Supporting Information

Tyner et al. 10.1073/pnas.0903233106

SI Experimental Procedures

Collection of Patient Samples and Cell Culture. All clinical samples were obtained with informed consent with approval by the Institutional Review Boards of Stanford University and Oregon Health and Science University. Patient 07-079 was enrolled on an IRB-approved protocol at Washington University in St. Louis (04-1065; Principal Investigator, Tim Graubert). Blood or bone marrow from patients was separated on a Ficoll gradient. Cells were cultured in R10 [RPMI-1640 medium supplemented with 10% FBS (Atlanta Biologicals), L-glutamine, penicillin/streptomycin (Invitrogen), and fungizone (Invitrogen)], supplemented with 10^{-4} M 2-mercaptoethanol (Sigma); 293 T17 and BA/F3 cells were obtained from American Type Culture Collection. HEL cells were obtained from the German National Resource Centre for Biological Material (DSMZ); 293 T17 cells were maintained in DMEM supplemented with 10% FBS (Atlanta Biologicals), L-glutamine, penicillin/streptomycin (Invitrogen), and fungizone (Invitrogen). HEL and BA/F3 cells were maintained in R10, with BA/F3 cells also requiring WEHI-conditioned media.

Cell Viability, Proliferation, and Colony Assays. For proliferation and viability assays, cells were incubated for 72 h (density per well of 5,000 for cell lines and 50,000 for primary cells) in the presence of dose gradients of thrombopoietin (Peprotech), AG490 (EMD Biosciences), or midostaurin (PKC412) (LC Laboratories), at which point a CellTiter 96 AQ_{ueous}. One solution cell proliferation assay was performed (Promega). For determination of factor-independent growth, BA/F3 cells were cultured in R10 in the absence or presence of midostaurin (0–250 nM). Total viable cells were determined daily by using PI exclusion on a Guava cell counter (Guava Technologies). Two independently derived clones of $MPL^{1886InsGG}$– expressing BA/F3 cells were tested for WEHI-independent growth capacity. Midostaurin was refreshed each day. For colony assays, 200,000 cells were incubated in AG490 or midostaurin in methocult containing IL-3, GM-CSF, and SCF (StemCell Technologies). Ten days later, colonies were counted manually with brightfield microscopy. For siRNA knockdown in BA/F3 cells, electroporation conditions were 400 V, 100 μs, and 1 pulse.

Small Interfering RNA Knockdown Using Tyrosine Kinase Library. Patient blood or bone marrow was prepared as above and 2.25×10^7 cells were resuspended in 4.2 mL of siPORT buffer (Ambion). Cells were aliquoted at 42 μL per well onto a 96-well electroporator (Ambion), and 2 μL of siRNA at 20 μM was added to each well [tyrosine kinase library (Dharmacon) with single and pooled nonspecific siRNA, as well as siRNA against EPHA5, EPHA6, src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation (SRMS), apoptosis-associated tyrosine kinase (AATK), LMTK3, N-RAS, K-RAS (all from Dharmacon) added separately]. Targeting sequences for each pool of 4 siRNA constructs for all positive hits are listed in Table S2. Cells were electroporated at 1110 V (equivalent of 150 V per well), 200 μs, 2 pulses, and 50,000 cells per well were replated into triplicate plates containing 100 μL per well of culture media. For determination of cell viability, cells were subjected to the CellTiter 96 AQ_{ueous} one solution cell proliferation assay (MTS) (Promega). All values were normalized to the median plate value.

Immunoblotting and Flow Cytometry. For BA/F3 stimulation, cells were serum starved overnight. Cells were stimulated for 15 min with 0–10 ng/mL recombinant thrombopoietin (Peprotech). For JAK2 silencing in primary cells, CMML cells were incubated with single or pooled JAK2 or nonspecific siRNA and electroporated at 150 V, 200 μs, and 2 pulses, and cultured for 48 h. All cells were lysed in sample buffer (75 mM Tris pH 6.8, 3% SDS, 15% glycerol, 8% β-mercaptoethanol, 0.1% bromophenol blue) and separated by SDS/PAGE. Proteins were transferred to PVDF membranes (Millipore) and subjected to immunoblot analysis with antibodies specific for MPL (Millipore), total or phospho-STAT5 (BD Biosciences), total or phospho JAK2, STAT3, ERK1/2, AKT (Cell Signaling), or β-actin (Millipore). For flow cytometry, cells from patient 07-079 were immunostained with antibodies specific for CD3 and CD33, and each single-positive population was sorted on a BD FACSAria. Genomic DNA was isolated (Qiagen) and sequencing performed as described below.

Cloning and Creation of Stable Cell Lines. For cloning of MPL, RNA was extracted from HEL cells (Qiagen) and reverse-transcribed into cDNA (SuperScript III ; Invitrogen) by using random hexamer primers. MPL was PCR amplified by using the forward primer, 5′ CACCACACAGTGGCGGAGAAGATG 3′, and the reverse primer, 5′ GCCTAATTGTGAGGGCAGAC 3′, and cloned into the Gateway vector, pENTR/D-TOPO (Invitrogen). MPL was then cloned into MSCV-IRES-GFP (MIG) by performing an LR recombination reaction. Introduction of the 2 base pair GG insertion (1886InsGG) was carried out by using the Quikchange XL-II mutagenesis kit (Stratagene), and MPL W515L was kindly provided by Ross Levine (Sloan-Kettering, New York). Retrovirus expressing MIG-MPL WT, 1886InsGG, or W515L was propagated in 293 T17 cells by cotransfection of each respective MIG-MPL construct with the EcoPack plasmid (kindly provided by Richard Van Etten, Tufts-New England Medical Center, Boston) by using Fugene (Roche). One milliliter of viral supernatant was mixed with polybrene (5 mg/mL), Hepes (7.5 mM), and 10^6 BA/F3 cells and centrifuged at $2,500 \times g$ for 90 min at 30 °C. GFP positive cells were sorted on a BD FacsAria (BD Biosciences) after 48 h.

