Supplemental Materials

Supplemental Methods

Anchored multiplex PCR for targeted next-generation sequencing of fusions

A detailed description of the Anchored Multiplex Technology is described elsewhere.[1] This assay consists of fusion unidirectional gene specific primers (GSPs) targeting specific exons in 62 genes known to be involved in chromosomal rearrangements. 346 GSPs ranging from 18 to 39 base pairs in length are provided by Archer™ and designed either in the 5'or 3' direction of the corresponding gene. GSPs, in combination with adapter-specific primers, enrich for known and novel fusion transcripts. Briefly, RNA was extracted followed by cDNA synthesis and cDNA undergoes end repair, dA tailing and ligation with half-functional Illumina molecular barcode adapters (MBC). These sequencing adapters contain molecular barcodes that allow for read de-duplication and quantitative analysis. Clean up after all enzymatic steps was performed using AMPURE XP magnetic beads. Cleaned ligated fragments were subject to two consecutive rounds of PCR amplifications using two sets of gene specific primers (GSP1 pool used in PCR1 and a nested GSP2 pool designed 3' downstream of GSP1, used in PCR2) and universal primers complementary to the Illumina adapters. This allowed for enrichment of fusion transcripts with the knowledge of only one of the gene partners. At the end of the two PCR steps the final targeted amplicons were ready for 2x150bp sequencing on an Illumina MiSeq sequencer. The Archer™ analysis software V5.0.4 was used for data analysis.

Histology

For histological analysis, samples were washed in PBS, fixed overnight in 4% paraformaldehyde (methanol free). Samples were washed in PBS once before being washed in water. Bones were incubated for 2 hours on ice in 8% HCl solution for decalcification. Samples were rinsed with water and stored in PBS before immunostaining. Tissues were fixed in 10% neutral buffered formalin

overnight and processed for paraffin embedding and sectioned at 4 μM. Sections were microwaved for 15 min in 0.01 M sodium citrate buffer pH 6 for antigen retrieval. Sections were blocked with 1.6% H2O2/PBS and then 10% normal horse serum/1% BSA and stained for 1 hour with mouse anti-CD45 (Dako M0701) at 1/200 dilution. Secondary antibody was used with biotinylated horse anti-mouse IgG (Vector Labs), followed by ABC reagent (Vector Labs), developed with DAB (Vector Labs) and counterstained with hematoxylin. Pictures were taken using an inverted microscope and analyzed with NIS-Elements software from Nikon.

Western Blotting

Anti-TrkA/B/C (EPR17341) and anti-phospho-TrkA/B/C (EPR19140) were purchased from Abcam. Anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (no. 9101), anti-p44/42 MAPK (ERK1/2) (137F5) (no.4695), anti-phospho-AKT (Ser473) (no.9271), anti-AKT (C67E7) (no.4691), anti-PLCγ1 (D9H10) (no.5690), anti-phospho- PLCγ1 (Tyr783) (no.2821), as well as the secondary antibodies anti- rabbit IgG-HRP (no. 7076) and anti-mouse IgG-HRP (no. 7074) were purchased from Cell Signaling Technology. Anti-β-Actin (A5441) was purchased from Sigma-Aldrich®. Cell lysates were prepared in RIPA buffer supplemented with Halt protease and phosphatase inhibitor cocktail (Thermo Scientific). Equal amounts of protein, as measured by the Bradford protein assay, were resolved in 4–12% Bis-Tris NuPage gradient gels (Life Technologies) and transferred electrophoretically on a polyvinylidene difluoride 0.45-m membrane. Membranes were blocked for 30 minutes at room temperature in 5% bovine serum albumin (BSA) in TBST before being incubated overnight at 4°C with the primary antibodies. All primary antibodies were diluted 1:1,000 in 5% BSA in TBST, except anti-β-actin, which was diluted 1:5,000 in 5% BSA in TBST. After three washes of 5 min in TBST, secondary antibodies were diluted 1:2,000 in 5% BSA in TBST and incubated for 1 h at room temperature. After another three washes in TBST, detection of the signal was achieved by incubating the membrane on an ECL solution from Millipore and exposure on autoradiography films from Denville Scientific (Metuchen, NJ, USA). In some instances, blots were

stripped for 30 minutes with Western Blot Stripping Buffer from Thermo Scientific (Rockford, IL). After re-blocking, the blots were re-probed with species appropriate secondary antibody to ensure complete stripping before using another primary antibody.

