Supplementary information

Gab2 is essential for transformation by FLT3-ITD in acute myeloid leukemia

Supplementary material and methods

Inhibitor treatment

Cells were treated with quizartinib (Selleck Chemicals), sorafenib (Santa Cruz Biotechnology) or DMSO and analyzed by Western Blotting or flow cytometry.

Western Blotting and immunoprecipitation (IP)

Western Blot analysis and Gab2 IP were performed as previously described.[1]

Generation of GAB2 knockdown cells

The knockdown of GAB2 in MOLM-13 cells was performed using the inducible pTRIPZ shRNA expression system as described previously[2]. The following shRNA clones (Dharmacon, Lafayette, USA) were used: pTRIPZ-Gab2-shRNAmir (V3THS_342925 = #1; V3THS_342929 = #2; V3THS_342930 = #3) and the control vector pTRIPZ non-silencing. Selection of MOLM-13 cells was commenced 24 h post infection (15 µg/ml puromycine, Sigma) (Fig. 1 D-F, Supplementary Fig. 2/3).

Flow cytometry

Cells were stained with Annexin-V-FITC, Propidium-Iodide and DAPI (all Biolegend) according to the manufacturer's protocols and analyzed by flow cytometry. At least 10,000 cells were counted per condition.

MOLM-13 cells and expression vectors

MOLM-13 cells were kindly provided by Prof. Justus Duyster, University of Freiburg, Freiburg (Germany) (Fig. 1A-D, Supplementary Fig. 1A-C). Cells were cultivated under water vapor saturated atmosphere at 37°C and 5% CO2 in RPMI-1640 supplemented with 10% FCS (Biochrom), 2 mM L-Glutamine, 100 U penicillin, 100 µg/ml streptomycin and 10 mM HEPES (all PAN-Biotech). The bicistronic expression vectors pMIG FLT3-WT, FLT3-ITD and FLT3 D838Y (numbering based on murine FLT3) were kind gifts of Prof. Justus Duyster, University of Freiburg, Freiburg (Germany) (Fig. 2A/B, Supplementary Fig. S4 A-D). The retroviral vector pMIG/p210Bcr-Abl was a kind gift of Dr. Sebastian Herzog, University of Freiburg, Freiburg (Germany) (Fig. 2 A/B, Supplementary Fig. S4 A-D).

Bone marrow isolation, transduction and cytokine deprivation

Gab2-proficient, -haploinsufficient or -deficient mice[3] were primed with 5-fluorouracil (5-FU; 125 mg/kg bodyweight i.p., Sigma-Aldrich) three days before sacrifice. After harvesting bone marrow cells (Fig. 2A/B, Supplementary Fig. S4A-D) they were immediately resuspended in IMDM (PAN-Biotech) supplemented with 20% FCS (Biochrom), 2 mM L-Glutamine, 100 U penicillin, 100 µg/ml streptomycin, 10 mM HEPES (all PAN-Biotech), 10 ng/ml human recombinant IL-6, 10 ng/ml murine-SCF (both PeproTech) and IL-3 (5% vol. conditioned medium of the X63Ag IL-3 cell line). After 8 h the cells were exposed to viral supernatants from transfected Plat-E cells as described previously[4,5] and spin infected (20 min, 290 g). A second spin infection was performed 24 h later. The infection rate was controlled 48 h post second spin infection by flow cytometry. Cytokine deprivation was performed seven days post infection (Fig. 2 A/B, Supplementary Fig. S4D). Therefore, the cells were washed three times with DPBS before the pellet was resuspended in deprivation medium (IMDM supplemented with 20% FCS, 2 mM L-Glutamine, 100 U penicillin, 100 µg/ml streptomycin, 10 mM HEPES). From this point on, cells were regularly monitored by microscopy and flow cytometry.

