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Phosphorylation of BECLIN-1 by BCR-ABL suppresses autophagy in chronic myeloid leukemia

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ABSTRACT

Autophagy is a genetically regulated process of adaptation to metabolic stress and was recently shown to be involved in the treatment response of chronic myeloid leukemia (CML). However, *in vivo* data are limited and the molecular mechanism of autophagy regulators in the process of leukemogenesis is not completely understood. Here we show that *Beclin-1* knockdown, but not *Atg5* deletion in a murine CML model leads to a reduced leukemic burden and results in a significantly prolonged median survival of targeted mice. Further analyses of murine cell lines and primary patient material indicate that active BCR-ABL directly interacts with BECLIN-1 and phosphorylates its tyrosine residues 233 and 352, resulting in autophagy suppression. By using phosphorylation-deficient and phosphorylation-mimic mutants, we identify BCR-ABL induced BECLIN-1 phosphorylation as a crucial mechanism for BECLIN-1 complex formation: interaction analyses exhibit diminished binding of the positive autophagy regulators UVRAG, VPS15, ATG14 and VPS34 and enhanced binding of the negative regulator Rubicon to BCR-ABL-phosphorylated BECLIN-1. Taken together, our findings show interaction of BCR-ABL and BECLIN-1 thereby highlighting the importance of BECLIN-1-mediated autophagy in BCR-ABL$^+$ cells.

Introduction

The BCR-ABL fusion kinase has been identified in more than 95% of chronic myeloid leukemia (CML) and 20% of acute lymphoblastic leukemia (ALL) cases.[1,2] Oncogenic BCR-ABL activates several aberrant kinase-dependent pathways including anti-apoptosis, proliferation and differentiation,[3,4] leading to the development of several successful tyrosine kinase inhibitors (TKI) in BCR-ABL$^+$ leukemia treatment.[5,6] However, there are still unsolved issues in TKI-based therapies for patients with CML: Suppression of the disease relies in most patients on continuous and lifelong TKI therapy[7,8] and disease relapse occurs due to emerged TKI resistance.[9-11] Thus, identification of additional important mediators could significantly improve CML therapy.

Autophagy is an evolutionarily conserved mechanism for the degradation of cytoplasmic components including organelles and proteins and plays an important role in cellular homeostasis. Because of its potential role in metabolism and cell survival, altered autophagy processes are critical for cancer cell fate. Several reports indicate

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that autophagy may be a promising target pathway in BCR-ABL$^+$ leukemia treatment.[12-18] However, the distinct role of autophagy in the process of leukemogenesis is not completely understood and crucial autophagy mediators have not been evaluated in *in vivo* leukemogenesis mouse models.

BECLIN-1, a master regulator of autophagy, is essential for the formation of the autophagosome and autolysosome as a part of the Rubicon, VPS15, VPS34, ATG14, UVRAG and BECLIN-1 complex.[19-23] An *in vitro* study has discovered that a treatment strategy combining TKI and inhibitors of BECLIN-1-mediated autophagy may be beneficial for BCR-ABL$^+$ CML therapy,[16] but *in vivo* data are missing and the molecular mechanisms underlying this effect remain unclear.

Methods

GST-pulldown assay, immunoprecipitation and Western blotting

All *Beclin-1* fragments were cloned into PGEX-4T2 vector, which were confirmed by Sanger sequencing. Those constructs were transformed into Bl21 competent cells, and a single clone was picked for culture in LB medium at 37°C with vigorous shaking. IPTG was added when the OD600 of the bacterial suspension reached 0.6. After an additional 2 hours (h) of incubation, bacterial cells were harvested, lysed using lysozyme and sonification and incubated for 3 h with glutathione-agarose beads in NETN buffer (0.5% NP40, 20 mM Tris/HCl, 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Benzamidin, protease inhibitor cocktail [Roche]) at 4°C. The beads were then incubated with K562 cell lysates over night at 4°C. Immunoprecipitation and Western blotting were performed as described previously.[24-26] Briefly, immunoprecipitation was performed by adding IP lysis buffer (40 mM HEPES, 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 50 mM NaF, 0.3% CHAPS, 1 mM sodium orthovanadate, 1 mM glycerolphosphate, protease inhibitor cocktail) to the cells for 1 h on ice. Pre-clearing of the lysates was performed using protein A or G agarose beads, followed by incubation with anti flag beads (Sigma) or antibody plus protein A or G beads (GE healthcare) overnight at 4°C. Protein extraction for Western blotting was performed using protein lysis buffer (10 mM Tris/HCl, 130 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 20 mM Na_2HPO_4/NaH_2PO_4, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 20 mM NaF, 1 mM glycerole 2-phosphate, protease inhibitor cocktail).

In vitro kinase assay

In vitro kinase assay was performed as described previously.[27] Briefly, recombinant active ABL (ProQinase GmbH) was incubated with 10 μCi [γ-^{33}P]ATP (PerkinElmer) and 1 μg recombinant GST-BECLIN-1 fragment in 50 μL kinase buffer (70 mM HEPES, 25 mM β-glycerophosphate, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 1.2 mM DTT, 50 μg/mL PEG20.000, and 1% DMSO). Reactions were incubated at 30°C for 40 min. Proteins were separated by 10% SDS-PAGE, and phosphorylation was visualized by autoradiography.

Flow cytometry

Flow cytometric staining was performed as previously described.[28-30]

Mice

Mice were caged in special caging system with autoclaved food and acidified water at the University of Freiburg in accordance with national and institutional guidelines for animal care. All animal studies have been approved by the Ethics committees of the University Medical Center Freiburg and the district government in Freiburg (approval no. 35-9185.81/G-13/05).

Statistics

Statistical comparisons were performed using GraphPad Prism 6 software. Detailed statistical tests and significance cutoffs are indicated in each figure legend. All data represent the mean ± standard error of the mean (SEM).

