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Supplemental Information

ADAM8 Is an Antigen of Tyrosine Kinase Inhibitor-Resistant Chronic Myeloid Leukemia Cells Identified by Patient-Derived Induced Pluripotent Stem Cells

Masashi Miyauchi, Junji Koya, Shunya Arai, Sho Yamazaki, Akira Honda, Keisuke Kataoka, Akihide Yoshimi, Kazuki Taoka, Keiki Kumano, and Mineo Kurokawa

Experimental Procedures

Transduction of CML cell lines

To obtain retrovirus supernatants, Plat-A packaging cells were transiently transfected with pMXs-ADAM8-flag-neo. 48 hr later, the viral supernatant was collected and utilized for infection. The vector-transduced cells were selected by medium containing G418 (0.8 mg/ml for K562 and 1.2 mg/ml for NCO2).

Immunoblotting

Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Membranes were probed with the following antibodies: anti-flag (Sigma), anti–c-Abl (Cell Signaling Technology) and anti–β-actin (Cell Signaling Technology). Blots were detected using an ImmunoStar Zeta (Wako Pure Chemical Industries) and an LAS-3000 image analyzer (Fujifilm), as recommended by the manufacturers.

Immunofluorescence analysis

Lin-/c-Kit+/BCR-ABL+ cells were purified from BM of a murine model of CML. A total of 2×10^4 to 5×10^4 cells were cytospun onto glass slides. The cells were fixed with 3.7% formaldehyde in PBS for 30 minutes, permeabilized by treatment with 0.2% Triton X in PBS for 10 minutes, and blocked with 1% BSA in PBS for 60 minutes. Then, the slides were incubated with rabbit anti–ADAM8 polyclonal antibody (bs-4195R; 1:100 dilution; Bioss ANTIBODIES) overnight at 4°C, followed by incubation with Alexa Fluor 555 goat anti-rabbit IgG (1:250 dilution; Thermo Fisher SCIENTIFIC) for 3 hr. After the cells were washed, they were treated with ProLong Gold Antifade Reagent with DAPI (Thermo Fisher SCIENTIFIC). Fluorescence images were captured with BZ-X710 All-in-One Fluorescence Microscope (KEYENCE).

Retrovirus production and a murine model of CML

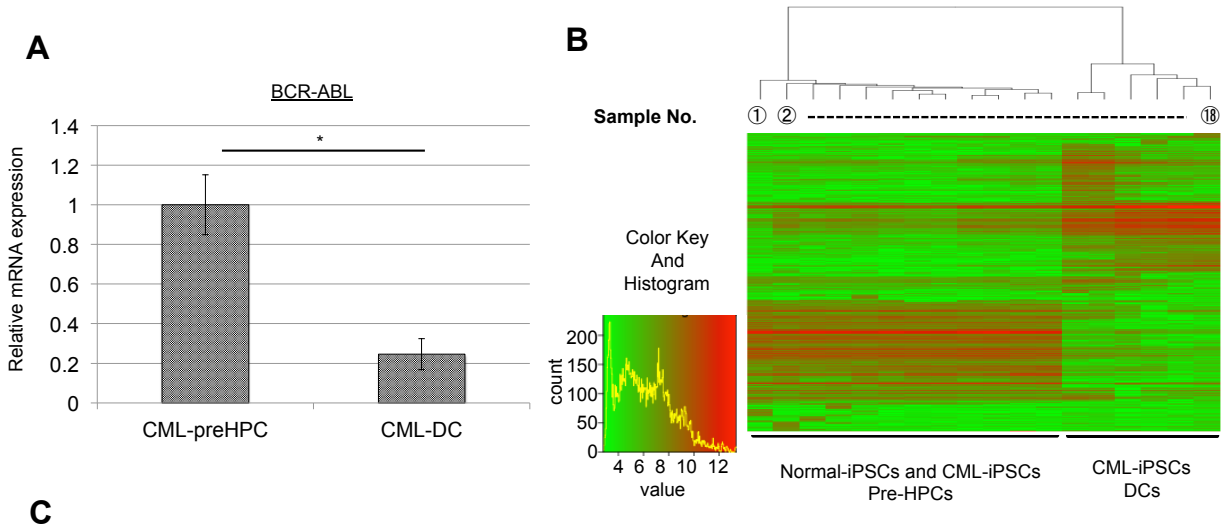
To obtain retrovirus supernatants, Plat-E packaging cells were transiently transfected with pGCDNsam-BCR–ABL-IRES-GFP. c-Kit+ sorted C57/B6 mouse BM cells were purified and incubated in α-MEM with 20% FCS, 1% PS and cytokines (50 ng/ml SCF, 50 ng/ml TPO, 10 ng/ml IL-6) at 37 °C in a 5% CO_2 incubator for 24 hr, as previously described (Sato *et al.*, 2014). Subsequently, cultured cells were infected with retrovirus in the presence of RetroNectin (Takara Bio Inc.). The infected cells were collected 48 h after retrovirus infection, and vector-transduced cells were injected into lethally irradiated (9.5 Gy) recipient mice.

Cell-cycle analysis

For cell-cycle analyses with anti-Ki67 antibody and Hoechst 33342, we followed the protocol described earlier with minor modification (Wilson *et al.*, 2008).

