Science Advances
AAAS

Supplementary Materials for

The role of the atypical chemokine receptor CCRL2 in myelodysplastic syndrome and secondary acute myeloid leukemia

Theodoros Karantanos, Patric Teodorescu, Brandy Perkins, Ilias Christodoulou, Christopher Esteb, Ravi Varadhan, Eric Helmenstine, Trivikram Rajkhowa, Bogdan C. Paun, Challice Bonifant, W. Brian Dalton, Lukasz P. Gondek, Alison R. Moliterno, Mark J. Levis, Gabriel Ghiaur, Richard J. Jones*

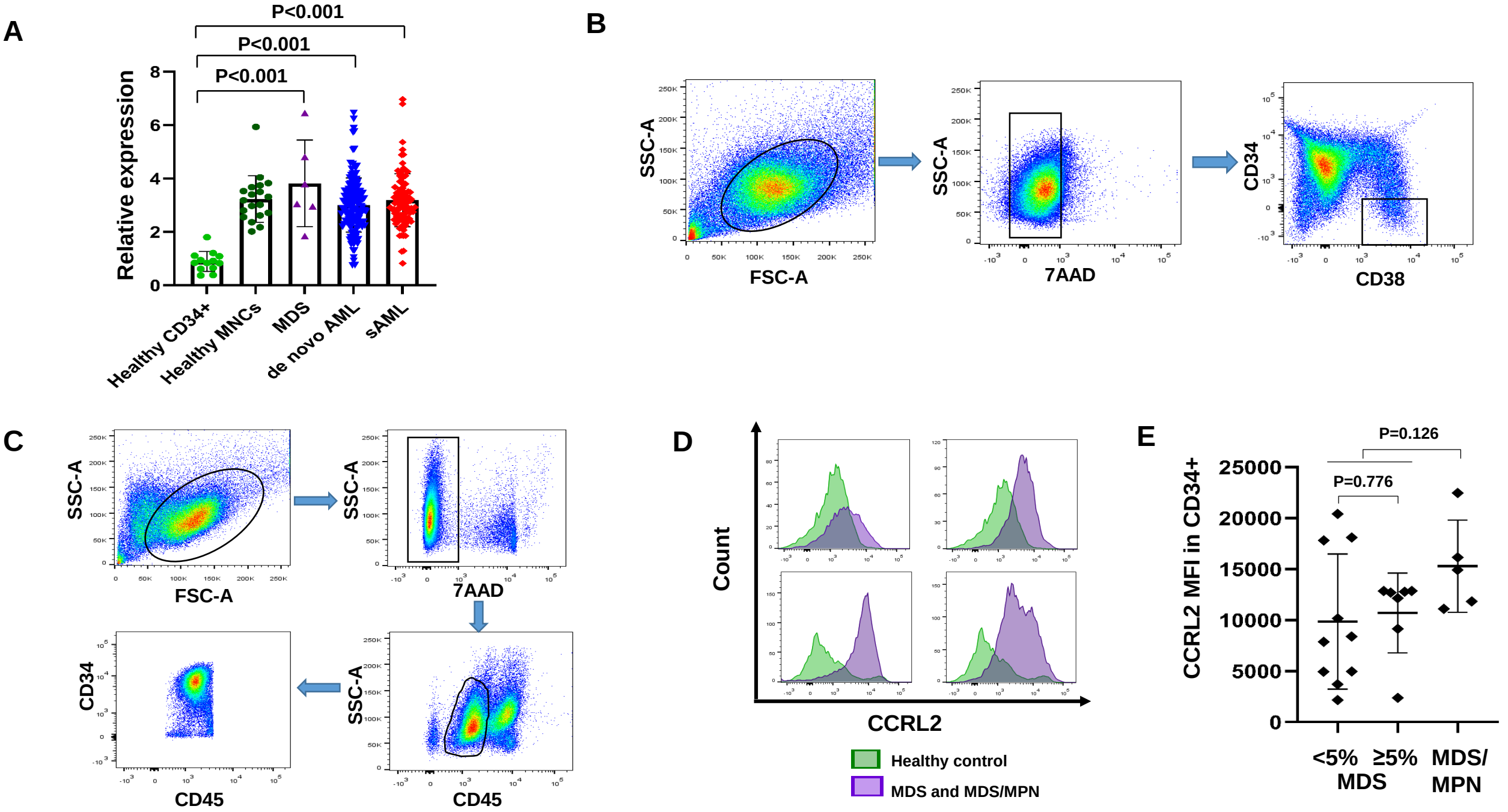
*Corresponding author. Email: rjjones@jhmi.edu

Published 18 February 2022, *Sci. Adv.* **8**, eabl8952 (2022)
DOI: 10.1126/sciadv.abl8952

This PDF file includes:

