INVENTORY FOR SUPPLEMENTARY INFORMATION

FIGURE LEGENDS

Supplemental Table 1. Co-occurring mutations in the T-ALL patient. All mutations in the patient that passed read quality filters and SNP exclusion criteria (MAF<0.001). Mutations were checked in COSMIC to determine if it was previously reported in a cancer case. Six mutations were investigated with Sanger sequencing of leukemic and normal DNA samples and determinations of germline or somatic status are listed. Sanger validation of Notch1 I1616N was faint in the leukemic sample, potentially indicating a subpopulation.

Supplemental Table 2. Antibodies used in this study. A list of all antibodies used in these experiments.

Supplemental Table 3. Complete results of the small-molecule inhibitor screen. Biologically replicate WT and R461C lines were run on a screen of 104 small-molecule kinase inhibitors as previously described[1]. R461C cells were IL-3 independent and tested in IL-3 free media, while WT cells were grown and tested in IL-3 supplemented media. Colorimetric viability readouts (MTS) at 72 hours are used to compute IC_{50} values. Average WT and R461C IC_{50} are compared and the table is sorted for percent difference between the two. The top ranked inhibitors are those most selective for CSF2RB R461C.

Supplemental Table 4. CSF2RB mutations identified in primary patient samples. A list of all CSF2RB missense mutations found in patients and tested in the Ba/F3 transformation assay; only R461C transformed Ba/F3 cells. The composition of the 449 leukemia cohort is listed by diagnosis type.

Supplemental Figure 1. CSF2RB R461C is discovered in a primary T-ALL sample. (A) Clinical details for the CSF2RB-mutated patient. (B) Deep sequencing reads of a primary T-ALL sample for the R461C mutation. (C) Sanger sequencing confirmation of the observed mutation following PCR amplification of the patient's leukemic gDNA. (D) Sanger sequencing of the same mutation using a day 79 minimal residual disease blood sample from the same patient indicates the mutation is germline.

Supplemental Figure 2. R461 is a conserved residue in CSF2RB homologues. (A) Taxonomic distribution of mammalian species used for multiple sequence alignment. (B) Multiple sequence alignment for mammalian CSF2RB homologues. Alignment performed using CLUSTALO, 25.2% of positions were identical.

Supplemental Figure 3. CSF2RB R461C transforms Ba/F3 cells. Validation of the capacity of CSF2RB R461C to rapidly transform Ba/F3 cells following IL-3 withdrawal, as shown in Figure 1A. Cells were infected using a MIG-CSF2RB construct by retrovirus, and then sorted for GFP+ cells. Outgrowth monitored by frequent counting of viable cells using Guava ViaCount (Millipore).

Supplemental Figure 4. CSF2RB R461C is enriched in Ba/F3 prior to gaining factor independence. (A) Ba/F3 cells transfected with WT or R461C CSF2RB and puromycin-selected were stained for CSF2RB and analyzed by flow cytometry. Percent of cells positive for surface CSF2RB displayed for three biological replicates. (B) Histograms for intensity of PE-conjugated CSF2RB across biologically replicate lines, with mean fluorescent intensity (MFI) for positive cells shown at right. Stained and unstained parental Ba/F3 cells shown are as controls. Dotted line drawn at WT peak for reference. (C) MFI of PE-CSF2RB for 10,000 cells in each sample (**p<0.01 WT vs R461C).

Supplemental Figure 5. Residue R461 lies within the transmembrane domain of CSF2RB in most predictive models. (A) Illustration of transmembrane domain prediction models for CSF2RB (predicted domain in orange, residue 461 shown in bold). Uniprot uses no single model but a combination of four. The TMPred and maximum hydrophobicity window approaches yielded differing results for the WT (orange) and R461C (green) sequence. (B) List of prediction platforms, results, and servers used in this study.

Supplemental Figure 6. R461C cells are more sensitive to JAK-inhibitors than CSF2RB WT cells. (A-C) Ba/F3 cells were exposed to increasing concentrations of JAK-inhibitor for 72 hours prior to assessment of viability by a colorimetric assay. Normalized viability shown across drug concentrations (*p<0.05, **p<0.01). (D) Ba/F3 lines were treated for 24 hours with indicated JAK-inhibitors (Ruxolitinib 350nM, Tofacitinib 350nM, AZD1480 1μM) and analyzed for apoptotic induction using Annexin V staining and flow cytometry (Guava PCA). 48 hour reads shown in Figure 2D.

