Oncogenic and drug-sensitive NTRK1 rearrangements in lung cancer

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We identified new gene fusions in patients with lung cancer harboring the kinase domain of the NTRK1 gene that encodes the high-affinity nerve growth factor receptor (TRKA protein). Both the MPRIP-NTRK1 and CD74-NTRK1 fusions lead to constitutive TRKA kinase activity and are oncogenic. Treatment of cells expressing NTRK1 fusions with inhibitors of TRKA kinase activity inhibited autophosphorylation of TRKA and cell growth. Tumor samples from 3 of 91 patients with lung cancer (3.3%) without known oncogenic alterations assayed by next-generation sequencing or fluorescence in situ hybridization demonstrated evidence of NTRK1 gene fusions.

Treatment with orally active kinase inhibitors crizotinib and erlotinib or gefitinib is superior to standard chemotherapy in patients with lung cancers that have ALK fusions or EGFR mutations, respectively1–12. Addition of oncogenes involving fusions of ROS1, RET, FGFR1, FGFR2 and FGFR3 have been identified in lung cancers and demonstrate great potential for therapeutic intervention3–9. These oncogenes also occur in several other common malignancies, expanding the potential relevance of this therapeutic approach9–12.

We performed a targeted next-generation DNA sequencing (NGS) assay on tumor samples from 36 patients with lung adenocarcinoma, involving the kinase domain of the NTRK1 gene, which encodes the TRKA receptor tyrosine kinase but no other potentially oncogenic alterations (Fig. 1, Supplementary Fig. 1 and Supplementary Table 1). We confirmed the exon junctions and mRNA expression by RT-PCR and cloning of the entire cDNAs (Supplementary Figs. 2–4). In the first case, the 5′ end of the myosin phosphatase–Rho-interacting protein gene (MPRIP) was joined with the 3′ end of NTRK1. MPRIP is involved in actin cytoskeleton regulation and has been implicated in a gene fusion in small-cell lung cancer, putatively causing early termination of TP53 (ref. 13). We detected expression of the fusion protein, RIP-TRKA (encoded by MPRIP-NTRK1), in 293T cells with exogenous expression of the MPRIP-NTRK1 cDNA, a malignant pleural effusion sample and early-passage lung cancer–derived cells (CUTO-3) derived from the same patient growing in culture (Supplementary Fig. 4). CUTO-3 cells demonstrated autophosphorylation of this previously uncharacterized protein at critical TRKA tyrosine residues14. MPRIP harbors three coiled-coil domains (CCDs), one of which mediates interaction with myosin phosphatase15. Gene partners of ALK, ROS1, and RET fusions often contain CCDs, which are known to mediate dimerization and consequent activation of the kinase domain; thus, it is likely that the CCDs contained within MPRIP-NTRK1 perform a similar function14,16 (Supplementary Fig. 5). The second case harbored a CD74-NTRK1 gene fusion. CD74, which encodes the major histocompatibility complex (MHC) class II invariant chain, is a known activating fusion partner of ROS1, and the CD74-TRKA protein is predicted to be localized in the plasma membrane17–19 (Supplementary Fig. 5).

We developed a fluorescence in situ hybridization (FISH) assay to detect chromosomal rearrangements within the NTRK1 gene (Supplementary Fig. 6a). Hybridization of these probes showed clear separation of the 5′ and 3′ probes in the tumor cells in the patient samples containing the MPRIP-NTRK1 or CD74-NTRK1 gene fusions, but not in the nontumor cells from those samples or a control sample (Fig. 1b and Supplementary Fig. 6b). Fusions between NTRK1 and TPM3, TFG or TPR have previously been identified in colorectal and thyroid cancers11,20. Although TPM3 (1q22-23) lies in close proximity to NTRK1 (1q21-22), FISH detected a separation in signals in the KM12 colorectal cancer cell line that harbors a TPM3-NTRK1 fusion (Supplementary Figs. 6c and 7). Using this FISH assay, 56 additional lung adenocarcinoma samples without detectable oncogenic alterations were screened for NTRK1 rearrangements, and one additional positive case was identified (Supplementary Table 2 and Supplementary Fig. 6d). Quantitative

