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Supplemental Information

Targeting the Extracellular Signal-Regulated Kinase 5 Pathway to Suppress Human Chronic Myeloid Leukemia Stem Cells

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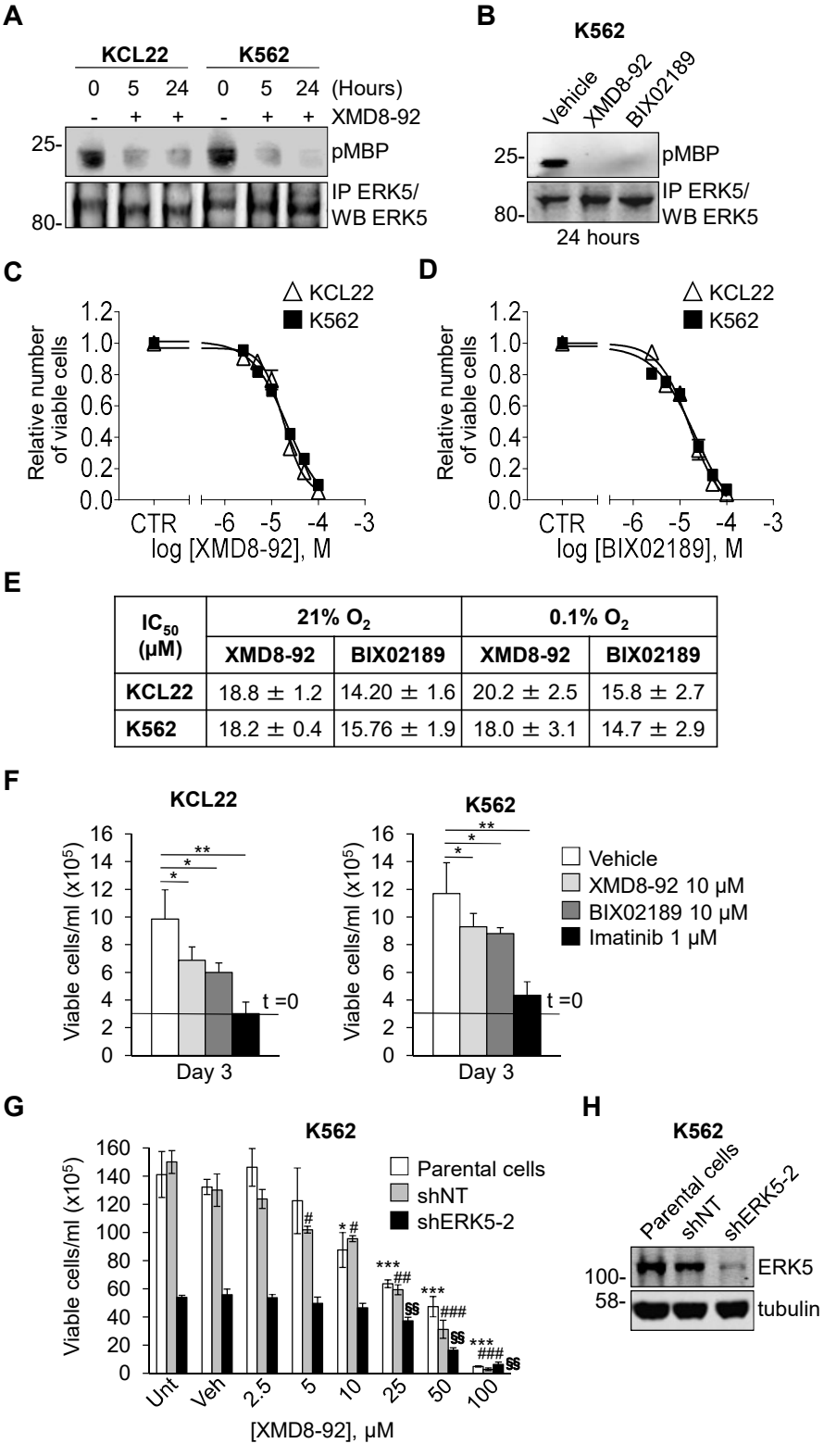
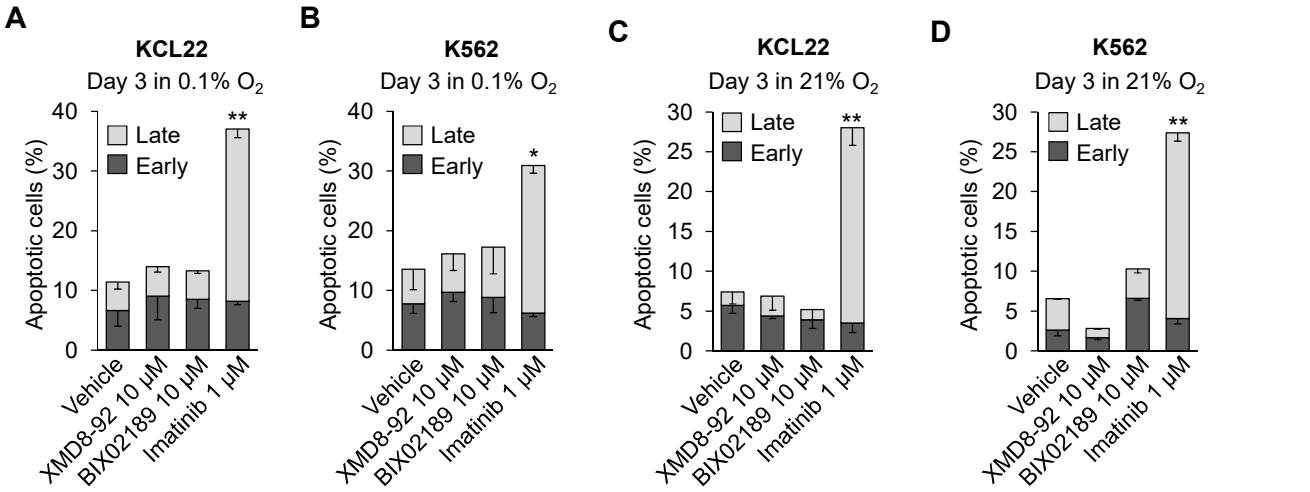
Supplementary Figure 1



