Leukemia (2013) **27,** 118–129

www.nature.com/leu

ORIGINAL ARTICLE

Gab2 signaling in chronic myeloid leukemia cells confers resistance to multiple Bcr-Abl inhibitors

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Grb2-associated binder 2 (Gab2) serves as a critical amplifier in the signaling network of Bcr-Abl, the driver of chronic myeloid leukemia (CML). Despite the success of tyrosine kinase inhibitors (TKIs) in CML treatment, TKI resistance, caused by mutations in Bcr-Abl or aberrant activity of its network partners, remains a clinical problem. Using inducible expression and knockdown systems, we analyzed the role of Gab2 in Bcr-Abl signaling in human CML cells, especially with respect to TKI sensitivity. We show for the first time that Gab2 signaling protects CML cells from various Bcr-Abl inhibitors (imatinib, nilotinib, dasatinib and GNF-2), whereas Gab2 knockdown or haploinsufficiency leads to increased TKI sensitivity. We dissected the underlying molecular mechanism using various Gab2 mutants and kinase inhibitors and identified the Shp2/Ras/ERK and the PI3K/AKT/mTOR axes as the two critical signaling pathways. Gab2-mediated TKI resistance was associated with persistent phosphorylation of Gab2 Y452, a PI3K recruitment site, and consistent with this finding, the protective effect of Gab2 was completely abolished by the combination of dasatinib with the dual PI3K/mTOR inhibitor NVP-BEZ235. The identification of Gab2 as a novel modulator of TKI sensitivity in CML suggests that Gab2 could be exploited as a biomarker and therapeutic target in TKI-resistant disease.

Leukemia (2013) **27,** 118–129; doi:10.1038/leu.2012.222

Keywords: Bcr-Abl; Gab2; TKI resistance; imatinib; rapamycin; 14–3–3

INTRODUCTION

The discovery of the oncogenic protein tyrosine kinase Bcr-Abl as the product of the Philadelphia chromosome (Ph^+) and the driver of chronic myeloid leukemia (CML) led to the development of the first clinically relevant tyrosine kinase inhibitor (TKI), imatinib mesylate (IM).[1] Bcr-Abl retains the catalytic domain of c-Abl, while the Bcr moiety recruits interaction partners such as the Grb2/Gab2 (Grb2-associated binder 2) complex into the operating range of the Abl kinase and therefore increases its substrate repertoire and 'biological portfolio'.[2–4] As a consequence, Bcr-Abl organizes a multimeric protein complex and activates various signaling pathways.[5]

CML therapy changed radically with the development of selective Bcr-Abl inhibitors, such as IM, which exploit the phenomenon of oncogene addiction.[6,7] Despite their success, however, IM or second generation inhibitors do not eradicate Bcr-Abl-positive CML stem cells, whose persistence in the bone marrow leads to the accumulation of additional (epi)genetic alterations that ultimately cause TKI resistance. This represents a clinical problem, either occurring within the first 18 months of treatment (primary resistance) or as acquired resistance of previously TKI-sensitive CML (secondary resistance).[1,8,9] In 60% of cases, secondary TKI resistance is caused by mutations within the Bcr-Abl fusion gene preventing the action of the inhibitor in the catalytic cleft of the enzyme.[1,8] This insight led to the development of second and third generation Bcr-Abl inhibitors such as the ATP-competitive compounds nilotinib (NL) and dasatinib (DS) and, more recently, the allosteric inhibitors GNF-2/5.[10] Bcr-Abl mutation-independent mechanisms of TKI resistance remain ill-defined at the molecular level. However, there is increasing evidence that aberrant activity or expression of components of the Bcr-Abl signaling network contributes to TKI resistance.[1,8] For example, overexpression or hyper-activity of Src family kinases (SFKs) mediate TKI resistance in CML.[11,12] These findings raise the possibility that combination of a TKI with inhibitors of other signaling pathways may improve CML therapy.[8] However, such strategies require a detailed knowledge of the individual components, their regulation and the architecture of the Bcr-Abl signaling network.

One critical signal transducer of Bcr-Abl is Gab2, a member of the Gab family of docking proteins.[4,13] Using multiple interaction motifs, Gab proteins couple growth factor and cytokine receptors to downstream effectors such as the Shp2/Ras/ERK (extracelluar signal-regulated kinase), PI3K/AKT/mTOR and JAK/STAT pathways.[14] The association of Gab proteins with receptor tyrosine kinases and other tyrosine-phosphorylated proteins is mediated *via* the Grb2 adapter.[14] For example, Grb2 bridges Bcr-Abl and Gab2 by binding *via* its central SH2 domain to

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Received 7 June 2012; revised 25 July 2012; accepted 27 July 2012; accepted article preview online 3 August 2012; advance online publication, 28 August 2012

phospho-tyrosine 177 (Y177) in the Bcr moiety, and with its C-terminal SH3 domain to specific binding sites in Gab2.[2,15] Consequently, the phenylalanine substitution of the putative Y177 auto-phosphorylation site or the mutation of the Grb2-binding motifs in Gab2 impair the recruitment and tyrosine phosphorylation of the docking protein by Bcr-Abl in Ba/F3 cells.[4] Furthermore, Gab2-deficient murine myeloid progenitors cannot be transformed by Bcr-Abl.[4]

The physiological role of Gab2 has been revealed by characterization of Gab2 $-/-$ mice, which exhibit defects in osteoclast and mast cell differentiation and mild abnormalities in several hematopoietic lineages.[14,16–19] However, Gab2 is also implicated in oncogenic signaling. Firstly, if uncoupled from PI3K-controlled negative feedback loops, Gab2 transforms non-hematopoietic cell types.[20,21] Secondly, aberrant expression of the human *GAB2* gene occurs in multiple solid tumor types and acute myeloid leukemia.[14] Functional studies in breast cancer and melanoma models indicate that dysregulated Gab2 signaling contributes to increased proliferation, reduced growth factor requirements and enhanced cellular motility.[14,22] Finally, retroviral transduction of bone marrow from Gab2-deficient (Gab2 $-/-$) mice revealed an essential role for this docking protein in the transforming properties not only of Bcr-Abl,[4] but also of Shp2/*PTPN*11 *gain-of-function* (g-o-f) mutants, which are associated with somatically acquired and Noonan syndrome-associated juvenile myelomonocytic leukemia.[23,24]

The pivotal role of Gab2 in Bcr-Abl signaling is further underscored by the observations that short hairpin (sh) RNA-mediated silencing of endogenous Gab2 inhibits proliferation and colony formation of $CD34^+$ cells from CML patients, but not their counterparts isolated from healthy donors.[13] These findings suggest that human CML might be addicted to Bcr-Abl-driven Gab2 signaling and identify Gab2 as a potential therapeutic target, for example, for CML patients whose tumors have acquired resistance to TKIs such as IM. Here, we further characterize the role of this docking protein in Bcr-Abl signaling and demonstrate for the first time that Gab2 signaling dictates the TKI sensitivity of CML cells, a finding that is of potential prognostic and therapeutic value.

MATERIALS AND METHODS

Cell lines

K562 and Ba/F3 cells were kind gifts from Dr Mark Guthridge, Adelaide (Australia) and Professor Michael Huber, Aachen (Germany), respectively. K562 and Ba/F3 cells were maintained in RPMI-based medium.[21]

Patient samples

Patient samples were collected after approval by the Ethics Committee of the Albert-Ludwigs-University Freiburg (protocol number: 345/10 and 119/10) and after written informed consent according to the declaration of Helsinki. The protocol for Gab2-specific quantitative immunohistochemistry (IHC) of bone marrow biopsies (BMBs) was published by us before,[25] but is provided with the additional details of the present study and protocols for the cultivation of primary human CML blast in Supplementary data. Note that in the present study Gab2 protein expression was examined in BMBs of patients at initial diagnosis (initial biopsy), under TKI treatment (remission control biopsy) and in the TKI-refractory cases also at disease relapse (relapse biopsy). Same numbers of myeloid cells were evaluated in all remission control BMBs to define the potential of Gab2 protein expression as a supplementary biomarker for disease progression.

Fluorescence-activated cell sorting (FACS)

FACS analysis was performed using a CyAN ADP flow cytometer and Summit 4.3 software (Beckman-Coulter, Brea, CA, USA). Cells were stained with 7-AAD, Annexin-V-Alexa-647 (Biolegend, San Diego, CA, USA) or Annexin-V-PE (Becton Dickinson, Franklin Lakes, NJ, USA) according to the protocol of the manufacturer.

Antibodies, biochemical analysis and western blotting

K562, Ba/F3 and primary CML cells were processed to total cellular lysates by lysing in normal lysis buffer and analyzed by western blotting as described previously.[26] A detailed list of antibodies is provided in Supplementary data.