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PCR demonstrated high NTRK1 kinase domain expression only in the tumors with the known NTRK1 rearrangements or in the KM12 cell line (Supplementary Fig. 8). Analysis of transcriptome data from The Cancer Genome Atlas of 230 lung adenocarcinomas failed to detect evidence of NTRK1 fusions (data not shown). A recent transcriptome study of 87 lung adenocarcinoma tumor samples, which

**Figure 1** Discovery and validation of oncogenic NTRK1 gene fusions in lung cancer samples. (a) Schematic of genomic rearrangement from tumor samples harboring MPRIP-NTRK1 and CD74-NTRK1 using the FoundationOne next-generation sequencing assay, including chromosomal breakpoints for each gene rearrangement. (b) Break-apart FISH analysis of MPRIP-NTRK1 and CD74-NTRK1 tumor samples showing clear separation of green (5′) and red (3′) signals corresponding to the NTRK1 gene. (c) TRKA (NTRK1) fusions are autophosphorylated and activate key downstream signaling pathways. Representative immunoblot analyses (n = 3) of cell lysates from 293T cells expressing RIP-TRKA and CD74-TRKA, but not their kinase-dead (KD) variants, display phosphorylation of critical tyrosine residues (Y496, Y680 and Y681) in TRKA (pTRKA) and activation of ERK (phosphorylation of T202 and Y204, pERK). TPM3-TRKA fusions support cellular proliferation. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay of Ba/F3 demonstrated that cells expressing empty vector (EV) or the kinase-dead variant of RIP-TRKA did not proliferate (n = 3). Data are expressed as mean ± s.e.m. (e) MPRIP-NTRK1 or CD74-NTRK1 gene fusions induce tumor growth. NIH3T3 cells expressing RIP-TRKA, RIP-TRKA-KD, CD74-TRKA, EML4-ALK or empty vector were injected into the flanks of nude mice and observed for tumor growth. Representative pictures taken at day 12 following injection are shown. The number of tumors induced in the injected animals is shown in parentheses (five mice injected per cell line, n = 1).

**Figure 2** Drug treatment inhibits activation of TRKA, downstream signaling and proliferation in cells expressing NTRK1 fusion. (a) RNAi knockdown of NTRK1 inhibits cell proliferation in a cell line harboring TPM3-NTRK1. KM12 cells were analyzed by MTS proliferation assay 1–5 d after transfection with two different NTRK1-specific siRNAs (siRNA 1 and siRNA 2) or scrambled siRNA (control) (n = 3). Data are expressed as mean ± s.e.m. (b) Ba/F3 cells expressing MPRIP-NTRK1 (RIP-TRKA) or EV were lysed after 5 h of treatment with the indicated doses of drugs (G, gefitinib 1,000 nM) or DMSO control (C). Phosphorylation of TRKA (Y496, Y680 and Y681) or ERK (T202 and Y204) was assessed by antibodies specific to the indicated tyrosine or threonine residues. (c) CUTO-3 lung cancer cells harboring the MPRIP-NTRK1 gene fusion were treated with the indicated doses and drugs and subjected to immunoblot analysis. (d) Ba/F3 cells expressing the MPRIP-NTRK1 fusion demonstrate inhibition of proliferation as measured by MTS assay using the pan-TRK inhibitor, ARRY-470, and the multikinase inhibitor, CEP-701, but not the EGFR inhibitor, gefitinib (n = 5). Data are expressed as mean ± s.e.m.
identified a set of previously uncharacterized fusions, did not include oncogenic fusions involving NTRK1 (J.-S. Seo, Seoul National University College of Medicine, personal communication)\(^1\).