Sequencing Analyses. Genomic DNA from patient samples (Qiagen) was used to sequence JAK2, K-RAS, and MPL by using previously described primers (1). For MPL exon 12, individual copies of the PCR product were cloned and sequenced. Total RNA (Qiagen) from patient 07-079 was used to synthesize cDNA (Invitrogen SuperScript III) with random hexamer primers and MPL was amplified by using the forward primer, 5′ CAGGACTACAGACCCCACAG 3′, and the reverse primer, 5′ AGCCTGCCTGTGGAGAAAG 3′. Individual copies were cloned and sequenced.

Allele-Specific PCR. Allele-specific PCR for KIT^{D816V} was performed as previously described (2). For $MPL^{1886InsGG}$, quantitative PCR was performed on genomic DNA by using SYBR Green qPCR SuperMix (Invitrogen) and a DNA Engine Opticon 2 system for real-time PCR (Bio-Rad) with the following primers: 5′ CAC ACT ACA GGA GAC TGA GGC 3′ and 5′ GGC TGC TGC CAA TAG CTT CC 3′.

Statistical Analyses. For RAPID screens, a Student's t test was carried out for each well in comparison to single and pooled

nonspecific controls. The mean of the 2-tailed P value was evaluated for significance. Data points exceeding 2 SDs of the mean below the mean value on the plate, and $P < 0.05$ were considered significant.

For cell proliferation, cell viability, and colony assays, a Student's t test was carried out for each dose or time point compared with the relevant control cell line or the no drug control.

1. Sjoblom T, et al. (2006) The consensus coding sequences of human breast and colorectal cancers. *Science* 314:268–274.
2. Corless CL, et al. (2006) Allele-specific polymerase chain reaction for the imatinib-resistant KIT D816V and D816F mutations in mastocytosis and acute myelogenous leukemia. *J Mol Diagn* 8:604–612.