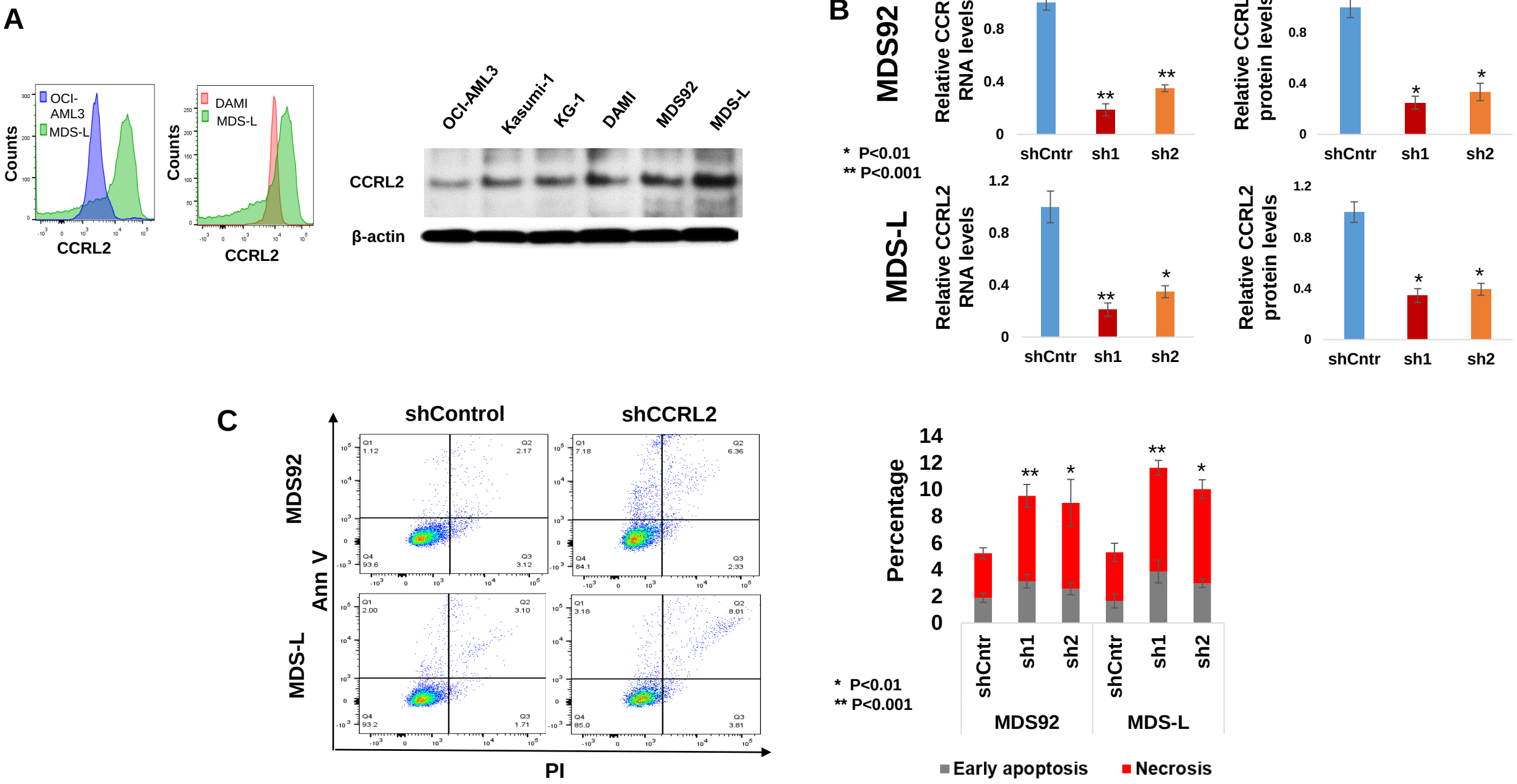
Figs. S1 to S7
Tables S1 to S3

Supplementary Figure 1



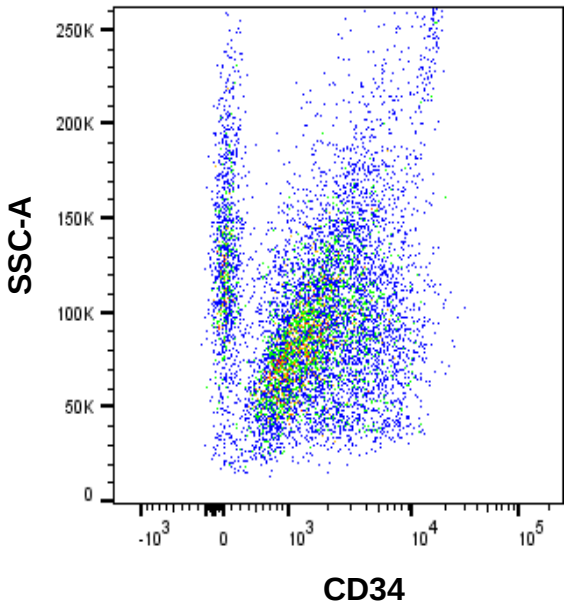
Supplementary Figure 1. A. The CCRL2 expression is higher in samples from MDS and MDS/MPN and CD34+ de novo and sAML compared to CD34+ cells from heathy controls (P<0.001) based data derived from the publicly available Beat AML database. **B.** Gating strategy for CD34+ and CD34+CD38- cells from healthy controls and MDS patients based on SSC-A/FSC-A and 7AAD. **C.** Gating strategy for CD34+ blasts from patients with AML based on SSC-A/FSC-A, and SSC-A/CD45. **D.** CCRL2 expression in representative samples of CD34+ cells from MDS and MDS/MPN patients in comparison to healthy controls **E.** No differences were observed in the CCRL2 protein levels expressed by CD34+ cells from patients with different subtypes of MDS (MDS with <5% blasts, MDS with >5% blasts and MDS/MPN).