Statistical analysis

Unless stated otherwise, bar graphs show the average of at least three independent experiments, error bars represent the standard error of the mean (s.e.m.) and asterisk indicates $P<0.05$ (Student's *t*-test).

RESULTS

Lowering Gab2 expression increases the efficacy of IM

We have recently reported by quantitative IHC of BMBs that the spatio-temporal Gab2 expression pattern correlates with distinct phases of disease progression in CML.[25] In agreement with this study, we identified a dataset[27] in the Oncomine database,[28] which indicates a strong tendency for elevated Gab2 transcript levels in the bone marrow and peripheral blood samples from CML patients during accelerated phase and blast crisis (BC) compared with chronic phase (Figure 1a). Similar findings for Gab2 protein expression were reported subsequently.[29] Furthermore, our analysis of Gab2 expression levels in various cancer cell lines using the recently published Cancer Cell Line Encyclopedia (CCLE)[30] revealed that Gab2 expression levels are elevated in CML cell lines compared with the other cancer cell lines (Supplementary Figure S1A). This observation correlates well with the fact that all these CML lines were derived from BC patients. The upregulation of mean Gab2 levels in samples or cell lines derived from advanced CML in these four independent studies and the fact that BC is particularly refractory to TKIs[9,27] led to our hypothesis that Gab2 might modulate TKI sensitivity. Thus, we established tetracycline-inducible knockdown and cDNA expression systems in K562 cells, a CML model cell line derived from a patient in BC. Among the 15 CML cell lines analyzed in the CCLE dataset, K562 cells showed intermediate Gab2 levels (Supplementary Figure S1B) and thus appear as particularly suited for modulation of its expression and subsequent phenotypic analysis. First, we addressed how the depletion of endogenous Gab2 would affect the cellular behavior and IM-responsiveness of CML cells. To this end, K562 cells were stably infected with a lentiviral construct for the inducible knockdown of Gab2 by microRNA-adapted shRNAs (shRNAmir) or with a non-silencing control vector (Supplementary Figure S2). Cells were exposed to the tetracycline analog doxycycline (dox) for 7 days (d) and the successful Gab2 knockdown was confirmed by western blotting (Figure 1b). In agreement with a previous report,[13] K562 cells responded to the induction of a Gab2-specific shRNAmir with a reduction of their proliferation rate compared with controls (Figure 1c).

Next, we determined the ability of IM to induce apoptosis of Gab2 knockdown and control cells by Annexin-V staining. Inducible knockdown of endogenously expressed Gab2 in K562 cells rendered the cells more sensitive towards IM, while vector control cells displayed the same IM-responsiveness in the presence or absence of dox (Figure 1d). Thus, depletion of endogenous Gab2 increases the efficacy of IM to induce cell death of K562 cells.

Inducible overexpression of Gab2 in K562 cells confers IM resistance

In order to analyze the effects of increased Gab2 expression in human CML cells, we generated K562 cells allowing the dox-inducible overexpression of Gab2 with a hemagglutinin (HA) tag at the C-terminus (Gab2-HA). As constitutive overexpression of wildtype Gab2 ($Gab2^{wt}$) induces megakaryocytic differentiation of

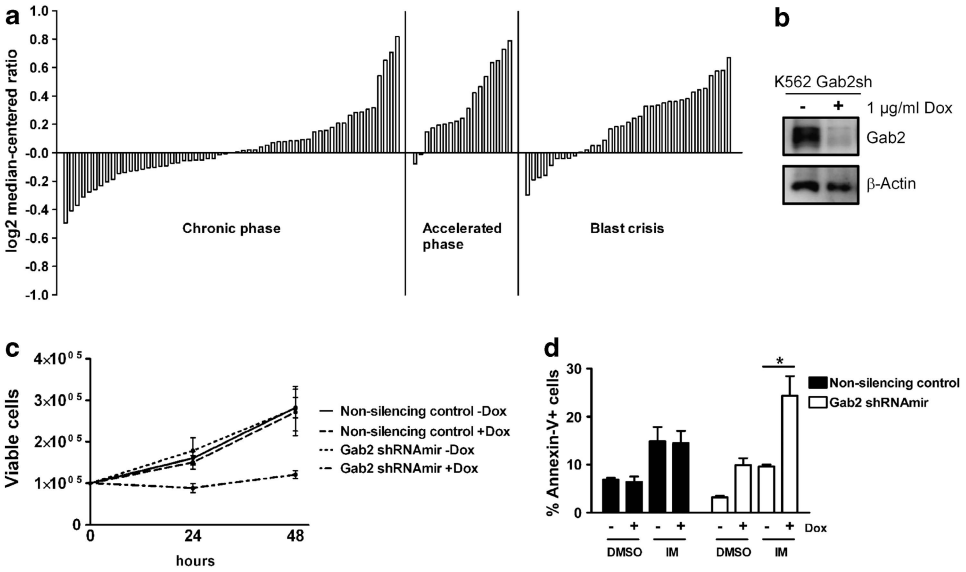


Figure 1. Lowering endogenous Gab2 levels reduces proliferation and increases IM sensitivity of CML cells. (**a**) Gab2 expression in BMBs and peripheral blood samples from 91 cases of CML in chronic phase (42 cases), accelerated phase (17 cases) and BC (32 cases) was analyzed and visualized using Oncomine (Compendia Bioscience, Ann Arbor, MI, USA) using the dataset by Radich *et al.*[27] (**b**) K562 cells infected with a construct for shRNAmir-mediated knockdown of Gab2 or a non-silencing control vector were exposed to 1 μg/ml dox for 7 d or left untreated. Successful inducible Gab2 knockdown was confirmed by western blotting. (**c**) K562 cells infected with a construct for shRNAmir-mediated knockdown of Gab2 or a non-silencing control vector were exposed to 1 μg/ml dox for 7 d or left untreated. Subsequently, 1×10^5 cells were plated and the number of viable cells was determined 24 and 48 h later. (**d**) K562 cells were infected with a construct for shRNAmir-mediated knockdown of Gab2 or a non-silencing control vector and treated with dox for 7 d (or left untreated). Thereafter, cells were exposed to IM (1 μM) or dimethylsulfoxide (DMSO) for 24 h, stained with Annexin-V and analyzed by FACS. Note that depletion of endogenous Gab2 sensitizes K562 cells towards IM.

K562 cells,[31] we first established a stable K562 subline (K562tet), which contains an improved tetracycline (tet)-regulated expression system for cDNAs (Supplementary Figure S3).[32] Subsequently, we stably transfected K562tet cells with tet-responsive expression vectors for Gab2 (pTET/Gab2-bsr) or empty control vectors. Cells were exposed to dox for 24 h and the expression of Gab2 was monitored by western blotting (Figure 2a and Supplementary Figure S3). IM sensitivity of Gab2-overexpressing cells was analyzed by FACS using two distinct read-outs for cell death, 7-AAD (Figure 2b) and Annexin-V (Supplementary Figure S4). In the absence of IM, the induction of the docking protein itself led to a slight increase in 7-AAD-positive cells, which might be explained by the aforementioned Gab2-induced differentiation events,[31] as differentiated megakaryocytes are unable to survive in culture in the absence of cytokines. Strikingly, the induction of exogenous Gab2 was sufficient to render K562 cells resistant to cell death induced by IM (Figure 2b and Supplementary Figure S4), its successor NL (Figure 2c) or the structurally unrelated allosteric Bcr-Abl inhibitor GNF-2 (Figure 2d). Moreover, Gab2-overexpressing cells showed a reduced activation of caspases 3 and 7 in response to IM treatment (Figure 2e). In summary, we report here for the first time that overexpression of Gab2 renders CML cells resistant to TKI-induced cell death.

To demonstrate the protective effect of Gab2 from IM in a distinct system, we transduced murine Ba/F3 cells with a retroviral expression vector encoding $p210^{Bcr\text{-}Abl}$. Proliferation of Ba/F3 cells is dependent on exogenous interleukin-3 (IL-3), but expression of Bcr-Abl renders them IL-3-independent. Consequently, Ba/F3 cells are widely used for the analysis of Bcr-Abl signaling and TKI development.[4,10] First, we infected Ba/F3 cells with a $p210^{Bcr\text{-}Abl}$ expression vector and selected stable pools. As expected, Ba/F3 cells infected with the $p210^{Bcr\text{-}Abl}$ expression vector, but not the empty control vector, survived in the absence of IL-3 (Supplementary Figure S5). Subsequently, $p210^{Bcr\text{-}Abl}$-expressing cells were infected with either a Gab2 expression vector or an empty control vector (Supplementary Figure S3) and tested for their IM sensitivity. In the absence of IL-3, IM caused a reduction in viability of Ba/F3 cells expressing $p210^{Bcr\text{-}Abl}$, while those co-expressing Gab2 were significantly protected from this TKI (Figure 2f). By contrast, $p210^{Bcr\text{-}Abl}$-expressing Ba/F3 cells super-infected with a retroviral vector for wildtype Lyn (Lyn^{wt}), whose overexpression is implicated in the TKI resistance of CML,[11,33] were not protected from IM. Nevertheless, a g-o-f mutant of Lyn (Lyn^{Y508F}) protected $p210^{Bcr\text{-}Abl}$-expressing Ba/F3 cells from IM (Figure 2f) as reported previously.[34] Interestingly, the phosphorylation of endogenous Gab2 at one of its three PI3K recruitment sites, Y452, was markedly increased by Lyn^{Y508F} (Figure 2g). Taken together, these data demonstrate that the expression level of Gab2 is a critical determinant for TKI sensitivity in the human CML cell line K562 and the murine Ba/F3 Bcr-Abl reconstitution system.

