SUPPLEMENTARY APPENDIX

FLT3-ITD cooperates with Rac1 to modulate the sensitivity of leukemic cells to chemotherapeutic agents via regulation of DNA repair pathways

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 doi:10.3324/haematol.2018.208843

Received: October 10, 2018.

Accepted: April 9, 2019.

Pre-published: April 11, 2019.

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SUPPLEMENTAL METHODS

Cell lines

Cells were cultured at 37 °C with 5% CO_2 in RPMI medium 1640 containing 10% fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin. Culture medium for TF-1 cells that are FLT3/ITD-negative was supplemented with GM-CSF (2 ng/ml, Peprotech, Rocky Hill, NJ, USA). Molm14 cells were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zelkulturen, Braunschweig, Germany). All other cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were freshly thawed from stocks that were confirmed to be free of mycoplasma and frozen in 2010. Cells stably expressing control shRNA and shRNA directed against DOCK2 were described previously.[1]

Reagents

Ara-C and 5-Fluorouracil (5-FU) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sorafenib was obtained from LC Laboratories (Woburn, MA, USA). NSC23766 was obtained from Tocris Bioscience (Ellisville, MO, USA). MK8776 was obtained from AdooQ BioScience LLC (Irvine, CA, USA). MK1775 and B02 were obtained from Selleck Chemicals (Houston, TX, USA). For *in vitro* studies, stock solutions of ara-C (50 mM), 5-FU (50 mM), NSC23766 (100 mM), sorafenib (10 μM), MK8776 (5 mM), MK1775 (100 mM) and B02 (10 mM) were prepared in dimethyl sulphoxide (DMSO). For *in vivo* studies, MK8776 (1 mg/ml) and MK1775 (1.5 mg/ml) solutions were prepared in 45% (w/v) (2-Hydroxypropyl)-β-cyclodextrin (Sigma-Aldrich), and ara-C (5 mg/ml) solution was prepared in Phosphate Buffered Saline (PBS).

Western blotting

Protein samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (BIO-RAD, Hercules, CA, USA), and detected by the indicated antibodies followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence. Antibodies against FLT3 (480), DOCK2 (365242), PIM-1 (13513), RAD51 (8349), phospho-JUN (S63; 822), JUN (1694), FOS (52), MYB (7874), and MEIS1/2 (25412) were obtained from Santa Cruz Biotechnology, Inc (Dallas, TX, USA). Antibodies against phospho-FLT3 (Y589/591; 3474), phospho-STAT5 (Y694; 9351), phospho-CHK1 (S345; 2341), CHK1 (2360), phospho-WEE1 (S642; 4910), WEE1 (4936), MSH2 (2017), and MLH1 (3515) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against phospho-Histone H2AX (S139; 05-636) were obtained from Millipore (Billerica, MA, USA). Antibodies against β-ACTIN (A5441) were obtained from Sigma-Aldrich. Western blot images were obtained using the ChemiDoc™ Touch Imaging System (BIO-RAD) and quantification of band densities was performed with Image Lab software (BIO-RAD).

Quantitative RT-PCR

Quantitative RT-PCR was performed using the CFX™96 Real Time System (BIO-RAD) according to the manufacture's instruction. The primer sequences used are listed in Supplemental Table 1.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using the EZ-Magna ChIP™ A/G kit (Millipore, Billerica, MA, USA), and the primer sequences were listed in Table S1.

***In vitro* drug treatment of mouse bone marrow cells**

Whole bone marrow cells from leukemic *Flt3*$^{+/ITD}$*; NHD13* and *Flt3*$^{+/+}$*; NHD13* mice were harvested as previously described.[2, 3] Cells were cultured at 1 x 10^6 cells/ml at 37 °C with 5% CO_2 in RPMI medium 1640 containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin. *Flt3*$^{+/ITD}$*; NHD13* cells were cultured in the presence of 10 mg/L insulin, 5.5 mg/L transferrin and 6.7 ng/L sodium selenite for 24 hr before apoptosis analysis. *Flt3*$^{+/+}$*; NHD13* were cultured for 48 hr before apoptosis analysis.

REFERENCES:

1. Wu M, Hamaker M, Li L, Small D, Duffield AS. DOCK2 interacts with FLT3 and modulates the survival of FLT3-expressing leukemia cells. *Leukemia* 2017; **31**: 688-96.
2. Greenblatt S, Li L, Slape C, *et al.* Knock-in of a FLT3/ITD mutation cooperates with a NUP98-HOXD13 fusion to generate acute myeloid leukemia in a mouse model. *Blood* 2012; **119**: 2883-94.
3. Lin YW, Slape C, Zhang Z, Aplan PD. NUP98-HOXD13 transgenic mice develop a highly penetrant, severe myelodysplastic syndrome that progresses to acute leukemia. *Blood* 2005; **106**: 287-95.

Table S1. Sequences of primers used in quantitative RT-PCR and ChIP assays.