Supplemental Table 1

Co-occuring mutations in the T-ALL patient

Gene	CDS change	Amino acid change	Type	COSMIC status	Somatic confirmation
CSF2RB	C1381T	R461C	Missense	Not found	Germline
PTEN	C737G	P246R	Missense	Full match	Somatic
NOTCH1	C6856GC	L2286A…	Frameshift insertion	Not found	Somatic
NOTCH1	T4847A	I1616N	Missense	Full match	Somatic (subpopulation)
LHCGR	T47A	L16Q	Missense	Full match	Germline
TRAT1	C361T	R121C	Missense	Full match	Germline
IGF1R	G361T	E121*	Nonsense	Not found	N/A (not tested)
LRRK2	G1033A	D345N	Missense	Not found	N/A (not tested)
MAST3	T2374C	S792P	Missense	Not found	N/A (not tested)
NEK5	T2120G	L707R	Missense	Not found	N/A (not tested)
PHKG2	G1174A	D392N	Missense	Not found	N/A (not tested)
POMK	T401G	V134G	Missense	Not found	N/A (not tested)

Supplemental Table 2

Antibodies used in this study

Target	Antibody	Vendor	Clone	Species	Primary Dilution	Secondary Antibody	Secondary Dilution	Predicted Size (kDa)
CSF2RB	sc-678	Santa Cruz	K-17	Rb	1:200	HRPαRb	1:5000	130
CSF2RB (PE-conjugated)	306104	Biolegend		Ms	1:50	n/a	n/a	n/a
CSF2RB (Immunoprecipitation)	sc-678	Santa Cruz	K-17	Rb	1:25	n/a	n/a	n/a
Normal rabbit IgG (Immunoprecipitation)	sc-2027	Santa Cruz		Rb	1:12.5	n/a	n/a	n/a
p-Tyrosine	05-321	Millipore	4G10	Ms	1:5000	HRPαMs	1:5000	n/a
p-STAT5 (Y694)	9351	Cell Signaling		Rb	1:1000	HRPαRb	1:5000	90
p-STAT3 (Y705)	9131	Cell Signaling		Rb	1:1000	HRPαRb	1:5000	79, 85
Total STAT3	9139	Cell Signaling		Ms	1:1000	HRPαMs	1:5000	79, 86
p-PI3 Kinase (p85 Y458, p55 Y199)	4228	Cell Signaling		Rb	1:1000	HRPαRb	1:5000	60, 85
p-mTor (S2448)	5536	Cell Signaling	D9C2 XP	Rb	1:1000	HRPαRb	1:5000	289
mTOR	2983	Cell Signaling	7C10	Rb	1:1000	HRPαRb	1:5000	289
p-S6 Ribosomal Protein (S235/236)	4858	Cell Signaling	D57.2.2E XP	Rb	1:1000	HRPαRb	1:5000	32
MEK1/2	9122	Cell Signaling		Rb	1:1000	HRPαRb	1:5000	45
p-p44/42 MAPK (ERK1/2) (T202/Y204)	9101	Cell Signaling		Rb	1:1000	HRPαRb	1:5000	42, 44
Total p44/42 MAPK (ERK 1/2)	9102	Cell Signaling		Rb	1:1000	HRPαRb	1:5000	42, 44
Total Jak2	3230	Cell Signaling	D2E12 XP	Rb	1:1000	HRPαRb	1:5000	125
GAPDH	AM4300	ThermoFisher	6C5	Ms	1:5000	HRPαMs	1:5000	36
Actin	T 6074	Sigma	B512	Ms	1:10000	HRPαMs	1:5000	54

Supplemental Table 4

Mutations found in primary leukemic samples*

Diagnosis	CCDS Change	Amino Acid Change	Location
AML	G145A	D49N	Extracellular
Ph- aCML	G553A	A185T	Extracellular
Ph+ CML	T626C	V209A	Extracellular
T-ALL	C1381T	R461C	Membrane spanning or adjacent
B-ALL	G1570T	V524L	Cytoplasmic
B-ALL	C1661T	T544M	Cytoplasmic
AMML - M4	G1940T	G647V	Cytoplasmic
AML - M5	G1940T	G647V	Cytoplasmic
B-ALL	C2497G	L833V	Cytoplasmic