SUPPLEMENTAL FIGURES

Supplemental Figure 1



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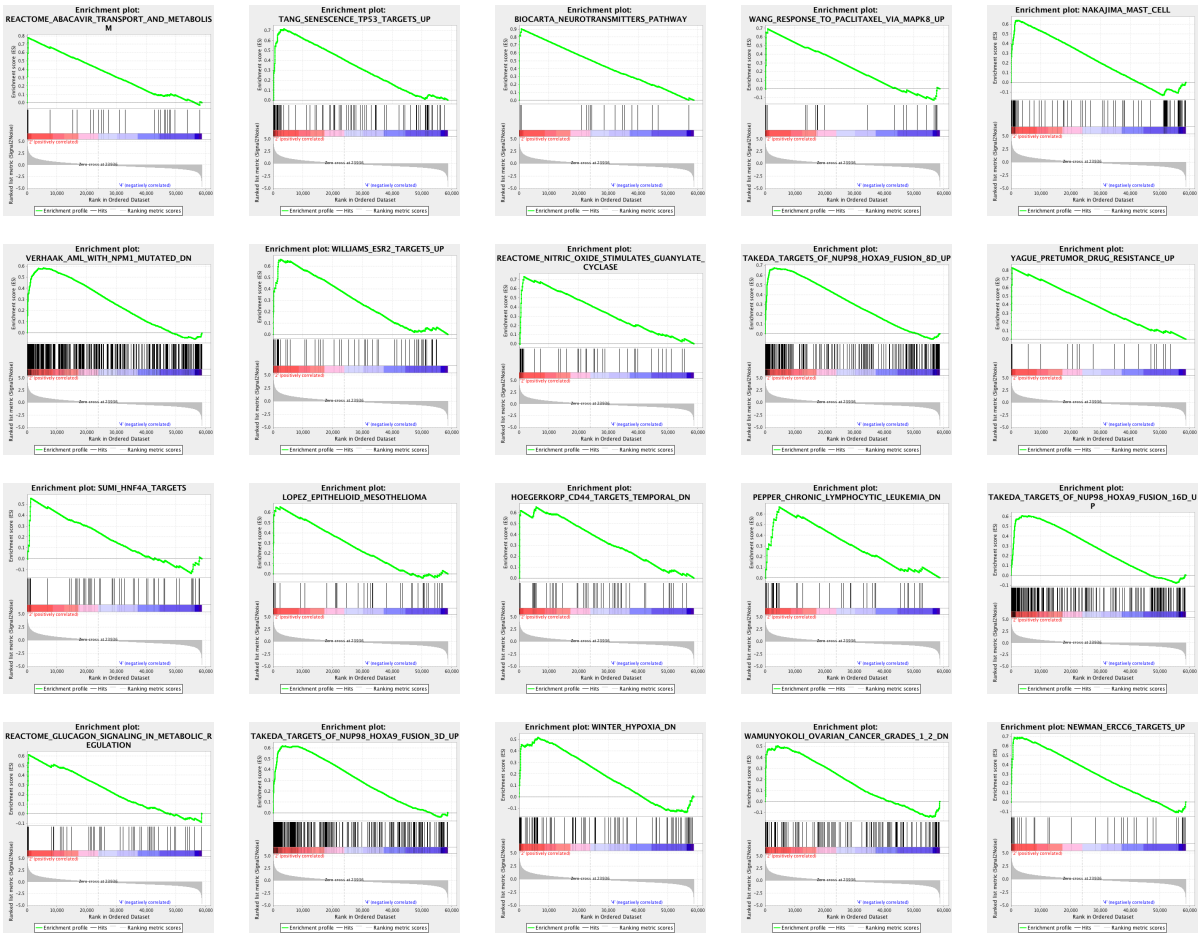

