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Letter
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Gab2 is Essential for Transformation by FLT3-ITD in Acute Myeloid Leukemia

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About 30% of acute myeloid leukemias (AML) are driven by constitutive activation of the FMS-like tyrosine kinase 3 (FLT3), either due to an internal tandem duplication (ITD) in the juxtamembrane domain or mutations in the tyrosine kinase domain (TKD). FLT3-ITD is associated with poor outcomes, whereas FLT3-TKD mutations usually confer a less aggressive course of the disease.[1] Targeting FLT3 has been the objective of multiple clinical trials resulting in the recent approval of midostaurin, the first multikinase inhibitor for the treatment of FLT3-mutant AML, by the Food and Drug Administration.[2] However, in single agent studies with FLT3 inhibitors clinical responses are characterized by an only transient reduction in peripheral blood and/or bone marrow blasts.[3] Thus, despite being an important key player in AML, FLT3 might cooperate with other signaling molecules in promoting leukemogenesis. The docking protein GRB2-associated binder 2 (Gab2) serves as an amplifier in the signaling network of growth factor and cytokine receptors.[4,5] Gab2 works as an assembly platform by binding to FLT3 via the adaptor Grb2,[5,6] thereby amplifying signaling into SHP2/Ras/ERK, PI3K/AKT, and STAT5 pathways leading to survival, proliferation, and migration.[5] However, it is not known whether Gab2 is similarly critical in FLT3-ITD-driven transformation as described for other oncogenic tyrosine kinases like Bcr-Abl.[7,8]

Based on previous studies, we started to analyze the interplay between FLT3 and Gab2 by treating the human FLT3-ITD-positive AML cell line MOLM-13 with the FLT3-selective inhibitor quizartinib (QZ) and analyzed Gab2 phosphorylation on Western Blot (Figs. 1A and S1, Supplemental Digital Content, http://links.lww.com/HS/A30), as well as the binding of Gab2 to known interactors like Grb2, SHP2, and p85 (PI3K) in a Gab2 immunoprecipitation (Fig. 1B and C). FLT3 inhibition reduces Gab2 phosphorylation on various sites, for example, the PI3K binding site Y452 and the SHP2 binding site Y643 (Figs. 1A and S1, Supplemental Digital Content, http://links.lww.com/HS/A30). Consequently and in line with these results, we observed less binding of SHP2 and of the p85 subunit of PI3K to Gab2 upon treatment with the FLT3 inhibitors QZ and sorafenib (SF) (Fig. 1B and C). Next, we established a Gab2 knockdown in MOLM-13 cells (Figs. 1D, S2, and S3A and B, Supplemental Digital Content, http://links.lww.com/HS/A30) using a vector allowing the doxycycline (dox) inducible expression of an shRNA together with turbo RFP (tRFP) from the same transcript. The knockdown of Gab2 lowers the activity of the Raf/MEK/ERK and PI3K/AKT/mTOR signaling pathways as shown by less phosphorylation of MEK, ERK, and their downstream target c-Fos, as well as less phosphorylation of the mTOR substrate S6K (Figs. 1D and S2, Supplemental Digital Content, http://links.lww.com/HS/A30). Consequently, we observed that the knockdown

Funding/support: This study was supported in part by the Excellence Initiative of the German Research Foundation (GSC-4, Spemann Graduate School, BIOSS EXC 294), the Heisenberg program (TB), the José Carreras Leukämie-Stiftung e.V. and the Deutsche Krebshilfe (Mildred-Scheel-Doktoranden-Program, 111815 to K.S.).
Disclosure: The authors have indicated they have no potential conflicts of interest to disclose.
KS and CS have contributed equally to this study.
TB and SH are co-senior authors.
All authors designed, analyzed, and discussed experiments. KS, CS, AG, FMU, and SH performed all cellular and biochemical experiments. KA, RT, and JH conducted the histological analysis. HB, HLP, and RZ provided clinical samples and/or expertise. SH wrote the manuscript together with KS, CS, HB, KA, and TB. All authors reviewed and commented on the manuscript and accepted its final version.
Supplemental Digital Content is available for this article.
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HemaSphere (2019) 3:2(e184)
Received: 22 January 2019 / Accepted: 25 January 2019
Citation: Sies K, Spohr C, Gründer A, Todorova R, Uhl FM, Huber J, Zeiser R, Pahl HL, Becker H, Aumann K, Brummer T, Halbach S. Gab2 Is Essential for Transformation by FLT3-ITD in Acute Myeloid Leukemia. *HemaSphere*, 2019;3:2. http://dx.doi.org/10.1097/HS9.0000000000000184

