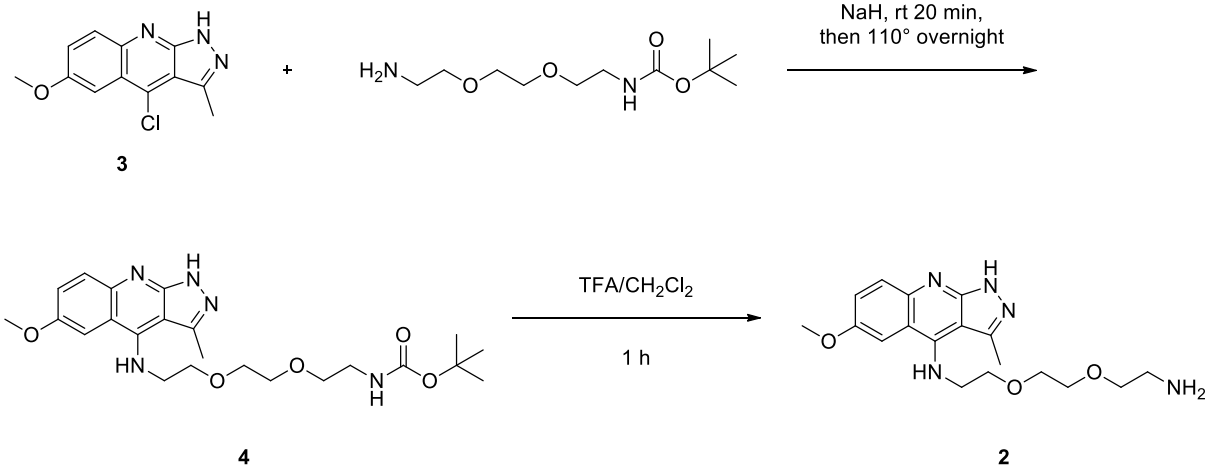
Supporting Information

General Information. NMR spectra were recorded on a Bruker Avance III 400 (Bruker, Billerica, MA, U.S.). Chemical shifts are given in ppm, and coupling constants are given in hertz. Mass spectra were recorded using a XeVo-UPLC-TQ-MS system (Waters, Milford, MA, U.S.). Purification by flash column chromatography (FCC) was done using silica gel 60 (Merck, Darmstadt, Germany), MPLC was performed on a Biotage Isolera system (Biotage, Uppsala, Sweden). The purity of the synthesized compounds was determined and confirmed by UPLC analysis. All synthesis chemicals were purchased from Sigma-Aldrich and Santa Cruz and used without further purification.

Compounds. SCH51344 was purchased from Calbiochem (EMD Millipore, Billerica, MA, USA). Racemic (*RS*)-crizotinib was obtained from Selleck Chemicals (Selleckchem, Houston, TX, USA), (*R*)-Crizotinib was purchased from Tocris (Tocris Bioscience, Bristol, UK), ChemieTek (ChemieTek, IN, USA), and Wuxi Apptec (Wuxi Apptec, Tianjin, China). (*S*)-Crizotinib was obtained from ChemFuture (ChemFuture PharmaTech, Jiangsu, China), and Wuxi Apptec (Wuxi Apptec, Tianjin, China). ABT-702 and KU55933 were purchased from Tocris (Tocris Bioscience, Bristol, UK), VE821 was obtained from Axon Medchem (Axon Medchem, Groningen, The Netherlands).