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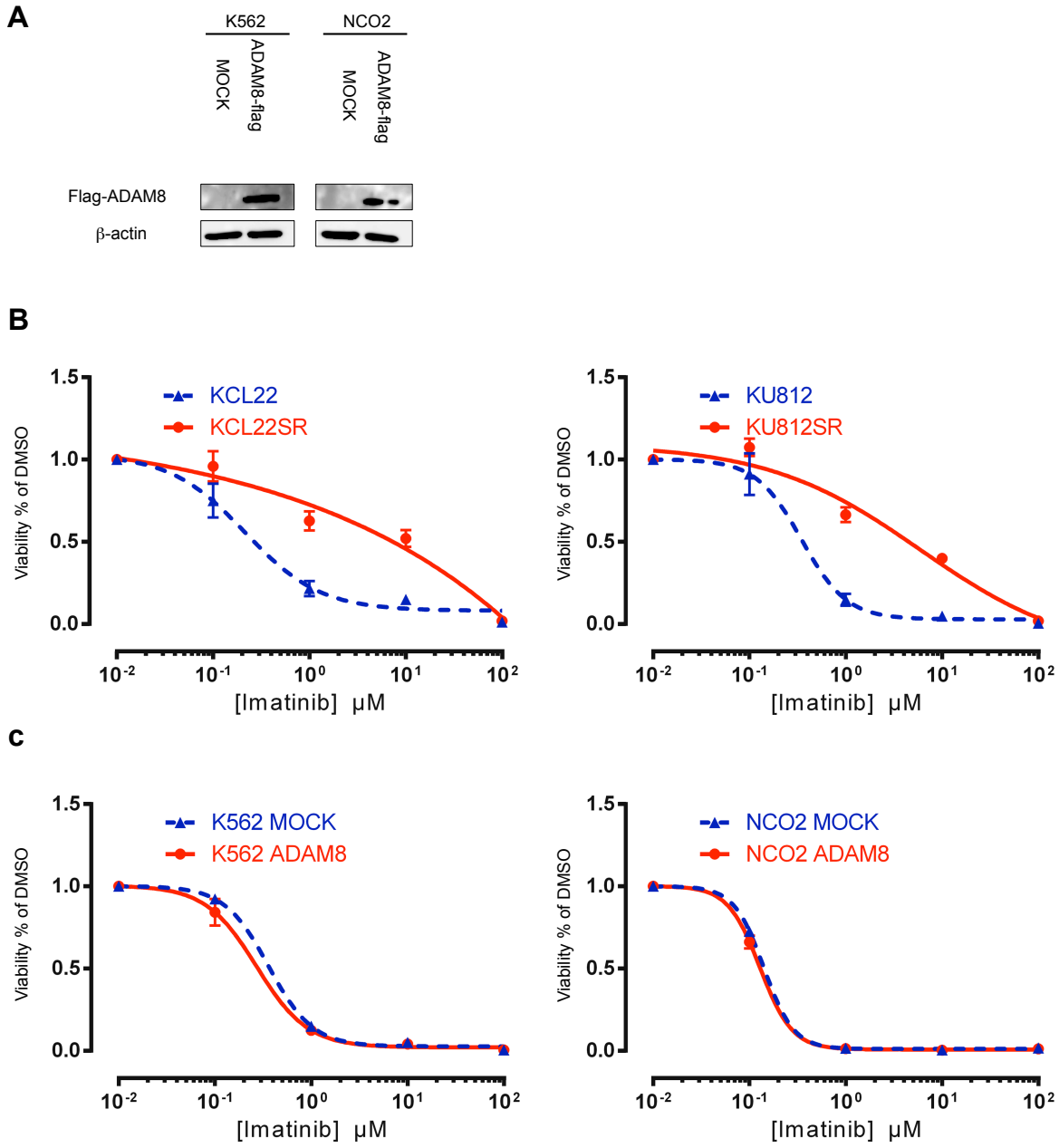

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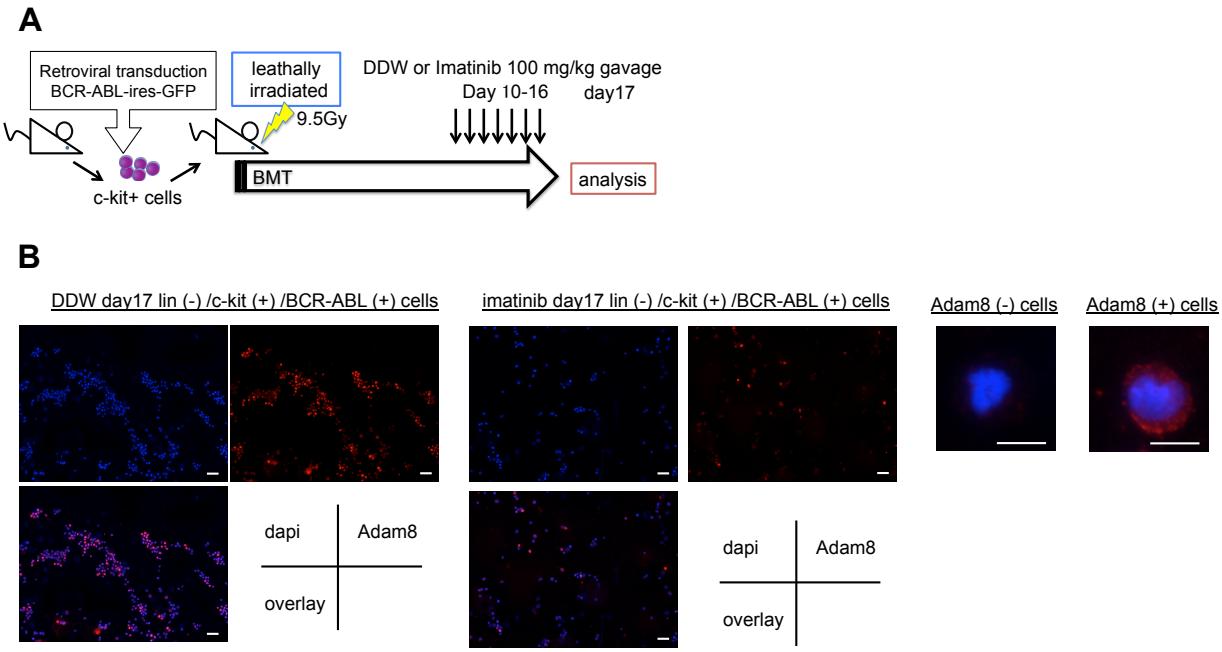
Supplemental Figure 3