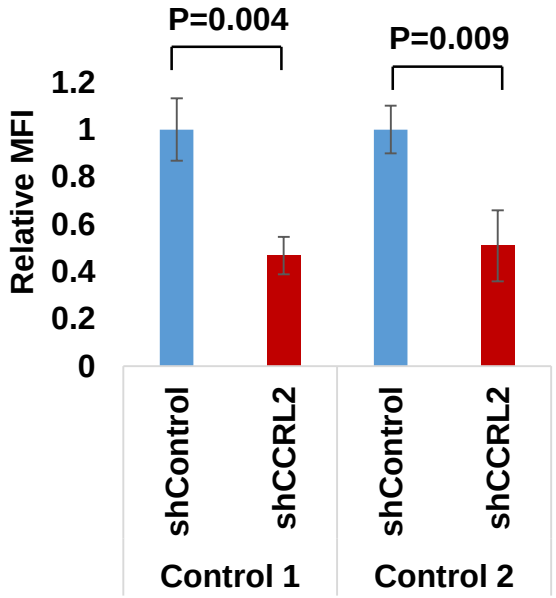
Supplementary Figure 2

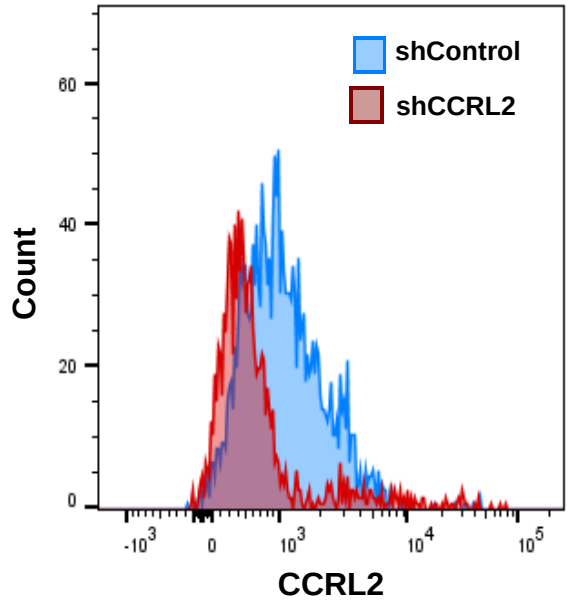


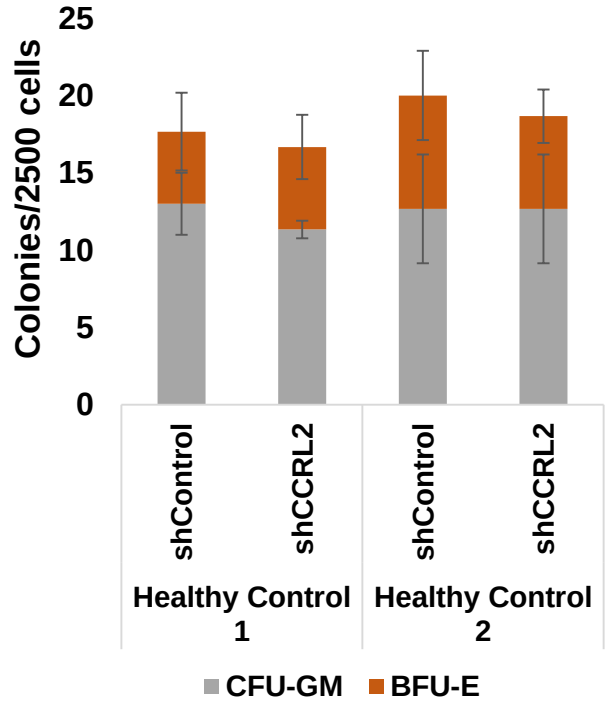
Supplementary Figure 2. A. Western blotting showing the protein levels of CCRL2 in de novo AML (OCI-AML3, Kasumi-1), sAML (KG-1, DAMI) and MDS (MDS92 and MDS-L) cell lines. **B**. CCRL2 was knocked down in MDS92 and MDS-L by transduction with 2 different shRNAs (sh1 and sh2) targeting CCRL2 using empty vector as control. The efficacy of the knockdown was assessed at the RNA and protein level. The RNA expression of CCRL2 was suppressed in MDS92 cells ($P<0.001$) and MDS-L cells ($P<0.001$-sh1, $P=0.003$-sh2). The protein expression of CCRL2 was suppressed in MDS92 cells ($P=0.002$-sh1, $P=0.009$-sh2) and MDS-L cells ($P=0.002$-sh1, $P=0.001$-sh2), n=3. **C.** CCRL2 knockdown increases the apoptotic rate of MDS92 cells ($P<0.001$-sh1 and $P=0.002$-sh2) and MDS-L cells ($P<0.001$-sh1 and $P=0.002$-sh2) based on Annexin V/PI staining.