Flow cytometry

All FACS antibodies (for both human and mouse studies) were purchased from BD-Pharmingen, Biolegend, or eBioscience. MNCs from patient samples, xenografted tissues or peripheral blood were stained with a combination of antibodies against human CD3 (hCD3, SK7/HIT3a), hCD14 (61D3/MφP9), hCD16 (3G8), hCD19[2] (HIB19), hCD33 (WM-53/P67.6), hCD34 (4H11/581), hCD38 (HIT2), hCD45 (HI30), hCD45RA (HI100), hCD90 (5E10), and hCD123 (7G3). Antibodies against mouse CD45.1 (mCD45.1; A20) and mTer119 were used to exclude host-derived cells. DAPI was used as a viability marker. Briefly, up to 1.5 million cells from freshly drawn bone marrow aspirate were stained with 3 10-“color” panels, washed, and acquired on Canto-10 cytometer (BD Biosciences, San Jose, CA). The results were analyzed with custom Woodlist software (generous gift of Wood BL, University of Washington). An abnormal population was identified by visual assessment of populations with antigen expression that was ‘different-from-normal’ as described in prior publications.[2]

Colony forming assays and in vitro and in vivo drug studies

Murine c-Kit$^+$ cells were resuspended in Iscove’s modified Dulbecco’s medium (Lonza) + 10% FBS (Mediatech) at a concentration of $5x10^5$ cells/mL in 3 mL of MethoCult® M3434 methylcellulose (STEMCELL Technologies), penicillin/streptomycin (Mediatech) and increasing doses of larotrectinib. Next, 1.1 mL of the MethoCult solution was plated into 35 x10-mm Petri dishes, in duplicate. Plates were placed into an incubator at 37°C and 5% CO2 for 7-14 days, and colony (defined as a cluster of >50 cells) counts were then determined under a microscope at 40X magnification.

For in vitro drug testing, Ba/F3 or 32D cells were tested for sensitivity to larotrectinib (gift from Loxo Oncology, South San Francisco, CA). Half-maximal inhibitory concentrations were determined by measuring cell viability after 72 hours of treatment via the CellTiter-Glo ATP luminescence assay (Promega).

For in vivo drug testing, vehicle solution was prepared by adding pharmaceutical-grade methylcellulose (4,000 centipoise, Fisher Chemical) in near-boiling water, then cooling to room temperature. Pharmaceutical-grade polysorbate 80 (Sigma-Aldrich) was then added to achieve a final concentration of 0.5% methylcellulose (w/v)/0.1% polysorbate 80 (v/v). The vehicle solution was sterilized by autoclaving for 15 minutes at 15 psi and 121°C. Larotrectinib suspension was prepared for dosing daily by vortexing larotrectinib in vehicle until a uniform suspension was observed. After engraftment of human cells reached >10%, mice were randomized to receive vehicle or larotrectinib at a dose of 150 mg/kg body weight/day via oral gavage. Mice were treated once daily for 7 days and then a bone marrow aspirate was performed and analyzed as above. Mice then received another 7 days of daily treatment and were euthanized for full analysis.

Supplemental Table 1. Demographics of cohort studied.

Disease	# Patient (N)	Gender	Age, median (range)
Acute lymphoblastic leukemia	659	M: 61.15%; F: 38.85%	24 (0 - 86)
Acute myeloid leukemia	1201	M: 55.61%; F: 44.39%	59 (0 - 88)
Soft tissue histiocytic neoplasm	78	M: 52.56%; F: 47.44%	27 (0 - 86)
Soft tissue histiocytic non-Langerhans	23	M: 56.52%; F: 43.48%	51 (0 - 77)
Multiple myeloma	1859	M: 59.57%; F: 40.43%	65 (0 - 88)
Myelodysplastic syndrome	744	M: 58.86%; F: 41.14%	67 (0 - 87)
Myeloproliferative neoplasm	350	M: 55.87%; F: 44.13%	24 (0 - 86)
Myelodysplastic/ Myeloproliferative neoplasm	52	M: 59.62%; F: 40.38%	64 (0 - 86)
Other*: DLBCL (n=481) CLL (n=249) Follicular lymphoma (n=147) Mantle cell lymphoma (n=103) Hodgkin's lymphoma (n=69) Waldenstrom's (n=54) Burkitt lymphoma (n=52) Aplastic anemia (n=42) HCL (n=19) APL (n=12) BPDCN (n=10) Mastocytosis (n=10) T-cell lymphomas (including AITL, Mycosis fungicides; Sezary syndrome and T-cell lymphoma NOS; n=455) NHL NOS (n=313) Leukemia NOS (n=176) Hematologic disorder NOS (n=153)	2,345	M: 61.51%; F: 38.49%	60 (0 - 88)
Total	7,311	M: 59.36%; F: 40.64%	61 (0-88)