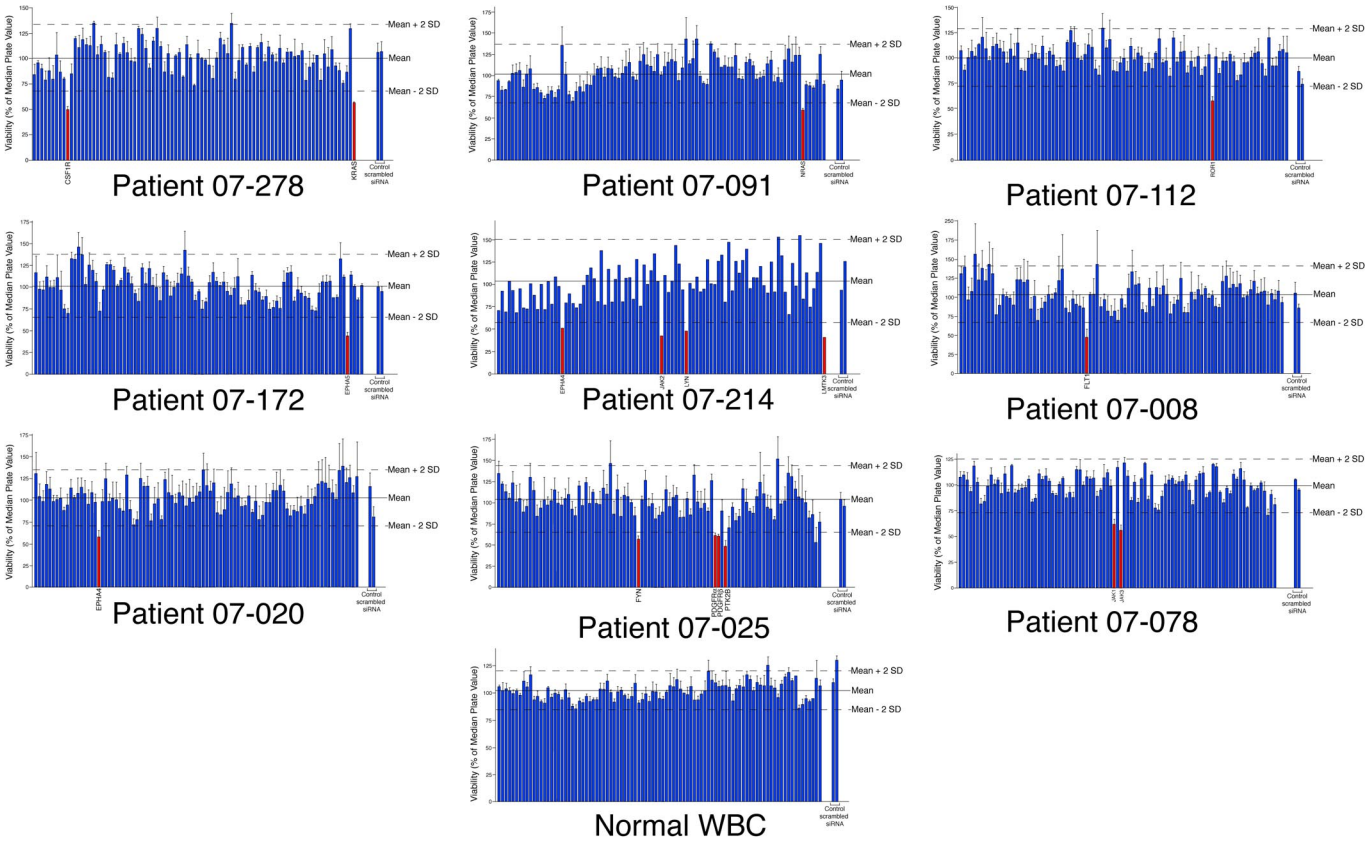

Fig. S1. RAPID identifies sensitivity to individual tyrosine kinases in cells from leukemia patients, but not normal white blood cells. RAPID analysis was performed on cells from 30 leukemia patients as well as 4 normal bone-marrow transplant donor cells. Sensitivity to silencing of an individual tyrosine kinase or *N-RAS*/*K-RAS* was observed in 10 of the 30 samples, whereas normal white blood cells did not exhibit sensitivity to targeting of any of the genes.

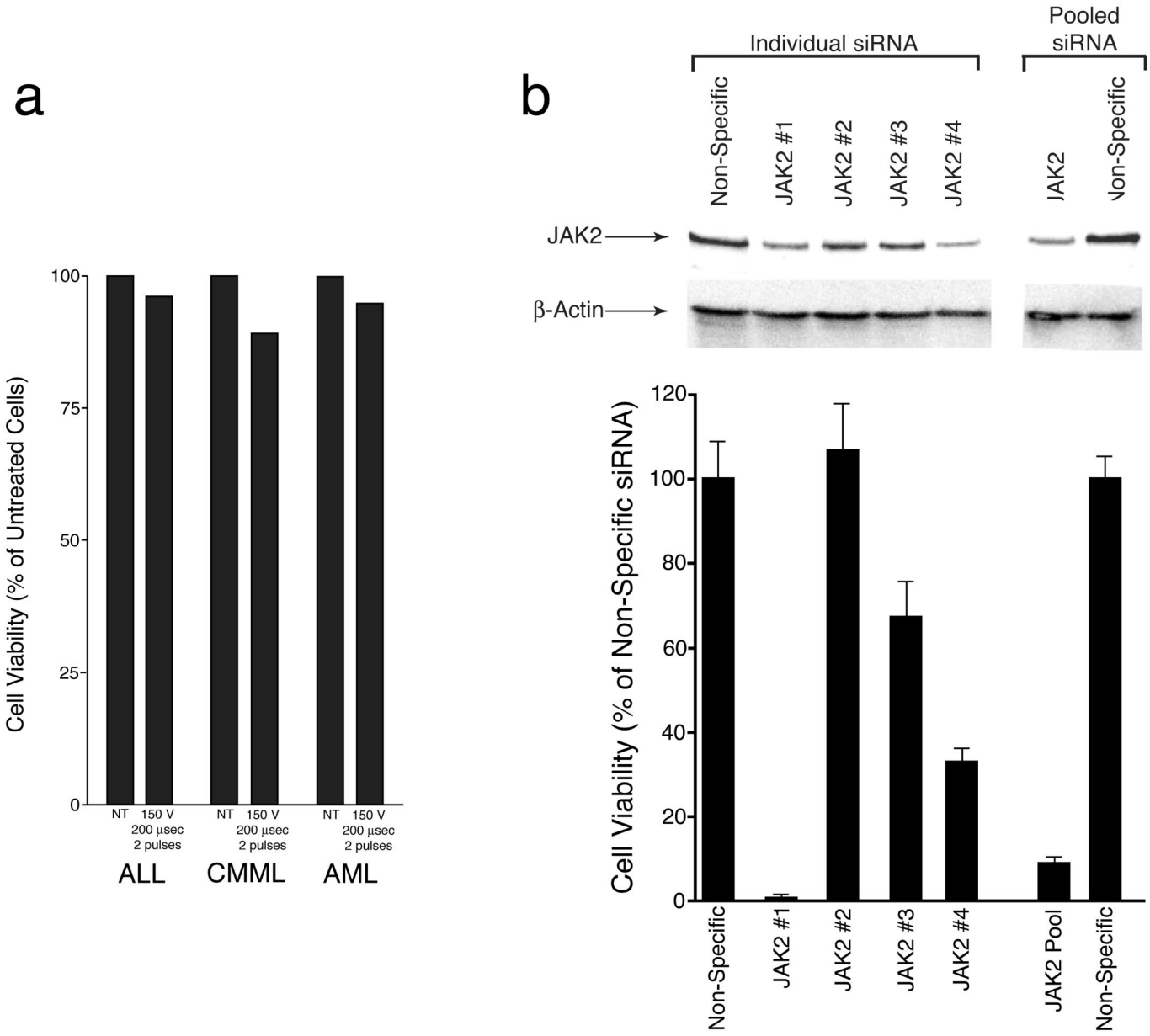

Fig. S2. Efficiency of siRNA gene silencing by electroporation without cell death in primary leukemia cells. (*A*) Cells from patients with acute lymphoblastic leukemia (ALL), chronic myelomonocytic leukemia (CMML), or acute myeloid leukemia (AML) were electroporated at 150 V, 200 μs for 2 pulses, and plated into culture medium for 2 days. Cell viability was measured by exclusion of propidium iodide. (*B*) Cells from a CMML patient were electroporated as in *A* in the presence of individual siRNAs targeting JAK2, a pool of all four JAK2 siRNAs, or nonspecific siRNA. Cells were plated into culture medium, and cells were lysed at 48 h. Whole-cell extracts were subjected to immunoblot analysis for JAK2 and β-actin. Transformed BA/F3 cells expressing MPL^{1886InsGG} (JAK2-dependent) were electroporated with individual or pooled siRNA targeting JAK2 or nonspecific siRNA. Cell viability was measured at 96 h using an MTS assay. Values are normalized to the respective nonspecific control and represent percent mean ± SEM (n = 6).