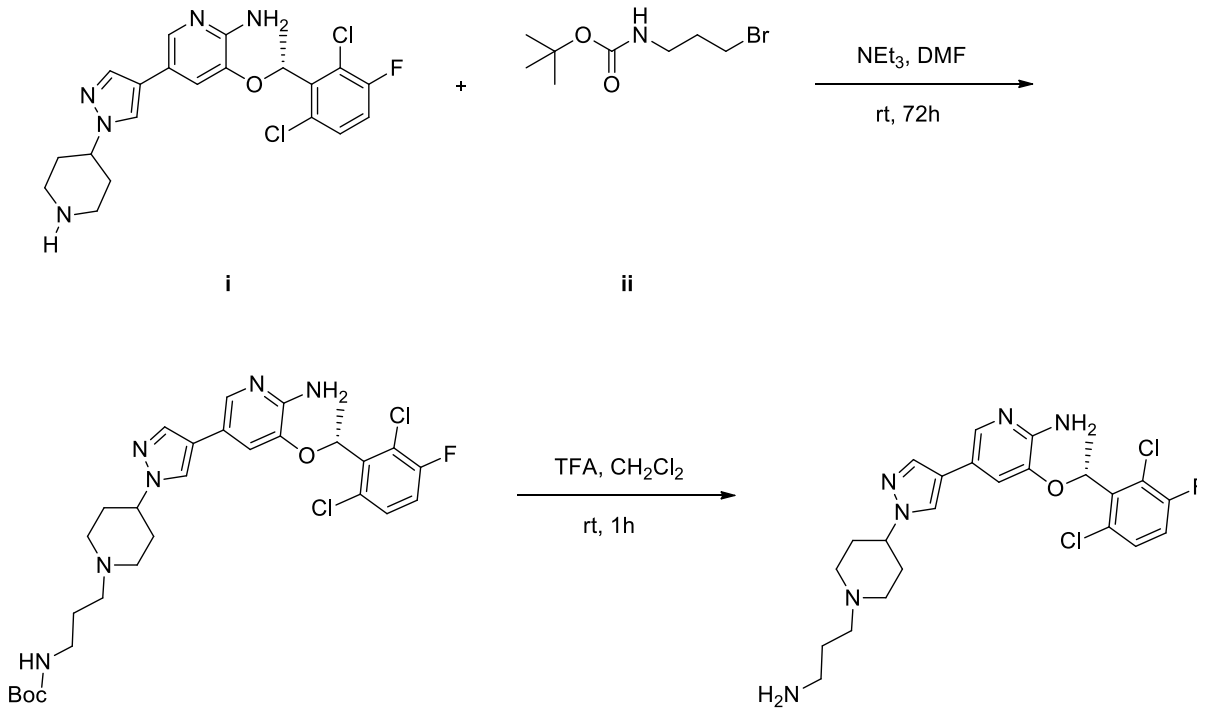
Synthesis of compound 2:



***tert*-Butyl (2-(2-(2-((6-methoxy-3-methyl-1*H*-pyrazolo[3,4-b]quinolin-4-yl)amino)-ethoxy)ethoxy)ethyl)carbamate (4).** According to literature,[1] sodium hydride (3.3 mg, 0.087 mmol, 60% dispersion) was added to *N*-Boc- 2,2'-(ethylenedioxy)diethylamine (723 mg, 2.8 mmol) and the mixture stirred for 20 min at room temperature. Upon addition of 4-chloro-6-methoxy-3-methyl-1*H*-pyrazolo[3,4-b]quinoline (**3**)[2] (18 mg, 0.07 mmol) the reaction was heated to 110 °C and stirred overnight. After cooling, water (5 mL) was added followed by extraction with ethyl acetate (3×10 mL). The organic layer was dried over sodium sulphate, filtered, and concentrated in vacuo. Flash column chromatography (dichloromethane/ethanol 10:1) gave a yellowish crystalline solid. Yield: 12.2 mg (37%). ^{1}H NMR (400 MHz, DMSO) δ 12.50 (s, 1H), 7.64 (d, *J* = 9.3 Hz, 1H), 7.55 (d, *J* = 2.7 Hz, 1H), 7.30 (dd, *J* = 9.3, 2.7 Hz, 1H), 6.68 (s, 1H), 6.21 (s, 1H), 3.88 (s, 3H), 3.82 (q, *J* = 5.5 Hz, 2H), 3.67 (t, *J* = 5.5 Hz, 2H), 3.55 – 3.49 (m, 2H), 3.44 (dd, *J* = 5.9, 3.5 Hz, 2H), 3.35 – 3.29 (m, 2H), 3.01 (q, *J* = 11.9, 6.0 Hz, 2H), 2.70 (s, 3H), 1.35 (s, 9H); MS ESI *m*/*z* 360 [M^+ + H].

