Supplementary Materials and Methods

GST pulldown and binding assay

Tribbles 1-3 constructs were amplified by PCR, ligated into pGEX 4T-1 (GE Healthcare) and expressed in BL21(DE3)pLysS cells (Stratagene)**.** Cells were lysed by sonication in buffer A (25mM Tris 7.5, 200mM NaCl, 5mM DTT and 5% Glycerol). The soluble lysate was incubated with Glutathione Sepharose beads (Amersham Biosciences), in the presence of protease inhibitors (Roche), for 3 hours at room temperature with gentle agitation. The beads were washed with buffer B (buffer A with 1M NaCl). ^{35}S labeled C/EBPα was generated using the TnT Coupled Transcription/Translation kit (Promega) according to the manufacturer's instructions. 5μL of GST-Tribbles 1, 2 or 3 beads were incubated with 5, 10 or 20μL of the IVTT mix in buffer A plus protease inhibitors (Roche), 1% BSA and 0.5% NP-40 to a final volume of 175 μL for one hour at room temperature. The beads were washed 5x with buffer B and resuspended in 20 μL of SDS loading buffer. 10μL of each sample was loaded into a standard protein gel. The gel was stained with Coomasie Blue, then fixed in a solution of 40% water, 50% MeOH and 10% acidic acid for one hour. The gel was incubated for 2-3 hours in a solution of 1M Sodium salycilate in 10% glycerol, then dried and visualized by autoradiography.

HeLa cell transfection

FLAG-tagged Trib2 and Trib3 and HA-tagged COP1 and C/EBPα were cloned into pcDNA3 and the integrity of the constructs was confirmed by sequencing. HeLa cells were transfected via Lipofectamine 2000 (Invitrogen) using 200 ng of each plasmid, except C/EBPα, where 75 ng was used. Cells were lysed in 2xSDS buffer 30 hours after transfection. MG132 (10μM) was added two hours prior to cell lysis.

Immunoprecipitation

For immunoprecipitation, 1 mg of precleared whole cell lysate was incubated overnight with anti-flag M2 agarose beads (Sigma). Agarose beads were washed 3 times with RIPA lysis buffer and immunoprecipitated proteins were eluted by boiling for 5 minutes with 2X SDS sample buffer.

32D stimulation and phospho-Erk analysis

32D cells were transduced with the indicated retroviral supernatants. 48 hours post-transduction, GFP+ cells were sorted and plated. Sorted cells were cytokine starved (IMDM, Gibco; 0.5% FCS; 100 U/mL penicillin, 100 ug/mL streptomycin, and 12 uM L-glutamine) for 14 hours. Cells were stimulated with 20 ng/mL IL-3 (Peprotech) for 15 minutes and harvested. For flow cytometric analysis, $4x10^6$ cells were fixed with 2% para-formaldeyde for 10 minutes at 37 ^{0}C, then spun down and resuspended in 1mL of cold 95% methanol and incubated on ice for 20 minutes. Cells were then washed with permeabilization buffer (PBS, 1% FCS, 0.1% azide, 0.2% saponin). Cells were stained with anti-phospho-Erk antibody (Cell Signaling) for primary, biotinylated goat-anti-rabbit (Cell Signaling) for secondary and streptavidin-PE (eBioscience) for tertiary stains.

Supplementary Figure Legends

Supplementary Figure S1. Structural alignment of Tribbles homologs. Comparison of the amino acid sequences of mouse Trib1, Trib2 and Trib3. Alignments were performed using GeneDoc software (http://www.flu.org.cn/en/download-47.html). Black and grey shaded residues indicate identical and similar sequences, respectively. The KD domain is indicated by the dotted line above the sequences and the COP1 binding site is indicated by the asterisks above the sequences.

Supplementary Figure S2. Immunophenotype of Trib1-induced AML. (A) Protein expression in 293T cells transfected with the indicated constructs. The FLAG-tagged Trib proteins were detected using an anti-FLAG antibody. The expressed proteins are indicated with arrows. (B) Flow cytometric analysis of Trib1-induced AML in BM and spleen. The Gr-1/CD11b and c-kit/F4/80 expression patterns were derived from the cells within the GFP-expressing gates shown in the middle panel.

