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Phosphorylation of Beclin 1 by BCR-ABL suppresses autophagy in CML

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Abbreviations

5-FU	5-fluorouracil
Abl	Abelson kinase
ALL	Acute lymphoid leukaemia
AML	Acute myeloid leukaemia
APS	Ammoniumpersulfat
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma-2
BCR	Breakpoint cluster region protein
BCR-ABL	Breakpoint cluster region-Abelson
BM	Bone marrow
BMT	Bone marrow transplantation
Bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
CML	Chronic myeloid leukemia
CNS	Central nervous system
DAPI	4,6-diamidino-2-phenylindole
DEPC	Diethylenpyrocarbonate
dH_2O	Distilled water
DMSO	Dimethyl sulpoxide
DNA	Deoxyribonucleic acid

DT	Diptheria Toxin
DTT	Dithiothreitol
EDTA	Ethylendiamin-N,N,N′,N′-tetraacetic acid
FCS	Foetal calf serum
FDA	Food and Drug Administration
FOXO	Forkhead box O
FITC	Fluorescein isothiocyanate
Grb2	Growth factor receptor bound protein 2
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
Hck	Haemopoietic cell kinase
HRP	Horseradish Peroxidase
HSC	Haemopoietic stem cell
IL-3	Interleukin-3
IL-6	Interleukin-6
IM	Imatinib mesylate
IP	Immunoprecipitation
LPS	Lipopolysaccharid
IPTG	Isopropyl-β-D-Thiogalactosid
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton

KD	Knockdown
KO	Knockout
MEFs	Mouse embryo fibroblast cell line
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PDGF-R	Platelet-derived growth factor receptors
PI	Propidium iodine
PI3K	Phosphatidylositol 3-kinase
p-Tyr	Phosphorylated tyrosine
PVDF	Polyvinylidene fluoride
RBC	Red blood cell
RNA	Ribonucleic acid
SCF	Stem cell factor
SDS	Sodium Dodecyl Sulfate
PMSF	Phenylmethylsulfonyl fluoride
SH	Src homology
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SOS	Son of Sevenless
TEMED	Tetramethylethylendiamin
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor

TRIS	Tris(hydroxymethyl)aminomethane
Ub	Ubiquitin
WB	Western blotting
WBC	White blood cell
WCL	Whole cell lysate
WT	Wild type

Abstract

The constitutively active chimeric tyrosine kinase BCR-ABL is critical for initiation, progression and maintenance of chronic myelogenous leukemia (CML). Imatinib and second-generation BCR-ABL tyrosine kinase inhibitors (TKIs) serve now as standard therapies for Ph^+ patients. However, disease persistence occurs frequently and one of the major reasons was considered to be insensitivity of CML stem cells to TKI treatment.

Recently accumulated evidence argues that, autophagy, a genetically regulated process of adaptation to metabolic stress, is involved in TKI-induced cell death. It is hypothesized, that TKI-induced autophagy could allow CML stem cells to become metabolically dormant enabling their survival under conditions that may mimic growth factor deprivation and thereby "counter" TKI-induced cell death. However, the molecular mechanism of TKI-induced autophagy in BCR-ABL^+ CML, as well as its role in malignant progression is poorly understood.

It has been shown that autophagy functions in CML *in vitro*, however, les is known *in vivo*. This study aimed to identify the precise role of autophagy and its effector molecules in a murine CML model. To identify the impact of autophagy in BCR-ABL-driven leukemia, a targeted genetic approach to knockdown Beclin 1 as a key regulator of autophagy in a CML mouse model was used. Interestingly, mice transplanted BCR-ABL expressing bone marrow harboring Beclin 1 knockdown showed a less aggressive disease with significantly lower WBC-count, leukemic burden and prolonged overall survival of the mice compared to mice transplanted with BCR-ABL + Beclin 1 WT BM.

To further test whether BCR-ABL regulates autophagy, LC3 was measured as a marker for autophagy in BCR-ABL^+ K562 cell. Interestingly, inhibition of BCR-ABL activity by nilotinib led to increased LC3-II expression and punctual LC3 accumulation, indicating that BCR-ABL activity can suppress autophagy. Next, the proteins involved in BCR-ABL mediated autophagosome formation were investigated. Recruitment of

VPS34, UVRAG and ATG14 to Beclin 1 was increased in case of nilotinib treatment and could thereby positively regulate autophagosome formation, whereas Rubicon, a negative regulator, was recruited less frequently to the Beclin 1 complex.

To further clarify the function of Beclin 1, biochemical analyses were performed. It showed that Beclin 1 binds to BCR-ABL independent of BCR-ABL kinase activity and Beclin 1 is phosphorylated by BCR-ABL. To test the impact of BCR-ABL mediated Beclin 1 phosphorylation on autophagy induction, Beclin 1 phospho-mimic (Y233E/Y352E) and phospho-deficient (Y233F/Y352F) mutations were generated. Interestingly, nilotinib treatment failed to induce autophagy in cells expressing the Beclin 1 phospho-mimic mutation, thereby highlighting the necessity of Beclin 1 in BCR-ABL-mediated autophagy. Expression of Beclin 1 mutants in *Beclin 1* knockout MEFs and K562 cells showed decreased binding of UVRAG, ATG14 and VPS34 to Beclin 1 Y233E/Y352E, suggesting an important role of Beclin 1 phosphorylation for complex stabilization and autophagy suppression. Interestingly, phospho-deficient Beclin 1 also delays BCR-ABL-driven leukemogenesis, demonstrating that Beclin 1 phosphorylation is crucial for BCR-ABL-mediated leukemogenesis. In contrast, deletion of *Atg5*, another central regulator of autophagy, did not impede disease onset or progression in the CML model.

Taken together, these findings identify Beclin 1 as a specific substrate of BCR-ABL, thereby highlighting the importance of Beclin 1 in BCR-ABL-mediated leukemogenesis and showing that autophagy induction in CML cells may be rather a specific Beclin 1-BCR-ABL interaction effect than a general microenvironmental stress phenomenon.

1 Introduction

1.1 Philadelphia chromosome

Over half a century ago, the Philadelphia chromosome or Philadelphia translocation was the first specific cytogenetic abnormality identified in a human malignancy which was called chronic myelogenous leukemia (CML) (Moorhead et al., 1960; Nowell and Hungerford, 1960). Later it was found that the translocation between chromosomes 9 and 22, causing the *ABL* gene from chromosome 9 to join the *BCR* gene on chromosome 22, thus forming the *BCR-ABL* fusion gene (Groffen et al., 1984). The Philadelphia chromosome is a hallmark of chronic myelogenous leukemia, and is also found in some patients with acute lymphoblastic leukemia (ALL) or acute myelogenous leukemia (AML) due to the same chromosomal translocation and molecular mechanisms as CML. Today, the strategy for CML treatment is to inhibit the BCR-ABL tyrosine kinase activation, as BCR-ABL fusion kinase has been reported to be the unique cause of CML (Kantarjian et al., 2007). In order to understand the mechanism of BCR-ABL-driven leukemogenesis, it is necessary to understand the functions of BCR and c-ABL.

1.2 ABL

The *c-ABL* oncogene belongs to the family of non-receptor tyrosine kinases, which is originally identified for its homology with *v-ABL* (Abelson murine leukemia virus) and has a function in causing an acute neoplastic transformation in mice (Abelson and Rabstein, 1970). The c-ABL tyrosine kinase and its transforming variants have been reported to function in tumorigenesis, which plays an important role in several cellular processes, including cell division, adhesion, differentiation, and response to stress (Van Etten, 1999). The mammalian *c-ABL* gene is ubiquitously expressed. It encodes two 145 kDa isoforms arising from alternative splicing of two distinct first exons (1a/I and 1b/IV in the human and mouse respectively); resulting in myristoylation sites (Ben-Neriah et al., 1986). In most cells, such as fibroblasts, the c-ABL protein is

mainly located in the nucleus, is bound to chromatin; but can also be found in the cytoplasm where it co-localizes with F-actin (Van Etten et al., 1989). In primary haematopoietic cells and neurons, c-ABL is located more in the cytoplasm (Shaul, 2000). The pattern of expression and the intracellular location of the c-ABL protein suggest that this molecule plays an important role in cellular biology and also exerts multiple functions in various cell compartments (Saglio and Cilloni, 2004). However, the exact role of c-ABL still needs further investigation.

The c-ABL structure is represented in Figure 1. As a non-receptor tyrosine kinase, the c-ABL protein possesses three SRC homology (SH) regions at the N-terminal extremity that mediates catalytic (SH1), phospho-tyrosine, and proline-rich sequence binding proteins (SH2-SH3), respectively (Laneuville, 1995). The C-terminal extremity of ABL protein contains a domain of interaction with F-actin, suggesting an important role of c-ABL in the mechanisms which regulate the variations of the cellular morphology and the intercellular adhesion (Woodring et al., 2003). The first exon of ABL1 is lost in the BCR-ABL fusion protein due to the translocation breakpoint on chromosome 9 (upstream of exon 2 of the *ABL1* gene). Several diverse functions have been attributed to c-ABL. The normal c-ABL protein is involved in the regulation of the cell cycle (Sawyers et al., 1994), in the cellular response to genotoxic stress (Yuan et al., 1999), and in the transmission of information about cellular environment through integrin signaling, but overall, it appears that the ABL protein serves as a cellular module that integrates signals from both extracellular and intracellular sources, influencing decisions with regard to cell cycle and apoptosis (Deininger et al., 2000).

1.3 BCR

The *BCR* gene is located on human chromosone 22. It is the site of breakpoints used in the generation of Philadelphia chromosome translocation. The BCR protein contains a coiled-coil domain, a serine/threonine kinase domain, and a region that binds to SRC-homology 2 domains ((Che et al., 2001; Pendergast et al., 1991), Figure 1). The center of the protein has a pleckstrin homology domain (PH) (Figure 1), which

is similar to the guanine nucleotide exchange factors (GEFs) that stimulate the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on Rho guanidine exchange factors (Denhardt, 1996). The C-terminal region of BCR has been reported to be activated by the GTPase function of the small GTP-binding protein Rac, indicating that BCR could be a target for regulation by Rac and has important implications for the role of BCR translocations due to the translocations-mediated C-teminal region of BCR loss in CML (Diekmann et al., 1991).

1.4 BCR-ABL

Three translocation breakpoints are mapped within the *BCR* gene (Figure 1). This leads to the expression of proteins with an apparent molecular weight of 185/190 kDa (termed BCR-ABL p185), 210 kDa (termed BCR-ABL p210), and a rare 230 kDa (termed BCR-ABL p230) form. These different BCR-ABL fusion proteins are expressed in different diseases. The p210 expression is the molecular hallmark of CML, while around two-thirds of patients express p185, and p230 is rarely expressed in neutrophilic-CML/chronic neutrophilic leukemia.

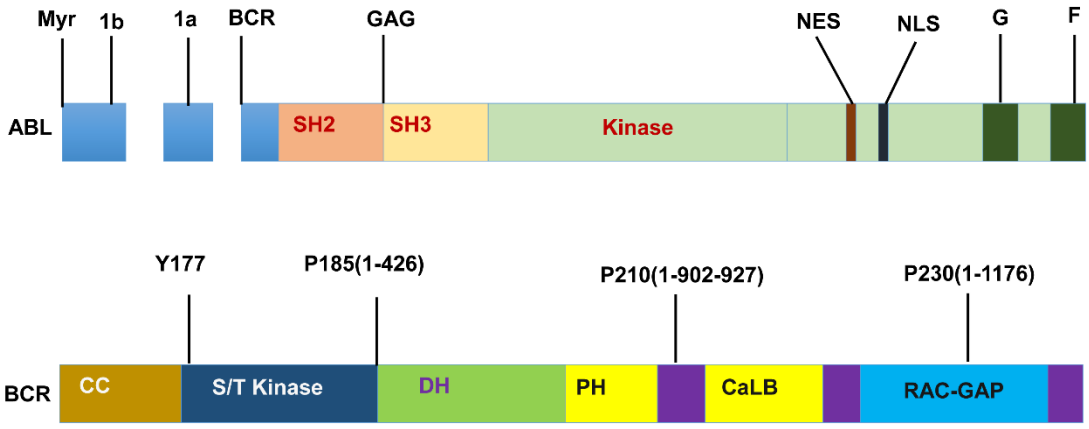

Figure 1. Schematic representation of the ABL and BCR structures.

ABL protein: Two isoforms of ABL (1a and 1b) are generated by alternative splicing of the first exon, and a site with myristoylation modification locates in 1b. Besides the spliced sequence, the ABL protein contains a SH3 domain, a SH2 domain, a tyrosine kinase domain, nuclear

localization signals (NLS), a nuclear exporting signal (NES), and an actin-binding domain (ABD) for both monomeric (G) and filamentous (F) forms of actin. BCR protein contains coiled-coil motif (CC), serine-threonine kinase (S/T kinase), Dbl homology domain (DH), pleckstrin homology domain (PH), putative calcium-dependent lipid binding site (CaLB) and RAC guanosine triphosphatase-activating protein (RAC-GAP) domain. BCR has one binding site for GRB2 and GRB10 at Y177. p185, p210 and p230 indicate the points at which BCR most commonly fuses to ABL. Reproduced with modification from (Ren, 2005).

1.5 Signaling of BCR-ABL

The aberrant tyrosine kinase activity of BCR-ABL has been shown to be necessary and sufficient to maintain the leukemia phenotype of CML (Daley et al., 1990; Heisterkamp et al., 1990; Kelliher et al., 1990). The activation of ABL tyrosine kinase is a primary event in the genesis of CML, making it the preferential target for drug therapy. Physiologically, the ABL protein transports between nucleus and cytoplasm; however, when fused to BCR, the fusion protein is mainly retained in the cytoplasm and loses the ability for transportation. The ABL tyrosine kinase is constitutively activated by BCR fusion leading to dimerization or tetramerization and subsequent autophosphorylation, which also increases the binding sites for the SH2 domains of other proteins (Pendergast et al., 1991; Pendergast et al., 1993). The BCR-ABL protein binds to several cytoplasmic proteins that are involved in critical cellular processes like the regulation of transcription, proliferation and apoptosis, for example, the perturbation of the RAS–mitogen-activated protein kinase (MAPK) leading to increased proliferation, the Janus-activated kinase from the (JAK)–STAT pathway leading to impaired transcriptional activity, and the phosphoinositide 3-kinase (PI3K)/AKT pathway resulting in increased apoptosis ((Melo and Deininger, 2004), Figure 2 and 3).

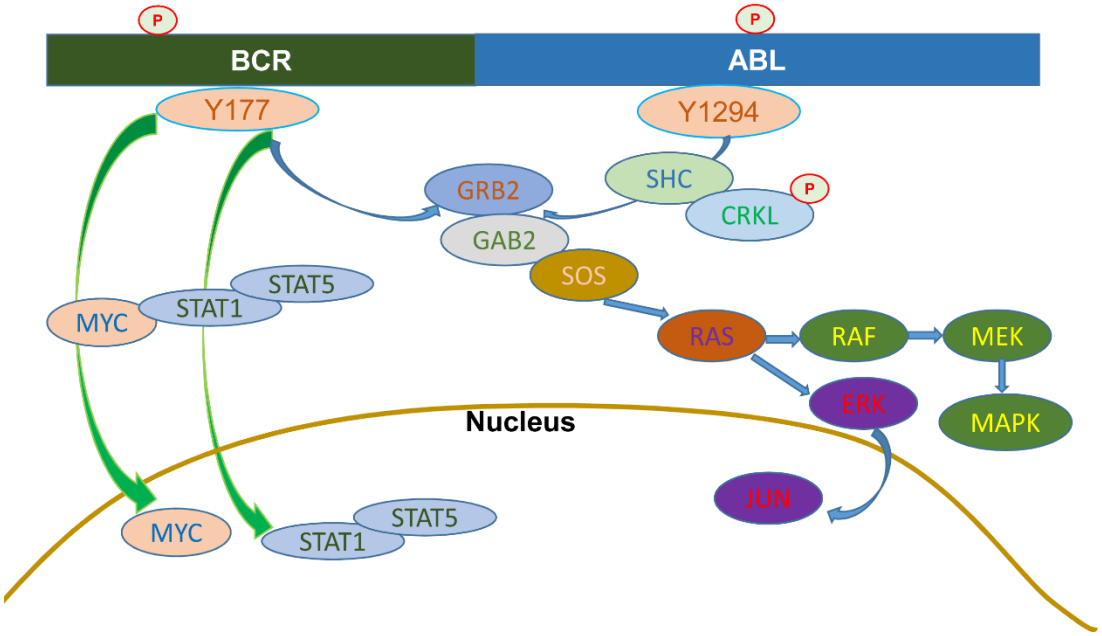

Figure 2. Schematic representation of the molecular pathway activated by BCR-ABL.

Phosphorylation at BCR Tyr177 is essential for BCR-ABL–mediated leukemogenesis. Transcription factors like STAT1 and STAT5 are involved in BCR-ABL signaling leading to the induction of cytokine independence. The protooncogene *MYC* is a target of the transforming activity of the BCR-ABL. The BCR-ABL/GRB2/SOS complex constitutively activates the RAS downstream pathway, thereby activating MEK1/2 and MAPK proteins and resulting in abnormal cell proliferation. Reproduced with modification from (Cilloni and Saglio, 2012).

As a part of BCR-ABL protein, BCR contains a tyrosine site (tyrosine 177) that interacts with the SH2 domain of the adaptor protein GRB2 (Figure 2-3). Moreover, phosphorylation of BCR at tyrosine 177 is crucial for BCR-ABL-mediated leukemogenesis, and the mutation of Y177 significantly destroys GRB2 interaction and impairs BCR-ABL-induced Ras activation mediated transformation *in vitro* and leukemogenesis *in vivo* (Pendergast et al., 1993; Zhang et al., 2001). The BCR-ABL/GRB2 complex recruits Son of Sevenless (SOS) to form BCR-ABL/GRB2/SOS complex resulting in conversion of the inactive GDP-bound form of Ras to its active GTP-bound state and the activation of the scaffold adapter

GRB2-associated binding protein 2 (Ren, 2005; Sattler et al., 2002). Consequently, the GRB2/SOS/GAB2 complex leads to constitutive activation of RAS downstream signal pathway MAPK/ERK/MET resulting in cell proliferation. In addition, the complex activates PI3K/AKT pathway, which promotes survival by suppressing the activity of the forkhead box O (FOXO) transcription factor, and enhances cell proliferation by inducing p27 proteosomal degradation and mTOR activation (Skorski et al., 1995). Besides GRAB2, a lot of protein mediated-signaling pathways are activated by BCR-ABL, including MYC, CRKL and STAT5 that are critical for BCR-ABL-dependent transformation and leukemogenesis (Sawyers et al., 1992; Senechal et al., 1996; Shuai et al., 1996). The protooncogene *MYC*, which is highly expressed in CML cells, is independent of the RAS pathway but directly upregulated by the ABL SH2 region. Moreover, *in vitro* inhibition of c-Myc impairs BCR-ABL transformation, and thereby impairs leukemogenesis (Sawyers et al., 1992).

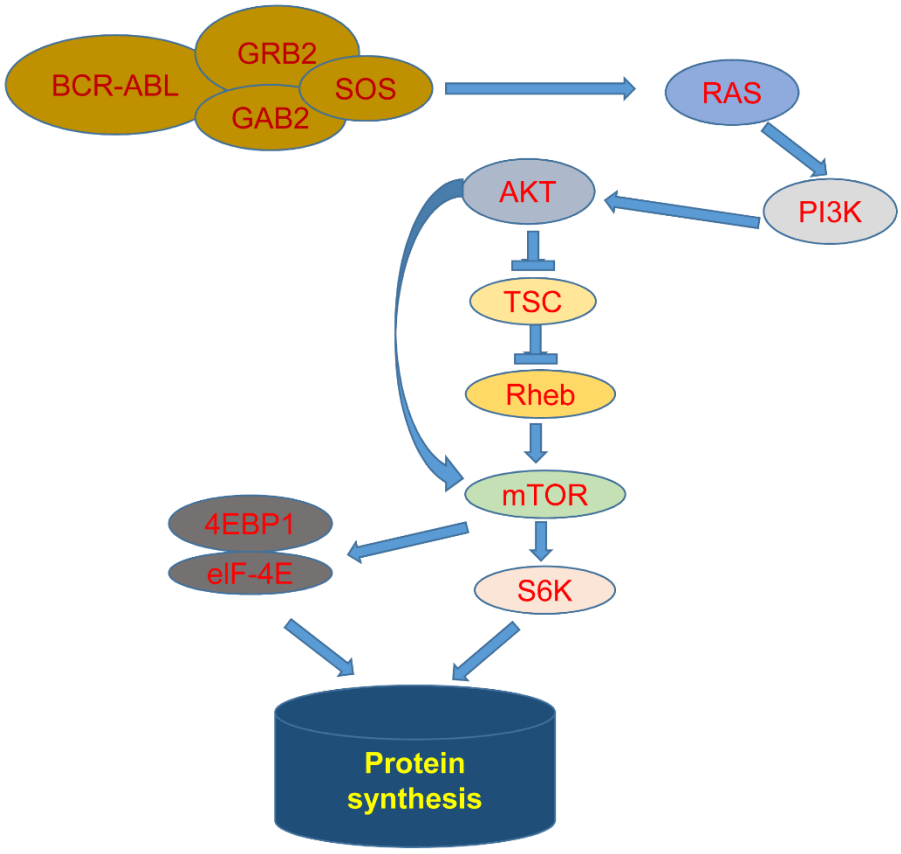

Figure 3. Schematic representation of BCR-ABL–mediated mTOR pathway activation.

PI3K/AKT signaling pathway is activated by BCR-ABL leading to mTOR activation, which is involved in protein synthesis. Reproduced with modification from (Cilloni and Saglio, 2012).

1.6 Treatment of BCR-ABL$^+$ Leukemia

BCR-ABL fusion gene is formed by chromosomal translocation, and is required for the pathogenesis of CML, and the tyrosine kinase activity of ABL is essential for BCR-ABL-mediated transformation, thereby the ABL kinase is an attractive target for therapeutic intervention (Deininger et al., 2005). In 2001, the Food and Drug Administration (FDA) proved the ABL kinase inhibitor imatinib for clinical use, which has proven high efficiency in CML treatment since. Imatinib is a 2-phenylaminopyrimidine tyrosine kinase inhibitor with specific activity for ABL, platelet derived growth factor receptor, c-KIT, and ABL-related protein (Druker, 2003). The pharmacological basis of the inhibition has been deduced by crystallographic studies. Imatinib binds to the amino acids of ATP binding site of the BCR-ABL tyrosine kinase leading to stabilization of the inactive and non-ATP-binding form of BCR-ABL, resulting in prevention of tyrosine autophosphorylation and activation of its substrates. This process ultimately results in shutting down the downstream signaling pathways involved in leukemogenesis (Savage and Antman, 2002). It has been documented that imatinib produces a 92%-98% decrease in CML colony growth without significantly inhibiting normal colony growth (Holtz et al., 2002). Clinically, 97% of patients achieve complete hematologic remissions, and 86% of newly diagnosed patients in the chronic phase achieve complete cytogenetic response after imatinib treatment (Goldman and Melo, 2003; Sawyers et al., 2002). However, some patients develop CNS (central nervous system) relapse and resistant mutations in BCR-ABL during ongoing imatinib therapy (Apperley, 2007; Pfeifer et al., 2003). Several reports have been shown that imatinib treatment causes a variety of resistance mutations ((Soverini et al., 2011), Figure 4). Thus, second generation BCR-ABL kinase inhibitors

like nilotinib, dasatinib, bosutinib and bafetinib were developed, which have exhibited efficacy in imatinib-resistant CML (Druker, 2008; Kantarjian et al., 2006; Quintas-Cardama et al., 2007; Talpaz et al., 2006; Weisberg et al., 2007). Nilotinib is designed to enhance selectivity and inhibition potency upon BCR-ABL, and clinical trial have demonstrated improved clinical response compared to imatinib (Kantarjian et al., 2011; Larson et al., 2012; Saglio et al., 2010; Weisberg et al., 2006). Although most of imatinib-resistance mutations can be targeted by second-generation inhibitors, there are still some mutations with unpredictable response to these inhibitors such as E255K, F317L and T351I (Table 1). Drug resistance mutations is an eminent problem for BCR-ABL$^+$ patients treatment, and impose an urgent need to design new strategies to overcome drug resistance. An alternative approach would be to target critical cellular processes that play important role in cell homeostasis like autophagy. Several reports have shown that targeting autophagy enhances TKI toxicity in BCR-ABL$^+$ cells. Thus, autophagy-related proteins could be involved in BCR-ABL-mediated leukemogenesis (Altman et al., 2011; Bellodi et al., 2009; Goussetis et al., 2012; Helgason et al., 2011; Yu et al., 2012). Therefore, it is interesting and important to understand why and how autophagy affects BCR-ABL-mediated leukemogenesis.

