Autophagic degradation determines the fate of T315I-mutated BCR-ABL protein

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Supplementary Information

Materials and Methods

Patient blood samples

Blood samples from newly diagnosed CML patients were collected following a protocol approval by the institutional review board and with the informed consent of the patients.

Cell culture and treatment

WT-, M351T-, Y253F- or T315I-BCR-ABL-transformed mouse pro-B Baf3 cell lines (Baf3p210) were established as reported previously,[1] and obtained as gifts from Dr. Brian J. Druker, Oregon Health and Science University Cancer Institute. WT-BCR-ABL-positive human ALL cell line TCCY and its imatinib-resistant clone having T315I-mutated BCR-ABL were gifted from Dr. Yuko Sato, International Medical Center of Japan. WT-BCR-ABL-harboring TCCY cell line was established from an ALL patient as reported previously.[2] The WT-BCR-ABL-harboring TCCY cells were treated with imatinib by gradually increasing the concentration (3-20 μM). The dead cells were washed out every 3 to 4 days, and the resistant subclones were isolated by limiting dilution. Cells were tested for mycoplasma contamination by using a MycoAlert Mycoplasma Detection Kit (LT07-118; Lonza, Rockland, ME, USA). Cells were cultured under an atmosphere of 95% air and 5% CO_2 at 37 °C in RPMI-1640 medium (189-02025; Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, St. Louis, MO, USA). Validation of the BCR-ABL protein levels and growth rate of WT and T315I cells in the steady state are shown in Figure S1.

AIC-47 was synthesized as reported earlier.[3] AIC-47, imatinib (I0936; Tokyo Chemical Industry, Tokyo, Japan), ponatinib (CS-0204; ChemScene, Monmouth Junction, NJ,

USA), Z36 (ab141757; Abcam, Cambridge, MA, USA), ABT-737 (ab141336; Abcam), 3-methyladenine (3-MA; Sigma-Aldrich), and chloroquine (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture medium at a final concentration of DMSO (<0.3%), which concentration showed no significant effect on the growth and differentiation of the cells (data not shown). Rapamycin and U0126 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Sequencing of the Abl kinase domain

The mutations of BCR-ABL were confirmed by sequencing of the Abl kinase domain. Primers for PCR amplification were *BCR-Forward* (5'-ACAGCATTCCGCTGACCATCAATAAG-3') and *ABL-Reverse* (5'-AGAACTTGTTGTAGGCCAG-3'). The PCR products were then subjected to automated sequencing by using primer *ABL-Forward* (5'-TCCCCCAACTACGACAA-3').

Western blotting

Protein extraction and Western blotting experiments were performed as described previously.[4] Antibodies against the following proteins were purchased from Cell Signaling Technology: Phospho-Abl (#2861), LC3B (#3868), p62 (#5114), Beclin-1 (#3495), Atg5 (#8540), Atg7 (#2631), phospho-mTOR (#5536), mTOR (#2983), Bcl-2 (#3498), Bcl-xL (#2764), Bad (#9239), phospho-SAPK/JNK (#4668), SAPK/JNK (#9258), phospho-p44/42MAPK (#4370), and p44/42MAPK (#4695). Antibodies against c-Abl (sc-131), p-mTOR (sc-293133), Bcl-2 (sc-509), p-Bcl-2 ser70 (sc-293128), Bik (sc-365625), and NOXA (sc-56169) were obtained from Santa Cruz Biotechnology

(Santa Cruz, CA, USA). Anti-Ras antibody (ab55391) was purchased from Abcam. The loading control was prepared by re-incubating the same membrane with anti-β-actin antibody (A5316; Sigma-Aldrich).

Real-time RT-PCR

Total RNA was isolated from cells by using a NucleoSpin miRNA kit (TaKaRa, Otsu, Japan) according to the manufacturer's protocol. The expression levels of mRNAs were determined as described previously.[3]

Tracking of *BCR-ABL* mRNA

The decay of *BCR-ABL* mRNA was traced by using a Click-iT RNA capture Kit (Invitrogen). WT- or T315I-BCR-ABL harboring Baf3p210 and TCCY cells were incubated overnight with 0.2 mM 5-ethynyl uridine (EU), which is naturally incorporated into nascent RNA. Total RNA was extracted from the cells after incubation for 0, 3, 6, 12, 24 or 48 h. EU-labeled RNA was purified and subjected to quantitative RT-PCR.