IC_{50} (µM)	21% O_2		0.1% O_2	
	XMD8-92	BIX02189	XMD8-92	BIX02189
KCL22	18.8 ± 1.2	14.20 ± 1.6	20.2 ± 2.5	15.8 ± 2.7
K562	18.2 ± 0.4	15.76 ± 1.9	18.0 ± 3.1	14.7 ± 2.9

Supplementary Figure 2



E

KCL22
Day 3 in 21% O_2

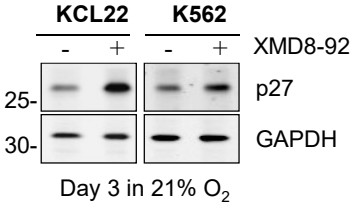
	Vehicle	XMD8-92
G0/G1	52.4 ± 6.9	74.4 ± 5.6*
S	40.8 ± 8.9	19.5 ± 5.0*
G2/M	6.8 ± 2.5	6.1 ± 2.1

F

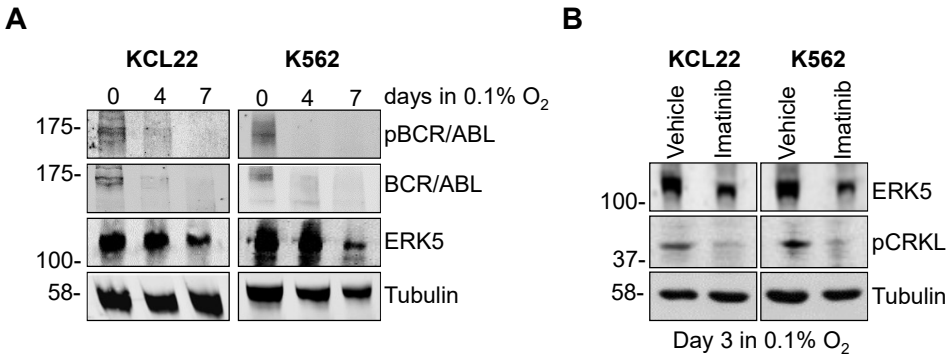
K562
Day 3 in 21% O_2

	Vehicle	XMD8-92
G0/G1	38.6 ± 1.8	50.4 ± 0.5*
S	59.8 ± 1.8	47.5 ± 0.6*
G2/M	1.6 ± 0.3	2.1 ± 0.3