Study approval

The studies using human samples were conducted according to the Declaration of Helsinki principles. All biological samples were obtained following written informed consent from the patient and upon approval by the Ethics Committee of the University Medical Center Freiburg.

Additional methodology is provided in the *Online Supplementary Materials and Methods.*

Results

Knockdown of *Beclin-1* delays BCR-ABL-mediated leukemogenesis *in vivo*

To further investigate the impact of autophagy in CML we examined the role of BECLIN-1, a master autophagy mediator, in BCR-ABL induced transformation and colony forming assays. *Beclin-1* was downregulated using a targeted genetic approach with an individualized micro RNA-based knockdown of *Beclin-1* in BCR-ABL-overexpressing Ba/F3 cells and bone marrow derived cells (BMDC). Specific knockdown of *Beclin-1* with two individually designed siRNA resulted in significantly lower proliferation of BCR-ABL transduced Ba/F3 cells compared to cells infected with a control miR sequence (Figure 1A-B). As the secondly designed *Beclin-1* miR resulted in the most efficient *Beclin-1* knockdown, we performed all further experiments solely with *Beclin-1* miR2. We could detect higher apoptosis levels, but no decrease in cell cycle rate in *Beclin-1* miR cells (Figure 1C, *Online Supplementary Figure S1A*).

Furthermore, we could show significantly lower colony formation in BCR-ABL-expressing primary BMDC with *Beclin-1* downregulating miR in comparison to control BMDC (Figure 1D-E).

Next, we examined the effects of *Beclin-1* knockdown in a CML mouse model *in vivo*. BMDC from 5-FU pretreated animals were infected with a vector expressing BCR-ABL and the specific *Beclin-1* (pMmiRBec–BCR-ABL) or control miR sequence (pMmiRCtrl–BCR-ABL) on one construct and under the LTR promoter. Survival of mice transplanted with BCR-ABL-expressing *Beclin-1* knockdown BMDC was sustained and significantly prolonged compared to the control group (median survival 28 *vs.* 50 days, $P<0.0001$) (Figure 1F). Furthermore, the white blood cell count (WBC) and the leukemic burden of mice transplanted with *Beclin-1* knockdown BCR-ABL BMDC was significantly lower (87.3 *vs.* 14.8 x 10^3/μL on day 17, $P<0.0001$) compared to the control group (Figure 1G).

Immunoblotting of splenocyte extracts of transplanted mice confirmed efficient downregulation of *Beclin-1* one month after transplantation (Figure 1H). Upon disease induction, fluorescence-activated cell sorting (FACS) analyses of transplanted animals showed no differences in the immune phenotype of the BCR-ABL induced disease by *Beclin-1* downregulation (*Online Supplementary Figure S1B-D*). To test whether the impact of *Beclin-1* knockdown on CML cells is due to a general effect of autophagy inhibition or more due to a specific role of BECLIN-1 in BCR-ABL induced diseases, we also deleted another main autophagy regulator, ATG5 in a CML mouse model: *Atg5*