***N*-(2-(2-(2-Aminoethoxy)ethoxy)ethyl)-6-methoxy-3-methyl-1*H*-pyrazolo[3,4-b]quinolin-4-amine (2).** Trifluoroacetic acid (40 µL) was added to a solution of compound 4 (8 mg, y mmol) in dichloromethane (4 mL) and the mixture was stirred at room temperature for 45 min. After removal of solvents, the crude product was purified by MPLC (dichloromethane/methanol 9:1) to give a yellow wax-like solid. Yield: 3 mg (48%). ^{1}H NMR (400 MHz, DMSO) δ 12.53 (s, 1H), 7.65 (d, *J* = 9.3 Hz, 1H), 7.57 (d, *J* = 2.7 Hz, 1H), 7.31 (dd, *J* = 9.3, 2.7 Hz, 1H), 6.25 (s, 2H), 3.89 (s, 3H), 3.83 (t, *J* = 5.6 Hz, 2H), 3.68 (t, *J* = 5.5 Hz, 2H), 3.61 – 3.37 (m, 7H), 2.83 (t, *J* = 5.3 Hz, 2H), 2.70 (s, 3H); MS ESI *m*/*z* 460 [M^+ + H].

Synthesis of (*S*)-crizotinib affinity probe:

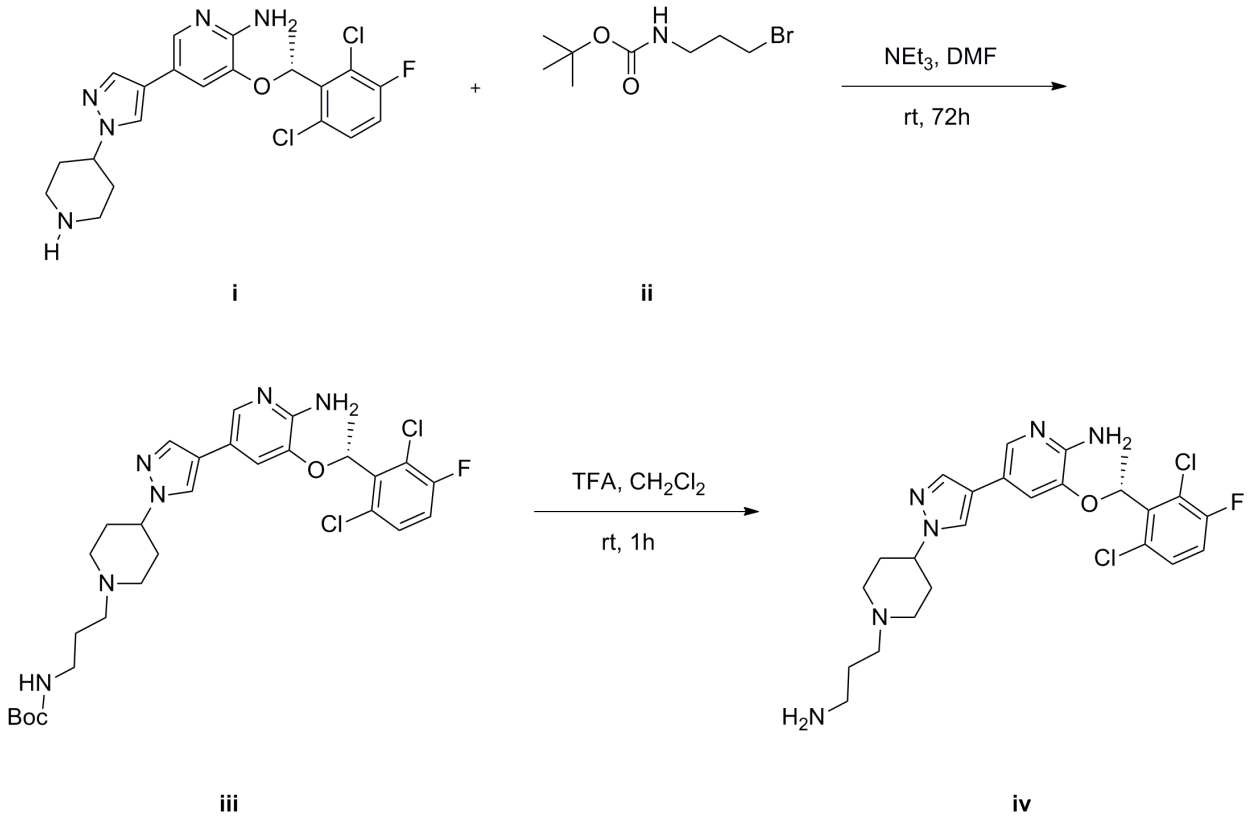


(*S*)-tert-butyl (3-(4-(4-(6-amino-5-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-3-yl)-1H-pyrazol-1-yl)piperidin-1-yl)propyl)carbamate (iii). To a solution of (*S*)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)-5-(1-(piperidin-4-yl)-1H-pyrazol-4-yl)pyridin-2-amine (**i**) (10 mg, 0.02 mmol) and triethylamine (4.5 mg, 0.04 mmol) in *N,N*-dimethylformamide (300 μL) tert-butyl (3-bromopropyl)carbamate (**ii**) (5.3 mg, 0.02 mmol) was added and the mixture was stirred at room temperature for 72 h. The reaction was diluted with water (5 mL) followed by extraction with ethyl acetate (3×10 mL). The organic layer was washed with brine (2×5 mL), dried over sodium sulphate, filtered, and concentrated in vacuo. Flash column chromatography (dichloromethane/ethanol 4:1) gave a white crystalline solid. Yield: 10.8 mg (80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (d, *J* = 1.7 Hz, 1H), 7.58 – 7.46 (m, 2H), 7.30 (dd, *J* = 8.9, 4.8 Hz, 1H), 7.04 (dd, *J* = 8.9, 7.9 Hz, 1H), 6.87 (d, *J* = 1.7 Hz, 1H), 6.07 (q, *J* = 6.7 Hz, 1H), 4.78 (s, 2H), 4.18 – 4.02 (m, 1H), 3.48 (s, 1H), 3.20 (d, *J* = 6.0 Hz, 2H), 3.05 (d, *J* = 11.8 Hz, 2H), 2.47

(t, J = 6.7 Hz, 2H), 2.27 – 1.95 (m, 6H), 1.85 (d, J = 6.7 Hz, 3H), 1.75 – 1.62 (m, 2H), 1.44 (s, 9H); MS ESI m/z 607 [M^+ + H].

(*S*)-5-(1-(1-(3-aminopropyl)piperidin-4-yl)-1H-pyrazol-4-yl)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-2-amine (iv). Trifluoroacetic acid (150 µL) was added to a solution of (*S*)-tert-butyl (3-(4-(4-(6-amino-5-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-3-yl)-1H-pyrazol-1-yl)piperidin-1-yl)propyl)carbamate (**iii**) (10 mg, 0.02 mmol) in dichloro-methane (300 µL) and the mixture was stirred at room temperature for 1 h. The solution was concentrated in vacuo, the residue resuspended in water (3 mL), and the pH was adjusted to 10 using 2M NaOH. After extraction with ethyl acetate (3×3 mL), the organic layer was dried over sodium sulphate, filtered, and concentrated in vacuo. The crude product was purified by MPLC (dichloromethane/ethanol 9:1) to give a brownish wax-like solid. Yield: 6.4 mg (77%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 1.5 Hz, 1H), 7.61 – 7.46 (m, 2H), 7.30 (dd, J = 8.9, 4.8 Hz, 1H), 7.04 (dd, J = 8.9, 7.9 Hz, 1H), 6.87 (d, J = 1.6 Hz, 1H), 6.07 (q, J = 6.7 Hz, 1H), 4.76 (s, 2H), 4.20 – 4.01 (m, 1H), 3.07 (d, J = 11.7 Hz, 2H), 2.81 (t, J = 6.5 Hz, 2H), 2.47 (t, J = 7.1 Hz, 2H), 2.23 – 1.94 (m, 6H), 1.85 (d, J = 6.7 Hz, 3H), 1.80 (s, 2H), 1.73 – 1.63 (m, 2H); MS ESI m/z 507 [M^+ + H].