Supplementary Figure S3. Trib3-transduced cells persist in Trib3 BMT mice. (A) The percentages of GFP+ cells in recipients of MigR1 (n = 4) and Trib3 (n = 7) BMT at the indicated time points. (B) Representative FACS profile of GFP+ cells in the peripheral blood of a MigR1 and Trib3 BMT at 6 weeks and 45 weeks post BMT. (C) Southern blot detection of proviruses in BM from Trib1 and Trib3 mice. DNA preparations were digested with Xba1 to detect intact provirus (Trib1: 4.0 kb, Trib3: 4.1kb, MigR1: 2.9kb)

Supplementary Figure S4. Trib2 but not Trib3 induce efficient degradation of C/EBPα in HeLa cells. Western blot for exogenous C/EBPα in HeLa cells transfected with the indicated FLAG-tagged Trib constructs and/or HA-tagged C/EBPα and HA-tagged COP1. C/EBPα and COP1 were detected with an anti-HA antibody, Trib2 and Trib3 were detected with an anti-FLAG antibody. β-actin was the protein loading control. The normalized C/EBPα expression amounts are shown at the bottom. Normalized C/EBPα expression was determined by the ratio of the C/EBPα to the β -actin signal (as determined by densitometry). These data were then normalized to the ratio in HeLa cells transfected with C/EBPα and Trib2 only (which was set at 1). Data are representative of two independent experiments.

Supplementary Figure S5. Trib1 and Trib2 strongly bind C/EBPα. (A) GST pull down of Trib1, Trib2, or Trib3 incubated with *in vitro* translated (IVT) ^{35}S C/EBPα. Top panel: autoradiograph of IVT ^{35}S C/EBPα. Bottom Panel: Coomassie stain of GST proteins (B) C/EBPα autoradiograph normalized to Trib-GST expression. Data are representative of 3 independent experiments.

Supplementary Figure S6. Trib1-3 associate with COP1. 293T cells transfected with the indicated constructs were immunoprecipitated with anti-FLAG beads. Western blot is shown for HA-COP1 (upper panel). The middle and lower panels show the whole cell lysates (WCL) for COP1 (HA) and Trib1-3 (FLAG).

Supplementary Figure S7. Trib proteins do not activate Erk1/2 acutely. 32D cells were transduced with MigR1, Trib1, Trib2 or Trib3 and sorted. Sorted cells were cytokine-starved for 14 hours and then stimulated with 20ng/mL of IL-3 for the time

indicated. The phosphorylation status of Erk1/2 was determined by flow cytometric analysis.