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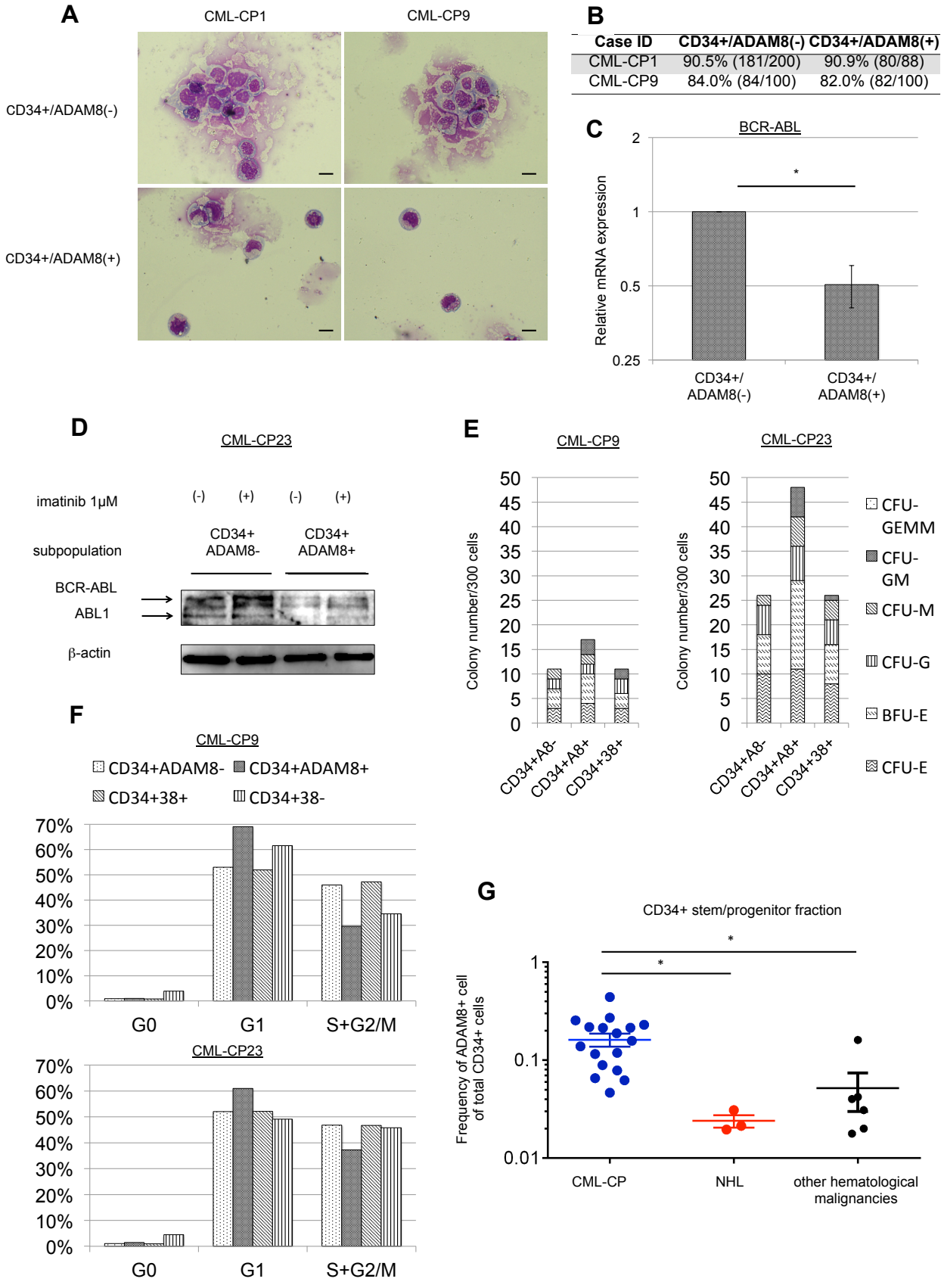
Supplemental Figure 4



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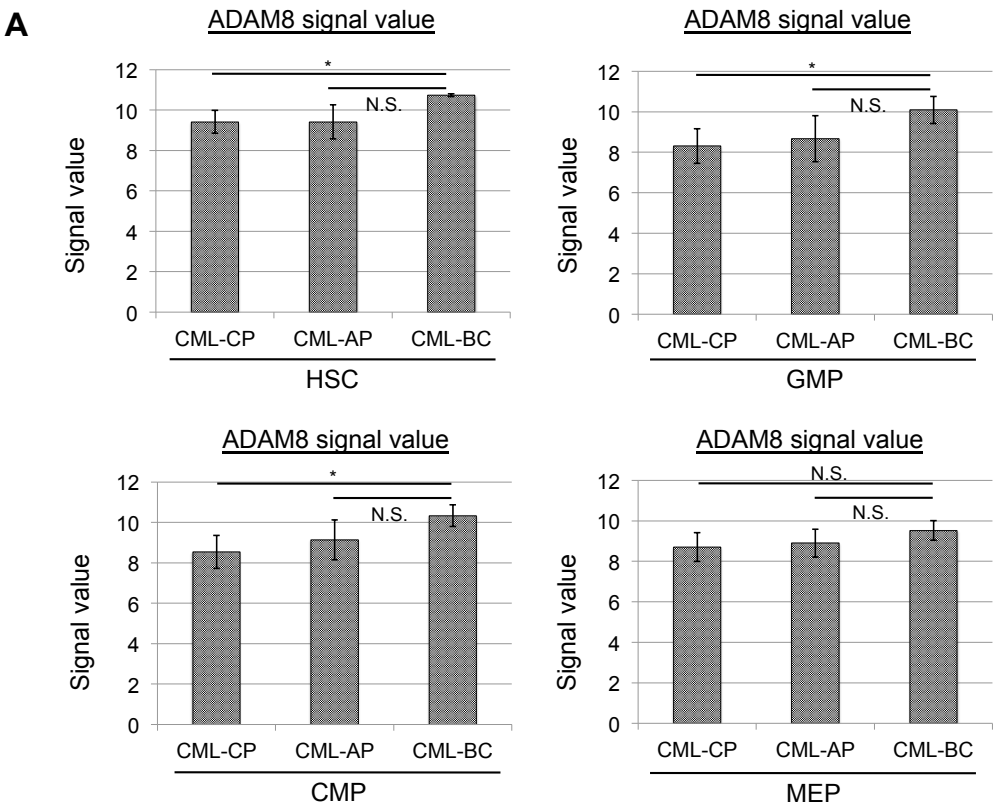
Supplemental Figure 5