Gene	Forward	Reverse
CHK1	GGTGCCTATGGAGAAGTTCAA	TCTACGGCACGCTTCATATC
FOS	GGGGCAAGGTGGAACAGTTAT	CCGCTTGGAGTGTATCAGTCA
JUN	TCCAAGTGCCGAAAAAGGAAG	CGAGTTCTGAGCTTTCAAGGT
MYB	GGCAGAAATCGCAAAGCTAC	CTGGCTGGCTTTTGAAGACT
DOCK2	AGCACAAAATGTTACAGGGCA	CCATCAGATCGTACATCATGGAC
FLT3	AGGATCAGGTGCTTTTGGAA	TGCCTCTCTTTCAGAGCTGTC
GAPDH	GAAGGTGAAGGTCGGAGTCA	AATGAAGGGGTCATTGATGG
MEIS1	AAATGCCTATCGATTTGGTGA	AACGAGTAGATGCCGTGTCA
MSH2	GCCATTTTGGAGAAAGGACA	CTCACATGGCACAAAACACC
MSH6	CCCCACCAGTTGTGACTTCT	TGTTGGGCTGTCATCAAAAA
MLH1	TGGGACGAAGAAAAGGAATG	GATCAGGCAGGTTAGCAAGC
MLL	GGAGATGGGAGGCTTAGGAA	AGGGCTCACAACAGACTTGG
PIM-1	GGCTCGGTCTACTCAGGCA	GCAAATCCGGTCCTTCTCCAC
RAD51	CTTTGGCCCACAACCCATTTC	ATGGCCTTTCCTTCACCTCCAC
WEE1	TGAAGAGGGCGATAGTCGTT	GCACTTGTGGTATCCGAGGT
ChIP	GGTAGCAGTGGCTAAGAGGAATAC	CTAAGAGGAGCTCAGGATGGCT

Table S2. Results of unpaired two-tailed t-tests between samples shown in Figure 4B.

P values:

	Cells with high γH2AX %		Mean fluorescence intensity	
Treatment	C	KD	C	KD
None vs MK8776	0.0445	0.0038	0.1227	0.0994
None vs MK1775	0.0922	<0.0001	0.2062	0.0033
None vs B02	0.0053	0.0001	0.0115	0.0005
None vs Ara-C	0.0056	<0.0001	<0.0001	<0.0001
Ara-C+MK8776 vs MK8776	<0.0001	<0.0001	<0.0001	<0.0001
Ara-C+MK8776 vs Ara-C	0.0004	<0.0001	0.0033	<0.0001
Ara-C+MK1775 vs MK1775	<0.0001	<0.0001	<0.0001	<0.0001
Ara-C+MK1775 vs Ara-C	<0.0001	0.0001	<0.0001	<0.0001
Ara-C+B02 vs B02	0.0104	<0.0001	0.003	<0.0001
Ara-C+B02 vs Ara-C	0.0866	0.0002	0.0018	0.0003
	C vs KD		C vs KD	
None	0.8115		0.0002	
MK8776	0.0046		<0.0001	
MK1775	0.0001		0.0006	
B02	0.0007		0.0019	
Ara-C	<0.0001		0.0062	
Ara-C+MK8776	<0.0001		<0.0001	
Ara-C+MK1775	<0.0001		<0.0001	
Ara-C+B02	<0.0001		<0.0001	

C: MV4;11 cells expressing control shRNA. KD: MV4;11 cells expressing an shRNA against DOCK2.

Figure S1.

