Acquired Resistance to the TRK Inhibitor Entrectinib in Colorectal Cancer

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ABSTRACT

Entrectinib is a first-in-class pan-TRK kinase inhibitor currently undergoing clinical testing in colorectal cancer and other tumor types. A patient with metastatic colorectal cancer harboring an LMNA–NTRK1 rearrangement displayed a remarkable response to treatment with entrectinib, which was followed by the emergence of resistance. To characterize the molecular bases of the patient’s relapse, circulating tumor DNA (ctDNA) was collected longitudinally during treatment, and a tissue biopsy, obtained before entrectinib treatment, was transplanted in mice (xenopatient), which then received the same entrectinib regimen until resistance developed. Genetic profiling of ctDNA and xenopatient samples showed acquisition of two point mutations in the catalytic domain of NTRK1, p.G595R and p.G667C. Biochemical and pharmacologic analysis in multiple preclinical models confirmed that either mutation renders the TRKA kinase insensitive to entrectinib. These findings can be immediately exploited to design next-generation TRKA inhibitors.

SIGNIFICANCE: We provide proof of principle that analyses of xenopatients (avatar) and liquid biopsies allow the identification of drug resistance mechanisms in parallel with clinical treatment of an individual patient. We describe for the first time that p.G595R and p.G667C TRKA mutations drive acquired resistance to entrectinib in colorectal cancers carrying NTRK1 rearrangements. Cancer Discov; 6(1); 1–9. © 2015 AACR.

See related commentary by Okimoto and Bivona, p. xxx.

INTRODUCTION

TRK receptors are a family of tyrosine kinases that comprises three members: TRKA, TRKB, and TRKC, encoded by the neurotrophic tyrosine kinase receptor, type 1 (NTRK1), NTRK2, and NTRK3 genes, respectively. Genomic rearrangements is the most common mechanism of oncogenic activation for this family of receptors, resulting in sustained cancer cell proliferation through activation of MAPK and AKT downstream pathways (1). Rearrangements of the NTRK1, NTRK2, and NTRK3 genes occur across different tumors, including colorectal cancers (2).
Entrectinib (RXDX-101, previously known as NMS-E628) is a potent pan-TRK, ALK, and ROS1 inhibitor, currently undergoing phase I clinical trials (3). During treatment with entrectinib, a patient with metastatic colorectal cancer harboring an LMNA–NTRK1 rearrangement showed a remarkable response. We reasoned that, as it has been shown for most targeted agents, response to entrectinib might be limited in time due to the emergence of acquired resistance. Nothing is presently known about the mechanisms of resistance to entrectinib and consequently further lines of treatment are not available. We postulated that it might be possible to identify the resistance mechanism(s) while the patient was being treated by analyzing circulating tumor DNA (ctDNA) and developing a xenopatient (avatar).

RESULTS

Acquired Resistance to TRKA Inhibition in a Patient with Colorectal Cancer

A molecular screen identified a genetic rearrangement involving exon 10 of NTRK1 and exon 11 of the LMNA genes (4) in a patient with metastatic colorectal cancer whose disease was intrinsically resistant to first-line FOLFOX, second-line FOLFIRI/cetuximab, and third-line irinotecan. We and others have previously reported that colorectal cancer cell models harboring NTRK1 translocations are sensitive to NTRK1 silencing and to TRKA (protein encoded by the NTRK1 gene) kinase inhibition (5–7). Based on this, the patient was enrolled in the phase I ALKA clinical trial (EudraCT Number 2012-000148-88) of the pan-TRK kinase inhibitor entrectinib, a first-in-class drug currently undergoing clinical testing (3). The patient received entrectinib on an intermittent dosing schedule of 4 days on/3 days off for 3 weeks followed by a week break in every 28-day cycle (4). Treatment was remarkably effective and well tolerated, leading to a partial response (PR) with 30% tumor shrinkage of multiple liver metastases that was demonstrated by an early CT scan assessment performed after 30 days of treatment. The clinical response lasted 4 months, followed by the emergence of drug resistance as evaluated by Response Evaluation Criteria in Solid Tumor (RECIST) progression (Fig. 1, top).