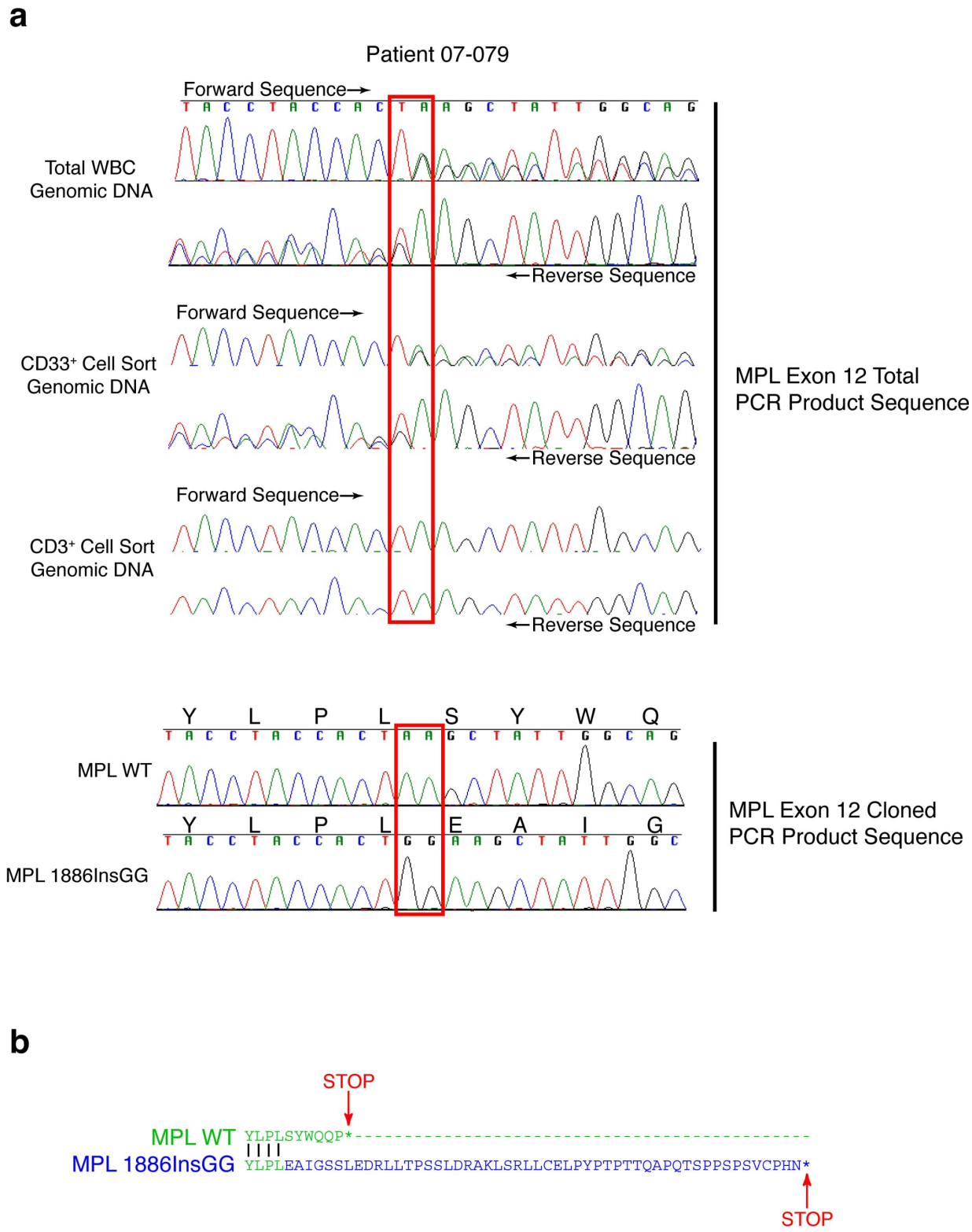

Fig. S3. Identification of a novel MPL mutation in patient 07-079. (*A*) MPL was sequenced in genomic DNA from patient 07-079 and found to have a 2-base-pair insertion (1886InsGG) in exon 12. Exon 12 PCR products were cloned and individual clones confirmed equal abundance of exon 12 WT and 1886InsGG in patient 07-079. Frozen viable cells from patient 07-079 were immunostained with antibodies specific for CD3 and CD33, and $CD3^+$ or $CD33^+$ cells were sorted by flow cytometry, and DNA was isolated from each cell population. Sequencing revealed that only the $CD33^+$ cell population exhibited the $MPL^{1886InsGG}$ mutation, indicating that it is an acquired, somatic mutation. (*B*) Hypothetical translation of the carboxy terminus of resultant proteins from MPL^{WT} and $MPL^{1886InsGG}$.