The activity of the Bcr-Abl/Grb2/Gab2 axis is modulated by 14–3–3 proteins

Gab proteins contain multiple protein–protein interaction and phosphorylation motifs, which control their signaling in- and output.[14] To dissect which structural features and effector pathways are involved in the protective effect of Gab2 from IM, we used various Gab2 mutants in K562tet cells.[21,26] As we have previously shown that EGF-triggered Gab2 signaling is terminated by 14–3–3 proteins,[21] we first expressed several Gab2 14–3–3 binding mutants in K562tet cells following the procedure outlined in Supplementary Figure S3. This panel comprises $Gab2^{2xR18}$, constitutively bound to its negative regulator 14–3–3 and therefore impaired in Grb2 binding, and its control mutant $Gab2^{2xKK}$ (see Supplementary Figure S6 online for details). The latter as well as the $Gab2^{2xA}$ protein lack the functional 14–3–3-binding motifs and represent g-o-f mutants.[21] As the Bcr-Abl-dependent tyrosine phosphorylation of murine Gab2 is

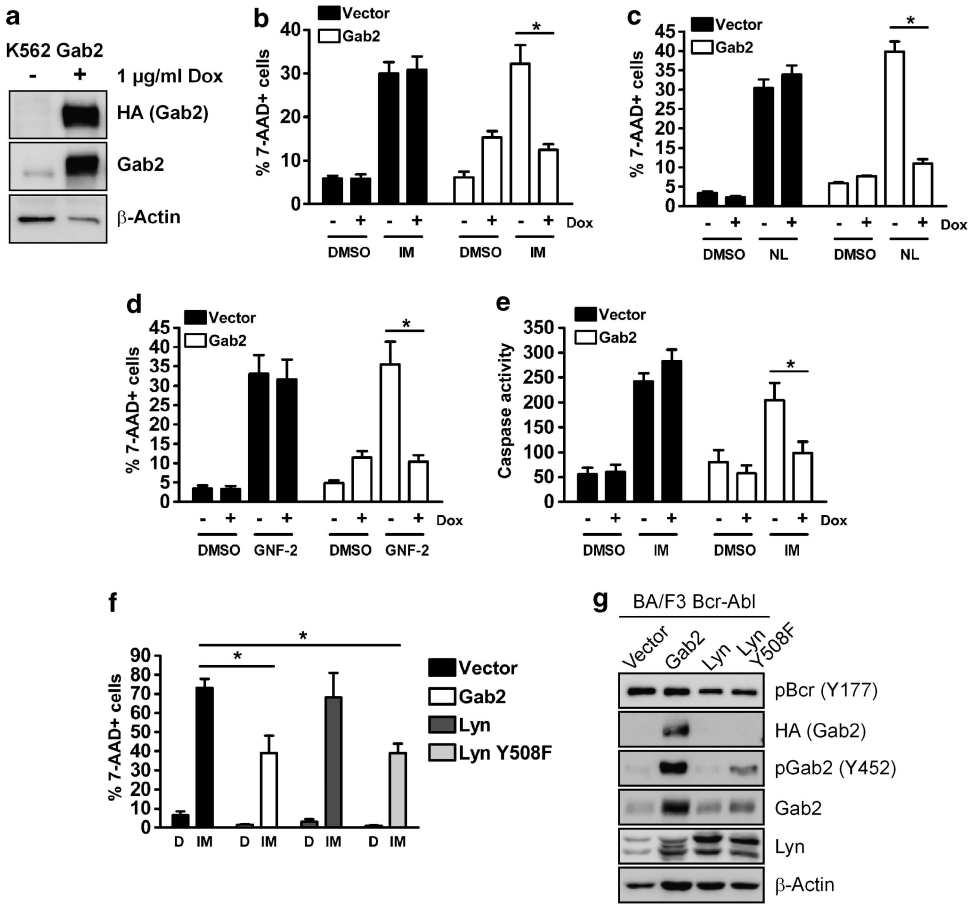


Figure 2. Inducible overexpression of Gab2 confers TKI resistance. (**a**) Western blot analysis of K562tet cells stably transfected with pTET/Gab2. Total cellular lysates were generated from cells treated with either dox for 24 h or left untreated. (**b**) K562tet cells stably transfected with pTET/Gab2 or 'empty' control vector were treated with dox for 24 h and then exposed to IM (1 µM) or dimethylsulfoxide (DMSO, D) for additional 72 h. Thereafter, the cells were stained with 7-AAD to monitor cell death. (**c**) Same experimental set-up as in (**b**) except that IM was replaced by 1 µM NL. (**d**) Same experimental set-up as in (**b**) except that IM was replaced by 1 µM of the allosteric Bcr-Abl inhibitor GNF-2. (**e**) K562tet cells stably transfected with pTET/Gab2 or 'empty' control vector were treated with dox for 24 h and then exposed to IM (1 µM) or DMSO for additional 24 h. Cells were processed to cellular lysates and caspase-3/7 activity was measured using a fluorometric assay as described in the Supplementary Material and methods section. (**f**) Ba/F3 cells were transduced with pBabe-Hygro-Bcr-Abl and subsequently with one of the following pWZL constructs: pWZL-Bsr-Gab2-HA, pWZL-Bsr-Lyn, pWZL-Bsr-Lyn^{Y508F} or pWZL-Bsr empty control vector. Cells were treated with 0.25 µM IM or D for 48 h. Cell death was monitored by 7-AAD staining. (**g**) Expression of Bcr-Abl, HA-tagged Gab2, Lyn and Lyn^{Y508F} in the Ba/F3 cells tested in (**f**) was monitored by western blotting.

dependent on the presence of intact Grb2-binding sites in both Gab2 and Bcr-Abl,[4] we also included the Grb2-binding deficient Gab2$^{\Delta Grb2}$ mutant.[26] First, we analyzed the tyrosine phosphorylation status of these Gab2 mutants and their signaling characteristics (Figures 3a and b). Indeed, as described for Ba/F3 cells,[4] HA-tagged, purified Gab2^{wt} is tyrosine phosphorylated in human K562 cells, while the Gab2$^{\Delta Grb2}$ mutant remains almost unphosphorylated on tyrosine residues. Importantly, the constitutively 14–3–3-bound Gab2^{2xR18} is hardly phosphorylated as well (Figure 3a and Supplementary S7A). By contrast, the g-o-f mutants Gab2^{2xA} and Gab2^{2xKK} display an even stronger tyrosine phosphorylation than Gab2^{wt} (Figure 3a and Supplementary Figure S7A). Importantly, only the signaling-competent Gab2^{wt}, Gab2^{2xA} and Gab2^{2xKK} proteins induced a marked increase in phosphorylated AKT and ERK (Figure 3b and Supplementary Figures S7B andC). Interestingly, only expression of Gab2^{wt} or the g-o-f mutants Gab2^{2xA} and Gab2^{2xKK} rendered K562 cells resistant to IM (Figure 3c) and GNF-2 (Supplementary Figure S7D), whereas cells expressing the signaling-impaired Gab2$^{2\times18}$ and Gab2$^{\Delta Grb2}$ mutants were hardly or not protected at all. Thus, the protective effect of Gab2 from TKIs is linked to its Grb2-binding ability and tyrosine phosphorylation status.