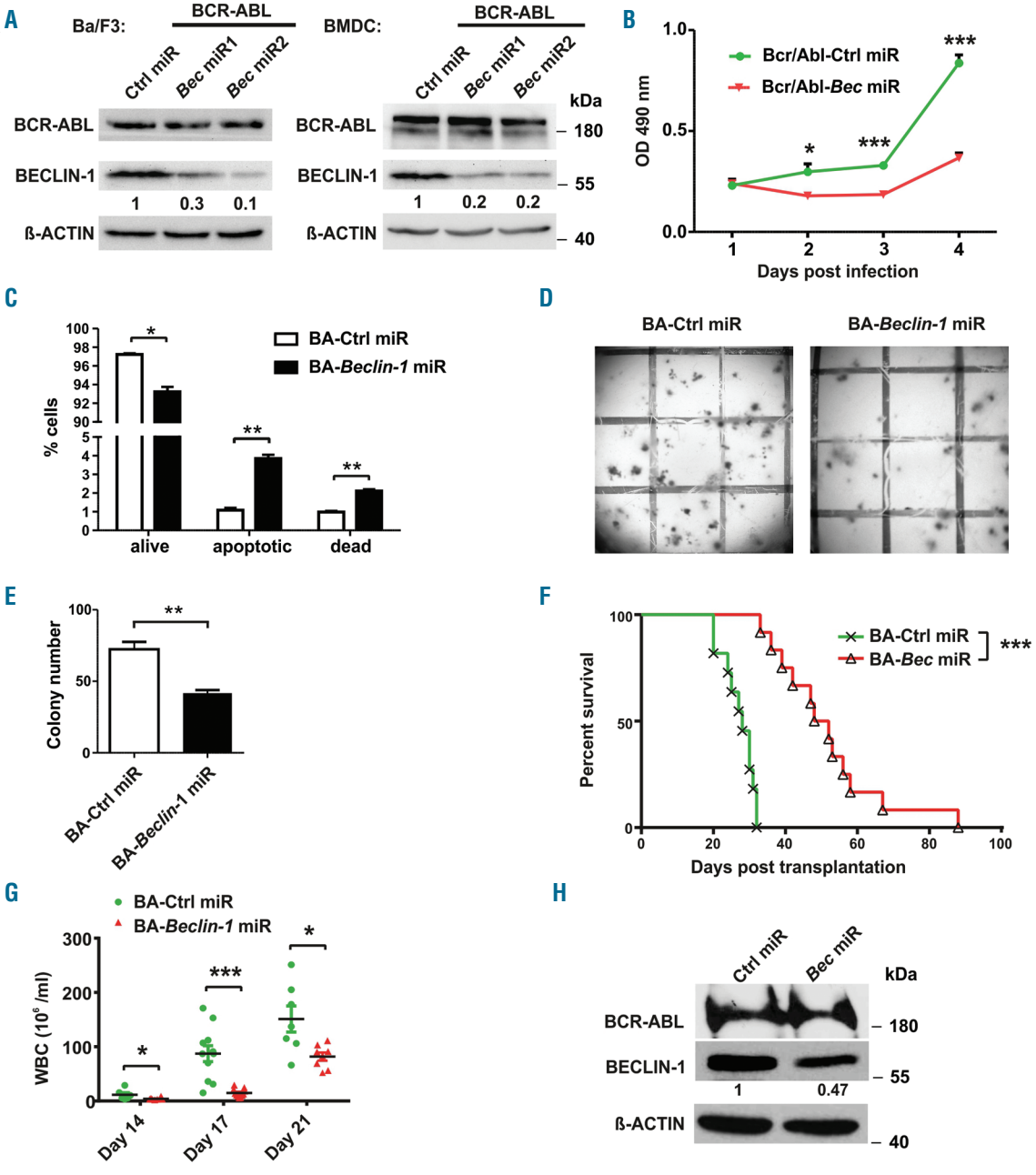

Figure 1. BECLIN-1 downregulation delays BCR-ABL-mediated proliferation *in vitro* and *in vivo*. (A) Immunoblot analyses were used to confirm downregulation of BECLIN-1 using two different *Beclin-1* directed miR in Ba/F3 cells and BMDC. (B) Cell proliferation measurement was performed by MTT assay in Ba/F3 cells infected with indicated construct towards IL-3 withdrawal, indicating that BCR-ABL-mediated cell proliferation is impaired by *Beclin-1* knockdown. ***P<0.001, *P<0.05, Student's *t*-test. (C) Statistical analysis of flow cytometric staining showing Annexin-V$^-$/ propidium iodide (PI)- ("alive"), Annexin-V$^+$/ PI$^-$("apoptotic") and Annexin-V$^+$/ PI$^+$ ("dead") Ba/F3 cells with indicated construct. **P<0.01, *P<0.05, Student's *t* test. (D) Methylcellulose (MC) colony formation assay of primary 5-FU enriched bone marrow cells showed impaired colony formation upon BCR-ABL expression in *Beclin-1* knockdown cells compared to control miR expressing cells. 1,000 EGFP$^+$ BMDC infected with the indicated construct were plated into methylcellulose in the absence of growth factors and colonies were quantified 10 days later. One representative well is shown. Three independent experiments were performed in doublets. Grid size is 5 x 5 mm. (E) Quantitation of the MC shown in (D) (72 *vs.* 55.7 colony-forming unit [CFU], *P<0.05, and 72 *vs.* 41.7 CFU, **P<0.01, respectively, student's *t*-test). (F) Kaplan-Meier curve demonstrates a significantly prolonged survival of mice transplanted with *Beclin-1* knockdown BCR-ABL+ BMDC compared to control mice (Median survival 28 *vs.* 50 days, ***P<0.001 in two independent transplantations, Log-rank test (n=11, control miR; n=13, *Beclin-1* miR)). (G) WBC from peripheral blood (PB) showed a significant reduction of leukemic progression in mice transplanted with *Beclin-1* knockdown cells (11.6 *vs.* 4 million/mL, day 14, *P<0.05; 87.3 *vs.* 14.8 million/mL, day 17, ***P<0.001; 151.1 *vs.* 81.9 million/mL, day 21, *P<0.05). (H) Efficient and durable knockdown of Beclin-1 was proven by immunoblot analyses of spleen cells of transplanted mice (day 27).

conditional knockout BMDC[31] were infected with a BCR-ABL-Cre fusion vector and transplanted into wild-type (wt) recipient mice. Interestingly, deletion of *Atg5* was not able to induce a delay in leukemia induction or progression of BCR-ABL transplanted mice (*Online Supplementary Figure S1E*). Furthermore, *Atg5* deletion had no influence on the WBC of the transplanted animals (*Online Supplementary Figure S1F*), despite efficient deletion of the floxed *Atg5* alleles upon Cre expression in BCR-ABL positive BMDC (*Online Supplementary Figure S1G*).

In order to exclude toxic effects of *Beclin-1* knockdown on normal hematopoiesis, we transplanted solely *Beclin-1* miR infected BMDC into mice, which exhibited no differences in survival, WBC or lineage phenotype compared to the control group (*Online Supplementary Figure S2A-G*).

Our results from the *in vivo* CML mouse model show a significant and specific impact of *Beclin-1* knockdown on CML disease induction.

Active BCR-ABL suppresses autophagy through the BECLIN-1 complex

It has been shown previously that BCR-ABL kinase inhibitors induce autophagy. Accordingly, inhibition of BCR-ABL kinase activity by nilotinib led to an induction of autophagy measured by increased LC3-II expression and punctual LC3 accumulation (*Online Supplementary Figure S3A-C*). To differentiate, whether the autophagy induction by nilotinib is caused by specific BCR-ABL inhibition or due to an unspecific nilotinib effect, we treated nilotinib-resistant Ba/F3-BCR-ABL-T315I cells with nilotinib and could show that this treatment failed to induce autophagy, suggesting that active BCR-ABL indeed sup-