Emergence of NTRK1 Mutations in ctDNA during Entrectinib Treatment

To unveil the molecular basis of acquired resistance to TRKA inhibition, we analyzed ctDNA, a form of liquid biopsy (8) we previously optimized to detect and monitor drug resistance in patients treated with targeted agents (9, 10). ctDNA extracted from plasma samples collected before treatment initiation and at clinical relapse was subjected to molecular profiling using the IRCC-TARGET panel, a next-generation sequencing (NGS) platform based on 226 cancer-related genes which we optimized to detect with high sensitivity mutations in ctDNA (10). Profiling of ctDNA at entrectinib resistance revealed two novel NTRK1 genetic alterations in the kinase domain of the protein, p.G595R and p.G667C, which were not detected in ctDNA obtained before initiation of therapy (Supplementary Tables S1 and S2). To monitor the NTRK1-mutated alleles in the patient’s plasma collected throughout the treatment, droplet digital PCR (ddPCR; refs. 11, 12) assays were designed for both mutations. As a means of tracking the overall disease, a ddPCR assay was also optimized to detect the LMNA–NTRK1 rearrangement in ctDNA.
Longitudinal analysis of plasma revealed that the p.G595R- and p.G667C-mutated alleles were initially absent in ctDNA but emerged in the circulation as early as 4 weeks upon initiation of treatment with entrectinib (Fig. 1). NTRK1 mutation frequencies continued to increase in ctDNA and peaked when clinical progression was radiologically confirmed (16 weeks after initiation of treatment). The profile of the LMNA–NTRK1 rearrangement in ctDNA paralleled tumor response and resistance to entrectinib (Fig. 1 and Supplementary Table S3).

**Secondary Resistance to Entrectinib in a Colorectal Cancer Xenopatient**

To functionally evaluate the mechanistic basis of resistance to entrectinib, a biopsy specimen gathered before initiation of treatment was transplanted subcutaneously in an immunocompromised mouse (xenopatient; see Supplementary Methods). Upon successful engraftment, the tumor was expanded in multiple mice, which were treated with dosage levels and schedules that matched clinically relevant exposure achievable in patients. Entrectinib induced remarkable tumor shrinkage in the xenopatient, whereas vehicle-treated tumors grew exponentially (Fig. 2A). After 3 weeks of drug dosing, one of the tumors treated with entrectinib rapidly developed resistance to TRKA inhibition (Fig. 2A). NGS-based molecular profiling of this resistant sample using the IRCC-TARGET panel unveiled the LMNA–NTRK1 rearrangement peculiar to the patient and the NTRK1 p.G595R mutation, which could not be detected in the untreated tumor.

**Figure 2.** Resistance to entrectinib in xenopatient and colorectal cancer cell models carrying NTRK1 translocations. **A,** biopsy specimen obtained from a thin needle biopsy of a patient with metastatic colorectal cancer harboring an LMNA–NTRK1 rearrangement was first implanted subcutaneously in an immunocompromised mouse and then expanded in multiple mice upon successful engraftment. Mice were treated with dosage levels and schedules (60 mg/kg, 4 days/week) that yielded clinically relevant exposure achievable in patients. After 3 weeks of treatment, a mouse (*#4*) in the treated arm relapsed. Blue and red lines, vehicle- and entrectinib-treated mice, respectively. **B,** proliferation assay of KM12 (carrying a TPM3–NTRK1 rearrangement) R1 cells made resistant to a low dose of entrectinib (300 nmol/L). Cell viability was assessed by measuring ATP content after 5 days of treatment. Sanger sequencing electropherogram of KM12 R1 shows NTRK1 p.G667C mutation. **C,** proliferation assay of KM12 (carrying an TPM3–NTRK1 rearrangement) R2 cells made resistant to a high dose of entrectinib (2 μmol/L). Cell viability was assessed by measuring ATP content after 5 days of treatment. Sanger sequencing electropherogram of KM12 R2 shows an NTRK1 p.G595R mutation.
Secondary Resistance to Entrectinib in Cells Carrying NTRK1 Rearrangements