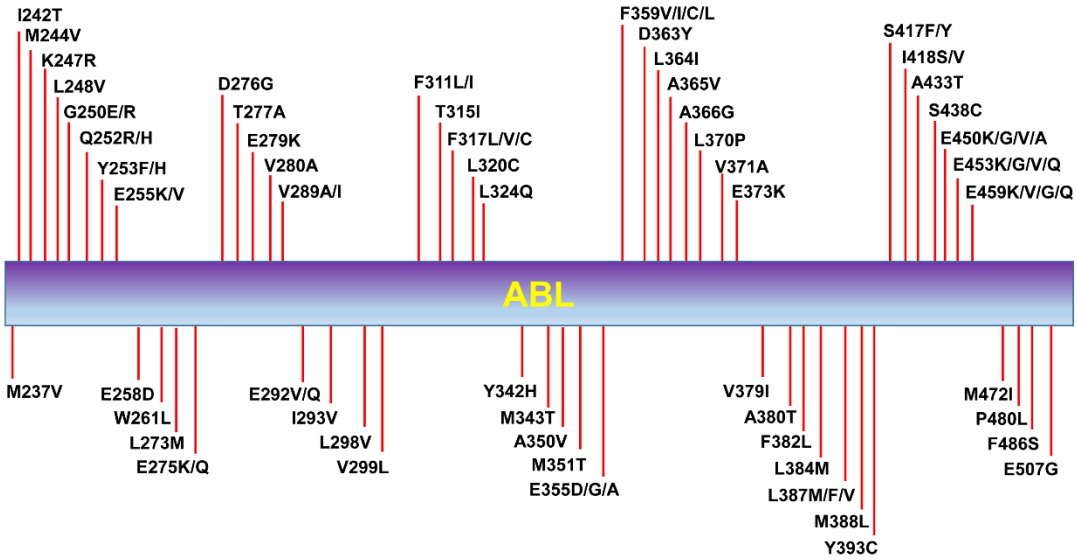

Figure 4. Map of all the amino acid substitutions in the BCR-ABL identified in clinical samples from patients reported to be resistant to imatinib (Soverini et al., 2011).

Table 1. BCR-ABL point mutations that show decreased sensitivity or resistance to nilotinib and dasatinib (Jabbour et al., 2010).

Sensitivity	Nilotinib	Dasatinib
Less sensitive or resistant	E255K/V Y253H F359C/V T315I	Q252H E255K/V V299L F317L T315A T315I

1.7 Autophagy

Three different types of autophagy have been described, including macroautophagy, microautophagy, and chaperone-mediated autophagy, and the term “autophagy” usually indicates macroautophagy unless otherwise specified (Mizushima, 2007). Autophagy means “self-eating”, and is an evolutionarily conserved catabolic mechanism for degradation of cytoplasmic components including organelles and proteins; it plays essential roles in survival, development and homeostasis (Mizushima, 2007; Yang and Klionsky, 2010). Cellular self-eating involves entrapment of cytoplasm and organelles in double-membrane vesicles called autophagosomes, followed by degradation in lysosomes. Subsequently, degraded materials are recycled into the building blocks for the synthesis of new biomolecules. Autophagy consists of a non-selective regulation mechanism that leads to lysosomal degradation of cytoplasmic materials and a selective regulation mechanism that specifically degrades targeted proteins and organelles (Mizushima et al., 2008; Rabinowitz and White, 2010). Defects in basal autophagy result in toxic accumulation of protein garbage and damaged organelles, whereas induced autophagy limits cell survival. In 1999, Beth Levine group first uncovered direct evidence that autophagy contributes to

malignancies: Overexpression of a critical autophagy regulator, Beclin 1, impairs cell proliferation *in vitro* and tumorigenesis *in vivo* (Aita et al., 1999; Liang et al., 1999). These findings turned on a light for research into the function of autophagy and autophagy related proteins in physiology and human diseases like cancer.

1.8 Molecular mechanisms of autophagy

Autophagy responds to environmental stimuli through regulatory factors that are involved in the autophagic machinery. These regulatory factors consist of homologues of products of the autophagy-related genes (*Atg*) originally identified in yeast (Table 2). Additionally, there are several non-ATG proteins involved in autophagy regulation (Table 3). From autophagosome formation to lysosomal degradation, autophagy basically consists of five stages: initiation, nucleation, expansion, maturation and degradation (Figure 5). ULK complex is required for the initiation of autophagy, including UNC51 like ULK1/2, ATG13, FIP200 (FAK family kinase-interacting protein of 200 kDa) and ATG101 (Alers et al., 2012). ULK1/2 is activated by starvation and phosphorylated substrates such as ATG13 and FIP200 (McAlpine et al., 2013), and inactivated by AMPK-mTOR pathway (Wirth et al., 2013). Besides the ULK complex, the Beclin 1 complex also plays an important role in autophagosome initiating, and is composed of VPS34, VPS15, Beclin 1 and ATG14. Once the autophagosome is initiated, the nucleation takes place, during which the ULK complex and Beclin 1 complex phosphorylate substrates (such as ATG13 and RB1CC1) to form the isolation membrane responsible for sequestering of the autophagic substrates. Following nucleation, the ATG16L1 complex, including ATG12, ATG5 and ATG16L1, is recruited to the membrane. Then, the complex functions as an E3-like ligase, thereby regulating the lipidation of LC3 and its family members GATE16 and GABARAP (GABA receptor-associated protein). Accordingly, the complex is associated with the autophagosomal membrane (Mizushima et al., 2011) to function in the closure of the isolation membrane, leading to form an autophagosome that causes sequestration/entrapment of cytoplasmic constituents. Sequentially,

autophagosomes dock and fuse with lysosomes to form autolysosomes. Although the molecular mechanism is still not clear, Beclin 1 and UVRAG are thought to be key regulators that play crucial roles in this process. Alternatively, amphisomes can be formed by fusing between endosomal vesicles (such as endosomes and multivesicular bodies) and autophagosomes. Then, it eventually docks with lysosomes. Finally, the hydrolyzation of sequestered/entrapped cellular contents takes place in the autolysosomes. Lysosomal enzymes degrade the inner membrane of autophagosomes together with the enclosed cargos, and the digested products are released back into the cytosol.

Table 2. The core ATG proteins (Lamb et al., 2013).

Protein	**Position in autophagic pathway**	**Alternative name and function**
ULK1 and ULK2	ULK complex	Atg1 orthologues; Ser/Thr kinases that mediate mTOR signaling and ATG9 cycling
ATG13	ULK complex	ULK1 and ULK2 substrate that also modulates the activity of the ULK complex
FIP200	ULK complex	Atg17 orthologue; ULK1 and ULK2 substrate that also modulates the activity of ULK complex
ATG101	ULK complex	Interacts with ULK1 and ATG13
Beclin 1	PI3K complex	Atg6 orthologue; part of the PI3K complex and also has a role in autophagy during initiation, formation and

		maturation
VPS34	PI3K complex	Catalytically active subunit of the PI3K complex
P150	PI3K complex	VPS15 orthologue; recruits the PI3K complex to membranes
ATG14L	PI3K complex	Atg14 orthologue; directs the PI3K complex to the omegasome; also known as Barkor
WIPI1 and WIPI2	PtdIns(3)P-binding protein	Atg18 orthologues; bind to PtdIns(3)P on the autophagosome
ATG3	LC3-phosphatidylethanolamine conjugation	Similar to the E2 ubiquitin conjugating enzyme; conjugates LC3 to phosphatidylethanolamine
ATG4	LC3-phosphatidylethanolamine conjugation	Cys protease that cleaves carboxy-terminal Gly residues from LC3 homologues and is also required to recycle LC3 from the autophagosome outer membrane
ATG7	LC3-phosphatidylethanolamine and ATG12 conjugation	Similar to E1 ubiquitin activating enzymes; activates ATG12 and LC3 homologues
LC3-A, LC3-B, LC3-C, GATE16,	LC3-phosphatidylethanolamine conjugation	Atg8 homologues; ubiquitin-like proteins that recruit cargo to autophagosomes and amy aid

GABARAPL1, GABARAPL2, and GABARAPL3		in membrane fusion
ATG5	ATG5-ATG12 conjugation	Conjugated to ATG12
ATG10	ATG5-ATG12 conjugation	Similar to E2 ubiquitin conjugating enzyme; links ATG12 to an internal Lys residue in ATG5
ATG12	ATG5-ATG12 conjugation	Ubiquitin-like protein conjugated to ATG5 that functions in the activation of ATG3
ATG16L1	ATG5-ATG12 conjugation	Binds to the ATG5-ATG12 conjugate and directs LC3 conjugation at the isolation membrane
ATG9A and ATG9B	Integral membrane proteins	Atg9 orthologues; required for autophagosome formation
ATG2A and ATG2B	Localize to omegasome	Atg2 orthologures; required for closure of isolation membranes to form autophagosomes

Table 3. Non-ATG proteins with roles in autophagy (Lamb et al., 2013).

Proteins	**Class**	**Alternative**
Vesicle coats and adaptors		
AGFG1	Clathrin adaptor	HRB; promotes membrane delivery to the endocytic route

AP2	Clathrin adaptor	Heterotrameric complex that binds clathrin and plasma membrane proteins, some of which are receptors
Clathrin	Coat	Forms outer protein coat on coated vesicles, three light chains and three heavy chains make up one clathrin unit in the coat
Kinases		
mTOR	Kinase	FRAP; requited for cell growth, regulates stress responses and inhibits autophagy
RAPTOR	Scaffold	Substrate binding subunit of mTORC1
AMPK	Kinase complex	Senses the availability of ATP in the cell and regulates mTOR activity through the TSC1-TSC2 complex and RAPTOR
Lipids, lipid-binding proteins and PI3K complex interactors		
DFCP1	Lipid-binding protein	Localizes to the omegasomes on starvation
GAPR1	Lipid-binding protein	Golgi-localized protein that may promote the localization of Beclin 1 to the Golgi
PtdIns(3)P	Lipid	Enriched on autophagosomal and endocytic membranes and serves to anchor the autophagic machinery to the isolation membrane
PtdIns(4,5)P2	Lipid	Plasma membrane lipid that is involved in singling
PLD1	Phospholipase	Phosphatidylcholine-specific phospholipase
SNX18	Sorting nexin	Promotes autophagosome formation by binding to and tabulating PtdIns(4,5)P2-positive membranes
VMP1	Beclin 1 effector	Interacts with Beclin 1

Tethering complexes		
Exocyst	Tether	Multisubunit complex with two distinct compositions; involved in the delivery of vesicles to plasma membrane
Exo84	Exocyst subunit	ExoC8; interacts with GIP-bound RALA and RALB and forms a complex with ATG proteins that promotes autophagy
SFCs	Exocyst subunit	FxoC2; interacts with RALB and forms complex with ATG proteins to inhibit autophagy
TRAPP complex	Tether	Multisubunit vesicle tethering complex and GEF for RAB1; involved in ER-Golgi and endosomal traffic and also functions in autophagosome formation
SNAREs		
STX7	t-SNARE	Mediates endocytic traffic from early endosomes to late endosomes and lysosomes; also has a role in the formation of ATG16L1-positive autophagosome precursors
STX8	t-SNARE	Mediates fusion of late endosomes; also has a role in the formation of ATG16L1-positive autophagosome precursors
STX17	t-SNARE	Autophagosome-localized SNARE protein with two transmembrane helices
VAMP3	v-SNARE	Cellubrevin; involved in the fusion of ATG9- and ATG16L1-positive membranes
VAMP7	v-SNARE	TIVAMP; required for late endosome and lysosome fusion and function, also involved in ATG16L1 precursor formation
VAMP1B	v-SNARE	Involved in ATG16L1 precursor formation and

		late endosome-lysosome fusion
Small GTPases and regulators		
OAIL1	RAB GAP	IBC1D25; RAB GAP and RAB33B effector
TBC1D7	RAB GAP	Interacts with the TSC1-TSC2 complex and enhances its RHEB GAP activity
TBC1D14	RAB GAP	RAB GAP and RAB11 effector
GRAF1	Small GTPase regulator	ARFGAP26; PtdIns(4,5)P2-binding protein that is involved in clathrin-independent endocytosis and autophagosome formation
ARF6	Small GTPase	GTP-binding protein that is involved in vesicle transport from the plasma membrane
Dyamin	Small GTPase	Member of mechanochemical protein family that tabulates and severs membranes in a GTP-dependent manner
RAB1	Small GTPase	Promotes ER-Golgi traffic and autophagosome formation
RAB11	Small GTPase	Positively regulates recycling endosome function, cytokinesis and autophagosome formation
RAB33B	Small GTPase	Involved in Golgi transport and promotes autophagosome maturation
RAGA, RAGB, RAGC and RAGD	Small GTPase	Required for amino acid dependent recruitment of mTORC1 to the lysosome
RALA and RALB	Small GTPase	Involved in exocytosis and cytokinesis; stabilizes the exocyst complex on membranes
RHEB	Small GTPase	Essential for mTORC1 activity

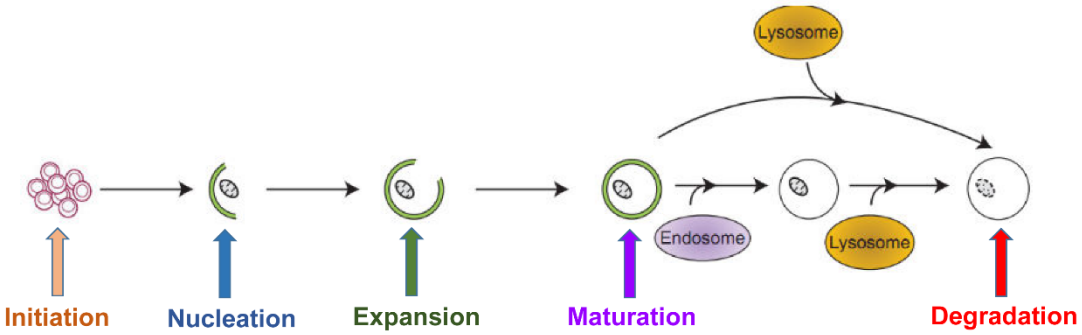

Figure 5. The process of autophagy in mammalian cells.

Initially, an isolation membrane is formed for sequestering the autophagic substrate. After the closure of the isolation membrane, autophagosomes are formed resulting in sequestration/entrapment of cytoplasmic constituents. Endosome and lysosome then fused with the autophagosome. Finally, lysosomal enzymes degrade the inner membrane of autophagosomes and the cargoes, and the digested materials are released back into the cytosol. Reproduced with modification from (Mah and Ryan, 2012; Mizushima, 2007).

1.9 Autophagy and cancer

Autophagy acts as a survival mechanism under stressful conditions to maintain cellular homeostasis by regenerating metabolic precursors and clearing subcellular garbage (Levine and Kroemer, 2008; Mizushima et al., 2008; Ravikumar et al., 2010). The first link between autophagy and cancer was made when it was observed that *Beclin 1* expression is monoallelically deleted in breast, ovarian, and prostate cancers. In addition, genetical re-expression of Beclin 1 in MCF7 cells results in impaired proliferation and tumorigenesis (Aita et al., 1999; Liang et al., 1999). More recently, a series of studies using genetically modified mouse models showed that lack autophagy regulators Beclin 1, Atg4C or BIF1 causes increased tumor formation (Marino et al., 2007; Qu et al., 2003; Takahashi et al., 2007; Yue et al., 2003). Interestingly, another autophagy regulator, Atg5, does not affect tumor formation in KO mouse model (Takamura et al., 2011). Furthermore, several non autophagy-related proteins that are not only well known in cancers but also reported to modulate autophagy like p53 and RAS, which illustrating that autophagy might be

involved in cancerigenesis (Elgendy et al., 2011; Tasdemir et al., 2008). Thus, the effects of autophagy regulators on tumorigenesis may be mediated by autophagy-independent mechanisms or by synergistic effects on autophagy and other cellular mechanisms.

1.10 Beclin 1

The Bcl2 interacting myosin-like coiled-coil protein (Beclin 1) is named after its interaction with the B-cell lymphoma 2 (Bcl2) protein (Liang et al., 1998). Beclin 1, also named Atg6, belongs to autophagy related family of proteins and shares ~30% sequence identity with its yeast ortholog ATG6/VPS30 (Klionsky et al., 2003). Beclin 1 is a 60-kDa protein containing a Bcl_2 homology domain (BH3), a coiled-coil domain (CCD) and an evolutionarily conserved domain (ECD). Beclin 1 is a core molecule of the phosphatidylinositol 3-kinase class III (PtdIns3KC3) complex, also called VPS34 complex. The complex is crucial for all vesicle-mediated sorting pathways related to lysosomes like autophagy. Furthermore, Beclin 1 acts as a platform for Beclin-1–Vps34 complex assembly and stimulates its activity (Yue and Zhong, 2010). Binding of Beclin 1 to PtdIns3KC3 via its ECD domain is required for the generation of PtdIns(3)P by the PtdIns3KC3 complex and the subsequent recruitment of additional Atg proteins that orchestrate autophagosome formation (Kihara et al., 2001). Interestingly, Beclin 1 forms large homo-oligomers via interaction of the CCD and BH3 domains (Adi-Harel et al., 2010; Ku et al., 2008). Beclin 1 multimerization is needed for the recruitment and consequent concerted action of other autophagy-inducing factors (Ku et al., 2008; Pattingre et al., 2005).

Generally, there are two different stable Beclin 1 complexes in both yeast and mammals, and Beclin 1-VPS34-VPS15 constitutes the central platform that binds to different proteins during different stages of autophagic signaling. After autophagosome formation is initiated by binding of ATG14 (forming complex I), ATG14 directs complex I to the phagophore assembly site (PAS) or endoplasmic reticulum from which autophagosomes emerge (Matsunaga et al., 2010). On the other

hand, the platform binds UVRAG to form complex II, which is also involved in autophagosome formation (Itakura et al., 2008). In addition, the Beclin 1 core complex function is negatively regulated by Rubicon, another Beclin 1 binding protein (Funderburk et al., 2010).

1.11 Beclin 1 and cancer

As a central autophagy regulator, Beclin 1 was first shown to be a tumor suppressor in breast cancer (Aita et al., 1999; Liang et al., 1999). Sequentially, it has been found that Beclin $1^{+/-}$ mice were prone to tumor formation, indicating that decreased Beclin 1 contributes to tumorigenesis (Qu et al., 2003). Consistent with these findings, several studies showed that phosphorylation of Beclin 1 by AKT and EGFR causes loss of function, contributing to tumor initiation (Wang et al., 2012; Wei et al., 2013). Interestingly, Beclin 1 knockdown inhibits cell proliferation and enhances TKI toxicity in a leukemic cell model (Yu et al., 2012), indicating that Beclin 1 might be a crucial regulator in blood cancer besides solid tumor. Recently, one surprising study showed that inactive EGFR is an inducer of autophagy via association with the autophagy inhibitor Rubicon, which in turn disassociates Beclin 1 from Rubicon, indicating that shutting down EGFR signaling in many types of cancers still cannot kill the cancers due to the survival mechanism of autophagy that is induced by inactive EGFR (Tan et al., 2015). Taken together, targeting Beclin 1-mediated autophagy might be an attractive approach for cancer therapy, especially drug resistant entities.

1.12 Aims of the study

Several BCR-ABL tyrosine kinase inhibitors were developed for clinical use providing significant success in the therapy of BCR-ABL^{+} leukemia. However, the development of secondary drug resistance in some patients has become an urgent problem, prompting a need for alternative treatment strategies. Recently, several groups reported that the autophagy pathway might be a promising and novel target for intervention in BCR-ABL-mediated leukemia (Altman et al., 2011; Bellodi et al., 2009;

Helgason et al., 2011; Yu et al., 2012). Because of potential role of autophagy in metabolism and cell survival, altering autophagy may provide a critical means to kill cancer cells. Beclin 1, a central regulator of autophagic pathway, is essential for autophagosome formation. Autophagy has been considered as a mechanism that plays a role in cancer; however, the exact role played by the process at various stages of cancer progression are not yet clear and in some cases are contradictory. This study aims to identify the role of Beclin 1-mediated autophagy in BCR-ABL$^+$ leukemia， which might aid in the development of novel strategies to overcome BCR-ABL$^+$ leukemia.

