Concomitant targeting of FLT3 and BTK overcomes FLT3 inhibitor resistance in acute myeloid leukemia through the inhibition of autophagy

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Abstract

Strategies to overcome resistance to FMS-like tyrosine kinase 3 (FLT3)-targeted therapy in acute myeloid leukemia (AML) are urgently needed. We identified autophagy as one of the resistance mechanisms, induced by hypoxia and the bone marrow microenvironment via activation of Bruton tyrosine kinase (BTK). Suppressing autophagy/BTK sensitized *FLT3*-mutated AML to FLT3 inhibitor-induced apoptosis. Furthermore, co-targeting FLT3/BTK/aurora kinases with a novel multi-kinase inhibitor CG-806 (luxeptinib) induced profound apoptosis in *FLT3*-mutated AML by co-suppressing FLT3/BTK, antagonizing autophagy, and causing leukemia cell death in *FLT3*-wildtype AML by aurora kinase-mediated G2/M arrest and polyploidy, in addition to FLT3 inhibition. Thus, CG-806 exerted profound anti-leukemia activity against AML regardless of *FLT3* mutation status. CG-806 also significantly reduced AML burden and extended survival in an *in vivo* patient-derived xenograft leukemia murine model of FLT3 inhibitor-resistant *FLT3*-ITD/TKD double-mutant primary AML. Taken together, these findings indicate that CG-806 has a unique mechanistic action and pre-clinical activity, which is presently undergoing clinical evaluation in both *FLT3* wildtype and mutant AML.

Introduction

Acute myeloid leukemia (AML) is a diverse group of hematologic malignancies characterized by clonal evolution and genetic heterogeneity.[1,2] Mutations in the FMS-like tyrosine kinase 3 (*FLT3*) gene are detected in approximately one-third of patients with newly diagnosed AML. These mutations include the common *FLT3* internal tandem duplication (*FLT3*-ITD) in approximately 20-25% of AML and point mutations in the tyrosine kinase domain (*FLT3*-TKD) in approximately 5-10% of AML cases. FLT3-targeted therapy represents an important paradigm in the management of patients with highly aggressive, *FLT3*-mutated AML. A number of FLT3 inhibitors have been developed in recent years, including the small molecular inhibitors sorafenib[3] and quizartinib.[4] While midostaurin and gilteritinib are approved by the Food and Drug Administration, all inhibitors show only limited efficacy in clearing leukemic blasts from the bone marrow (BM) microenvironment and inducing sustained remissions, resulting in relapse and/or resistance.[4,5] Thus, it is of paramount importance to understand the underlying mechanisms of this resistance. We and others have reported that acquired secondary *FLT3*-TKD mutations, including mutations of residues D835, Y842, and F691, which have been identified in relapsed patients receiving FLT3-targeted therapy, can contribute to resistance.[6-8] It has also been reported that sorafenib-induced macroautophagy, hereafter referred to as autophagy, through induction of endoplasmic reticulum stress and 5' AMP-activated protein kinase (AMPK)-dependent mammalian target of rapamycin complex 1 (mTORC1) inhibition in liver cancer cells,[9] and in human myeloid dendritic cells.[10] Thus, AMPK-mTORC1 is potentially a key player in sorafenib-induced autophagy,[11] which could be associated with resistance to FLT3 inhibitors.

Autophagy is a process of intracellular degradation of proteins, organelles, etc. in response to various stressors, including chemotherapy in leukemia.[12] In the cancer context, autophagy has a dual role both as a tumor initiator, by inducing DNA damage and genetic instability, and as a

tumor promoter, by providing cancer cells with the necessary nutrients for survival.[13] As autophagy has an adaptive tumorigenic function, it may also provide leukemia cells with a mechanism of resistance to FLT3 inhibitor-mediated cytotoxicity. Evidence has shown that autophagy sustains the *FLT3*-ITD-dependent proliferation of leukemic cells through the activation of transcription factor 4 (ATF4). Targeting either autophagy or ATF4 reduces AML tumor burden in mice.[14] In addition, we reported that autophagy targeting sensitized AML to chemotherapy,[15] implying an association between autophagy and chemoresistance. Thus, inhibition of autophagy may be a potentially effective therapeutic strategy in AML by overcoming resistance to FLT3-targeted therapy.

In the present study, we observed upregulation of phospho-Bruton tyrosine kinase (BTK) levels and an increase of ATF4 accompanying autophagy in FLT3 inhibitor-resistant leukemia cell lines and in primary AML samples from patients who had received sorafenib monotherapy and developed resistance during a clinical trial of FLT3-targeted therapy. In addition, conditions mimicking the BM microenvironment (e.g., hypoxia and the presence of mesenchymal stem cells [MSC]) also triggered an increase of autophagy in *FLT3*-mutated AML cells. By repressing autophagosome-lysosome fusion or BTK activation, we enhanced quizartinib-induced apoptosis in FLT3 inhibitor-resistant leukemia cells. Of note, blockade of BTK/FLT3 with a small molecule multi-kinase inhibitor, CG-806, exerted impressive anti-leukemia activity against FLT3 inhibitor-resistant leukemias *in vitro* and *in vivo* in a patient-derived xenograft (PDX) leukemia murine model engrafted with FLT3-inhibitor-resistant primary AML, suggesting that co-targeting BTK and FLT3 may provide a novel strategy for preventing or overcoming FLT3 inhibitor resistance.

Methods

Cell lines and patients' samples

The human AML cell lines MOLM14, MV4-11 (harboring *FLT3*-ITD mutations), OCI-AML3 and THP-1 (harboring *FLT3* wildtype [WT]); the murine leukemia cell lines Ba/F3 (harboring different *FLT3* mutations including ITD, TKD or ITD+TKD double mutations or *FLT3* WT); and MSC were used for this study. Details of the cell lines and culture conditions are provided in the *Online Supplementary Methods*. All cell lines were validated by STR DNA fingerprinting using the AmpFISTR Identifier kit according to the manufacturer's instructions (Applied Biosystems cat. 4322288).

AML patients' samples were obtained after written informed consent following institutional guidelines of the University of Texas MD Anderson Cancer Center and in accordance with the principles of the Declaration of Helsinki. Mononuclear cells were purified from primary samples by Ficoll-Hypaque (Sigma-Aldrich) density-gradient centrifugation and were cultured in RPMI 1640 culture medium supplemented with 10% fetal bovine serum before treatment.