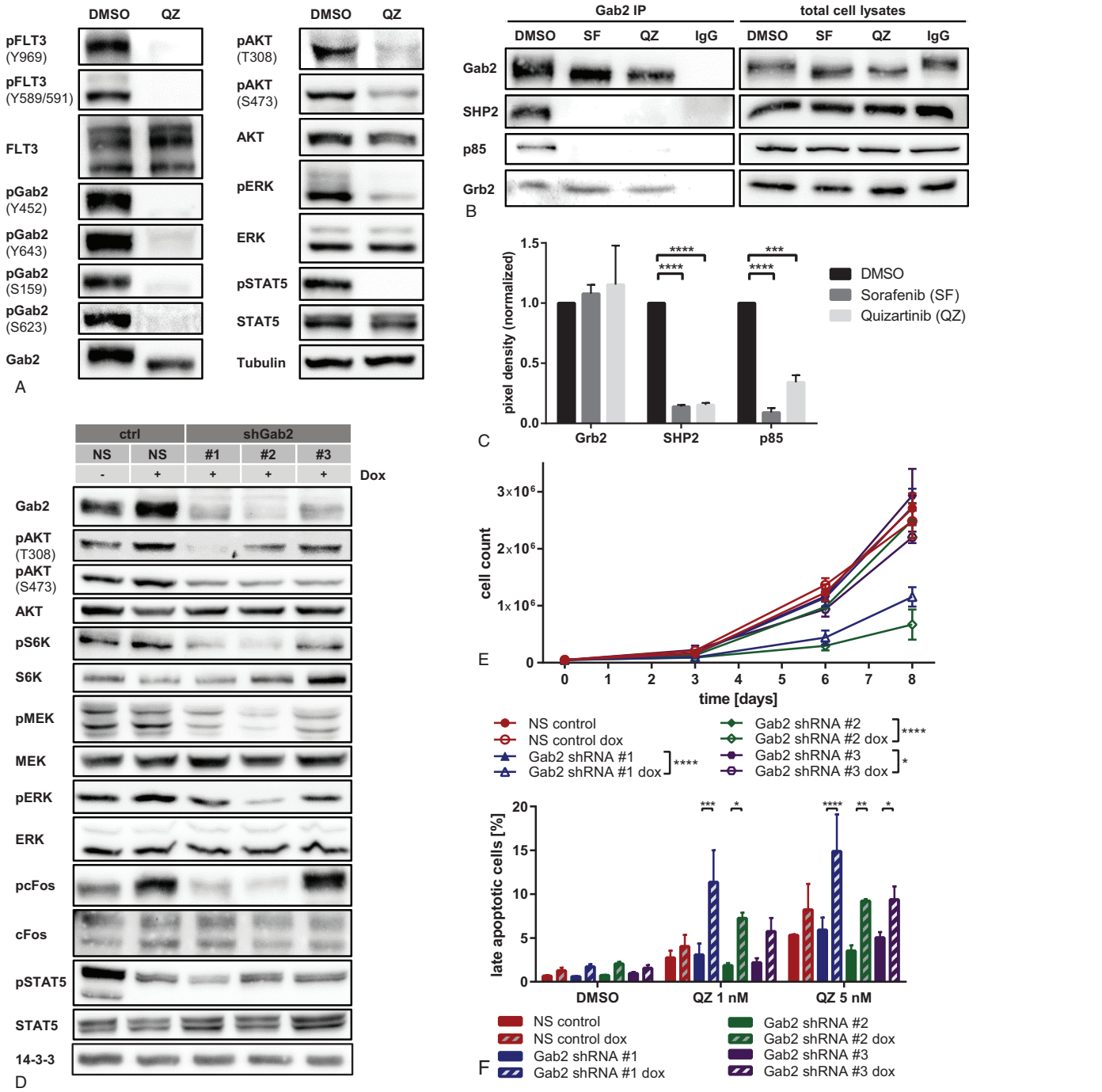

Figure 1. The inhibition of FLT3 affects Gab2 phosphorylation, as well as its binding to downstream signaling proteins, and knockdown of Gab2 leads to decreased proliferation and increased quizartinib sensitivity. (A) MOLM-13 cells were treated with quizartinib (QZ; 10 nM) for 4 hours and subjected to Western Blot analysis using the indicated antibodies. Shown is one representative Western Blot. Quantification of 3 independent experiments can be found in the supplementary information (Supplemental Digital Content, http://links.lww.com/HS/A30). (B) MOLM-13 cells were treated with QZ (10 nM) and sorafenib (SF; 10 μM) for 4 hours. Subsequently, Gab2 was immunoprecipitated and the cells were analyzed by Western Blotting using the indicated antibodies. Shown is one representatative Western blot out of three. (C) Quantification of the Western Blot shown in (B). (D) MOLM-13 cells stably infected with shRNAs mediating a Gab2 knockdown (no. 1–3) or a nonsilencing (NS) control were induced with doxycycline (dox; 0.5 μg/mL) for 3 days. Cells were starved in 1% FCS the night before lysis and finally analyzed by Western Blotting using the indicated antibodies. Shown is one representatative Western blot out of three. Quantification can be found in the supplementary information (Supplemental Digital Content, http://links.lww.com/HS/A30). (E and F) MOLM-13 cells (as described in (D)) were induced with dox (1 μg/mL) for 7 days and (E) subjected to a proliferation assay or (F) subsequently exposed to QZ or DMSO for 24 hours. In (E), cells were afterward double-stained with 4′,6-diamidine-2-phenylindole (DAPI) and Annexin-V and analyzed by flow cytometry. Late apoptotic cells are defined as double positive for Annexin-V and DAPI. (C, E, F) Shown is the mean and SEM of three independent experiments. Statistics were calculated using 2-way analysis of variance with uncorrected Fisher LSD. DMSO = dimethyl sulfoxide, FCS = fetal calf serum, Gab2 = GRB2-associated binder 2, LSD = least significant difference, SEM = standard error of mean.