List of antibodies

Antibodies used for Western Blotting in this study were: anti-phospho-FLT3 Y589/591 (30D4), anti-phospho-FLT3 Y969 (C24D9), anti-FLT3 (8F2), anti-phospho-GAB2 Y452 (C33G1), anti-phospho-GAB2 S159, anti-GAB2 (26B6), anti-phospho-AKT T308, anti-phospho-AKT S473, anti-AKT, anti-phospho-ERK (p44/42 MAPK Erk1/2) Thr202/Tyr204, anti-ERK (p44/42 MAPK), anti-phospho-Stat5 Tyr694, anti-STAT5, anti-PI3 Kinase p85, anti-phospho-p70 S6 Kinase Thr389 (1A5), anti-p70 S6 Kinase (49D7), anti-phospho-MEK1/2 Ser217/221 (41G9), anti-MEK1/2, anti-phospho-c-Fos Ser32 (D82C12), anti-c-Fos (9F6) (all Cell Signaling Technology), anti-Tubulin, anti-pan 14-3-3 (H-8) (both Santa Cruz Biotechnology), anti-SHP2, anti-Grb2 (both Becton Dickinson), anti-phospho-GAB2 Y643, anti-phospho-GAB2 S623 (both Sigma-Aldrich)) and anti-GAPDH (Abcam). The antibody for the GAB2 immunoprecipitation (IP) was purchased from Santa Cruz Biotechnology (Gab2 H-6).

Histology

The study included 21 formalin-fixed and paraffin-embedded bone marrow biopsies (BMB) of healthy individuals (n = 4) or patients with FLT3-ITD (n = 4), FLT3-TKD (n = 4) and FLT3 wt/NPM1 (n = 9). Biopsies were stained with chloroacetatesterase (ACE) as described elsewhere.[6] Human sample collection and analysis were approved by the ethics committee of the University of Freiburg, Germany (protocol numbers 176/16) and an informed consent was given by the patients and healthy donors.

Gab2 immunohistochemistry (IHC)

Serial 3-μm-thick sections of bone marrow biopsies (BMBs) were deparaffinized in xylene and graded alcohols, followed by specific antigen retrieval by cooking of the sections in “Target Retrieval Solution” (Dako; pH 6.1)

in a steamer, as described previously[7]. Blocking in flex peroxidase (Dako) was performed for 10 min. After incubation with the primary antibody for 30 min at room temperature (Anti-GAB2 antibody [EPR2869] (ab108423) Abcam; 1:50), the corresponding secondary antibody (Flex/Rabbit Linker ready-to-use; Dako) was added for 15 min. Following a 20 min incubation with Flex/HRP (ready-to-use; Dako) sections were washed and incubated with Flex DAB chromogen (1:50; Dako) for 10 min and counterstained with hematoxylin (Waldeck) for 1 min and mounted. Quantitative evaluation of Gab2-specific IHC was carried out by examining 100 myeloid cells or blasts, respectively, in each of 3 random high-power fields per case (total n = 300 cells per BMB) assessing the amount of cells being Gab2 negative or positive in cytoplasma or nucleus. To exclude unspecific and/or background reactions, adequate controls were performed as described previously[7]. Briefly, negative controls were prepared by replacing Gab2 with an immunoglobulin isotype antibody showing complete negative staining. In addition, an alternative Gab2 antibody (Anti-GAB2 (26B6); Cell Signaling Technologies) was used showing a similar Gab2 staining pattern in bone marrow biopsies.

Statistics

The quantitative data are presented as mean ± SEM. Statistics were calculated using the indicated statistical test. A p-value of $p \leq 0.05$ was considered statistically significant (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$).

Supplementary tables

Patient data

Table S1: Data of the patients, whose bone marrow biopsies were analyzed in Fig. 2c/d.

#	Sex	Age at biopsy (years)	Disease stage at biopsy	FLT3 status	NPM1	Karyotype	Perinuclear/nuclear Gab2 positive blasts [%]
1	M	74	Induction failure	ITD	WT	45,XY,-7 [20]	95.6
2	F	44	Relapse	ITD	Mut	46,XX [20]	96.3
3	F	46	Initial diagnosis	ITD	WT	46,XX,t(3;5)(q25;q34)[19]	87
4	F	37	Initial diagnosis	ITD	WT	46,XX[20]	97.6
5	F	32	Initial diagnosis	TKD	WT	46,XX,1dmin[2], 46,XX[18]	95.3
6	M	63	Initial diagnosis	TKD	WT	46,XY[21]	98
7	M	73	Initial diagnosis	TKD	WT	46,XY [22]	96
8	M	25	Initial diagnosis	TKD	WT	46,XY[10]	99.3
9	M	64	Initial diagnosis	WT	Mut	46,XY[20]	98
10	F	58	Initial diagnosis	WT	Mut	46,XX[20]	93
11	F	39	Initial diagnosis	WT	Mut	46,XX[23]	97
12	M	57	Initial diagnosis	WT	Mut	46,XY[20]	96
13	F	49	Initial diagnosis	WT	Mut	46,XX[20]	100
14	M	65	Initial diagnosis	WT	Mut	46,XY[20]	92
15	F	69	Initial diagnosis	WT	Mut	46,XX[20]	97
16	F	32	Initial diagnosis	WT	Mut	46,XX[20]	100
17	M	69	Initial diagnosis	WT	Mut	46,XY[21]	98