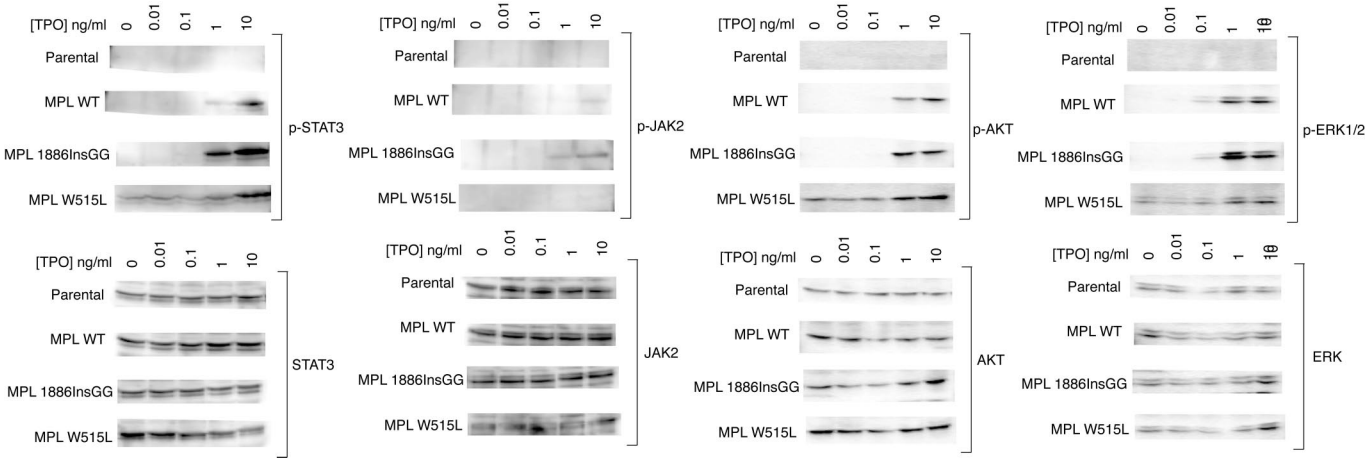

Fig. S4. MPL^{1886InsGG} is hypersensitive to thrombopoietin. Parental BA/F3 cells or those stably expressing MPL WT, 1886InsGG, or W515L were serum starved overnight and stimulated for 15 min with 0–10 ng/mL thrombopoietin. Whole-cell lysates were subjected to immunoblotting with antibodies specific for total or phospho-JAK2, STAT3, AKT, and ERK.

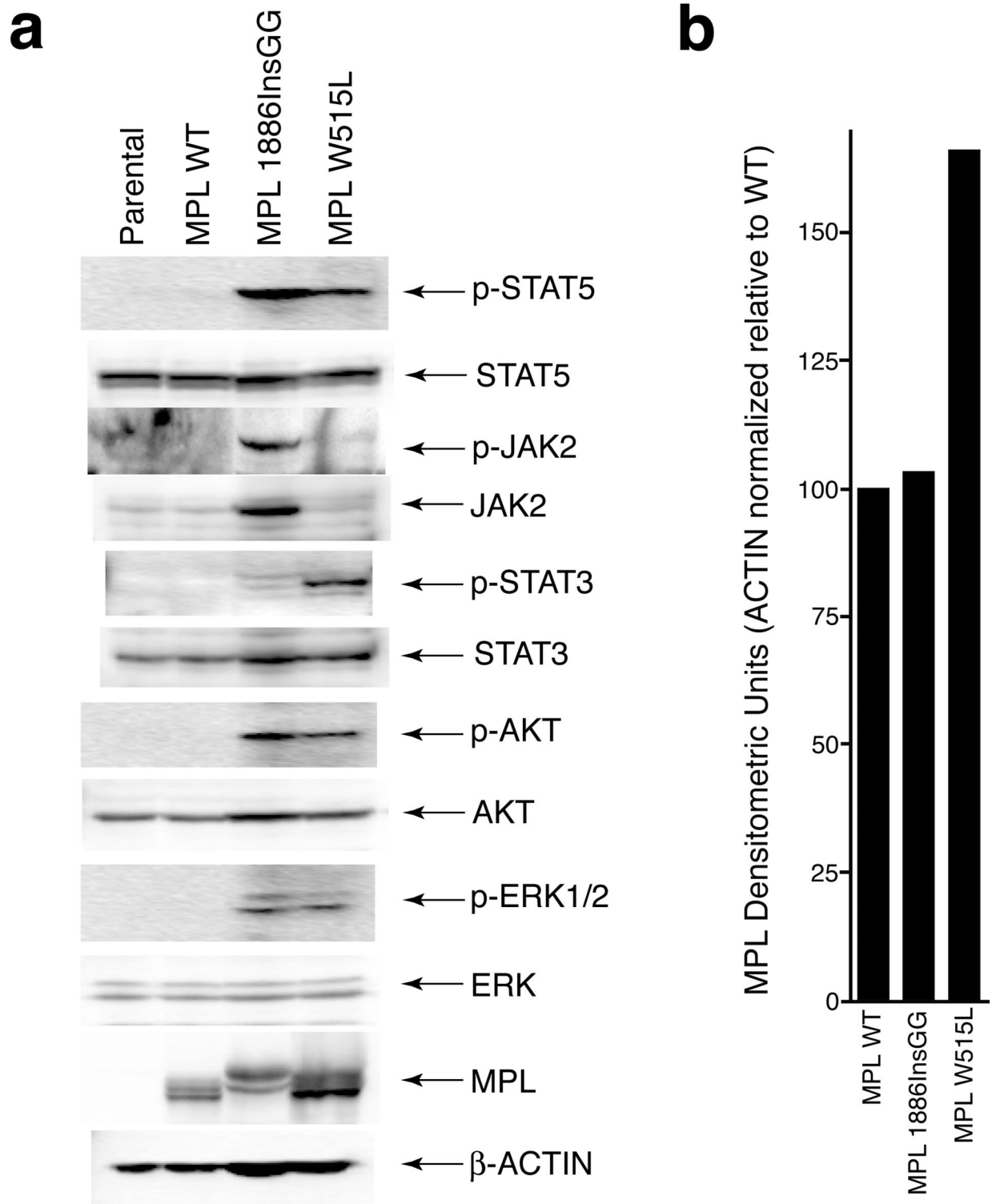

Fig. S5. MPL^{1886InsGG} is constitutively active. (*A*) Factor-independent BA/F3 cells expressing MPL 1886InsGG or W515L as well as parental BA/F3 cells or those stably expressing MPL WT, were serum starved overnight. Whole-cell lysates were subjected to immunoblotting with antibodies specific for total or phospho-JAK2, STAT5, STAT3, AKT, and ERK as well as MPL and β-actin. (*B*) Densitometric analysis of MPL levels from *A*. MPL levels were normalized to actin and levels of actin-normalized MPL WT, 1886InsGG, and W515L are shown relative to MPL WT.