* Leukemia patient cohort consists of 449 total leukemias:
184 MPN, 147 AML, 108 ALL (92 B-ALL, 16 T-ALL, 5 undefined lymphoid leukemias), 10 other

Supplemental Figure 1

A

Clinical details for CSF2RB-mutated T-ALL patient

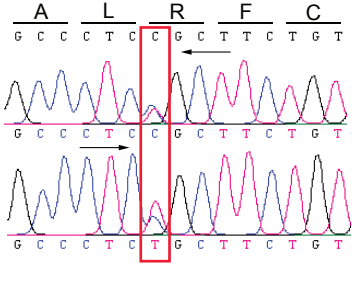
Age	14
Gender	F
Diagnosis	Immature T-ALL
WBC blasts per µl	511,000
Karyotype	46, XX, t(7;11)(q34;p12), inc[15]
Immunophenotype Markers	CD1a-,CD2+, Cytoplasmic CD3+, Surface CD3-, weak CD4+, weak CD5+, CD7+, CD8+, CD10+, CD99+, CD117-

B

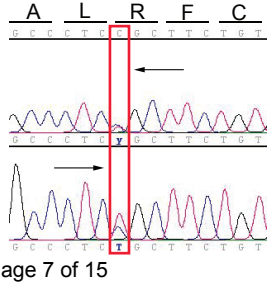
Deep sequencing results - CSF2RB R461C

	Reads	Percent Total	Forward Reads	Reverse Reads
WT allele	48	46%	22	26
Mut allele	57	54%	20	37
Total	105			