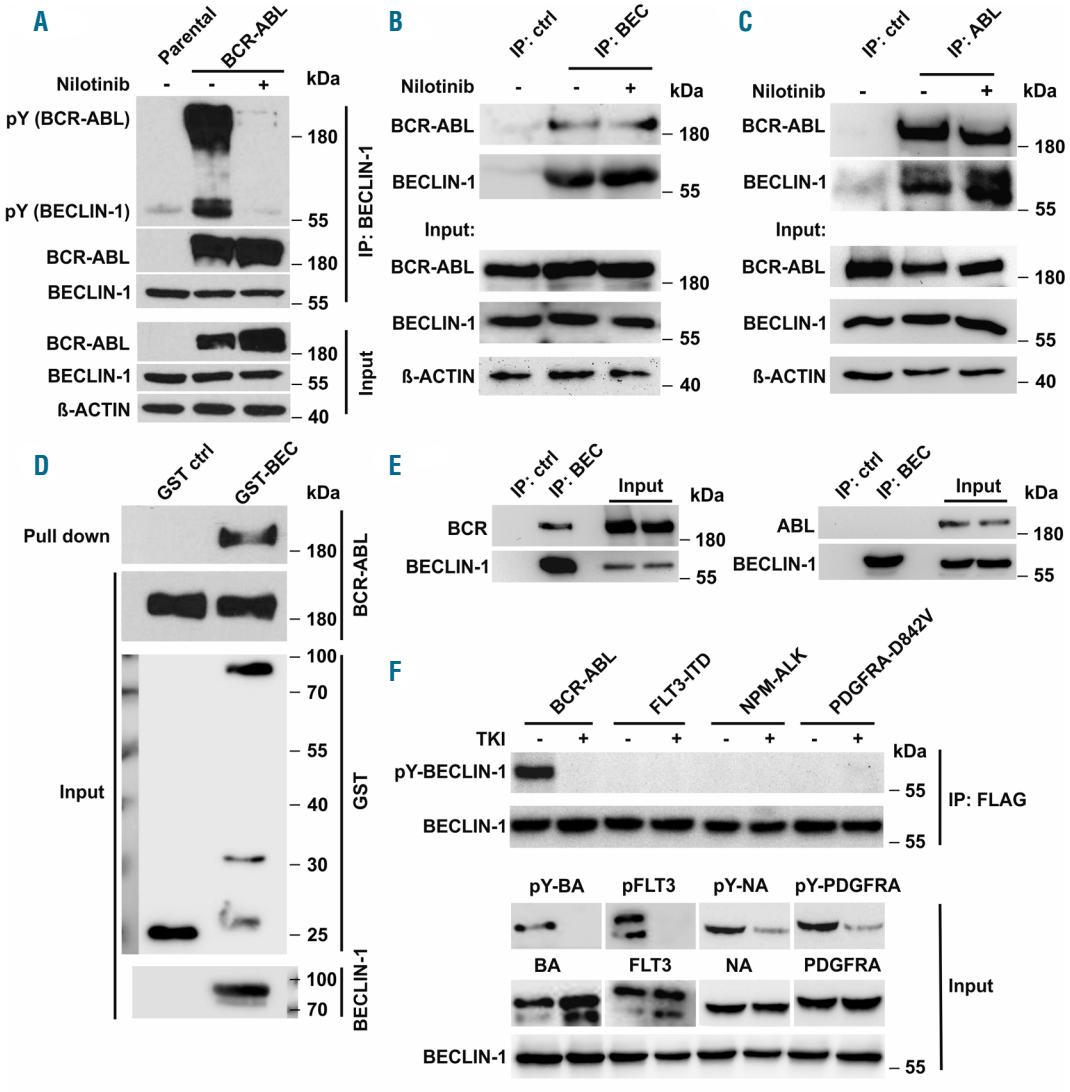

Figure 2. BCR-ABL interacts with and phosphorylates BECLIN-1. (A) Co-immunoprecipitation analyses in BCR-ABL-tranfected HEK293T cells demonstrate, that BCR-ABL strongly phosphorylates and binds BECLIN-1. A phosphotyrosine antibody was used for phospho-BECLIN-1 and phospho-BCR-ABL detection. (B) Immunoprecipitation of BCR-ABL with BECLIN-1 and (C) BECLIN-1 with ABL in K562 cells confirms the interaction reciprocally and at endogenous level. (D) GST-pull-down of BCR-ABL with recombinant GST-Beclin-1 in K562 cells corroborated the interaction between BCR-ABL and BECLIN-1. (E) BECLIN-1 immunoprecipitation in *Beclin-1* transfected HEK293T cells demonstrates that BECLIN-1 is interacting with BCR but not with ABL. (F) BECLIN-1 is exclusively phosphorylated by BCR-ABL among several oncogenic tyrosine kinases in HEK293T cells. For TKI treatment, specific inhibitors (nilotinib for BCR-ABL, sorafenib for FLT3-ITD and PDGFRA-D842V, and TAE684 for NPM-ALK) were added into medium four hours before cell harvest.

presses autophagy. Moreover, addition of interleukin-3 (IL-3), which rescues cells from nilotinib-induced cell death but does not rescue BCR-ABL inhibition, could not block autophagy (*Online Supplementary Figure S3D-F*).

Based on our mouse model data, we hypothesized that BECLIN-1 may be an essential player in autophagy suppression by BCR-ABL. BECLIN-1 has a crucial role in autophagosome formation as being part of the UVRAG-VPS15-ATG14-VPS34-RUBICON-BECLIN-1 complex. Interestingly, we could show that the formation of the UVRAG-VPS15-ATG14-VPS34-RUBICON-BECLIN-1 complex was altered in a BCR-ABL positive human cell line (K562) after nilotinib treatment (*Online Supplementary Figure S3G*): recruitment of positive regulators of autophagosome formation (VPS15, VPS34, UVRAG and ATG14) to BECLIN-1 was increased upon BCR-ABL inhibition, whereas the recruitment of the negative regulator RUBICON to BECLIN-1 was impaired after nilotinib treatment. These results indicate that BCR-ABL kinase activity modulates the BECLIN-1 complex composition and thereby leads to autophagy suppression.

BCR-ABL interacts with BECLIN-1

Next, we aimed to investigate how BCR-ABL kinase activity modulates BECLIN-1 complex composition. We found that BCR-ABL strongly binds to BECLIN-1, independent of ABL kinase activity.

Furthermore, BECLIN-1 was tyrosine phosphorylated in a complex with kinase active BCR-ABL indicating that BCR-ABL may directly phosphorylate BECLIN-1 (Figure 2A). Immunoprecipitation of endogenous BCR-ABL in K562 cells confirmed the BCR-ABL/BECLIN-1 interaction (Figure 2B-C) and GST-pulldown-assays using purified BECLIN-1 suggest that BCR-ABL and BECLIN-1 may bind directly to each other (Figure 2D).