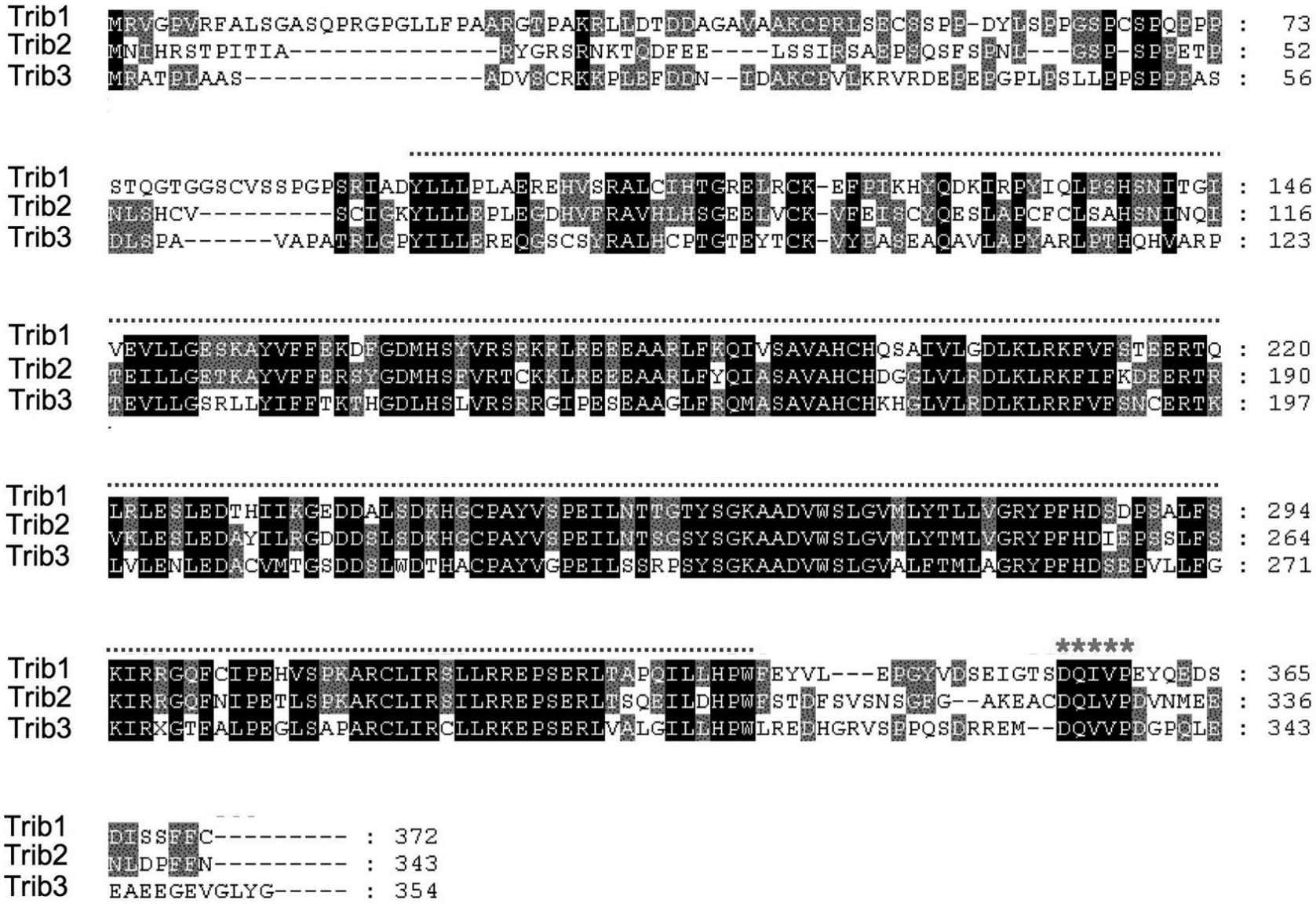
Trib1
Trib2
Trib3
MRVGPVRFALSGASQPRGPGLLFPAARGTPAKRLLDTDDAGAVAARCPRLSECSSPP-DYLSPPGSPCSPQPPP : 73
MNIHRSTPITIA--------------RYGRSRNKTQDFEE----LSSIRSAEPSQSFSPNL---GSP-SPPETP : 52
MRATPLAAS----------------ADVSCRKKPLEFDDN--IDARCPVLKRVRDEPEPGPLPSLLPPSPPPAS : 56
STQGTGGSCVSSPGPSRIADYLLLPLAEREHVSRALCIHTGRELRCK-EFPIKHYQDKIRPYIQLPSHSNITGI : 146
NLSHCV---------SCIGKYLLLEPLEGDHVFRAVHLHSGEELVCK-VFEISCYQESLAPCFCLSAHSNINQI : 116
DLSPA------VAPATRLGPYILLEREQGSCSYRALHCPTGTEYTCK-VYEASEAQAVLAPYARLPTHQHVARP : 123
VEVLLGESKAYVFFEKDFGDMHSYVRSRKRLREEEAARLFKQIVSAVAHCHQSAIVLGDLKLRKFVFSTEERTQ : 220
TEILLGETKAYVFFERSYGDMHSFVRTCKKLREEEAARLFYQIASAVAHCHDGGLVLRDLKLRKFIFKDEERTR : 190
TEVLLGSRLLYIFFTKTHGDLHSLVRSRRGIPESEAAGLFRQMASAVAHCHKHGLVLRDLKLRRFVFSNCERTK : 197
LRLESLEDTHIIKGEDDALSDKHGCPAYVSPEILNTTGTYSGKAADVWSLGVMLYTLLVGRYPFHDSDPSALFS : 294
VKLESLEDAYILRGDDDSLSDKHGCPAYVSPEILNTSGSYSGKAADVWSLGVMLYTMLVGRYPFHDIEPSSLFS : 264
LVLENLEDACVMTGSDDSLWDTHACPAYVGPEILSSRPSYSGKAADVWSLGVALFTMLAGRYPFHDSEPVLLFG : 271
*****
KIRRGQFCIPEHVSPKARCLIRSLLRREPSERLTAPQILLHPWFEYVL---EPGYVDSEIGTSDQIVPEYQEDS : 365
KIRRGQFNIPETLSPKAKCLIRSILRREPSERLTSQEILDHPWFSTDFSVSNSGFG--AKEACDQLVPDVNMEE : 336
KIRKGTFALPEGLSAPARCLIRCLLRKEPSERLVALGILLHPWLREDHGRVSPPQSDRREM--DQVVPDGPQLE : 343
DISSFFC--------- : 372
NLDPEFN--------- : 343
EAEEGEVGLYG----- : 354