To assess whether the mechanism of resistance was patient specific or contingent on the peculiar NTRK1 rearrangements, independent models of acquired resistance to entrectinib were established. The KM12 colorectal cancer cell line harbors a distinct genetic rearrangement involving exon 10 of the NTRK1 and exon 7 of TPM3 genes (5, 7) and is also highly sensitive to entrectinib (Fig. 2B and C). Independent batches of parental (sensitive) KM12 cells were exposed to either acute constant dose (R2) or escalating doses (R1) of entrectinib until resistant derivatives emerged (Fig. 2B and C; see Supplementary Methods). Molecular profiling of the cells that became resistant to lower concentrations of entrectinib until resistant derivatives emerged (Fig. 2B and C; see Supplementary Methods). Molecular profiling of the cells that became resistant to lower concentrations of entrectinib until resistant derivatives emerged (Fig. 2B and C).

To further evaluate the mechanisms of entrectinib resistance, we engineered Ba/F3 cells to express ETV6–TRKA. In this model system, the ETV6 domain mimics the dimerization effect of TRK fusion partners that occur in human tumors. Ba/F3 cells engineered to express ETV6–TRKA became exquisitely sensitive to entrectinib (Supplementary Fig. S2). ETV6–TRKA Ba/F3 cells were then exposed to entrectinib treatment until resistant derivatives emerged and were analyzed as described above. Remarkably, upon development of resistance, Ba/F3 also acquired the p.G595R mutation in the kinase domain of TRKA when a high dose of entrectinib was applied, whereas the p.G667C allele emerged in the presence of a lower dose of the drug (Supplementary Fig. S2). Analogous to what we observed in KM12, the two mutations were found in independent pools of Ba/F3 cells, indicating they do not co-occur in the same cells.

NTRK1 p.G595R and p.G667C Mutations Drive Resistance to TRK Inhibitors

We then examined the impact of the p.G595R and p.G667C variants on the three-dimensional (3-D) structure of the TRKA catalytic domain (see Supplementary Methods). The binding model of entrectinib with wild-type (WT) TRKA highlighted that entrectinib makes extensive hydrogen bonding as well as hydrophobic interactions with the protein in the ATP pocket where p.G595S and p.G667C residues are located (Fig. 3A). The p.G595R and p.G667C mutations create steric hindrance that either abrogates binding (p.G595R) or reduces the binding affinity (p.G667C) of entrectinib to the TRKA catalytic pocket (Fig. 3B and C, respectively).

We next assessed whether and to what extent mutations in the kinase domain of NTRK1 drive resistance to TRKA inhibition. We engineered Ba/F3 cells expressing wild-type, p.G595R, or p.G667C TPM3–TRKA fusion proteins. We then measured the sensitivity of NTRK1-mutated cells to TRK inhibitors currently in clinical development. LOXO-101 is a TRK inhibitor in a phase I trial for patients with advanced solid tumors with NTRK alterations (NCT02122913); TSR-011 is presently undergoing a phase I trial for patients with advanced solid tumors or lymphomas with NTRK alterations (NCT02048488).

As shown in Supplementary Fig. S3, Ba/F3 cells harboring the NTRK1 translocation become highly sensitive to TRK inhibitors (Supplementary Fig. S3A and S3B; Supplementary Table S4). On the contrary, NTRK1 p.G595R or p.G667C mutations are resistant to entrectinib, LOXO-101, and TSR-011 (Supplementary Fig. S3C and S3D, respectively). Of potential clinical relevance, and in line with previous results, NTRK1 p.G595R appears to be more potent in conferring resistance than p.G667C.

These results are indeed consistent with the observation that entrectinib and LOXO-101 retain a partial effect on p.G667C (IC_{50} = 61 and 524 nmol/L, respectively) but are totally ineffective on p.G595R (IC_{50} > 1000 nmol/L) in Ba/F3–engineered cells (Supplementary Table S4).

Alignment of the TRKA kinase domain with clinically targeted tyrosine kinases, such as ALK, ROS, EGFR, MET, and KIT, showed that the glycine residues at positions 595 and 667 lie in a conserved region (Supplementary Fig. S4A and S4B, respectively), and are analogous to residues previously found to be associated with secondary resistance to other kinase inhibitors, such as erlotinib, crizotinib, and imatinib (Fig. 3D and E, respectively).