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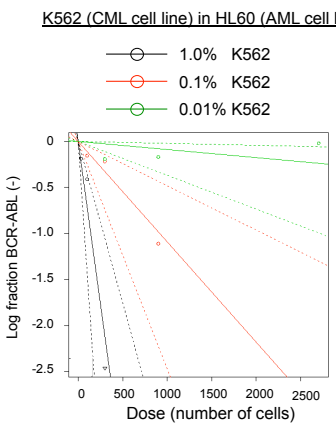
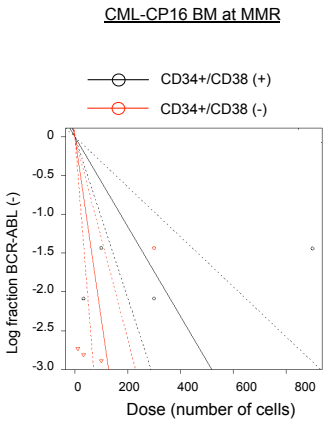
Supplemental Figure 6

A





1.0% K562

Number of cells per well	Number of BCR-ABL positive well
300	6/6
100	2/6
33	1/6
Estimated frequency (95% CI)	1/142 (1/285-1/70.7)

0.1% K562

Number of cells per well	Number of BCR-ABL positive well
900	4/6
300	1/6
100	1/6
Estimated frequency (95% CI)	1/917 (1/2078-1/405.2)

0.01% K562

Number of cells per well	Number of BCR-ABL positive well
2700	0/6
900	1/6
300	1/6
Estimated frequency (95% CI)	1/11397 (1/47762-1/2719.9)

*** ***

CD34+/CD38(+)

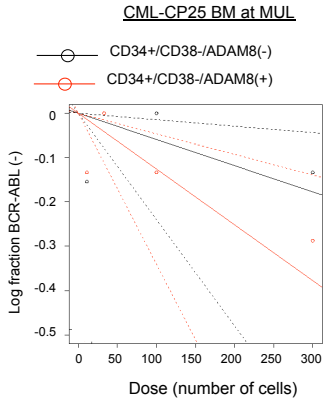
Number of cells per well	Number of BCR-ABL positive well
900	6/8
300	7/8
100	6/8
33	7/8
Estimated frequency (95% CI)	1/172.8 (1/310.4-1/96.3)

CD34+/CD38(-)

Number of cells per well	Number of BCR-ABL positive well
300	6/8
100	8/8
33	8/8
11	8/8
Estimated frequency (95% CI)	1/42.6 (1/76.7-1/23.8)

***

B



CD34+/CD38-/ ADAM8(-)

Number of cells per well	Number of BCR-ABL positive well
300	1/8
100	0/8
33	0/8
11	1/7
Estimated frequency (95% CI)	1/1691 (1/6864-1/417)

CD34+/CD38(-)/ ADAM8(+)

Number of cells per well	Number of BCR-ABL positive well
300	2/8
100	1/8
33	0/8
11	1/8
Estimated frequency (95% CI)	1/795 (1/2152-1/294)

C

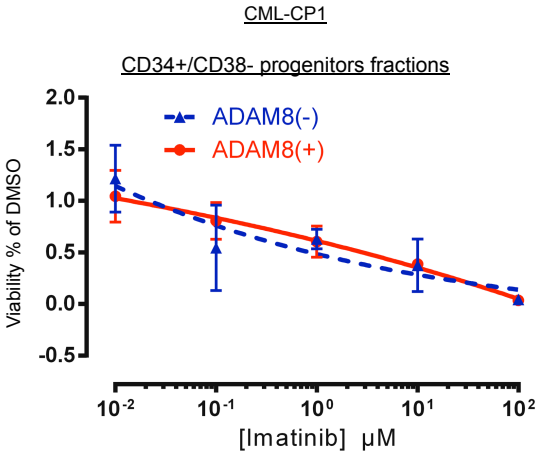


Figure S1. Gene expression profiling of CML-pre-HPCs. Related to Figure 3
(A) The expression of mRNA levels of BCR-ABL in CML-pre-HPCs and CML-DCs. Real-time quantitative PCR revealed that CML-pre-HPCs showed distinct expression levels of BCR-ABL from CMP-DCs. Data show means ± s.d., n = 3 independent experiments. (*P* values: two-tailed Student's *t*-test; * $P < 0.05$)
(B) Hierarchical Clustering analysis of gene expression. Samples are included two major clusters, one consists of pre-HPCs and another consists of DCs. All samples are listed in Supplemental Table 2.
(C) GSEA analysis of CML-pre-HPCs. 20 of all 23 gene sets enriched from 3267 curated gene sets (false discovery rate q-value < 0.05) are shown. All 23 gene sets enriched from 3267 curated gene sets are descbed in Supplemental Table 3.