To formally prove that these fusion proteins are oncogenic, we expressed *MPRIP-NTRK1* and *CD74-NTRK1* cDNA constructs in three other noncancer cell lines commonly used to assess oncogenicity—293T cells, NIH3T3 fibroblasts and Ba/F3 cells—and assessed them for oncogenic traits expected in these cells. We observed expression of the appropriate-sized chimeric proteins and TRK autophosphorylation, as seen in the CUTO-3 cells\(^1\) (Figs. 1c and 2b and Supplementary Figs. 4, 9 and 14). Introduction of a *NTRK1* kinase-dead mutation did not result in TRKA autophosphorylation or in increased phosphorylation of extracellular signal–related kinases 1 and 2 (ERK1/2) (Figs. 1c and Supplementary Fig. 9). *MPRIP-NTRK1* and *CD74-NTRK1*, but not their kinase-dead counterparts, induced interleukin-3 (IL-3)-independent proliferation of Ba/F3 cells (Fig. 1d). Similarly, *MPRIP-NTRK1* and *CD74-NTRK1* supported anchorage-independent growth of NIH3T3 cells and formed tumors in nude mice, and *CD74-NTRK1* induced a light-refractory appearance of NIH3T3 cells (Fig. 1e and Supplementary Figs. 10 and 11). Knockdown of *TPM3-NTRK1* in KM12 cells reduced proliferation, further supporting the role of *NTRK1* fusions as oncogenes (Fig. 2a and Supplementary Fig. 12).

Given the prior success of treatment with kinase inhibitors in patients with cancers harboring *ALK* and *ROS1* fusions, we asked whether *NTRK1* fusions might provide a similar target in patients with lung cancer or other malignancies. ARRY-470 is a selective kinase inhibitor with nanomolar activity against TRKA, TRKB and TRKC but no other notable kinase inhibition below 1,000 nM (Supplementary Fig. 13 and Supplementary Table 3). Lestarutinib (CEP-701) and crizotinib also have activity against TRKA in addition to other kinases\(^2,3\). Treatment of cells expressing *MPRIP-NTRK1* or *CD74-NTRK1* with ARRY-470, CEP-701 and, to a lesser extent, crizotinib inhibited autophosphorylation of RIP-TRKA and CD74-TRKA (Fig. 2b and Supplementary Figs. 9 and 14a). Activation of the mitogen-activated protein kinase (MAPK) and AKT kinase pathways was also inhibited in Ba/F3 cells expressing exogenous *MPRIP-NTRK1* or *CD74-NTRK1* (Fig. 2b and Supplementary Fig. 14a). Phosphorylation of endogenously expressed RIP-TRKA in CUTO-3 and tropomyosin 3 (TPM3)-TRKA in KM12 cells was similarly inhibited by all three drugs (Fig. 2c and Supplementary Fig. 15a).

Inhibition of proliferation of Ba/F3 cells expressing *NTRK1* gene fusions was greatest with CEP-701 and ARRY-470 (Fig. 2d and Supplementary Fig. 14b). Crizotinib was a less potent inhibitor, although in a range similar to that seen for inhibition of ephrin-derm microtubule-associated protein-like 4–anaplastic lymphoma kinase (EML4-ALK) or syndecan-4–c-ros oncogene 1, receptor tyrosine kinase (SDC4-ROS1) (ref. 3) (Supplementary Fig. 16). The less potent effects of crizotinib on cell proliferation are consistent with decreased inhibition of phosphorylated TRKAs (pTRKA) and downstream phosphorylated ERK1/2 (pERK1/2) (Fig. 2b and Supplementary Fig. 14a). All three drugs also inhibited colony formation of NIH3T3 cells expressing *NTRK1* fusions in soft agar (Supplementary Fig. 10). KM12 cells were similarly sensitive to ARRY-470 and CEP-701, but less so to crizotinib (Supplementary Fig. 15b). ARRY-470 did not inhibit proliferation of Ba/F3 cells expressing other oncogene targets (epidermal growth factor receptor (EGFR), *ALK* or ROS1) or of lung and colorectal cell lines that do not harbor an *NTRK1* fusion (Supplementary Fig. 17). All three drugs induced cell-cycle arrest in G1 and apoptosis of KM12 cells (Supplementary Fig. 18).