Immunocytochemistry

WT- or T315I-BCR-ABL-harboring Baf3p210 cells were incubated with AIC-47 for 24 h and then immunostained with LC3 (M152-3; MBL, Nagoya, Japan) and c-Abl (sc-131; Santa Cruz Biotechnology) antibodies as described previously.[4] The nuclei were stained with Hoechst33342. The cells were viewed with a LSM710 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Supplementary references for Materials and methods

1. La Rosee P, Johnson K, Corbin AS, et al. In vitro efficacy of combined treatment depends on the underlying mechanism of resistance in imatinib-resistant Bcr-Abl-positive cell lines. Blood. 2004;103(1):208-215.
2. Kano Y, Akutsu M, Tsunoda S, et al. In vitro cytotoxic effects of a tyrosine kinase inhibitor STI571 in combination with commonly used antileukemic agents. Blood. 2001;97(7):1999-2007.
3. Shinohara H, Taniguchi K, Kumazaki M, et al. Anti-cancer fatty-acid derivative induces autophagic cell death through modulation of PKM isoform expression profile mediated by bcr-abl in chronic myeloid leukemia. Cancer letters. 2015;360(1):28-38.
4. Shinohara H, Kumazaki M, Minami Y, et al. Perturbation of energy metabolism by fatty-acid derivative AIC-47 and imatinib in BCR-ABL-harboring leukemic cells. Cancer letters. 2016;371(1):1-11.

Supplementary Figures

Fig. S1

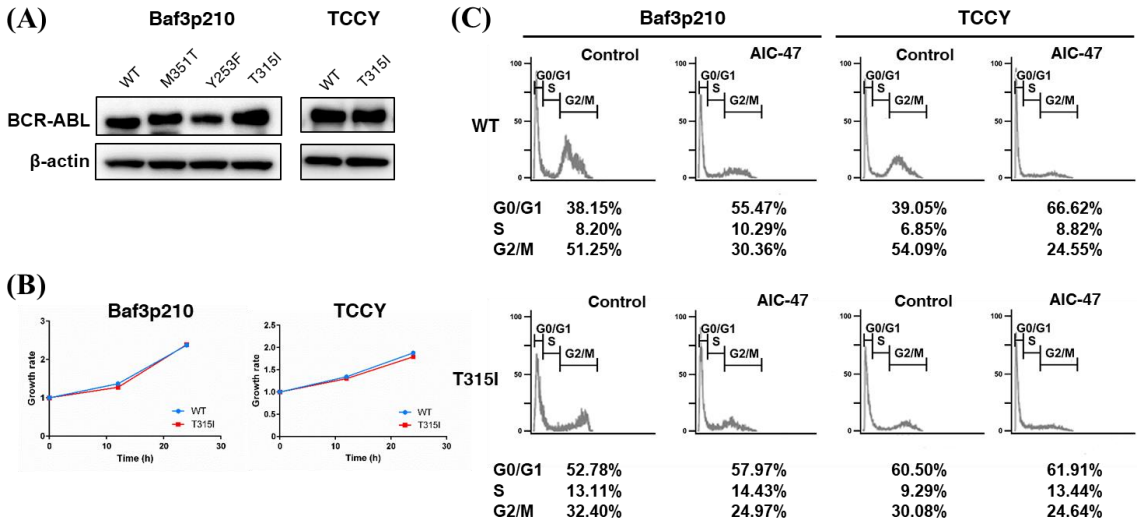

Figure S1

(A) Validation of expression levels of BCR-ABL protein in Baf3p210 and TCCY cells at steady state. **(B)** Validation of growth rate of Baf3p210 and TCCY cells at steady state. The growth rate was not significantly different between WT- and T315I-BCR-ABL-harboring cells **(C)** Effect of AIC-47 on cell-cycle profile of Baf3p210 and TCCY cells. WT- or T315I-BCR-ABL-harboring Baf3p210 and TCCY cells were treated with 10 μM AIC-47 for 24 h. DNA histograms were analyzed by PI staining and flow cytometry. Percent cells in each phase of the cell-cycle are indicated under the panels.