Supplementary Figure 3



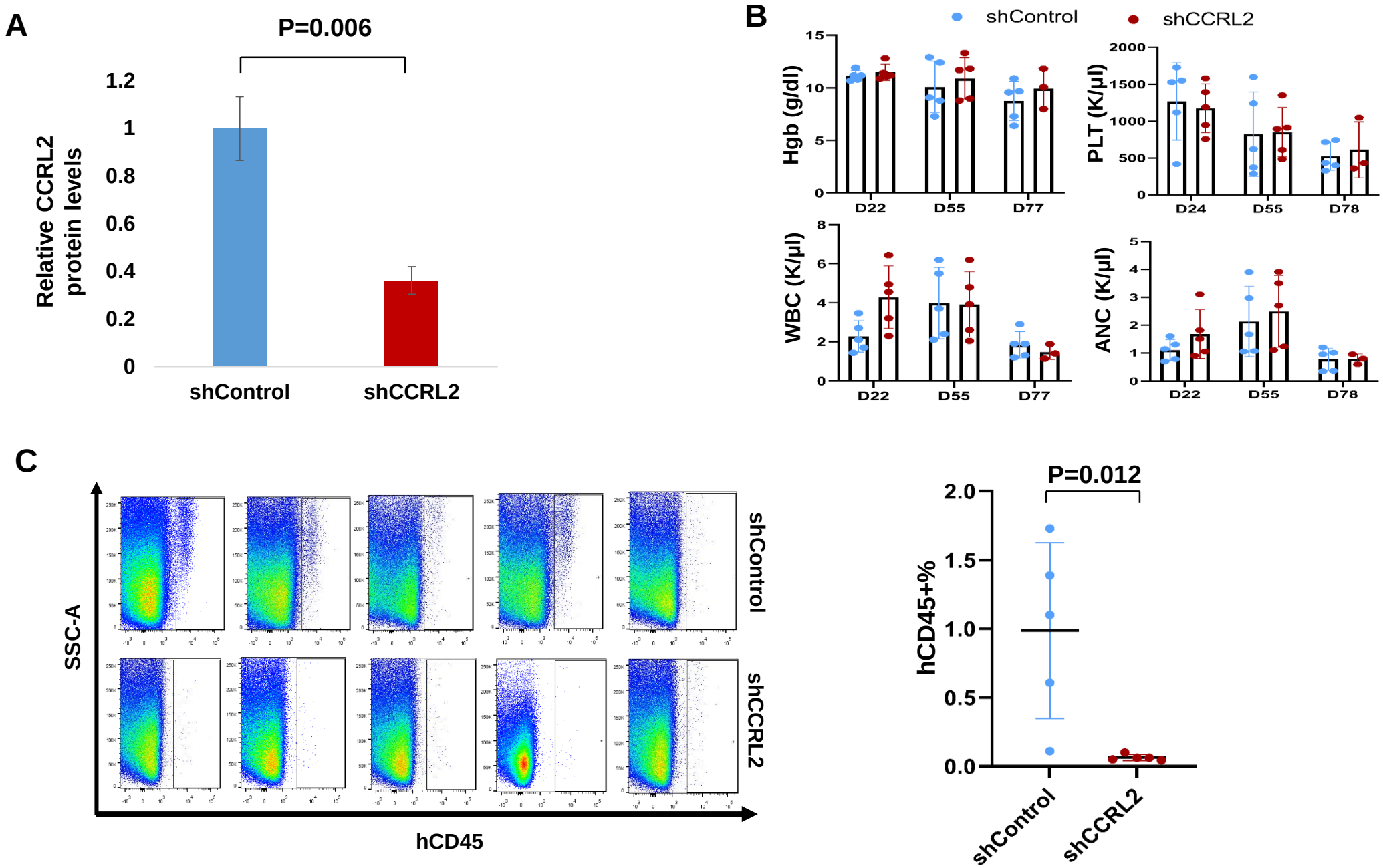






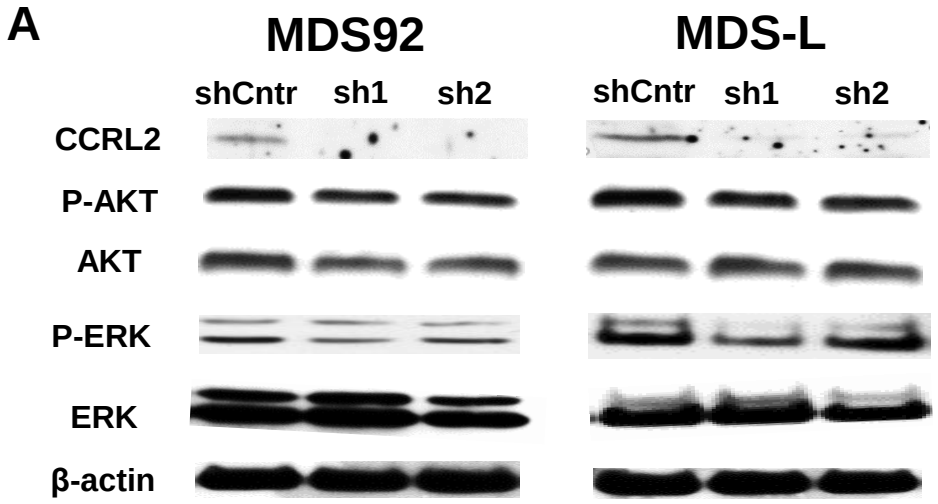
Supplementary Figure 3. CD34+ cells sorted from bone marrow aspirates from two healthy controls were transduced with shControl and shCCRL2 lentiviruses. CCRL2 expression was assessed by flow cytometry. CCRL2 knockdown did not significantly affect the clonogenicity of healthy CD34+ cells, n=3.