Gab2 modulates IM sensitivity via the Shp2/ERK and the PI3K/AKT pathways

Next, we dissected the downstream effector pathways by which Gab2 mediates TKI resistance. To this end, we expressed Gab2 mutants with defective PI3K (Gab2$^{\Delta p85}$) or Shp2 (Gab2$^{\Delta Shp2}$) recruitment sites (Supplementary Figure S6) in K562 cells in an inducible fashion. Previous work by several laboratories has shown that the Gab2$^{\Delta p85}$ and Gab2$^{\Delta Shp2}$ mutants fail to activate the PI3K/AKT or Ras/ERK pathway, respectively, in several cell types.[14,17,26] This is also the case for K562 cells (Figure 3d). Moreover, the Gab2$^{\Delta Shp2}$ mutant is hyper-phosphorylated at the PI3K recruitment site Y452, suggesting that Gab proteins serve as a substrate for bound Shp2, as discussed previously.[14] Importantly, the Gab2$^{\Delta p85}$ and Gab2$^{\Delta Shp2}$ mutants fail to confer the same degree of IM resistance as the wildtype protein, indicating that both effector pathways are involved in the protective effect mediated by the docking protein (Figure 3e).

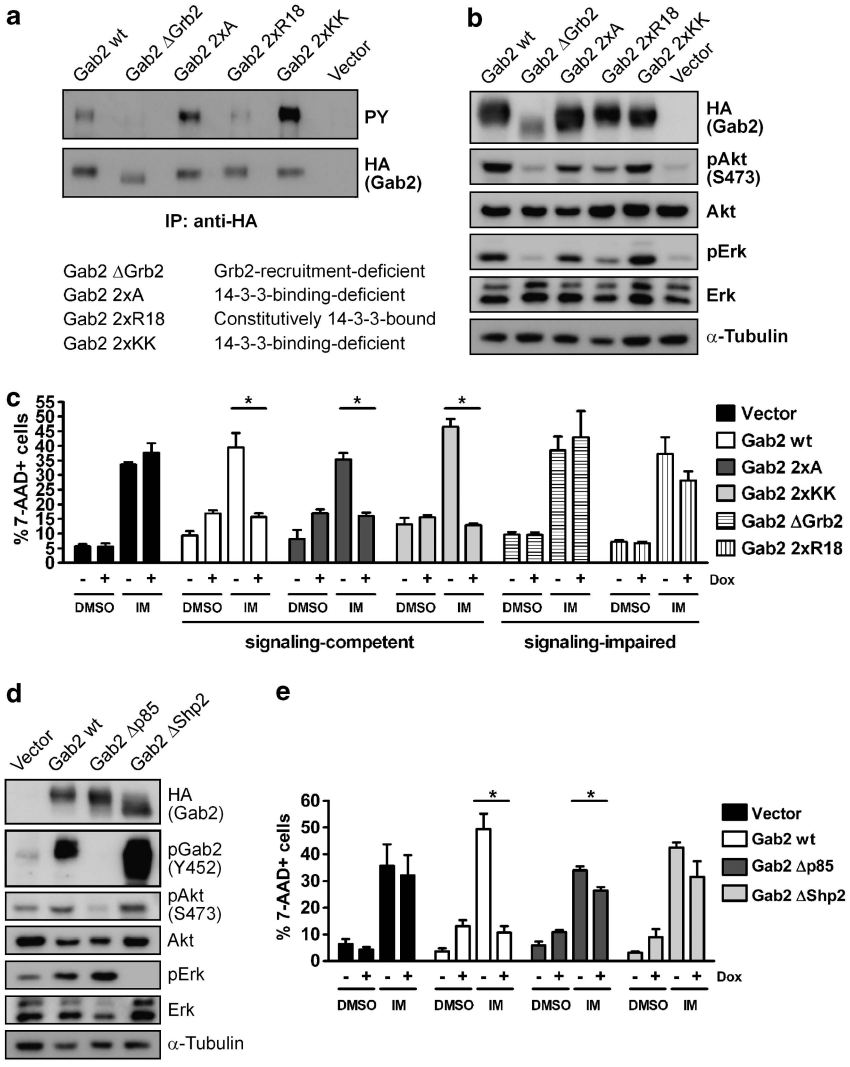


Figure 3. Gab2-mediated TKI resistance is dependent on the interactions with Grb2, p85 and Shp2 and is counteracted by 14–3–3-binding. (**a**) K562tet cells harboring the indicated dox-inducible expression constructs were treated with dox (1 μg/ml) for 24 h. Signaling complexes organized by the indicated Gab2 proteins were purified using anti-HA antibodies and subjected to a western blot analysis. Note that the signaling-impaired $Gab2^{\Delta Grb2}$ and $Gab2^{2xR18}$ mutants are hardly phosphorylated, whereas $Gab2^{wt}$, $Gab2^{2xA}$ and $Gab2^{2xKK}$ display a prominent steady-state tyrosine phosphorylation. (**b**) K562tet cells harboring the indicated dox-inducible expression constructs were treated with dox (1 μg/ml) for 24 h. Total cellular lysates (TCLs) were analyzed by western blotting using the indicated antibodies. Note that compared with cells expressing $Gab2^{wt}$, $Gab2^{2xA}$ and $Gab2^{2xKK}$ those expressing $Gab2^{\Delta Grb2}$ and $Gab2^{2xR18}$ exhibit no or only a modest increase in pAKT and pERK levels. (**c**) K562tet cells stably transfected with the indicated inducible Gab2 expression vectors or 'empty' control vector were treated with 1 μg/ml dox for 24 h and then exposed to IM (1 μM) or dimethylsulfoxide (DMSO) for additional 72 h. Thereafter, the cells were stained with 7-AAD to monitor cell death. Note that only signaling-competent Gab2 mutants confer protection from IM. (**d**) K562tet cells harboring the indicated dox-inducible expression constructs were treated with dox (1 μg/ml) for 24 h. TCLs were analyzed by western blotting using the indicated antibodies. (**e**) K562tet cells stably transfected with the indicated Gab2 expression vectors or 'empty' control vector were treated with 1 μg/ml dox for 24 h and then exposed to IM (1 μM) or DMSO for additional 72 h. Thereafter, cells were stained with 7-AAD to monitor cell death.

Effect of TKIs on Gab2 phosphorylation and downstream signaling Interestingly, while increasing doses of IM led to a decrease in overall tyrosine phosphorylation of Gab2, the phosphorylation of Y452 was only slightly affected by the drug regardless of whether Gab2 was ectopically (Figure 4a and Supplementary Figure S8) or endogenously expressed (Supplementary Figure S9).

As observed previously in other cell types,[14] increased Gab2 levels in K562 cells led to augmented activation of the steady-state Ras/ERK, PI3K/AKT and STAT5 pathways (Figure 4a and Supplementary Figure S8), which have been all implicated in IM resistance.[8,35,43] Although Bcr-Abl-driven phosphorylation of ERK in Gab2-overexpressing cells was only slightly reduced by increasing concentrations of IM, the phosphorylation of STAT5 was strongly affected by this TKI. Importantly, the phosphorylation of AKT at its key regulatory residues T308 and S473 was not significantly affected by IM (Figure 4a and Supplementary Figure S8). Together with the persistence of Y452 phosphorylation in IM-treated cells, this finding suggests that Gab2 maintains high levels of PI3K signaling in the presence of this TKI. As SFKs, in particular Lyn, have been implicated in the tyrosine phosphorylation of Gab2 (Figure 2g) and also in IM resistance[33,36] and as the PI3K recruitment site Y452 is embedded within a potential phosphorylation motif for SFKs, we next asked how the dual Abl/SFK-inhibitor DS would affect the cellular viability of K562tet/Gab2 cells and the phosphorylation status of the docking protein. Administration of DS at doses as low as 0.1 μM led to a stronger