Synthesis of (*R*)-crizotinib affinity probe:



The (*R*)-crizotinib affinity probe was prepared in an analogous manner as described for the (*S*)-enantiomer. Experimental data are reported below.

(*R*)-tert-butyl (3-(4-(4-(6-amino-5-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-3-yl)-1H-pyrazol-1-yl)piperidin-1-yl)propyl)carbamate (iii). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (d, *J* = 1.3 Hz, 1H), 7.52 – 7.38 (m, 2H), 7.23 (dd, *J* = 8.9, 4.8 Hz, 1H), 6.98 (dd, *J* = 8.9, 7.9 Hz, 1H), 6.80 (d, *J* = 1.7 Hz, 1H), 6.00 (q, *J* = 6.7 Hz, 1H), 5.24 (s, 1H), 4.81 (s, 2H), 4.15 – 3.99 (m, 1H), 3.14 (d, *J* = 5.9 Hz, 2H), 3.00 (d, *J* = 11.2 Hz, 2H), 2.42 (t, *J* = 6.8 Hz, 2H), 2.18 – 1.89 (m, 6H), 1.78 (d, *J* = 6.7 Hz, 3H), 1.72 – 1.58 (m, 2H), 1.37 (s, 9H); MS ESI *m*/*z* 607 [M^+ + H].

(*R*)-5-(1-(1-(3-aminopropyl)piperidin-4-yl)-1H-pyrazol-4-yl)-3-(1-(2,6-dichloro-3-fluorophenyl)ethoxy)pyridin-2-amine (iv). ^{1}H NMR (400 MHz, DMSO) δ 7.95 (d, *J* = 0.5 Hz, 1H), 7.76 (d, *J* = 1.8 Hz, 1H), 7.58 (dd, *J* = 9.0, 5.0 Hz, 1H), 7.45 (t, *J* = 8.7 Hz, 1H), 6.91 (d, *J* = 1.7 Hz, 1H), 6.09 (q, *J* = 6.6 Hz, 1H), 5.63 (s, 2H), 4.10 (td, *J* = 11.0, 5.5 Hz, 1H), 3.39 (dd, *J* =

14.0, 7.0 Hz, 3H), 2.95 (d, *J* = 11.5 Hz, 2H), 2.74 (t, *J* = 6.6 Hz, 2H), 2.38 (t, *J* = 6.9 Hz, 2H), 2.10 – 1.90 (m, 6H), 1.81 (d, *J* = 6.6 Hz, 3H), 1.67 – 1.57 (m, 2H); MS ESI *m*/*z* 507 [M^+ + H].

Immobilization and affinity purification. Drug-affinity matrices were prepared essentially as described previously.[3] Briefly, 25 nmol of compound was immobilised on 50 μL NHS-activated Sepharose 4 Fast Flow beads (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Affinity chromatography and elution were performed in duplicate as reported previously,[4] using 10 mg total cell lysate as protein input per replicate.

Solution tryptic digestion and peptide purification. After elution, enriched proteins were reduced with dithiothreitol, cysteine residues alkylated by incubation with iodoacetamide and the samples digested with modified porcine trypsin (Promega, Madison, WI). Three percent (and multiples thereof) of the digested eluates were purified and concentrated by C18 reversed-phase material for subsequent duplicate analysis by gel-free one-dimensional liquid chromatography mass spectrometry (1D-LCMS). Details of the LCMS methodology are as previously described.[5]

Protein identification. Peak extraction and conversion of RAW files into the MGF format for subsequent protein identification was performed with msconvert (ProteoWizard Library v2.1.2708). An initial database search was performed with broader mass tolerance to re-calibrate the mass lists for optimal final protein identification. For the initial protein database search, Mascot (www.matrixscience.com, version 2.3.02) was used. Error tolerances on the precursor and fragment ions were ±10 ppm and ±0.6 Da, respectively, and the database search limited to fully-tryptic peptides with maximum 1 missed cleavage, carbamidomethyl cysteine and methionine oxidation set as fixed and variable modifications, respectively. The Mascot peptide ion score threshold was set to 30, and at least 3 peptide identifications per protein were required. Searches were performed against the human UniProtKB/SwissProt database (www.uniprot.org release 2012-05) including all protein isoforms.