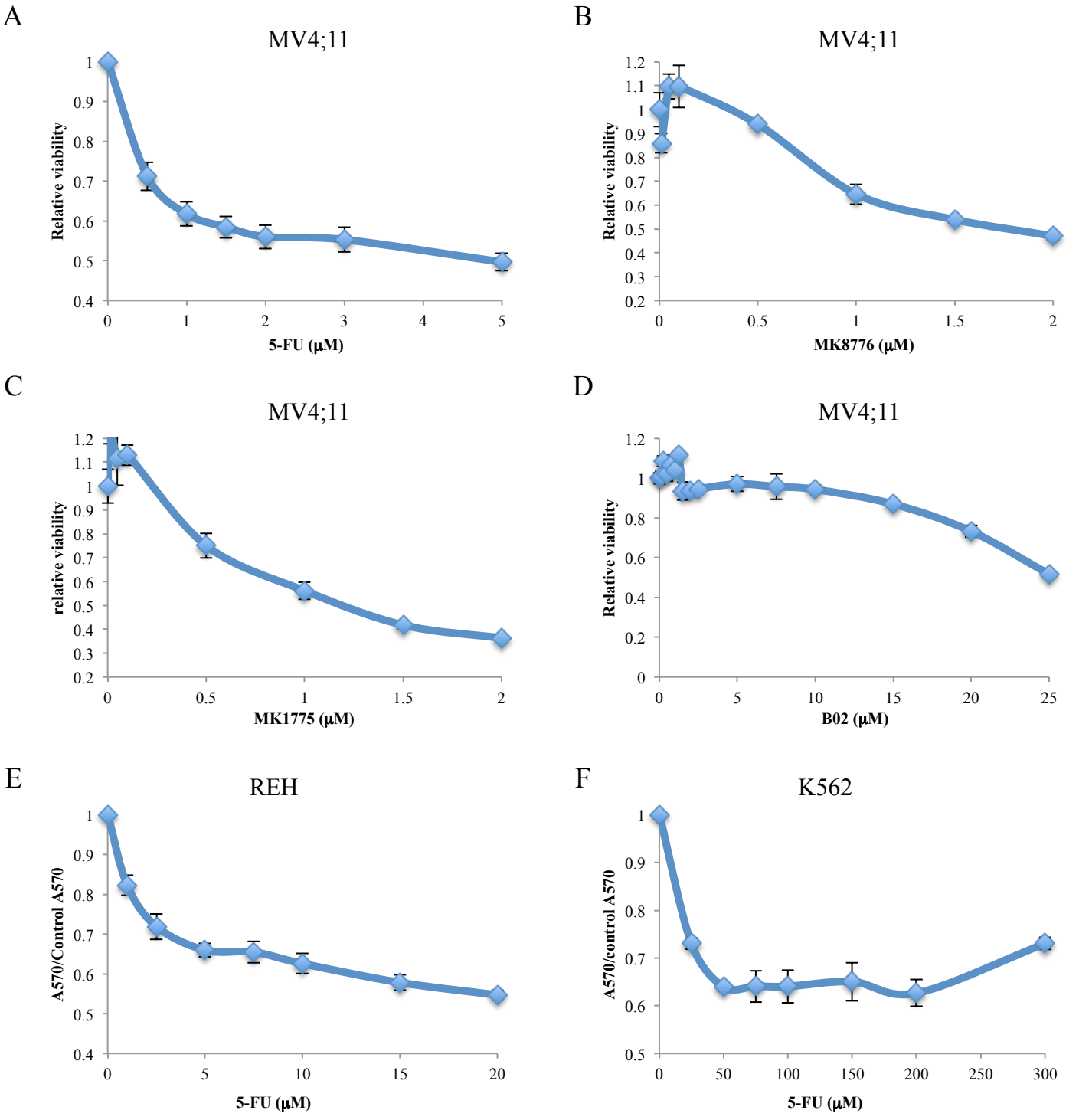


Figure S1. MTT assays of MV4;11 cells in response to 5-FU (A), MK8776 (B), MK1775 (C), and B02 (D), and REH (E) and K562 (F) cells in response to 5-FU. All measurements were performed 48 hr after the addition of drugs.

Figure S2.