Abbreviations:
M, male; F, female; ITD, internal tandem duplication; TKD, mutation in the tyrosine kinase domain;
WT, wildtype = no ITD, no TKD mutation

Genetic data are from disease stage at biopsy, except data marked in grey letters which are from initial diagnosis

Supplementary figures

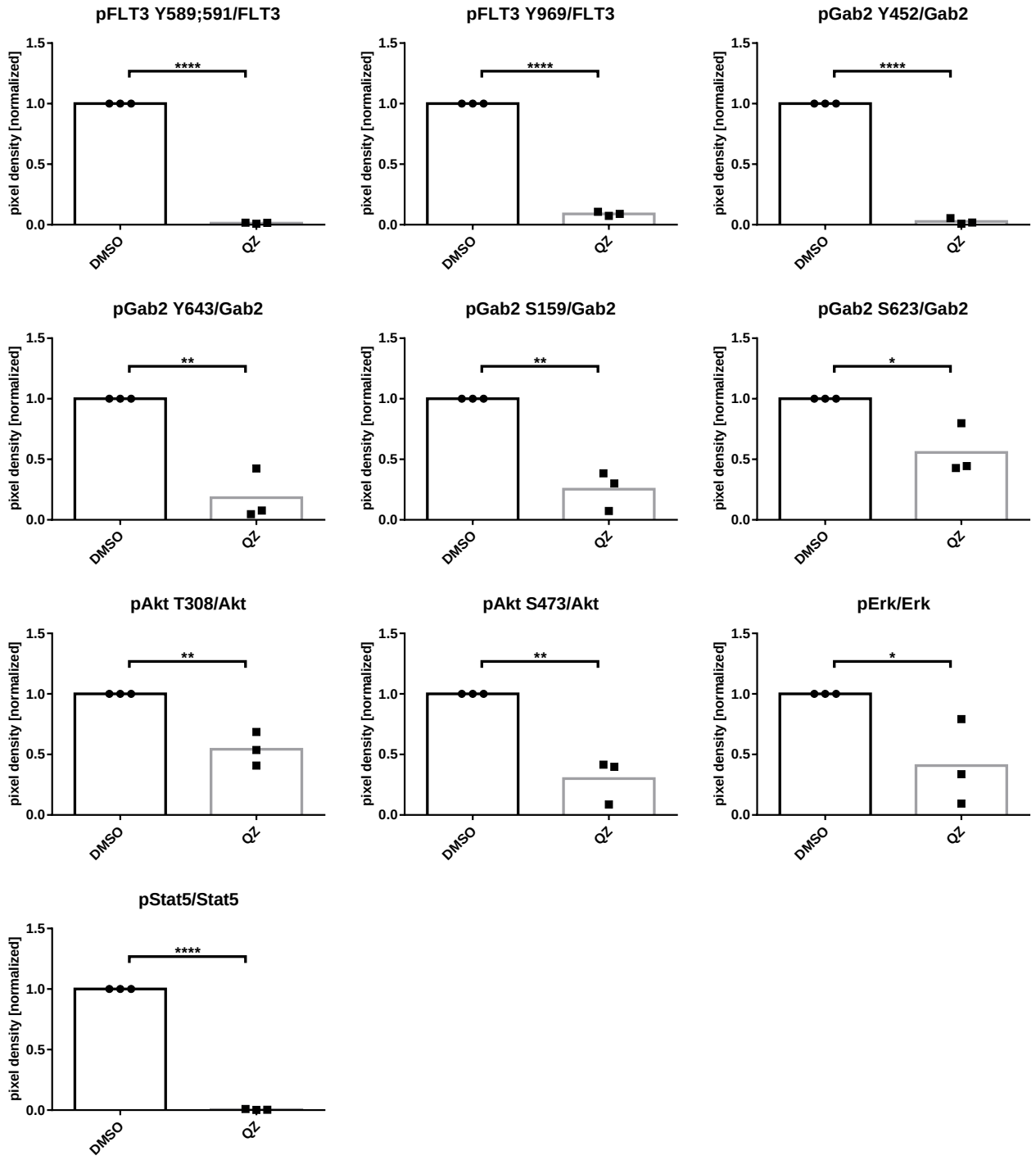


Figure S1

Quantification of Western Blots shown in Figure 1A and two independent replicates (total n=3). All proteins were normalized to Tubulin (loading control) on each membrane. Subsequently, phospho-proteins were normalized to the corresponding total protein. Statistics were calculated using unpaired t-tests.