To mechanistically study the impact of NTRK1 resistant alleles, we established two cell lines, one from the xenopatient treated with vehicle, and the other from the xenopatient who became resistant to entrectinib (Fig. 4A). Both cell lines displayed the LMNA–NTRK1 translocation found in the patient tumor (Fig. 4B), but only cells derived from the xenotumor that had become resistant to entrectinib carried the p.G595R allele (Fig. 4C). Both cell lines displayed a pharmacologic response to entrectinib analogous to that observed in the corresponding xenopatients (Fig. 4D). Biochemical characterization confirmed that NTRK1 secondary mutations render the corresponding proteins insensitive (or only marginally sensitive) to entrectinib and capable of activating downstream signaling in the presence of the drug (Fig. 4E and F). We next asked whether the tumor cell that had become resistant remained dependent on the expression of TRKA. Indeed, siRNA-mediated suppression of mutant NTRK1 in resistant cells induced apoptosis, similar to the knockdown of WT NTRK1 in sensitive cells (Fig. 4G).

DISCUSSION

A subset of colorectal cancers carries NTRK1 translocations, which also occur in other tumor types, such as lung tumors and thyroid carcinomas (6, 13–15). The TRK inhibitor entrectinib induced a remarkable clinical response in a patient with a metastatic colorectal cancer carrying a LMNA–NTRK1 translocation, whose disease was intrinsically refractory to
three prior lines of therapy, including anti-EGFR inhibition (4). However, after 4 months of treatment, resistance developed in this patient. The entrectinib half-life is 17 to 44 hours, and the intermittent dosing regimen may have promoted or anticipated the development of resistance due to incomplete treatment coverage of the patient. Nevertheless, it is still unknown whether or not continuous dosing will affect the emergence and/or the type of acquired mutations.

In this work, we sought to identify mechanisms of resistance to entrectinib, as this is key to the development of additional lines of therapy for patients carrying NTRK1 rearrangements. The most commonly used approach to study resistance to targeted therapies involves molecular profiling of tissue biopsy obtained at progression. However, tumor heterogeneity and tissue sampling limit the effectiveness of this strategy. In addition, tissue biopsies are not always feasible and are associated with nonnegligible risks (16). Most importantly, even when the biopsy reveals emergence of alleles that were not present before treatment, their functional role in driving resistance remains to be formally established using functional assays. This requires significant experimental efforts, and the timeframe is not compatible with further treatment of the patient from whom the biopsy was obtained. We find that coupling pharmacologic analyses of xenopatients with molecular profiles of liquid biopsies allows the identification of resistance mechanisms in parallel with clinical treatment of individual patients, thus potentially enabling decisions on subsequent treatment options.

We report for the first time that acquisition of p.G595R and p.G667C mutations in the kinase domain of TRKA drives secondary resistance to TRK inhibition in colorectal cancer cells carrying NTRK1 rearrangements. Both mutations were detected in patient plasma obtained at progression, suggesting that both are indeed associated with acquired resistance...
Figure 4. Biochemical and pharmacologic characterization of xenopatient-derived colorectal cancer cells. A, colorectal cancer cells were established from a vehicle-treated xenopatient (sensitive to entrectinib) and from the tumor grown in xenopatient #4 that became resistant to entrectinib treatment in vivo. B, Sanger sequencing electropherogram shows LMNA–NTRK1 genetic rearrangements in both xenopatient-derived cell lines. C, cells derived from the xenopatient that developed resistance to entrectinib display NTRK1 p.G595R mutation. D, drug proliferation assay of LMNA–NTRK1-rearranged colorectal cancer cells. Entrectinib-sensitive cells established from vehicle-treated xenopatient are indicated with black line; entrectinib-resistant cells established from resistant xenopatient are indicated with the red line. Cell viability was assessed by measuring ATP content after 5 days of treatment. E, sensitive and resistant xenopatient-derived cells were treated with 1 μmol/L entrectinib for 16 hours; after that, protein lysates were analyzed by Western blot. F, sensitive and resistant xenopatient-derived cells were treated with 1 μmol/L entrectinib for 48 hours; after that, protein lysates were analyzed by Western blot. G, RNAi knockdown of WT NTRK1 in xenopatient-derived sensitive cells and mutated NTRK1 in xenopatient-derived resistant cells induces apoptosis, as shown by cleaved PARP (c.PARP). Protein lysates were analyzed by Western blot 3 days after transfection with NTRK1-specific pooled siRNAs, scrambled siRNA, or transfection reagent (mock).
Entrectinib, LOXO-101 and TSR-011 were obtained from Ignyta.