C

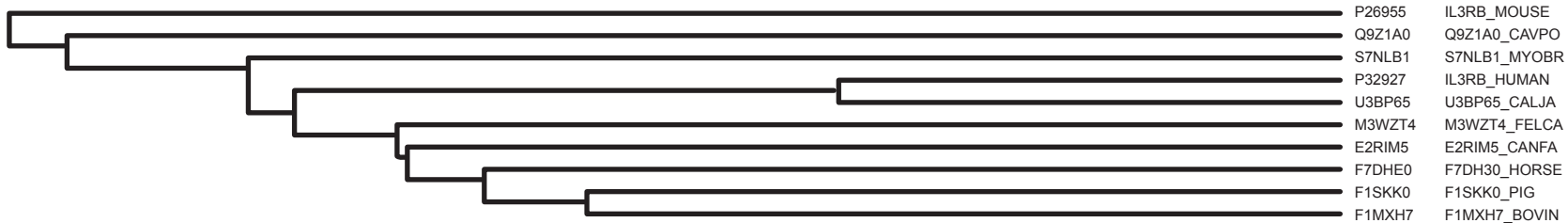


D



Supplemental Figure 2

A



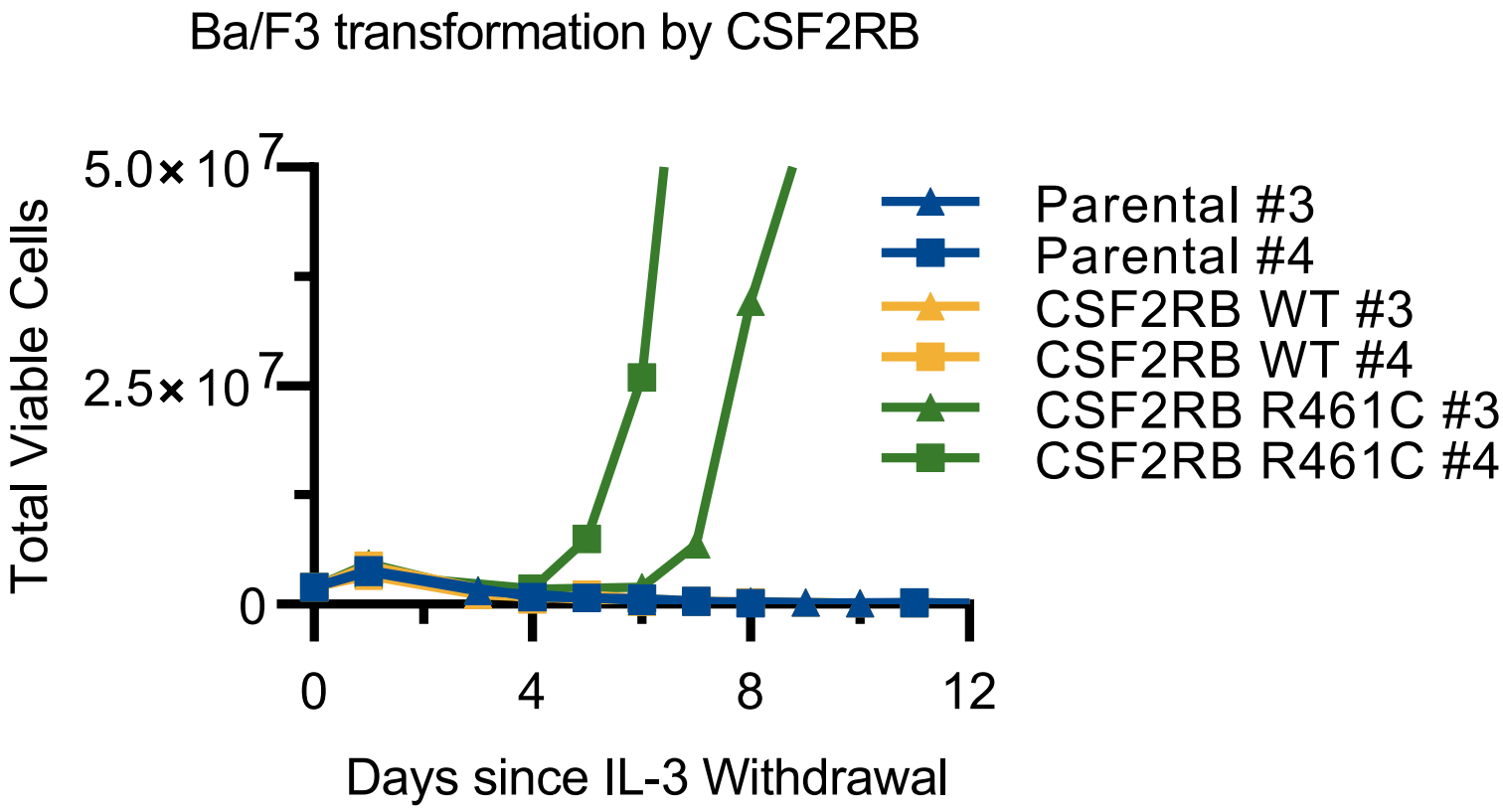
B

* - Single, fully conserved residue

: - Conservation between groups of strongly similar properties (> 0.5 Gonnet PAM 250 matrix)

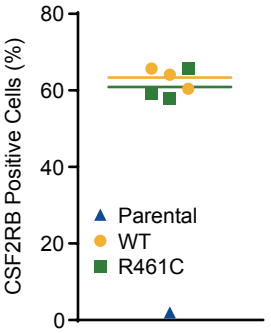
. - Conservation between groups of weakly similar properties (≤ 0.5 Gonnet PAM 250 matrix)