Compounds

Quizartinib, ibrutinib and SNS-314 were purchased from Selleckchem (Houston, TX, USA). CG-806 (luxeptinib) was provided by Aptose Biosciences (San Diego, CA, USA). The molecular structures of the kinase inhibitors are shown in *Online Supplementary Figure S1*. Chloroquine was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Apoptosis assays

Cells were treated with drugs or an equivalent amount of dimethylsulfoxide for 48-72 h. Cells were harvested and stained with annexin V-fluorescein isothiocyanate/propidium iodide according to the manufacturer's instructions. Apoptosis induction was analyzed by measuring annexin V positivity and propidium iodide positivity with fluorescence activated cell sorting as described previously.[16]

Cell cycle and polyploidy analysis

Cell cycle progression was measured using flow cytometric analysis of DNA content and BrdU incorporation. A DNA histogram was plotted to show the cell cycle, and diploid and polyploid distributions were measured with a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA). The details are provided in the *Online Supplementary Methods*.

BTK and ATG7 knockdown

BTK protein was knocked down by transfecting BTK short interfering (si)RNA into MOLM14 cells. ATG7 protein, an essential effector enzyme for canonical autophagy, was knocked down either by electroporation transfection of ATG7 siRNA into MV4-11 cells or lentiviral transduction of ATG7 short hairpin (sh)RNA into OCI-AML3 cells. Details are provided in the *Online Supplementary Methods*.

Patient-derived xenograft murine leukemia model

A primary BM specimen was collected with informed consent from an AML patient who had been treated with sorafenib and developed resistance (harboring *FLT3*-ITD/D835 mutations) and was xenografted using the University of Texas MD Anderson Cancer Center Institutional Review Board protocol Lab02-395. Nod.Cg-PrkdcscidIL2rgtm1Wjl/SzJ (NSG) mice were purchased from Jackson Laboratories and handled according to Institutional Animal Care and Use Committee-approved protocol #00001303-RN01. The leukemia cells were administered intravenously into the NSG mice at a dose

of 3.5x10^6 cells/mouse. The mice were randomly separated into two groups, one given the vehicle (15% Transcutol plus 85% PEG400, n=13 mice) and the other given the treatment (CG-806 at a dose of 100 mg/kg, n=13 mice). The vehicle or CG-806 was given orally every day for 5 days on, followed by 2 days off/week starting from when the leukemia cells reached about 1% engraftment in the blood (day 27 in this case). Leukemia cell engraftment was monitored by measuring hCD45$^+$/mCD45$^-$ cells in mouse peripheral blood by flow cytometry and the mice's body weight and vital signs were monitored simultaneously. Three mice from each group were sacrificed 72 days after injection of the leukemia cells. Peripheral blood, BM and spleen were collected and leukemia cell engraftment was assessed by determining the hCD45$^+$/mCD45$^-$ cell population by flow cytometry. The survival curve was plotted and analyzed by GraphPad Prism 7 using the Kaplan-Meier method.[17]

Statistical analyses

The Student *t* test or two-way analysis of variance was used to analyze immunoblot and cell apoptosis data. A *P* value ≤0.05 was considered statistically significant. All statistical tests were two-sided and the results are expressed as the mean of triplicate samples/experiments ± standard deviation or 95% confidence intervals (error bars). The efficacy of CG-806 with respect to survival was estimated by the Kaplan-Meier method,[17] and log-rank analysis was used to test for differences in survival.

Results

Upregulation of autophagy is associated with resistance to FLT3 inhibitors and is further increased by hypoxia and co-culture with mesenchymal stem cells

Since leukemia cells bearing TKD or ITD/TKD double mutations are resistant to certain FLT3 inhibitors, in comparison to cells with only ITD mutations,[6-8] we sought to determine whether autophagy was associated with the resistance. Since LC3-II, but not LC3-I, is bound to phosphatidylethanolamine (PE) in the autophagosome membrane and closely correlates to the number of autophagosomes, therefore serving as a good indicator of autophagosome formation,[18] we determined autophagy levels by measuring LC3-II (or LC3-II/I ratios) based on semi-quantitative analyses of immunoblotting data of murine leukemia cell lines harboring *FLT3* WT or *FLT3* mutations (ITD or ITD+TKD dual mutations). The results demonstrated that the autophagy levels were positively associated with upregulation of ATF4 and Beclin-1, which is another inducer of autophagy (Figure 1A). High autophagy levels were associated with high values of IC_{50} (50th percentile of the maximal inhibitory concentration) to FLT3 inhibition as well, especially in cells with *FLT3*-TKD mutations (*Online Supplementary Table S1*). The cells with higher autophagy levels also had higher levels of phospho-FLT3 or phospho–ERK, which are associated with resistance to FLT3 inhibitors.[14,19] Interestingly, high phospho-BTK levels were observed in cells with high autophagy levels, implying a hitherto unknown correlation between the two proteins in these cell lines (Figure 1A).

We further compared autophagy levels in paired primary AML samples before and after the administration of sorafenib in a clinical trial. The patients were given sorafenib and either did not respond to the therapy or died during treatment (*Online Supplementary Table S2*). The resistant samples (showing upregulation of phospho-FLT3 after sorafenib treatment in comparison to pre-treatment levels in these *FLT3* mutated samples) had higher LC3-II levels than their pre-treatment counterparts, a phenomenon accompanied by upregulation of phosphorylated Unc-51-like autophagy-activating kinase 1 (ULK1) in addition to upregulation of ATF4 and phospho-BTK (Figure 1B). The phosphorylation of ULK1 at Ser556 is AMPK-dependent and is required for the activation of downstream autophagy.[20,21] The results imply that sorafenib resistance may be associated with upregulation of autophagy, which is likely related to upregulation of ATF4 and phospho-ULK1 as well as phospho-BTK.