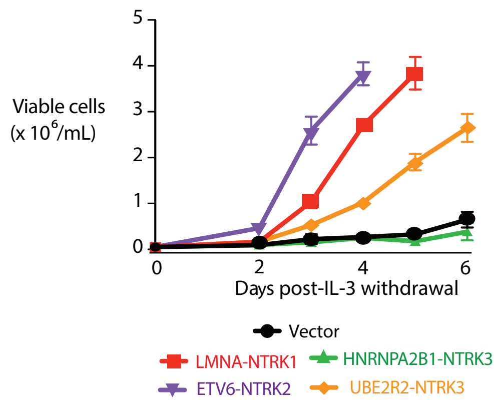
*DLBCL = diffuse large B-cell lymphoma; CLL = chronic lymphocytic leukemia; CML = chronic myeloid leukemia; CMML = chronic myelomonocytic leukemia; HCL = hairy cell leukemia; APL = acute promyelocytic leukemia; BPDCN = blastic plasmacytoid dendritic cell neoplasm; AITL = angio-immunoblastic T-cell lymphoma, and NOS = not otherwise specified.

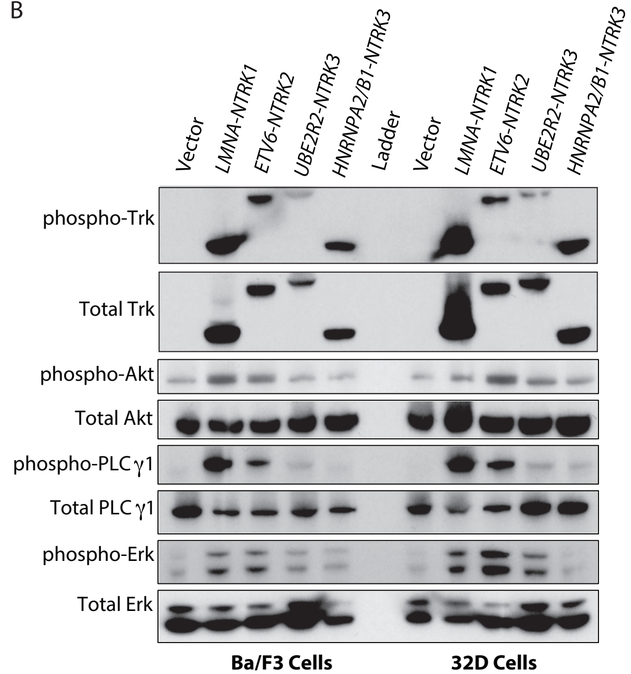
Supplemental Table 2. Detailed blood and marrow parameters pre- and post-larotrectinib treatment.

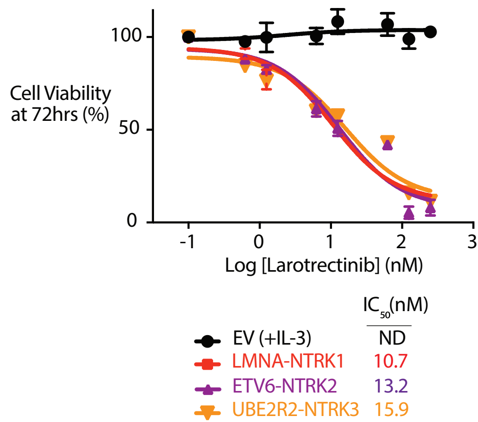
	WBC (k/μL)	% BM blasts	ANC (k/μL)	Hgb (g/dL)	PLT (k/μL)
Pre-larotrectinib	99	54	8	7	66
Post-larotrectinib	17	20	2	8	102

Abbreviations: ANC=absolute neutrophil count; BM= bone marrow; Hgb= hemoglobin; PLT= platelet; WBC= white blood cell count.