**Patient’s Sample Collection**

Patient’s plasma and tumor biopsy were obtained through protocols approved by the local Ethical Committee at Ospedale Niguarda Ca’ Granda, Milan, Italy. The study was conducted according to the provisions of the Declaration of Helsinki, and the patient signed informed consent before sample collection. The liver biopsy was subcutaneously implanted in NOD-SCID mouse according to a study protocol approved by the Ethical Committee at Ospedale Niguarda Ca’ Granda, Milan, Italy, and the animal procedures approved by the Ethical Commission of the Institute for Cancer Research and Treatment and by the Italian Ministry of Health.

**ddPCR Analysis**

Isolated circulating free DNA was amplified using ddPCR Supermix for Probes (Bio-Rad) with the LMNA-TRK1 translocation, NTRKI p.G595R and NTRKI p.G667C assays (sequences of custom-designed probes are listed in Supplementary Table S5). ddPCR was then performed according to the manufacturer’s protocol, and the results are reported as a percentage or fractional abundance of mutant DNA alleles to total (mutant plus wild-type) DNA alleles. DNA template (8–10 μL) was added to 10 μL of ddPCR Supermix for Probes (Bio-Rad) and 2 μL of the primer and probe mixture. This reaction mix was added to a DG8 cartridge, together with 60 μL of Droplet Generation Oil for Probes (Bio-Rad) and used for droplet generation. Droplets were then transferred to a 96-well plate (Eppendorf) and then thermal cycled with the following conditions:

- Stage 1: 94°C for 10 minutes
- Stage 2: 94°C for 30 seconds, 60°C for 1 minute, repeated 20 times
- Stage 3: 98°C for 10 minutes
- Stage 4: 98°C for 1 minute, 60°C for 1 minute, repeated 10 times
- Stage 5: 98°C for 10 minutes

**Cell Line Authentication**

KM12 colorectal cancer cells were obtained from the NCI60 cell line bank and authenticated in May 2011. The genetic identity of the cell line was last checked not fewer than 3 months before performing experiments by the Cell ID System and by Gene Print 10 System (Promega), through short tandem repeats (STR) at 10 different loci (D5S818, D13S317, D7S820, D16S539, D21S11, vWA, TH01, TPOX, CSF1PO, and amelogenin). Amplicons from multiplex PCRs were separated by capillary electrophoresis (3730 DNA Analyzer; Applied Biosystems) and analyzed using GeneMapperID software from Life Technologies. Cell lines were tested and resulted negative for Mycoplasma contamination with the Venor GeM Classic Kit (Minerva Biolabs).

**Establishment of Primary Colorectal Cancer Cell Lines**

Primary colorectal cancer cell lines were established from tumor tissues obtained from patient-derived xenografts. Tumor tissues were dissociated into single-cell suspension by mechanical dissociation using the gentleMACS Dissociator (Miltenyi Biotec) and enzymatic degradation of the extracellular matrix using the Tumor Dissociation Kit (Miltenyi Biotec) according to the manufacturer’s instructions. The cell suspension was then centrifuged at 1,200 rpm for 5 minutes. Supernatants were removed and cell pellets were resuspended with DMEM/F12 medium containing 10% FBS. This process was repeated three times. Then, cell suspensions were filtered through a 70-μm cell strainer (Falcon) and resuspended with culture medium DMEM-F12 containing 2 mmol/L L-glutamine, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), gentamicin 50 μg/mL, and 10 μmol/L ROCK inhibitor Y-27632 (Selleck Chemicals Inc.).

**Methods**

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TRKA Mutations and Resistance to Entrectinib in Colorectal Cancer

Kinase Domain Alignment

The amino-acidic sequences of human TRKA [P04629], ALK [Q9UM73], ROS1 [P08822], EGFR [P00533], KIT [P10721], and MET [P08581] were obtained from the UniprotKB database (26). Their kinase domains were aligned using the MUSCLE tool (27) and results were post-processed using Jalview (28).