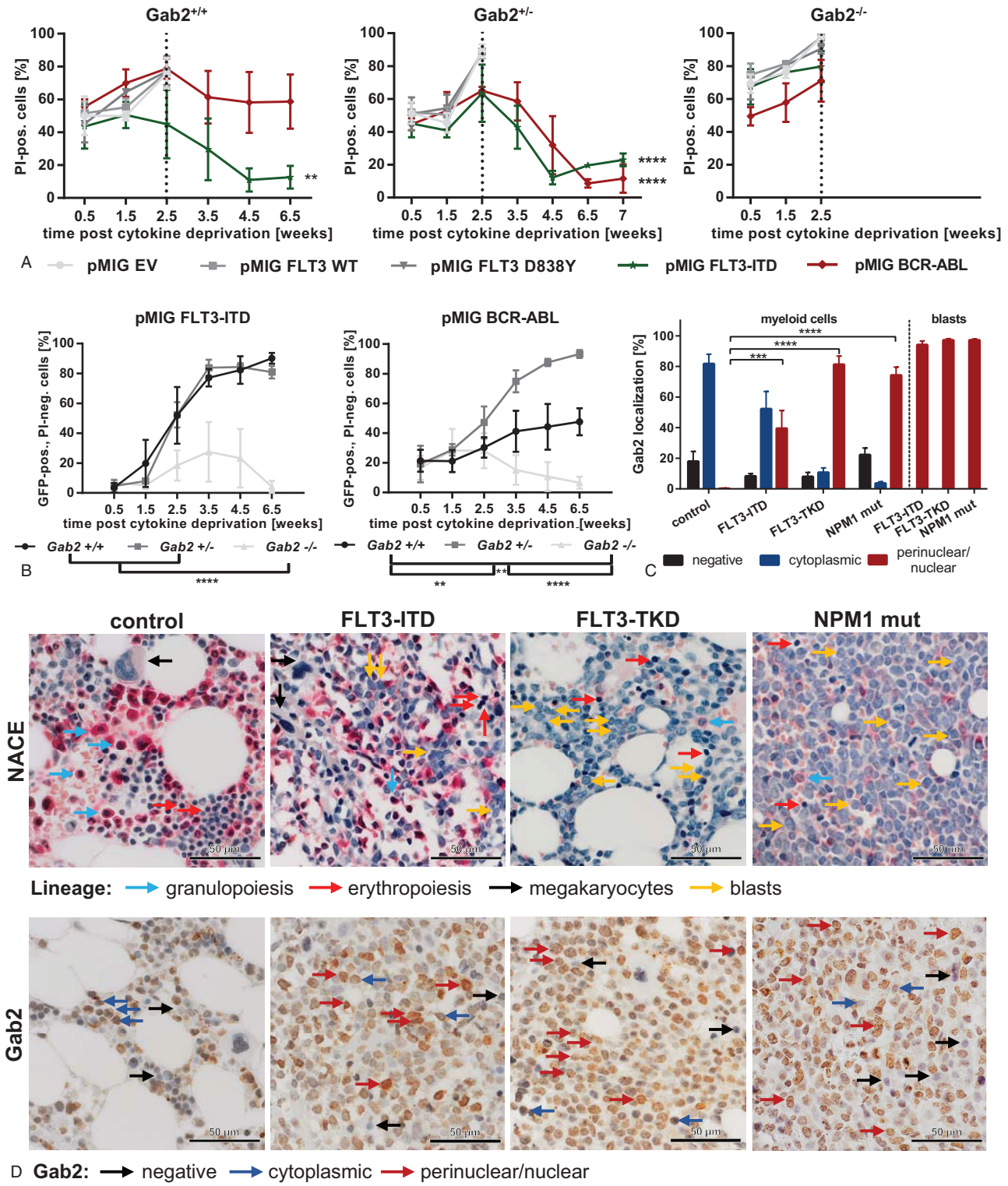

Figure 2. Gab2 is required for the FLT3-ITD mediated transformation of primary murine bone marrow cells and is highly expressed in blasts from FLT3- and NPM1-mutant AML patients. Murine bone marrow cells with different Gab2 genotypes were transduced with the indicated constructs and analyzed for their viability. Shown is the mean percentage and SEM of propidium-iodide (PI) positive cells over time. The dotted line indicates the time point at which cells that were declared dead have been excluded from further analysis. (B) Enrichment analysis of infected (GFP-positive) cells from (A). Shown is the mean percentage and SEM of GFP-positive, PI-negative cells over time. (A and B) *Gab2*$^{+/+}$ and *Gab2*$^{-/-}$ n=3; *Gab2*$^{+/-}$ n=4. Statistics were calculated using a 2-way ANOVA with corrected Tukey test. (C) Quantification of the subcellular Gab2 localization in bone marrow sections of AML patients and controls as shown in (D). Control n=4, FLT3-ITD n=4, FLT3-TKD n=4, FLT3-WT/NPM1 n=9. Shown is the mean percentage and SEM of Gab2-positive myeloid cells and blasts. Relevant statistics were calculated using a 2-way ANOVA with corrected Tukey test. (D) Chloracetatesterase (NACE) and immunohistochemical Gab2 staining of bone marrow sections from AML patients and healthy controls, respectively. Selected cells are indicated with arrows. AML=acute myeloid leukemia, ANOVA=analysis of variance, EP=erythropoiesis, FLT3=FMS-like tyrosine kinase 3, Gab2=GRB2-associated binder 2, GFP=green fluorescent protein, GP=granulopoiesis, ITD=internal tandem duplication, MK=megakaryocytes, NACE=naphthol-AS-D-chloroacetate esterase, SEM=standard error of mean, TKD=tyrosine kinase domain.