Figure S2. Overexpression of ADAM8 in CML cell lines. Related to Figure 4
(A) Overexpression of ADAM8-flag in CML cell line. Expression of ADAM8 is confirmed in two CML cell lines.
(B) Viability assay of TKI-resistant cell lines which was previously reported. Viability assay distinguish TKI-resistant cell lines from sensitive cell lines. Data show means ± s.d., n = 3 independent experiments.
(C) Viability assay of CML cell lines which express ADAM8. Overexpression of ADAM8 has no effect on TKI-sensitivity in CML cell lines. Data show means ± s.d., n = 3 independent experiments.

Figure S3. The expression of Adam8 in a murine CML model. Related to Figure 4
(A) Experimental scheme for analysis of Adam8 in a murine CML model.
(B) Immunofluorescence microscopy of Adam8 in Lin-/c-Kit+/BCR-ABL+ BM of a murine CML model. Scale bars: 50 μm (left), 10 μm (right).
(C) The frequency of Adam8+ cells does not show significant difference between DDW cohort and imatinib cohort. Data show means ± s.e.m. n = 2 mice.

Figure S4. Characterization of ADAM8+/CD34+ cells in primary samples of newly diagnosed CML-CP patients. Related to Figure 4
(A) Microscopy of CD34+/ADAM8+ cells in BM of CML-CP patients at diagnosis. Scale bars: 10 μm.
(B) FISH analysis of CD34+/ADAM8+ cells. The frequency of *BCR-ABL*+ cells does not show significant difference between CD34+/ADAM8+ cells and CD34+/ADAM8- cells.
(C) The expression of mRNA levels of BCR-ABL in CD34+/ADAM8+ cells. CD34+/ADAM8+ cells had lower expression levels of BCR-ABL than CD34+/ADAM8-. Data show means ± s.e.m., n = 2 patients. (*P* values: two-tailed Student's *t*-test; * $P < 0.05$)
(D) The expression of protein levels of BCR-ABL in CD34+/ADAM8+ cells. CD34+/ADAM8+ cells had lower expression levels of BCR-ABL than CD34+/ADAM8-.
(E) CFC assay of CD34+/ADAM8+ cells. CFC assay revealed the distinct colony-forming capacity of CD34+/ADAM8+ cells. Data show means, n = 2, technical replicates.
(F) Cell-cycle analysis of CD34+/ADAM8+ cells. CD34+/ADAM8+ cells accumulated in G1 phase of cell cycle. Data show means, n = 2, technical replicates.

(G) ADAM8 expression in BM of patients with CML-CP (n = 18), NHL (n = 3) and other hematological malignancies (AML: n = 1, ALL; n = 1, MDS; n = 1, CMML; n = 3) on FCM analysis. The frequency of ADAM8+ cells is significantly higher in CD34+.
Data show means ± s.e.m. n = shown above, patients. (*P* values: two-tailed Student's *t*-test; * $P < 0.05$)

Figure S5. ADAM8 expression in patients with the progressive phase of CML. Related to Figure 5
(A) The expression of ADAM8 in primary sample with progressive phase of CML. Published array data (GSE47927) showed that primary samples from CML-BC highly expressed ADAM8 in the subpopulations. HSC: hematopoietic stem cell, CMP: common myeloid progenitor, GMP: granulocyte monocyte progenitor, MEP: megakaryocyte erythroid progenitor. Data show means ± s.d. CML-CP; n = 6, CML-AP; n = 4, CML-BC; n = 2 patients.

Figure S6. The frequency of residual CML cells in ADAM8+/CD34+/CD38+ subpopulation of CML-CP patients with optimal TKI response under the treatment of TKI. Related to Figure 6
(A) Limiting dilution assay of CML cell lines and CD34+/CD38- fractions as controls. Data of cell line and a patient show n = 1. (CI: confidential interval).
(*P* values: chi-square test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)
(B) The frequency of residual CML cells in ADAM8- and ADAM8+ subpopulation among CD34+/CD38- fraction. Data of a patient show n = 1.
(C) TKI-sensitivity of ADAM8+/CD34+/CD38- cells in a patient with newly diagnosed CML-CP. Data show means ± s.d. n = 3 technical replicates.

SUPPLEMENTAL TABLES

Table S1. List of all ES-like clones obtained

	Clone No.	Stem cell gene expression	Integration check	Teratoma formation	G-banded chromosomal analysis	BCR-ABL expression
Healthy	12	○	○	○	46 XY	
donor	14	○	○	○	46 XY	
	17	○	○	○	46 XY	
CML-CP	1	○				
patient No.1	2					
	3	○	○	○	46 XY t(9;22)	○
	4	○				○
	5	○	○	○	46 XY t(9;22)	○
	6	○				
	7	○				
	8	○	○		46 XY t(9;22)	○
	9 to 14	○				○
CML-CP	1	○			46 XY	×
patient No. 2	2	○	○			×
	3	○	○	○	46 XY	×
	4	○	○	○	46 XY t(9;22)	○
	5					×
	6	○				×
	7					×
	8	○			46 XY t(9;22)	○