Figure S1

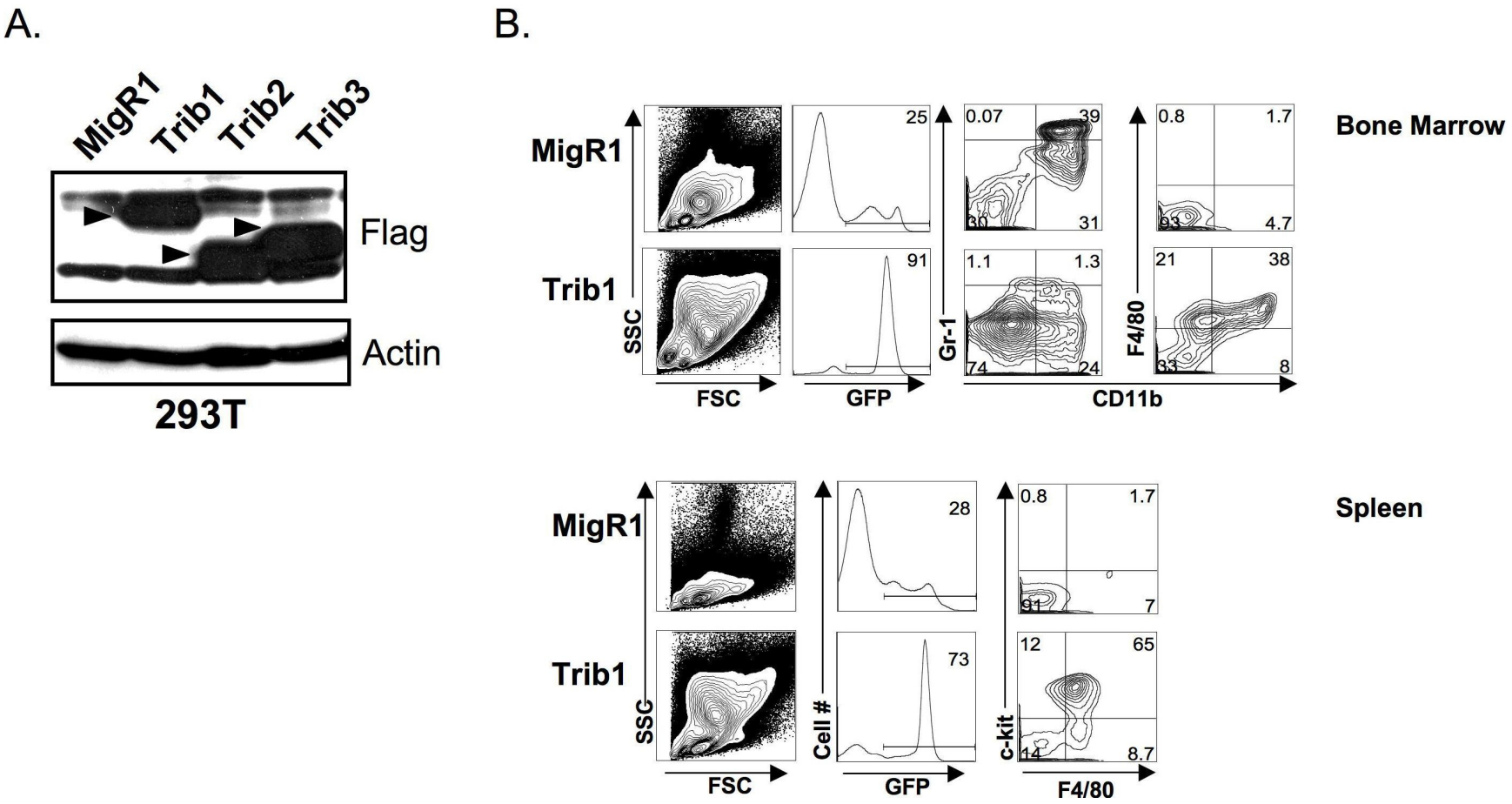
A.
MigR1
Trib1
Trib2
Trib3
Flag
Actin
293T
B.
MigR1
Trib1
SSC
FSC
GFP
Gr-1
CD11b
F4/80
Bone Marrow
Cell #
c-kit
Spleen