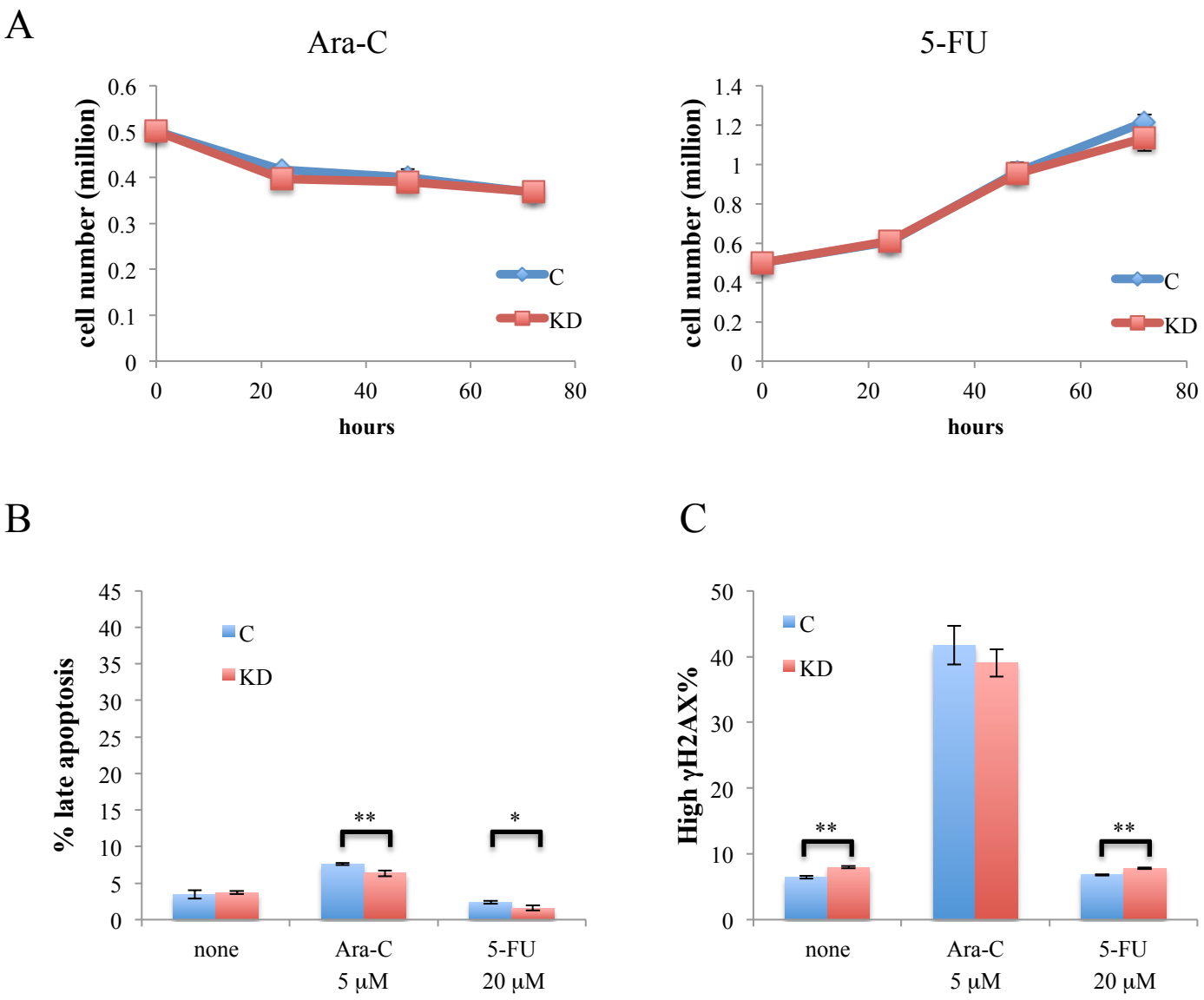
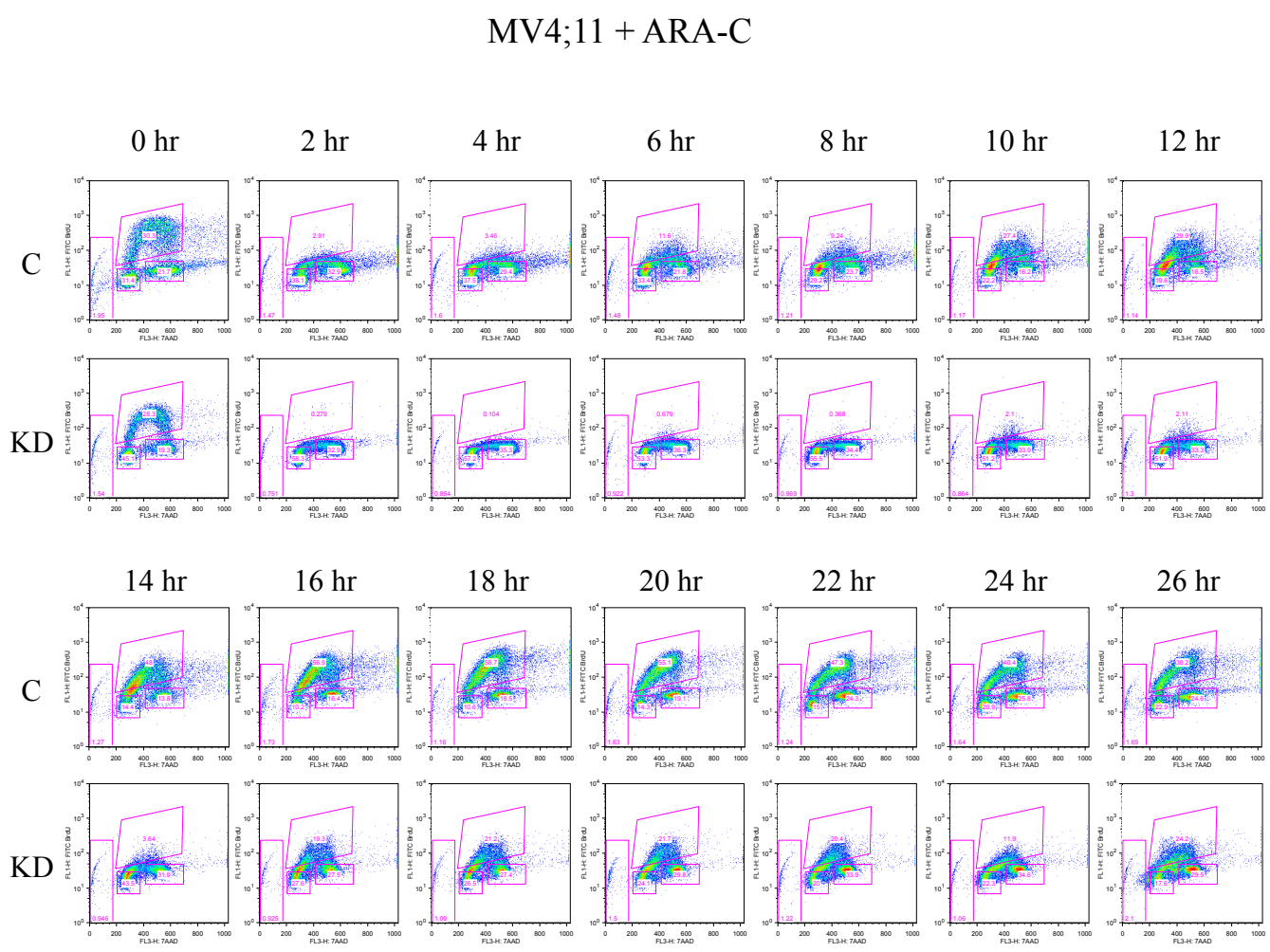
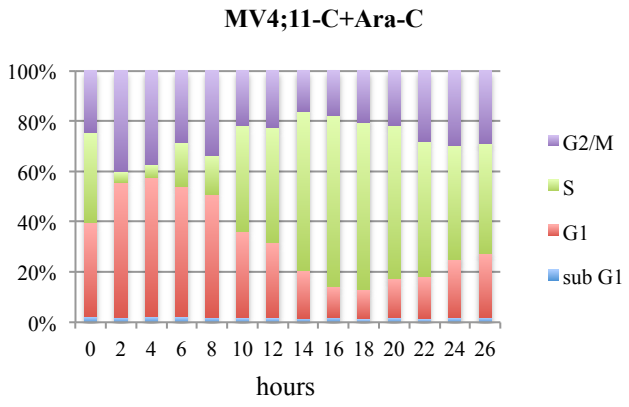