We could also detect BCR-ABL/BECLIN-1 co-localization using immunofluorescence staining (*Online Supplementary Figure S4A*). To investigate which region of BCR-ABL binds to BECLIN-1, we performed binding assays by overexpressing either *BCR* or *ABL* together with *Beclin-1* in 293T cells. Immunoprecipitations revealed that BCR interacts with BECLIN-1 but not ABL (Figure 2E).

BCR-ABL directly phosphorylates BECLIN-1 at specific tyrosine residues Y233 and Y352

We next investigated, whether BECLIN-1 is exclusively phosphorylated by BCR-ABL. Interestingly, all other tested oncogenic kinases (FLT3-ITD, NPM-ALK and PDGFRA-D842V) failed to induce BECLIN-1 phosphorylation, implying that BECLIN-1 is a specific substrate of BCR-ABL and not a general target of oncogenic tyrosine kinase signaling (Figure 2F). Moreover, we were able to confirm BECLIN-1 tyrosine phosphorylation in all tested samples of primary CML patient material, whereas BECLIN-1 phosphorylation was absent in healthy control samples (*Online Supplementary Figure S4B*).

To test whether BECLIN-1 is a direct target of BCR-ABL we performed an *in vitro* kinase assay, and identified specific phosphorylation in two distinct regions of BECLIN-1: One spanning amino acid (aa) region 141 - 277 and another aa region 338 - 450 (Figure 3A). Furthermore, we generated a series of tyrosine residue mutants to determine specific BECLIN-1 tyrosine residues phosphorylated by BCR-ABL. Strong phosphorylation by BCR-ABL could be detected on BECLIN-1 tyrosine residues Y233 and Y352, whereas Y162 and Y338 show minor phosphorylation (Figure 3B). Western blot analyses of single and double phosphorylation-deficient mutants of those distinct BECLIN-1 tyrosine residues validated our results (Figure 3C) and demonstrated that BCR-ABL phosphorylates BECLIN-1 specifically at tyrosine residues Y233 and Y352. Interestingly, tyrosine Y352 (AA352-355 YCSG) is part of a STAT5 Src Homology 2 (SH2) domain binding motif (Y[VLTFIC]xx).[37]

Phospho-mimic mutant *Beclin-1* Y233E/Y352E suppresses autophagy through BECLIN-1 complex alterations whereas the phospho-deficient *Beclin-1* Y233F/Y352F mutant induces autophagy

To evaluate whether phosphorylation of BECLIN-1 regulates autophagy, we generated a BECLIN-1 phosphorylation-mimic (Y233E/Y352E) and a phosphorylation-deficient mutant (Y233F/Y352F). In an LC3 puncta assay in K562 cells, we found that the phosphorylation-mimic BECLIN-1 mutant suppresses autophagy, whereas the phosphorylation-deficient BECLIN-1 mutant induces increased autophagy (Figure 4A-B). By immunoblotting, we could confirm that the phosphorylation-mimic mutant Y233E/Y352E decreases autophagy, whereas expression of the phosphorylation-deficient mutant Y233F/Y352F induces autophagy (Figure 4C). Our findings therefore suggest that phosphorylation of BECLIN-1 by BCR-ABL suppresses autophagy induction.

Next we sought to know, whether the impaired autophagy induction of the phospho-mimic mutant Y233E/Y352E may be due to an altered recruitment of complex components to BECLIN-1. It has been shown recently, that lack of BECLIN-1 leads to downregulation of the BECLIN-1 complex binding partners.[38] Indeed, *Beclin-1* deficient, BCR-ABL expressing MEF showed downregulation of BECLIN-1 binding partners, which could be rescued by re-expression of either wt *Beclin-1* or both phospho-mutants ((BEC FF and EE). Furthermore, expression of the *Beclin-1* Y233E/Y352E mutant leads to decreased UVRAG and ATG14 levels, whereas Rubicon levels were increased compared to phosphorylation-deficient BECLIN-1 cells (Figure 4D).

From our results we hypothesized that the phosphorylation status of BECLIN-1 is important for the stabilization and recruitment of the different binding partners to the BECLIN-1 complex. Interestingly, co-immunoprecipitation of BECLIN-1 complex components revealed that autophagy activating proteins like VPS15, VPS34 and ATG14 were recruited less to the phospho-mimic BECLIN-1 (BEC EE) complex compared to the phospho-deficient BECLIN-1 (BEC FF) complex (Figure 4E, *Online Supplementary Figure S4C*). These results indicate that the altered autophagy by the two mutants is due to altered binding capacities of positive regulators to the BECLIN-1 core complex and thereby alters BECLIN-1 complex activity.

Next we asked, whether expression of the phospho-mimic *Beclin-1* mutant could overcome the TKI-induced BCR-ABL inhibition-mediated autophagy induction and indeed, expression of *Beclin-1 EE* Y233E/Y352E impaired nilotinib-induced autophagy measured by LC3-II expression (Figure 4F) and LC3 puncta accumulation (Figure 4G-H). BCR-ABL inhibition by nilotinib was not able to enhance the autophagy-stimulatory effect of the phosphorylation-deficient BECLIN-1 Y233F/Y352F mutant com-

pared to wt BECLIN-1, pointing to a BECLIN-1-specific autophagy regulation by BCR-ABL (Figure 4F-H).

In further BECLIN-1 complex analyses, we could demonstrate that the resistance of the phospho-mimic *Beclin-1* mutant to nilotinib-induced autophagy is caused by an altered composition of the BECLIN-1 core complex with an impaired recruitment of the activation components ATG14, UVRAG, VPS15 and a gain of the negative regulator RUBICON to the BECLIN-1 core complex (*Online Supplementary Figure S5A*). BECLIN-1 phosphorylation with subsequent resistance to TKI-induced autophagy may thereby provide a novel explanation of how leukemic cells can escape autophagy-induced cell death and develop TKI resistance.