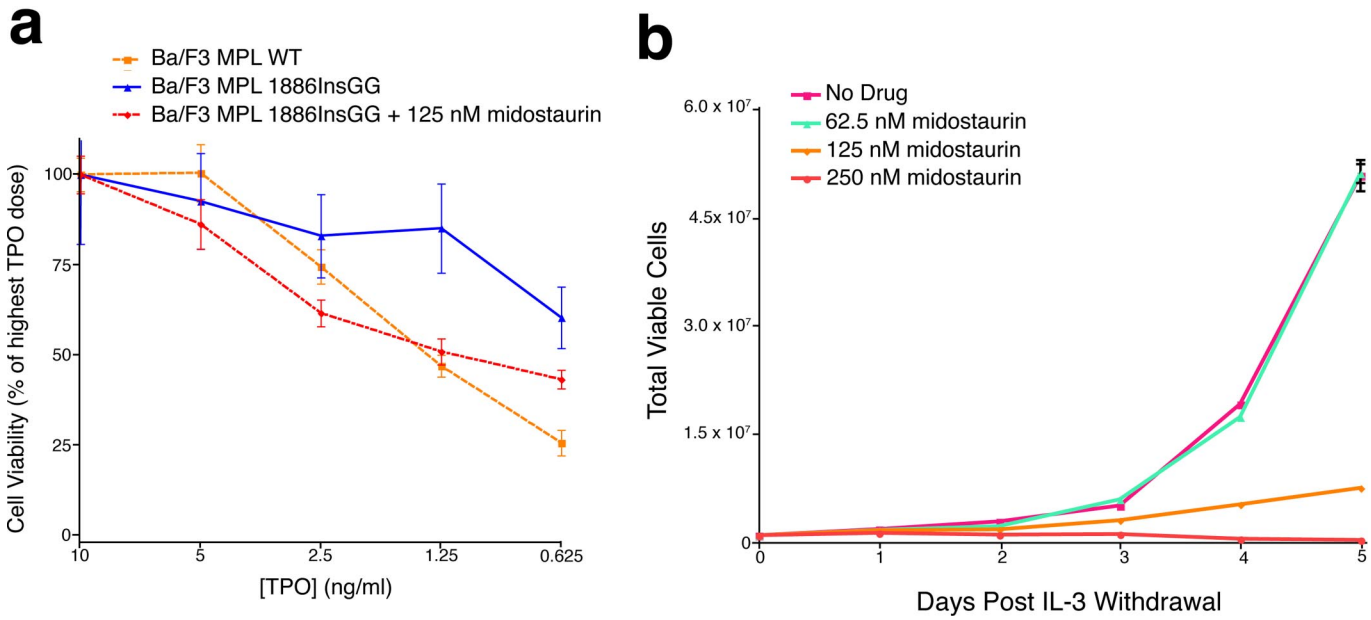

Fig. S6. Midostaurin restores Ba/F3 $MPL^{1886InsGG}$ to MPL^{WT} phenotype. (*A*) A cell proliferation assay was performed with Ba/F3 cells expressing MPL WT or 1886InsGG over a dose gradient of thrombopoietin in the absence or presence of midostaurin (125 nM). Cell viability was measured at 72 h using an MTS assay. (*B*) Ba/F3 cells expressing $MPL^{1886InsGG}$ were placed into media lacking IL-3 with 0, 62.5, 125, or 250 nM midostaurin. Cells were counted and fresh midostaurin was added daily.