2. Materials and methods

2.1 Materials

2.1.1 Reagents

2-Mercaptoethanol	Sigma-Aldrich, Taufkirchen
Acrylamid/Bisacrylamid Gel 30	Carl Roth, Karlsruhe
Agarose	Carl Roth, Karlsruhe
Aluminumhydroxide Gel	Sigma-Aldrich, Taufkirchen
Ammoniumchloride	Fluka, Taufkirchen
Ammonium bicarbonate	Sigma-Aldrich, Taufkirchen
APS	Sigma-Aldrich, Taufkirchen
Ampicillin	Sigma-Aldrich, Taufkirchen
Aqua ad injectabilia, steril	Braun, Melsungen
Bacto™ Agar	BD Biosciences, Heidelberg
Bacto™ Trypton	BD Biosciences, Heidelberg
Bromine phenole blue	Sigma-Aldrich, Taufkirchen
BSA, Fraktion V	Carl Roth, Karlsruhe
Calcimchloride	Merck, Darmstadt
Calciumhydrogencarbonat	Sigma-Aldrich, Taufkirchen
Chloroform	Sigma-Aldrich, Taufkirchen
Complete™ Protease Inhibitor Cocktail	Roche Diagnostics, Penzberg
Coomassie Brilliant Blue	Sigma-Aldrich, Taufkirchen
DEPC	Fluka, Taufkirchen
DMSO	Sigma-Aldrich, Taufkirchen
Disodium phosphate	Merck, Darmstadt
DTT	Promega, Heidelberg
dNTP-Mix, 10 mM	Thermo Fisher Scientific, Darmstadt
Glacial acetic acid	Carl Roth, Karlsruhe
Ethanol	Sigma-Aldrich, Taufkirchen
Ethidiumbromid	Carl Roth, Karlsruhe

EDTA	Fluka, Taufkirchen
Formaldehyde	Sigma-Aldrich, Taufkirchen
Glucose	Carl Roth, Karlsruhe
Glutaraldehyde 2,5%	Fluka, Taufkirchen
Glycerol	Fluka, Taufkirchen
Glycerol-2-phosphate	Sigma-Aldrich, Taufkirchen
Glycine	Merck, Darmstadt
HEPES	Fluka, Taufkirchen
Isopropanol	Merck, Darmstadt
IPTG	Sigma-Aldrich, Taufkirchen
Calciumchloride	Merck, Darmstadt
Calcium bicarbonate	Sigma-Aldrich, Taufkirchen
LPS	Sigma-Aldrich, Taufkirchen
Magnesiumchloride	Carl Roth, Karlsruhe
Methanol	Merck, Darmstadt
N,N-Dimethylformamid	Merck, Darmstadt
Sodium Acetate	Fluka, Taufkirchen
Sodium Azid	Sigma-Aldrich, Taufkirchen
Sodium Chloride	Carl Roth, Karlsruhe
Sodium Citrate	Fluka,Taufkirchen
Sodium Dihydrogen Phosphat	Merck, Darmstadt
SDS	Carl Roth, Karlsruhe
Sodium Fluorid	Fluka, Taufkirchen
Sodium Hydroxide	Merck, Darmstadt
Sodium Ortho Vanadate	Sigma-Aldrich, Taufkirchen
Sodium pyrophosphate	Fluka, Taufkirchen
Permeabilization Buffer (10x)	eBioscience, San Diego, USA
PMSF	Roche, Mannheim
Polybrene	Sigma-Aldrich, Taufkirchen

Propidiumiodid	Sigma-Aldrich, Taufkirchen
TEMED	Fluka, Taufkirchen
TRIS	Carl Roth, Karlsruhe
Triton X-100	Sigma-Aldrich, Taufkirchen
Milk powder	Fluka, Taufkirchen
Tween 20	Fluka, Taufkirchen
Xylolcyanol	Sigma-Aldrich, Taufkirchen

2.1.2 Medium and supplements for cell culture

β-Mercaptoethanol, 50mM	Gibco/Invitrogen, Darmstadt
DMEM, cell culture medium	Thermo Fisher Scientific, Darmstadt
FCS Gold	PAA, Pasching, Austria
G418 (Neomycin)	Calbiochem, Darmstadt
Puromycin	Calbiochem, Darmstadt
Penicillin and streptomycin 100x	Thermo Fisher Scientific, Darmstadt
PBS	Thermo Fisher Scientific, Darmstadt
HANK`s BSS	PAA, Pasching, Austria
EBSS	Thermo Fisher Scientific, Darmstadt
L-Glutamin, 100x	Thermo Fisher Scientific, Darmstadt
IL-3	PeproTech, Hamburg
IL-6	PeproTech, Hamburg
SCF	PeproTech, Hamburg
Hygromycin	Calbiochem, Darmstadt
DT	Calbiochem, Darmstadt
Lipofectamin™ 2000	Thermo Fisher Scientific, Darmstadt
MethoCult®	StemCell Technologies, Canada
Opti-Mem®	Thermo Fisher Scientific, Darmstadt
PBS, sterile	Thermo Fisher Scientific, Darmstadt
RPMI 1640 (high Glucose, Glutamine)	Thermo Fisher Scientific, Darmstadt

Trypsin-EDTA- DMEM	Thermo Fisher Scientific, Darmstadt
EBSS	Thermo Fisher Scientific, Darmstadt

2.1.3 Enzymes

2.1.3.1 Restriction enzymes

EcoR1 (100 U/μl)	Thermo Fisher Scientific, Darmstadt
Mfe1(10 U/μl)	Thermo Fisher Scientific, Darmstadt
Sal1(15 U/μl)	Thermo Fisher Scientific, Darmstadt
HindIII (10 U/μl)	Thermo Fisher Scientific, Darmstadt
SalI (10 U/μl)	Thermo Fisher Scientific, Darmstadt
Dpn I(10U/μl)	Thermo Fisher Scientific, Darmstadt
BamHI (100 U/μl)	Thermo Fisher Scientific, Darmstadt
Xho1 (100 U/μl)	Thermo Fisher Scientific, Darmstadt

2.1.3.2 DNA polymerases

Pfu-DNA polymerase	Thermo Fisher Scientific, Darmstadt
Taq-DNA polymerase	Thermo Fisher Scientific, Darmstadt

2.1.3.3 Other enzymes

T4-DNA Ligase	Roche, Mannheim
Phosphatase	Roche, Mannheim

2.1.4 Radioactive substances

^{33}P-γ-ATP	PerkinElmer, Baesweiler

2.1.5 Materials and kits for western blotting

PVDF membrane (Immobilon P)	Millipore, Schwalbach
CL-XPosure™ Film	Thermo Fisher Scientific
Amersham Hyperfilm MP	GE Healthcare Life Sciences
SuperSignal West Pico Substrate	Thermo Fisher Scientific

SuperSignal West Dura Substrate	Thermo Fisher Scientific
SuperSignal West Femto Substrate	Thermo Fisher Scientific

2.1.6 Antibodies and Beads

Antibodies for FACS analysis

Mouse BD Fc Block™ (Product No. 553141)	BD Bioscience
Anti-Mouse CD45 APC (Product No. 17-0451-83)	eBioscience
Anti-Mouse CD45 PE-Cy7 (Product No. 25-0451-82)	eBioscience
Anti-Mouse CD11b PE (Product No. 61-0112-82)	eBioscience
Anti-Mouse Gr-1 PE-Cy7 (Product No. 25-5931-82)	eBioscience
Anti-Human/Mouse B220 PB (Product No. 48-0452-82)	eBioscience
Anti-Mouse Thy-1.2 APC (Product No. 46-0903-82)	eBioscience

Antibodies for western blotting

Mouse anti ABL (Product No. 554148)	BD Bioscience
Mouse anti Actin (Product No. A5316)	Sigma Aldrich
Rabbit anti ALK (Product No. 3363)	Cell Signaling Technology
Rabbit anti ATG14 (Product No. PD026)	MBL International
Rabbit anti BCR (Product No. 3902)	Cell Signaling Technology
Mouse anti Beclin 1 (Product No. NBP1-00085)	Novus Biologicals
Rabbit anti Beclin 1 (Product No. 3495)	Cell Signaling Technology
Mouse anti FLAG (Product No. F1804)	Sigma Aldrich
Rabbit anti FLAG (Product No. PA1-984B)	Thermo Fisher Scientific
Rabbit anti FLT3 (Product No. 06-647)	Merck Millipore
Rabbit anti pFLT3 (Product No. 3464)	Cell Signaling Technology
Mouse anti phosphotyrosine 4G10 (Product No. 05-321)	Merck Millipore
Mouse anti phosphotyrosine PY20 (Product No. 610000)	BD Bioscience
Rabbit anti LC3 (Product No. 3868)	Cell Signaling Technology
Rabbit anti PDGFRA (Product No.07-276)	Merck Millipore

Rabbit anti UVRAG (Product No. 5320)	Cell Signaling Technology
Rabbit anti VPS15 (Product No. PA5-21848)	Thermo Fisher Scientific
Rabbit anti VPS34 (Product No. 4263)	Cell Signaling Technology
Anti-Mouse IgG, HRP-linked (Product No. 7076)	Cell Signaling Technology
Anti-Rabbit IgG, HRP-linked (Product No. 7074)	Cell Signaling Technology

Antibodies for immunoprecipitation

Mouse anti ABL (Product No. 554148)	BD Bioscience
Rabbit anti FLAG (Product No. PA1-984B)	Thermo Fisher Scientific
Mouse anti Beclin 1 (Product No. sc-48341)	Santa Cruz Biotechnology
Rabbit anti Beclin 1 (Product No. sc-11427)	Santa Cruz Biotechnology

Antibodies for immunofluorescence

Rabbit anti BCR (Product No. 3902)	Cell Signaling Technology
Mouse anti Beclin 1 (Product No. NBP1-00085)	Novus Biologicals
Rabbit anti LC3 (Product No. 3868)	Cell Signaling Technology

Anti-Mouse IgG Alexa Fluor® 488 (Product No.A-11001) Thermo Fisher Scientific

Anti-Mouse IgG Alexa Fluor® 610 (Product No.A-20980) Thermo Fisher Scientific

Anti-Rabbit IgG Alexa Fluor® 488 (Product No.A-11034) Thermo Fisher Scientific

Anti-Rabbit IgG Alexa Fluor® 610 (Product No.A-20981) Thermo Fisher Scientific

Beads

Anti-FLAG® M2 Affinity Gel (Product No. A2220)	Sigma Aldrich
Glutathione Sepharose (Product No. 17-5279-01)	GE Healthcare
Protein A Sepharose (Product No. 17-0780-01)	GE Healthcare
Protein G Sepharose (Product No.17-0618-01)	GE Healthcare

2.1.7 Cell lines

HEK293T	Human embryonic kidney cell line, DSMZ

NIH3T3	Murine embryonic fibroblast cell line, DSMZ
ΦNX-Eco	Retroviral ecotropic virus packaging cell line
Ba/F3	Murine pro B cell line, DSMZ
K562	Human myelogenous leukemia cell line, DSMZ
Beclin 1 KO MEFs	Beclin 1 knockout MEFs, gift from Dr. Yue

2.1.8 Vectors and DNA constructs

MSCV-IRES-GFP	Gift from Dr. Pear
MSCV-IRES-Berry	Gift from Dr. Brummer
pLMP	Open biosystems, Darmstadt
PGEX-4T-2	Amersham Biosciences, Freiburg
pCNDA4- *Flag-Beclin 1*	Addgene, USA
pBABE-puro-*mCherry-EGFP-LC3B*	Addgene, USA

2.1.9 Bacterial Strains

E.Coli DH5α™	Thermo Fisher Scientific, Darmstadt
OneShot®BL21 Star™ *E.coli*	Thermo Fisher Scientific, Darmstadt

2.1.10 Oligonucleotides for PCR (5′→3′)

pMIBerry *Beclin 1* cloning

BamH1-Forward: CGGGATCCATGGAAGGGTCTAAGACGTCCAAC

BamH1-Reverse: CGGGATCCTCACTTGTCATCGTCATCCTTGTAGTC

pGEX-*Beclin 1* cloning

BamH1-Forward: CGGGATCCGAAGGGTCTAAGACG

EcoR1-Reverse: CGGAATTCTCATTTGTTATAAAATTG

pGEX-*Beclin 1 aa1-115* cloning

BamH1-Forward: CGGGATCCGAAGGGTCTAAGACG

EcoR1-Reverse: CGGAATTCTCATCTTCGGCTGAGCTTCTC

pGEX-*Beclin 1 aa1-135* cloning

BamH1-Forward: CGGGATCCGAAGGGTCTAAGACG

EcoR1-Reverse: CGGAATTCTCATGGGTGATCCACATCTGT

pGEX-*Beclin 1 aa141-277* cloning

BamH1-Forward: CGGGATCCGATACTCTTTTAGACCAG

EcoR1-Reverse: CGGAATTCTCACCAGATGTGGAAGGTTGC

pGEX-*Beclin 1 aa278-337* cloning

BamH1-Forward: CGGGATCCCACAGTGGACAGTTTGG

EcoR1-Reverse: CGGAATTCTCATGAATGGTTTCCGTAAGG

pGEX-B*eclin 1 aa338-450* cloning

BamH1-Forward: CGGGATCCTATCTGGAGTCTCTGACAG

EcoR1-Reverse: CGGAATTCTCATTTGTTATAAAATTG

miR30-based shRNA cloning into pLMP vector

Sal1-Forward: CAGAAGGGTCGACAAGGTATATTGCTGTTGACAGTGAGCG

MfeI-Reverse: CTAAAGTAGCCCCTTCAATTGCGAGGCAGTAGGCA

miR30-based shRNA cloning into MIG-*BCR-ABL*

BamH1-Forward: TGAGGATCCTAGGGATAACAGGGTAATTG

BamH1-Reverse: ATGGGATCCAAAAAAGTGATTTAATTTATACC

Atg5 wild type allele detection

Forward: GAATATGAAGGCACACCCCTGAAATG

Reverse: GTACTGCATAATGGTTTAACTCTTGC

Atg5 deleted allele detection

Forward: CAGGGAATGGTGTCTCCCAC

Reverse: GTACTGCATAATGGTTTAACTCTTGC

2.1.11 Oligonucleotides for site directed mutagenesis (5′→3′)

Beclin 1 tyrosine sites mutagenesis

Y162F

Forward: GTGTCAGAACTTCAAACGCTGTTTG

Reverse: CAAACAGCGTTTGAAGTTCTGACAC

Y229F

Forward: GAGGAAGCTCAGTTTCAGAGAGAA

Reverse: TTCTCTCTGAAACTGAGCTTCCTC

Y233F

Forward: CAGAGAGAATTCAGTGAATTTAAAC

Reverse: GTTTAAATTCACTGAATTCTCTCTG

Y233E

Forward: CAGAGAGAAGAGAGTGAATTTAAAC

Reverse: GTTTAAATTCACTCTCTTCTCTCTG

Y256F

Forward: CCAGATGCGTTTTGCCCAGACGCAG

Reverse: CTGCGTCTGGGCAAAACGCATCTGG

Y328F

Forward: GAAATTTCAGAGATTCCGACTTGTTCC

Reverse: GGAACAAGTCGGAATCTCTGAAATTTC

Y352F

Forward: GAGCTGCCGTTATTCTGTTCTGGG

Reverse: CCCAGAACAGAATAACGGCAGCTC

Y352E

Forward: GAGCTGCCGTTAGAGTGTTCTGGG

Reverse: CCCAGAACACTCTAACGGCAGCTC

Y394F

Forward: GTTTTTGTCTTCCCTTCAGGATGCATG

Reverse: CATCCATCCTGAAGGGAAGACAAAAAC

Y413F

Forward: GTGGCGGCTCCTTTTCCATCAAAACC

Reverse: GGTTTTGATGGAAAAGGAGCCGCCAC

2.1.12 Oligonucleotides for sequencing (5′→3′)

Beclin 1 seq-303: GGAGGCATCTGATG

Beclin 1 seq-933: GTTGCTGCTCCATG

pGEX-5': GGGCTGGCAAGCCACGTTTGGTG

IRES-Reverse: CCTCACATTGCCAAAAGACG

MSCV-5': CCCTTGAACCTCCTCGTTCGACC

2.1.13 Molecular markers for nucleic acids and proteins

GeneRuler™ 1kb DNA Ladder	ThermoFisher scientific, Darmstadt
GeneRuler™ High Range RNA Ladder	ThermoFisher scientific, Darmstadt
PageRuler™ Prestained Protein Ladder	ThermoFisher scientific, Darmstadt

2.1.14 Mouse Strains

Wild-type Balb/c and C57BL/6 mice were purchased from Charles River

Atg5 knockout mice (kindly provided by Dr. Huber)

2.1.15 Materials and kits for molecular biology

Bio-Rad Protein Assay Kit	Bio-Rad, München
DNeasy® Blood & Tissue Kit	Qiagen, Hilden
Microvette® 300Z	Sarstedt, Nümbrecht
Plasmid Maxiprep Kit	MACHEREY-NAGEL, Düren
GeneJET Plasmid Miniprep Kit	ThermoFisher scientific, Darmstadt
GeneJET Gel Extraction Kit	ThermoFisher scientific, Darmstadt
GeneJET PCR Purification Kit	ThermoFisher scientific, Darmstadt
Rapid DNA Ligation Kit	Roche, Manheim
SuperSignal® Substrate	ThermoFisher scientific, Darmstadt

2.1.16 Instruments

Agarose gel Electrophoresis chamber	Biometra, Göttingen
CO2-Incubator SW J 500 TV BB	ThermoFisher scientific, Darmstadt
Digital watch LC 1200 S	Satorius, Göttingen
ChemoCam Imager	INTAS Science Imaging Instruments
ELISA Reader Sunrise	Tecan, Crailsheim
Optimax	Protec, Oberstenfeld
Flow cytometer (EPICS®XL)	Beckman-Coulter, Krefeld
Fluorescence microscope	Olympus Optical Co., Hamburg
Thermo heater 5436	Eppendorf, Hamburg
Incubator-Shaker Innova 4000	New Brunswick Scientific, USA
Cold centrifuge J2-HS, Rotor JA-14	Beckman, Fullerton, USA
Cold centrifuge 5417R, 5810R	Eppendorf, Hamburg
Light microscope Axiovert 25	Zeiss, Jena
LKB Ultraspec III, Spectrophotometer	Pharmacia,Uppsala, Schweden

Magnetic stirrer IKAMG RH	Janke & Kunkel, Staufen
Micro96 Harvester	Skatron Instruments, Norwegen
Mikroscope V 200	Hund, Wetzlar
Multi-Gel long electrophoresis chamber	Biometra, Göttingen
PCR-Thermocycler Primus 96	Peqlab, Erlangen
pH-Meter Φ32	Beckman, Fullerton, USA
Refrigerated Incubator-Shaker Innova	New Brunswick Scientific, USA
Sterile workbench, HeraSafe	ThermoFisher scientific, Darmstadt
Scintillation counter (LS65000)	Beckman, Fullerton, USA
Stromgenerator, Powerpack P25	Biometra, Göttingen
Tabletop centrifuge 5417R	Eppendorf, Hamburg
Transfer Electrophoresis Unit	Hoefer, San Fransisco, USA
Trio-Thermoblock	Biometra, Göttingen
Ultra-Turrax T8	IKA®-Werke, Staufen
Ultracentrifuge, Rotor VTI 80	Beckman, Fullerton, USA
UV crosslinker 2400	Stratagene, La Jolla, USA
UV-Lamp TI 2	Biometra, Göttingen
Vortex Genie2	Scientific Industries, USA
Water bath 1083	GFL, Burgwedel

2.1.17 Buffers

Coomassie-staining solution	0.25% Coomassie-blue 45% Methanol 10% glacial acetic acid
Coomassie-destaining solution	45% Methanol 10% glacial acetic acid
DEPC-H_2O	0.1% DEPC in A.d.

	O.N after autoclave
DNA-loading buffer (6x)	30% Glycerol (v/v) 0.25% Bromphenolblue (w/v)
FACS-buffer	0.1% BSA in PBS
Luria-Bertani (LB) Medium	1% Bacto-Tryptone 0.5% Bacto-beef extract 1% NaCl in A.d. With 1 M NaOH (pH 7)
Protein lysis-buffer	10 mM Tris/HCl (pH 7.5) 130 mM NaCl 5 mM EDTA 0.5% Triton X-100 20 mM Na_2HPO_4/NaH_2PO_4 (pH 7.5) 10 mM Sodiumpyrophosphate (pH 7) 1mM Sodiumorthovanadate 20 mM NaF 1mM Glycerol-2-Phosphate 1 Protease-inhibitor cocktail tablet Add 10 ml A.d.
CHAPS lysis buffer	40 mM HEPES (pH 7.4) 120 mM NaCl 1 mM EDTA 10 mM pyrophosphate 50 mM NaF

	0.3% CHAPS
	1 mM sodium orthovanadate
	1 mM glycerolphosphate
	1 Protease-inhibitor cocktail tablet
	Add for 10 ml A.d.
NETN-buffer	0.5% (v/v) NP_{40}
	20 mM Tris/HCl (pH 8)
	100 mM NaCl
	1 mM EDTA
	1 mM PMSF
	1 mM Benzamidin
	1 Protease-inhibitor cocktail tablet
	Add 10 ml A.d.
RBC lysis buffer	150 mM NH_4Cl
	1 mM $KHCO_3$
	0.1 mM Na_2EDTA, pH 7.3 in A.d.
RNA-loading buffer (5x)	0.2% Bromphenolblau
	4 mM EDTA
	7.2% Formaldehyd (37%)
	20% Glycerol
	3% Formamid
	40% RNA-Laufpuffer in DEPC-H_2O
SDS-electrophoresis buffer	25 mM Tris
	192 mM Glycine
	0.1% SDS in A.d.

SDS-loading buffer (2x)	1 M Tris/HCl (pH 6.8) 200 mM DTT 4% SDS 0.2% Bromphenolblue 20% Glycin in A.d.
SSC-buffer (20x) (Standard saline citrate)	0.3 M Sodium citrate HCl (pH 7) 3 M NaCl
TAE-buffer (10x)	0.4 M Tris 1.1% Acetic acid 2% 0.5M EDTA (pH 8) in A.d.
TNE-buffer	10 mM Tris (pH 8) 100 mM NaCl 1 mM EDTA
Transfer buffer	25 mM Tris 192 mM Glycin 0.1% SDS in A.d. 20% Methanol in A.d.
Stacking gel buffer SDS-PAGE (4x)	1.5 M Tris (pH 8.8) 0.4% SDS in A.d.
Separating gel buffer for SDS-PAGE (4x)	0.5 M Tris (pH 6.8) 0.4% SDS in A.d.

Standard kinase assay buffer (2.5x)	125 mM HEPES-NaOH (pH7.5) 7.5 mM $MgCl_2$ 7.5 mM $MnCl_2$ 7.5 uM Na-orthovanadate 2.5 mM DTT
Kinase-dilution-buffer (10x)	500 mM HEPES-NaOH (pH7.5) 2.5 mg/ml PFG20000 10 mM DTT

2.2 Methods

2.2.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is a technology used to amplify a single copy or a few copies of DNA (Mullis et al., 1986). It is based on the ability of DNA polymerases to replicate an ssDNA template with the help of a small DNA fragment, the so-called primer. The polymerases used for PCR originate from thermophilic bacteria like Thermus aquaticus (Taq), which means they are stable at temperatures as high as 98 °C.

In the initial PCR step, the template dsDNA is denatured at 98 °C, followed by an annealing step in which the primers are allowed to align on the ssDNA at a lower temperature. The polymerase extends the 3'-ends of these primers by linking single nucleotides to a new DNA strand complementary to the template strand.

These three basic steps are repeated in a PCR cycler device up to 35 times, exponentially increasing the number of DNA molecules in the solution, as the number of template molecules doubles with each cycle. Preparation for a PCR reaction is listed following (Table 4).

Table 4. PCR reaction preparation

Content	Volume

10x PCR Pfu Buffer	5 µl
dNTPs mix (1000 µM)	1 µl
Pfu polymerase	1 µl
Forward primer (10 pM)	2 µl
Reverse primer (10 pM)	2 µl
DNA Template	1µl
Nuclease free dsH_2O	38 µl
Total	50 µl

2.2.2 Enzyme digestion

Preparation of DNA for traditional cloning methods is dependent upon restriction enzyme digestion to generate compatible ends of the DNA fragments for subsequent ligation. Restriction endonucleases are enzymes that digest DNA double strands, resulting in sticky ends or blunt ends. They recognize a 6-8 basepair palindromic DNA sequence. In this study, FastDigest XhoI, ECORI, BamHI, MfeI, SalI, HpaI, and AgeI (Thermo Scientific) were used according to the manufacturer's protocol (Table 5). For vector digestion, 1 µg of vector DNA was mixed with 10x FastDigest buffer, enzyme and water and incubated for 20 min at 37 °C.