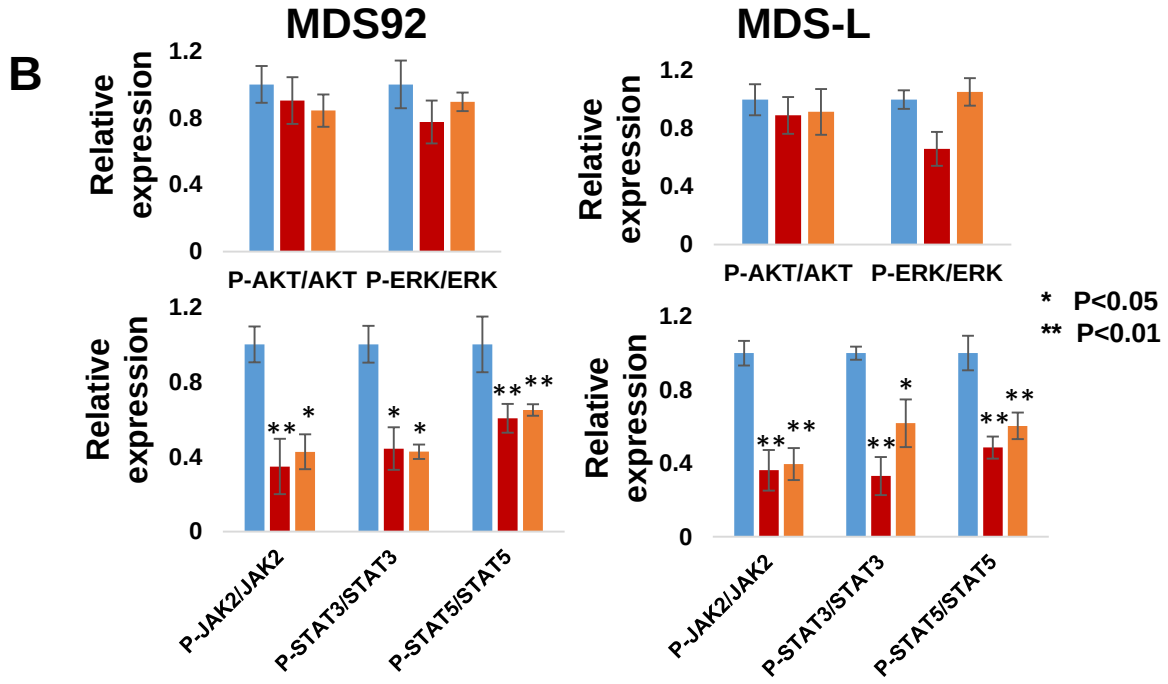
Supplementary Figure 4

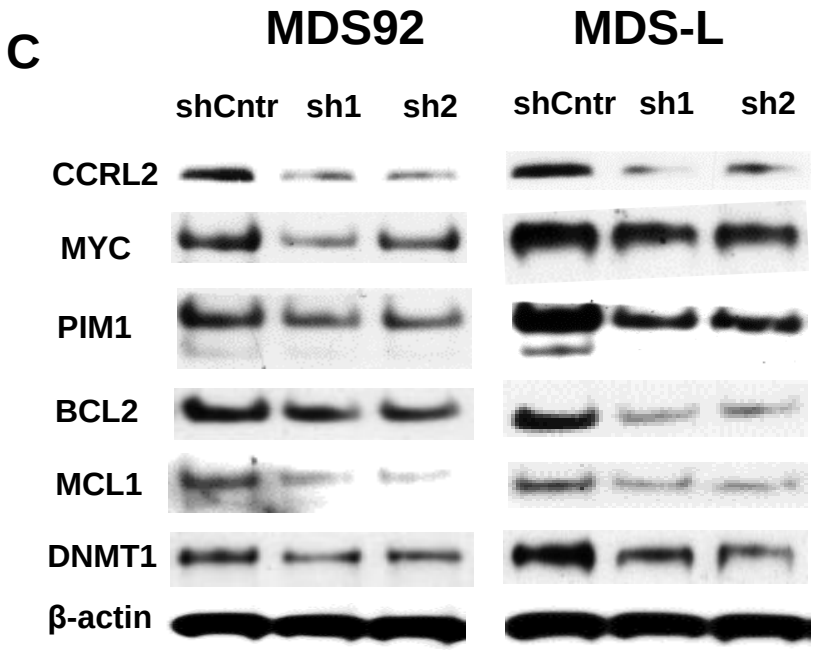


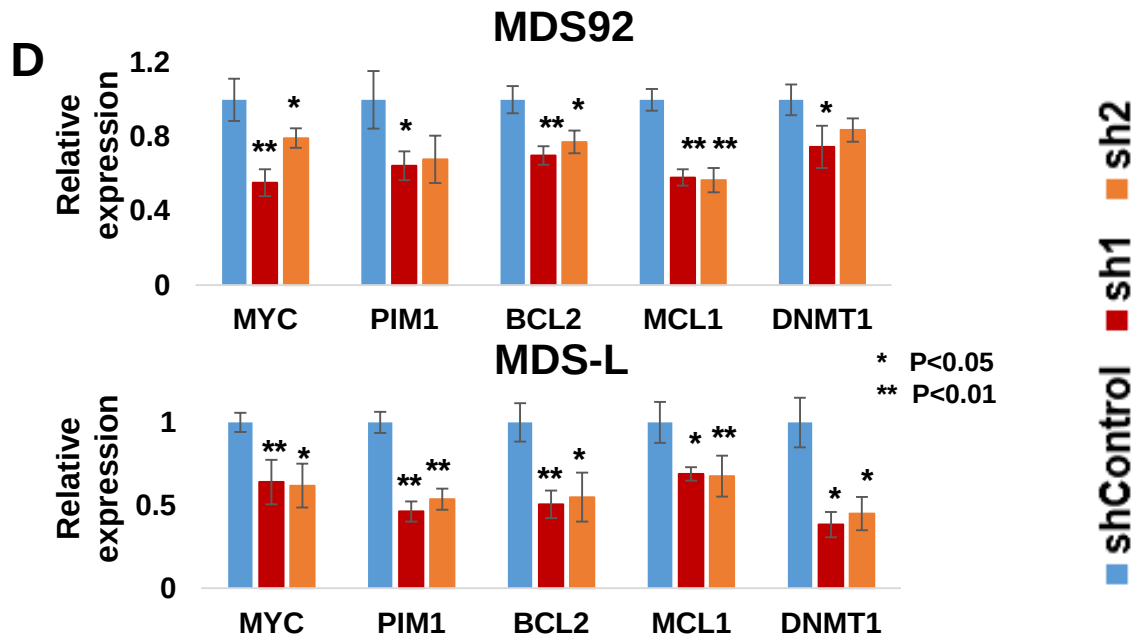
Supplementary Figure 4. A. The protein levels of CCRL2 is significantly lower in MDS-L cells transduced with shCCRL2 compared to MDS-L cells transduced with shControl (P=0.006) following selection to puromycin for 10 days. **B.** Hemoglobin (Hgb), platelets (PLT), total white blood cells (WBC) and absolute neutrophil counts (ANC) in mice injected with MDS-L cells transduced with shControl and shCCRL2 at 24, 55 and 78 days after the injection. **C.** The percentage of human CD45+ cells is higher in the spleens of mice injected with MDS-L cells transduced with shControl and shCCRL2 (P=0.012), n=5. Graphs show the mean value and standard deviation.