The index patient (*MPRIP-NTRK1*) had no standard therapies and no clinical trials of potentially effective TRKA inhibitors available; therefore, the patient consented to treatment with crizotinib (250 mg twice daily) outside of a clinical trial. The patient showed a minor radiographic response with a decrease in serum levels of tumor marker CA125 but experienced disease progression after ~3 months (Supplementary Fig. 19). This modest clinical activity of crizotinib is consistent with the *in vitro* activity that we observed and could be caused by non–TRKA kinase effects.

We have identified new recurrent oncogenic *NTRK1* fusions in a subset of patients (3 out of 91, 3.3%) with lung adenocarcinoma that did not contain other common oncogenic alterations. Our study further highlights the utility of targeted NGS to discover drug-sensitive genetic alterations in patients with lung cancer. Based on our data, clinical studies of selective TRKA inhibitors in *NTRK1*–rearranged non–small-cell lung cancer are warranted.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** cDNA sequences are available in GenBank under accession codes KF724384 and KF724385.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

R.C.D., P.A.J. and V.A.M. conceived of the research idea. R.C.D. and P.A.J. designed experiments, took responsibility for the oversight of the project and wrote the manuscript. A.V. and D.E. designed and performed immunoblotting, generated Ba/F3 and NIH3T3 cells expressing exogenous *NTRK1* fusion constructs, performed flow cytometry and MTS assays and contributed to writing of the manuscript. D.L. and P.J.S. designed and performed NGS assays. M.V.-G. designed FISH probes and interpreted FISH experiments. S.K. and S.M. performed and analyzed FISH experiments. A.T.L. and M.C. performed cloning of RT-PCR fusion experiments and contributed to interpretation of the data. K.D.D. performed *in vitro* analyses and contributed to interpretation of the data. A.B.P. performed *in vivo* experiments and contributed to interpretation of data. P.S.H., L.A.G. and G.K. performed bioinformatics analyses. J.H. and S.W.A. designed and profiled ARRY-470. E.M.B. and M.B. collected clinical data. J.K., H.S. and G.K. performed bioinformatics analyses and contributed to interpretation of the data. A.B.P. performed *in vivo* experiments and contributed to interpretation of data. K.D.D. performed *in vitro* analyses and contributed to interpretation of the data. A.B.P. performed *in vivo* experiments and contributed to interpretation of data.

**COMPETING FINANCIAL INTERESTS**

The authors declare competing financial interests: details are available in the online version of the paper.
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ONLINE METHODS

Patient tumor samples. Colorado Multiple Institutional Board (IRB) or Dana-Farber Cancer Institute IRB approval was obtained for all patients in this study. All patients provided written informed consent. FoundationOne testing and FISH analyses were performed in CLIA-certified laboratories. The index patient who underwent treatment with crizotinib consented to treatment outside of a clinical trial.

Next-generation DNA sequencing. DNA was extracted from 40 µm of formalin-fixed paraffin-embedded (FFPE) or frozen tissue using the Maxwell 16 FFPE Plus LEV DNA Purification kit (Promega) and quantified using the PicoGreen fluorescence assay (Invitrogen). Library construction was performed as previously described using 50–200 ng of DNA sheared by sonication to ~100–400 base pairs before end repair, dA addition and ligation of indexed Illumina sequencing adaptors24. Enrichment of target sequences (3,320 exons of 182 cancer-related genes and 37 introns from 14 genes recurrently rearranged in cancer) were accomplished using TruSeq Nano Paired-End Sample Preparation Kit (Illumina) and the Nextera XT (Illumina) dual-indexed PCR master mix. Paired-end sequencing of samples was performed on an Illumina HiSeq 2500 platform to an average of 200 million reads per sample. Reads were mapped to the human genome (hg19) using BWA-MEM (version 0.7.5) and re-mapped using the publicly available SAMtools, Picard and Genome Analysis Toolkit25,26. Genomic rearrangements were detected by clustering chimeric reads mapped to targeted introns.