Table 5. Fast digestion preparation

Content	Volume
cDNA	x µl
Enzyme 1	1 µl
Enzyme 2	1 µl
10x FastDigest buffer	3 µl
Nuclease free dsH_2O	26-x µl
Total	30 µl

2.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis is used for the separation, identification, purification and isolation of mixed population of DNA or proteins in an agarose matrix. Agarose is heated in TAE buffer and forms a polysaccharide matrix at room temperature. This matrix is used to separate different sized DNA molecules, as smaller molecules will migrate faster through the matrix than larger molecules. DNA and molecular weight markers are loaded onto the gel in combination with DNA sample buffer. The anionic DNA molecules are forced to migrate towards the anode with the application of electric current. The resulting DNA bands are visualized using DNA intercalating agents, such as ethidium bromide or GelRed™ (Biotium), which can be visualized using 320 nm UV light.

2.2.4 Gel extraction

After a PCR reaction or restriction enzyme digestion, the DNA fragment of interest is separated by agarose gel electrophoresis. Then, the desired DNA fragments can be extracted from the gel using a sterile scalpel with the help of an UV lamp. To prevent DNA damage, the UV lamp exposure should be kept to a minimum. The DNA is subsequently purified from the gel slices using the GeneJET Gel Extraction Kit (Thermo Scientific), according to the manufacturer's instructions.

2.2.5 Dephosphorylation of vector DNA

DNA ligases join linear DNA fragments together via covalent bonds. DNA ligation creates a phosphodiester bond between the 3' hydroxyl group of one nucleotide and the 5' phosphate of another. For efficient ligation, DNA strands must be prevented from self-ligating (self-circularization and concatenation) by dephosphorylation of DNA ends. Alkaline phosphatase acts as a catalyst to remove the 5'-phosphoryl termini required by ligases, preventing self-ligation of DNA.

After gel extraction and purification of a digested DNA fragment, 1 μl rAPid Alkaline Phosphatase (Roche), 10x buffer and water are added to the DNA and incubated at

37 °C for 30 min (Table 6). Afterwards, the reaction mix is incubated for 2 min at 75°C to inactivate the enzyme.

Table 6. Dephosphorylation preparation

Content	Volume
Vector DNA	x µl, up to 1 µg
rAPid Alkaline Phosphatase Buffer	2 µl
rAPid Alkaline Phosphatase	1 µl
Nuclease free dsH_2O	17-x µl
Total	20 µl

2.2.6 DNA ligation

The generation of recombinant DNA plasmids involves the linkage of linear vector DNA with the insert fragment to create a circular molecule (Sambrook et al., 2001). The Rapid DNA Ligation Kit (Roche) is used to covalently link the 5'-phosphate residue of one DNA molecule and the 3'-hydroxyl group of another molecule. Vector DNA and fragment DNA are mixed in a 1:3 – 1:7 ratio and the ligation reaction is performed at room temperature for 30 min (Table 7). Ligated circular DNA is then used to transform competent bacteria. As a ligation control, the linear vector is treated with ligase without the presence of the insert.

Table 7. DNA ligation preparation

Content	Volume
Vector DNA	1 µl
Insert DNA	8 µl
T4 DNA Ligation Buffer	10 µl
T4 DNA Ligase	1 µl
Total	20 µl

2.2.7 Bacterial transformation

The ligated vector DNA is introduced into bacteria using a quick heat shock. This transformed bacteria will then amplify the vector. 50 µl chemically competent E.coli DH5α™ (Thermo Scientific) are thawed on ice and incubated together with 5 µl ligation mix or 0.5 µg DNA for 30 min at 4 °C. The plasmids attach to the bacterial membrane and are taken up during a heat shock at 37 °C for 45 s. After 2 min of chilling on ice, the bacteria are plated on LB agar plates containing ampicillin and incubated overnight at 37 °C. Bacteria, which have taken up a re-circularized vector containing the *β-lactamase* gene, are resistant to ampicillin. These bacteria form visible colonies and are then picked to inoculate 5 ml (Mini-prep) or 200 ml (Maxi-prep) LB medium.

2.2.8 Plasmid isolation

The purification of plasmid DNA from bacteria is performed in small scales for analytic purposes (Mini-prep) or in larger amounts (Maxi-prep) to generate material for cell transfections. The GeneJET Plasmid Miniprep Kit (Thermo Scientific) or NucleoBond Xtra Maxi Kit (Macherey-Nagel) is used according to the manufacturer's instructions. Initially, the bacteria are pelleted and lysed under alkaline conditions. Bacterial proteins, lipids and chromosomal DNA in the lysate are precipitated and removed by a filter (Maxi-prep) or centrifugation (Mini-prep). The smaller plasmid DNA remains in solution and is bound to a silica-membrane. After a series of washing steps the plasmid DNA is eluted with water. Up to 1-2 mg of DNA can be obtained from 200 ml bacterial suspension using the Maxi-prep kit.

2.2.9. Cell Lines and Cell Culture

Murine Ba/F3 cell line was grown in RPMI1640 medium containing 10% fetal calf serum, 200 U/ml penicillin and 200 µg/ml streptomycin (Gibco), in the presence of murine interleukin-3 (mIL-3). K562 cell line was cultured in RPMI1640 medium containing 10% fetal calf serum, 200 U/ml penicillin and 200 µg/ml streptomycin. The HEK293T cell line, the Beclin 1 knockout MEFs and the NIH3T3 cell line were

maintained in Dulbecco modified eagle medium (Gibco) containing 10% fetal calf serum, 200 U/ml penicillin and 200 µg/ml streptomycin.

2.2.10 Lipofectamin transfection and retrovirus collection

A number of different methods are used to introduce foreign DNA into eukaryotic cells, known as cell transfection. Examples include electroporation, calciumphosphate transfection, lipofection and viral transduction.

In the present study, murine cells were stably transfected using retroviral supernatants. The virus production was performed using the Phoenix E packaging cell line, which originated from HEK-293T cells by stably incorporating plasmids encoding for the viral structure genes (Pear et al., 1993). The retroviral packaging signal is not present on these plasmids, in order to prevent viral self-replication. Instead, it has to be transfected using another DNA vector to initiate the virus assembly. Here, the MSCV-IRES-EGFP (MIG) or MSCV-IRES-Berry (MIB) vector was used, which contain the retroviral packaging site, transcription and processing elements, and the gene of interest. The plasmid was introduced into the PhoenixE cells using Lipofectamine2000 (Invitrogen). This reagent forms cationic lipids, which bind the anionic DNA. The lipids are either actively taken up by the cells or merge with the cell membrane.

2×10^6 Phoenix E cells stably expressing the retroviral genes were transfected with 10 µg vector DNA in a 60 mm dish. In two separate vials 500 µl Opti-MEM were mixed with 20 µl Lipofectamine2000 and the DNA, respectively. After 5 min at room temperature, both mixtures were put together and incubated for another 20 min at room temperature. The lipo-DNA complex was then added to the cells and replaced with fresh medium after 24 h. The virus supernatant was collected every 12 hours after that.

2.2.11 cDNA constructs and generation of stable cell lines

Initially, 97 bps oligonucleotides (miR30-based shRNA) were synthesized by MWG-Biotech. SacI1 and Mfe1 restriction sites were added to the oligonucleotide by PCR amplification, and the purified PCR product was digested with both enzymes. The resulting DNA fragment was ligated into the pLMP vector, which was linearized using Xho1 and EcoR1 restriction previously. As a second step, the miR sequence was again amplified by a PCR reaction using the pLMP-miR plasmid as a template in order to add BamH1 restriction sites on both 5' and 3' ends. Finally, the BamH1 digested-PCR product and the MIG-*BCR-ABL* plasmid were ligated. All the constructs were verified by DNA sequencing. The following PCR program was used for both PCR reactions.

Initial denaturation	95 °C 3 min	
Denaturation	95 °C 30 s	30x
Annealing	54 °C 30 s	
Extension	72 °C 30 s	
Final extension	72 °C 10 min	
Hold	4 °C until analysis	

The Beclin 1 tyrosine mutations were generated by PCR-based site-directed mutagenesis (Quickchange, Stratagene, Heidelberg, Germany). Primers are described in the section 2.1.11. All the constructs were verified by DNA sequencing. The following PCR program was used

Initial denaturation	95 °C 3 min	
Denaturation	95 °C 30 s	18x
Annealing	60 °C 1 min	
Extension	72 °C 13 min	
Final extension	72 °C 10 min	
Hold	4 °C until analysis	

Flag-Beclin 1 was cloned into MIB by using BamH1 enzyme. The construct was verified by DNA sequencing. The following PCR program was used:

Initial denaturation	95 °C 3 min	
Denaturation	95 °C 30 s	30x
Annealing	55 °C 45 s	
Extension	72 °C 90 s	
Final extension	72 °C 10 min	
Hold	4 °C until analysis	

Beclin 1 different domain was generated by PCR and subcloned into the pGEX4T.2 vector. This vector contains Glutathione S-transferase (GST) upstream, with which the EcoRI and BamH1 enzymes were used for subcloning to keep inserts in the frame. All the constructs were verified by DNA sequencing. The following PCR program was used:

Initial denaturation	95 °C 3 min	
Denaturation	95 °C 30 s	30x
Annealing	55 °C 30 s	
Extension	72 °C 30 s	
Final extension	72 °C 10 min	
Hold	4 °C until analysis	

Flag-Beclin 1 WT, *Flag-Beclin 1*-Y233E/Y352E and *Flag-Beclin 1*-Y233F/Y352F constructs were stably expressed in K562 cells and Beclin 1 KO MEFs using retroviral infection.

2.2.12 Kinase inhibition studies

In this study, kinases inhibitors were used to block the activation of various kinases, including Flt3-ITD, BCR-ABL, PDGFRA and NPM-ALK. Imatinib, nilotinib and TAE684 were obtained from Selleckchem. Sorafenib was purchased from American Chemicals Custom Corporation (San Diego, CA, USA). Imatinib, nilotinib, TAE684 and sorafenib were dissolved in dimethyl sulfoxide (DMSO) as a stock solution of 10mM.

2.2.13 Western blotting

Western blotting is a robust protein analysis technique that identifies cellular proteins by using specific antibodies. The proteins are previously separated according to their size by gel electrophoresis. As a first step, cells of interest are harvested and lysed with lysis buffer for 30 min on ice. The cell debris is then pelleted by centrifuging the lysate for 20 min with 13000g at 4 °C, protein lysate is then mixed with SDS sample buffer. Antibodies typically recognize a small portion of the protein of interest and this domain may reside within the 3D conformation of the protein.

To enable access of the antibody to this portion, it is necessary to unfold the protein, i.e. denature it. Protein lysates are boiled at 95 °C for 5 min for denaturation, and then are loaded on the SDS-PAGE gel.

After gel electrophoresis, separated proteins on the gel are transferred to a Polyvinylidene fluoride (PVDF) membrane with the Trans-Blot Turbo Transfer System (Bio-Rad). The PVDF membrane is soaked in methanol briefly for its activation, and then the membrane is soaked in transfer buffer for several minutes until it is completely wet. The filter papers are all pre-wet in transfer buffer before use. Then the transfer sandwich is assembled. Proteins were transferred at 25V for 30 minutes.

After transfer, the membranes are then blocked with 5% milk or 5% bovine serum albumin for 30 min at room temperature. The membrane is incubated with the primary antibody diluted in blocking buffer for overnight at 4 °C. On the second day, membrane is rinsed three times with PBS-Tween washing buffer. Subsequently, the membrane is incubated with the secondary antibody conjugated to horse reddish peroxidase (Cell signaling technology). Protein bands are visualized using chemiluminescent substrates system.

2.2.14 Immunoprecipitation

The cell extract for immunoprecipitation is prepared adding CHAPS lysis buffer to the cells for 1 hour on ice. Pre-clearing of the lysates is performed using protein A or G agarose beads, depending on the species in which the first antibody was raised (Table 8). This step is followed by incubation with the primary antibody of interest for

overnight at 4 °C. In this study, Flag IPs were performed using agarose conjugated anti FLAG beads (Sigma) by incubation for overnight at 4 °C, and Beclin 1 IPs were performed using anti Beclin 1 antibodies (Santa Cruz) together with protein beads. Immunoprecipiates are washed three times with lysis buffer before they are eluted from the beads using SDS sample buffer.

Table 8. The affinity of beads to different species

Species immunoglobulin isotype	Protein A	Protein G
Mouse IgG 1	+	+++
Mouse IgG 2a	+++	+++
Mouse IgG 2b	++	++
Mouse IgG 3	+	+
Rabbit all isotypes	+++	++

Key: +++ = Strong binding, ++ = Medium binding, + = Weak binding

2.2.15 Stripping

In order to re-incubate the PVDF membrane with another primary antibody, the membrane has to be stripped off the antibodies used in a previous detection first. The membrane is incubated in stripping buffer (0.2 M NaOH) for about 10 min with shaking at room temperature. The stripping buffer can remove antibodies bound to the membrane without disturbing the proteins. The membrane is washed briefly in PBST and re-blocked in blocking buffer for 30 min before incubation with another primary antibody.

2.2.16 Bone marrow transduction and transplantation model

The bone marrow research is highly dependent on *in vivo* experimentation, because *in vitro* techniques do not mimic these complicated *in vivo* systems. Therefore, bone marrow transplantation is an ideal model for leukemia-related research. In this study, murine bone marrow is harvested from female donor mice 4 days after injection of 150 mg/kg 5-fluorouracil (Ribosepharm, Munich, Germany) and stimulated overnight in Iscove modified Dulbecco medium (Gibco) supplemented with 20% FCS and growth factors (10 ng/mL mIL-3, 10 ng/mL mIL-6, and 50 ng/mL mSCF). Bone marrow cells are infected by spin infection (1200g, 32°C, 90 minutes) using retroviral supernatant supplemented with growth factors and 4 µg/mL polybrene (Sigma). Subsequently, the cells are resuspended in Hanks balanced salt solution (Sigma) and injected into the tail vein of lethally irradiated (800 rad) female recipient mice. Animals that received a transplant are monitored for signs of disease by serial measurement of peripheral blood (PB) counts. All animals are caged in a special caging system (Thoren Caging Systems, Hazleton, PA) with autoclaved food and acidified water. All procedures were reviewed and approved by the university's supervisory animal care committee.

2.2.17 *Atg5* deletion analysis in BMT murine model

To study the effect of autophagy related protein, ATG5, on BCR-ABL-mediated leukemogenesis, $Atg5^{flox/flox}$ and $Atg5^{wt/wt}$ mice (Liu et al., 2012) were used. Bone morrow from $Atg5^{flox/flox}$ and $Atg5^{wt/wt}$ mice were infected with MIG-*BCR-ABL-Cre* construct. Then BM transplantation took place as described in 2.2.16. To assess the Cre-loxP system-mediated deletion of the *Atg5* allele in the murine model, genotyping analysis was performed. 10 mg spleen of each group was cut up into small pieces and digested using proteinase K. Genomic DNA was then isolated using the DNeasy Blood & Tissue Kits from QIAGEN according to the manufacturer’s instructions.

After genomic DNA isolation from spleen, PCR-based genotyping was used for *Atg5* deletion analysis. PCR primers were described in 2.1.10 and The following PCR program was used:

Initial denaturation 95 °C 3 min

Denaturation	95 °C 30 s	30x
Annealing	60 °C 30 s	
Extension	72 °C 45 s	
Final extension	72 °C 10 min	
Hold	4 °C until analysis	

2.2.18 Analysis of transplanted mice

Hemoglobin (Hb), hematocrit (HCT), platelets, and white blood cell counts (WBC) were determined using an automated counter (SCIL vet abc, Heska, Fort Collins, CO). Single cell suspension of spleen was prepared using a 100µm cell strainer, and flushing the bones isolated single cell suspension of BM. This step was followed by RBC lysis with RBC lysis buffer for 15 min at room temperature. Cells were frozen in 90% FBS and 10% DMSO. For flow cytometry, cells were washed in PBS with 1% BSA (FACS buffer). To avoid "background" staining caused by Fc receptors that are expressed in many cell types, FC block was used. The cells of interest were then stained with monoclonal antibodies in PBS with 1% BSA for 30 minutes in dark. After washing with FACS buffer three times, cells were re-suspended in FACS buffer containing PI to discriminate viable/non-viable cells. A gating strategy to identify living leukocytes from an organ cell suspension is depicted in Figure 6. Flow cytometry was performed on a FACS cytometer (antibodies are described in 2.1.6). At least 10,000 events were acquired, and data was analyzed using FlowJo V10.

To analyze expression of BCR-ABL and Beclin 1 in transplanted mice, freshly isolated spleen cells (day 21 post BMT) were boiled in SDS loading buffer (95 °C for 10 min) and analyzed using the Western Blot method (2.2.13).

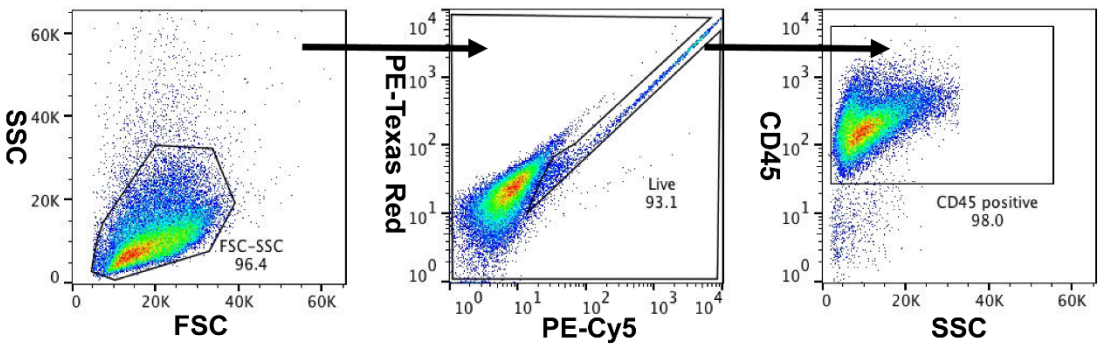

Figure 6. Gating strategy for living leukocytes from transplanted murine organ by flow cytometry.

The FSC-SSC gate (left panel). Dead cells are stained with PI, and live cells show a low signal from PE-Cy5 and PE-Texas Ted channels (middle panel). Leukocytes are distinguished from erythrocytes by staining with anti CD45 antibody (right panel). These cells are further analyzed by staining with antibodies derived against lineage marker antigens

2.2.19 Proliferation assay

The non-radioactive cell proliferation assay is a colorimetric technique that determines the quantity of viable cells in proliferation or chemosensitivity assays. To evaluate the effect of Beclin 1 on BCR-ABL-driven cell proliferation, the MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium, CellTiter 96; Promega, Madison, WI))-based method was used according to manufacturer's instructions. Briefly, 100 µl cell suspension containing 10,000 cells was plated into each well of a 96 well plate. After the cells had grown for a defined time, 20 µl of MTS reagent was added into each well. The absorption of formazan at 490 nm was detected by TECAN plated reader after 1 hour incubation at 37 °C. Measurements were performed in triplicates.

2.2.20 *In vitro* kinase assay

The *in vitro* kinase assay uses a purified kinase to transfer radioactive-labeled ATP to an extracted substrate. In the present study, purified active ABL kinase was obtained from Proqinase, and GST-Beclin 1 mutants were used as substrates. The Beclin 1

mutants were expressed and purified from BL21 cells. The *In vitro* kinase assay was performed in 50 µl of kinase buffer containing 60 mM HEPES pH 7.9, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 µm Na_3VO_4, 125 mm DTT, 20 µM ATP, ^{33}P-γ-ATP. After the kinase reaction for 30 min at 30 °C, gel electrophoresis was used to separate the radiolabeled ATP from the radiolabeled substrate, and the amount of phosphorylated substrate was quantified using standard autoradiography.

2.2.21 GST purification and GST binding studies

Wild type Beclin 1, Beclin 1 aa1-115, aa1-135, aa141-277, aa278-337 and aa338-450 recombinant proteins were expressed and purified from BL21 cells. GST fusion proteins are incubated for 3 h with glutathione-agarose beads in binding buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0, and 5mM EDTA) at 4 °C. After washing three times in binding buffer, the beads were then incubated with their potential binding proteins for 3 h at room temperature. The beads were then washed three times and bound proteins were eluted with SDS sample buffer afterwards.

2.2.22 Immunofluorescence analysis with confocal microscopy

Immunofluorescence staining is employed in order to detect LC3 puncta or distribution of BCR-ABL and Beclin 1 within the cells. In this study, K562 cells or Ba/F3 cells were fixed by 4% formaldehyde. Poly-L-lysine coated multi-spot microscope slides were prepared in advance; poly-L-lysine is adhesive and it facilitates binding of suspension cells to glass microscopic slides. Fixed cells adhere to the slides after 30 min at 37 °C. After a washing step using PBS, blocking and permeabilisation were performed by incubation of the cells with PBS containing 10% goat serum and 0.3% Triton-X-100 solution for 1 hour at room temperature. The fixed and permeabilised cells were then incubated with the primary antibody overnight at 4 °C. Subsequently, the slides were stained using an Alexa Fluor®-coupled secondary antibody (2.1.6) for 1h at room temperature in the dark. The slides were washed four times for 5 min with PBS and then air-dried. Anti-fade DAPI was added to

the slide to stain nuclei and a coverslip placed on the top. The slides were stored at 4 °C until analysis. All slides were analyzed using the Leica confocal microscopy system.

2.2.23 Statistics

The results are shown as the mean ± standard deviation of the mean (mean ± SD). Unless otherwise stated, all statistical analyses were performed with the GraphPad Prism 6 software using the two-sided unpaired Student's *t*-test and the assumption of normality was checked. *P*-values less than 0.05 were considered to be statistically significant.

3 Results

3.1 *Beclin 1* knockdown delays BCR-ABL-mediated leukemogenesis

Since TKI-caused drug resistance became an urgent problem in CML patients, great effort has been made in the development of alternative therapy strategies in the treatment of BCR-ABL$^+$ leukemia. Similar to other oncogenic kinases, constitutively active BCR-ABL mimics growth factor stimulation of the cells. As a consequence, BCR-ABL$^+$ cells escape cell cycle control, as well as programmed cell death. Another effect of constitutively active BCR-ABL is deregulation of homeostatic pathways like autophagy. Autophagy is considered an essential mechanism for cancer progression and transformation. However, whether autophagy has a role in the promotion or suppression of cancerogenesis, is still unresolved.