We further assessed whether the BM microenvironment modulates autophagy levels in leukemia cells. The BM niche provides a sanctuary for AML cells and protects them from targeted therapies.[22] *FLT3*-mutant MOLM14 cells were cultured in BM niche-mimicking conditions (i.e. hypoxia [1% oxygen tension] or in the presence of MSC) *in vitro* for 48 h. The BM niche-mimicking conditions modestly upregulated LC3-II/I and Beclin-1 levels and these effects were accompanied by an increase of phospho-BTK and hypoxia inducible factor-1α (HIF-1α) (Figure 1C). Co-culture of the MOLM14 cells with MSC also led to a significant increase in autophagosomes as compared to the number in MOLM14 cells alone in normoxia without MSC (Figure 1D, E). These observations suggest a possible association between autophagy and hypoxia or MSC co-culture and the protection (resistance) afforded by the BM microenvironment against AML therapies.

Chloroquine enhances quizartinib-induced apoptosis and partially abrogates the protection of acute myeloid leukemia cells mediated by mesenchymal stem cells

To validate if autophagy levels were associated with FLT3 inhibitor resistance, we sought to sensitize AML cells to FLT3 inhibitor-induced killing by reducing autophagy. We first tested the sensitivity to apoptosis induction by using the second-generation FLT3 inhibitor quizartinib and impairing autophagy with chloroquine, a lysosomotropic agent that inhibits lysosomal degradation of the autophagosome.[23] The presence of chloroquine sensitized cells to quizartinib-induced apoptosis, as determined by annexin

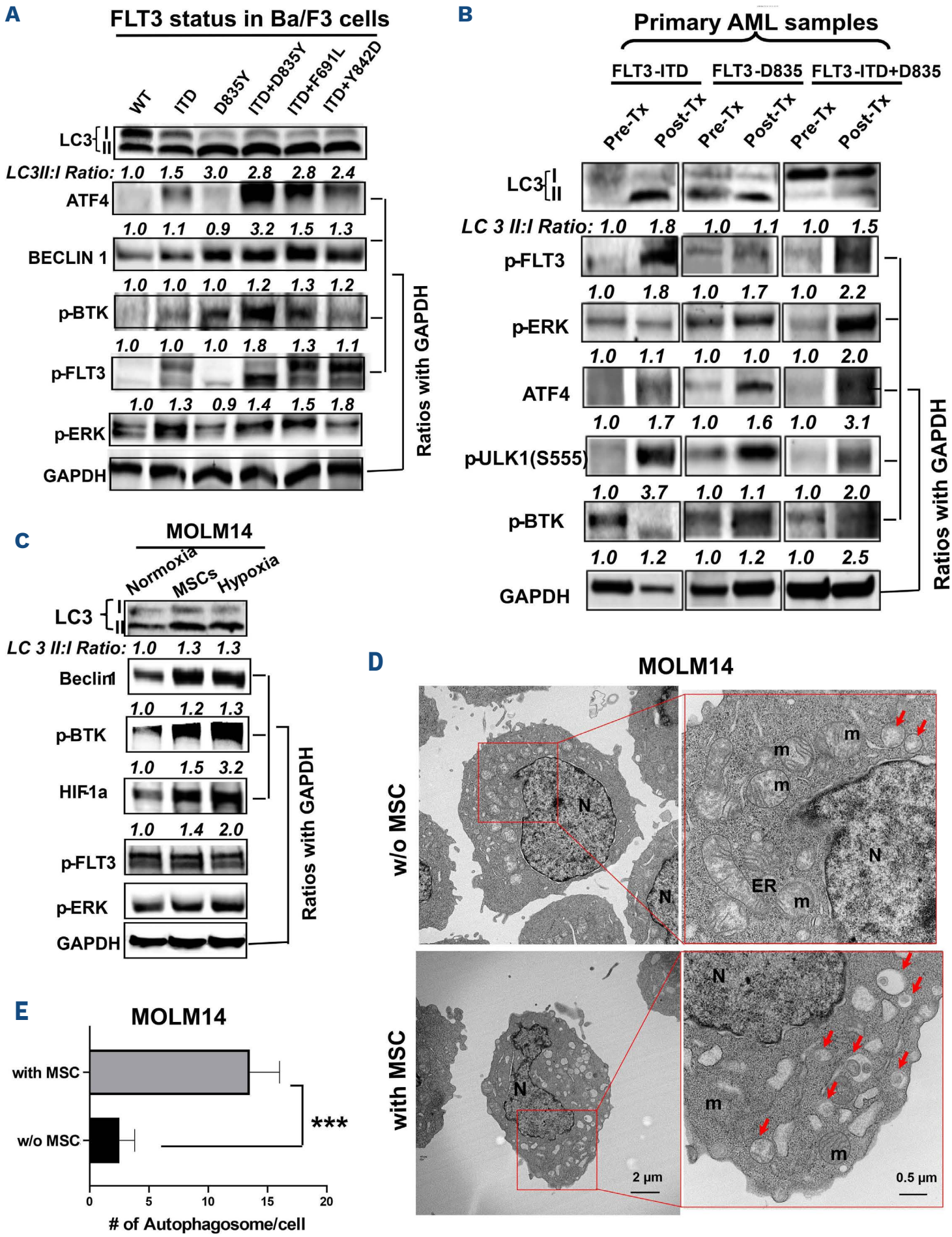

Figure 1. Upregulation of autophagy is associated with resistance to FLT3-targeted therapy, which further increases in hypoxia and mesenchymal stem cell co-culture of *FLT3*-mutated acute myeloid leukemia. (A) Basal expression levels of autophagy-related proteins was assessed by immunoblotting in a panel of murine leukemia cell lines with different *FLT3* mutational status. (B) Autophagy-related proteins were investigated in samples from patients with acute myeloid leukemia, which were collected before and after administration of sorafenib in a clinical trial of sorafenib. (C) *FLT3*-ITD-mutated MOLM14 cells were cultured together with mesenchymal stem cells (MSC) or in hypoxic conditions for 48 h and autophagy-related proteins were analyzed by immunoblotting. (D) MOLM14 cells were co-cultured with or without (w/o) MSC for 48 h and the ultrastructure of autophagosomes was observed with transmission electron microscopy (TEM). Nuclei, endoplasmic reticulum, and mitochondria are marked. Red arrows indicate autophagosomes. Left panel: scale bar represents 2 μm. Right panel: scale bar represents 0.5 μm. (E) A statistical analysis of autophagosome numbers based on TEM observation of at least 20 cells per sample. The numbers shown in the western blot data are from semi-quantitative analyses of protein levels, comparing them to the control samples of each group. ***P<0.001. WT: wildtype; ITD: internal tandem duplication; Tx: treatment with sorafenib; N: nuclei; ER: endoplasmic reticulum; m: mitochondria; MSC: mesenchymal stem cells; w/o: without.