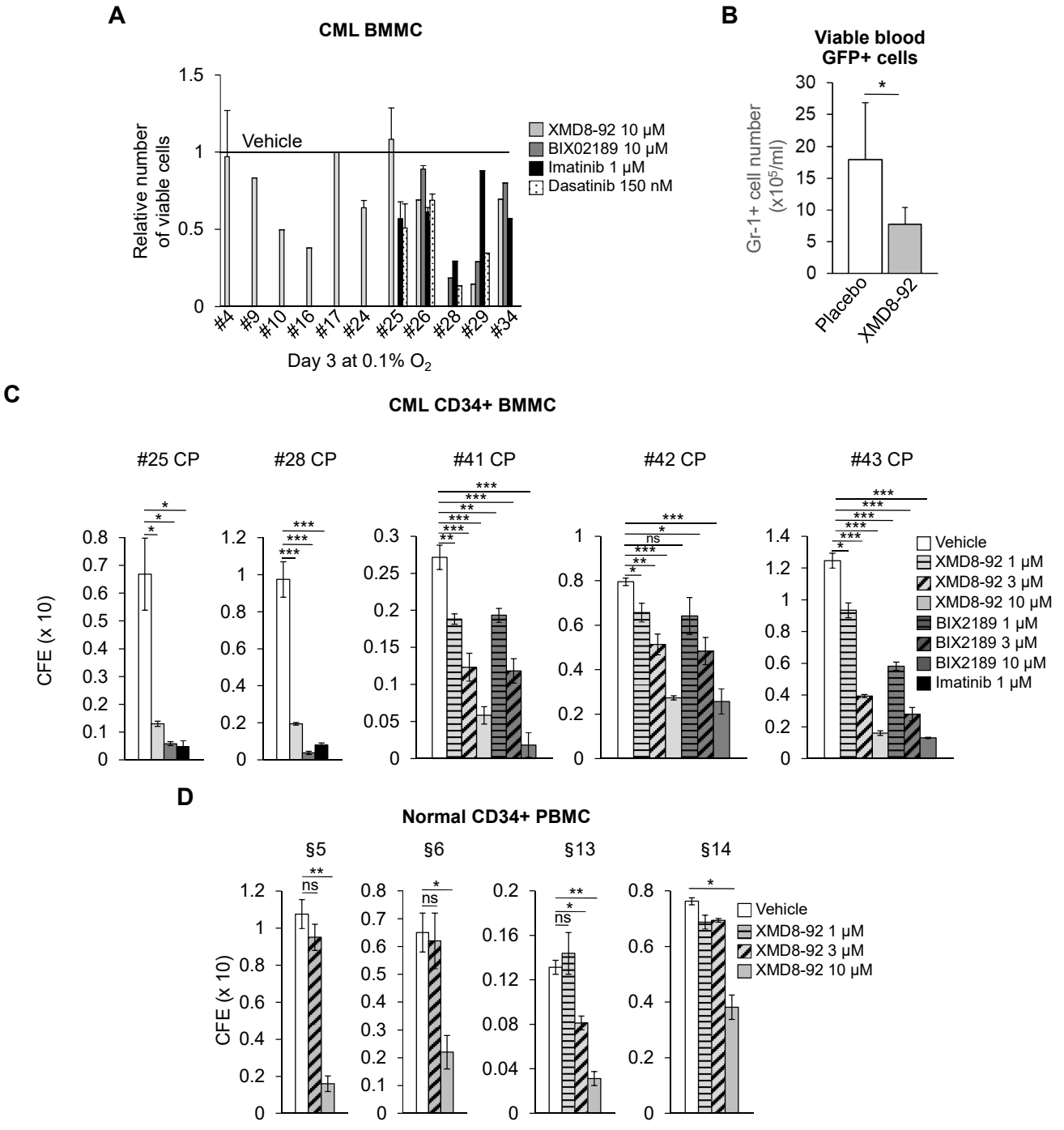
G



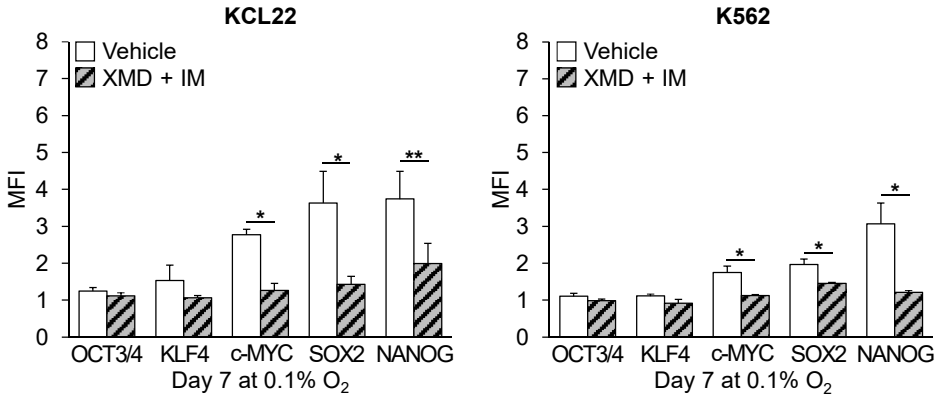
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Targeting the Extracellular signal-Regulated Kinase 5 pathway to suppress human chronic myeloid leukemia stem cells