Previously, *Beclin 1* was reported as a tumor suppressor gene. Specifically, overexpression of Beclin 1 is beneficial for cancer treatment in breast cancer and ovarian cancer. However, leukemia related gene array analysis with clinical samples showed that *Beclin 1* expression is upregulated in CML patient samples compared to healthy samples (fold change: 1.22; Q-value: <0.1%); this result gives a clue into the biology of CML and could provide the basis for identification of Beclin 1 as a new therapeutic target (Diaz-Blanco et al., 2007). Beclin 1 acts as a critical regulator of autophagy and cancer. Yet, little is known about the function and regulation of Beclin 1 in BCR-ABL-mediated leukemia. To find out the role of Beclin 1 in BCR-ABL-mediated leukemia, a retroviral vector system composed of *miR30-shRNA*-mediated *Beclin 1* downregulation in combination with *BCR-ABL* expression, was established (Figure 7a). Consistent with previous reports, *Beclin 1* knockdown enhances TKI toxicity on BCR-ABL expressing Ba/F3 cells (Figure 10). Moreover, as it is shown in Figure 7b-c, *Beclin 1* knockdown significantly impairs cell proliferation compared to the control group. Similarly, bone morrow colony formation assays further confirmed the effect of Beclin 1 downregulation on BCR-ABL-mediated cell proliferation (Figure 7d-f). Next, Beclin 1 knockdown was investigated in BCR-ABL-mediated leukemogenesis *in vivo*. To first exclude the effect of Beclin 1

downregulation on hematopoietic stem cells, *Beclin 1 miR* alone was transplanted into mice. As expected, there is no influence on mice survival, WBC count, or lineage phenotype (Figure 8a-g). Consequently, *BCR-ABL* and *Beclin 1 miR* were transplanted into mice. Results showed longer survival rates and low WBC counts in Beclin 1 downregulated mice compared to control mice (Figure 7g-h). FACS analysis confirmed that all the mice in the either control group or the Beclin 1 downregulation group achieved myeloid leukemia disease (Figure 9). Additional immunoblotting with the primary spleens showed the vector system works well with significant downregulation of Beclin 1 in *Beclin 1 miR* group and equal expression of BCR-ABL in both groups (Figure 7i). Autophagy suppression caused by BCR-ABL-mediated Beclin 1 phosphorylation inferred a loss of Beclin 1 function. Furthermore, an analysis of clinical samples suggested that upregulation of Beclin 1 in BCR.ABL^{+} leukemia is likely to be even worse for patients (Diaz-Blanco et al., 2007). This might explain why *Beclin 1* knockdown dramatically delays BCR-ABL-driven leukemogenesis.

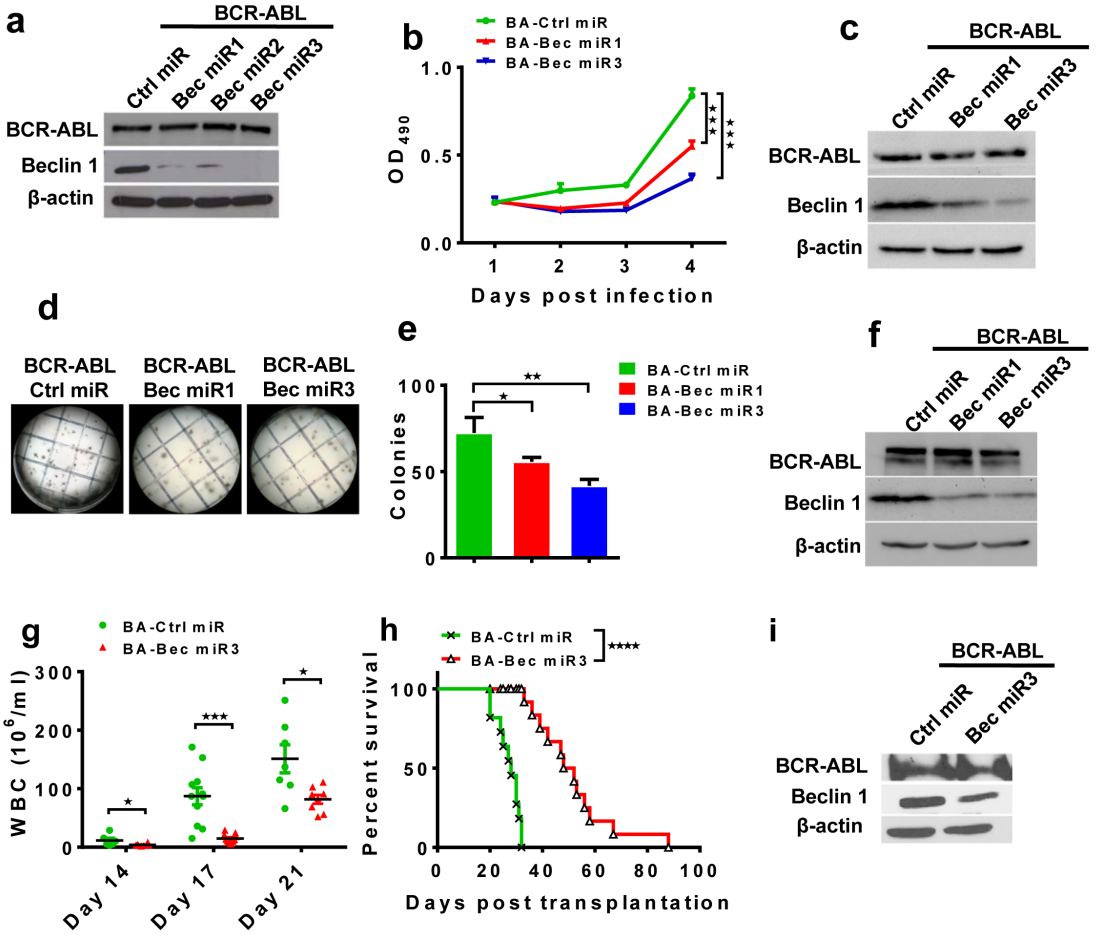

Figure 7. Beclin 1 downregulation delays BCR-ABL-mediated leukemogenesis *in vitro* and *in vivo*.

(a) Expression of Beclin 1 and BCR-ABL was analyzed by Western blotting in Ba/F3 cells infected with indicated constructs. (b) Cell proliferation was performed by MTT assay in Ba/F3 cells infected with indicated constructs towards IL3 withdraw. (c) Western blotting was used for confirming expression of Beclin 1 and BCR-ABL in cells shown in (b). (d) Bone marrow methylcellulose colony formation assay utilized for cell proliferation measurement. Bone marrow cells infected with indicated constructs were plated into methylcellulose in the absence of growth factors, and colonies were quantified 10 days post plating. (e) Quantification of colonies shown in (d). (f) Western blotting was used for confirming expression of Beclin 1 and BCR-ABL in cells shown in (d). (g) White blood cells from peripheral blood of transplanted mice were counted. (h) Mice survival curve was analyzed by Kaplan-Meier test. (i) Expression of Beclin 1 and BCR-ABL was evaluated with transplanted mice spleen.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, Student's *t* test or Kaplan-Meier test.

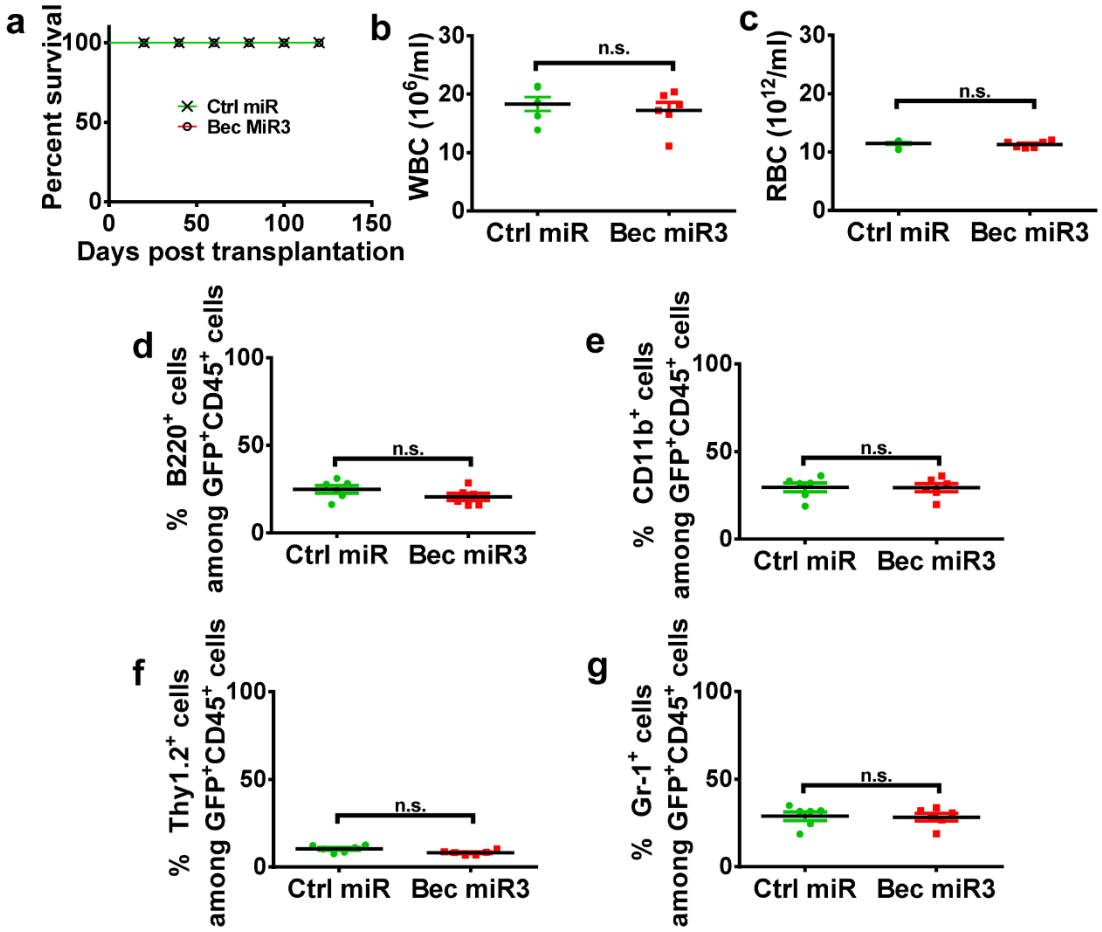

Figure 8. Beclin 1 downregulation does not influence hematopoietic cells in the BM transplantation model.

Mice transplanted with *Beclin 1 miR3* alone showed normal (a) survival rate, (b) WBC amount, (c) RBC amount, (d-g) percentage of lineage markers (anti-CD45, anti-B220, anti-Gr1 and anti-CD11b) compared to control mice.

n.s., no significance, Student's *t* test or Kaplan-Meier test.

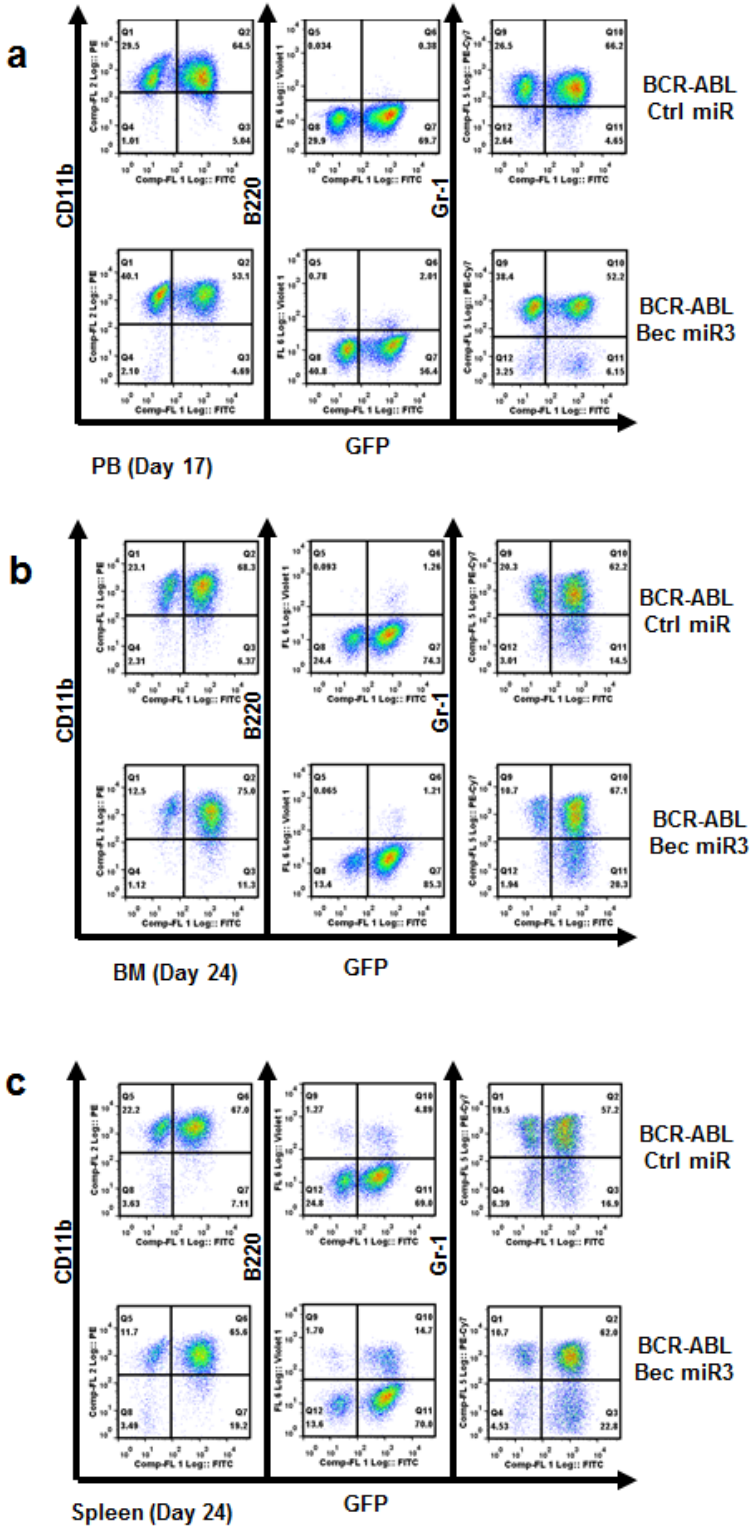

Figure 9. Lineage analysis of transplanted mice.

(a) Peripheral blood, (b) bone marrow, and (c) spleen cells were harvested from transplanted mice, and FACS analysis was performed by staining with anti-CD45, anti-B220, anti-Gr1 and anti-CD11b.

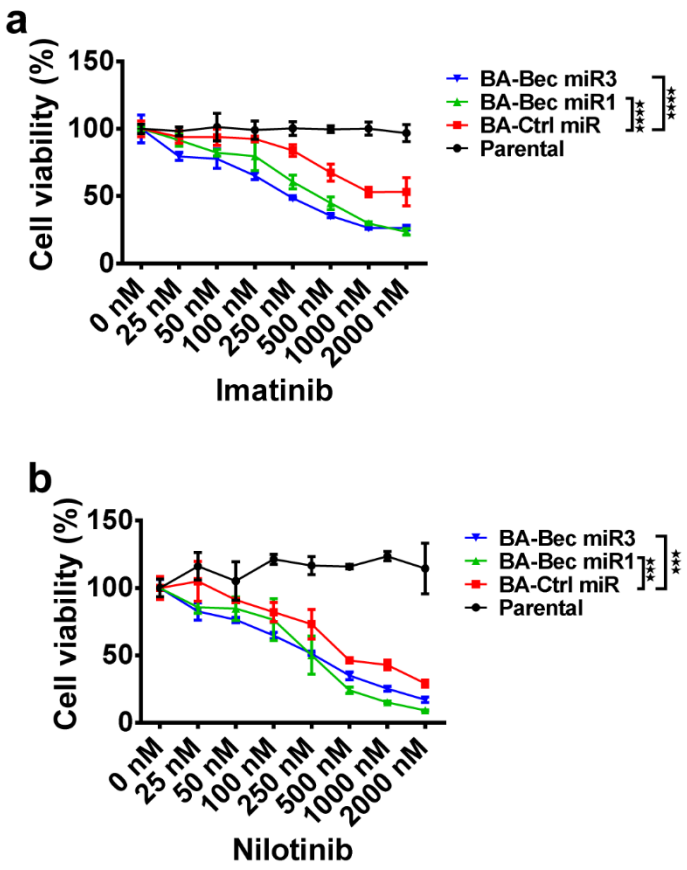

Figure 10. Beclin 1 downregulation enhances TKI toxicity in BCR-ABL expressing Ba/F3 cells.

MTT assay was used to identify cell viability in Ba/F3 cells transduced with indicated construct upon (a) imatinib and (b) nilotinib treatment.

3.2 *Atg5* deletion does not influence BCR-ABL-driven leukemogenesis

Autophagy is a crucial mechanism to maintain cell homeostasis, and there are several proteins involved in this cellular regulation. Beclin 1 is suggested to be an important molecule in BCR-ABL-mediated leukemia. Beclin 1 is a specific autophagy-related protein in BCR-ABL-mediated leukemogenesis, but could there be another autophagy regulator having the same effect? To investigate this, autophagy-related protein, Atg5, was considered as an experimental objective. ATG5 is crucial for isolation membrane formation in autophagy process. Interestingly, *Atg5* deletion using the *Atg5 Cre-Flox* system cannot alter progression of leukemic disease caused by BCR-ABL (Figure

11a-c). In summary, Beclin 1 is a specific target of BCR-ABL revealing an essential role in BCR-ABL-driven leukemia. Targeting Beclin 1 might be one potential possibility for BCR-ABL^{+} leukemia treatment.

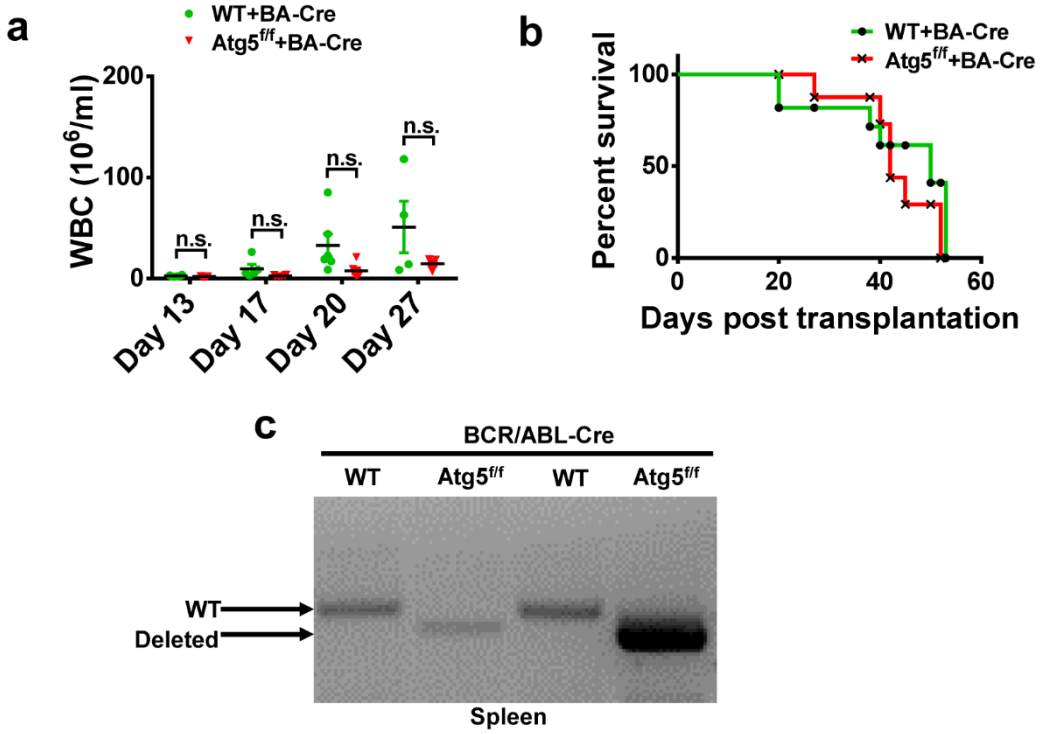

Figure 11. *Atg5* deletion does not affect BCR-ABL-mediated leukemogenesis *in vivo*.

Bone marrow cells from Atg5 $^{wt/wt}$ and Atg5$^{flox/flox}$ mice were infected with *BCR-ABL-Cre*, and the transplantation was taken place afterwards. (a) WBC amount was measured on indicated day. (b) Survival rate was analyzed for leukemogenesis progression evaluation. (c) Genome DNAs of spleens from transplanted mice were isolated, and PCR reaction was used for *Atg5* deletion confirmation.

n.s., no significance, Student's *t* test or Kaplan-Meier test.

3.3 Active BCR-ABL suppresses autophagy

In recent years, several methods have been developed in order to directly detect autophagy. Among these methods, LC3-II blotting and LC3 puncta staining are most widely used for autophagy detection. LC3 family proteins include MAP1-LC3, GATE-16, and GABARAP, which are the mammalian homologues of yeast Atg8.

These proteins can be found on both the isolation membrane and mature autophagosomes and are frequently conjugated with phosphatidylethanolamine (PE) (Kabeya et al., 2000). LC3 includes two different forms, LC3-I and LC3-II, the conversion of the endogenous LC3-I to LC3-II is related with autophagosome formation. In SDS-PAGE, LC3-II migrates faster than LC3-I due to its hydrophobicity. Consequently, immunoblotting of LC3 usually gives two bands: LC3-I (apparent mobility, 18 kD) and LC3-II (apparent mobility, 16 kD) (Kabeya et al., 2000). LC3 is located on both the outer and inner membrane of autophagosomes. After the fusion of the autophagosome with lysosomes, LC3 on the inner membrane is degraded. This process leaves less LC3 on the autolysosome membrane, resulting in a reduced number of LC3 puncta. Thus, staining of LC3 is simple and highly specific as a measure of autophagosome quantity (Mizushima et al., 2010).

In this study, the BCR-ABL^{+} cell line K562 was used to study the subcellular localization of LC3 and the levels of LC3-II to identify a possible role of BCR-ABL in autophagy induction. Treatment of the cells with the BCR-ABL kinase inhibitor nilotinib resulted in autophagy induction as visualized by an increased conversion of LC3-I to LC3-II (Figure 12a). The number of LC3 puncta per cell was also enhanced (Figure 12b-c). To further evaluate whether autophagy induction is caused directly by nilotinib or BCR-ABL kinase activity inhibition, autophagy induction was determined in murine Ba/F3 cells expressing WT BCR-ABL or a drug resistance mutant (BCR-ABL-T315I). Nilotinib treatment induces autophagy in the presence of mIL3 in Ba/F3-BCR-ABL cells, but not in Ba/F3-BCR-ABL-T315I cells (Figure 12d-f). These results point towards a specific effect of BCR-ABL in autophagy, rather than an unspecific effect of TKI treatment. Thus, these experiments suggest that active BCR-ABL suppresses autophagy.