RNA extraction from formalin-fixed paraffin-embedded and frozen tissues. RNA was isolated from FFPE or frozen tumor samples as described previously27. Briefly, FFPE samples were processed using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion) following deparaffinization in xylene and washed with 100% ethanol before Protease K digestion. Extraction of RNA from frozen tissue was accomplished using TRIReagent (Ambion). Alternatively, tumors from patients with non–small cell lung cancer obtained at surgery were snap-frozen in liquid nitrogen, embedded in optimal cutting temperature (OCT) medium and sectioned. RNA was prepared using Trizol (Invitrogen) followed by RNeasy MinElute cleanup kit (Qiagen).

RT–PCR and sequencing of MPRIP-NTRK1 and CD74-NTRK1. RT–PCR of MPRIP-NTRK1 was carried out using the SuperScript III First-Strand Synthesis System (SSIII RT) from Invitrogen with a NTRK1 primer located in exon 15 (‘NTRK1 Y490R1’) followed by PCR using the same reverse primer, NTRK1 Y490R1, and a primer to MPRIP located in its third coil-coiled domain (‘MPRIP CC3F1’). PCR products were resolved on an agarose gel, and the fragments were excised and treated with ExoSapIT (Affymetrix) before being sequenced by the University of Colorado Cancer Center DNA Sequencing and Analysis Core using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 1.1 (Applied Biosystems) using the same forward and reverse primer as in the RT–PCR reaction. For CD74-NTRK1, reverse transcription was carried out using the QuantTect Reverse Transcription Kit (Qiagen). PCR of the resulting cDNA was performed using the primers ‘CD74 exon 3 FOR’ and ‘NTRK1 exon 15 REV’. Primers used for RT–PCR and sequencing are available in Supplementary Table 4. The reference sequences used for exon alignment are National Center for Biotechnology Information (NCBI) reference sequences NM_002529.3 (NTRK1), NM_015134.3 (MPRIP) and NM_001025159.2 (CD74).

Cloning full-length MPRIP-NTRK1, CD74-NTRK1 and TPM3-NTRK1. cDNA was generated from each patient tumor sample using the following procedures. For the MPRIP-NTRK1 construct, cDNA was transcribed using the SSIII RT kit from Invitrogen along with a primer located at the end of NTRK1 (NTRK1stopR2). This cDNA was used to amplify two separate overlapping fragments that were used to generate full-length MPRIP-NTRK1 by overlap-extension PCR using the two fragments alone for ten cycles and then adding the MPRIPstart and NTRK1stopR1 primers for an additional 30 cycles of PCR amplification. The resulting 4-kb PCR product was gel-isolated and confirmed by Sanger sequencing. A 3’ hemagglutinin (HA) tag was added to MPRIP-NTRK1 using PCR amplification with primers harboring the HA-encoding sequence. The amplified product was subsequently cloned into the pCDH-CMV-MSC1-EF1-Puro lentiviral plasmid (System Biosciences). Full-length TPM3-NTRK1 was amplified from KM12 CDNA using TPM3start RI and NTRKStopNotI primers and cloned into the lentiviral plasmid as described above. The National Center for Biotechnology Information (NCBI) reference sequence used for TPM3 is NM_153649.3. For the CD74-NTRK1 construct, cDNA was transcribed with Quantscript Reverse Transcriptase (Qiagen). Full-length CD74-NTRK1 was amplified using the primers ‘CD74 FOR’ and ‘NTRK1 REV’ using AccuPrime Taq DNA Polymerase (Invitrogen) and cloned into the pDNA-Dual vector (BD Biosciences) and recombined into IPI1520 retroviral vector as previously described27. The full-length cDNA of each gene was confirmed by sequencing. Primers used for cloning are available in Supplementary Table 4.

Quantitative PCR of NTRK1. Relative-quantification PCR (RQ-PCR) assay of the NTRK1 tyrosine kinase domain (Hs01021011_m1; Applied Biosystems) was used to evaluate its level of mRNA expression. The relative quantification method (Δ∆CT) in the StepOnePlus Real-Time PCR system (Applied Biosystems) was used with β-glucuronidase (GUSB) (Applied Biosystems) as an endogenous control. All samples were evaluated in triplicate.