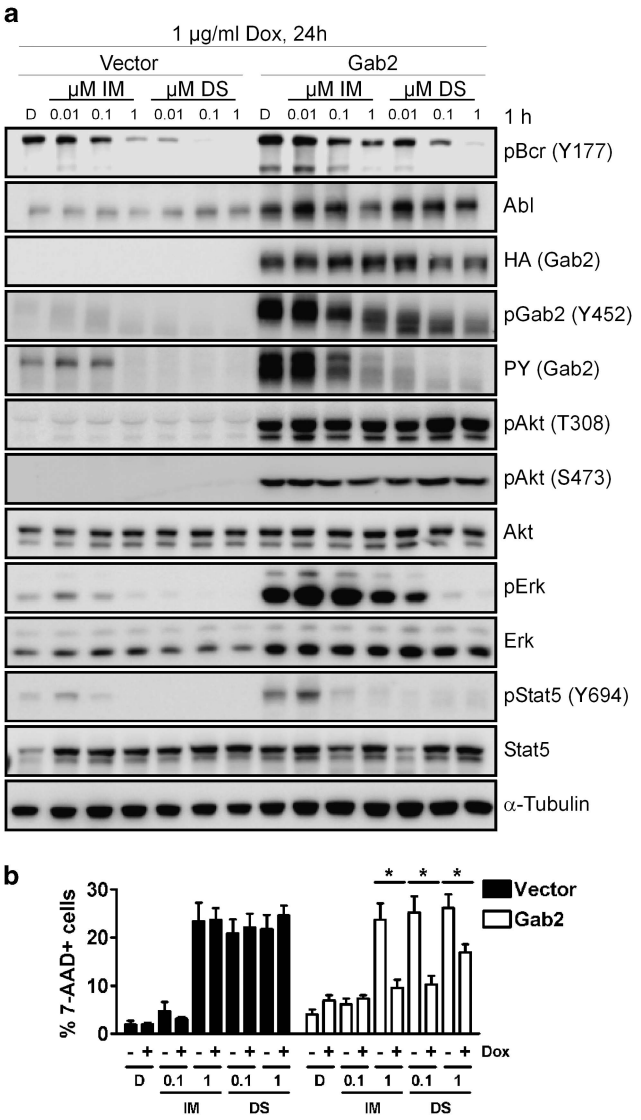


Figure 4. The PI3K/AKT and Shp2/ERK pathways are involved in Gab2-induced TKI resistance. (**a**) K562tet cells stably transfected with the inducible Gab2^{wt} expression vector or 'empty' control vector were treated with 1 µg/ml dox for 24 h and then exposed to the indicated doses of IM, DS or dimethylsulfoxide (DMSO, D) for 1 h. Total cellular lysates were analyzed by western blotting using the indicated antibodies. (**b**) K562tet cells stably transfected with the indicated Gab2 expression vectors or 'empty' control vector were treated with 1 µg/ml dox for 24 h and then exposed to IM, DS (0.1/1 µM) or DMSO (D) for additional 72 h. Thereafter, the cells were stained with 7-AAD to monitor cell death.

inhibition of overall Gab2 tyrosine phosphorylation, ERK and STAT5 activation than that observed with IM (Figure 4a and Supplementary Figure S8). Surprisingly, however, phosphorylation of AKT at T308 and S473 was hardly affected by this and even higher concentrations of DS and Gab2 phosphorylation on Y452 decreased only slightly (Figure 4a and Supplementary Figure S8).

Consistent with our earlier demonstration that signaling *via* both Shp2/Ras/ERK and PI3K promotes TKI resistance, and the relative effects of IM and DS on ERK activation, Gab2-over-expressing cells exhibited still a significant resistance to DS (Figure 4b).

Higher Gab2 expression levels correlate with TKI-refractory disease

The Gab2-mediated TKI resistance in our K562 and Ba/F3 models prompted us to assess the expression pattern of the docking protein in primary, p210$^{Bcr\text{-}Abl}$-positive CML BMBs by IHC. We identified 20 well-characterized CML patients with clinical follow-up, of whom 10 cases were secondary TKI-refractory and 10 cases were TKI-sensitive (Table 1). From these patients, we included BMBs at initial diagnosis, at remission control and at relapse in our analyses. With these samples, we observed that the Gab2 expression pattern in myeloid cells differs between TKI-refractory and TKI-sensitive remission control biopsies taken under TKI treatment (Figures 5a–q). Consequently, we conducted a quantitative analysis of 300 myeloid cells in all evaluated remission control BMBs, a cell-based procedure that removes any potential bias caused by distinct sample cellularities. Strikingly, secondary TKI-refractory, relapsing patients, who all achieved at least a complete hematological response (Table 1), showed a significantly higher frequency of Gab2-positive myeloid cells compared with TKI-sensitive patients in their remission control BMBs ($P<0.0001$; Figure 5r). By contrast, Gab2-positive myeloid cells were similarly frequent in TKI-refractory and TKI-sensitive patients at initial diagnosis. This correlation is another strong argument that Gab2 contributes to development and/or maintenance of TKI resistance in some CML patients and that this may be detected at remission control BMBs using Gab2 IHC as a biomarker. Furthermore, this analysis clearly supports our functional *in vitro* data.

The resilience of Y452 phosphorylation to TKIs in the K562tet/Gab2 model invites for an analysis of the Gab2 phosphorylation status in primary CML samples. Therefore, we resorted to p210$^{Bcr\text{-}Abl}$-positive BC PBLs (peripheral blood leukocytes) from CML patient No. 8 of our cohort, who developed the Bcr-Abl^{G250E} mutant under IM treatment (Supplementary Figure S10A). This Bcr-Abl mutant displays a partial resistance to IM, but remains sensitive to NL and DS.[37] BC PBLs from this patient were grown either in the presence or absence of IL-3 and SCF (stem cell factor), treated with TKIs and analyzed by western blot (Figure 6a). This analysis demonstrates for the first time the phosphorylation of Gab2 at Y452 in a primary CML sample. Most strikingly, Y452 phosphorylation persisted under the influence of all the three Bcr-Abl inhibitors, even under NL and DS treatment. By contrast, NL and DS efficiently inhibited the auto-phosphorylation of Bcr-Abl on Y177, whereas, commensurate with the G250E mutation in Bcr-Abl and the established diagnosis of IM resistance, IM had no effect on this phosphorylation event in cultured PBLs. Interestingly, compared with starved PBLs, Bcr-Abl Y177 phosphorylation was increased in cells cultured in IL-3 and SCF-containing medium. This indicates that this residue, which is widely believed to represent an auto-phosphorylation site of Bcr-Abl, is also modulated by cytokine receptor signaling in primary CML cells, as suggested previously.[38] Taken together, this case of IM-resistant CML further supports the concept that Gab2 is highly expressed at advanced disease stages and confirms our K562 cell-based discovery that the phosphorylation of Y452 is hardly affected by clinically relevant TKIs.

Reduction of Gab2 expression sensitizes primary cells to TKIs

In order to test our hypothesis that Gab2 knockdown sensitizes primary CML blasts to TKI treatment, we infected BC cells from patient No. 8 of our cohort with lentiviral pLeGOhU6 vectors[39] containing either a non-silencing control or a Gab2 shRNA construct (Supplementary Figure S10). In full agreement with our knockdown studies in K562 cells, we show that these primary human CML blasts display a greater TKI sensitivity compared with their counterparts infected with the empty control vector (Figure 6b). In addition, we investigated the TKI sensitivity of primary murine bone marrow cells expressing Bcr-Abl. We reasoned that Bcr-Abl-transformed bone marrow cells from

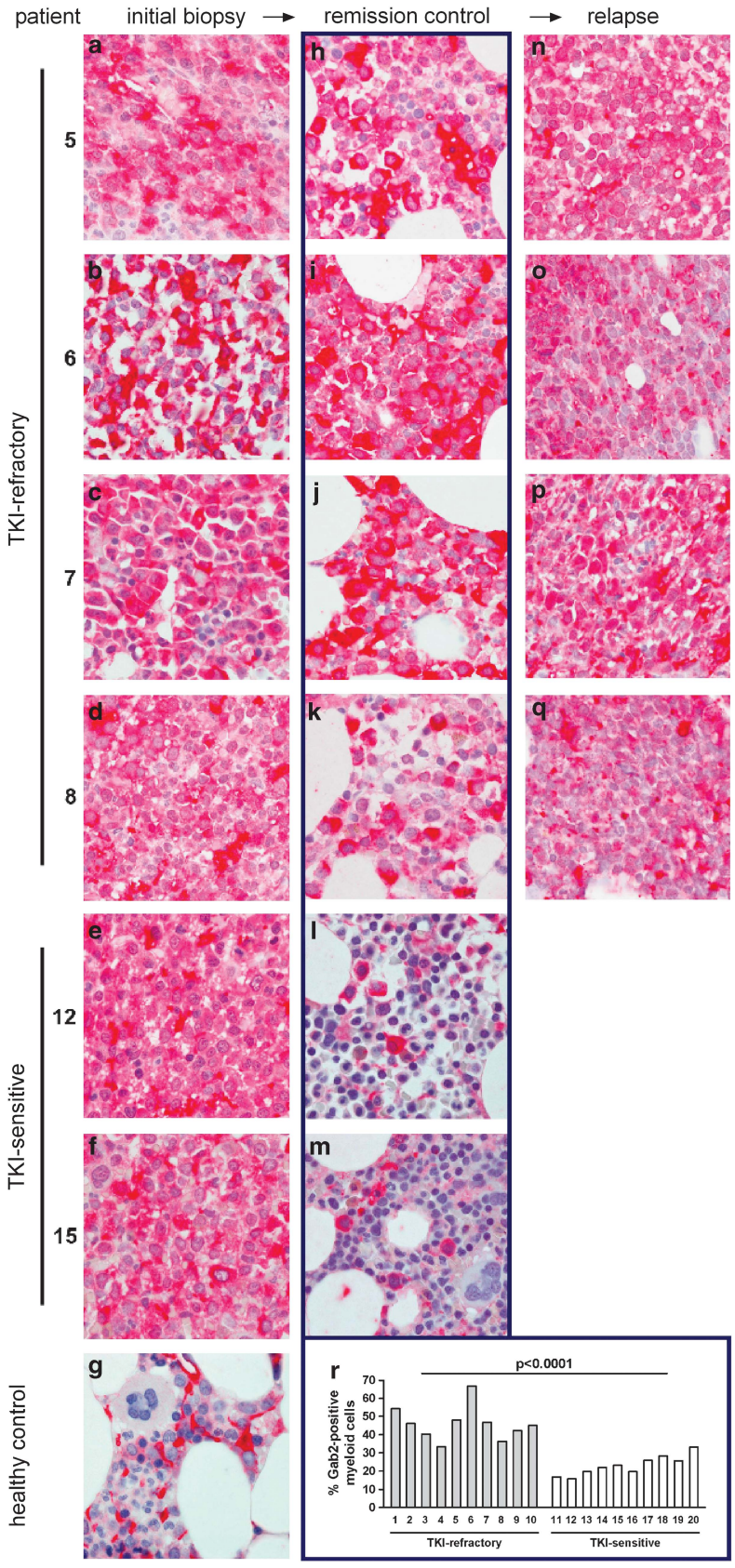
Gab2 $-/-$ [16] or even Gab2 $+/-$ mice might be more sensitive to TKIs than those from Gab2 $+/+$ mice due to a gene dosage effect. Indeed, cells from Gab2 $+/-$ mice displayed $<50\%$ of Gab2 protein expression compared with those from wildtype mice (Figures 6c and d). Next, we infected 5-fluoruracil-primed bone marrow corresponding to all the three genotypes with a bicistronic retroviral expression vector encoding both $p210^{Bcr\text{-}Abl}$ and GFP (green fluorescent protein). In agreement with a previous study[4] showing that cells from a distinct Gab2 $-/-$ strain cannot be efficiently transformed by Bcr-Abl, we failed to enrich GFP-positive cells from this genotype as well. Nevertheless, we were successful in expansion of pMIG/Bcr-Abl-infected Gab2 $+/+$ and Gab2 $+/-$ cells. In Figure 6e, we show for the first time that pMIG/Bcr-Abl-transduced Gab2 $+/-$ cells were sensitive to TKIs even in the presence of cytokines, while wildtype control cells displayed only a marginal increase in cell death. This is in