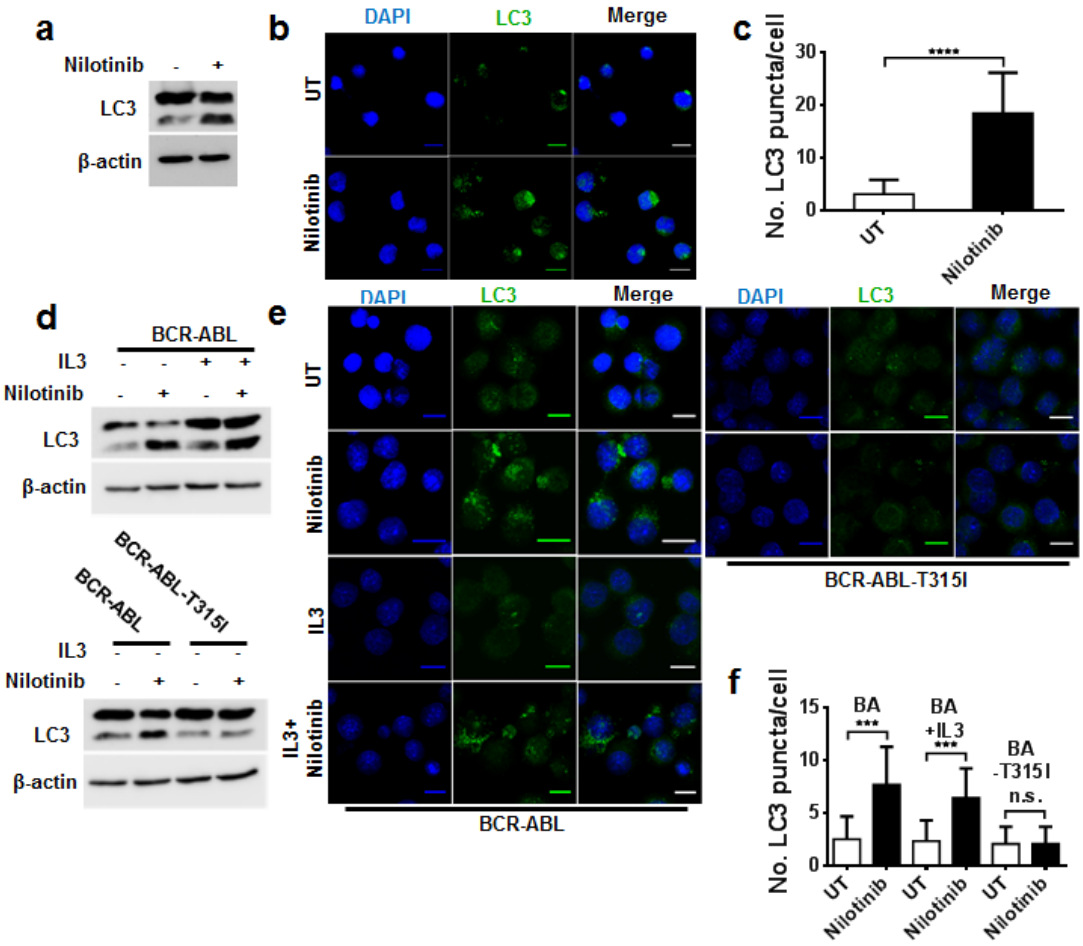

Figure 12. Active BCR-ABL suppresses autophagy.

(a) Accumulation of autophagosome-associated LC3-II in nilotinib-treated K562 cells. The Western blot shows endogenous LC3-I and LC3-II levels. Actin was measured as loading control. (b) Formation of LC3 puncta in *mCherry-EGFP-LC3* transduced K562 cells. *LC3*-transduced K562 cells were treated for 12h with or without 2 μM nilotinb, and cells were stained with anti-LC3 antibody. Nuclei were counterstained with DAPI. Scale bar, 10μm. (c) Quantitation of LC3 puncta. *mCherry-EGFP-LC3* transduced K562 cells with or without nilotinib treatment were used for LC3 puncta quantification. Bars are mean± SD. ****p < 0.0001, Student's *t* test. (d) Nilotinib treatment induces autophagy in BCR-ABL-expressing Ba/F3 cells in presence/absence of IL3, but not in BCR-ABL-T315I-expressing Ba/F3 cells. (e) Immunostaining of LC3 puncta in Ba/F3 cells with nilotinib treatement. Cells were treated for 12h with or without 2 μM nilotinb, and cells were stained with anti-LC3 antibody. Nuclei were

counterstained with DAPI. Scale bar, 10μm. (f) Quantitation of LC3 puncta with in BCR-ABL$^+$ Ba/F3 cells. LC3 puncta were counted. Bars are mean ± SD. ****p < 0.0001, Student's *t* test.

3.4 BCR-ABL-mediated autophagy suppression leads to alteration of the Beclin 1 complex

Several lines of evidence suggest a direct role of BCR-ABL in the regulation of autophagy. Initially, BCR-ABL was proven to activate PI3K/AKT signaling, which is considered to be a pathway inhibiting autophagy (Kharas et al., 2008; Klejman et al., 2002). Furthermore, it has been shown that targeting autophagy proteins increases cell death caused by TKIs in BCR-ABL$^+$ cells (Bellodi et al., 2009; Yu et al., 2012). It has also been demonstrated that TKI treatment triggers autophagy in BCR-ABL$^+$ cells (Bellodi et al., 2009; Sheng et al., 2011). Nevertheless, the mechanism of BCR-ABL's involvement in the process of autophagy process requires further investigation. Beclin 1, a central player in autophagy has been reported to be a crucial mediator in several cancers (Liang et al., 1999; Qu et al., 2003; Wang et al., 2012; Wei et al., 2013; Yue et al., 2003). Furthermore, oncogenic kinases EGFR and AKT are able to phosphorylate Beclin 1 causing Beclin 1 loss-of-function, thereby negatively regulating autophagy (Wang et al., 2012; Wei et al., 2013). Beclin 1 has a crucial role in autophagosome formation, linking it to several diseases (such as Alzheimer disease, heart disease and breast cancer) which are caused by the deregulation of the Beclin 1-VPS34-VPS15-UVRAG-Rubicon-ATG14 complex (He and Levine, 2010; Kang et al., 2011; McKnight and Zhenyu, 2013). These findings indicate that Beclin 1, VPS34, VPS15, UVRAG and ATG14 act as positive regulators of autophagy, whereas Rubicon is a negative regulator.

In the present study, the recruitment of VPS15, VPS34, UVRAG, ATG14 and Rubicon to Beclin 1 was investigated in nilotinib-treated K562 cells (Figure 13). The levels of VPS15, VPS34, UVRAG and ATG14 binding to Beclin 1 were increased in nilotinib treated cells and could thereby positively regulate autophagosome formation. The negative regulator Rubicon was less recruited to the Beclin 1-complex. Taken

together, these findings indicate that BCR-ABL-mediated autophagy suppression is regulated by alterations in Beclin 1 complex formation.

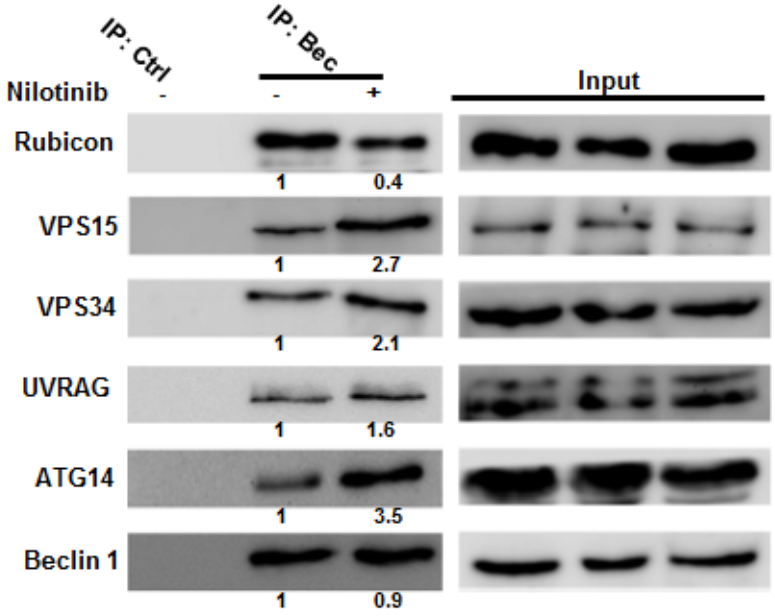

Figure 13. BCR-ABL activation alters Beclin 1 affinity to binding partners of the Beclin 1 complex.

Immunoprecipitation of indicated proteins (ATG14, UVRAG, VPS34，VPS15 and Rubicon) with Beclin 1 in K562 cells with/without nilotinib treatment. The numbers indicate the fold change.

3.5 BCR-ABL is directly binding to Beclin 1

The structure of the majority of signaling proteins consists of a catalytic domain and one or several protein binding domains (Vogel et al., 2004). Proteins possessing binding domains without catalytic activity may act as adaptor, scaffold or anchoring proteins to assemble protein complexes (Davison, 1993). This complex formation is often crucial for downstream signal transduction.

To evaluate whether Beclin 1 could be a binding partner of BCR-ABL, HEK293T cells were transfected with BCR-ABL-expressing vector. Immunoprecipitation of Beclin 1 showed that BCR-ABL strongly phosphorylates Beclin 1, and Beclin 1 interacts with BCR-ABL. This interaction is independent of BCR-ABL kinase activity, as nilotinib-treated cells also show BCR-ABL-Beclin 1 interaction (Figure 14a). To further confirm this result in cells expressing endogenous BCR-ABL, immunoprecipitation of

either Beclin 1 or ABL in K562 cells was performed. Again, strong interaction of Beclin 1 and BCR-ABL could be observed (Figure 14b-c). Next, it should be determined whether BCR-ABL binds to Beclin 1 directly or if adaptor proteins mediate the interaction. A GST pull down provided confirmation that the BCR-ABL-Beclin 1 interact directly (Figure 14d). Consistent with the binding experiments, immunofluorescence staining further confirmed the co-localization of BCR-ABL and Beclin 1 in K562 cells (Figure 15).

The precise site of interaction between two proteins is critical for the downstream signaling pathways triggered by the interaction. Therefore, it should be determined which region of BCR-ABL is binding to Beclin 1. As an initial experiment, either wild type BCR or wild type ABL were overexpressed together with Beclin 1 in HEK293T cells. Surprisingly, immunoprecipitation and western blotting suggested that BCR, but not ABL, interacts with Beclin 1, (Figure 16a). Consequently, the binding site of Beclin 1, which interacts with BCR-ABL, should be resolved. To address this question, different fragments of Beclin 1 were cloned and fused with GST. These short fragments were used to pull down BCR-ABL in K562 cell lysates. BCR-ABL binding could only be observed in pulldowns using N-terminal, but not the C-terminal fragments of Beclin 1 (Figure 16b). These results indicate that BCR-ABL is binding to Beclin 1 directly, and that the BCR part of BCR-ABL acts as an adaptor to bring BCR-ABL and Beclin 1 together. This interaction ultimately leads to Beclin 1 phosphorylation.

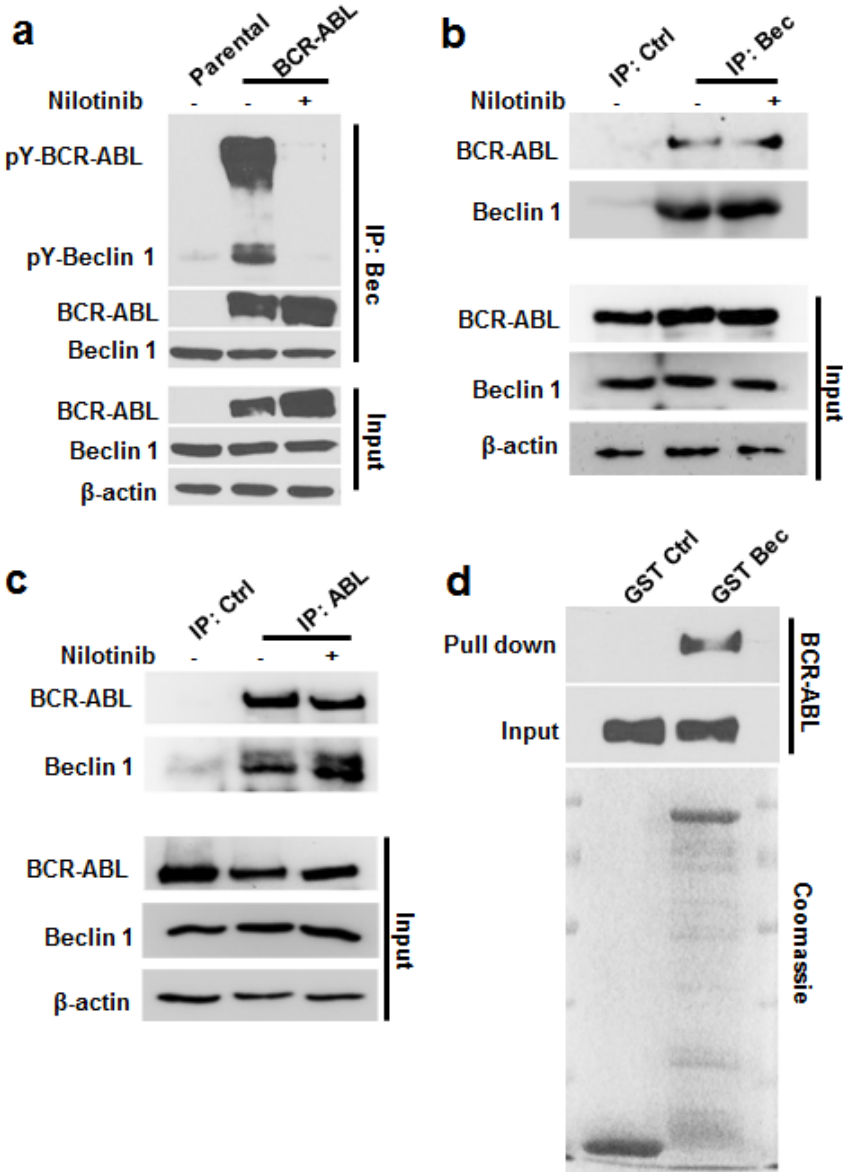

Figure 14. BCR-ABL binds to Beclin 1 directly.

(a) Immunoprecipitation of BCR-ABL with Beclin 1 in nilotinib-treated HEK293T cells transfected with *BCR-ABL*. A phosphotyrosine antibody was used to detect phosphorylated Beclin 1 and phosphorylated BCR-ABL. (b) Immunoprecipitation of BCR-ABL with anti-Beclin 1 antibody in K562 cells. (c) Immunoprecipitation of Beclin 1 with anti-ABL antibody in K562 cells. (d) GST pull down of BCR-ABL with recombinant GST-Beclin 1 in K562 whole lysate.

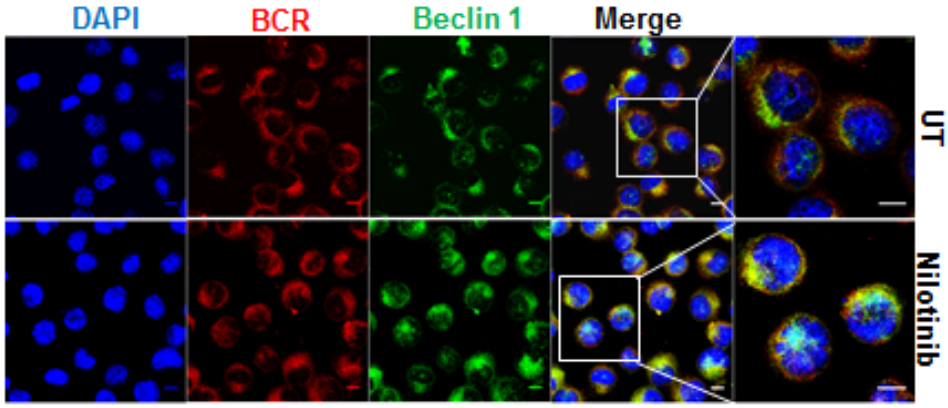

Figure 15. Co-localization of BCR-ABL and Beclin 1.

Confocal microscopy shows co-localization of BCR-ABL and Beclin 1 in K562 cells. The cells were stained with anti-BCR antibody (Red) and anti-Beclin 1 antibody (Green). Nuclei were counterstained with DAPI. Scale bar, 10μm.

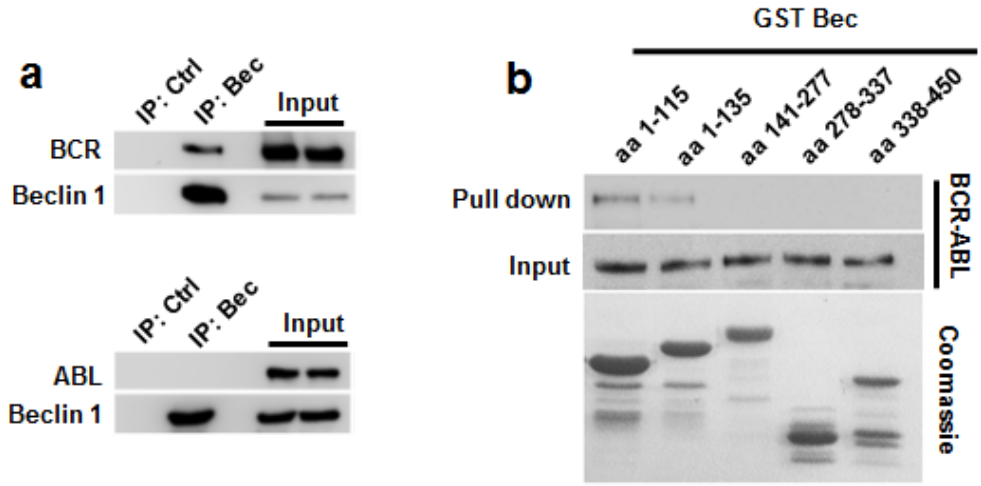

Figure 16. BCR but not ABL is binding to N-tail of Beclin 1.

(a) Immunoprecipitation with Beclin 1 in HEK293T cells transfected with *Beclin 1* and either *BCR* or *ABL*. (b) Pull-down of BCR-ABL with recombinant GST-Beclin 1 fragments in K562 whole lysate.

3.6 Beclin 1 is a substrate of BCR-ABL

Protein phosphorylation is a reversible and covalent post-translational modification serving to modify the activity of the phosphorylated molecule. Phosphorylation of signaling molecules affects basal cellular processes such as proliferation,

differentiation, motility, metabolism or cellular transport processes. The results from the previous section suggest that Beclin 1 is a substrate of BCR-ABL kinase. Next it should be tested whether other oncogenic kinases are also able to phosphorylate Beclin 1. Similar to BCR-ABL, FLT3-ITD, NPM-ALK and PDGFRA-V842D are also frequent occurrences in hematopoietic malignancies (Demoulin and Montano-Almendras, 2012; Drexler et al., 2000; Levis and Small, 2003). These proteins were co-expressed together with Flag-Beclin 1 in HEK293T cells. Strikingly, immunoprecipitation and western blotting showed that only BCR-ABL could phosphorylate Beclin 1 among all the oncogenic kinases tested (Figure 17). These results indicate that Beclin 1 is a substrate of BCR-ABL.

3.7 BCR-ABL phosphorylates Beclin 1 at tyrosine residues Y233 and Y352

As shown in section 3.3, Beclin 1 is phosphorylated in the presence of BCR-ABL. However, it remains unclear, whether this phosphorylation is a direct activity of the BCR-ABL kinase or if the reaction is catalyzed by another kinase, which is recruited by BCR-ABL. Therefore, an *in vitro* kinase assay was performed, using purified GST-Beclin 1 fragments as substrates for recombinant ABL kinase. The screen identified Beclin 1 phosphorylation by BCR-ABL at two fragments, one spanning aa 141-aa 277 and another aa 337-aa 450 (Figure 18). To further examine which exact tyrosine residues are phosphorylated by BCR-ABL, these Beclin 1 mutants were overexpressed in HEK293T cells together with BCR-ABL and used for immunoprecipitation with an anti-FLAG antibody. In mutants lacking all tyrosine residues except Y233 or Y352, BCR-ABL-mediated phosphorylation could still be detected, indicating that these sites are specifically phosphorylated by BCR-ABL (Figure 19a). To confirm these results, a mutant lacking both tyrosine residues was generated. Beclin 1 phosphorylation was still detectable with a mutation at either Y233 or Y352, whereas Beclin 1 mutated at both tyrosine residues remain completely non-phosphorylated (Figure 19b). These data suggest that BCR-ABL phosphorylates Beclin 1 directly at Y233 and Y352. The results indicate the possibility that BCR-ABL

interferes with autophagy by altering Beclin 1 phosphorylation. Thus, Beclin 1-dependent autophagosome complex formation may be impaired.

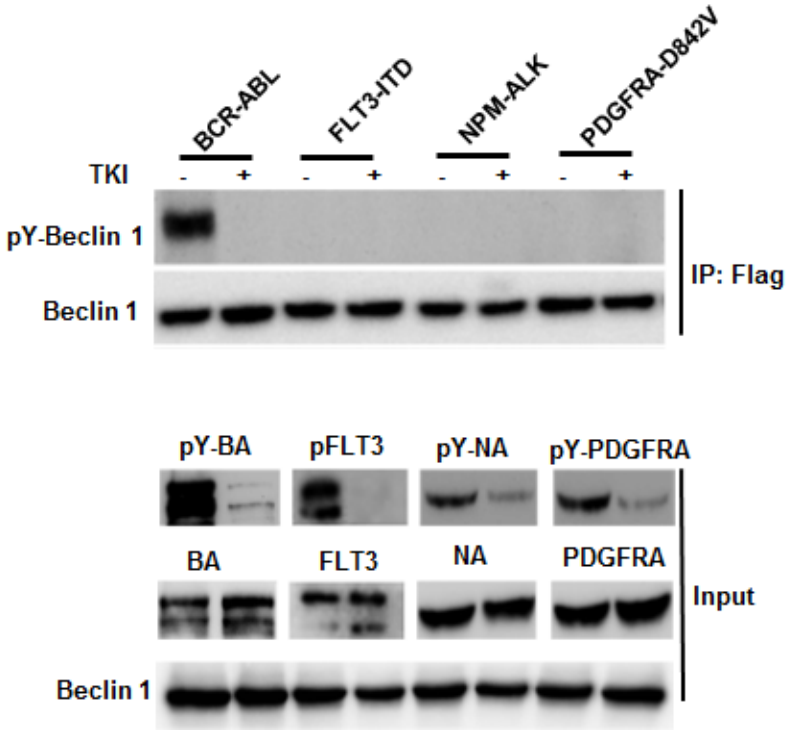

Figure 17. Beclin 1 is a substrate of BCR-ABL.

Various oncogenic kinases are tested for their Beclin 1 phosphorylation activity. Beclin 1 was immunoprecipitated from extracts of HEK293T cells transfected with *Beclin 1* together with either *BCR-ABL*, *Flt3-ITD*, *NPM-ALK*, or *PDGFRA-D842V*. An anti-phosphotyrosine (pY) antibody was used to detect Beclin 1 phosphorylation. For TKI treatment, inhibitors specific to the respective kinases (nilotinib for BCR-ABL, sorafenib for FLT3-ITD and PDGFRA-D842V, and TAE684 for NPM-ALK) were added to the cells 4 hours before cell harvest.

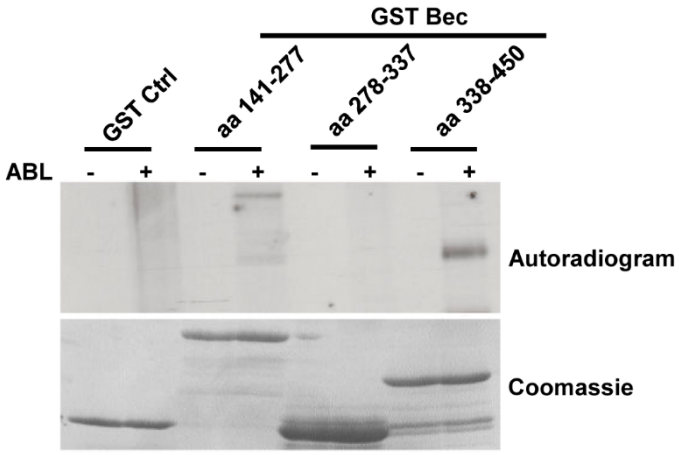

Figure 18 Beclin 1 is directly phosphorylated by BCR-ABL.