V staining in Ba/F3-*FLT3*-ITD and Ba/F3-ITD/D835Y mutant cell lines (Figure 2A). Immunoblot analysis confirmed that chloroquine enhanced quizartinib-induced apoptosis (shown as an increase of cleaved caspase 3) in *FLT3*-ITD mutant MOLM14 cells and in FLT3 inhibitor-resistant Ba/F3-ITD+D835Y cells, an effect that was accompanied by increased suppression of FLT3 and its downstream signaling pathways (i.e. p-ERK, p-mTOR and p-S6K) (Figure 2B). As expected, LC3-II was not decreased in the presence of chloroquine. In fact, chloroquine acts on autolysosome degradation to impair autophagic flux by decreasing autophagosome-lysosome fusion instead of reducing autophagosome formation, which was demonstrated in a previous study.[18] Further investigations showed that chloroquine partially abrogated MSC-mediated protection and resensitized leukemia cells to quizartinib-induced apoptosis in co-culture of leukemia cells with MSC (Figure 2C), suggesting that pharmacological disruption of autophagosome-lysosome fusion resensitizes *FLT3*-mutated leukemia cells to FLT3-targeted therapy.

BTK inhibition sensitizes *FLT3*-mutated leukemia cells to quizartinib-induced killing

Since we observed an association between phospho-BTK and the autophagy-related proteins Beclin-1 and LC3-II in cells resistant to FLT3 inhibitors, in both relapsed primary AML patients' samples and in *FLT3*-ITD-mutant MOLM14 cells under hypoxic culture or in the presence of MSC (Figure 1B, C), we sought to investigate whether suppression of BTK sensitized cells to quizartinib-induced apoptosis. BTK inhibition, achieved using the BTK-specific inhibitor ibrutinib,[24] significantly enhanced quizartinib-induced apoptosis in both *FLT3*-ITD-mutant and ITD/D835Y mutant cells in the presence or absence of MSC (Figure 3A, B, *Online Supplementary Figure S2*). Immunoblot analysis further demonstrated that ibrutinib suppressed autophagy and abrogated MSC-mediated protection through suppression of phospho-BTK and its downstream phospho-ERK, -mTOR, and -S6K signaling pathways (Figure 3C, *Online Supplementary Figure S3*). Furthermore, knockdown of BTK by siRNA markedly reduced levels of the autophagy

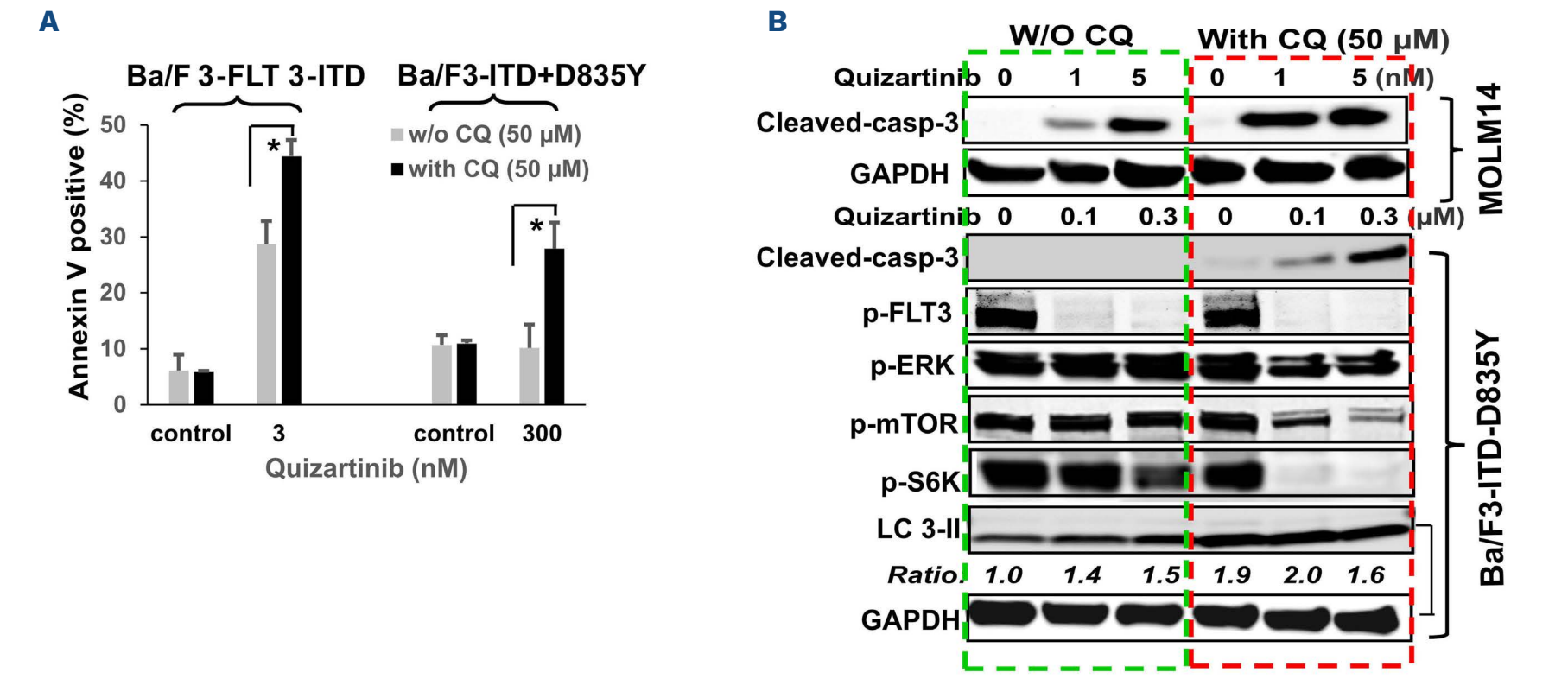

Figure 2. Autophagy inhibition with chloroquine enhances quizartinib-induced apoptosis and partially abrogates mesenchymal stem cell-mediated protection. (A) Murine leukemia cell lines harboring either *FLT3*-ITD or *FLT3*-ITD/D835Y mutations were exposed to increasing doses of quizartinib for 48 h in the presence or absence of chloroquine *in vitro*. Apoptosis induction was then assessed by measuring annexin V positivity by flow cytometry. (B) *FLT3*-ITD-mutated MOLM14 cells and Baf3-ITD/D835Y cells were exposed to increasing doses of quizartinib for 24 h in the presence (red box) or absence (green box) of chloroquine and then subjected to immunoblot analysis. (C) MOLM14 cells were exposed to the indicated doses of quizartinib for 48 h in co-culture with (yellow box) or without mesenchymal stem cells (blue box) in the presence or absence of chloroquine (50 μM). Apoptosis induction was assessed by measuring annexin V positivity by flow cytometry. ***P<0.001; ****P<0.0001 (two-way analysis of variance). Error bars represent the standard deviation from three independent experiments. w/o: without; CQ: chloroquine.