Supplementary Figure 5



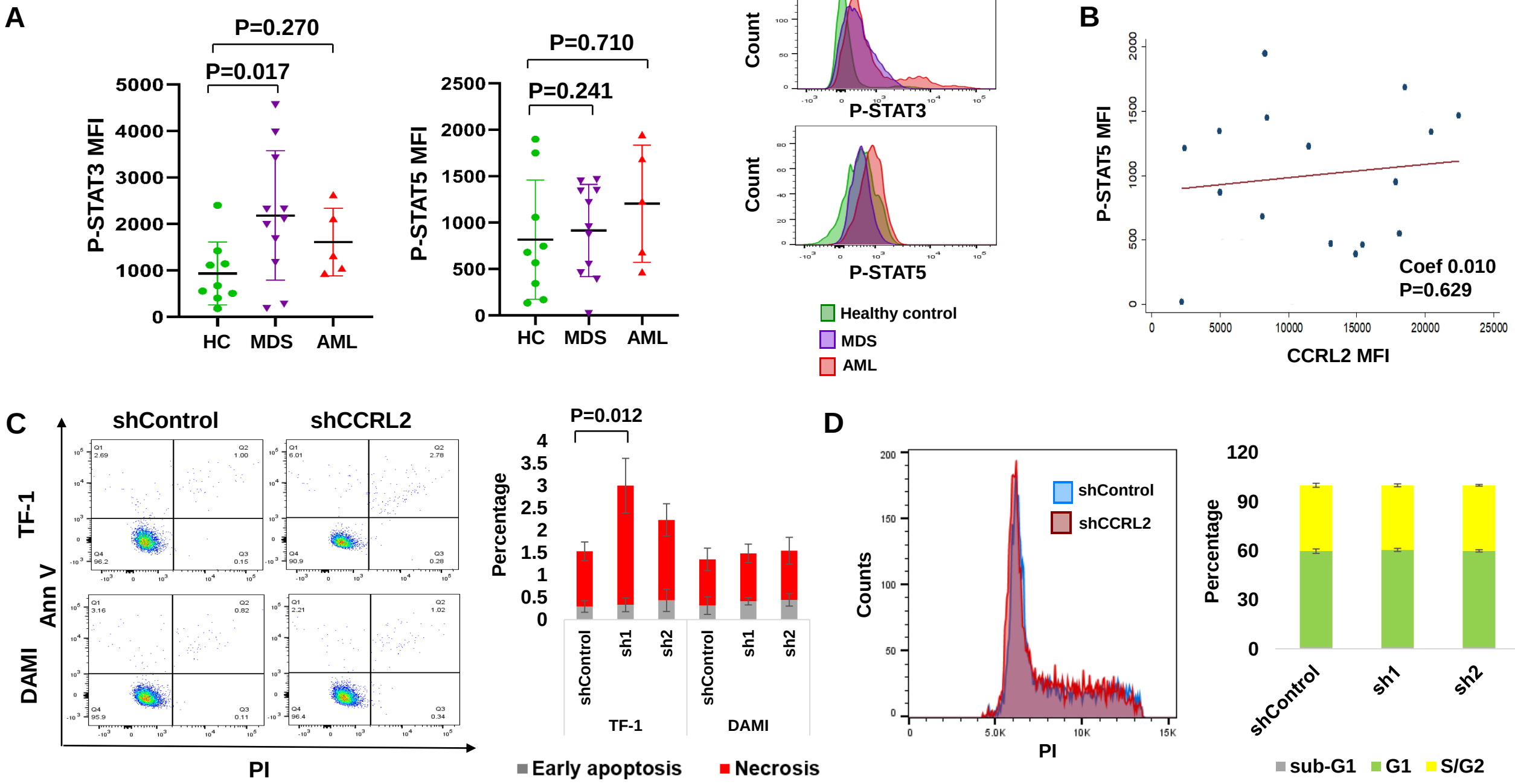






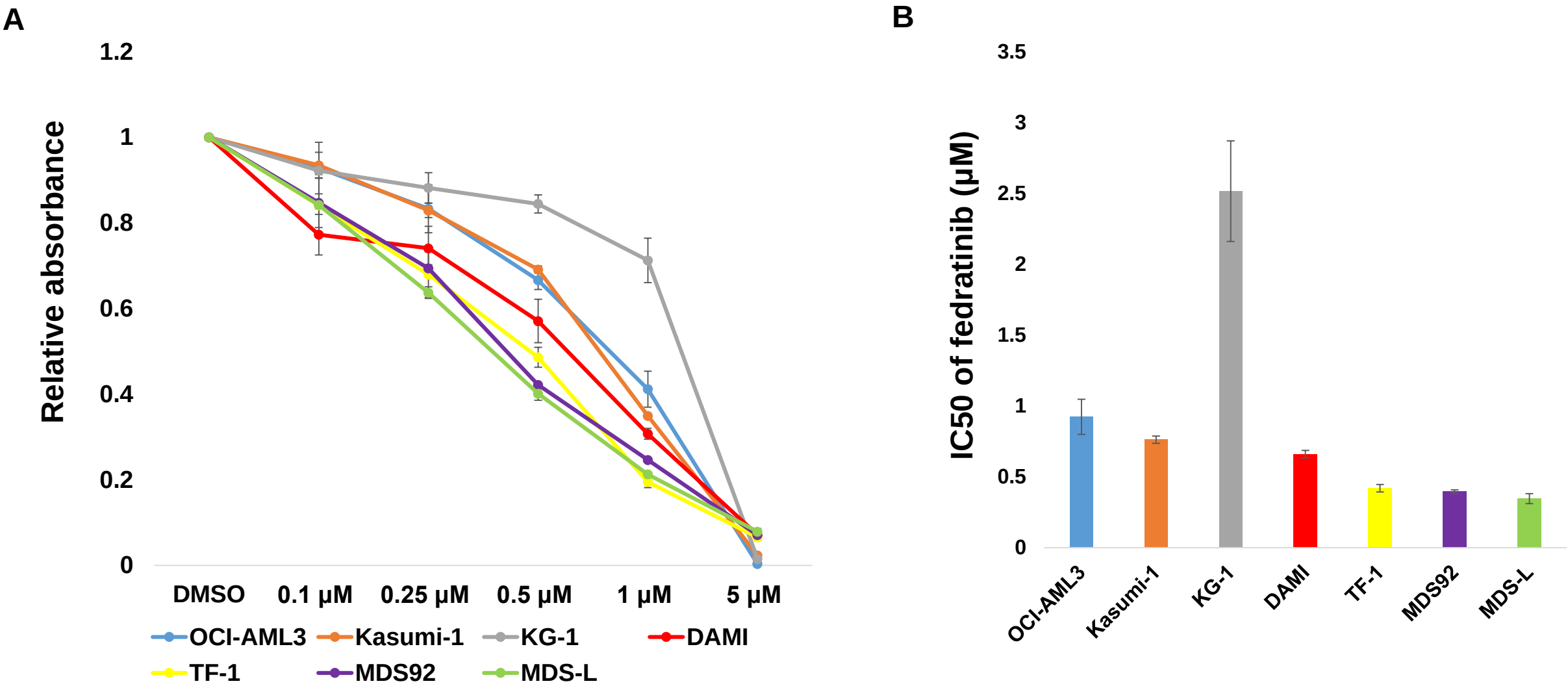
Supplementary Figure 5. A. Representative western blotting showing the effect of CCRL2 knockdown by two different shRNAs (sh1 and sh2) in the phosphorylation of AKT (Ser473) and ERK1/2 (Thr202/Tyr204) in MDS92 and MDS-L with empty vector (shControl) as control. **B.** No significant differences were noted in the phosphorylation of AKT (Ser473) and ERK1/2 (Thr202/Tyr204). CCRL2 knockdown significantly decreases the phosphorylation of JAK2 (Tyr1007/1008), STAT3 (Tyr105) and STAT5 (Tyr694) in MDS92 and MDS-L cells, n=3. **C.** Representative western blotting showing the effect of CCRL2 knockdown on the protein levels of the JAK2/STAT target genes: MYC, PIM1, BCL2, MCL1 and DNMT1. **D.** The relative protein levels of the JAK2/STAT target genes: MYC, BCL2, PIM1, MCL1 and DNMT1 in MDS92 and MDS-L transduced with shControl and shCCRL2 shRNAs. CCRL2 knockdown suppresses the protein levels of the JAK2/STAT target genes in both MDS cell lines, n=3. Graphs show the mean value and standard deviation.