Supplemental Figure 3

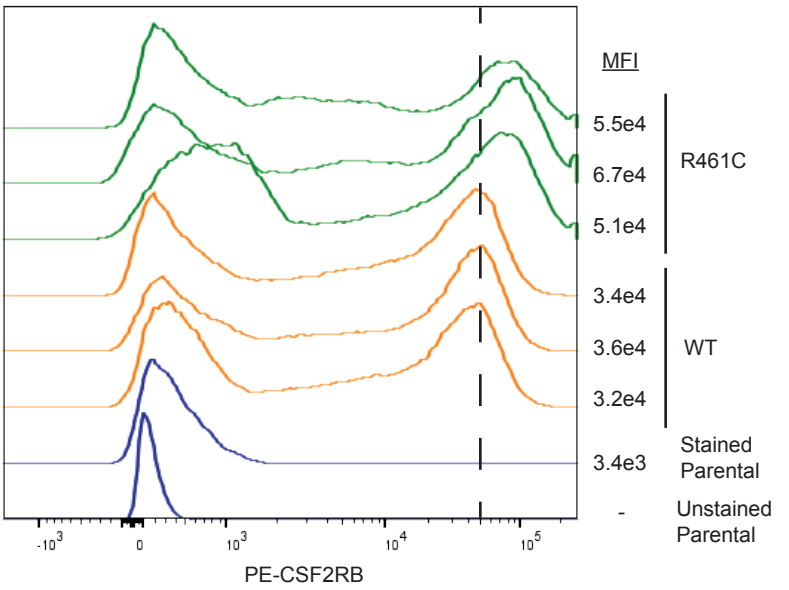


Supplemental Figure 4

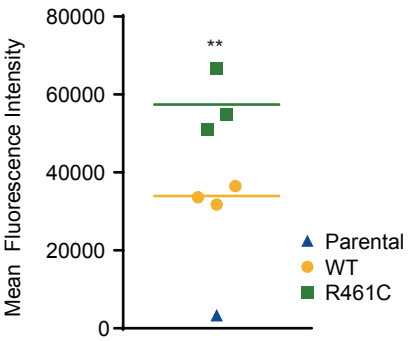
A



B

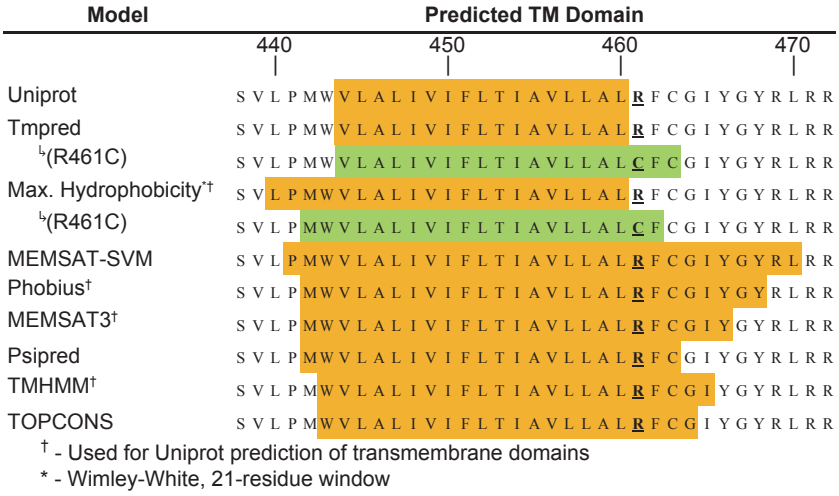


C



Supplemental Figure 5

A



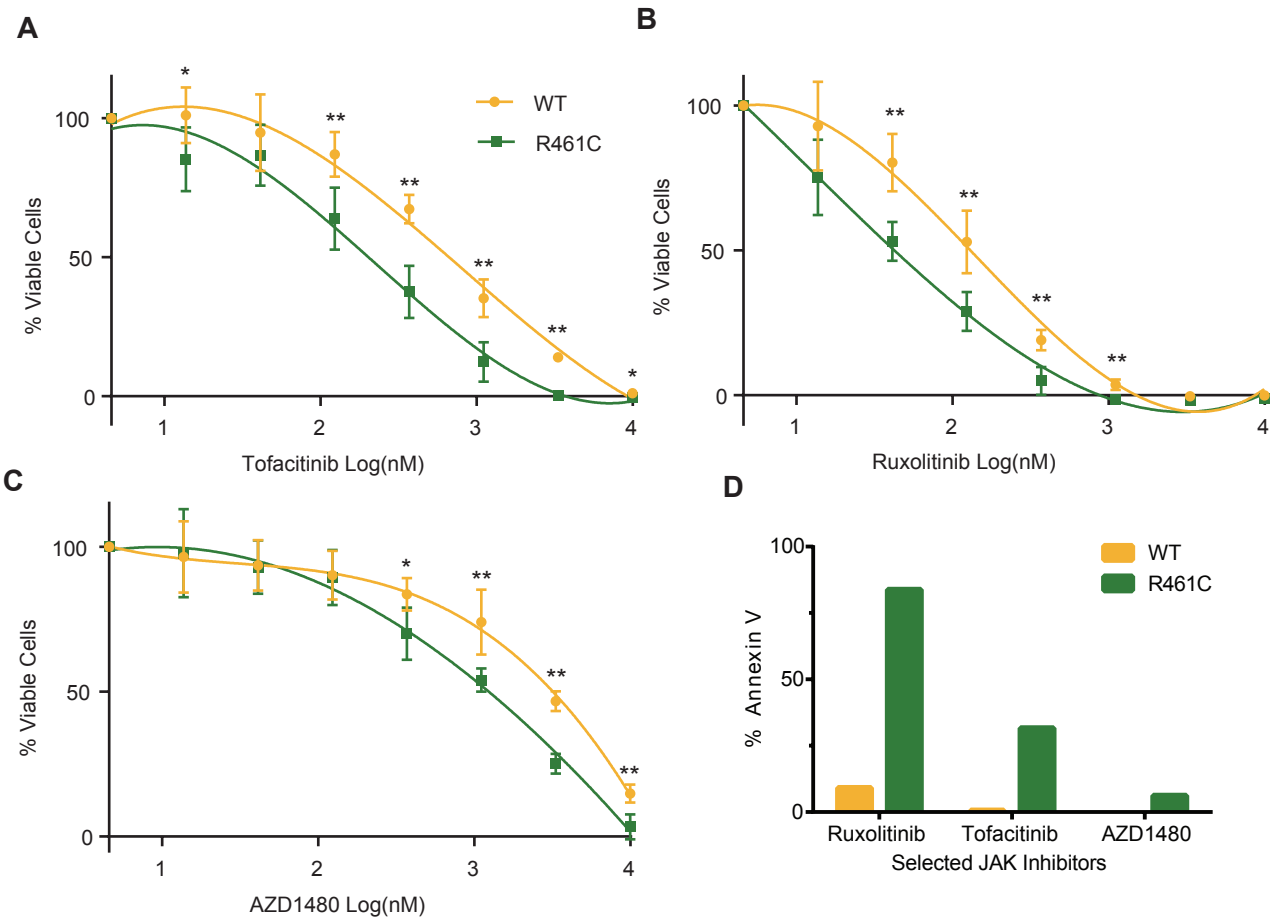
† - Used for Uniprot prediction of transmembrane domains

* - Wimley-White, 21-residue window

B

Model	Website	Predicted TM domain WT	Predicted TM domain R461C
Uniprot[2]	www.uniprot.org/uniprot/P32927	444-460	n/a
Tmpred[3]	www.ch.embnet.org/software/TMPRED_form.html	444-460	444-463
Max. Hydrophobicity[4]	http://www.tulane.edu/~biochem/WW/PepDraw/	440-460	442-462
MEMSAT-SVM[5-8]	bioinf.cs.ucl.ac.uk/psipred/	441-470	441-468
Phobius[9,10]	phobius.sbc.su.se/	442-468	442-468
MEMSAT3[5-8]	bioinf.cs.ucl.ac.uk/psipred/	442-466	442-466
Psipred[8,11]	bioinf.cs.ucl.ac.uk/psipred/	442-463	442-462
TMHMM[12,13]	www.cbs.dtu.dk/services/TMHMM/	443-465	443-465
TOPCONS[14]	topcons.cbr.su.se	443-464	443-464