Disclosure of Potential Conflicts of Interest

R. Wild has ownership interest (including patents) in I Reyny, Inc. A. Bardelli is a consultant/advisory board member for Horizon Discovery, Trovagene, and Biocrates. No potential conflicts of interest were disclosed by the other authors.

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Supplementary Material and Methods

Cell culture and generation of resistant cells
KM12 cells were cultured in RPMI medium (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and grown in a 37°C and 5% CO₂ air incubator. KM12 entrectinib-resistant derivatives were obtained by exposing cells to a chronic acute dose of 2 µM (named R2) or to escalating doses of entrectinib (named R1) until resistant derivatives emerged. Ba/F3-ETV6-TRKA cells (kind gift from Nerviano Medical Sciences) were cultured in RPMI medium (Invitrogen) supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and grown in a 37°C and 5% CO₂ air incubator. Ba/F3-ETV6-TRKA cells were treated with 30-100 nM of entrectinib initially in duplicates. The viability of Ba/F3-ETV6-TRKA cells was monitored for two to three weeks after initial treatment until the resistant populations emerged.

Xenopatient
The patient was a 75 year-old woman with metastatic CRC progressing without having had any objective response to standard previous therapies, presenting with an intact primary colon tumor, peritoneal carcinomatosis and liver metastases in segments 7 and 5 of 9.0
cm and 8.5 cm, respectively, and a right adrenal gland deposit of 2.2 cm. The primary tumor biopsied in August 2013 was colon adenocarcinoma and a liver biopsy was performed prior to provision of informed consent for molecular screening of actionable targets on March 2014. An immunohistochemical (IHC) screening ad hoc for ALK/ROS-1/TRKA abnormalities was performed within the ALKA phase 1 clinical trial. The liver biopsy was subcutaneously implanted in 8-week-old NOD-SCID mouse (from Charles River Laboratory) according to a study protocol approved by Ethical Committee at Ospedale Niguarda Ca' Granda, Milano, Italy. The patient's tumor biopsy sample took about 1 month to engraft, after that the tumor was passaged and expanded for one generation until production of two cohorts. These were randomized according to average tumor size of 400 cubic millimeters and treated with vehicle alone (3 mice), and entrectinib (5 mice). Treatments schedule was based on oral gavage at 60 mg/kg/day for four days/week (Monday to Thursday, as usual patients’ administration of the ALKA phase 1 clinical trial. Of note, continuous daily dosing is now considered the preferred schedule for entrectinib). Caliper measurements were taken once a week. Entrectinib was suspended in distilled sterile water containing 0.5% methylcellulose (Sigma Aldrich) and 1% Tween-80 (Sigma Aldrich). All animal procedures were approved by the Ethical Commission of the Institute for Cancer Research and Treatment (IRCC) and by the Italian Ministry of Health.

**Drug proliferation assay**

CRC cell lines were seeded at different densities (3-5 x10³ cells/well) in 100 μl complete growth medium in 96-well plastic culture plates at day 0. The following day, serial dilutions of entrectinib were added to the cells in serum-free medium, while DMSO-only treated cells were included as controls. Plates were incubated at 37°C in 5% CO₂ for 3-5 days, after which cell viability was assessed by measuring ATP content through Cell Titer-Glo® Luminescent Cell Viability assay (Promega). Luminescence was measured by Perkin
Elmer Victor X4 or BMG ClarioStar plate reader. IC50s were determined by 4-parameter curve fit with variable slope (Prism6).

**Western blotting analysis**

Prior to biochemical analysis, all cells were grown in their specific media supplemented with 10% FBS. Cells were treated with indicated concentrations of entrectinib for 16 or 72 hours. Total cellular proteins were extracted by solubilizing the cells in EB buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 2 mM EGTA; all reagents were from Sigma-Aldrich, except for Triton X-100 from Fluka) in the presence of 1 mM sodium orthovanadate, 100 mM sodium fluoride and a mixture of protease inhibitors. Extracts were clarified by centrifugation and normalized with the BCA Protein Assay Reagent kit (Thermo). Western blot detection was performed with enhanced chemiluminescence system (GE Healthcare) and peroxidase conjugated secondary antibodies (Amersham). The following primary antibodies were used for western blotting (all from Cell Signaling Technology, except where indicated): anti-phospho TRKA (Tyr674/675) (SantaCruz); anti-TRK (SantaCruz); anti-phospho AKT (Ser473); anti-AKT; anti-phospho-p44/42 ERK (Thr202/Tyr204); anti-p44/42 ERK; anti-phospho-MEK1/2 (Ser217/221), anti-MEK1/2; anti-PARP; anti-actin (Millipore).