In vitro kinase assay using recombinant ABL kinase and purified GST-Beclin 1 fragments. Fragments containing Beclin 1 aa141-277 and aa338-450 are phosphorylated by ABL kinase

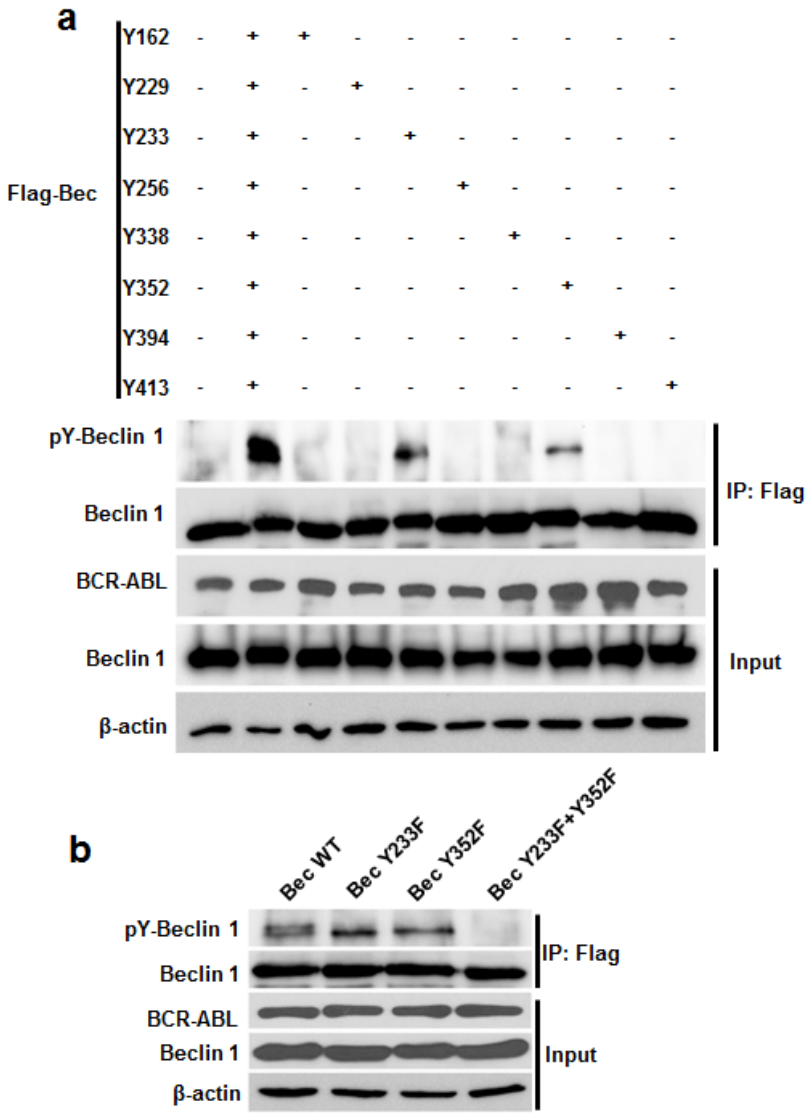

Figure 19. BCR-ABL specifically phosphorylates Beclin 1 at tyrosine residues Y233 and Y352.

(a) Identification of BCR-ABL-mediated Beclin 1 phosphorylation sites. Site directed mutagenesis was used to generate Beclin 1 mutants. In each mutant construct, one tyrosine site was left unmutated, while all others were mutated to phenylalanine. Immunoprecipitation with anti-FLAG antibody in HEK293T cells transfected with *Flag-Beclin 1* mutations and *BCR-ABL*. An anti-phosphotyrosine antibody was used to detect Beclin 1 phosphorylation. (b) Immunoprecipitation with anti-FLAG in HEK293T cells transfected with *Flag-Beclin 1* mutations and *BCR-ABL*. An Anti–phosphotyrosine antibody was used to detect Beclin 1 phosphorylation.

3.8 Beclin 1 phosphorylation-mimic mutant suppresses autophagy

Protein phosphorylation plays a central role in several cellular processes. To evaluate the impact of Beclin 1 phosphorylation on autophagy, a so-called "phosphorylation-mimic mutant" of Beclin 1 was generated. Phosphomimetics are amino acid substitutions that mimic a phosphorylated protein. In this study, a glutamic acid replaces a tyrosine for phosphorylation mimic, and phenylalanine replaces a tyrosine for phosphorylation deficiency. These Beclin 1 Y233E/Y352E mutant and phosphorylation-deficient Beclin 1 Y233F/Y352F mutants were used to further study the role of Beclin in autophagy regulation. Recently it was shown that the expression of UVRAG, VPS34 and ATG14 is decreased in Beclin-deficient MEF cells (McKnight et al., 2014). Here, overexpression of Beclin 1 mutants together with BCR-ABL showed via western blotting that both wild type Beclin 1 and Beclin 1 mutants can rescue the expression of molecules of Beclin 1 complex. However, Beclin 1 phosphorylation rescued less UVRAG and ATG14, but more Rubicon compared to Beclin 1 phosphorylation-deficiency mutant, pointing that Beclin 1 phosphorylation leads to its function change for stabilizing the binding partner molecules (Figure 20). It is well documented that Beclin 1 plays a major role in autophagy. Thus, it is very important to identify whether Beclin 1 phosphorylation affects autophagy. In K562 cells, phosphorylation-mimic mutant Beclin 1 Y233E/Y352E significantly suppresses autophagy with decreased expression of LC3-II, whereas phosphorylation-deficiency mutant Beclin 1 Y233F/Y352F induces autophagy with increased expression of LC3-II compared to control (Figure 21a). Similarly, LC3 puncta assay was used to confirm that phosphorylation of Beclin 1 suppresses autophagy, while non-phosphorylation of Beclin 1 induces autophagy (Figure 21b-c). These results indicate that phosphorylation causes loss-of-function Beclin 1 leading to impaired autophagy induction. Above, we have shown that BCR-ABL-mediated Beclin 1 phosphorylation could suppress autophagy through a reduction in recruitment of Beclin 1 binding-partners to Beclin 1. To find out the mechanism which Beclin 1

phosphorylation-mimic mutant or Beclin 1 phosphorylation-deficiency mutant employ to regulate autophagy, the Beclin 1 complex was investigated. Interestingly, it was observed that less recruitment of UVRAG, VPS15, VPS34 and ATG14 and more recruitment of Rubicon to wild-type Beclin 1 and Beclin 1 Y233E/Y352E compared to Beclin 1 Y233F/Y352F (Figure 21d). Taken together, Beclin 1 phosphorylation negatively regulates Beclin 1-mediated autophagy by Beclin 1 complex alterations that might be caused by a Beclin 1 phosphorylation-mediated conformational change. This suggests that constitutive Beclin 1 tyrosine phosphorylation enhances autophagy suppression in cells with active BCR-ABL.

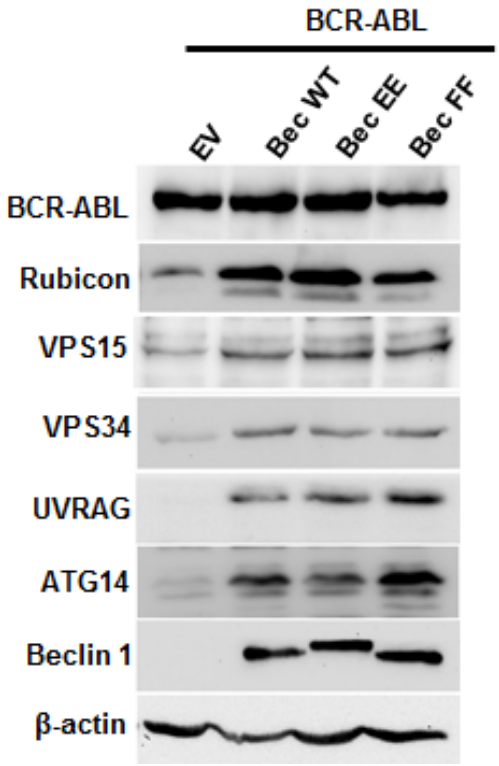

Figure 20. BCR-ABL-mediated Beclin 1 phosphorylation alters stability of Beclin 1-binding partners.

Western blotting was used for ATG14, UVRAG, VPS34 and Rubicon expression investigation in *Beclin 1* knockout MEFs transduced with *BCR-ABL* and indicated *Beclin 1* construct.

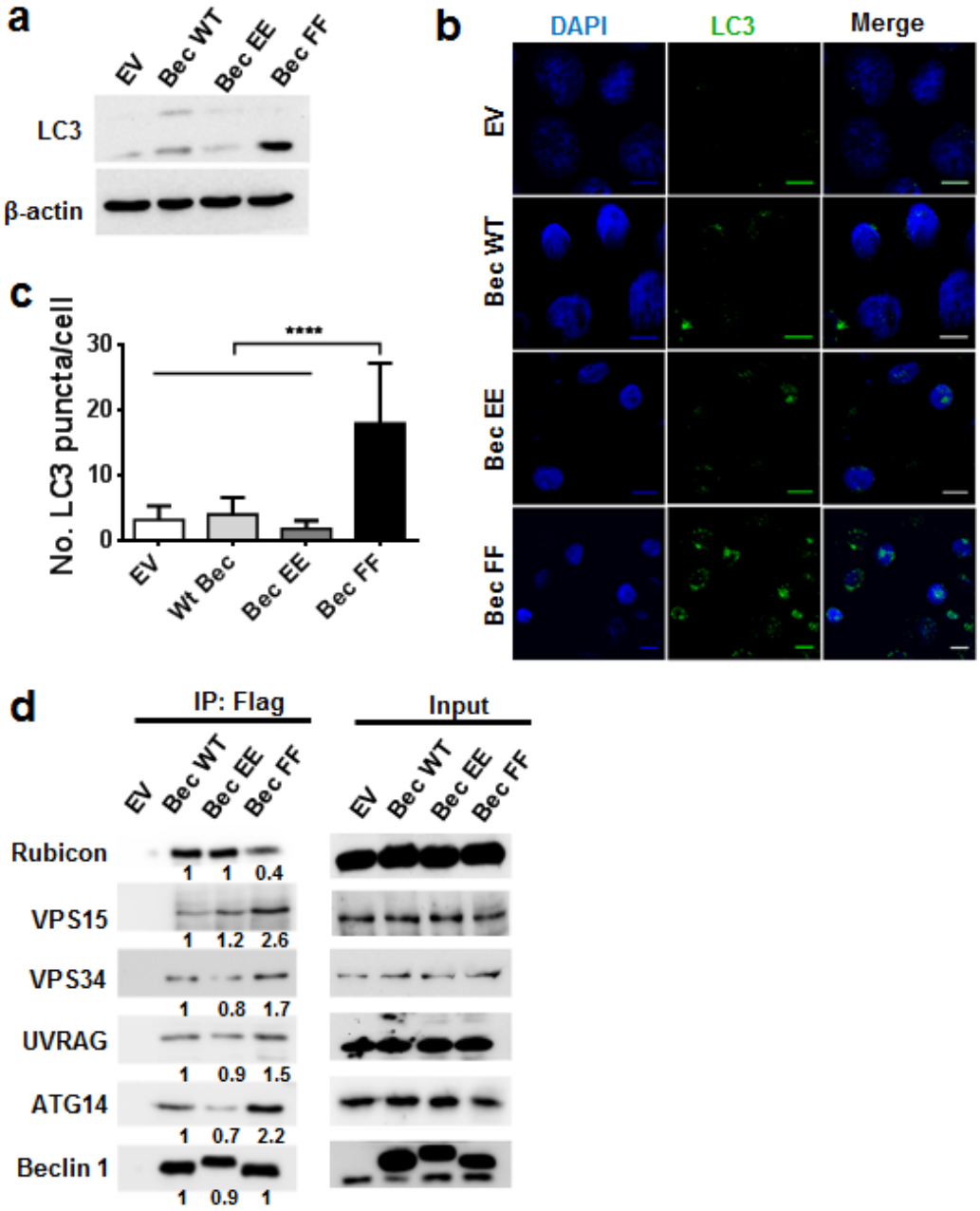

Figure 21. Effect of Beclin 1 tyrosine phosphorylation on autophagy.

(a) Beclin 1 tyrosine phosphorylation-deficiency mutant induces autophagy. LC3 level was evaluated by Western blotting in indicated Beclin 1 mutant-transduced K562 cells. (b) LC3 puncta was measured by confocal microscopy in K562 cells transduced with *mCherry-EGFP-LC3* and indicated *Beclin 1* construct. Cells were stained with anti-LC3 antibody, and nuclei were counterstained with DAPI. Scale bar, 10μm. (c) Quantification of LC3 puncta. K562 cells transduced with *mCherry-EGFP-LC3* and indicated *Beclin 1* construct were used for LC3 puncta counting. Bars are mean ± SD. ****$p < 0.0001$, student's *t* test. (d)

Beclin 1 tyrosine phosphorylation site mutations alter Beclin 1 binding-partners recruitment. Immunoprecipitation of indicated protein (ATG14, UVRAG, VPS34，VPS15 and Rubicon) with Flag in K562 cells transduced with indicated *Flag-Beclin 1* construct.

WT, wild-type Flag-Beclin 1; FF, Flag-Beclin 1 Y233F/Y352F; EE, Flag-Beclin 1 Y233E/Y352E

3.9 Beclin 1 phosphorylation-mimic mutant is resistance to BCR-ABL inhibition mediated autophagy

Since drug resistance became a major problem for BCR-ABL$^+$ leukemia treatment, scientists have made great efforts to circumvent this resistance. However, the exact mechanism is still not completely clear. As it is shown above, TKI-mediated BCR-ABL inhibition can trigger autophagy. To further identify whether Beclin 1 phosphorylation mutants could alter autophagy upon TKI treatment, LC3-II expression, as well as LC3 puncta were measured. From Figure 22a-c, phosphorylation-mimic Beclin 1 Y233E/Y352E impairs BCR-ABL inhibition-mediated autophagy induction in case of nilotinib treatment, which was confirmed by measurement of LC3-II expression and LC3 puncta. Next, Beclin 1 immunoprecipitation could show that Beclin 1 Y233E/Y352E-caused resistance to BCR-ABL inhibition mediated autophagy induction is due to less ATG14, UVRAG, VPS15 recruitment and more Rubicon recruitment to Beclin 1 complex (Figure 22d). Thus, Beclin 1 tyrosine dephosphorylation may be essential for TKI-induced autophagy.

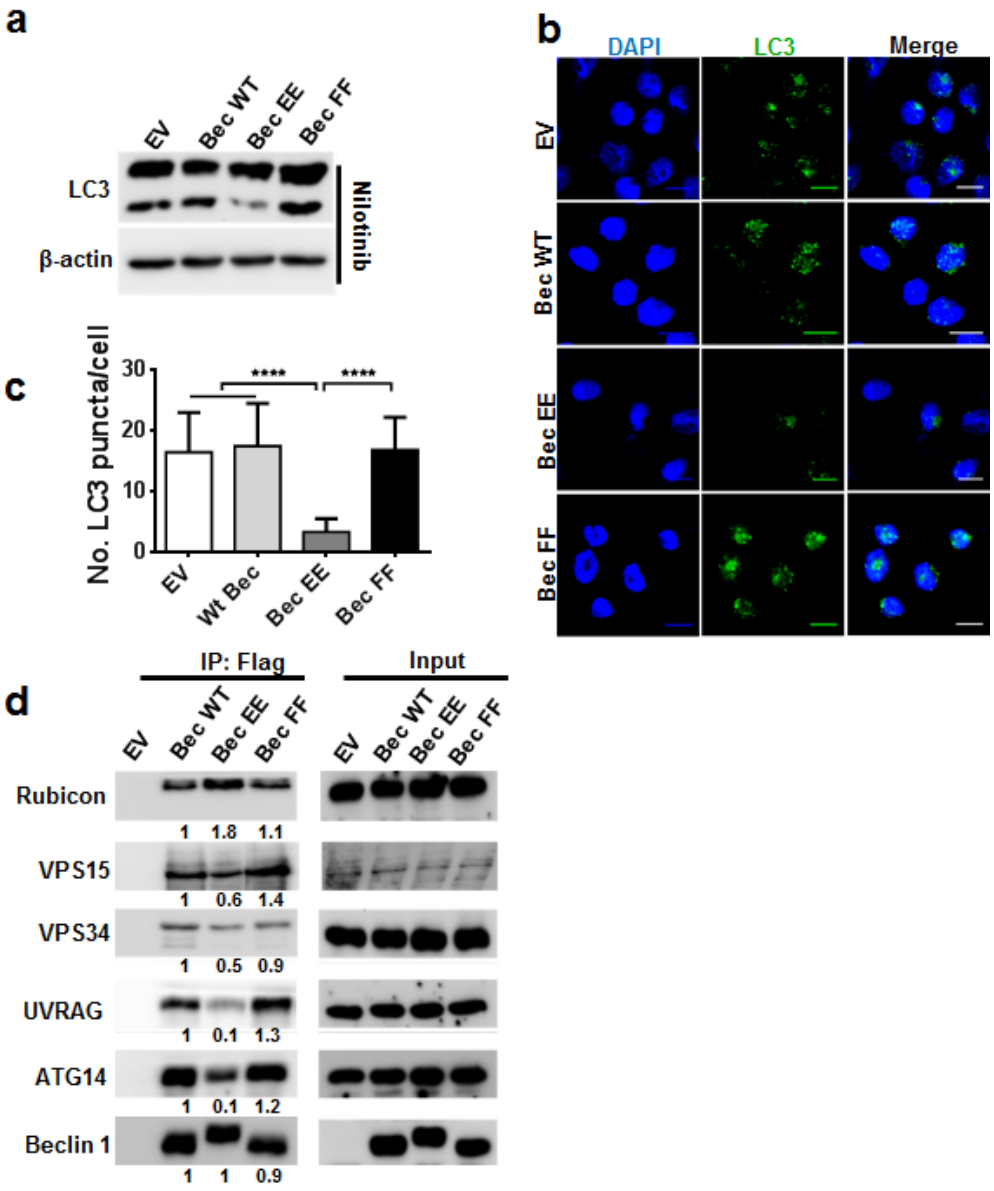

Figure 22. Effect of Beclin 1 tyrosine phosphorylation on autophagy upon nilotinib treatment.

(a) Beclin 1 tyrosine phosphorylation-deficiency mutation induces autophagy. LC3 level was evaluated by Western blotting in indicated Beclin 1 mutant-transduced K562 cells. (b) LC3 puncta was measured by confocal microscopy in K562 cells transduced with *mCherry-EGFP-LC3* and indicated *Beclin 1* construct upon nilotinib treatment. Cells were stained with anti-LC3 antubody, and nuclei were counterstained with DAPI. Scale bar, 10μm. (c) Quantitation of LC3 puncta. K562 cells transduced with *mCherry-EGFP-LC3* and indicated *Beclin 1* construct were used for LC3 puncta measurement. Bars are mean ± SD. ****p <

0.0001, Student's *t* test. (d) Beclin 1 tyrosine phosphorylation site mutations alter Beclin 1 binding-partners recruitment towards nilotinib treatment. Immunoprecipitation of indicated protein (ATG14, UVRAG, VPS34 and Rubicon) with Flag in K562 cells transduced with indicated *Flag-Beclin 1* construct.

3.10 Beclin 1 tyrosine phosphorylation does not affect starvation-mediated autophagy

It has been well documented that starvation is a standard treatment for autophagy induction. Recently, it was showed that Beclin 1 S90 phosphorylation is involved in starvation-mediated autophagy induction (Wei et al., 2015). To test whether Beclin 1 tyrosine phosphorylation at Y233 and Y352 can influence starvation-mediated autophagy, LC3 was measured in K562 cells in case of starvation treatment. Starvation induces autophagy measured by increased LC3-II expression and accumulation of LC3 puncta in K562 cells (Figure 23a-c), indicating that active BCR-ABL could not suppress starvation-mediated autophagy. To further confirm the effect of Beclin 1 phosphorylation on starvation-mediated autophagy, the K562 cells expressing tyrosine phosphorylation-mimic Beclin 1 (Beclin 1 Y233E/Y352E) and phosphorylation-deficient Beclin 1 (Beclin 1 Y233F/Y352F) were starved. LC3 measurement showed that starvation triggers autophagy in both Beclin 1 tyrosine phosphorylation-mimic mutant and Beclin 1 phosphorylation-deficiency mutant (Figure 23d-f). These data suggest that Beclin 1 tyrosine phosphorylation is not involved in starvation-mediated autophagy.

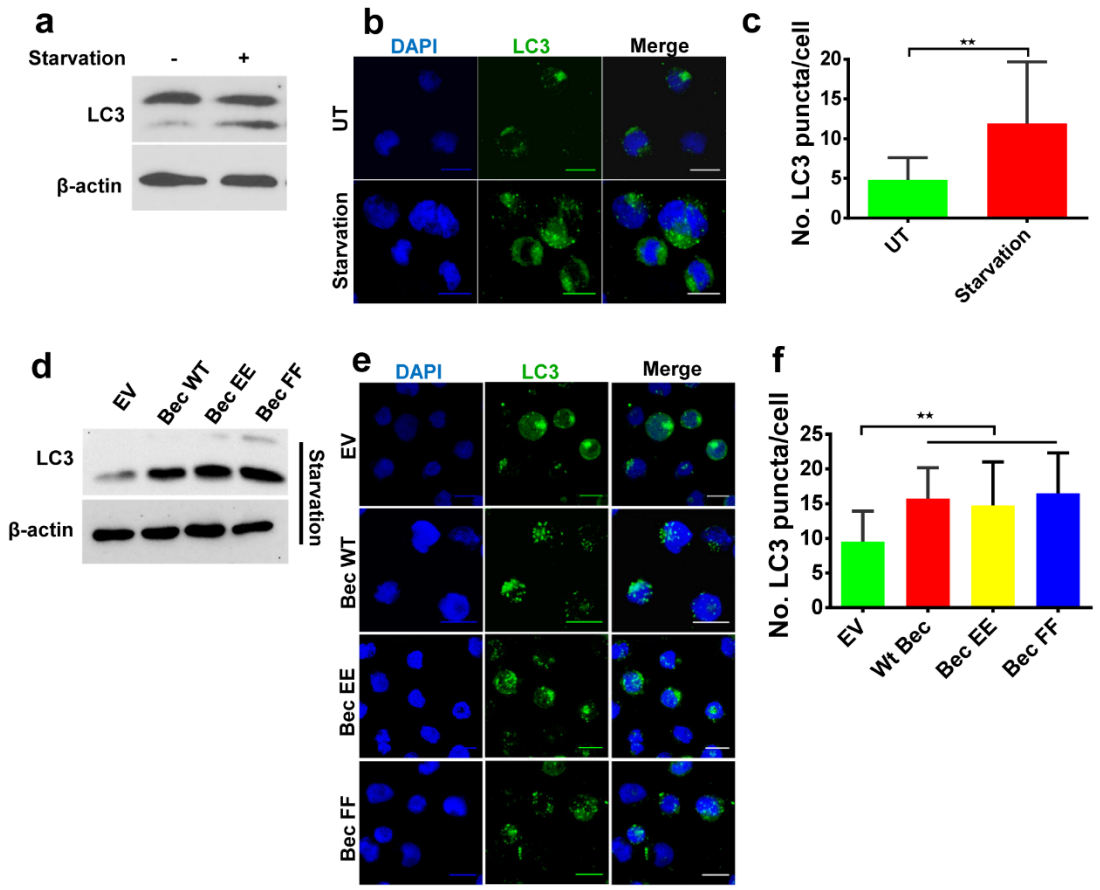

Figure 23. Beclin 1 tyrosine phosphorylation does not influence starvation-induced autophagy.