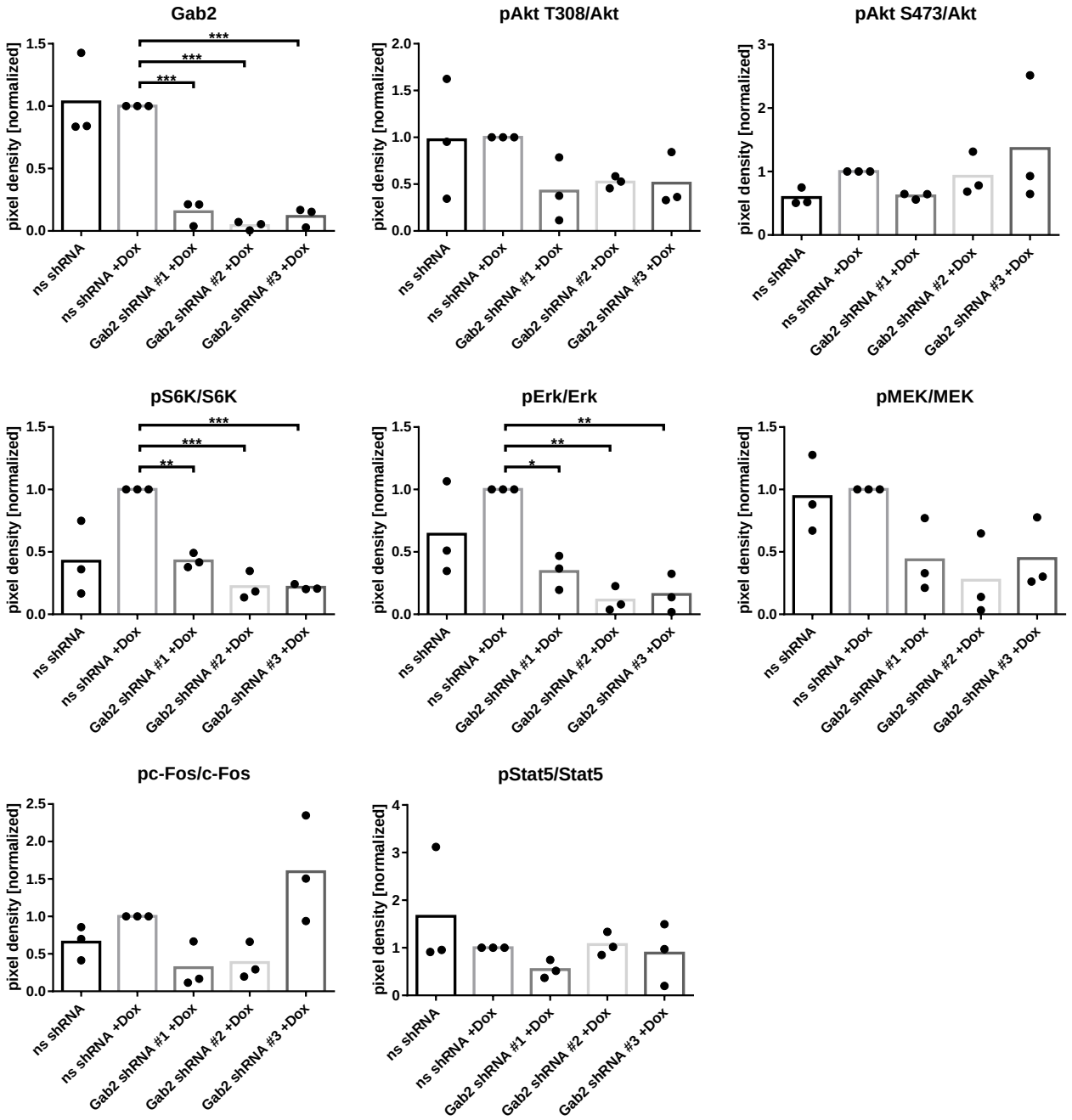
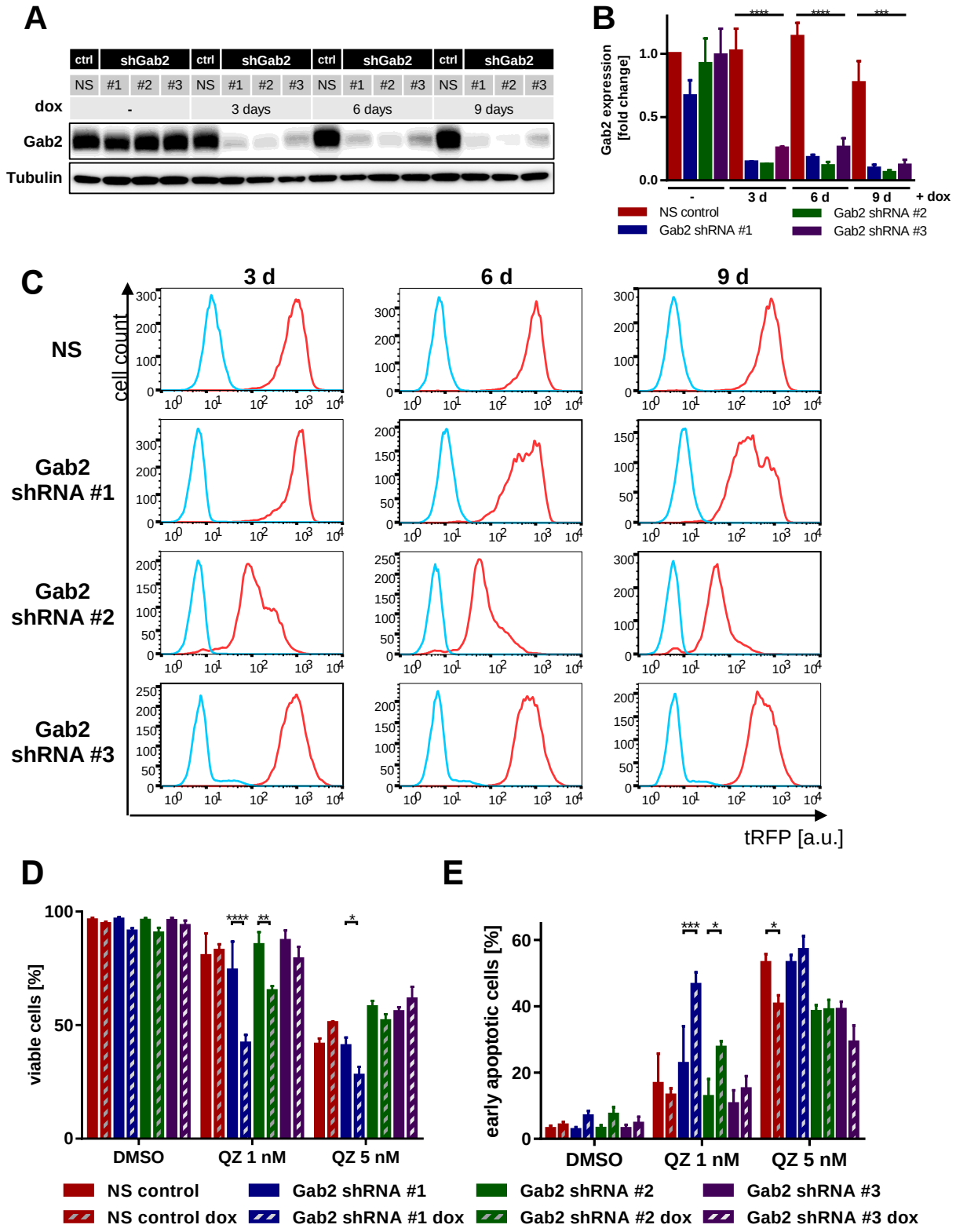


Figure S2

Quantification of Western Blots shown in Figure 1D and two independent replicates (total n=3). All proteins were normalized to 14-3-3 (loading control) on each membrane. Subsequently, phospho-proteins were normalized to the corresponding total protein. Statistics were calculated using One-way ANOVA with Tukey's multiple comparison.