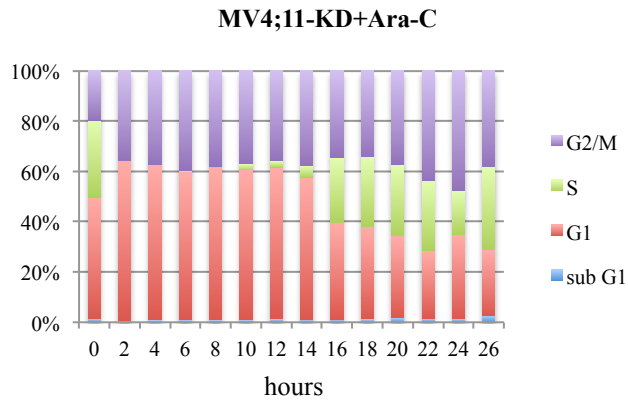
Figure S2. Effects of DOCK2 knockdown on the sensitivity of K562 cells to Ara-C and 5-FU. (A) Proliferation of K562 cells in the presence of Ara-C (5 μM) and 5-FU (20 μM). (B) DOCK2 knockdown in K562 cells resulted in slight decrease in the fraction of cells in late apoptosis in the presence of Ara-C (5 μM) and 5-FU (20 μM). Cells were treated for 72 hr. (C) Compared with control K562 cells, the fraction of DOCK2 KD cells harboring elevated γH2AX level was similar in the presence of Ara-C (5 μM), and slightly elevated in the presence of 5-FU (20 μM). Cells were treated for 18 hr. C: cells expressing a control shRNA; KD: cells expressing an shRNA against DOCK2.

Figure S3.

A


MV4;11 + ARA-C
0 hr
2 hr
4 hr
6 hr
8 hr
10 hr
12 hr
C
KD
14 hr
16 hr
18 hr
20 hr
22 hr
24 hr
26 hr
C
KD
FL3-H: 7AAD
FL1-H: FITC BrdU


MV4;11-C+Ara-C
100%
80%
60%
40%
20%
0%
0 2 4 6 8 10 12 14 16 18 20 22 24 26
hours
G2/M
S
G1
sub G1


MV4;11-KD+Ara-C
100%
80%
60%
40%
20%
0%
0 2 4 6 8 10 12 14 16 18 20 22 24 26
hours
G2/M
S
G1
sub G1