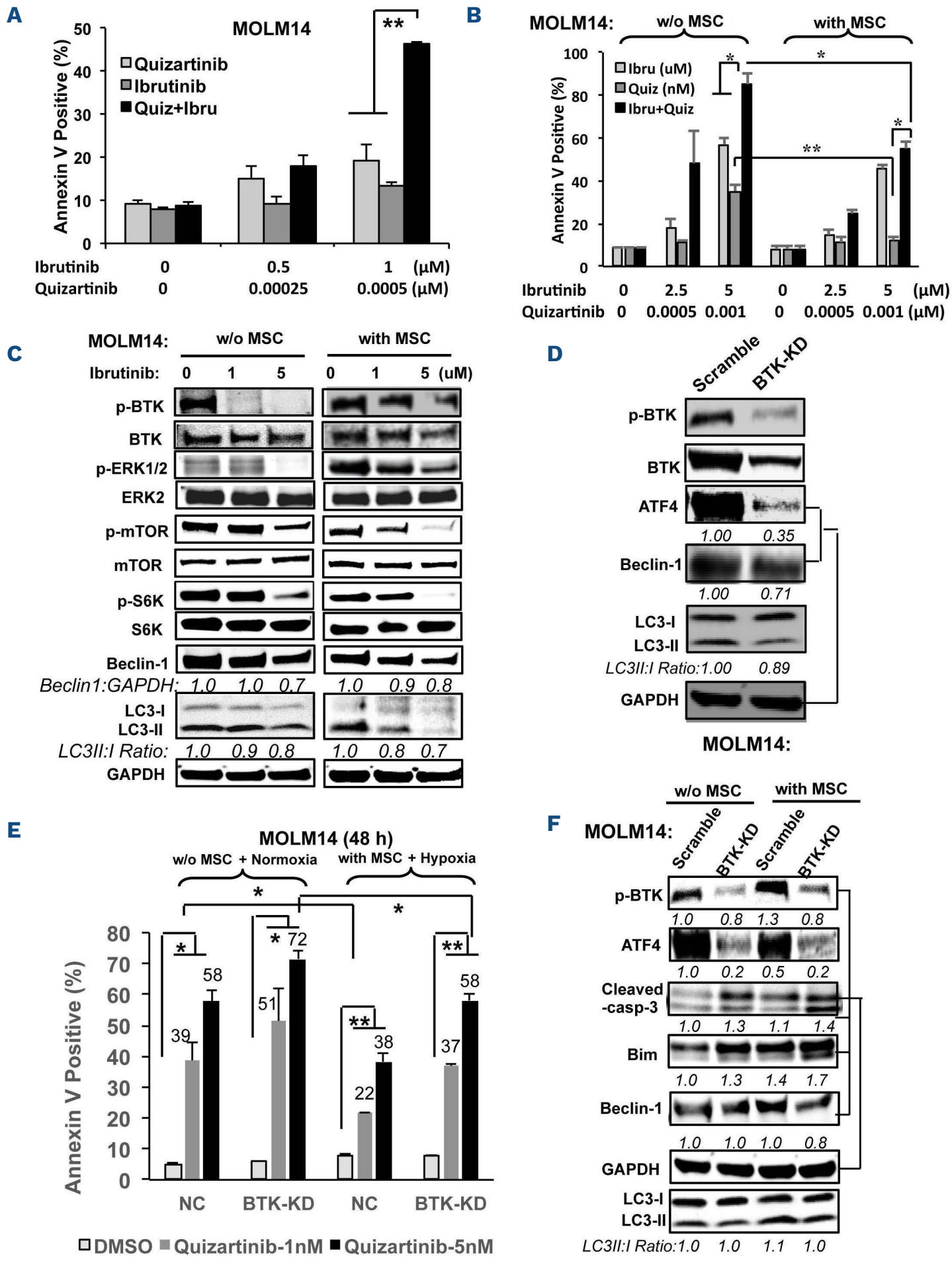

Figure 3. BTK inhibition sensitizes leukemic cells to quizartinib-induced killing and abrogates mesenchymal stem cell-mediated protection in *FLT3*-mutated leukemia cells. (A) MOLM14 cells were exposed to ibrutinib, quizartinib or their combination for 48 h and apoptosis induction was assessed by measuring annexin V positivity by flow cytometry. (B) MOLM14 cells were treated with quizartinib, ibrutinib or their combination for 48 h in the presence or absence of mesenchymal stem cells (MSC), and annexin V positivity was measured by flow cytometry. (C) MOLM14 cells were exposed to ibrutinib for 24 h in the presence or absence of MSC, and protein levels were analyzed by immunoblotting. The numbers are from semi-quantitative analyses of protein levels in comparison to the control samples of each group. (D) MOLM14 cells were transfected with BTK siRNA for 48 h, and protein expression was determined by immunoblotting. (E) BTK knockdown (BTK-KD) MOLM14 cells and cells transfected with scrambled RNA were exposed to quizartinib in the presence or absence of MSC for 48 h. Apoptosis induction was assessed by measuring annexin V positivity by flow cytometry. The numbers over the individual bars indicate exact levels of apoptosis induction. (F) BTK knockdown and MOLM14 cells transfected with scrambled RNA were co-cultured with or without MSC for 24 h, followed by immunoblot analysis. **P*<0.05; ***P*<0.01. Error bars of the flow cytometry data represent the standard deviation from three independent experiments. Quiz: quizartinib; Ibru: ibrutinib; w/o: without; KD: knockdown; NC: normal control (transfected with scrambled RNA); DMSO: dimethylsulfoxide.

inducers ATF4 and Beclin-1 (Figure 3D), triggered similar levels of quizartinib-induced apoptosis, abrogated MSC-mediated protection, increased the pro-apoptotic protein Bim and cleaved caspase-3, although it showed only marginal modulation of Beclin-1 and LC3-II levels (Figure 3E, F). However, BTK knockdown by siRNA was marked by decreased ATF4 levels (Figure 3D), suggesting a correlation between BTK levels and autophagy modulation. At least, these findings suggest that high BTK activity is associated with resistance to FLT3 inhibitors, and that this effect could be overcome by suppressing BTK activation, implying that BTK is also involved in the modulation of autophagy.