**siRNA screening**

The siRNA targeting reagents were purchased from Dharmacon, as a SMARTpool of four distinct siRNA species targeting different sequences of the TRKA transcript. Cell lines were grown and transfected with SMARTpool siRNAs using RNAiMAX (Invitrogen) transfection reagents following manufacturer's instructions. Briefly RNAi screening conditions were as follows: on day one siRNA were distributed in each well of a 6-well plate at final concentration of 20 nmol/L. Transfection reagent was diluted in OptiMEM and
aliquoted at 250µl/well; after 20 minutes of incubation, 2ml in media without antibiotics were added to each well. After 3 days, total cellular proteins were extracted to perform western blot analysis. Each plate included the following controls: mock control (transfection lipid only), scramble (AllStars, Qiagen) as negative control.

**Plasma Samples Collection**

At least 10 mL of whole blood were collected by blood draw using EDTA as anticoagulant. Plasma was separated within 5 hours through 2 different centrifugation steps (the first at room temperature for 10 minutes at 1,600 × g and the second at 3,000 × g for the same time and temperature), obtaining up to 3 mL of plasma. Plasma was stored at -80°C until ctDNA extraction.

**ctDNA isolation and genome equivalents quantification (GE/ml plasma)**

ctDNA was extracted from plasma using the QIAamp Circulating Nucleic Acid Kit (QIAGEN) according to the manufacturer's instructions. 6 µl of ctDNA were used as template for each reaction. All samples were analyzed in triplicate. PCR reactions were performed using 10 µl final volume containing 5 µl GoTaq®qPCR Master Mix, 2X with CXR Reference Dye (Promega) and LINE-1 [12,5µmol] forward and reverse primers. DNA at known concentrations was also used to build the standard curve. Primer sequences are listed in Supplementary Table S5.

**3-Dimensional modelling of TRKA**

The binding mode of entrectinib with wild type TRKA was obtained using Glide docking implemented in Maestro (Maestro Release 2015-1, Schrodinger, Inc, New York, NYC, 2015). Receptor coordinates were downloaded from PDB (PDB code: 4PMT) and properly prepared by adding protons, sampling water orientations and finally full energy
minimization. Both p.G595R and p.G667C mutated models were built with Maestro and subsequently subjected to energy minimization.

Supplementary Figures and Tables

Supplementary Figure S1. **LMNA-NTRK1** genetic rearrangement detected in patient’s plasma and xeno. NGS analysis using the IRCC-TARGET panel retrieved an in-frame gene fusion event between exon 11 of the LMNA gene and the exon 10 of the NTRK1 gene in patient’s plasma (A) and xeno (B).
Supplementary Figure S2. Acquisition of mutations in the TRKA kinase domain drives secondary resistance to entrectinib in Ba/F3 TRKA WT cells. Proliferation assay of Ba/F3-ETV6-TRKA WT, Ba/F3-ETV6-TRKA G667C and Ba/F3-ETV6-TRKA G595R cells. Cell viability was assessed by measuring ATP content after 3 days of treatment with the indicated concentrations of entrectinib.
Supplementary Figure S3. *NTRK1* mutations confer resistance to TRKA inhibition.