Supplemental Figure 6



References

1. Tyner JW, Yang WF, Bankhead A, 3rd, Fan G, Fletcher LB, Bryant J, et al. Kinase pathway dependence in primary human leukemias determined by rapid inhibitor screening. Cancer research. 2013 Jan 1;73(1):285-96. PubMed PMID: 23087056. Pubmed Central PMCID: PMC3537897. Epub 2012/10/23. eng.
2. Consortium TU. UniProt: a hub for protein information. Nucleic acids research. 2015 January 28, 2015;43(D1):D204-D12.
3. Hofmann K, Stoffel W. TMBASE - A database of membrane spanning protein segments. Biol Chem Hoppe-Seyler. 1993;374(166).
4. White SH, Wimley WC. Hydrophobic interactions of peptides with membrane interfaces. Biochimica et biophysica acta. 1998 Nov 10;1376(3):339-52. PubMed PMID: 9804985. Epub 1998/11/07. eng.
5. Jones DT, Taylor WR, Thornton JM. A model recognition approach to the prediction of all-helical membrane protein structure and topology. Biochemistry. 1994 Mar 15;33(10):3038-49. PubMed PMID: 8130217. Epub 1994/03/15. eng.
6. Jones DT. Improving the accuracy of transmembrane protein topology prediction using evolutionary information. Bioinformatics (Oxford, England). 2007 Mar 1;23(5):538-44. PubMed PMID: 17237066. Epub 2007/01/24. eng.
7. Nugent T, Jones DT. Transmembrane protein topology prediction using support vector machines. BMC Bioinformatics. 2009;10:159. PubMed PMID: 19470175. Pubmed Central PMCID: PMC2700806. Epub 2009/05/28. eng.
8. Buchan DW, Minneci F, Nugent TC, Bryson K, Jones DT. Scalable web services for the PSIPRED Protein Analysis Workbench. Nucleic acids research. 2013 Jul;41(Web Server issue):W349-57. PubMed PMID: 23748958. Pubmed Central PMCID: PMC3692098. Epub 2013/06/12. eng.
9. Kall L, Krogh A, Sonnhammer EL. A combined transmembrane topology and signal peptide prediction method. Journal of molecular biology. 2004 May 14;338(5):1027-36. PubMed PMID: 15111065. Epub 2004/04/28. eng.
10. Kall L, Krogh A, Sonnhammer EL. Advantages of combined transmembrane topology and signal peptide prediction--the Phobius web server. Nucleic acids research. 2007 Jul;35(Web Server issue):W429-32. PubMed PMID: 17483518. Pubmed Central PMCID: PMC1933244. Epub 2007/05/08. eng.
11. Jones DT. Protein secondary structure prediction based on position-specific scoring matrices. Journal of molecular biology. 1999 Sep 17;292(2):195-202. PubMed PMID: 10493868. Epub 1999/09/24. eng.
12. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. Journal of molecular biology. 2001 Jan 19;305(3):567-80. PubMed PMID: 11152613. Epub 2001/01/12. eng.
13. Sonnhammer EL, von Heijne G, Krogh A. A hidden Markov model for predicting transmembrane helices in protein sequences. Proceedings / International Conference on Intelligent Systems for Molecular Biology ; ISMB International Conference on Intelligent Systems for Molecular Biology. 1998;6:175-82. PubMed PMID: 9783223. Epub 1998/10/23. eng.
14. Tsirigos KD, Peters C, Shu N, Kall L, Elofsson A. The TOPCONS web server for consensus prediction of membrane protein topology and signal peptides. Nucleic acids research. 2015 Jul 1;43(W1):W401-7. PubMed PMID: 25969446. Pubmed Central PMCID: PMC4489233. Epub 2015/05/15. eng.

Supplemental Materials & Methods

Patient samples and genomic analysis

Clinical samples were obtained with informed consent approved by the Institutional Review Boards of Oregon Health & Science University and Erasmus University Medical Center - Sophia Children's Hospital. Bone marrow or blood samples from patients with acute leukemia were separated using a Ficoll gradient followed by red blood cell lysis. Cells were cultured in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS, Atlanta Biologicals), L-glutamine (Invitrogen), fungizone (Invitrogen), penicillin/streptomycin (Invitrogen), and 10^{-4}M 2-mercaptoethanol (Sigma).

Genomic DNA was isolated from cryopreserved patient sample material using Qiagen DNeasy columns. DNA was fragmented by sonication using an S2 Sonicator (Corvaris). Fragmented DNA was then processed according to the SeqEZ protocol (Nimblegen/Roche), which is based on the TruSeq protocol (Illumina). Solution capture was performed using a custom DNA probe capture library previously described[1]. The libraries were sequenced on a HiSeq 2000 sequencer (Illumina) followed by FASTQ assembly using the CASAVA pipeline (Illumina). Sequence capture, library preparation, and deep sequencing were performed by the OHSU Massively Parallel Sequencing Shared Resource.

Sanger sequencing of CSF2RB mutations was confirmed by PCR amplification of CSF2RB exons 10 and 11 using M13-tagged primers (Exon10&11F gtaaaacgacggccagCCCTGAGGTCGATTTCCC, Exon10&11R caggaaacagctatgaccGGACAGAGACAAGAGAGGCAG) followed by sequencing with M13 forward (GTAAAACGACGGCCAGT) and reverse (CAGGAAACAGCTATGACC) primers.