Table 1. Characteristics of secondary TKI-refractory and TKI-sensitive CML patients

	Patient Number	*Gender*	*Phase and age (years) at initial diagnosis*	*Number of therapy lines before TKI*	*Therapy before remission control*	*Type of remission*	*Therapy before relapse*	*Bcr-Abl mutational status*	*Drugs before TKI (cave therapy lines include combination therapy such as AraC IFN)*
TKI-refractory	1	F	CP, 55	3	TKI	CHR, CCR, additional cytogenetic aberrations	TKI		IFN, Anagrelide, Litalir, AraC
	2	M	CP, 41	2	TKI, DLI (2x)	CHR, CCR	TKI		Anagrelide, allo-HCT
	3	F	CP, 52	2	TKI	CHR (no cytogenetic response)	TKI		IFN, Hydroxyurea
	4	M	BC, 34	2	TKI	CHR, (molecular: Bcr-Abl $+$)	TKI	V299L	IFN, Hydroxyurea
	5	M	CP, 42	2	TKI	CHR	TKI		IFN, Hydroxyurea
	6	M	CP, 41	3	TKI	CHR, MCR	TKI	T315I	Hydroxyurea, auto-HCT, IFN, Vincristin
	7	M	CP, 59	2	TKI	CHR, cytogenetic relapse	TKI	Q252H	IFN, auto-HCT (BuCy)
	8	M	CP, 42	0	TKI	CHR, molecular relapse	TKI	G250E	
	9	M	CP, 45	0	TKI	CHR, no cytogenetic response, interphase-FISH 90% Ph^+	TKI		
TKI-sensitive	10	M	CP, 33	3	TKI	CHR	TKI	M224V, G250E	Hydroxyurea, auto-HCT, IFN
	11	M	CP, 59	0	TKI	CHR, CCR, MMR	TKI		
	12	M	CP, 43	1	TKI	CHR, CCR, no MMR	TKI		Hydroxyurea
	13	M	CP, 35	1	TKI	CHR, MCR	TKI		IFN, AraC
	14	M	CP, 35	0	TKI	CHR, MCR	TKI		
	15	M	CP, 39	1	TKI	CHR, CCR, MMR	TKI		Hydroxyurea
	16	W	CP, 48	0	TKI	CHR, CCR, CMR	TKI		
	17	M	CP, 41	1	TKI	CHR, MMR	TKI		Hydroxyurea
	18	M	AP, 44	1	TKI	CHR, CCR, MMR	TKI		Hydroxyurea
	19	M	CP, 66	0	TKI	CHR, MMR	TKI		
	20	M	CP, 59	1	TKI	CHR, nearly MMR	TKI		Hydroxyurea

Abbreviations: allo-HCT, allogeneic hematopoietic cell transplantation; AP, accelerated phase; AraC, cytarabin; auto-HCT, autologous hematopoietic cell transplantation; BC, blast crisis; BuCy, busulfan-cyclophosphamide; CCR, complete cytogenetic remission; CHR, complete hematological response; CMR, complete molecular remission; CP, chronic phase; DLI, donor lymphocyte infusions; F, female; FISH, fluorescence *in situ* hybridization; IFN, interferon; M, male; MMR, major molecular remission; Ph^+, Philadelphia chromosome; TKI, tyrosine kinase inhibitor.

Figure 5. TKI-refractory CML cases exhibit more Gab2-positive myeloid cells than TKI-sensitive cases under TKI treatment. Quantitative Gab2 immunohistochemistry (red) of BMBs of CML cases and a healthy control case (original magnification $\times 1000$; see Supplementary data and Reference[25] for protocol details). (**a–f**) Initial biopsies of CML cases. (**g**) Healthy control. (**h–m**) CML remission control BMBs under TKI treatment. (**n–q**) CML remission control BMBs at disease relapse. (**r**) Summary of quantitative and statistical evaluation of Gab2 staining in 300 myeloid cells in each remission control BMB. Note the similar Gab2 expression pattern of myeloid cells at initial diagnosis (**a–f**) and the significantly higher frequency of Gab2-positive myeloid cells in the remission control BMBs of TKI-refractory cases (**h–k**) compared with TKI-sensitive cases (**l, m**), with quantification given in (**r**). Note that quantification of Gab2 immunohistochemistry of remission controls was carried out by examining 100 myeloid cells in each of three random high power fields (magnification $\times 600$) per case (total $n = 300$ cells per BMB). Shown is the percentage of Gab2-positive myeloid cells for each case. TKI-refractory cases show significantly more Gab2-positive myeloid cells compared with TKI-sensitive cases (exact Wilcoxon test used at two-sided significance level of 5%).



agreement with a recent report showing that human CML blasts are protected from TKIs by cytokines.[40] Taken together, our experiments involving primary human CML blasts and primary murine bone marrow cells further support our concept that Gab2 expression levels determine the efficacy of Bcr-Abl inhibitors.

The combination of DS with inhibitors of the PI3K/AKT/mTOR pathway effectively kills Gab2-overexpressing K562 cells

The concept of synthetic lethality, which describes the lethal outcome of the combination of two otherwise tolerated genetic or functional alterations, is increasingly applied to drug resistance phenomena in oncology. As Gab2 mediates TKI resistance *via* the Ras/ERK and PI3K/AKT/mTOR pathways (Figures 3 and 4), we predicted that the simultaneous application of DS and an inhibitor of the PI3K/AKT/mTOR pathway would abolish the protective effect of Gab2. Given the persistence of AKT phosphorylation in IM- and DS-treated K562 cells, one potential effector molecule of the Gab2-PI3K axis might be mTOR, an important player in human cancers.[41] Furthermore, the mTOR isoform TORC1 can be targeted with clinically approved inhibitors such as rapamycin, which also acts synergistically with IM in the growth suppression of K562 and Bcr-Abl-transduced Ba/F3 cells[42] and is tested in combination with IM in clinical trials.

As shown in Figure 7a and Supplementary Figure S11, DS and rapamycin inhibited the Gab2-augmented phosphorylation of ERK and the mTOR substrate S6K, respectively. Furthermore, their combination abolished Gab2-induced activation of both signal transducers, whereas AKT phosphorylation was unaffected. As AKT inhibits cell death by multiple pathways and as rapamycin only inhibits mTORC1 but not mTORC2, we applied the dual PI3K/mTOR inhibitor NVP-BEZ235 (BEZ).[41] Application of BEZ strongly decreased phosphorylation of both S6K and AKT, whereas Gab2-mediated ERK activation was not affected. However, the combination of DS and BEZ inhibited the activating phosphorylation events on ERK, S6K and AKT (Figure 7b and Supplementary Figure S11). By contrast, the combination of IM and rapamycin, or IM and BEZ, reduced AKT and/or S6K, but not ERK, activation (Figures 7a and b and Supplementary Figure S11).