Recently, it has been shown that BECLIN-1 S90 phosphorylation is involved in starvation-mediated autophagy.[39] To test whether BECLIN-1 phosphorylation at Y233/Y352 can influence starvation-mediated or rapamycin-mediated autophagy, we starved K562 cells or treated them with rapamycin and found that cellular autophagy is induced in *Beclin-1* wt cells (*Online Supplementary Figure S5B-D*) and no differences could be demonstrated in K562 cells expressing either BECLIN-1 Y233E/Y352E or BECLIN-1 Y233F/Y352F (*Online Supplementary Figure S5E-H*). These results indicate that tyrosine phosphorylation of BECLIN-1 at Y233 and Y352 is not involved in starvation- or rapamycin-mediated autophagy but rather seems to be specific for tyrosine kinase-mediated autophagy processes.

Discussion

Recently, several studies have suggested that autophagy, a mechanism maintaining cellular homeostasis, plays an essential role in CML. However, the precise machinery of autophagy in CML development is not completely understood and crucial autophagocytotic mediators have not been investigated *in vivo* for their role in leukemogenesis in relevant CML mouse models.

Here, we define a molecular mechanism of autophagy suppression by BCR-ABL-specific BECLIN-1 phosphorylation. Silencing of *Beclin-1* by siRNA technology led to a significantly prolonged survival of BCR-ABL transplanted mice, whereas no profound differences could be found for *Atg5* deletion. Binding of BECLIN-1 to BCR-ABL led to phosphorylation at tyrosine residues Y233 and Y352, alteration of the BECLIN-1 interactome, and suppression of autophagy function.

Several active oncogenic kinases were demonstrated to serve as negative regulators of autophagy processes, whereas inhibition of oncogenic tyrosine kinases can reverse this effect. Until now, some links of BCR-ABL to autophagy processes have been described: BCR-ABL activates the PI3K/AKT signaling pathway, which is considered as a pathway inhibiting autophagy. Furthermore, TKI treatment itself triggers autophagy in BCR-ABL$^+$ cells and TKI-induced cell death can potentially be increased by targeting autophagy proteins in addition. Recently, it was demonstrated that Ponatinib-resistant CML cells can