A
ctrl
shGab2
NS
#1
#2
#3
dox
-
3 days
6 days
9 days
Gab2
Tubulin
B
Gab2 expression [fold change]
1.0
0.5
0.0
****
***
-
3 d
6 d
9 d
+ dox
NS control
Gab2 shRNA #1
Gab2 shRNA #2
Gab2 shRNA #3
C
3 d
6 d
9 d
NS
Gab2 shRNA #1
Gab2 shRNA #2
Gab2 shRNA #3
cell count
tRFP [a.u.]
D
viable cells [%]
100
50
0
****
**
*
DMSO
QZ 1 nM
QZ 5 nM
E
early apoptotic cells [%]
60
40
20
0
***
*
*
DMSO
QZ 1 nM
QZ 5 nM
NS control
Gab2 shRNA #1
Gab2 shRNA #2
Gab2 shRNA #3
NS control dox
Gab2 shRNA #1 dox
Gab2 shRNA #2 dox
Gab2 shRNA #3 dox

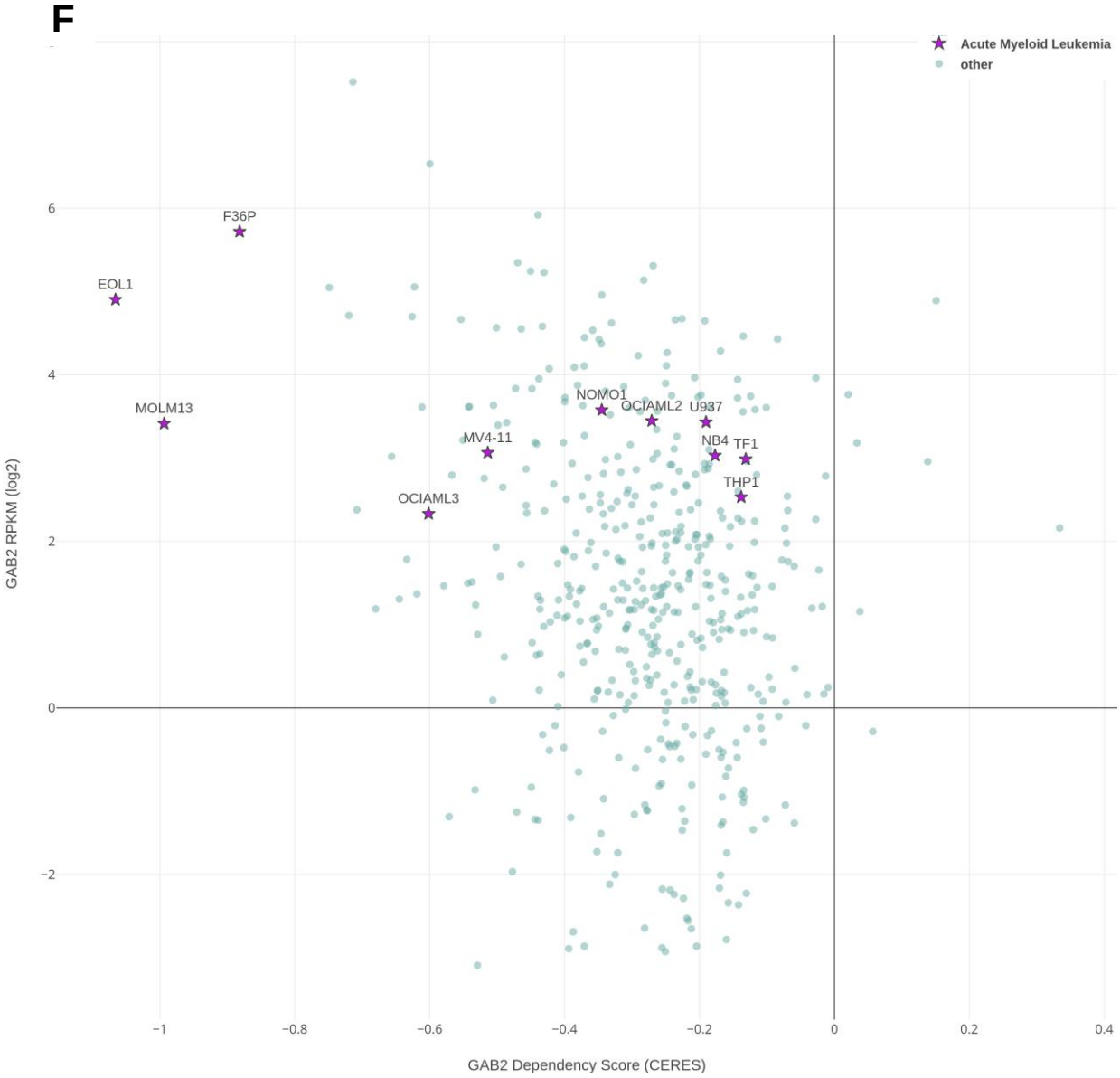


Figure S3

(A) MOLM-13 cells stably infected with shRNAs mediating a Gab2 knockdown (#1 - #3) or a non-silencing (NS) control were induced with dox (1 µg/ml) for the indicated durations and analyzed by Western Blotting. **(B)** Quantification of the knockdown shown in (A). **(C)** MOLM-13 cells stably infected with shRNA-mediated Gab2 knockdown constructs (#1 - #3) or a non-silencing (NS) control were induced with dox (1 µg/ml) for three, six or nine days (shown in red) or left untreated (shown in blue) and analyzed for the co-expressed tRFP by flow cytometry. Shown is one representative time course out of three experiments. **(D/E)** Addendum to Fig. 1 (F). Proportion of viable (DAPI negative, Annexin-V negative) cells (D) and early apoptotic (Annexin-V positive, DAPI negative) cells (E) after treatment with DMSO or the FLT3 inhibitor QZ. Shown is the mean of three independent biological replicates, error bars represent standard error of the mean (SEM). Statistics were calculated using non-matching two-way ANOVA with uncorrected Fisher's LSD test. **(F)** Correlation analysis of Gab2 expression and Gab2 dependency using the DepMap data explorer with the public 18Q3 expression and CRISPR datasets [8,9]. AML cell lines are highlighted with a star. RPKM = Reads per kilo base per million mapped reads; CERES = a computational method to estimate gene dependency levels from CRISPR-Cas9 essentiality screens while accounting for the copy-number-specific effect, as well as variable sgRNA activity.