of Gab2 impaired proliferation of MOLM-13 cells compared with noninduced controls (Fig. 1E). Supporting this, the proportion of cells with lower shRNA expression, indicated by tRFP coexpression, increased over time (Fig. S3C, Supplemental Digital Content, http://links.lww.com/HS/A30). These observations are in line with a study showing similar results in the FLT3-ITD-positive MV4-11 cell line.[5] In addition, we analyzed and correlated Gab2 expression and dependency using the DepMap data explorer with the public 18Q3 expression and CRISPR datasets.[9,10] Our correlation shows that MOLM-13 and MV4-11, among other AML cell lines, have high Gab2 expression levels and that both cell lines show *Gab2* dependency in a CRISPR proliferation screen (Fig. S3F, Supplemental Digital Content, http://links.lww.com/HS/A30). Next, we determined the influence of Gab2 on tyrosine kinase inhibitor (TKI) sensitivity using QZ. Importantly, Gab2 knockdown rendered MOLM-13 cells more sensitive toward QZ (Figs. 1F and S3D and E, Supplemental Digital Content, http://links.lww.com/HS/A30). We previously observed that Gab2 alters TKI sensitivity in the context of chronic myeloid leukemia (CML).[11,12] Furthermore, others and we previously reported that Gab2 is essential for Bcr-Abl-mediated transformation of murine bone marrow cells in vitro[7] and in vivo.[13] These data hint at a more general phenomenon of Gab2 downstream of hyper-activated kinases. We therefore aimed to investigate whether Gab2 has a similar role for FLT3-mediated transformation. Thus, we infected bone marrow cells from *Gab2*-proficient, -haploinsufficient, or -deficient mice with bicistronic vectors coexpressing green fluorescent protein (GFP) and wildtype (FLT3-WT) or mutant FLT3 (FLT3-ITD or FLT3-TKD).[14] Gab2 expression levels were validated via Western Blot (Fig. S4A, Supplemental Digital Content, http://links.lww.com/HS/A30) and comparable infection rates were controlled by flow cytometry (Fig. S4B, Supplemental Digital Content, http://links.lww.com/HS/A30). As expected, FLT3-WT does not harbor oncogenic potential and the infected cells died within a few days after cytokine deprivation regardless of their *Gab2* genotype (Fig. 2A). By contrast, FLT3-ITD-infected cells survived cytokine independently in a *Gab2*-proficient or -haploinsufficient background (Fig. 2A) and GFP-positive cells enriched over time (Fig. 2B). Strikingly, FLT3-ITD was not able to transform Gab2-deficient cells (Fig. 2A and B), indicating an essential role for Gab2 in FLT3-ITD-mutant AML. Flow cytometry data were underlined by microscopy observations (Fig. S4D, Supplemental Digital Content, http://links.lww.com/HS/A30) and FLT3 expression was validated via Western Blot (Fig. S4C, Supplemental Digital Content, http://links.lww.com/HS/A30). We also tested the FLT3-TKD mutation D838Y, but none of the cells survived cytokine-independently, regardless of their *Gab2* genotype. This finding may be linked to the differences in the clinical outcome of FLT3-ITD- versus FLT3-TKD-positive AMLs,[1] as well as to differences in factor-independent growth in culture and murine bone marrow transplantation models.