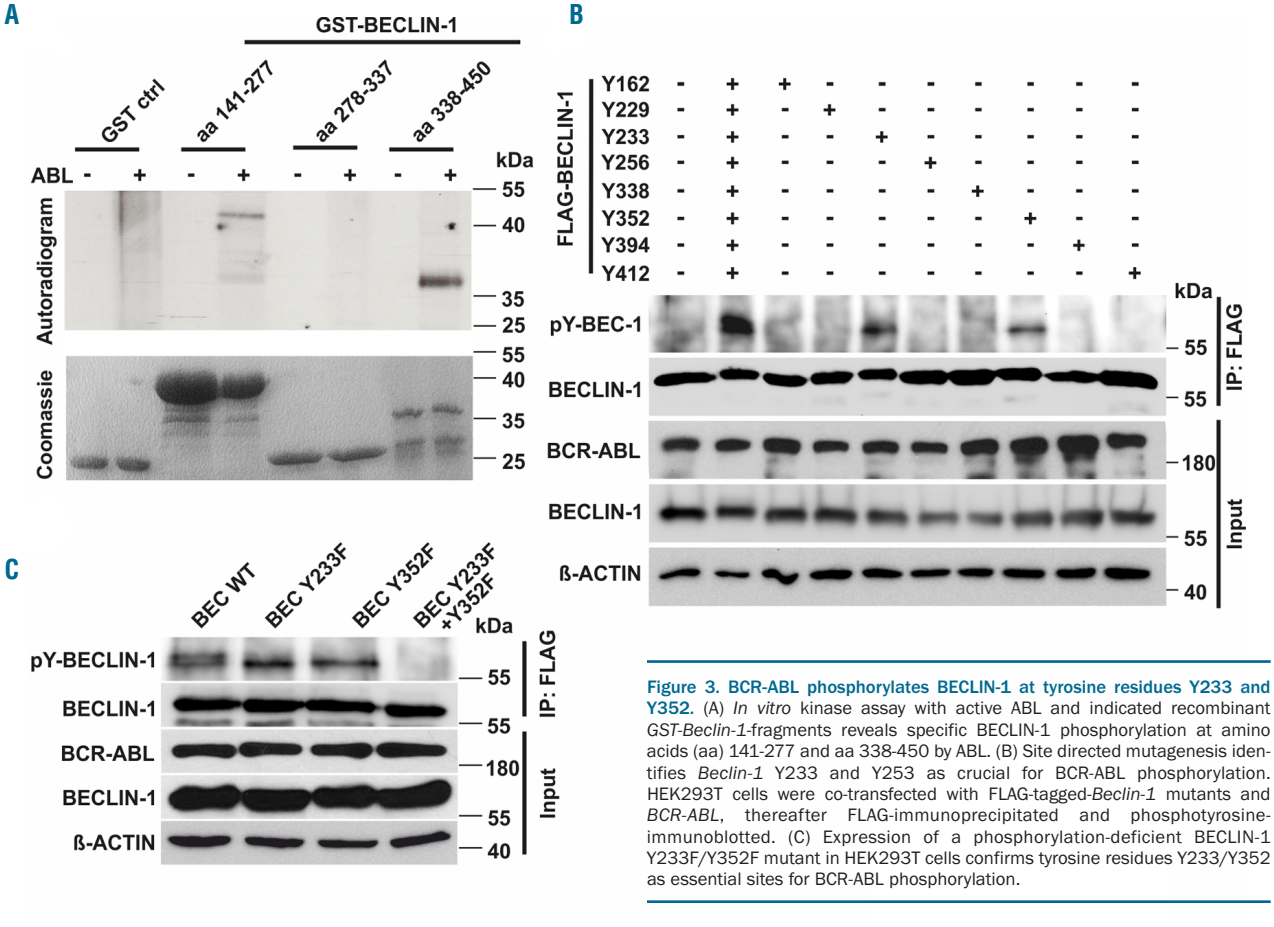

Figure 3. BCR-ABL phosphorylates BECLIN-1 at tyrosine residues Y233 and Y352. (A) *In vitro* kinase assay with active ABL and indicated recombinant *GST-Beclin-1*-fragments reveals specific BECLIN-1 phosphorylation at amino acids (aa) 141-277 and aa 338-450 by ABL. (B) Site directed mutagenesis identifies *Beclin-1* Y233 and Y253 as crucial for BCR-ABL phosphorylation. HEK293T cells were co-transfected with FLAG-tagged-*Beclin-1* mutants and *BCR-ABL*, thereafter FLAG-immunoprecipitated and phosphotyrosine-immunoblotted. (C) Expression of a phosphorylation-deficient BECLIN-1 Y233F/Y352F mutant in HEK293T cells confirms tyrosine residues Y233/Y352 as essential sites for BCR-ABL phosphorylation.