Figure S2

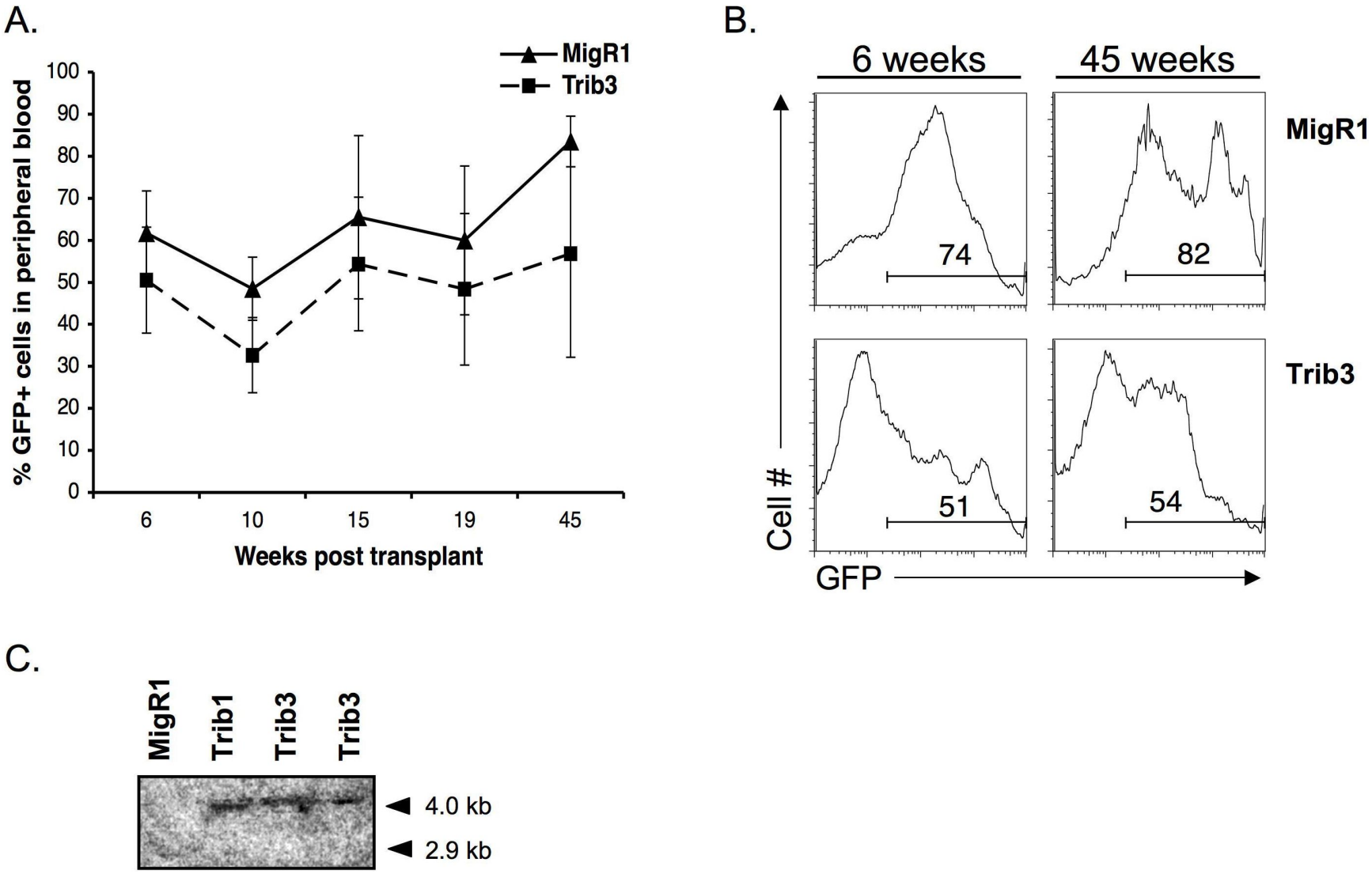
A.
MigR1
Trib3
% GFP+ cells in peripheral blood
100
90
80
70
60
50
40
30
20
10
0
6
10
15
19
45
Weeks post transplant
B.
6 weeks
45 weeks
MigR1
Trib3
74
82
51
54
Cell #
GFP
C.
MigR1
Trib1
Trib3
Trib3
4.0 kb
2.9 kb