Table S2. Hierarchical Clustering analysis of gene expression profiles

Sample No.	Sample	Subpopulation	Treatment
1	Normal-iPSCs H_1	pre-HPCs	imatinib 2.5 μM
2	CML-iPSCs Pt 1_2	pre-HPCs	imatinib 2.5 μM
3	Normal-iPSCs H_2	pre-HPCs	imatinib 2.5 μM
4	Normal-iPSCs H_2	pre-HPCs	DMSO
5	CML-iPSCs Pt 1_2	pre-HPCs	DMSO
6	Normal-iPSCs H_1	pre-HPCs	DMSO
7	CML-iPSCs Pt 2	pre-HPCs	DMSO
8	CML-iPSCs Pt 2	pre-HPCs	imatinib 2.5 μM
9	Normal-iPSCs P t2	pre-HPCs	DMSO
10	Normal-iPSCs Pt 2	pre-HPCs	imatinib 2.5 μM
11	CML-iPSCs Pt 1_1	pre-HPCs	DMSO
12	CML-iPSCs Pt 1_1	pre-HPCs	imatinib 2.5 μM
13	CML-iPSCs Pt 1_1	DCs	DMSO
14	CML-iPSCs Pt 1_1	DCs	imatinib 2.5 μM
15	CML-iPSCs Pt 1_2	DCs	DMSO
16	CML-iPSCs Pt 1_2	DCs	imatinib 2.5 μM
17	CML-iPSCs Pt 2	DCs	DMSO
18	CML-iPSCs Pt 2	DCs	imatinib 2.5 μM

Table S3. GSEA analysis for CML-pre-HPCs compared to CML-DCs in the absence of imatinib. 23 gene sets enriched from 3267 curated gene sets (C2) are listed (FDR q-value < 0.05).

No. of gene sets	Names of gene sets	FDR q-value
1	REACTOME_ABACAVIR_TRANSPORT_AND_METABOLISM	0.041
2	TANG_SENESCENCE_TP53_TARGETS_UP	0.041
3	BIOCARTA_NEUROTRANSMITTERS_PATHWAY	0.041
4	WANG_RESPONSE_TO_PACLITAXEL_VIA_MAPK8_UP	0.041
5	NAKAJIMA_MAST_CELL	0.041
6	VERHAAK_AML_WITH_NPM1_MUTATED_DN	0.041
7	WILLIAMS_ESR2_TARGETS_UP	0.041
8	REACTOME_NITRIC_OXIDE_STIMULATES_GUANYLATE_CYCLASE	0.041
9	TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_8D_UP	0.041
10	YAGUE_PRETUMOR_DRUG_RESISTANCE_UP	0.041
11	SUMI_HNF4A_TARGETS	0.041
12	LOPEZ_EPITHELIOID_MESOTHELIOMA	0.041
13	HOEGERKORP_CD44_TARGETS_TEMPORAL_DN	0.045
14	PEPPER_CHRONIC_LYMPHOCYTIC_LEUKEMIA_DN	0.045
15	TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_16D_UP	0.045
16	REACTOME_GLUCAGON_SIGNALING_IN_METABOLIC_REGULATION	0.044
17	TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_3D_UP	0.044
18	WINTER_HYPOXIA_DN	0.046
19	WAMUNYOKOLI_OVARIAN_CANCER_GRADES_1_2_DN	0.046
20	NEWMAN_ERCC6_TARGETS_UP	0.046
21	CHEN_NEUROBLASTOMA_COPY_NUMBER_GAINS	0.046
22	WIERENGA_STAT5A_TARGETS_DN	0.048
23	OUILLETTE_CLL_13Q14_DELETION_UP	0.048

Table S4. Characteristics of patients

Table shows information of patients at the time of diagnosis, whose samples were used in the present study.

Samples used for the establishment of CML-iPSCs.

Case No.	Use	Age	Gender	Ph1 (G-band)	FISH *BCR-ABL*	BCR-ABL type
CML-CP 1	FCM	66	Male	20/20 (100%)	N/A	p210
CML-CP 2	FCM	59	Female	20/20 (100%)	180/200 (90%)	p210 (b3a2)
CML-CP 3	FCM	66	Male	20/20 (100%)	193/200 (96.5%)	p210 (b3a2)
CML-CP 4	FCM Establishment of CML-iPSCs Pt.2	22	Male	20/20 (100%)	171/200 (85.5%)	p210 (b3a2)
CML-CP 5	FCM	54	Female	20/20 (100%)	194/200 (97%)	p210 (b3a2)
CML-CP 6	FCM	40	Male	20/20 (100%)	N/A	p210 (b3a2)
CML-CP 7	FCM	66	Male	20/20 (100%)	188/200 (94%)	p210 (b2a2)
CML-CP 8	FCM	62	Female	19/20 (95%)	N/A	p210 (b3a2)
CML-CP 9	FCM	56	Male	20/20 (100%)	N/A	p210 (b2a2)
CML-CP 10	FCM	65	Male	20/20 (100%)	192/200 (96%)	p210 (b2a2)
CML-CP 11	FCM	61	Female	20/20 (100%)	196/200 (98%)	p210 (b3a2)
CML-CP 12	FCM	58	Male	19/20 (95%)	N/A	p210 (b3a2)
CML-CP 13	FCM	33	Male	16/20 (80%)	N/A	p210 (b3a2)
CML-CP 14	FCM	51	Female	17/20 (85%)	N/A	p210 (b2a2)
CML-CP 15	FCM	65	Male	20/20 (100%)	N/A	p210 (b3a2)
CML-CP 16	FCM Limiting dilution assay	42	Male	20/20 (100%)	N/A	p210 (b2a2)
CML-CP 17	FCM	75	Female	20/20 (100%)	192/200 (96%)	p210 (b2a2)
CML-CP 18	FCM	54	Female	20/20 (100%)	N/A	p210 (b3a2)
CML-CP 19	FCM	63	Female	20/20 (100%)	186/200 (93%)	p210 (b3a2)
CML-CP 20	FCM	51	Female	19/20 (95%)	192/200 (96%)	p210 (b3a2)
CML-CP 21	FCM	35	Male	20/20 (100%)	N/A	p210
CML-CP 22	FCM	86	Male	20/20 (100%)	193/200 (96.5%)	p210 (b2a2)
CML-CP 23	FCM	23	Female	20/20 (100%)	N/A	p210 (b3a2)
CML-CP 24	Limiting dilution assay	67	Female	20/20 (100%)	N/A	p210 (b3a2)
CML-CP 25	Limiting dilution assay	49	Female	20/20 (100%)	N/A	p210 (b3a2)
CML-CP 26	Establishment of CML-iPSCs Pt.1	44	Male	20/20 (100%)	N/A	p210 (b3a2)