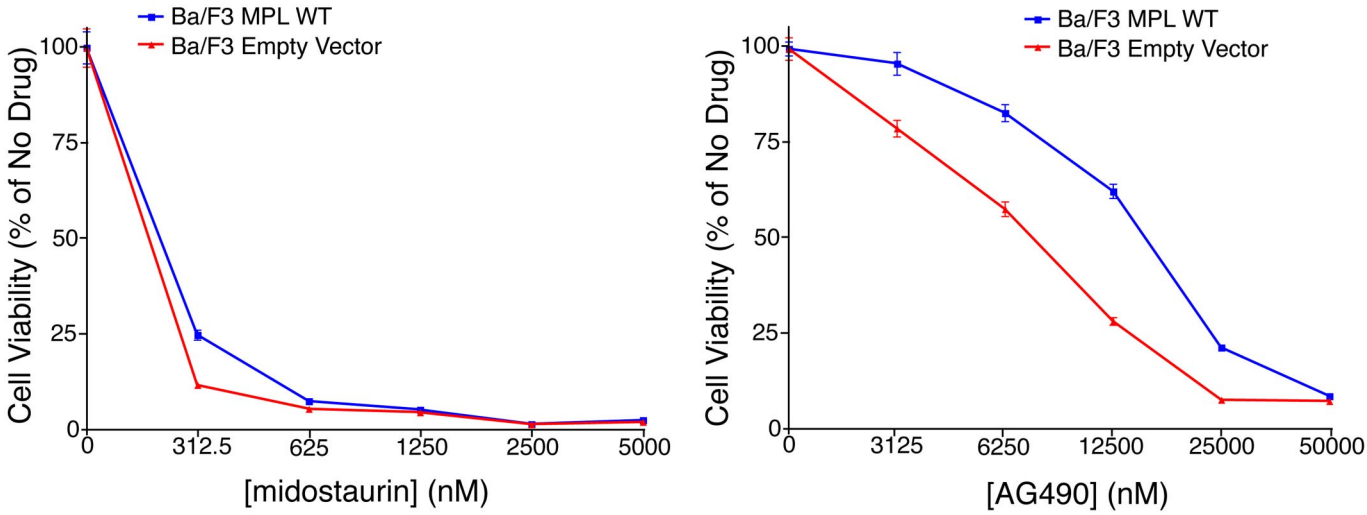

Fig. S7. JAK2-dependent parental BA/F3 cells are sensitive to JAK2 inhibition. A cell proliferation assay was performed with BA/F3 cells expressing MPL WT or empty vector over a dose gradient of midostaurin or AG490. Cell viability was measured at 72 h using an MTS assay.

Table S1. Complete RAPID results on patient 07-079

Gene	Viability	SE	*t* test 1	*t* test 2	Average of *t* test
ABL1	128	6	0.1340	0.3939	0.2640
ABL2	106	6	0.4862	0.1866	0.3364
ACK1	110	10	0.7658	0.5854	0.6756
ALK	118	4	0.8163	0.5552	0.6858
AXL	110	9	0.7719	0.4963	0.6341
BLK	113	3	0.7613	0.4137	0.5875
BMX	105	2	0.2510	0.0319	0.1415
BTK	94	2	0.0659	0.0274	0.0466
TP53RK	99	3	0.0956	0.0773	0.0864
CSF1R	98	2	0.0190	0.0908	0.0549
CSK	115	3	0.9399	0.0729	0.5064
DDR1	98	5	0.2032	0.0468	0.1250
DDR2	120	5	0.6568	0.5932	0.6250
STYK1	104	7	0.4125	0.1689	0.2907
EGFR	105	5	0.4041	0.0669	0.2355
EPHA1	111	5	0.6729	0.3198	0.4963
EPHA2	115	4	0.9541	0.0981	0.5261
EPHA3	111	2	0.5819	0.1037	0.3428
EPHA4	97	2	0.0205	0.0943	0.0574
EPHA7	122	9	0.5634	0.7165	0.6399
EPHA8	99	1	0.0501	0.0648	0.0574
EPHB1	92	6	0.1440	0.0342	0.0891
EPHB2	96	8	0.2755	0.1070	0.1913
EPHB3	88	7	0.1243	0.0475	0.0859
EPHB4	88	10	0.2049	0.1093	0.1571
EPHB6	100	3	0.1888	0.0137	0.1012
ERBB2	93	6	0.1844	0.0247	0.1045
ERBB3	88	5	0.0982	0.0221	0.0602
ERBB4	116	9	0.9234	0.9679	0.9457
FER	109	6	0.2909	0.5526	0.4217
FES	103	5	0.3185	0.0291	0.1738
FGFR1	101	6	0.2895	0.0677	0.1786
FGFR2	107	12	0.6637	0.4921	0.5779
FGFR3	98	9	0.3690	0.1425	0.2557
FGFR4	93	7	0.1720	0.0911	0.1315
FGR	97	11	0.3618	0.1977	0.2798
FLT1	104	5	0.3765	0.1117	0.2441
FLT3	104	3	0.2674	0.0282	0.1478
FLT4	112	2	0.5691	0.3895	0.4793
FRK	107	9	0.4920	0.5050	0.4985
FYN	84	7	0.1238	0.0302	0.0770
HCK	87	6	0.1073	0.0305	0.0689
IGF1R	100	4	0.0623	0.1555	0.1089
INSR	103	5	0.3016	0.0853	0.1935
ITK	94	13	0.3705	0.2420	0.3062
JAK1	81	9	0.1296	0.0528	0.0912
JAK2	34	4	0.0118	0.0009	0.0064
JAK3	87	3	0.0580	0.0089	0.0334
KDR	114	8	0.8917	0.8560	0.8738
LMTK2	114	8	0.8872	0.8044	0.8458
KIT	117	1	0.7965	0.9733	0.8849
LCK	102	5	0.3000	0.0284	0.1642
LTK	81	8	0.1034	0.0371	0.0702
LYN	96	8	0.2713	0.0643	0.1678
MATK	92	6	0.1536	0.0281	0.0908
MERTK	90	7	0.1539	0.0478	0.1009
MET	110	4	0.5657	0.2964	0.4310
MST1R	92	8	0.1888	0.0709	0.1298
MUSK	95	3	0.1293	0.0031	0.0662
NTRK1	116	11	0.9129	0.9828	0.9478
NTRK2	109	8	0.6588	0.3774	0.5181
NTRK3	100	1	0.0933	0.0445	0.0689
PDGFRA	83	9	0.1332	0.0669	0.1000
PDGFRB	89	8	0.1740	0.0827	0.1284