RNA sequencing. Paired-end RNA sequencing was performed as previously described28. RNA FASTQ files were aligned and splice junctions mapped using TopHat28 and analyzed for fusion reads using the Broad Institute Cancer Genome Analysis Tools Suite (http://www.broadinstitute.org/cancer/cga/) and http://www.broadinstitute.org/cancer/software/genepattern/modules/RNA-seq/).

Cell lines and reagents. NIH3T3, HEK-293T and A549 cells were purchased from ATCC. NIH3T3, HEK-293T and Ba/F3 were previously described30. The lung cancer cell lines A549, H3122, H1650, H1299 and HCC78 were previously described30–33. The colorectal cancer cell lines KM12, HCT116, HCT15, HT29 and SW837 were previously described34. Ba/F3 cells expressing the mutant E88ER, allele, E746_A750del, were previously described27. The lymphoblastoid cell line GM09948 (Coriell Cell Repository) was used for genomic mapping in FISH studies. All cancer cell lines were maintained in RPMI medium with 10% FBS. NIH3T3 and Ba/F3 cells transduced with full-length NTRK1 were supplemented with 100 ng/ml and 200 ng/ml β nerve growth factor (β-NGF) (R&D Systems), respectively. Crizotinib and gefitinib were purchased from Selleck Chemicals, CEP-701 from Sigma Aldrich or Santa Cruz Biotechnology and K252a from Tocris, and ARRY-470 was supplied by Array BioPharma. Antibodies to total AKT (clone 40D4, 1:2,000), AKT pSer473 (clone D3A7, 1:2,000), AKT pThr202/Tyr204 (clone D13.14.4E, 1:5,000), total STAT3 (clone 1B4H6, 1:5,000), STAT3 pY705 (clone D3A7, 1:2,000), PARP (clone 46D11, 1:5,000) and AKT pSer473 (clone D9E, 1:5,000) were purchased from Santa Cruz Biotechnology.

All cancer cell lines were maintained in RPMI medium with 10% FBS. NIH3T3 and Ba/F3 cells transduced with full-length NTRK1 were supplemented with 100 ng/ml and 200 ng/ml β nerve growth factor (β-NGF) (R&D Systems), respectively. Crizotinib and gefitinib were purchased from Selleck Chemicals, CEP-701 from Sigma Aldrich or Santa Cruz Biotechnology and K252a from Tocris, and ARRY-470 was supplied by Array BioPharma. Antibodies to total AKT (clone 40D4, 1:2,000), AKT pSer473 (clone D3A7, 1:2,000), AKT pThr202/Tyr204 (clone D13.14.4E, 1:5,000), total STAT3 (clone 1B4H6, 1:5,000), STAT3 pY705 (clone D3A7, 1:2,000), PARP (clone 46D11, 1:5,000) and AKT pSer473 (clone D9E, 1:5,000) were purchased from Cell Signaling Technology. Total TRKA (clone C-14, 1:1,000), γ-glutamylate 3-phosphate dehydrogenase (clone MAB374, 1:5,000) and α-tubulin (clone TU-02, 1:5,000) were purchased from Santa Cruz Biotechnology.

Derivation and propagation of CUTO-3 cells. The patient gave written informed consent for the derivation of a cancer cell line. Pleural fluid from the index patient harboring the MPRIP-NTRK1 gene was collected and mononuclear cells were isolated by centrifugation through a ficoll gradient (Thermo Scientific). Cells were seeded onto a 25-cm flask and cultured in serum-free ACL4 medium to inhibit outgrowth of normal stromal cells35. Once the tumor cell became the predominant cell type in the culture flask, the culture was subjected to differential trypsinization in order to dislodge the remaining minor population of stromal cells. After this enrichment process, tumors cells were cultured in ACL4 medium supplemented with 5% heat-inactivated FBS and routinely passaged using this medium. The CUTO-3 cells were later adapted to grow in RPMI 1640 with 10% FBS for ease of culturing and experimentation.