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. A, B) *Pharmacological inhibition of ERK5 activity.* KCL22 or K562 cells were treated with DMSO (Vehicle), 10 μM XMD8-92 or 10 μM BIX02189 for the indicated times and lysed. Immunoprecipitated ERK5 was subjected to nonradioactive *in vitro* kinase assay. The drugs impaired ERK5 ability to phosphorylate its substrate myelin basic protein (MBP). Migration of molecular weight markers is indicated (kDa). **C-E)** *Determination of XMD8-92 and BIX02189 IC50.* Dose-response curves of XMD8-92 (C) and BIX02189 (D) in KCL22 or K562 cells after 72 hours of treatment or DMSO (CTR) in 21% O_2. Values are means ± SD of data from three independent experiments normalized to matched vehicle-treated sample. (E) IC50 values for XMD8-92 and BIX02189 in CML cells treated for 72 hours in 21% or 0.1% O_2. Values represent means ± SD of data from three independent experiments. **F)** *Effects of MEK5/ERK5 inhibitors on KCL22 and K562 cell number in 21% O_2.* Cells were treated with DMSO (Vehicle) or inhibitors at the indicated concentrations from time 0 and viable cells were counted on day 3. Values represent means ± SD of

data from three independent experiments; *p < 0.05; **p < 0.01. **G, H)** *Effects of XMD8-92 on the number of viable ERK5-silenced CML cells.* (G) Parental or transduced K562 cells with non-targeting control (shNT) or one ERK5-targeting shRNA (shERK5-2) and left untreated (Unt) or treated with DMSO (Veh) or XMD8-92 at the indicated concentration from time 0 and viable cells were counted on day 3. Values are means ± SD of three independent experiments. *p ≤ 0.05; ***p ≤ 0.001; ns, not significant. (H) Immuno-blotting from total cell lysates of parental or transduced K562 cells with shNT or shERK5-2. Tubulin is loading control. Migration of molecular weight markers is indicated (kDa).

Supplementary Figure 2. *Effects of MEK5/ERK5 inhibitors on KCL22 and K562 apoptosis and cycle phase distribution*. **A-D)** Cells were incubated in 0.1% (A, B) or 21% O_2 (C, D) and treated with DMSO (Vehicle) or inhibitors at the indicated concentrations from time 0. The percentages of cells in early or late apoptosis were measured by Annexin V test and flow cytometry on day 3 of incubation. Values represent means ± SD of data from three independent experiments; *p < 0.05, **p < 0.01 versus vehicle. **E, F)** Cell cycle phase distribution obtained from cells treated as C and D; data from three independent experiments are shown (means ± SEM) in the Table; *, p ≤ 0.05 versus vehicle. **G**) Immuno-blotting of total lysates of cells treated as in C and D; GAPDH is a loading control; migration of molecular weight markers (kDa) is indicated; representative images from four independent experiments are shown.

Supplementary Figure 3. A) *Effects of incubation in 0.1% O_2 on the expression of ERK5 protein in CML cell lines***.** KCL22 or K562 cells were incubated in 0.1% O_2 for indicated times. Immuno-blotting from total cell lysates was then performed. Tubulin is a loading control. Migration of molecular weight markers is indicated (kDa). **B)** *Effects of imatinib on the expression of ERK5 protein in CML cell lines in low oxygen.* KCL22 or K562 cells were incubated in 0.1% O_2 and treated with DMSO (Vehicle) or 1 μM imatinib from time 0 to day 3. Immuno-blotting from total cell lysates was

then performed. Tubulin is a loading control. Migration of molecular weight markers is indicated (kDa).