Gene	Viability	SE	*t* test 1	*t* test 2	Average of *t* test
PTK2	89	8	0.1486	0.1005	0.1245
PTK2B	73	7	0.0575	0.0179	0.0377
PTK6	100	5	0.1231	0.1916	0.1573
PTK7	95	1	0.0327	0.0460	0.0394
PTK9	108	7	0.2922	0.5095	0.4009
PTK9L	105	5	0.1644	0.3042	0.2343
RET	100	2	0.1608	0.0093	0.0851
ROR1	97	6	0.2447	0.0301	0.1374
ROR2	88	4	0.0761	0.0106	0.0434
ROS1	74	6	0.0601	0.0140	0.0370
RYK	92	2	0.0637	0.0134	0.0385
SRC	102	4	0.1885	0.0903	0.1394
SYK	80	2	0.0207	0.0180	0.0194
TEC	84	1	0.0125	0.0230	0.0177
TEK	89	3	0.0117	0.0630	0.0373
TIE	84	2	0.0034	0.0412	0.0223
TNK1	108	4	0.4766	0.2334	0.3550
TXK	100	5	0.2383	0.0649	0.1516
TYK2	115	1	0.9419	0.5658	0.7539
TYRO3	104	5	0.3555	0.0689	0.2122
YES1	98	6	0.1995	0.1046	0.1521
ZAP0	110	1	0.3441	0.2532	0.2987
N-RAS	103	7	0.4142	0.1397	0.2770
K-RAS	110	1	0.2901	0.3144	0.3023
EPHA5	100	2	0.1229	0.0549	0.0889
EPHA6	93	6	0.1772	0.0415	0.1093
SRMS	86	10	0.1869	0.0809	0.1339
AATK	85	6	0.0817	0.0452	0.0635
LMTK3	85	6	0.0770	0.0341	0.0556
CONTROL SINGLE	115	4	NA	NA	NA
CONTROL POOL	117	4	NA	NA	NA

NA, not applicable.

Table S2. siRNA targeting sequences

Gene	Targeting sequence
CSF1R	GGAGAGCUCUGACGUUUGA
CSF1R	CAACAACGCUACCUUCCAA
CSF1R	CCACGCAGCUGCCUUACAA
CSF1R	GGAGAGAGCGGGACUAUAC
EPHA4	GGUCUGGGAUGAAGUAUUU
EPHA4	GAAUGAAGUUACCUUAUUG
EPHA4	GAACUUGGGUGGAUAGCAA
EPHA4	GAGAUUAAAUUCACCUUGA
EPHA5	GGAAAGACGUGUCAUAUUA
EPHA5	GAGAAUGGCUCUUUAGAUA
EPHA5	GAACAGCCUUCAAGAAAUG
EPHA5	CCAGAAACAUCUUAAUCAA
FLT1	GAGCAAACGUGACUUAUUU
FLT1	CCAAAUGGGUUUCAUGUUA
FLT1	CAACAAGGAUGCAGCACUA
FLT1	GCCGGAAGUUGUAUGGUUA
FYN	GGAAUGGACUCAUAUGCAA
FYN	CAAAGGAAGUUUACUGGAU
FYN	GCUCUGAAAUUACCAAAUC
FYN	CGCAUGAAUUAUAUCCAUA
JAK1	CCACAUAGCUGAUCUGAAA
JAK1	UGAAAUCACUCACAUUGUA
JAK1	UAAGGAACCUCUAUCAUGA
JAK1	GCAGGUGGCUGUUAAAUCU
JAK2	GAGCAAAGAUCCAAGACUA
JAK2	GCCAGAAACUUGAAACUUA
JAK2	GAUCCUGGCAUUAGUAUUA
JAK2	ACAGAAUGCUGGAACAAUA
JAK3	GCGCCUAUCUUUCUCCUUU
JAK3	CCAGAAAUCGUAGACAUUA
JAK3	CCUCAUCUCUUCAGACUAU
JAK3	UGUACGAGCUCUUCACCUA
LMTK3	GAACAGCGAGCAGAUCAAA
LMTK3	AGAAGACGCCCGAGAGUUG
LMTK3	GCAAGAUGGUCUCCUUCCA
LMTK3	CGAGAUGCCACGACUAUUC
LYN	AGACUCAACCAGUACGUAA
LYN	AGAUUGGAGAAGGCUUGUA
LYN	GCGACAUGAUUAAACAUUA
LYN	GAUCCAACGUCCAAUAAAC
PDGFRA	GCACGCCGCUUCCUGAUAU
PDGFRA	CAUCAGAGCUGGAUCUAGA
PDGFRA	GGCCUUACUUUAUUGGAUU
PDGFRA	GAGCUUCACCUAUCAAGUU
PDGFRB	GAAAGGAGACGUCAAAUAU
PDGFRB	GGAAUGAGGUGGUCAACUU
PDGFRB	CAACGAGUCUCCAGUGCUA
PDGFRB	UGACAACGACUAUAUCAUC
PTK2B	GAACAUGGCUGACCUCAUA
PTK2B	GGACCACGCUGCUCUAUUU
PTK2B	GGACGAGGACUAUUACAAA
PTK2B	GAGGAAUGCUCGCUACCGA
NRAS	CACCAUAGAGGAUUCUUAC
NRAS	CAAGUGUGAUUUGCCAACA
NRAS	AUACGCCAGUACCGAAUGA
NRAS	AAAGCGCACUGACAAUCCA
KRAS2	CGAAUAUGAUCCAACAAUA
KRAS2	UAAGGACUCUGAAGAUGUA
KRAS2	GACAAAGUGUGUAAUUAUG
KRAS2	GCUCAGGACUUAGCAAGAA
ROR1	GCAAGCAUCUUUACUAGGA
ROR1	GAGCAAGGCUAAAGAGCUA
ROR1	GAGAGCAACUUCAUGUAAA
ROR1	GAGAAUGUCCUGUGUCAAA