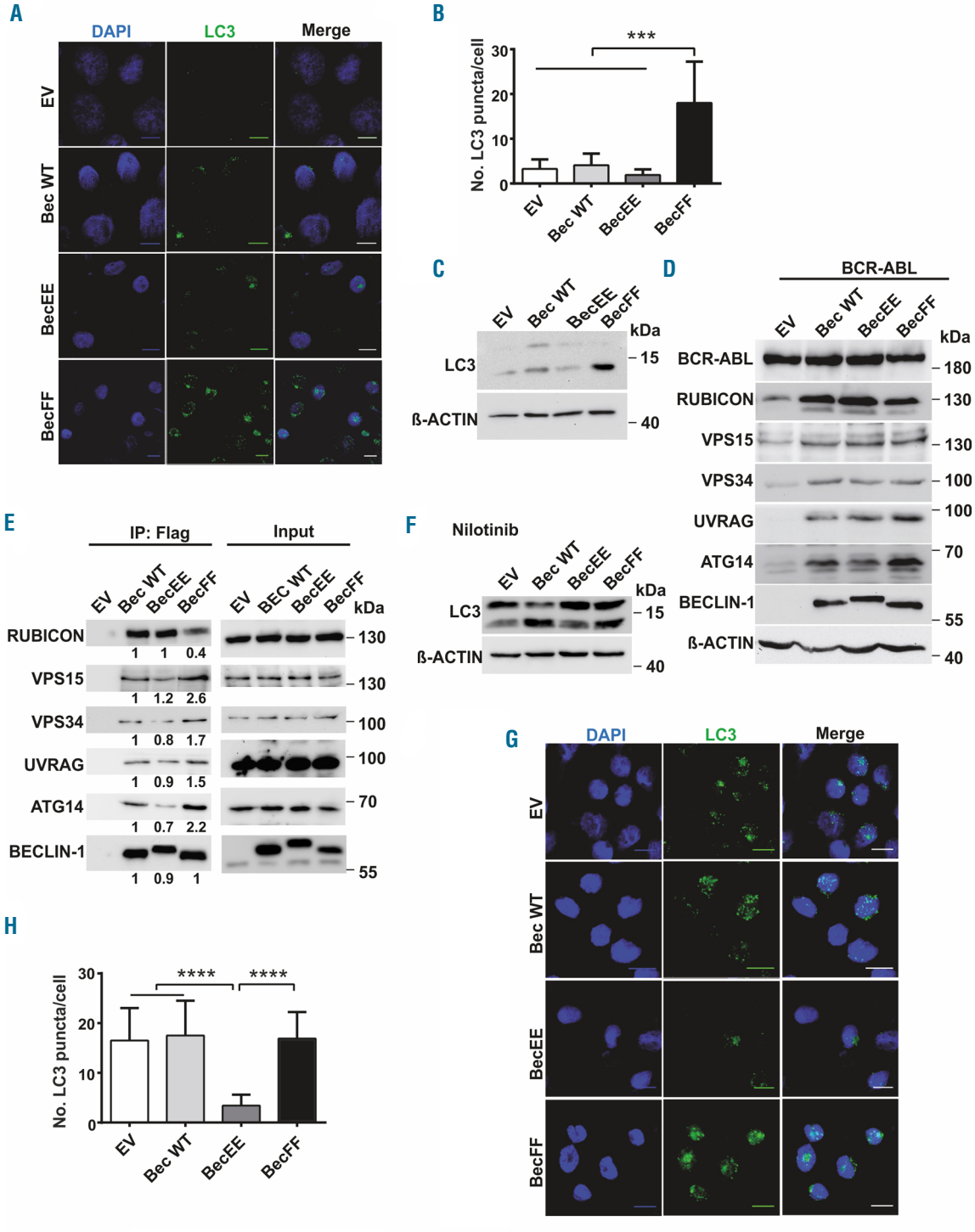

Figure 4. BCR-ABL mediated BECLIN-1 phosphorylation leads to suppression of autophagy and altered BECLIN-1 complex formation. (A) Confocal microscopy of K562 cells reveals significant induction of autophagy in BECLIN-1 Y233F/Y352F-expressing cells indicated by increased number of LC3 puncta. (B) Statistical analysis of LC3 puncta count in *Beclin-1*-mutant-transduced K562 cells (3.3, 4.1, 1.9, and 18 units respectively; ****P<0.0001 Student's t-test; Scale bar 10 μm). (C) Upregulation of LC3-II in K562 cells transduced with the phosphorylation-deficient *Beclin-1* Y233F/Y352F mutant. (D) Immunoblot analyses were used for the detection of ATG14, UVRAG, VPS34 and RUBICON expression levels in *Beclin-1* knockout MEF transduced with BCR-ABL and the indicated *Beclin-1* construct. (E) Immunoprecipitation of FLAG-tagged phosphorylation-deficient BECLIN-1 Y233F/Y352F mutant (BecFF) in K562 cells indicates altered autophagy complex formation compared to the phosphorylation-mimic BECLIN-1 mutant (BecEE). (F) *Beclin-1* tyrosine phosphorylation-mimic mutation blocks nilotinib-induced autophagy seen by reduced LC3-II levels compared to empty vector, *Beclin-1* wt, and *Beclin-1* FF. LC3 levels were evaluated by immunoblotting in the indicated *Beclin-1* mutant-transduced K562 cells. (G) LC3 puncta formation was measured using confocal microscopy in K562 cells transduced with mCherry-EGFP-LC3 and the indicated *Beclin-1* construct upon nilotinib treatment. Cells were stained with anti-LC3 antibody and nuclei were counterstained with DAPI. Scale bar, 10 μm. (H) Quantitation of LC3 puncta (16.5, 17.5, 3.4, and 16.9 dots/cell, respectively; P<0.0001 for comparison of *Beclin-1* EE to empty vector [EV], *Beclin-1* wt and *Beclin-1* FF). K562 cells transduced with mCherry-EGFP-LC3 and the indicated *Beclin-1* constructs were used for LC3 puncta measurement via Olympus ScanR screening station. Bars represent the mean±SD. ****P<0.0001 by Student's t-test. EV: empty vector; wt: wild-type *FLAG-Beclin-1*; EE: *FLAG-Beclin-1* Y233E/Y352E; FF: *FLAG-Beclin-1* Y233F/Y352F.

acquire BCL-ABL-independent resistance through autophagy inhibition by activation of mTOR.[46] Therefore, we hypothesize that BECLIN-1 may play similar roles in the resistance-acquired signaling cascade. BECLIN-1 is a central autophagy mediating protein in mammalian cells, and EGFR and AKT kinase have been shown to phosphorylate BECLIN-1 leading to inactivation of the protein and suppression of autophagy. Interestingly, active EGFR has been shown to phosphorylate the same tyrosine residues Y233/Y352 as BCR-ABL, further highlighting the importance of these tyrosine residues for autophagy suppression.[42] In line with these previous findings, we have demonstrated in the present study that BCR-ABL phosphorylates BECLIN-1 and thereby suppresses autophagy. Interestingly, this effect is mediated by the alteration of BECLIN-1 affinity to BECLIN-1 binding partners (ATG14, VPS34 and VPS15), known as the BECLIN-1 core complex. Our results therefore provide a novel explanation for the suppression of autophagy in CML and expand our knowledge regarding BECLIN-1-associated pathogenic mechanisms in BCR-ABL$^+$ leukemia.

A role for BECLIN-1 has been proposed in various malignancies, such as breast or lung carcinomas. However, the precise role of BECLIN-1 in tumorigenesis remains unclear: On one hand, *Beclin-1* is considered as a tumor suppressor and its overexpression is favorable for treatment of various solid tumors.[51-54] Furthermore, low expression of *Beclin-1* is a marker of poor prognosis and enhanced aggressiveness in breast cancer[55] and loss of one *Beclin-1* allele leads to enhanced tumor development in mice. On the other hand, our present study demonstrates that knockdown of *Beclin-1* prolongs the survival of BCR-ABL$^+$ leukemic mice, which is consistent with a previous *in vitro* study in CML cell lines.[16] Gene array analysis of CML patient samples revealed upregulated *Beclin-1* levels in CML patients compared to healthy controls (fold change: 1.22; *q*-value: <0.1%).[56] Importantly, we could show that CML patients exhibit a significant increase of phosphorylated BECLIN-1 levels. Targeting BECLIN-1 in specific approaches might thereby represent an elegant and alternative treatment option for TKI-resistant or intolerant CML patients by rendering CML cells sensitive to targeted therapies.

Recently, a study uncovered a kinase-independent role of EGFR in autophagy, showing that inactive oncogenic EGFR reversely triggers autophagy.[57] These findings support the hypothesis that cells can develop TKI-resistance through autophagy induction, which might be caused or even triggered through the inactive oncogenic kinase itself. This sheds some light on the role of autophagy on cellular survival rather than cell death and gives a rationale to explore the combinatory effect of kinase inhibitors with autophagy inhibitors. A number of drugs (Chloroquine, Bafilomycin A1, MAPK inhibitors and PI3K inhibitors) were described to have inhibitory effects on autophagy, however, most of them are poorly selective, limiting their therapeutic application. Therefore, the development of highly specific and selective autophagy inhibitors remains a mandatory necessity for the successful evaluation of the therapeutic combination therapy with TKI in CML. Moreover, accurate target identification among major autophagy players is fundamental for successful therapeutic application: BECLIN-1 seems an attractive target, as its role in kinase-driven cancer is not only shown for solid cancers but also for hematopoietic malignancies in our study. For ATG5, we were not able to prove essential function in CML development in mice, whereas Liu *et al*. were able to demonstrate a crucial role of the protein in MLL-AF9 mediated AML induction in mice.[61] ATG3 expression was shown to be indispensable for effective CML progression.[14] These results indicate that autophagy induction is highly specific and stringently regulated, not only depending on the particular malignancy, but also on the involvement of the master regulators.

Taken together, in this study we uncover a crucial role of BECLIN-1 in BCR-ABL mediated transformation *in vivo* and were able to identify a molecular mechanism by which BCR-ABL kinase activity regulates autophagy. We show that BCR-ABL binds and phosphorylates BECLIN-1 on tyrosine residues 233 and 352, thereby leading to alterations of the UVRAG-VPS15-ATG14-VPS34-RUBICON-BECLIN-1 complex. Moreover, the BCR-ABL/ BECLIN-1 interaction suppresses autophagy and thereby bypasses the negative effect of autophagy on cancer cell survival and proliferation. Importantly, these data may be of clinical relevance, as CML patients exhibit upregulated BECLIN-1 phosphorylation levels. Our findings provide a novel link between BCR-ABL and BECLIN-1 and shed some light on how specific oncogenes influence autophagy.

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