Figure S3

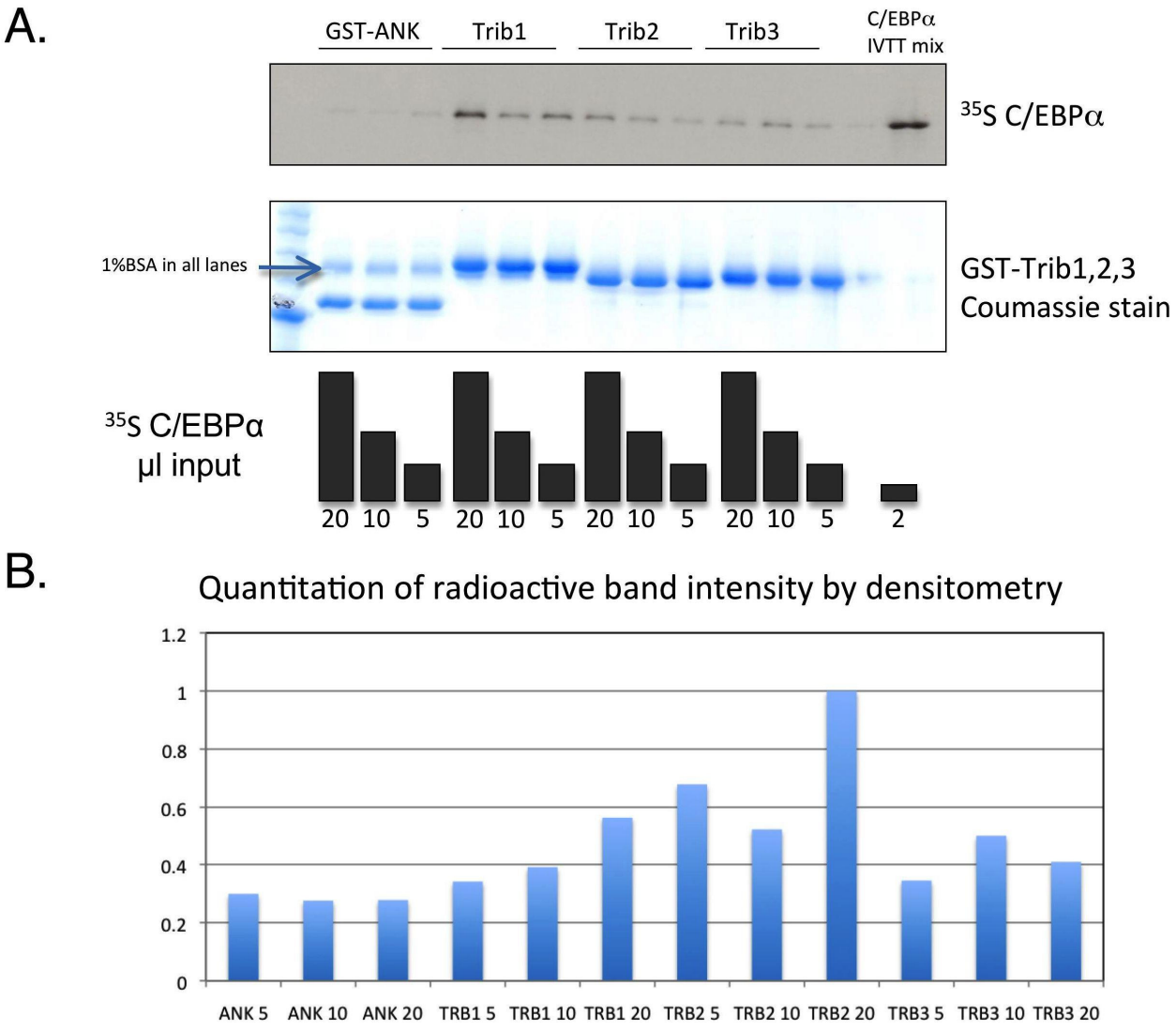
A.
GST-ANK
Trib1
Trib2
Trib3
C/EBPα
IVTT mix
35S C/EBPα
1%BSA in all lanes
GST-Trib1,2,3
Coumassie stain
35S C/EBPα
µl input
20 10 5 20 10 5 20 10 5 20 10 5 2
B.
Quantitation of radioactive band intensity by densitometry
1.2
1
0.8
0.6
0.4
0.2
0
ANK 5
ANK 10
ANK 20
TRB1 5
TRB1 10
TRB1 20
TRB2 5
TRB2 10
TRB2 20
TRB3 5
TRB3 10
TRB3 20

Figure S4

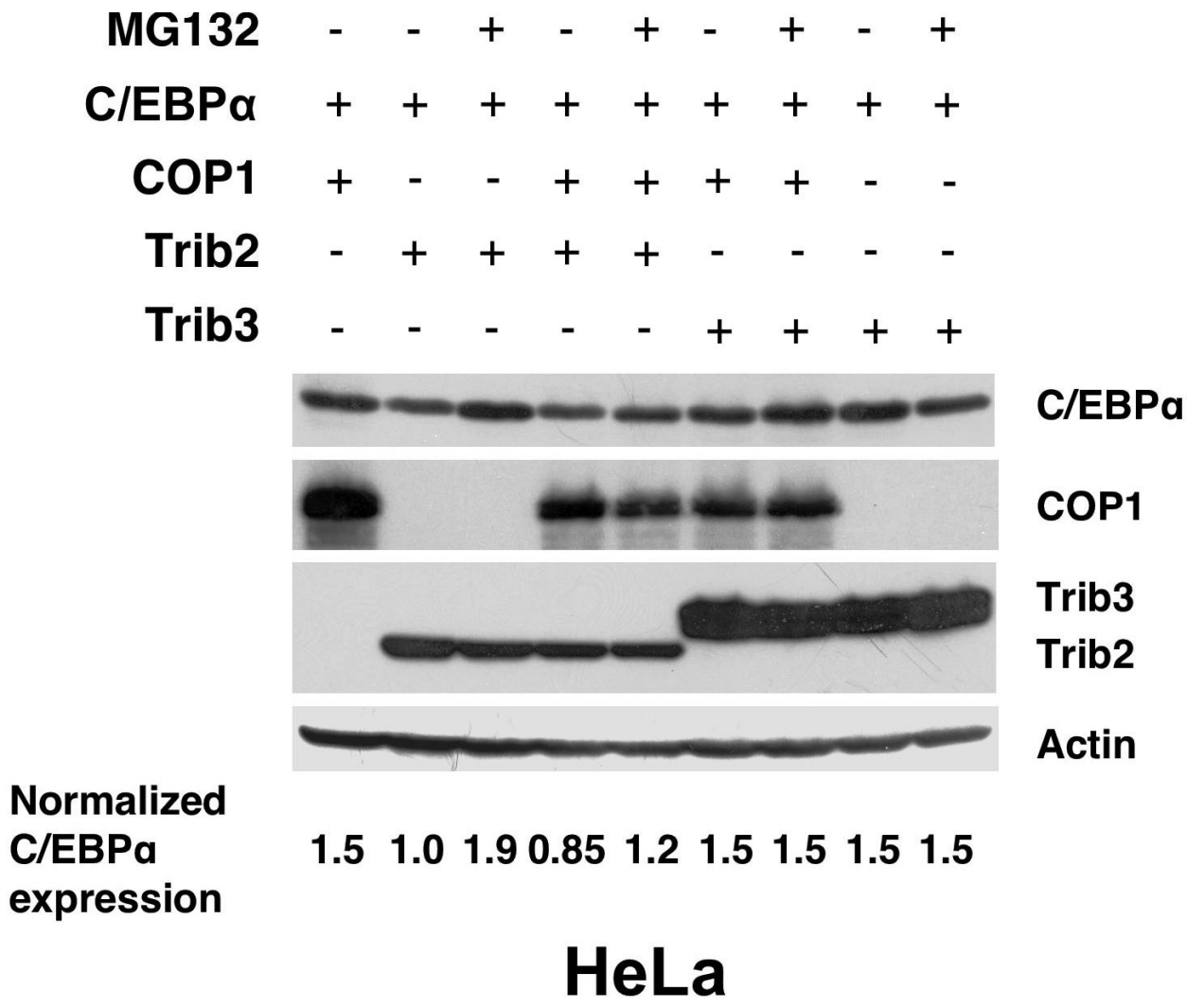
MG132 - - + - + - + - +
C/EBPα + + + + + + + + +
COP1 + - - + + + + - -
Trib2 - + + + + - - - -
Trib3 - - - - - + + + +
C/EBPα
COP1
Trib3
Trib2
Actin
Normalized C/EBPα expression
1.5 1.0 1.9 0.85 1.2 1.5 1.5 1.5 1.5
HeLa