We next analyzed the effect of the combinatorial treatments on cell viability. As shown in Figure 7c, application of rapamycin or BEZ alone hardly affected the viability of K562 cells regardless of their Gab2 expression level, while the combination of rapamycin with either IM or DS reduced Gab2-mediated TKI resistance. Importantly, a greater reversal of resistance to either TKI was observed upon combination with BEZ, and the combination of DS with BEZ resulted in the complete loss of the protective effect of exogenous Gab2 from cell death. Furthermore, combining BEZ with DS was more effective than with IM ($P = 0.049$). Taken together, our data indicate that simultaneous blocking of the Gab2-augmented Ras/ERK and PI3K/AKT/mTORC1/2 axes is necessary and sufficient to overcome Gab2-mediated TKI resistance.

DISCUSSION

Alterations in the CML signaling network are increasingly implicated in TKI resistance as alternative or additional events to Bcr-Abl mutations.[1,8] Here, we show for the first time that Gab2 represents a critical modulator of cellular TKI sensitivity. Using biochemical, pharmacological and genetic approaches, we demonstrate that Gab2 confers TKI resistance by amplifying the

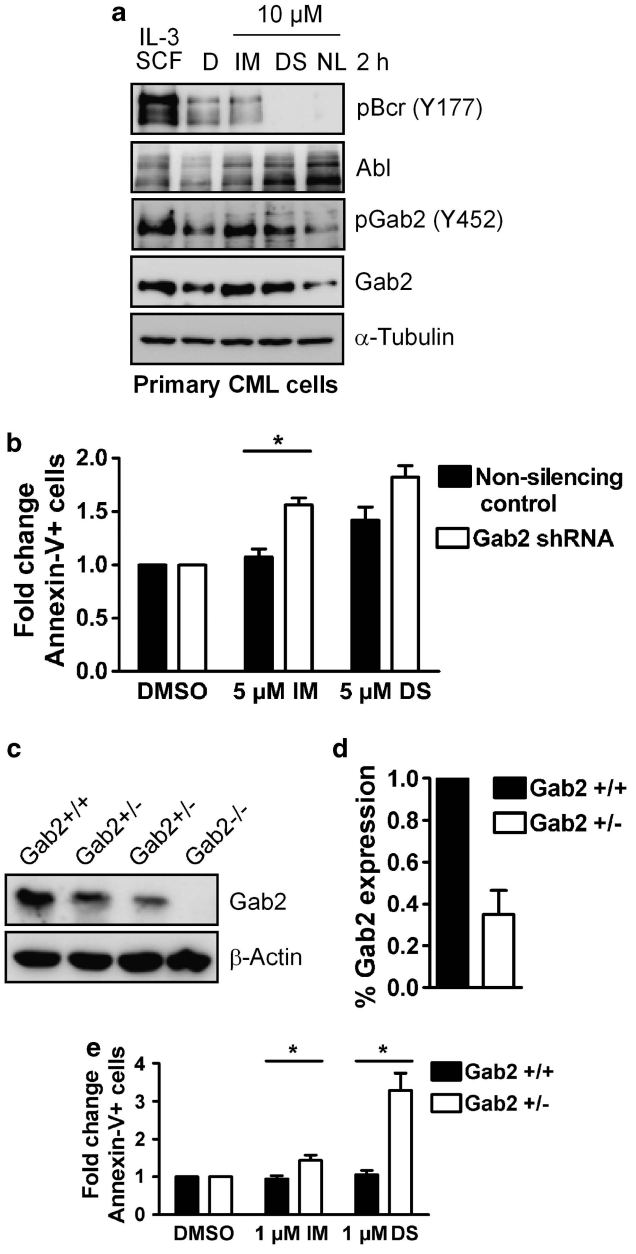


Figure 6. Reduction of Gab2 expression sensitizes primary cells to TKIs. (**a**) PBLs from a CML patient in BC (patient No. 8 of our cohort) were exposed to the indicated TKIs or the vehicle dimethylsulfoxide (DMSO, D) for 2 h. Lane 1: cells maintained in full-growth medium supplemented with 10 ng/ml recombinant human IL-3 and SCF. Lanes 2–5: serum and cytokine starvation for 5 h before TKI treatment. Total cellular lysates (TCLs) were analyzed by western blotting using the indicated antibodies. (**b**) Blast crisis PBLs from the same CML patient as in (**a**) were infected with a lentiviral construct for shRNA-mediated knockdown of Gab2 (pLeGOhU6-Gab2sh-G). Cells were subjected to the indicated treatments for 48 h and cell death of GFP-positive cells was analyzed using Annexin-V staining. Shown is the fold change of Annexin-V-positive cells compared with the vehicle control. (**c**) Murine splenocytes with the indicated genotypes were processed to TCLs and analyzed by western blotting using the indicated antibodies. (**d**) Western blot quantification of Gab2 expression levels. Note that Gab2 expression is reduced by $>50\%$ in Gab2 $+/-$ cells compared with wildtype controls. (**e**) Murine bone marrow cells with the indicated genotypes were infected with retroviral pMIG/p210$^{Bcr\text{-}Abl}$ expression vectors. Cells were exposed to 1 µM IM, 1 µM DS or DMSO for 48 h in the presence of IL-3. Cell death of GFP-positive cells was analyzed using Annexin-V staining. Shown is the fold change of Annexin-V-positive cells compared with the vehicle control.

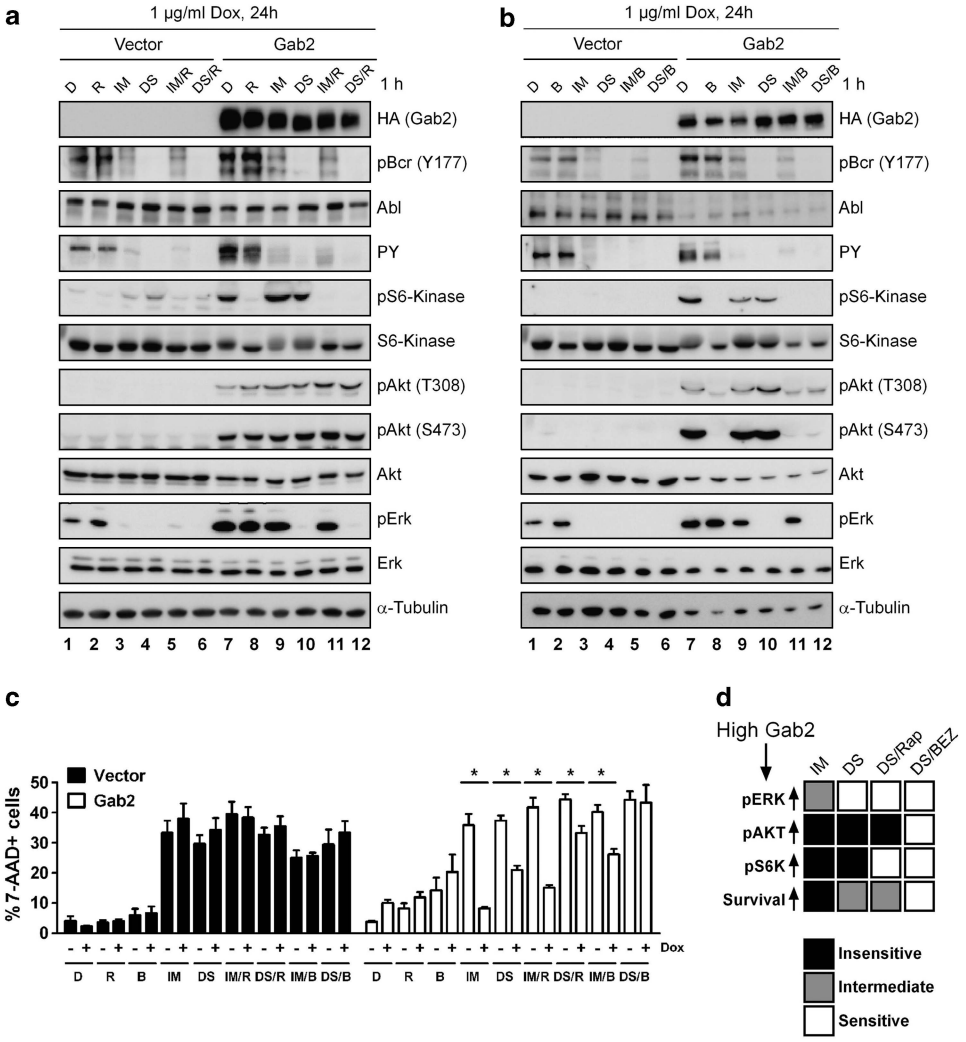


Figure 7. DS treatment in combination with inhibitors of the PI3K/AKT/mTOR pathway effectively kills Gab2-overexpressing K562 cells. (**a, b**) K562tet cells stably transfected with the inducible Gab2^{wt} expression vector or 'empty' control vector were treated with dox for 24 h and then exposed to the indicated treatments for 1 h (D, dimethylsulfoxide (DMSO); R, rapamycin: (100 nM); B, NVP-BEZ235: (2.5 μM); IM: (1 μM) and DS: (1 μM). Total cellular lysates were analyzed by western blotting using the indicated antibodies. (**c**) K562tet cells stably transfected with the indicated inducible Gab2^{wt} expression vectors or 'empty' control vector were treated with dox for 24 h and then exposed to the same treatments as in (**a, b**) for additional 72 h. Thereafter, the cells were stained with 7-AAD to monitor cell death. (**d**) Table summarizing the effects of the inhibitor treatments applied in this study on Gab2-mediated signaling and TKI resistance.