Fig. S2

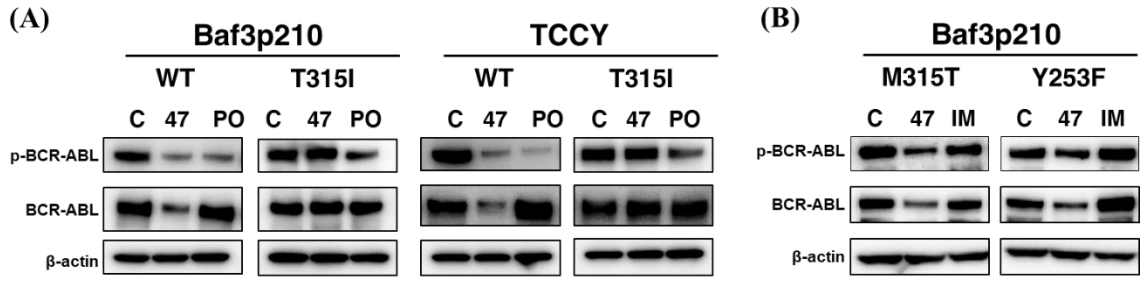

Figure S2

(A) Effects of 3rd TKI ponatinib on phosphorylation and expression of WT- and T315I-BCR-ABL in Baf3p210 and TCCY cells. The cells were treated with DMSO (Control; C), AIC-47 (47; 10 μM) or ponatinib (PO; 0.1 nM) for 48 h, followed by Western blotting analysis. **(B)** Effects of AIC-47 or imatinib on phosphorylation and expression of M351T- and Y253F-BCR-ABL in Baf3p210 cells. The cells were treated with DMSO (Control; C), AIC-47 (47; 10 μM) or imatinib (IM; 0.25 μM) for 48 h, followed by Western blotting analysis.

Fig. S3

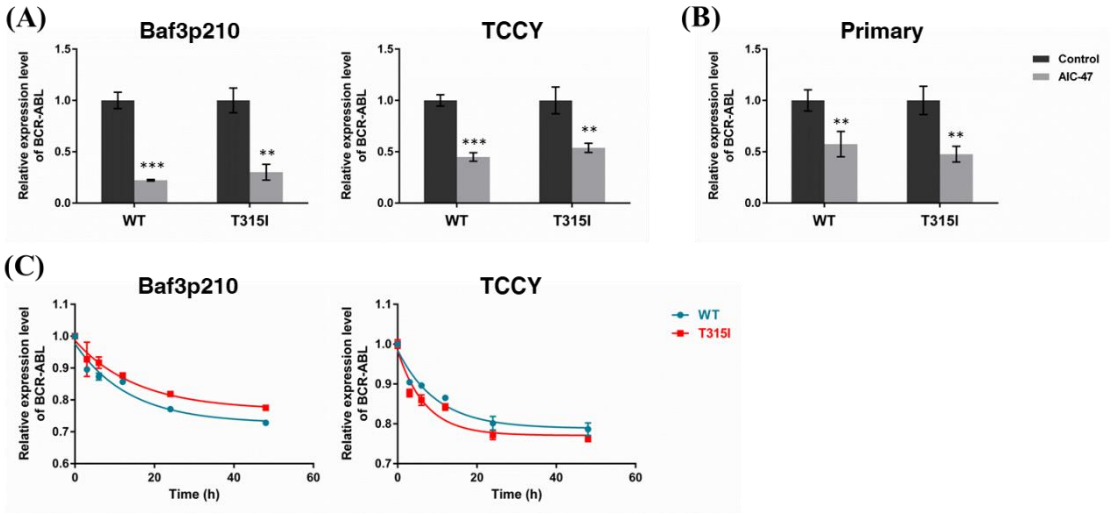

Figure S3

(A) Expression levels of *BCR-ABL* mRNA in Baf3p210 and TCCY cells. Cells were treated DMSO (Control) or AIC-47 (10 μM) for 24 h. Data are expressed as means ± SD of 3 different experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Control (Student's t test). **(B)** Expression levels of *BCR-ABL* mRNA in human primary CML cells from newly diagnosed patients. Cells were treated with DMSO (Control) or AIC-47 (10 μM) for 24 h. Data are expressed as means ± SD of 3 different experiments. $^{**}P < 0.01$ vs. Control (Student's t test). **(C)** Decay of *BCR-ABL* mRNA in the steady state. WT- or T315I-BCR-ABL-harboring Baf3p210 and TCCY cells were incubated overnight with 0.2 mM 5-ethynyl uridine (EU). EU-labeled RNA was purified and subjected to RT-PCR. Data are expressed as means ± SD of 3 different experiments.