Figure S5

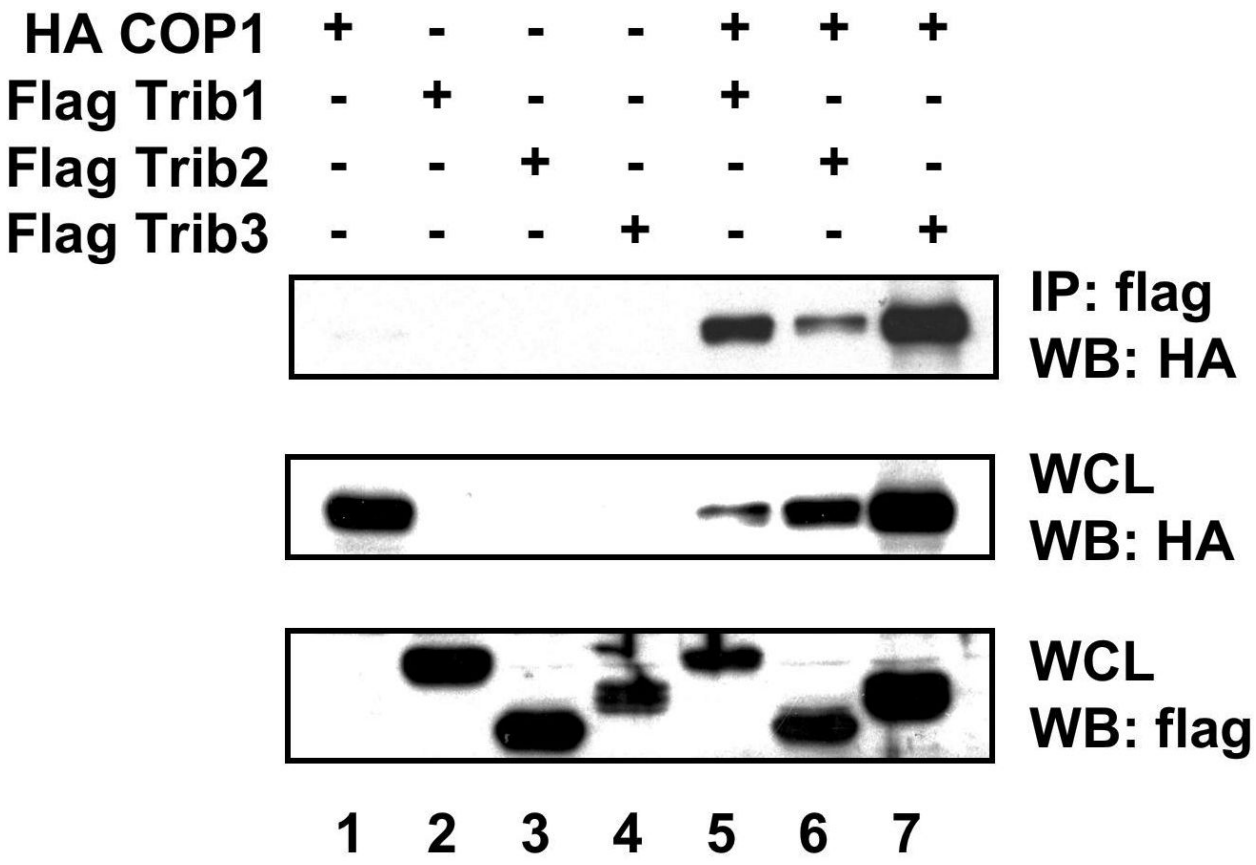
HA COP1 + - - - + + +
Flag Trib1 - + - - + - -
Flag Trib2 - - + - - + -
Flag Trib3 - - - + - - +
IP: flag
WB: HA
WCL
WB: HA
WCL
WB: flag
1 2 3 4 5 6 7