Co-targeting FLT3 and BTK with the multi-kinase inhibitor CG-806 abolishes mesenchymal stem cell- and hypoxia-mediated protection and induces apoptosis in *FLT3*-mutated leukemia cells

We further tested the anti-leukemia efficacy of a novel small molecule kinase inhibitor CG-806, which has multi-kinase inhibitory activity against FLT3, BTK, and aurora kinases (AURK) at low IC_{50} values (0.82, 5.0 and 0.38 nM against FLT3 ITD, BTK and aurora A, respectively) in a cell-free system (*Online Supplementary Table S3*). CG-806 has much lower IC_{50} values than most commercially available FLT3 inhibitors, including the Food and Drug Administration-approved small molecule FLT3 inhibitors midostaurin and gilteritinib, especially in AML cells with *FLT3*-ITD/TKD double mutations (*Online Supplementary Table S4*). CG-806 also demonstrated marked pro-apoptotic efficacy in AML cell lines and patients' samples harboring these mutations and in *FLT3* WT cells as well (*Online Supplementary Figures S3* and *S4*). Further investigations demonstrated that CG-806 completely abrogated MSC-mediated chemoprotection and triggered the induction of apoptosis in *FLT3*-ITD-mutant leukemias, accompanied by suppression of autophagy (Figure 4A-C, *Online Supplementary Figure S5*). ATG7 knockdown enhanced CG-806-induced apoptosis in FLT3-ITD mutant MV4-11 cells (Figure 4D, *Online Supplementary Figure S6*), suggesting an association between autophagy impairment and sensitivity to CG-806. Mechanistically, CG-806 profoundly suppressed FLT3, BTK, c-Myc, and ATF4 (Figure 4E, *Online Supplementary Figure S7*). Of note, CG-806 alone had stronger pro-apoptotic activity than could be induced by co-targeting FLT3 and autophagy, or BTK, with quizartinib and chloroquine or ibrutinib, respectively, in primary AML patients' samples (Figure 4F, *Online Supplementary Figure S8*).

CG-806 induces G2/M arrest and promotes polyploidy through aurora kinase inhibition in *FLT3* WT leukemia cells

Since CG-806 exerted profound anti-leukemia effects in Baf3-*FLT3* cells and primary AML samples with mutant *FLT3* (*Online Supplementary Figures S3* and *S4*), we further investigated whether the mechanism of triggering apoptosis in *FLT3*-mutated cells also applied to *FLT3* WT cells (i.e., targeting FLT3/BTK to suppress autophagy). Unexpectedly, CG-806 did not suppress autophagy in *FLT3* WT cells. Instead, it increased the LC3-II:I ratio ($P<0.001$) and this was accompanied by upregulation of the pro-autophagic proteins Beclin-1, ATG7, and phospho-ULK1 after exposure to CG-806 for 48 h (Figure 5A, *Online Supplementary Figure S9*). CG-806 predominantly triggered suppression of phospho-aurora kinases, especially aurora B and C, but had less effect on the modulation of phospho-FLT3 and -BTK signaling (*Online Supplementary Figure S10*). In addition, CG-806 showed enhanced activity against proliferating cells. The IC_{50} values were 3.88, 11.81, and 21.99 nM in the *FLT3* WT cells THP-1, OCI/AML3, and Kasumi-1, respectively. Interestingly, CG-806 had much weaker apoptogenic effects in *FLT3* WT cell lines such as THP-1 and Kasumi-1, and the EC_{50} (concentration producing half-maximal response) could not be reached even at micromolar concentrations (*data not shown*).

Cell cycle analysis by BrdU incorporation and propidium iodide staining revealed a marked increase in G2/M cells and polyploidization, with *FLT3* WT leukemia cells having a tetraploid DNA content even after very low doses of CG-806 (Figure 5B-D). Immunoblot analysis demonstrated that CG-806 induced upregulation of the anti-proliferative proteins p53, p21[25] and the DNA repair-related protein γH2AX in addition to decreasing PLK1 and phosphorylated-histone H3 -CDC25c, and -CDK2, which are closely associated with induction of polyploidization[26] (Figure 5E). To determine whether the effects of CG-806 on cell cycle progression and autophagy regulation were off-target or specific to aurora kinase inhibition, a specific aurora kinase inhibitor SNS-314[27] was used for reference in the *FLT3* WT cells. The results revealed induction of G2/M arrest, polyploidy and a slight increase in autophagy levels (Figure 5F, *Online Supplementary Figure S11*), implying an association between aurora kinase inhibition and CG-806-induced cell cycle perturbation and polyploidization in *FLT3* WT cells.

Impairment of autophagy re-sensitizes *FLT3* WT cells to CG-806-induced pro-apoptotic effects

To better understand the roles of autophagy and polyploidy in CG-806-induced killing of *FLT3* WT leukemia cells, we suppressed autophagy by chloroquine or ATG7 knockdown using shRNA in *FLT3* WT leukemia cells (*Online Supplementary Figure S12*). Inhibition of autophagy by chloroquine or ATG7 knockdown profoundly re-sensitized cells to CG-806-induced apoptosis (Figure 6A-C), suggesting an association between autophagy levels and sensitivity to inhibition of FLT3/BTK/aurora kinases. Meanwhile, inhibition of autophagy also reduced CG-806-induced polyploidy, but only modestly modulated G2/M

