.

*Histological analysis*

Tissues were fixed in buffered 10% formalin, embedded in paraffin, sectioned at 5-µm thickness, and stained with hematoxylin and eosin (H&E) for light microscopic evaluation at the Molecular Histopathology Laboratory, Laboratory Animal Sciences Program, Frederick National Laboratory. In addition, 5-µm sections were mounted on charged slides and stained on Leica Biosystems' BondRX auto-stainer with antibody for caspase-3 (Cell Signaling Technology, #9661; 1:100, 60 minutes) to detect apoptotic cell death as well as Ki67 (Cell Signaling Technology, #12202; 1:200, 30 minutes) to measure cell proliferation.

H&E- and immunohistochemistry-stained slides were blinded and scanned using an Aperio AT2 scanner (Leica Biosystems). Image analysis was performed using HALO imaging analysis software (Indica Labs), cytonuclear algorithm module to determine the percentage of nucleated cells that were positive for each marker. Tumor regions were annotated to exclude artifacts and severe necrotic regions.

*Merlin phosphorylation modelling*

Several structures of Merlin are available in the Protein Data Bank, including PDB IDs 4ZRJ (Homo sapiens, 302 residues with quality score 0.873); 6CDS (Homo sapiens, 325 residues with quality score 0.913); 4P7I (Mus musculus, 294 residues with quality score 0.866). However, none of these structures offer a complete model (number of matching residues indicated), which required building the missing or disordered regions by homology modeling. Initial models were obtained using YASARA (http://www.YASARA.org, RRID:SCR_017591), I-TASSER (RRID:SCR_014627) (3), Rosetta (4), Phyre2 (5), and SwissModel (6) to produce three-dimensional scaffolds for the missing regions. Finally, the models obtained from all the modeling engines were combined using YASARA HM_build macro to form a hybrid model with Z-score -0.056. The hybrid model was refined against the templates using Feedback Restrain Molecular Dynamics (7,8). The final model show all the phosphorylated sites considered is accessible to the solvent. DUET (9) was used to assess the impact of single point mutations in the structure stability, replacing the Ser or Thr by Asp to simulate the presence of a strong negative charge, but the reported results were mostly insensitive to the mutation and were discarded.

*Molecular dynamics simulations*

Molecular dynamics simulations were performed to more accurately estimate the impact of phosphorylation in the structure. Molecular dynamics simulations were performed with YASARA using the md_run macro with standard parameters and YASARA's md_analyze protocol to monitor the stabilization of the trajectories and compute RMSD, RMSF, and DCCM differences (i.e., comparing a mutant vs. WT or different substrates bound to the internal binding site, see Supplementary Fig. S5). Trajectories were run for more than 500 ns for each model. Stabilization of all the monitors was obtained after 125 ns for all trajectories, and the most sensitive monitors, computed over the last 100 ns of trajectory, are reported (Fig. S5, Supplementary Table S3).

Molecular dynamics simulations were performed to accurately estimate the impact of phosphorylation in Merlin structure. Merlin structure was very stable in all models irrespective of the phosphorylation site considered, except for the region from E474 to K533, which shows large displacements and deformation during the dynamics and seems to be affected by phosphorylation. This was especially evident for the phosphorylation of S518, which is known to affect stability. The effect appears to propagate from the flexible E474 - K533 region to the helices extending from L290 to M308 and S572 to L582. Of note, the nucleation of a helical motif extending from K515 to E524 benefits from the interaction of the phosphorylated S518 and K515 and is facilitated by the dislodging of the E474 - K533 region. The helix's stabilization seems to interfere with the structure's ability to regain its initial pose during the molecular dynamics simulations. A similar behavior is observed in the phosphorylated T272 model, where a large displacement of the T480 to I495 region was captured during the molecular dynamics trajectory (Supplementary Fig. S5), yet the structure can recover to a geometry similar to the initial pose in less 15 nanoseconds. This can be partially explained by the lack of arrangements similar to the S518 - K515 interaction in the other phosphorylated models considered. On the contrary, some of the models reveal transient interactions by the phosphorylated groups across secondary structure elements, which contributes to stabilizing the local geometry. We quantified the overall behavior of the models using the Root Mean Square Fluctuation (RMSF), the differences in the radius of gyration (RG), and differences in the dynamic cross-correlation matrices (Supplementary Fig. S5 and Supplementary Table S3).

## Supplementary Methods References

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