[14] In accordance with earlier studies,[7,8] Gab2 was also essential for Bcr-Abl-mediated transformation of murine bone marrow cells (Fig. 2A and B). To explore the role of Gab2 in human AML pathology, we stained Gab2 in bone marrow biopsies of AML patients harboring either an FLT3-ITD or an FLT3-TKD mutation (Fig. 2C and D, Supplementary Table S1, Supplemental Digital Content, http://links.lww.com/HS/A30). Furthermore, we included samples from FLT3-WT/NPM1-mutant AML to see whether Gab2 expression might be a feature specific to AML driven by constitutively active signaling molecules (Fig. 2C and D, Supplementary Table S1, Supplemental Digital Content, http://links.lww.com/HS/A30). Strikingly, we observed a strong Gab2 staining in samples from patients with FLT3-ITD, FLT3-TKD, and NPM1 mutations, respectively (Fig. 2C), thus supporting a biologically relevant role for Gab2 in FLT3- and NPM1-mutant AML. Additionally, the subcellular localization of Gab2 changed from cytoplasmic, in myeloid cells from healthy controls, to a perinuclear/nuclear appearance in immature myeloid cells and blasts. This phenomenon is in coherence with our previous findings in blasts from CML patients[15] and points toward a role of Gab2 in the nucleus/perinuclear region. Supporting this, Osawa et al reported the nuclear translocation of Gab1 and identified a nuclear localization signal,[16] which is highly conserved and also present in Gab2, except for one exchange of lysine to arginine that should not affect its function. Despite the different transformation potential of FLT3-ITD and FLT3-TKD mutations, we were not able to detect differences in their Gab2 staining pattern, suggesting that Gab2 might also be important in FLT3-TKD-mutant AML. Furthermore, our observation that FLT3-WT/NPM1-mutant AML cases show a similar Gab2 staining even points toward a more general role of Gab2 in AML.

In summary, our data identify Gab2 as a critical component for the FLT3-ITD-mediated transformation of murine bone marrow cells. Its knockdown impairs proliferation and increases QZ sensitivity in MOLM-13 cells. Finally, we showed that Gab2 is highly expressed in myeloid cells of patients with FLT3- and NPM1-mutant AML compared with healthy controls.

Therefore, our current data invite for further evaluation of Gab2 as a biomarker for TKI sensitivity or even as a new therapeutic target in AML. In addition, our work contributes to the emerging role of Gab2 as an essential signaling hub in various leukemia entities. Beside AML, Gab2 has been described to be critical for the development of CML[7,11,13] and more recently also for juvenile myelomonocytic leukemia.[17] Realization of pharmacological implications of the role of Gab2 call for novel approaches, for example, targeting Gab2 protein-protein interactions,[18] as Gab2 has no enzymatic activity.

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