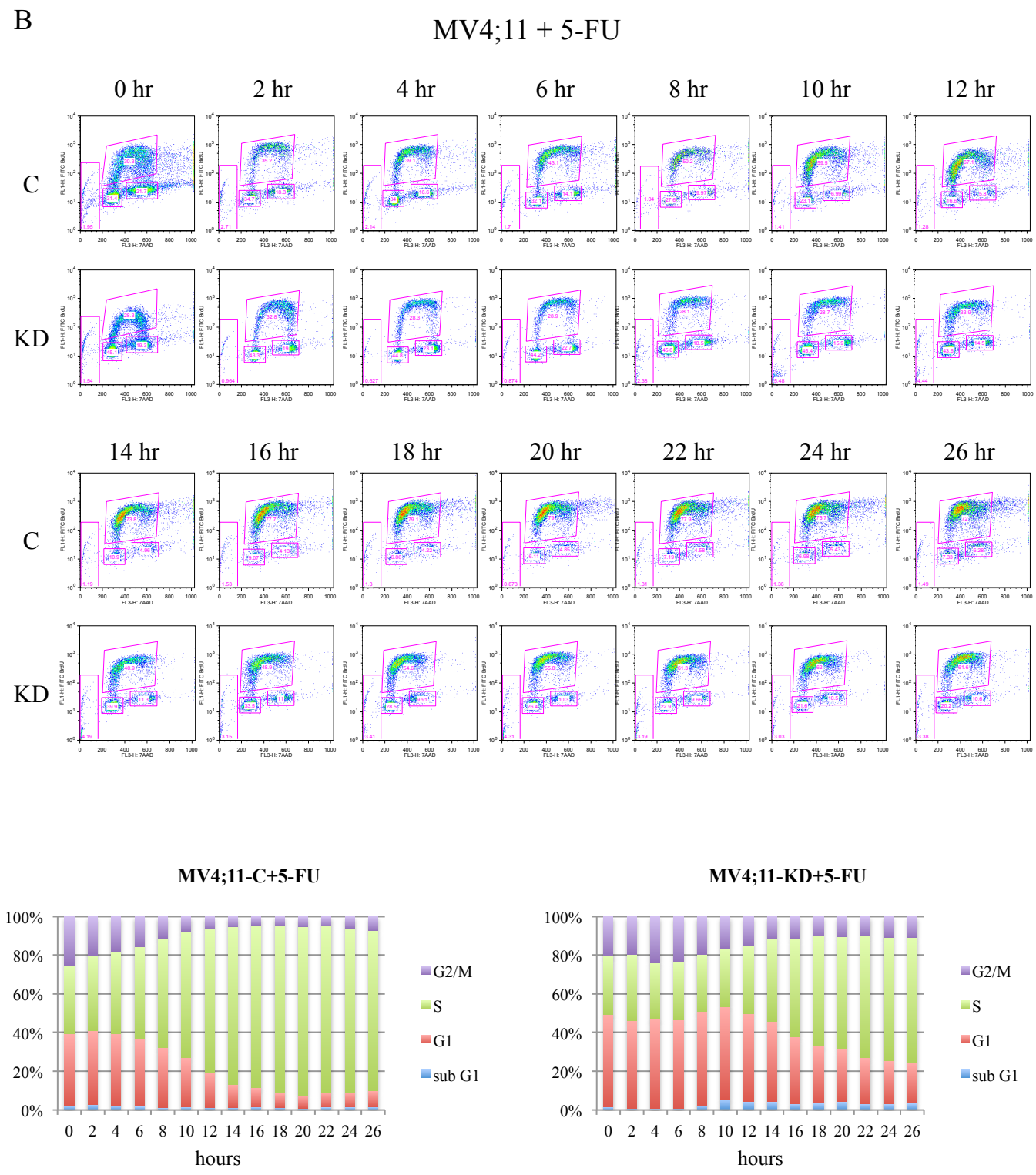


Figure S3. BrdU assays of MV4;11 cells after treatment with Ara-C (A) and 5-FU (B). At each time point, cells were pulse labeled with 10 μM of BrdU for 30 min before harvest. The percentage of cells in each cell cycle phase is indicated in the bottom panel. C: cells expressing a control shRNA; KD: cells expressing an shRNA against DOCK2.

Figure S4.