(a) LC3 expression level was detected in K562 cells with/without starvation. (b) LC3 puncta was measured by confocal microscopy in K562 cells transduced with *mCherry-EGFP-LC3* upon starvation. Scale bar, 10μm. (c) Quantification of LC3 puncta in K562 cells with/without starvation. Bars are mean ± SD. ****p < 0.0001, Student's *t* test. (d) LC3 expression level was detected in K562 cells transduced indicated *Beclin 1* construct towards starvation. (e) LC3 puncta were measured by confocal microscopy in K562 cells transduced with *mCherry-EGFP-LC3* and indicated *Beclin 1* construct upon starvation. Scale bar, 10μm. (f) Quantitation of LC3 puncta in K562 cells transduced with *mCherry-EGFP-LC3* and indicated *Beclin 1* construct upon starvation. Bars are mean ± SD (⩾1000 cells analyzed per sample). ****p < 0.0001, Student's *t* test.

3.11 Beclin 1 phosphorylation is crucial for BCR-ABL-driven leukemogenesis

As it is demonstrated above, BCR-ABL-mediated Beclin 1 phosphorylation plays a negative role in autophagy, and targeting Beclin 1 might be a potential strategy for BCR-ABL$^+$ leukemia treatment. To further confirm the function of Beclin 1 phosphorylation in BCR-ABL-mediated leukemogenesis, *Beclin 1 miR* plus *BCR-ABL* together with *Beclin 1* phosphorylation mutants were employed in a bone morrow transplantation mouse model. Wild type Beclin 1 mice and Beclin 1 phosphorylation mice show much more aggressive disease with more WBC amount and shorter life span compared to empty control group and the Beclin 1 non-phosphorylation mutant group, and the mice in *Beclin 1* non-phosphorylation mutant group are similar to the mice in the control group (Figure 24). The difference between “phosphorylation” group and “non-phosphorylation” group is small, especially in the mice survival experiment. This might be because Beclin 1 FF is not a complete function-null mutant and it might have some unknown function that is still involved in BCR-ABL-mediated leukemogenesis. Another possibility might be that human Beclin 1 (which was transplanted) cannot mimic the murine Beclin 1 function exactly, although the human Beclin 1 and murine Beclin 1 have highly similar amino acid sequences. Last but not least, these data partially explain the critical role that Beclin 1 phosphorylation plays in BCR-ABL-mediated leukemogenesis.

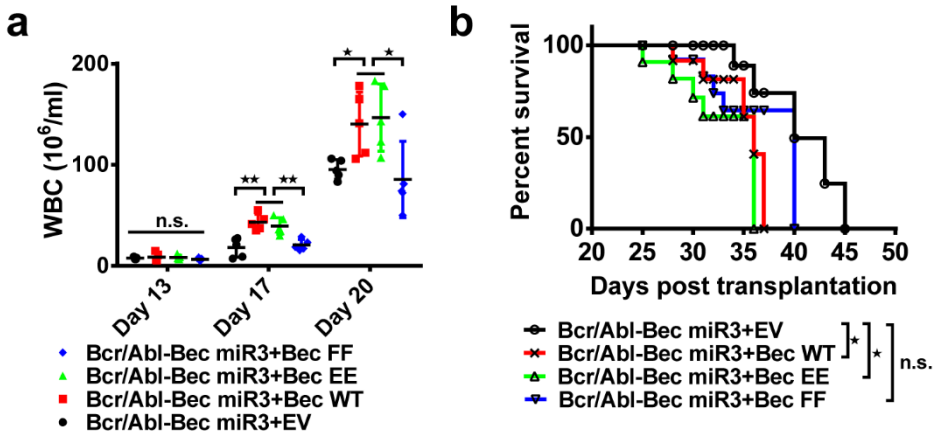

Figure 24. Beclin 1 phosphorylation is crucial for BCR-ABL-mediated leukemogenesis in BM transplantation model.

Beclin 1 miR based endogenous *Beclin 1* knockdown plus re-expression of indicated *Beclin 1* mutant together with *BCR-ABL* expression in bone morrow transplantation mouse model. (a) At indicated time post transplantation, mice from wild type Beclin 1 and Beclin 1 phosphorylation mutant group were taken for WBC measurement. (b) Mice life span was measured.

n.s., no significance, *p < 0.05, **p < 0.01 , Student's *t* test or Kaplan-Meier test.

4 Discussion

4.1 An alternative treatment strategy is needed for diseases driven by oncogenic kinases

In 2001, the FDA approved the first kinase inhibitor, imatinib, for clinical use. Since then, more and more specific inhibitors have become a front-line treatment option in several diseases including cancer. However the appearance of drug resistance has emerged as a great challenge for the therapy of kinase-driven diseases. These great achievements encourage scientists to focus on kinase inhibitor development and mechanism of kinase inhibitors for diseases treatment. Thus, numerous studies are now trying to understand the mechanism of drug resistance. Furthermore, highly specific second and third generation inhibitors are now available. However, problems of side effect or secondary drug resistance mutations still need to be addressed, these problems also appear in BCR-ABL$^+$ leukemia therapy (Apperley, 2007; Khorashad et al., 2013). To overcome or avoid the drug resistance, alternative strategies are urgently needed. A promising strategy has been proposed in focusing on BCR-ABL downstream signaling molecules. Another possibility is to target pathways that are aberrantly regulated by BCR-ABL. Therefore a better understanding of the involvement of BCR-ABL in various cellular mechanisms is required.

A series of screens on drug resistant oncogenic kinases have been conducted aiming to find alternative strategies in oncogenic kinase-driven disease therapy (Albers et al., 2013; Kancha et al., 2011a; Kancha et al., 2011b; Kancha et al., 2009; von Bubnoff et al., 2009; von Bubnoff et al., 2011; von Bubnoff et al., 2006; von Bubnoff et al., 2010). As an example, constitutively activate FLT3-ITD is a frequent occurrence in AML patients, imparting poor prognosis (Thiede et al., 2002). A number of FLT3 inhibitors have been tested in FLT3-ITD$^+$ AML treatment, however drug resistance has prevented effective treatment of the disease. HSP90 inhibition in combination with FLT3 inhibition was shown to effectively decrease cell proliferation *in vitro*, which might be a promising approach to overcome this issue (Yu et al., 2014). FLT3-ITD is a

client kinase for the HSP90 chaperone, and HSP90 inhibitor treatments resulted in degradation of inhibitor-resistant FLT3-ITD mutants. In case of BCR-ABL, TKI-caused drug resistance currently is a major problem in CML therapy. To overcome it, several strategies have been tested and shown favorable effects, such as the treatment by HSP90 inhibitor (Peng et al., 2007; Radujkovic et al., 2005), histone deacetylase inhibitor (Carew et al., 2007; Nimmanapalli et al., 2003), proteasome inhibitor (Gatto et al., 2003; Yu et al., 2003) or arsenic trioxide (Du et al., 2006; Goussetis et al., 2012). Therefore, selectively inducing cellular toxicity may prevent the emergence of secondary drug resistance in the leukemia patients. These findings suggest that targeting multiple properties of oncogenic kinases might be a potential therapeutic choice to overcome drug resistance.

Exposing oncogenic kinase transformed cells to TKIs induces cellular stress (Blume-Jensen and Hunter, 2001; Filosto et al., 2013), a process that might drive the development of drug resistance mutations. In order to develop new therapy strategies, it is not only necessary to uncover the signaling properties of the resistance mutation, but also critical to understand the mechanism, which leads to the acquisition of drug resistance mutations. Autophagy is a mechanism maintaining cellular homeostasis, providing the cell with a means to survive under stress conditions. Therefore, autophagy might be an early mechanism involved in development of drug resistance mutations. A better understanding of the contribution of autophagy in the survival mechanism under TKI-mediated stress conditions is critically required, which may provide novel and efficient approaches to overcome drug resistance.

4.2 Autophagy is involved in BCR-ABL-mediated leukemia induction via the central regulator Beclin 1

Stimulation of the cells by extracellular molecules like growth factors induces a signal that is transmitted inside the cell, leading to responses such as increased proliferation or differentiation. Oncogenic kinase like BCR-ABL can mimic this stimulation to activate downstream signaling pathways leading to aberrant regulation of basal cellular processes. Autophagy is a mechanism, which includes the degradation of

damaged organelles and misfolded proteins under stress conditions. In this study, it was found that BCR-ABL activation suppresses autophagy, indicating that BCR-ABL signaling alternates the cellular homeostasis balance. The autophagy induction was dependent on kinase activity of BCR-ABL leading to disruption of the balance. In line with these observations, several previous studies showed that TKI treatment induces autophagy in BCR-ABL^{+} cell line or patient samples (Bellodi et al., 2009; Helgason et al., 2011; Yu et al., 2012), indicating that autophagy induction is beneficial for BCR-ABL^{+} leukemia treatment. This TKI-related autophagy induction could re-modify the cellular homeostasis and get the aberrant mechanism back to balance. Every result has a reason; there must be some mediators between BCR-ABL and autophagy suppression.

As reviewed above, many proteins function in autophagy. Here, the proteins involved in BCR-ABL mediated autophagosome formation were investigated. Recruitment of VPS34, UVRAG and ATG14 to Beclin 1 was increased in case of nilotinib treatment and could thereby positively regulate autophagosome formation, whereas Rubicon, a negative regulator, was less recruited to the Beclin 1 complex. This observation suggested that the proteins of the Beclin 1 complex are crucial for BCR-ABL-mediated autophagy suppression. It has been demonstrated that Beclin 1 has significant tumor suppression effect in breast cancer, and Beclin 1$^{+/-}$ mice were prone to spontaneous malignancies, indicating that decreased Beclin 1 contributes to tumorigenesis (Aita et al., 1999; Liang et al., 1999; Qu et al., 2003). Those reports may explain why autophagy induction is beneficial for cancer therapy. The Beclin 1 core complex (VPS34, VPS15, ATG14, UVRAG and Rubicon) is a main regulator of autophagy and alteration of this complex strongly decreases autophagic activity (Funderburk et al., 2010; He and Levine, 2010). A central function of the Beclin 1 core complex is the control of cellular VPS34 lipid kinase activity that is essential for autophagy and other membrane trafficking processes. In this complex, Beclin 1-VPS15-VPS34 acts as a scaffold; UVRAG or ATG14 positively regulate autophagy function, whereas Rubicon acts as a negative regulator. Inhibition of BCR-ABL alternates the Beclin 1

interactome with more recruitment of VPS34, UVRAG and ATG14 to Beclin 1 and less recruitment of Rubicon to Beclin 1 leading to autophagy induction. These findings indicate that Beclin 1-mediated autophagy alternation might be a potential mechanism involved in BCR-ABL-mediated disease induction.

Genetically modified mice with defective autophagy in the whole body or specific tissues have been reported to be susceptible to pathogen infection, muscle damage, neurodegeneration and chronic inflammation, that can eventually lead to development of cancer (Cherra and Chu, 2008; Liang et al., 1998; Masiero et al., 2009; White et al., 2010; Yuk et al., 2012). In humans, defects in autophagy genes have been associated with the development of various pathologic conditions, including cancer (Kang et al., 2009; Knaevelsrud et al., 2010; Liang et al., 1999). Nevertheless, it is always unclear whether the defective phenotype is attributed to gene-specific autophagy independent functions or dysfunctional autophagy. In this study, a miR-30 based-Beclin 1 genetical approach was used in BCR-ABL$^+$ cell model and mice model. It was observed that Beclin 1 downregulation decreases the proliferation of BCR-ABL$^+$ cells both *in vitro* and *in vivo*. It was also found that Beclin 1 knockdown results in less aggressive disease with more WBC amount and longer lifespan in BCR-ABL transplanted mice. These findings uncover that autophagy regulator Beclin 1 is essential for BCR-ABL-mediated leukemia. Targeting Beclin 1 might be a promising strategy for BCR-ABL$^+$ leukemia therapy. Contrarily, the Beclin 1 overexpression has favorable effect in several solid tumors, such as breast cancer, ovarian cancer and hepatocellular carcinoma (Cai et al., 2014; Liang et al., 1999; Qiu et al., 2014). In this study, we could show that Beclin 1 is phosphorylated in BCR-ABL$^+$ leukemia. Hence, the phosphorylation status of Beclin 1 may explain why Beclin 1 behaves differently in distinct cancer types.

4.3 BCR-ABL mediated loss-of-function of Beclin 1 leads to autophagy suppression

BCR-ABL contains several domains that function as binding sites for adaptor proteins leading to the activation of a number of downstream signaling pathways

(Quintas-Cardama and Cortes, 2009; Ren, 2005). Surprisingly it could be demonstrated that the BCR part, but not the ABL kinase part, of BCR-ABL is binding directly to Beclin 1, and that this interaction leads to Beclin 1 phosphorylation. It is likely that that the BCR part serves as an “adaptor” to get ABL kinase and Beclin 1 in close proximity, which is a prerequisite for BCR-ABL-mediated Beclin 1 phosphorylation. Without a doubt, BCR-ABL is a quite strong kinase that can phosphorylate many molecules. Nevertheless, “adaptor BCR”-mediated interaction between BCR-ABL and Beclin 1 may make the phosphorylation happen easily.

Protein modifications like phosphorylation, acetylation, methylation, and ubiquitylation are essential processes in cellular homeostasis (Bannister and Kouzarides, 2011; Welchman et al., 2005). In the present study, it could be shown that BCR-ABL induces alterations in Beclin 1 complex formation, leading to suppression of autophagy. These findings raise the question how Beclin 1 regulates autophagy and what the causes that alter the affinity of Beclin 1 to its complex members are. Beclin 1 is directly phosphorylated by BCR-ABL at tyrosine residues 233 and 352, which are critical regulatory sites of Beclin 1 function in autophagy (Wei et al., 2013), resulting in autophagy suppression. Previous reports have shown that AKT-mediated phosphorylation of Beclin 1 (S234 and S295) and EGFR-mediated phosphorylation of Beclin 1 (Y229, Y233 andY352) function in autophagy inhibition, oncogenesis, and alteration of Beclin 1 interactome. (Wang et al., 2012; Wei et al., 2013). Several lines of evidence indicate that Beclin 1 phosphorylation leads to suppression of autophagy due to Beclin 1 complex dysregulation (Wang et al., 2012; Wei et al., 2015; Wei et al., 2013). Reduced autophagic activity has been described to contribute to tumor formation in breast cancer, ovarian cancer, lung cancer and CML. In a study of EGFR-mediated Beclin 1 phosphorylation, overexpression of a Beclin 1 tyrosine phosphomimetic (Y229E/Y233E/Y352E) in lung cancer mouse model results in decreased autophagy, increased cellular proliferation, and accelerated tumor growth (Wei et al., 2013). In this study, overexpression of Beclin 1 tyrosine phosphomimetic (Y233E/Y352E) in BCR-ABL+Beclin 1 miR-transplanted mice results in much more

aggressive disease with more WBC amount and less survival time, whereas overexpression of Beclin 1 tyrosine phosphodeficiency (Y233F/Y352F) is similar to empty vector group and contrasts the observed effect of the group bearing a phosphomimetic Beclin1. Phosphorylation of Beclin 1 may play a crucial role in BCR-ABL-mediated leukemogenesis, and the phosphorylation can serve as an oncogenic event in BCR-ABL$^+$ leukemia. These findings indicate that Beclin 1 is not only important in autophagy induction, but also critical for tumor suppression. Thus, Beclin 1 phosphorylation could serve as a clinical relevant marker in various oncogenic kinases-mediated disorders.

4.4 Beclin 1 is a target of BCR-ABL

Autophagy is an esoteric mechanism (Baehrecke, 2005; Debnath et al., 2005; Hippert et al., 2006): On one hand, it may be considered as a cell survival factor to promote cell proliferation. On the other hand, it can also be viewed as a pathway suppressing cell growth. Complicating this situation further, stress events always induce autophagy, but whether this is a death mechanism or potent function for cell preservation is not clear (Mizushima, 2007). Some studies have demonstrated high basal autophagic levels in some cancer cells; for example, autophagy is required for tumorigenic growth in pancreatic cancers (White, 2012; Yang et al., 2011). One interesting study showed that inactive EGFR is an autophagic inducer. Yet, active EGFR is an autophagic inhibitor, pointing towards an activated-kinase-independent role of EGFR in autophagy. These results may explain chemoresistance after TKI treatment in tumors harboring activating EGFR mutations with the expression of inactive, non-mutant EGFR (Tan et al., 2015).

Here it was shown that Beclin 1 can be phosphorylated by BCR-ABL, but not other oncogenic kinases like FLT3-ITD, PDGFRA-V842D or NPM-ALK, indicating that Beclin 1 is a specific target of BCR-ABL. In a murine transplantation model, *Beclin 1* knockdown dramatically delays BCR-ABL-driven leukemogenesis, whereas *Atg5* deletion does not affect BCR-ABL mediated leukemogenesis. These data suggested

that Beclin 1, but not Atg5, is a target of BCR-ABL. These results also point towards the role of Beclin 1 beyond autophagy induction in BCR-ABL-mediated leukemia. Furthermore, it could be that Beclin 1 associated autophagy pathway is essential for BCR-ABL-mediated leukemogenesis. These findings are in agreement with previous studies that propose targeting autophagy as a promising strategy for treatment of malignancies (Bellodi et al., 2009; Tan et al., 2015; Wei et al., 2013; Yu et al., 2012). Thus, autophagy might be a potential target in the development of novel strategies to overcome TKI drug resistance. Additionally, the present study showed that Beclin 1 downregulation enhances TKI toxicity in BCR-ABL expressing cells. A possible explanation is that oncogenic kinase-driven cells use autophagy induction to attenuate cellular stress triggered by TKI-treatment. Therefore, autophagy acts as mechanism for the cancer cells to escape apoptosis. Targeting autophagy could potentially impair this survival mechanism.

4.5 Future perspectives

Autophagy is an essential mechanism for cell survival, implicating a role of this process in malignant diseases. Although a number of autophagy-related proteins have been reported, it still remains unclear how these proteins regulate cellular processes and how autophagy contributes to cellular survival. The present study uncovers a crucial role for Beclin 1 phosphorylation in BCR-ABL-driven leukemia. It provides further insights into the mechanism of BCR-ABL-mediated autophagy suppression. It could be demonstrated that BCR-ABL directly phosphorylates Beclin 1 upon Beclin 1 complex alteration, indicating that Beclin 1-mediated autophagy inhibition plays an important role in BCR-ABL$^+$ leukemia. These findings provide a rationale that targeting autophagy, especially Beclin 1, as a potential alterative strategy in BCR-ABL$^+$ leukemia therapy.

As it is shown (Figure 25), these findings demonstrate a link between oncogenic signaling and the core autophagic machinery, by which BCR-ABL binds to Beclin 1, leading to its tyrosine phosphorylation and consequent inhibition of its autophagy

function by alteration of its affinity to ATG14, UVRAG, VPS34 and Rubicon. BCR-ABL signaling may be mechanistically linked to autophagy inhibition and tumorigenesis through regulation of the Beclin 1 complex. These findings also demonstrate a specific plausible mechanism suppressing autophagy in human cancer. Cross-talk between oncogenic kinases and autophagy proteins might represent a fundamental mechanism underlying the regulation of mammalian cell growth control and cancer. Furthermore, it is becoming necessary to evaluate whether Beclin 1 or Beclin 1-mediated autophagy regulates BCR-ABL$^+$ hematopoietic stem cells proliferation, self-renewal, and differentiation; and whether Beclin 1 or Beclin 1-mediated autophagy directly functions in BCR-ABL-mediated aberrant signaling pathways resulting in cell proliferation and cell death. Therefore, the development of a relevant autophagic inhibitor, specifically a Beclin 1 inhibitor, is critical, in combination with TKIs, they could potentially lead to eradication of CML stem cells and provide a benefit for the BCR-ABL$^+$ patients.

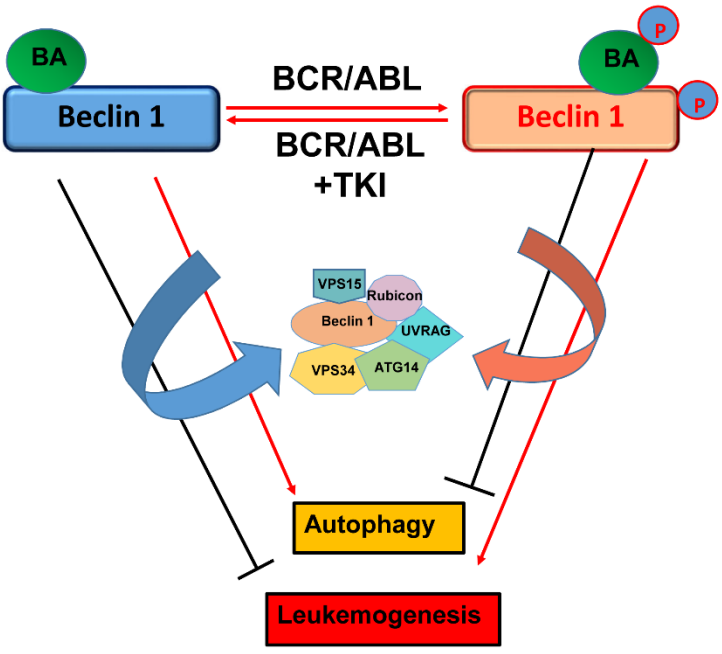

Figure 25. Schematic representation of Beclin 1 involvement in BCR-ABL-mediated autophagy suppression.

BCR-ABL binds to Beclin 1 leading to Beclin 1 phosphorylation, and although this binding is independent of BCR-ABL activation, an active BCR-ABL binds and suppresses autophagy through Beclin 1 complex via alternation of Beclin 1 affinity to Beclin 1-binding partners. Beclin 1 is a specific target of BCR-ABL, which is crucial for BCR-ABL-driven leukemogenesis.

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The role of JAK kinases in oncogenic CSF3R mutation-mediated leukemogenesis. Saig, Germany. Work shop 2015.

Beclin 1 is essential to allow BCR-ABL-mediated leukemogenesis. Saig, Germany. Work shop 2014.

HSP90 inhibitors overcome drug resistance caused by FLT3 kinase domain mutations. Vienna, Austrian. DGHO 2013.

Publications

Publications related to PhD work

1. **Yu C**, et al. Phosphorylation of Beclin 1 by BCR-ABL suppresses autophagy in CML. (Submitted)
2. **Yu C**, et al. Unresponsiveness of CSF3R point mutations to JAK kinases inhibition but not truncation mutations. (Manuscript in preparation)
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DECLARATION

Erklärung nach § 8 der Promotionsordnung der Albert-Ludwigs-Universität Freiburg für die Fakultät für Biologie und die Medizinische Fakultät im Fachbereich „Molekulare Medizin“

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe der Quelle gekennzeichnet. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe von Vermittlungs- beziehungsweise Beratungsdiensten (Promotionsberater oder anderer Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Die Bestimmungen der Promotionsordnung der Fakultät für Biologie und der Medizinischen Fakultät für Absolventen des interfakultären Diplomstudienganges Molekulare Medizin der Universität Freiburg sind mir bekannt; insbesondere weiß ich, dass ich vor Vollzug der Promotion zur Führung des Doktortitels nicht berechtigt bin.

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Place, date Signature