Cell culture

Ba/F3 cells were obtained from ATCC and grown in RPMI 1640 medium with 10% FBS, L-glutamine, fungizone, penicillin-streptomycin, and15% WEHI-conditioned medium (a source of IL3).

Ba/F3 transformation assay

The CSF2RB R461C mutation was cloned as previously described[1] into the pMXs-IRES-Puro plasmid. 2.5e7 pro-B Ba/F3 cells were electroporated in a 0.4mm cuvette at 300V for two 25ms pulses with 40µg of plasmid. Stably transfected cells were selected using two weeks of continual 2µg/ml puromycin selection. Parental Ba/F3 cells or those stably expressing CSF2RB WT or R461C were washed three times and re-suspended in RPMI 1640 with 10% FBS, L-glutamine, fungizone and penicillin-streptomycin. Viable cell counts were obtained using a propidium iodide exclusion on a Guava Personal Cell Analysis System (Millipore). Genomic DNA was harvested from outgrown lines and the expressed transgene was confirmed by Sanger sequencing.

Immunoblot analysis

Immunoblotting was performed as previously described[1] (all antibodies used in this study are listed in Supplemental Table 1). Prior to lysis, cells were starved overnight in 0.1% FBS. Cells were lysed in Cell Lysis Buffer (Cell Signaling) containing Complete Mini Protease Inhibitor Cocktail Tablets (Roche), Phosphatase Inhibitor Cocktail 2 (Sigma) and Phenylmethanesulfonyl fluoride solution (Sigma) and quantitated using a Bradford protein assay (Bio-Rad). Most samples were mixed with a concentrated protein loading dye containing 8% 2-mercaptoethanol prior to loading; for non-reducing gels the protein lysate was mixed with a 2-mercaptoethanol-free dye. Immunoprecipitations were performed with anti-CSF2RB or rabbit IgG isotype control.

Cycloheximide time course

Ba/F3 cells were treated with 100µg/ml cycloheximide in DMSO for the indicated times before washing in PBS with 0.5% FBS and staining for 45 minutes with PE-conjugated anti-CSF2RB antibody. Cells were analyzed by flow cytometry (BD FACSAria IIIu and BD LSR II) for mean fluorescence intensity and normalized to untreated controls.

Transmembrane domain prediction

Consensus coding sequences for wildtype and R461C CSF2RB were analyzed by the indicated modeling programs. Top predictions that maintained a single transmembrane domain and type-I orientation are listed.

Small-molecule kinase inhibitor screen

A library (previously described[2]) of 104 small-molecule kinase inhibitors across three 384-well plates was used to determine inhibitors that specifically inhibited the growth of R461C-expressing cells. Cells were maintained in their culture media, where only WT cells were supplemented with 15% WEHI-conditioned media. Cells were plated at 400 cells per well in 50µl total volume (8,000 cells per mL) and incubated for 3 days at 37°C, 5% CO_2 and then subjected to a CellTiter 96 AQ_{ueous} One solution, tetrazolium-based, cell proliferation assay (Promega). All values were normalized to cells incubated in the absence of drug and IC_{50} values were calculated from the resulting kill curves of each drug gradient. Biologically replicate WT and R461C lines were run on this assay, with R461C-specific drugs identified as drugs with the largest percent difference in IC_{50} between WT and R461C cells.

References

1. Maxson JE, Gotlib J, Pollyea DA, Fleischman AG, Agarwal A, Eide CA, et al. Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. The New England journal of medicine. 2013 May 9;368(19):1781-90. PubMed PMID: 23656643. Pubmed Central PMCID: PMC3730275. Epub 2013/05/10. eng.

2. Tyner JW, Yang WF, Bankhead A, 3rd, Fan G, Fletcher LB, Bryant J, et al. Kinase pathway dependence in primary human leukemias determined by rapid inhibitor screening. Cancer research. 2013 Jan 1;73(1):285-96. PubMed PMID: 23087056. Pubmed Central PMCID: PMC3537897. Epub 2012/10/23. eng.