input of Bcr-Abl into the Shp2/Ras/ERK and PI3K/AKT/mTOR/S6K pathways (Figure 7d and Supplementary Figure S12). The dependency on these pathways is illustrated by the necessity to inhibit both axes of Gab2 signaling in order to overcome Gab2-mediated TKI resistance. This concept explains the inability of Gab2$^{\Delta p85}$ or Gab2$^{\Delta Shp2}$ to provide full protection from Bcr-Abl inhibition and the distinct effects of IM and DS on Gab2-mediated cell survival. Only DS abolishes Gab2-induced ERK phosphorylation, most likely *via* an indirect effect, whereas the Gab2/PI3K axis withstands both TKIs (Figure 4, Supplementary Figures S7, 8 and 9). Most importantly, simultaneous treatment with DS and BEZ inhibited phosphorylation of ERK, S6K and AKT and completely overcame Gab2-mediated resistance (Figure 7). This finding further emphasizes the potential of the new, dual PI3K/mTOR inhibitor and is of particular importance, as TKI-resilient phosphorylation of Gab2 at its PI3K recruitment site Y452 was not only observed in K562, but also in primary CML cells (Figure 6a).

It should be noted that our results do not rule out a contribution of additional downstream effectors, like STAT5, which interacts with Gab2 in several cell types.[18] Indeed, STAT5 is implicated in TKI resistance by recent studies using murine CML models and patient samples.[43] Although we observed a slight increase of STAT5 phosphorylation upon Gab2 overexpression (Figure 4a and Supplementary Figure S13A), this phosphorylation event was highly sensitive to TKI treatment (Figure 4a). This finding argues against an important role of phospho-STAT5 in Gab2-mediated TKI resistance. Moreover, knockdown of Gab2 does not affect phosphorylation of STAT5 (Supplementary Figure S13B) and Gab2 overexpression hardly augments STAT5 phosphorylation in Ba/F3 cells (Supplementary Figure S13C).

As shown by the inability of the phosphorylation-impaired Gab2 mutants Gab2$^{\Delta Grb2}$, Gab2^{2xR18}, Gab2$^{\Delta p85}$ and Gab2$^{\Delta Shp2}$ to confer TKI resistance (Figure 3 and Supplementary Figure S12B), the protective effect of Gab2 still requires input from tyrosine kinases, which substitute for the inhibited Bcr-Abl oncoprotein during TKI treatment. This is of particular importance as SFKs mediate TKI resistance and persistent Gab2 tyrosine phosphorylation in CML cell line models and patient samples.[11,12,33,44] Indeed, Gab2-

overexpressing cells displayed a higher sensitivity to the dual Src/Abl inhibitor DS compared with IM, NL and GNF-2, suggesting that a cooperation of Gab2 and SFKs might contribute to TKI resistance. However, the fact that Gab2 phosphorylation on Y452 withstands DS implies that other kinases apart from Bcr-Abl and SFKs may be involved (Figures 4 and 6). Consequently, the identification of additional Y452 kinases may provide new therapeutic targets in CML. In this regard, it should be noted that Gab2 tyrosine phosphorylation is driven by other growth factor and cytokine signals,[14,45] which contribute to primary TKI resistance by promoting survival and expansion of Ph^+ stem or progenitor cells in the bone marrow.[40,46] These observations and the prominent Gab2 expression in blood and bone marrow samples from CML patients with advanced disease (Figures 1a and 5 and references[25,27]) support a model in which Ph^+ cells might be protected in the bone marrow niche by the signaling output of tyrosine-phosphorylated Gab2, a common denominator of both Bcr-Abl and cytokine receptor signaling. The increased survival and proliferation of Gab2-overexpressing cells in this environment may facilitate acquisition of additional secondary resistance mechanisms such as Bcr-Abl mutations.

Our data highlight the possibility to target Gab2 in CML therapy. In addition to kinases upstream of Gab2, protein–protein interactions mediating the formation of the Bcr-Abl/Grb2/Gab2 complex could be exploited therapeutically. This is further underscored by our finding that cells expressing the signaling-impaired $Gab2^{\Delta Grb2}$ or $Gab2^{2xR18}$ mutants are not protected from TKIs (Figure 3). The recent structural analysis[15] of the interaction between the C-terminal SH3 domain of Grb2 with Gab2 could provide the basis for the development of drugs that selectively disrupt Grb2/Gab2 complexes. Alternatively, the formation of the Bcr-Abl/Grb2/Gab2 complex could be prevented by small molecule-mediated stabilization of the Gab2/14–3–3 interaction.[21] The feasibility of targeting the Bcr-Abl/Grb2/Gab2 axis is further supported by the growth inhibition of CML cell models observed upon overexpression of a Grb2 decoy[47] or following RNAi-mediated depletion of Grb2.[48] However, in contrast to the embryonic lethal Grb2-knockout,[49] Gab2-deficient mice display a mild phenotype,[14,17,18] which suggests that blocking Gab2 expression or function might be well tolerated.

Our data also have more immediate, diagnostic implications. Although this concept still requires clinical validation in a larger cohort or by meta-analyses, our present work highlights the possibility that expression or phosphorylation levels of Gab2 could be developed as a companion biomarker assisting in patient stratification and predicting therapeutic outcome to already approved inhibitors. Indeed, we showed that remission control BMBs of TKI-refractory patients were associated with higher frequencies of Gab2-positive myeloid cells compared with TKI-sensitive patients (Figure 5r). We posit that the observed maintenance of Gab2 staining is linked to the persistence of Ph^+ cells in the bone marrow, indicating an incomplete remission. Given the well-documented role of Gab2 downstream of cytokine receptors, such Ph^+ cells with high Gab2 expression might be particularly suited to resist TKI therapy. Consequently, quantitative IHC of Gab2 in remission control BMBs may be a supportive predictive tool. Furthermore, we and others have recently reported that increased Gab2 levels are associated with disease progression.[25,27,29] Finally, the successful inhibition of Gab2-mediated TKI resistance by simultaneous treatment with DS and BEZ emphasizes how characterization of the Bcr-Abl signaling network can inform the rational design of therapies that overcome resistance to TKI monotherapy.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Dr Alexander Kühnemund and members of the Brummer laboratory for helpful discussions, Andreas Geissler for advice on the caspase assays and Katja Thurig, Christine Capri and Jennifer Schwarz for technical assistance. TB is supported by the German Research Foundation (DFG) through the Emmy-Noether-Program (BR3662/1-1) and EXC 294 *BIOSS*. FUW is funded by the Excellence Initiative of the DFG (GSC-4; Spemann Graduate School for Biology and Medicine) and EXC 294 *BIOSS*. RZ is supported by the DFG (ZE 872/1–1 and Heisenberg Fellowship ZE 872/2–1). SL, MW, RZ and TB are also supported by the DFG funded Collaborative Research Center 850. HLP gratefully acknowledges support by the DFG (Pa 611/5-1 and Pa 611/6-1) as well as the NIH (PO1 CA108671). RJD is supported by the National Health and Medical Research Council of Australia. DS is supported by the Austrian Academy of Sciences through an APART fellowship. KA is supported by a fellowship of the medical faculty of the University of Freiburg.

AUTHOR CONTRIBUTIONS

FUW, SH, SS and SB performed all cellular and biochemical experiments. KA conducted the IHC analyses. KA, SL, HLP, MW, CFW and RZ provided clinical samples and expertise. PA, DS and JMP provided critical reagents and mice. All authors contributed to the design, analysis and discussion of experiments. TB wrote the manuscript together with FUW, SH and RJD. All authors reviewed the manuscript.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)