The initial peptide identifications were used to deduce independent linear transformations for precursor and fragment masses that would minimize the mean square deviation of measured masses from theoretical. Re-calibrated mass list files were searched against the same human protein database by a combination of Mascot and Phenyx (GeneBio, SA, version 2.5.14) search

engines using narrower mass tolerances (±4 ppm and ±0.3 Da). One missed tryptic cleavage site was allowed. Carbamidomethyl cysteine was set as a fixed modification and oxidized methionine was set as a variable modification. To validate the proteins, Mascot and Phenyx output files were processed by internally-developed parsers. Proteins with ≥2 unique peptides above a score T_1, or with a single peptide above a score T_2 were selected as unambiguous identifications. Additional peptides for these validated proteins with score > T_3 were also accepted. For Mascot searches, the following thresholds were used: T_1=14, T_2=40 and T_3=10; Phenyx thresholds were set to 4.2, 4.75 and 3.5, respectively (P-value < 10^{-3}). The validated proteins retrieved by the two algorithms were merged, any spectral conflicts discarded and grouped according to shared peptides. A false discovery rate (FDR) of <1% for protein identifications and <0.1% for peptides (including the ones exported with lower scores) was determined by applying the same procedure against a database of reversed protein sequences.

Bioinformatic analysis. Non-specific binders were filtered from the drug pull-downs using the SAINT software (version 2.3.4).[6] Using protein spectral counts as a measure of protein abundance and comparing the data of a real pull-down versus the negative control experiments, SAINT calculates the probability of a prey protein to be a real bait interactor. We also compared SAINT probabilities with the fold-reduction of spectral count upon free compound competition representing a magnitude of effect. Fold-reduction was computed as the ratio of median spectral counts observed in pull-downs with/without competition. In each condition, 4 spectral counts were available for the median (2 biological replicates and 2 technical for each). Specificity according to the CRAPome database content was obtained from the CRAPome web site submitting pulled down protein UniProt accession codes and defined for each protein as

1-(number of samples containing the protein / total number of samples in the database)

(343 samples in the database at the time of making the queries).

Kinase assays. For most assays, kinase-tagged T7 phage strains were prepared in an E. coli host derived from the BL21 strain. E. coli were grown to log-phase and infected with T7 phage and incubated with shaking at 32°C until lysis. The lysates were centrifuged and filtered to remove cell debris. The remaining kinases were produced in HEK-293 cells and subsequently tagged

with DNA for qPCR detection. Streptavidin-coated magnetic beads were treated with biotinylated small molecule ligands for 30 minutes at room temperature to generate affinity resins for kinase assays. The liganded beads were blocked with excess biotin and washed with blocking buffer (SeaBlock (Pierce), 1% BSA, 0.05% Tween 20, 1 mM DTT) to remove unbound ligand and to reduce nonspecific binding. Binding reactions were assembled by combining kinases, liganded affinity beads, and test compounds in 1x binding buffer (20% SeaBlock,0.17x PBS, 0.05% Tween 20, 6 mM DTT). All reactions were performed in polystyrene 96-well plates in a final volume of 0.135 ml. The assay plates were incubated at room temperature with shaking for 1 hour and the affinity beads were washed with wash buffer (1x PBS, 0.05% Tween 20). The beads were then re-suspended in elution buffer (1x PBS, 0.05% Tween 20, 0.5 μM non-biotinylated affinity ligand) and incubated at room temperature with shaking for 30 minutes. The kinase concentration in the eluates was measured by qPCR.