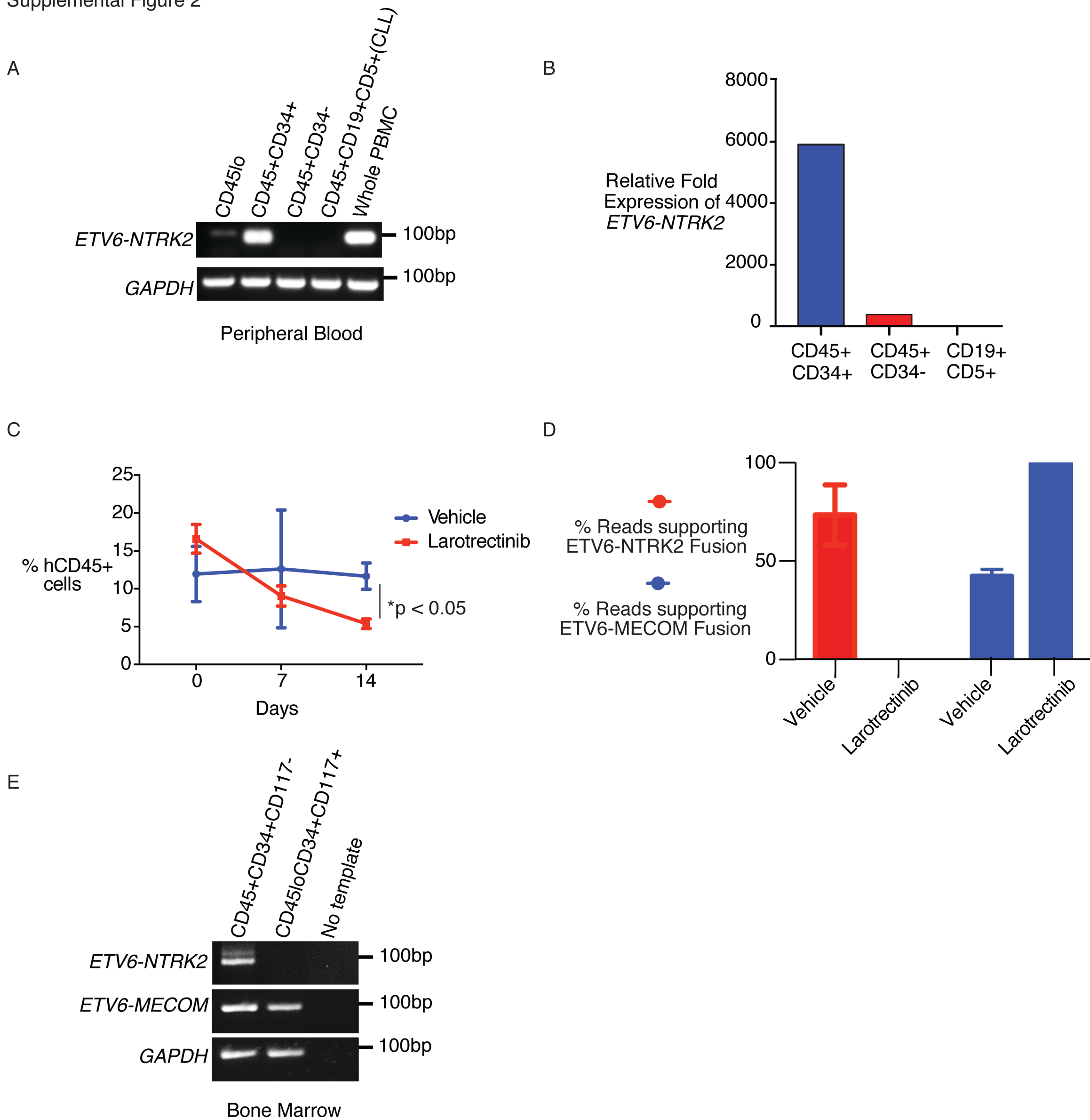
Supplemental Figure 1







Supplemental Figure 1. Differential transforming effects of TRK fusions found in hematologic malignancies. (A) Growth of Ba/F3 cells in media lacking IL-3 either expressing the indicated fusion protein or transduced with the empty vector as negative control. **(B)** Western blot performed on lysates from Ba/F3 cells or 32D cells expressing the indicated fusion protein or transduced with the empty vector as negative control show varying degrees of increased signaling in pAKT, pERK and pPLCγ1 relative to control cells (vector). **(C)** Cell viability of IL-3 independent Ba/F3 cells expressing TRK fusions after 72 hours of larotrectinib or vehicle treatment. Error bars represent mean and standard deviation from triplicate samples. The IC_{50} is calculated from the slope of the log inhibitor vs. response curve.



Supplemental Figure 2. Expression of *ETV6-NTRK2* fusion in acute myeloid leukemia (AML) patient. (A) FACS-sorted cells from the peripheral blood of the patient were subjected to RT-PCR to detect the *ETV6-NTRK2* fusion. **(B)** Relative expression of the *ETV6-NTRK2* fusion as measured by qRT-PCR in sorted populations from A. **(C)** Percentage of human hematopoietic cells in mouse BM at 0, 7, and 14 days of treatment with vehicle or larotrectinib. **(D)** *ETV6-NTRK2* or *ETV6-MECOM* supporting reads from targeted RNA-seq of hCD45 cells from the PDX at day 14. **(E)** FACS-sorted cells from the bone marrow of the patient were subjected to RT-PCR to detect the *ETV6-NTRK2* fusion and the *ETV6-MECOM* fusion. Error bars represent mean and standard deviation from triplicate samples. Differences were calculated using a two-sided Student's t-test.

REFERENCES

1. Zheng Z, Liebers M, Zhelyazkova B, et al. Anchored multiplex PCR for targeted next-generation sequencing. *Nat Med*. 2014;20(12):1479-1484.
2. Getta BM, Devlin SM, Levine RL, et al. Multicolor Flow Cytometry and Multigene Next-Generation Sequencing Are Complementary and Highly Predictive for Relapse in Acute Myeloid Leukemia after Allogeneic Transplantation. *Biol Blood Marrow Transplant*. 2017;23(7):1064-1071.