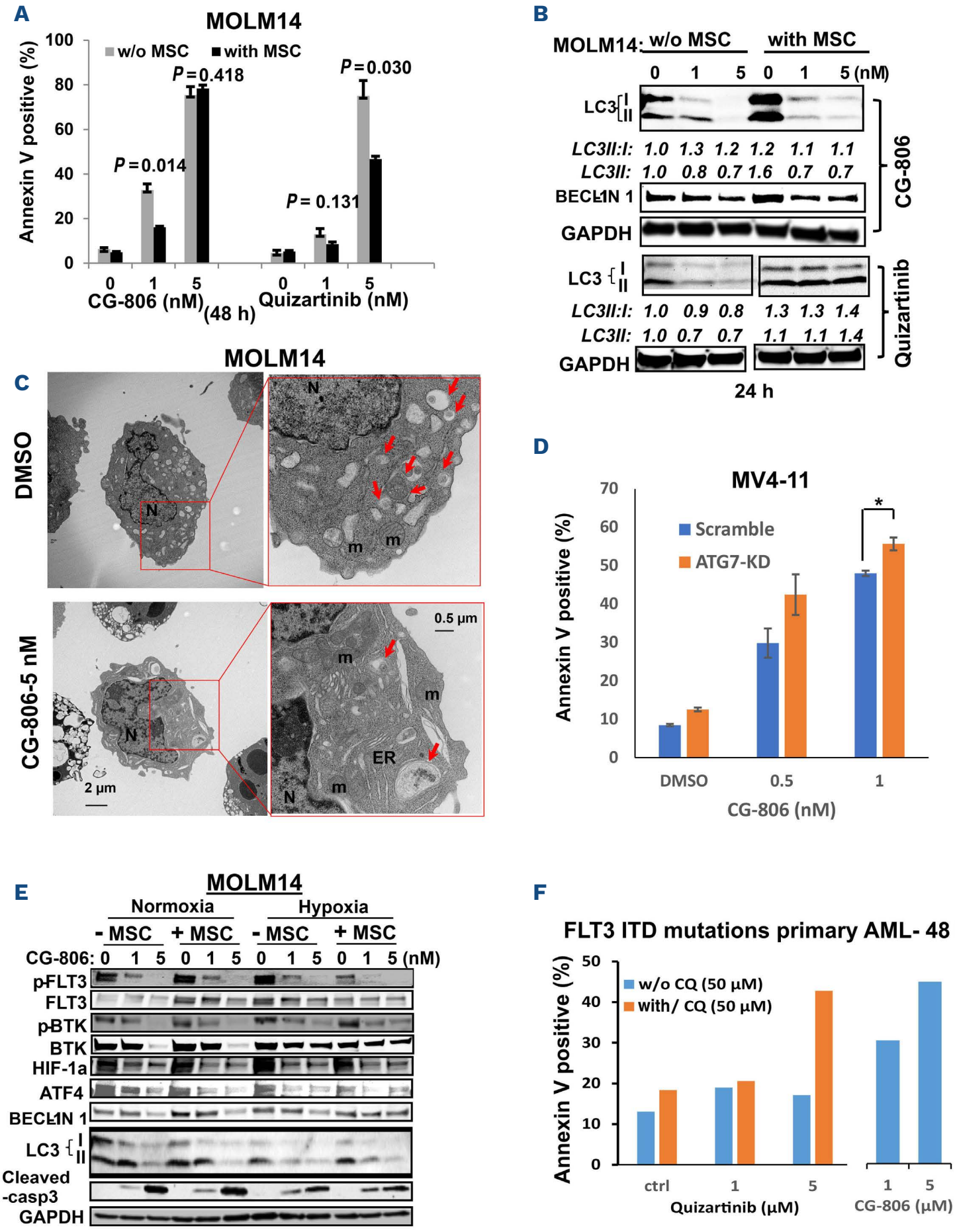

Figure 4. The FLT3/BTK inhibitor CG-806 abolishes mesenchymal stem cell/hypoxia-mediated protection and induces apoptosis in *FLT3*-mutated leukemia cells. (A) MOLM14 cells were exposed to either CG-806 or quizartinib for 48 h in the presence or absence of mesenchymal stem cells (MSC), and apoptosis induction was assessed by measuring annexin V positivity by flow cytometry. Data are from three independent experiments. (B) MOLM14 cells were exposed to either CG-806 or quizartinib for 24 h in the presence or absence of MSC and then cellular proteins were assessed by immunoblot analysis. (C) MOLM14 cells were exposed to CG-806 for 24 h and then cellular ultrastructure was observed using transmission electron microscopy. Nuclei, endoplasmic reticulum, and mitochondria are marked; the red arrows indicate autophagosomes. Left panel: the scale bar represents 2 μm. Right panel: the scale bar represents 0.5 μm. (D) *FLT3*-ITD-mutated MV4-11 cells were transfected with ATG7 siRNA or scrambled siRNA for 48 h and then exposed to CG-806 for an additional 48 h. Apoptosis induction was assessed by measuring annexin V positivity by flow cytometry. (E) MOLM14 cells were exposed to CG-806 in either normoxic or hypoxic conditions in the presence or absence of MSC for 24 h, and then subjected to immunoblot analysis. (F) *FLT3*-ITD-mutated primary AML blasts were exposed to either CG-806 or quizartinib *ex vivo* for 48 h. Apoptosis induction was assessed by measuring annexin V positivity by flow cytometry. w/o: without; DMSO: dimethylsulfoxide; N: nuclei; ER: endoplasmic reticulum; m: mitochondria; KD: knockdown.