Compound Handling and Kinase Binding Constant Determiantion. An 11-point 3-fold serial dilution of each test compound was prepared in 100% DMSO at 100x final test concentration and subsequently diluted to 1x in the assay (final DMSO concentration = 1%). Most Kds were determined using a compound top concentration = 30,000 nM. If the initial Kd determined was < 0.5 nM (the lowest concentration tested), the measurement was repeated with a serial dilution starting at a lower top concentration. A Kd value reported as 40,000 nM indicates that the Kd was determined to be >30,000 nM. Binding constants (Kds) were calculated with a standard dose-response curve using the Hill equation:

Response = Background + {(Signal – Background)/[1 + (KdHill Slope / DoseHill Slope)]}

The Hill Slope was set to -1. Curves were fitted using a non-linear least square fit with the Levenberg-Marquardt algorithm.

Expression and purification of MTH1 for crystallization. The expression construct was transformed into *E. coli* BL21 (DE3) competent cells containing the pRARE2 plasmid from commercial Rosetta cells. Colonies from the transformation were used to inoculate 100 mL of LB media containing 34 μg/ml chloramphenicol and 50 μg/ml kanamycin. The culture was grown overnight in a baffled shaker flask at 37 °C with shaking. This culture was used to

inoculate LB media by adding 10 ml of culture to 1 L of LB (containing 50 μg/ml kanamycin) in baffled shaker flasks. When the culture had an OD600 of approximately 0.6 the temperature was reduced to 18 °C and protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to 0.5 mM. The culture was left shaking at 18 °C overnight before the cell pellets were harvested by centrifugation. The cells were resuspended in Binding Buffer (20 mM imidazole, 500 mM NaCl, 50 mM HEPES pH 7.4, 5% glycerol) with the addition of 0.5 mM tris(2-carboxyethyl)phosphine (TCEP) and 0.2 mM phenylmethanesulphonyl fluoride (PMSF). The resuspended cells were stored at -20 °C.

The resuspended cells were thawed and lysed by sonication. Polyethyleneimine was added to a concentration of 0.15% and the lysate was centrifuged at 4 °C to remove insoluble material. The supernatant was loaded onto 7.5 ml of nickel-chelating resin. The resin was washed with Binding Buffer, and Binding Buffer containing 40 mM imidazole and then 60 mM imidazole. The protein was eluted with Binding Buffer containing 250 mM imidazole. The hexahistidine tag was removed by overnight treatment with TEV protease at 4 °C. The digested sample was concentrated to 5 ml volume and loaded onto a Superdex200 gel filtration column (HiLoad 16/60, GE Healthcare) pre-equilibrated in GF Buffer (50 mM HEPES pH 7.5, 300 mM NaCl, 0.5 mM TCEP). Fractions containing MTH1 were pooled and passed through a column of 2.5 ml nickel-chelating resin. The flow-through and an elution with GF Buffer containing 10 mM imidazole were combined. The protein identity was verified by electrospray ionization time-of-flight mass spectrometry (Agilent LC/MSD).
The MTH1 complexes were prepared by adding (*R*)- or (*S*)- crizotinib to dilute protein solution at an approximate molar ratio of 10:1. The MTH1:crizotinib complexes were concentrated together by ultrafiltration to a protein concentration of 20 mg/ml.

Crystallization and data collection. MTH1 complexes were crystallised by the sitting drop vapour diffusion method using 150 nL drops as detailed in Extended Data Fig. 5a. All crystals were cryo-protected in reservoir solution with the addition of 25% (v/v) ethylene glycol and flash-frozen in liquid nitrogen. X-ray diffraction data was collected at 100 K using 0.9795Å wavelength X-rays at the DIAMOND synchrotron, beamlines I04 and I02.

Structure determination and refinement. The diffraction images were processed using MOSFLM.[7] The integrated data were scaled and merged using AIMLESS[8] and the CCP4 suite of programs.[9] The structures were solved by molecular replacement using PHASER.[10] All structural models were built using COOT[11] and refined using REFMAC5.[12] Ligand restraints were generated with PRODRG.[13] Molprobity[14] was used for structure validation. There were no Ramachandran outliers in either structure. Data collection and refinement statistics can be seen in Extended Data Fig. 5b.

Supplementary References

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