Lentivirus or retrovirus production and cell transduction. MPRIP-NTRK1 or the kinase-dead variant was introduced into cells by lentivirus as previously described36. NIH3T3 cells transduced with lentivirus were cultured in DMEM medium with 5% FCS and 0.75 µg/ml puromycin. Ba/F3 cells transduced with lentivirus were cultured as above with 2 µg/ml puromycin and with or without
1 ng/ml IL-3 (R&D Systems). Alternatively, CD74-NTRK1 was introduced into cells using retrovirus as previously described\textsuperscript{27}. Polyclonal cell lines were established by puromycin selection. Cell proliferation and growth were performed as previously described\textsuperscript{27,36}.

**Mouse xenograft studies.** NIH3T3 cells (1 × 10\textsuperscript{6}) harboring the indicated expression vectors were resuspended in Matrigel (BD Biosciences) and injected subcutaneously into athymic nude mice (gift from J. DeGregori). Mice were monitored three times weekly for tumor formation and sacrificed when tumors reached approximately 2 cm × 2 cm. Approval for the use of animals in this study was granted by the University of Colorado Institutional Animal Care and Use Committee.

**Immunoblotting.** Immunoblotting was performed as previously described\textsuperscript{30}. Briefly, cells were lysed in RIPA buffer with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific) and diluted in loading buffer (LI-COR Biosciences). Membranes were scanned and analyzed using the Odyssey Imaging System and software (LI-COR). Alternatively, immunoblotting was performed according to the antibody manufacturer’s recommendations using chemiluminescent detection (Perkin Elmer). All western blot images are representative of at least three independent experiments.

**Proliferation assays.** All assays were performed as previously described by seeding 1,000 cells per well, drug treatments were performed 24 h after seeding and Cell Titer 96 MTS (Promega) was added 72 h later or as described previously\textsuperscript{27,30,36}. IL-3 was removed from Ba/F3 cells 48 h before seeding.

**Soft agar assays.** Anchorage-independent growth was measured by seeding 100,000 cells per well of soft agar in six-well plates as previously described\textsuperscript{30}. Medium was changed every 4 d for 2 weeks. Quantification was performed with MetaMorph Offline Version 7.5.0.0 (Molecular Devices).

**Fluorescence in situ hybridization.** FFPE tissue sections were submitted to a dual-color FISH assay using the laboratory developed NTRK1 break-apart probe (3’ NTRK1 (SpectrumRed) and 5’ NTRK1 (SpectrumGreen)). The prehybridization treatment was performed using the reagents from the Vysis Paraffin Kit IV (Abbott Molecular). Hybridization and analysis was performed as previously described\textsuperscript{30}. Samples were deemed positive for NTRK1 rearrangement if ≥15% of tumor cells demonstrated an isolated 3’ signal or a separation of 5’ and 3’ signals that was greater than one signal diameter.

**siRNA transfection.** KM12 cells were transfected with 30 nM NTRK1 Silencer Select siRNAs (Life Technologies) using siPORT NeoFX transfection reagent (Life Technologies) at 4 µL/mL.

**Flow cytometry.** Cell-cycle analysis was performed as previously described\textsuperscript{3}. Apoptosis was measured using the Vybrant apoptosis YO-PRO/PI kit (Invitrogen). Briefly, KM12 cells were seeded 24 h before treatment at 500,000 cells per well before trypsinization and staining.

**Immunohistochemistry.** Immunohistochemical studies for thyroid transcription factor-1 (TTF-1) and thyroglobulin were performed using standard procedures to exclude the possibility of a thyroid carcinoma, which can also express TTF-1 (Supplementary Fig. 20). Antibody against TTF-1 (Cell Marque, Cat. #CMC-573) was applied at 1:100 dilution and thyroglobulin (Signet, Cat. #228-13) was applied at 1:25 dilution and incubated at 37 °C for 32 min. Detection for TTF-1 was performed using Ventana multiview (UltraView) and detection for thyroglobulin using Ventana Avidin-Biotin (iView).

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