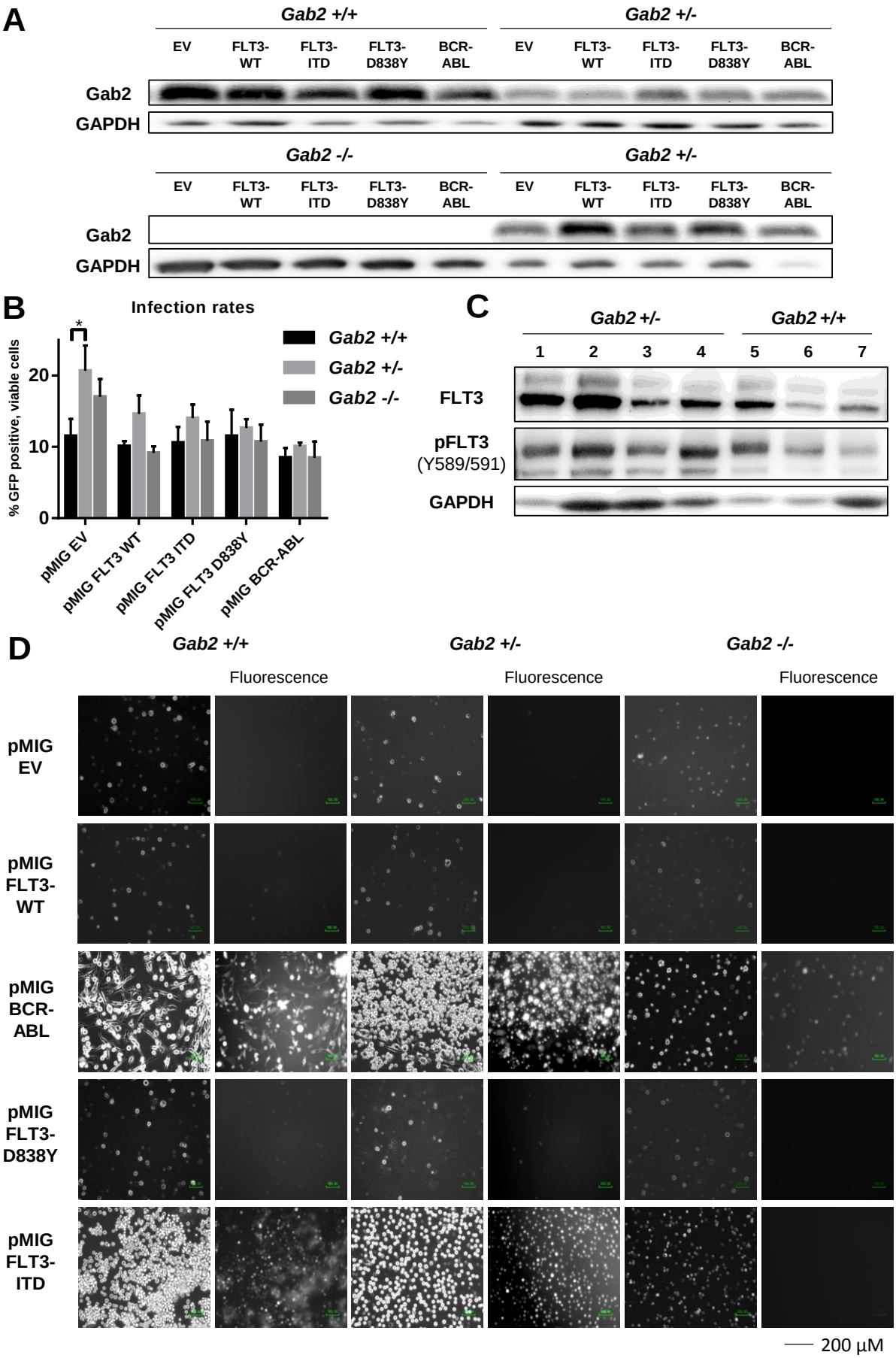

A
Gab2 +/+
Gab2 +/-
EV
FLT3-WT
FLT3-ITD
FLT3-D838Y
BCR-ABL
Gab2
GAPDH
Gab2 -/-
B
Infection rates
% GFP positive, viable cells
Gab2 +/+
Gab2 +/-
Gab2 -/-
pMIG EV
pMIG FLT3 WT
pMIG FLT3 ITD
pMIG FLT3 D838Y
pMIG BCR-ABL
C
1 2 3 4 5 6 7
FLT3
pFLT3 (Y589/591)
GAPDH
D
Fluorescence
pMIG EV
pMIG FLT3-WT
pMIG BCR-ABL
pMIG FLT3-D838Y
pMIG FLT3-ITD
200 µM

Figure S4

(A) Total cell lysates of murine bone marrow cells infected with the indicated vectors were analyzed by Western Blotting using the indicated antibodies. **(B)** Quantification of the flow cytometry analysis of pMIG-infected bone marrow cells coexpressing GFP 48 h post infection. n = 3 for *Gab2*$^{+/+}$ and *Gab2*$^{-/-}$ and n = 4 for *Gab2*$^{+/-}$ mice. Statistical analysis was performed using an unmatched two-way ANOVA with uncorrected Fisher's LSD test. **(C)** Total cell lysates of the FLT3-ITD transformed bone marrow cells of *Gab2*$^{+/-}$ (#1-4) and *Gab2*$^{+/+}$ (#5-7) mice under cytokine deprivation were analyzed by Western Blotting using the indicated antibodies. **(D)** Representative photographs of the murine bone marrow cells infected with the indicated vectors taken three weeks after cytokine deprivation.

Supplementary references

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9. Map BICD, (2018) CDS. Cancer Dependency Map, CRISPR Avana dataset 18Q3 (Avana_public_18Q3). @figshare; 2018.