Supplementary Figure 4. A) *Effects of MEK5/ERK5 inhibitors on the number of viable primary CML cells.* BMMC explanted from 11 CML patients were incubated at 0.1% O_2 in cultures treated with DMSO (Vehicle) or the indicated inhibitors and viable cells were counted at day 3. (A) Values are data from single experiments (performed in duplicate when error bars, indicating SD, are present) normalized for the respective vehicle-treated control. These data are averaged in Figure 3A. **B)** *Effects of XMD8-92 on leukemic PB WBC in vivo.* CML mice (mice/group: n=6) were treated twice daily with XMD8-92 (50 mg/Kg) or placebo and euthanized after 1 additional day. The number of GFP+ (leukemic) PB myeloid (Gr-1+) cells are shown as means ± SD. *, $p < 0.05$. **C, D)** *Effects of XMD8-92 on CFA of primary CML or normal CD34+ cells.* $CD34^+$-enriched BMMC from CML patients (C) or $CD34^+$-enriched PBMC from healthy donors (D) were plated in methylcellulose containing medium and treated with DMSO (Vehicle) or inhibitors at the indicated concentrations from time 0 and the number of colonies was scored after 7 days. CFE values are means ± SD of data from single experiments performed in duplicate; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ns, not significant.

Supplementary Figure 5. Effects of the pharmacological inhibition of ERK5 on the expression of stem cell markers. KCL22 or K562 cells were incubated in 0.1% O_2 and treated with DMSO (Vehicle) or the combination (XMD + IM) of XMD8-92 (10 μM) and imatinib (1 μM) for 7 days and the expression of stem cell markers was then measured by flow cytometry. Values are means ± SD of mean fluorescence intensity (MFI) data obtained from four independent experiments; *, $p < 0.05$; **, $p < 0.01$.

Supplementary Table 1. Specification of antibodies used in the experiments.

Protein	Use	Source	Notes	Cat. No.	Company
pABL-Y245	WB	rabbit polyclonal		#2862	Cell Signaling Technology, Danvers, MA, USA
c-ABL	WB	rabbit polyclonal		#2861	Cell Signaling Technology, Danvers, MA, USA
pERK5-T218/Y220	WB	rabbit polyclonal		#3371	Cell Signaling Technology, Danvers, MA, USA
ERK5	WB	rabbit polyclonal		#3372	Cell Signaling Technology, Danvers, MA, USA
ERK5	IP	rabbit polyclonal	C-20	c-1284	Santa Cruz Biotechnology, Santa Cruz, CA, USA
p27Kip1	WB	rabbit polyclonal		#2552	Cell Signaling Technology, Danvers, MA, USA
pCRKL-Y207	WB	rabbit polyclonal		#3181	Cell Signaling Technology, Danvers, MA, USA
VINCULIN	WB	mouse monoclonal		V9131	Sigma-Aldrich St. Louis, MO, USA
GAPDH	WB	goat polyclonal	V-18	sc-20357	Santa Cruz Biotechnology, Santa Cruz, CA, USA
TUBULIN	WB	mouse monoclonal		sc-32293	Santa Cruz Biotechnology, Santa Cruz, CA, USA
OCT3/4	FC	mouse monoclonal	C-10	sc-5279	Santa Cruz Biotechnology, Santa Cruz, CA, USA
KLF4	FC	mouse monoclonal		#09-0021	Stemgent, San Diego, CA, USA
c-MYC	FC	rabbit polyclonal		#9402	Cell Signaling Technology, Danvers, MA, USA
SOX2	FC	rabbit monoclonal		#3579	Cell Signaling Technology, Danvers, MA, USA
NANOG	FC	rabbit monoclonal		#4903	Cell Signaling Technology, Danvers, MA, USA
CD26-PE	FC	mouse monoclonal	2A6	#12-0269	Affymetrix eBioscience, San Diego, CA, USA
pMBP	WB	mouse monoclonal	P12	#05-429	Merck Millipore, Billerica, MA, USA
CD34-FITC	FC	mouse monoclonal	4H11[APG]	#21270343	Immuno Tools, Friesoythe, Germany

WB, western blotting; FC, flow cytometry.

Supplementary Table 2. Lentiviral shRNA sequences used in gene knockdown.

Clone number	Region	Sequence
control vector TRC1.5-pLKO.1	none	5’-CCGGCAACAAGATGAAGAGCACCAACTC GAGTTGGTGCTCTTCATCTTGTTGTTTTT-3’
TRCN0000010271	ERK5 CDS	5’CCGGCCAGTCCAACCTACCAGTCCTCTCG AGAGGACTGGTAGGTTGGACTGGTTTTT-3’
TRCN0000010275	ERK5 CDS	5’-CCGGGCCAAGTACCATGATCCTGATCTCG AGATCAGGATCATGGTACTTGGCTTTTT-3’

CDS, coding sequence.