Fig. S4

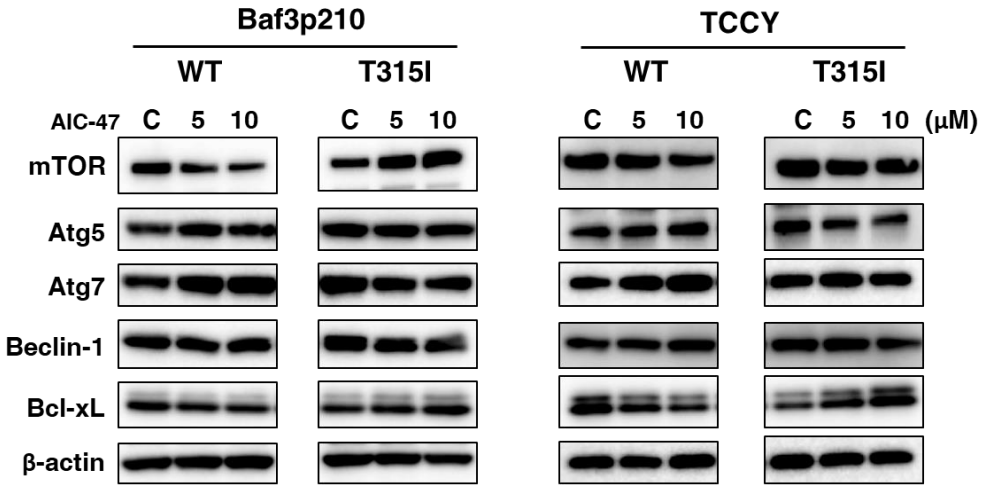

Figure S4

Effects of AIC-47 on expression of mTOR, Atg5, Atg7, Beclin-1, and Bcl-xL in Baf3p210 and TCCY cells. The cells were treated with DMSO (Control; C), AIC-47 (5 or 10 μM) for 24 h, followed by Western blotting analysis.

Fig. S5

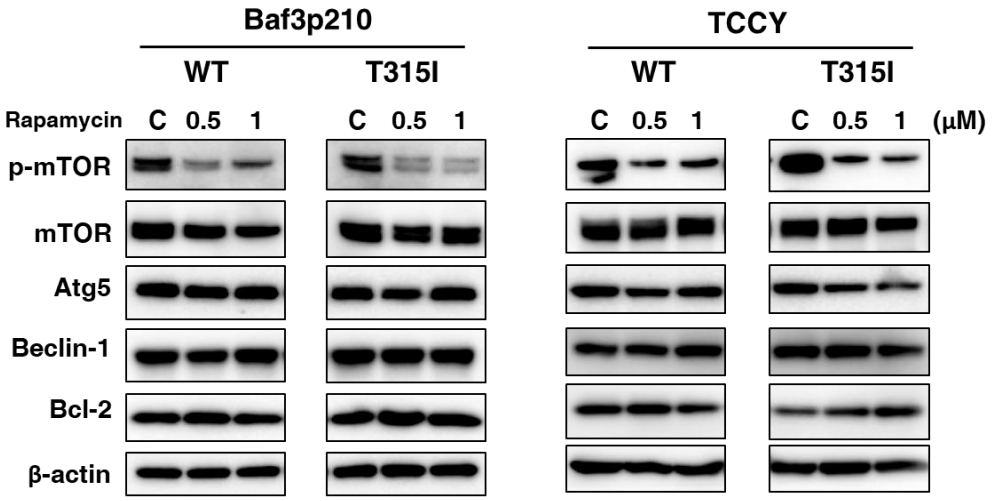

Figure S5

Effects of rapamycin on phosphorylation and expression of mTOR, Atg5, Beclin-1, and Bcl-2 in Baf3p210 and TCCY cells. The cells were treated with DMSO (Control; C), rapamycin (0.1 or 0.5 μM) for 24 h, followed by Western blotting analysis.