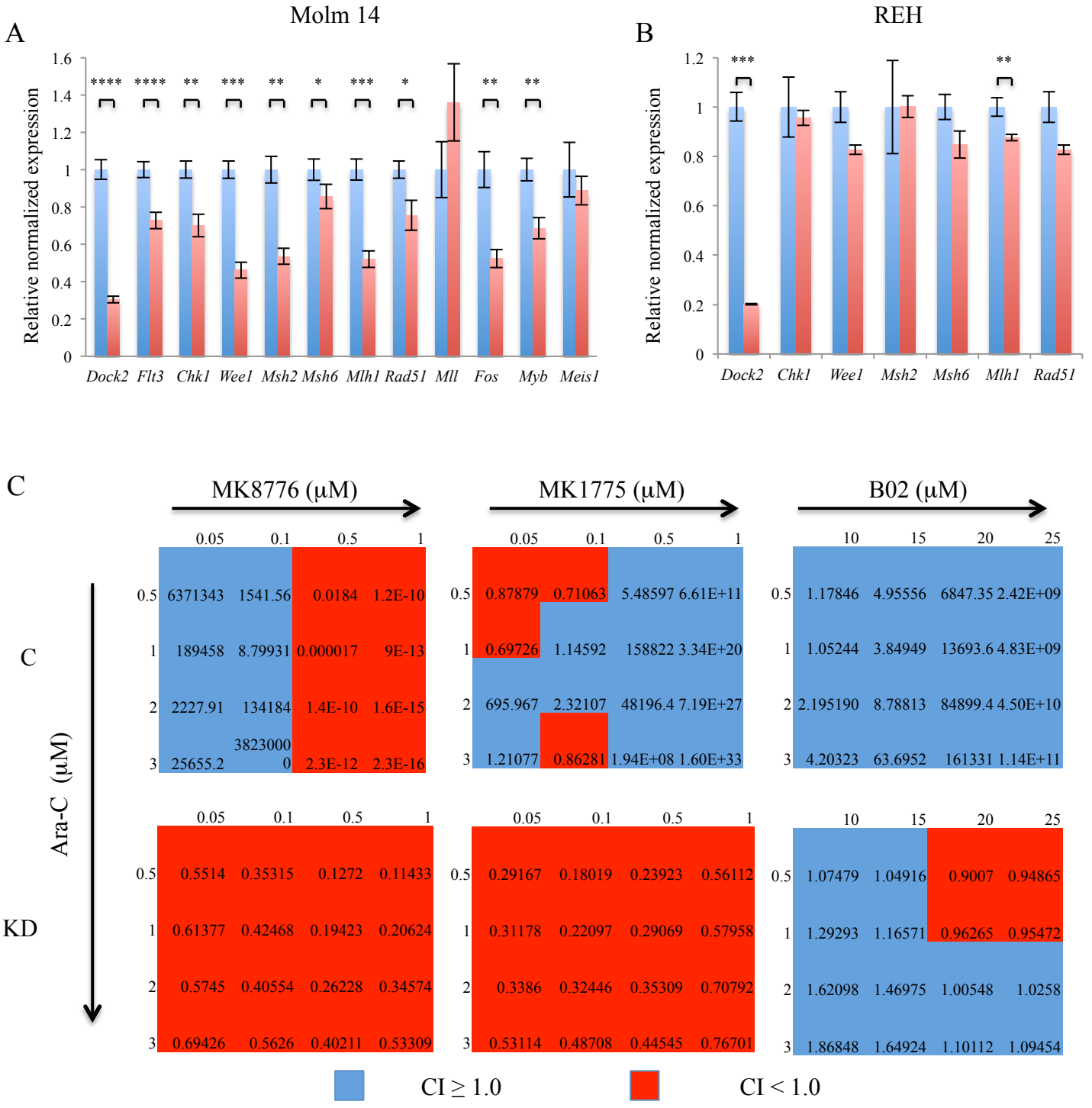


Figure S4. (A) Quantitative RT-PCR analysis revealed decreased expression of *Flt3, Chk1, Wee1, Msh2, Msh6, Mlh1, Rad51, Fos* and *Myb* in Molm 14 cells when DOCK2 expression was suppressed. The level of each transcript was normalized based on that of control cells. (B) Quantitative RT-PCR analysis of the expression of *Dock2, Chk1, Wee1, Msh2, Msh6, Mlh1* and *Rad51* in REH cells. The level of each transcript was normalized based on that of control cells. (C) CI values of Ara-C in combination with MK8776, MK1775 or B02 in MV4;11 cells, based on percentage of apoptotic cells. C: cells expressing a control shRNA; KD: cells expressing an shRNA against DOCK2.

Figure S5.