Table S5. List of antibodies for flow cytometery

Each table shows antibodies used for mouse cells and human cells.

Mouse

Epitope	Clone	Fluorophore	Supplier
Gr-1	RB6-8C5	Biotin	BioLegend
Mac-1	M1/70	Biotin	BioLegend
B220	RA3-6B2	Biotin	BioLegend
TER-119	TER-119	Biotin	BioLegend
CD3ε	145-2C11	Biotin	BioLegend
CD4	GK1.5	Biotin	BioLegend
CD8a	53-6.7	Biotin	BioLegend
CD127	A7R34	Biotin	BioLegend
Sca-1	E13-161.7	APC-Cy7	BioLegend
c-Kit	2B8	PE-Cy7	BioLegend

Human

Epitope	Clone	Fluorophore	Supplier
CD34	581	PE-Cy7	Biolegend
	4H11	APC	eBioscience
CD38	HIT2	FITC	Biolegend
CD43	DFT1	PE	BECKMAN COULTER
	4-29-5-10-21	PerCP-eFluor 710	eBioscience
CD45	H130	APC	Biolegend
CD90	5E10	Alexa Fluor 647	Biolegend
ADAM8	REA331	PE	Miltenyi Biotec
	REA331	APC	Miltenyi Biotec
IgG isotype	MOPC-21	Alexa Fluor 647	Biolegend
IgG isotype	679.1Mc7	PE	BECKMAbN COULTER

Table S6. List of primers

Gene	Use	Forward	Reverse
IL1RL1	real-time qPCR	ATGGGGTTTTGGATCTTAGCAAT	CACGGTGTAACTAGGTTTTCCTT
MEIS1	real-time qPCR	GATATAGCCGTGTTCGCCAAA	CGGTGGCAGAAATTGTCACAT
ADAM8	real-time qPCR	CGATGATGCTGCCTGCGATTG	CGCAGGTGGAGGGTGAAGTT
BCR-ABL	real-time qPCR	AACTCCAGACTGTCCACAGCA	AACGAGCGGCTTCACTCA
18s RNA	real-time qPCR	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
OCT3/4	RT-PCR	CCCCAGGGCCCCATTTTGGTACC	ACCTCAGTTTGAATGCATGGGAGAGC
OCT3/4 (transgene)	RT-PCR	CATTCAAACTGAGGTAAGGG	TAGCGTAAAAGGAGCAACATAG
KLF4	RT-PCR	ACCCATCCTTCCTGCCCGATCAGA	TTGGTAATGGAGCGGCGGGACTTG
KLF4 (transgene)	RT-PCR	CCACCTCGCCTTACACATGAAGA	TAGCGTAAAAGGAGCAACATAG
SOX2	RT-PCR	TTCACATGTCCCAGCACTACCAGA	TCACATGTGTGAGAGGGGCAGTGTGC
SOX2 (transgene)	RT-PCR	TTCACATGTCCCAGCACTACCAGA	TTTGTTTGACAGGAGCGACAAT
L-MYC	RT-PCR	GCGAACCCAAGACCCAGGCCTGCTCC	CAGGGGGTCTGCTCGCACCGTGATG
L-MYC (transgene)	RT-PCR	GGCTGAGAAGAGGATGGCTAC	TTTGTTTGACAGGAGCGACAAT
LIN28	RT-PCR	AGCCATATGGTAGCCTCATGTCCGC	TCAATTCTGTGCCTCCGGGAGCAGGGTAGG
LIN28 (transgene)	RT-PCR	AGCCATATGGTAGCCTCATGTCCGC	TAGCGTAAAAGGAGCAACATAG
EBNA-1 (transgene)	RT-PCR	ATCAGGGCCAAGACATAGAGATG	GCCAATGCAACTTGGACGTT
GAPDH	RT-PCR	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
BCR-ABL	Pre-amplification	AGAAGCTTCTCCCTGACATCCG	GGTACCAGGAGTGTTTCTCCAGACTG
	one-step RT-PCR	TGAAACTCCAGACTGTCC	TCAGACCCTGAGGCTCAAAG
ACTINB	Pre-amplification	CCAACCGCGAGAAGATGAC	TAGCACAGCCTGGATAGCAA
	one-step RT-PCR	CGCGAGAAGATGACCCAGAT	CACAGCCTGGATAGCAACGT

SUPPLEMENTAL REFERENCES

Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C. F., Eshkind, L., Bockamp, E., *et al.* (2008) Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair, *Cell*, 135, 1118–1129.