Proliferation assay of Ba/F3 (A), Ba/F3-TPM3-TRKA WT (B), Ba/F3-TPM3-TRKA G595R (C) and Ba/F3-TPM3-TRKA G667C (D). Cell viability was assessed by measuring ATP content after 3 days of treatment with indicated concentrations of entrectinib, LOXO-101 or TSR-011.
**Supplementary Figure S4. Homology alignment of NTRK1 p.G595 and p.G667 variants.** The alignments of amino acid sequences show that NTRK1 mutation p.G595 (A) and p.G667 (B) are conserved among 6 clinically relevant tyrosine kinases listed in the figure. Conserved residues among the all aligned kinase domains are highlighted in grey scale colors depending on their level of identity (dark grey: high identity; white: low identity).
Supplementary Table S1. NGS analysis of NTRK1 gene in patient’s plasma and xeno. The table lists the wild type (WT) and mutated (MUT) reads and fractional abundance (%) of mutations detected by NGS (IRCC-TARGET panel) analysis from plasma collected at baseline and resistance to entrectinib and from tumors grown in resistant and vehicle mice.

<table>
<thead>
<tr>
<th>Variant</th>
<th>MUT reads</th>
<th>WT reads</th>
<th>Fractional abundance (%)</th>
<th>MUT reads</th>
<th>WT reads</th>
<th>Fractional abundance (%)</th>
<th>MUT reads</th>
<th>WT reads</th>
<th>Fractional abundance (%)</th>
<th>MUT reads</th>
<th>WT reads</th>
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<td>300</td>
<td>0</td>
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Supplementary Table S2. NGS analysis of patient’s derived samples. The table shows mutations identified using the IRCC-TARGET-NGS panel analysis in the patient’s plasma ctDNA (obtained at entrectinib resistance), the resistant xeno and cells established from resistant xeno. To uncover somatic mutations, we compared each resistant sample to its sensitive counterpart as previously described (Siravegna et al, Nat Med 2015), and identified base-pair mismatches (Fisher’s Test) with fractional abundance above 1.5%. Mutations were then called only when supported by a 5% statistical significance and their occurrence was checked in the COSMIC database. Mutations were annotated (from left to right) according to gene name, the variant effect (synonymous, non-synonymous, stop-loss/gain), protein change (variant), number of wildtype (WT) or mutated (MUT) reads and the allelic frequencies (fractional abundance). Every somatic mutation was validated by visual examination using BAM files.
## Supplementary Table S3. Summary of serial ctDNA analyses.

Circulating tumor DNA (ctDNA) was isolated from serial blood draws collected before initiation of entrectinib (19 MAR 2014), and until treatment was terminated (14 JUL 2014). Each time point was analyzed by droplet digital PCR (ddPCR). The number of Genome Equivalents (GE), mutated (MUT) and wild type (WT) events and fractional abundance (%) are listed.

<table>
<thead>
<tr>
<th>GE/ml plasma</th>
<th>Sample ID</th>
<th>Date</th>
<th>Target</th>
<th>ddPCR Copies/ml Translocation</th>
<th>ddPCR Mut events</th>
<th>ddPCR WT events</th>
<th>Poisson Corrected (95% C.I.) Fractional Abundance (%)</th>
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<td>32211268</td>
<td>19 MAR 2014</td>
<td>LMNA-NTRK1</td>
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<td>2708390</td>
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<td></td>
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<td>--</td>
<td>74</td>
<td>2010</td>
<td>3.7 (4.5-2.95)</td>
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</table>
Supplementary Table S4. The p.G595R and p.G667C mutations confer resistance to multiple TRK inhibitors. Ba/F3, Ba/F3-TPM3-TRKA WT, Ba/F3-TPM3-TRKA G595R and Ba/F3-TPM3-TRKA G667C were treated with indicated TRK inhibitors for 3 days. IC50 values, determined by 4-parameter curve fit with variable slope, are listed in the Table.

<table>
<thead>
<tr>
<th></th>
<th>IC50 (nM)</th>
<th>Ba/F3-TPM3-TRKA WT</th>
<th>Ba/F3-TPM3-TRKA G595R</th>
<th>Ba/F3-TPM3-TRKA G667C</th>
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Supplementary Table S5. NTRK1 p. G595R, p.G667C and LMNA-NTRK1 fusion probes for ddPCR. Primers and probes used for ddPCR analysis are listed in the Table.

<table>
<thead>
<tr>
<th></th>
<th>NTRK1 p.G667C ddPCR custom probes</th>
<th>NTRK1 p.G595R ddPCR custom probes</th>
<th>LMNA-NTRK1 fusion ddPCR custom probes</th>
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<td>Reverse Sequence</td>
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<td>MUT Probe Sequence</td>
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