Supplementary Figure 6



Supplementary Figure 6. A. The STAT3 phosphorylation is higher in CD34+ cells from MDS patients compared to healthy controls (*P*=0.017) while no differences were observed in the phosphorylation of STAT5 in CD34+ cells from healthy controls, MDS and AML patients, n=8 healthy controls, n=11 MDS, n=5 AML. **B.** The expression of CCRL2 was not correlated with the phosphorylation of STAT5 in CD34+ cells from MDS and AML patients (Coef 0.009, *P*=0.750). n=11. Graphs show the mean value and standard deviation. **C.** CCRL2 knockdown with sh1 mildly increases the apoptosis in TF-1 cells (*P*=0.012 with sh1 and *P*=0.134 with sh2) while it did not affect the apoptotic rate of DAMI cells (*P*=0.653 with sh1 and *P*=0.528 with sh2). **D.** CCRL2 knockdown did not significantly affect the cell cycle progression in DAMI cells.

Supplementary Figure 7



Supplementary Figure 7. A. MTT assay for the assessment of the effect of fedratinib in various MDS and AML cell lines, n=3. **B.** The IC50 of fedratinib in various MDS and AML cell lines. MDS-L and MDS92 are the most sensitive cell lines to fedratinib, n=3.

SUPPLEMENTARY TABLES

Supplementary Table 1. Characteristics of healthy individuals, MDS and MDS/MPN and AML patients.

	Healthy individuals (N=12)	**MDS and MDS/MPN (N=22)**	**AML (N=13)**
Median age (range)	44.5 years (18 – 54 years)	66.0 years (46 – 79 years)	59.0 years (20 – 70 years)
Gender	Women (7) Men (5)	Women (10) Men (12)	Women (5) Men (8)
Disease subtype		MDS <5% blasts (10) MDS >5% blasts (7) MDS/MPN (5)	De novo (7) sAML (6)

Supplementary Table 2. Univariate linear regression analysis of the association of clinical and molecular characteristics of patients with MDS and MDS/MPN with excess blasts (≥5%) and sAML with CCRL2 expression (N=15)

Characteristics	**Coef**	**95% CI**	**P-value**
Male sex	3928.64	-20277.9 – 28135.20	0.731
Age	**-1092.92**	**-2326.72 – (-203.11)**	**0.025**
% Blasts	**399.68**	**69.23 – 730.12**	**0.021**
Number of somatic mutations	921.95	-3596.96 – 6440.86	0.724
Karyotype risk			
Very good/good	1		
Intermediate	5140.42	-24028.13 – 34308.96	0.708
Poor/Very poor	-3912.50	-30296.44 – 22471.44	0.752
Highest variant allele frequency	262.00	-114.40 – 638.39	0.157
TP53 mutation	-6184.09	-30220.90 – 17852.72	0.588
DNMT3A mutation	14.58	-26873.00 – 26902.16	0.999
TET2 mutation	-566.86	-48650.00 – 37314.24	0.780
JAK2 mutation	-5667.89	-48650.00 – 37314.24	0.780
RAS mutation	17323.33	-7479.942 – 42126.61	0.155
FLT3 mutation	**59274.64**	**34828.76 – 83720.53**	**<0.001**
SETBP1 mutation	-298.93	-43414.61 – 42816.75	0.988
RUNX1 mutation	-4062.30	-26746.96 – 18622.36	0.705
ASXL1 mutation	-9673.64	-33293.58 – 13946.31	0.392
IDH1/2 mutation	1770.833	-25095.80 – 28637.50	0.889
EZH2 mutation	25335.00	-15020.37 – 65690.37	0.198
SRSF2 mutation	-6945.42	-33508.99 – 19620.17	0.582
SF3B1 mutation	-7609.615	-38917.88 – 23698.65	0.608

Supplementary Table 3. Comparison of the entire MDS and MDS/MPN cohort to the MDS patients included in the CD34+CD38- analysis.

	Entire MDS and MDS/MPN cohort	**MDS patients included in the CD34+CD38- analysis**	**P-value**
Median age (range)	66.0 years (46.0 – 79.0)	62.0 years (46.0 – 78.0 years)	0.363
Male gender	12 (0.55)	6 (0.6)	0.781
Median blasts percentage (range)	0.02 (0.01 – 0.20)	0.04 (0.01 – 0.20)	0.605
Median IPSS-R (range)	4 (1 – 7.5)	5.25 (1 – 7.5)	0.489