Fig. S6

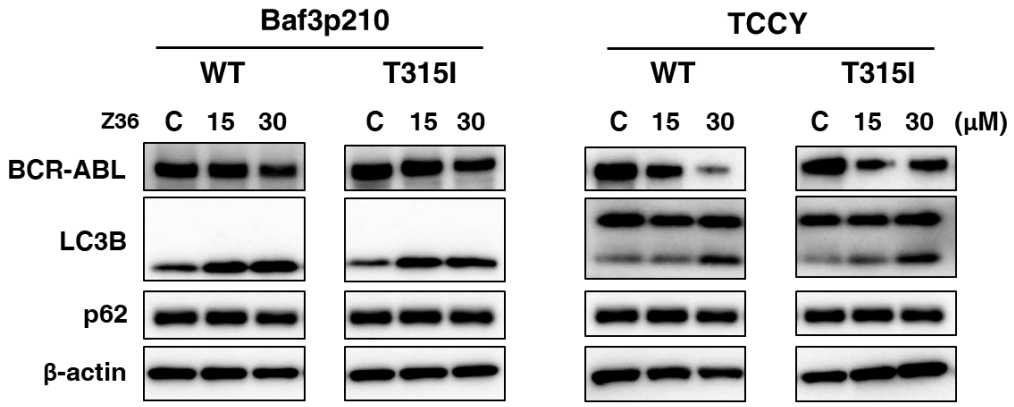

Figure S6

Effect of autophagy inducer Z36 on autophagy flux and expression of BCR-ABL. Conversion of LC3B and expression of BCR-ABL and p62 in Baf3p210 and TCCY cells treated with Z36 (15 or 30 μM) for 24 h were examined by Western blotting analysis.

Fig. S7

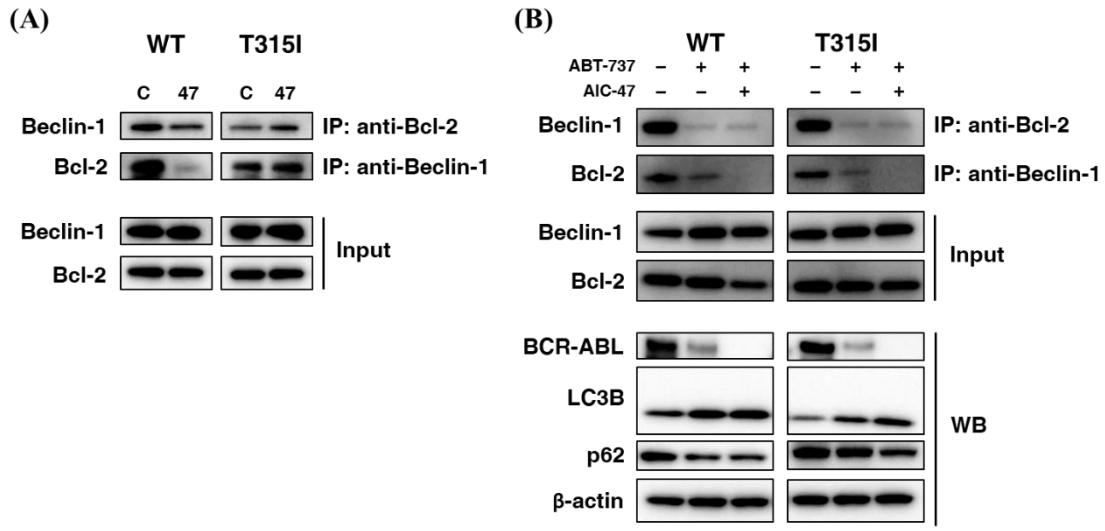

Figure S7

(A) Immunoprecipitation of primary CML patient cell lysates. Cells were treated with AIC-47 (5 μM) for 12 h. The expressed Bcl-2 or Beclin-1 was pulled down by antibodies corresponding to each protein. The binding of each protein was detected by Western blotting analysis. **(B)** Effect of Bcl-2/Beclin-1 dissociation on autophagy flux and expression of BCR-ABL. Baf3p210 cells were pretreated with ABT-737 (200 nM) for 12 h, and then treated with AIC-47 (5 μM) for 24 h. Immunoprecipitated Bcl-2 or Beclin-1, conversion of LC3B, and expression of BCR-ABL and p62 were detected by Western blotting analysis.