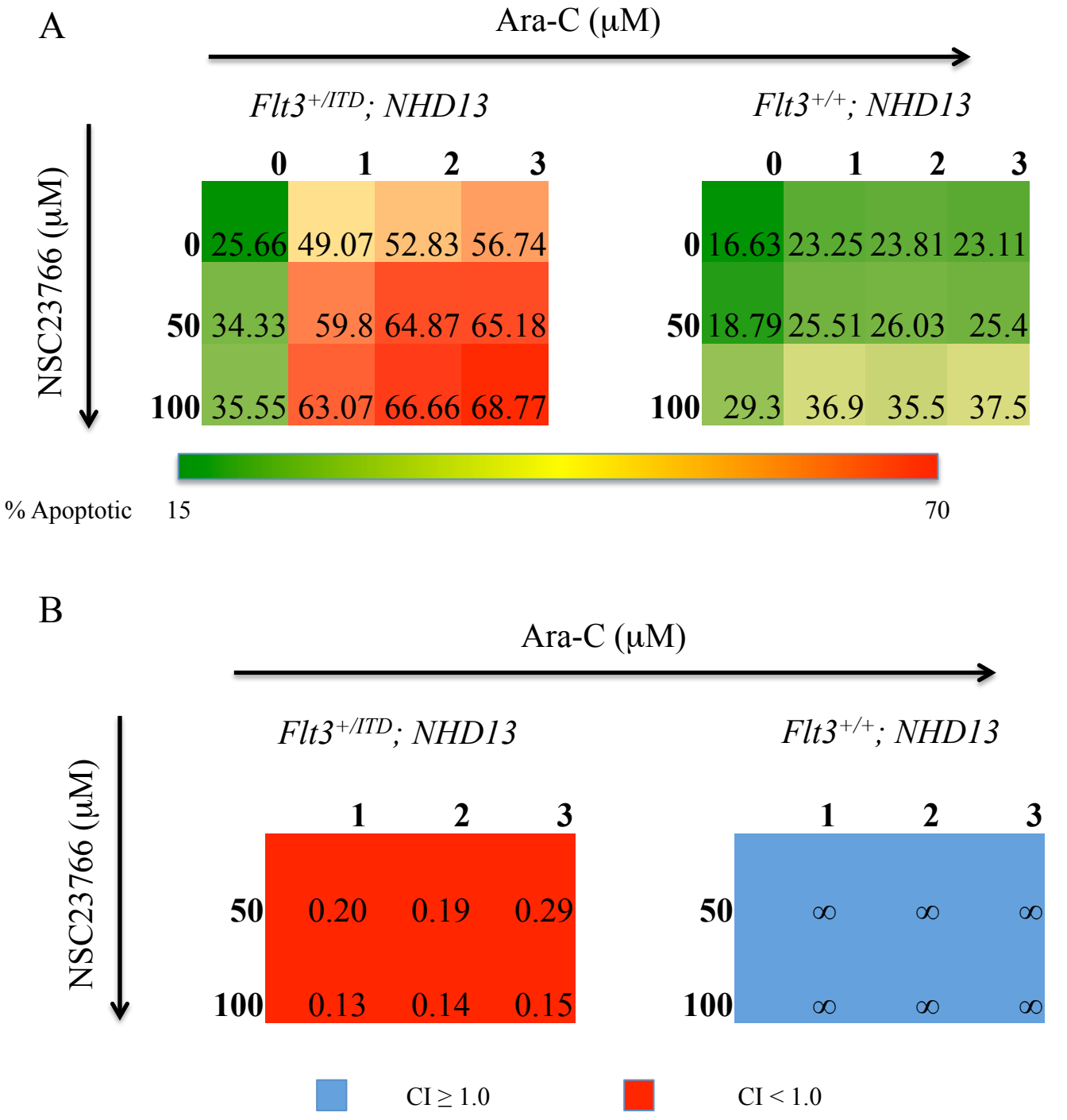


Figure S5. Suppression of Rac1 activity increased the sensitivity of leukemic *Flt3*$^{+/ITD}$*; NHD13* mouse bone marrow cells to Ara-C treatment, while no synergistic effects were observed in leukemic *Flt3*$^{+/+}$*; NHD13* mouse bone marrow cells. (A) Compared with *Flt3*$^{+/+}$*; NHD13* mouse bone marrow cells, *Flt3*$^{+/ITD}$*; NHD13* mouse bone marrow cells exhibited an increased fraction of apoptotic cells upon treatment with the Rac1 inhibitor NSC23766 in the presence of Ara-C. (B) NSC23766 and Ara-C acted synergistically to cause apoptosis in leukemic *Flt3*$^{+/ITD}$*; NHD13* mouse bone marrow cells, but not *Flt3*$^{+/+}$*; NHD13* mouse bone marrow cells.

Figure S6.

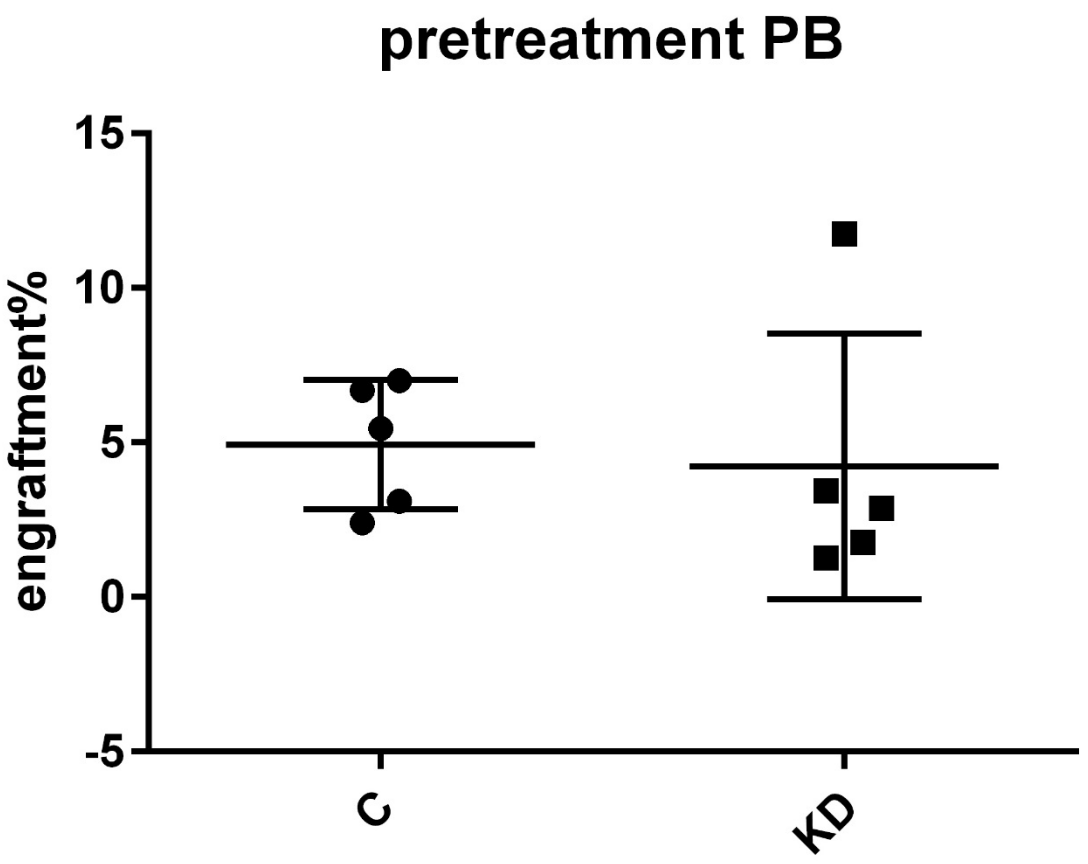


Figure S6. Pre-treatment PB blast percentage of NSG mice transplanted with MV4;11 cells expressing a control shRNA (C) and an shRNA against DOCK2 (KD). PB blast percentage was measured on day 12 for control mice and day 49 for DOCK2 KD mice respectively.