Figure S6

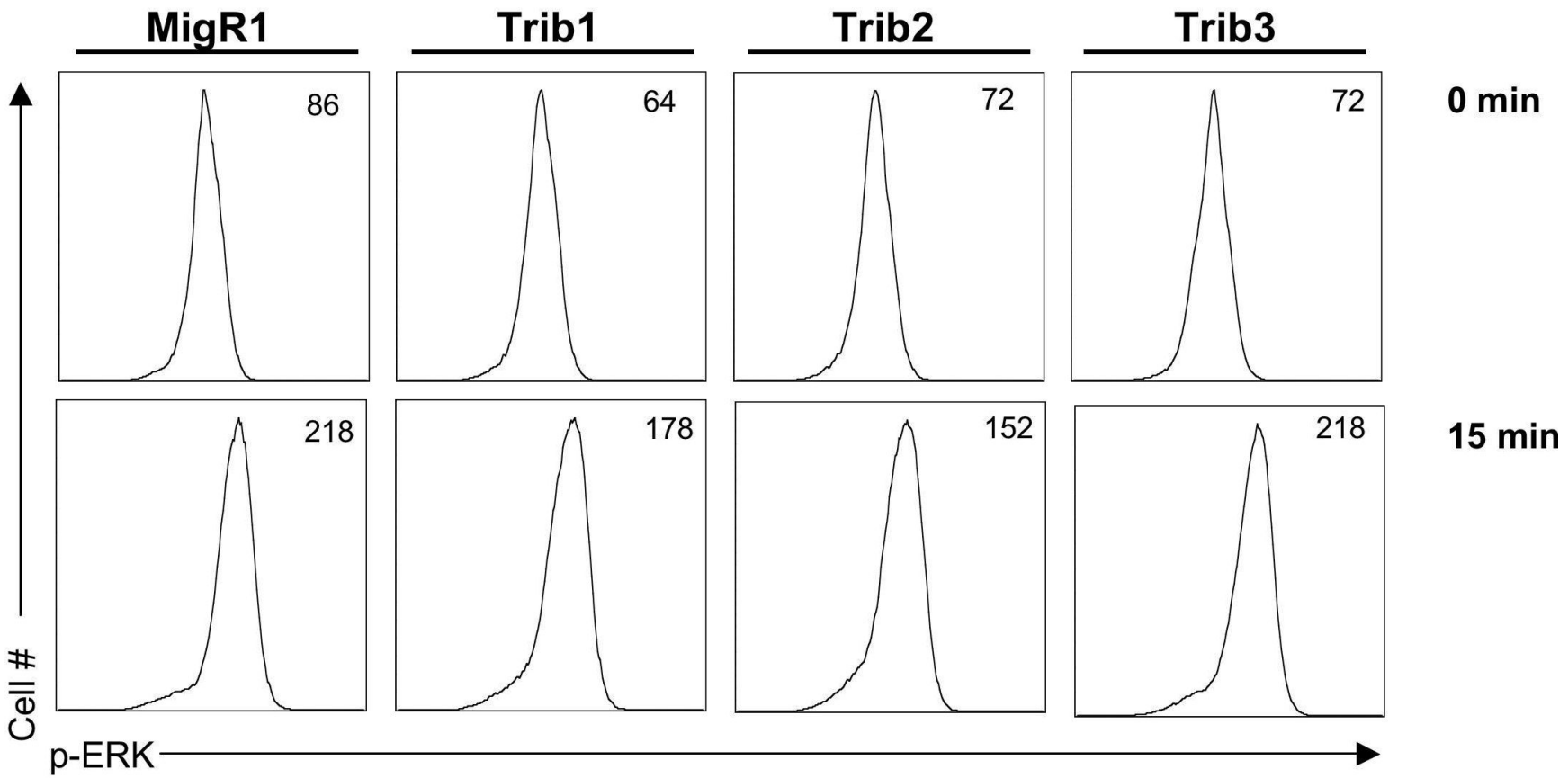
MigR1
Trib1
Trib2
Trib3
86
64
72
72
0 min
218
178
152
218
15 min
Cell #
p-ERK

Figure S7