Fig. S8

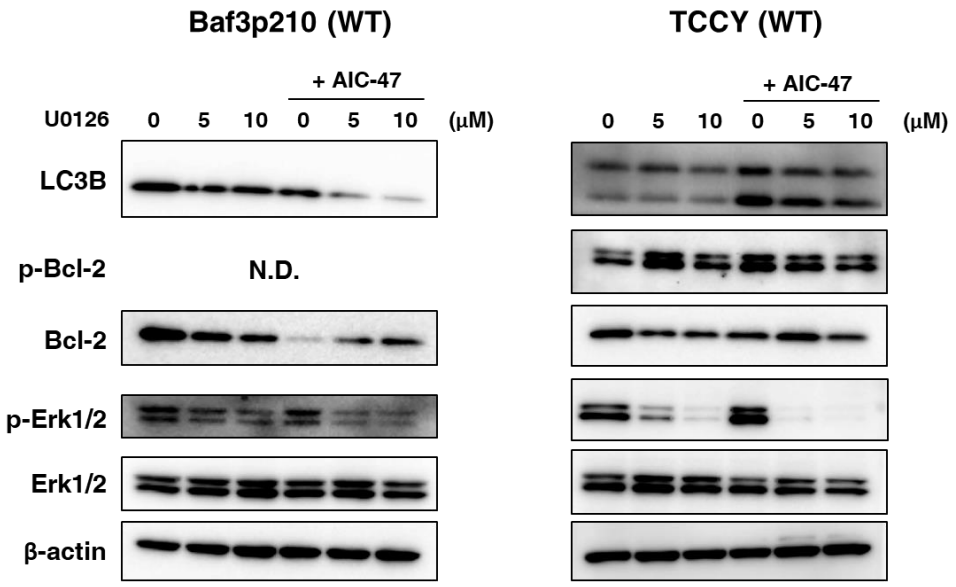

Figure S8

Effect of inhibition of MAPK/Erk signaling by MEK inhibitor U0126 on the phosphorylation of Bcl-2 and autophagy flux. WT-BCR-ABL-harboring cells were pretreated with U0126 (5 or 10 μM) for 4 h, and then treated with DMSO or AIC-47 (5 μM) for 24 h. The phosphorylation and expression of LC3B, Bcl-2, and Erk were examined by Western blotting analysis. Anti-phospho-Bcl-2 antibody could not detect phosphorylated Bcl-2 of mouse origin.

Fig. S9

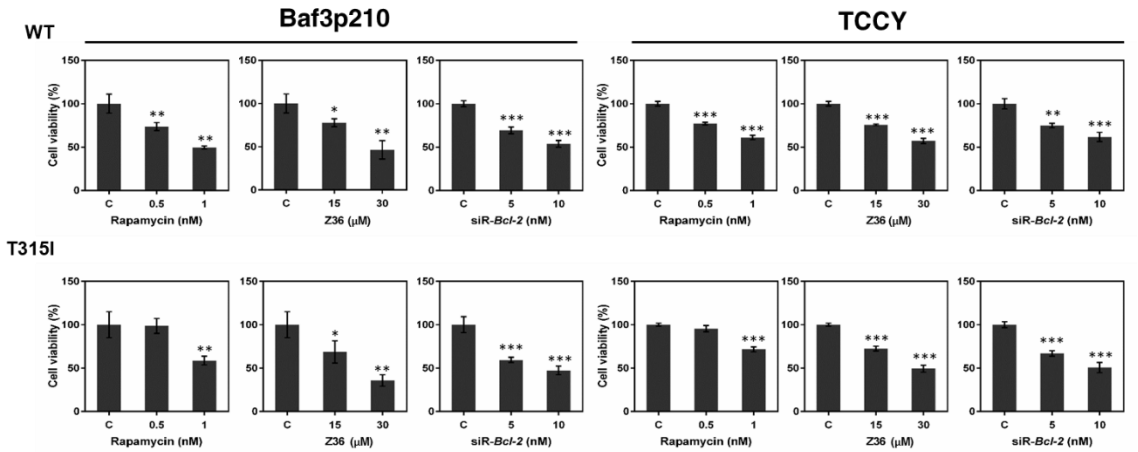

Figure S9

Effect of rapamycin, Z36 and siR-*Bcl-2* on the cell viability. Baf3p210 and TCCY cells were treated with rapamycin (0.5 or 1 nM) or Z36 (15 or 30 μM) for 24 h. Cells were transfected with siRNA for *Bcl-2* for 72 h. Viable-cell numbers were measured by performing the trypan-blue dye-exclusion test. Data are expressed as means ± SD of 3 different experiments. The cell viability of the Control is indicated as 100%. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. Control (Student's t test).