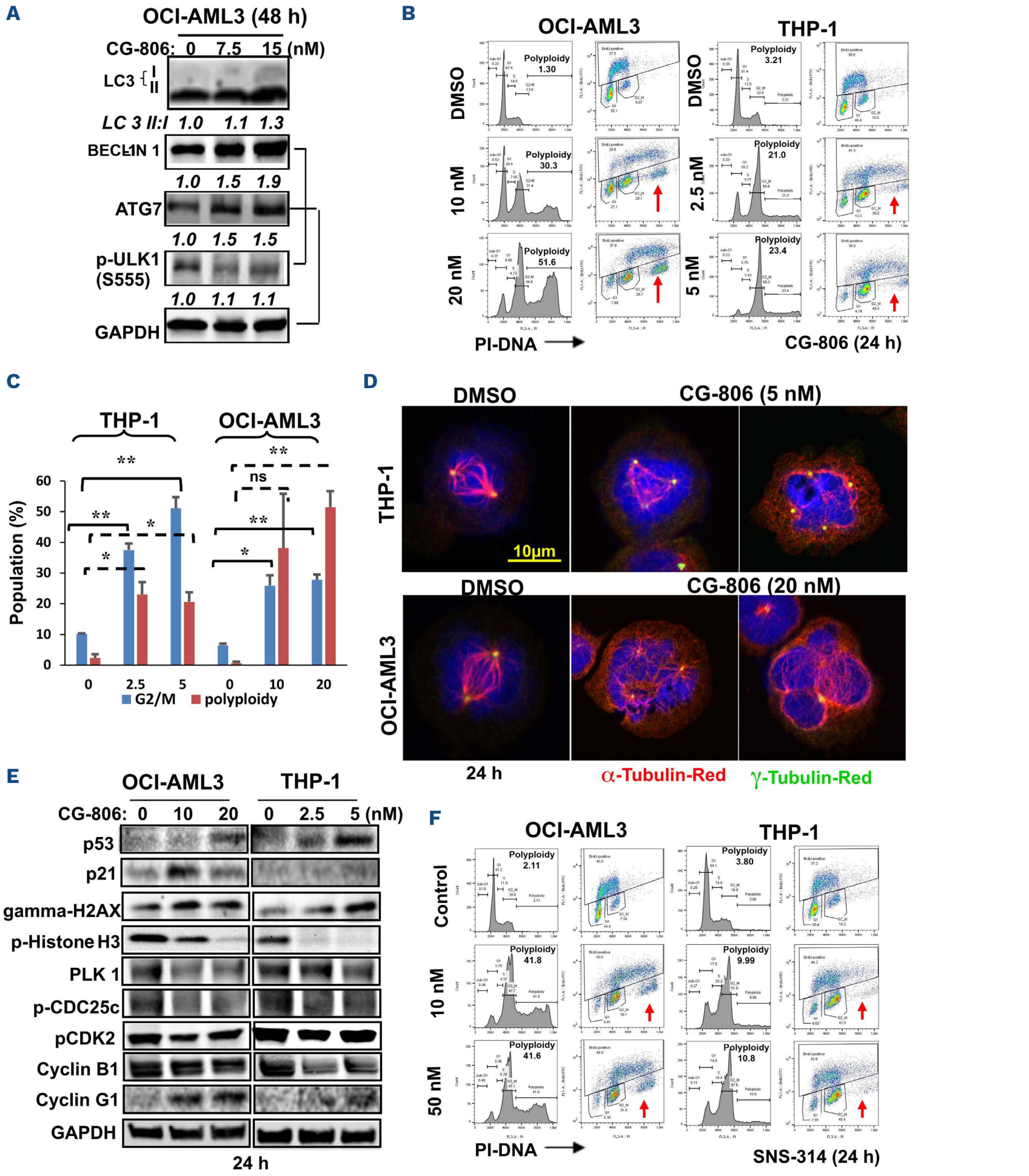

Figure 5. CG-806 induces G2/M arrest and polyploidy and is accompanied by suppression of pro-autophagic proteins through aurora kinase inhibition in *FLT3*-WT leukemia cells. (A) The *FLT3* WT leukemia cell line OCI/AML3 was exposed to CG-806 for 48 h and autophagy-related proteins were analyzed by immunoblotting. (B) The graphs show data representative of three independent experiments in which cells were stained with propidium iodide and anti-BrdU antibodies and analyzed by flow cytometry. Polyploidy is indicated by red arrows. (C) Percentage of cells in G2/M and polyploidy distribution, expressed as the mean of three independent experiments. Error bars represent the standard error of mean. Statistical analysis was carried out using the *t* test based on three independent experiments. (D) *FLT3* WT cells were treated with the indicated concentrations of CG-806 for 24 h and analyzed by immunofluorescence using γ-tubulin (green) and α-tubulin (red) antibodies. 4',6-Diamidino-2-phenylindole staining (blue) indicates a cell's nucleus (scale bar represents 10 μm). (E) The *FLT3* WT cells were exposed to CG-806 for 24 h and then the protein levels were assessed by immunoblotting. (F) *FLT3* WT cells were exposed to the BTK inhibitor SNS-314 for 24 h and DNA content was measured by flow cytometry. Red arrows indicate polyploidy. The data are from three independent experiments. DMSO: dimethylsulfoxide; PI: propidium iodide.

cells (Figure 6D, *Online Supplementary Figure S13*). Nevertheless, these results suggest that inhibition of autophagy enhances CG-806-triggered, pro-apoptotic effects and partially abolishes CG-806-induced polyploidy in *FLT3* WT cells.

CG-806 induces marked *in vivo* anti-leukemia effects in acute myeloid leukemia resistant to FLT3 inhibitors

We tested the pro-apoptotic effects of CG-806 and quizartinib in an *FLT3*-ITD/D835-mutated primary AML sample *ex vivo* by isolating hCD45^{+}/mCD45^{-} leukemia cells from the peripheral blood of a mouse xenografted with leukemia cells from a patient resistant to FLT3 inhibitors and exposed the cells to either agent for 48 h. The resistant leukemia cells were indeed resistant to quizartinib, but were sensitive to CG-806 (*Online Supplementary Figure S14*). We next tested the anti-leukemia activity of CG-806 in a PDX murine model by injecting *FLT3*-ITD/D835-mutated primary AML blasts via the tail vein. CG-806 markedly reduced leukemia cell burden in the peripheral blood, spleen, and BM after several weeks of drug administration (Figure 7A-C, *Online Supplementary Figure S15*). We observed a statistically significant prolongation of survival from a median of 72 days for controls to 113 days for the CG-806-treated (100 mg/kg) group (Figure 7D). Of note, the engrafted leukemia cells also exhibited a decrease in autophagy, as evidenced by LC3-II-PE fluorescence after CG-806 treatment (Figure 7E), suggesting that autophagy suppression through BTK inhibition has a benefit in overcoming FLT3 inhibitor resistance in AML.

Discussion

The mechanisms associated with autophagy and drug resistance during FLT3-targeted therapy of AML have not been extensively explored. In this study, we confirmed that leukemia cells harboring resistance-related *FLT3* mutations have higher basal levels of autophagy as well as higher phospho-FLT3, implying an association between autophagy and drug resistance during FLT3-targeted therapy. Mechanistically, there was a notable association between auto-

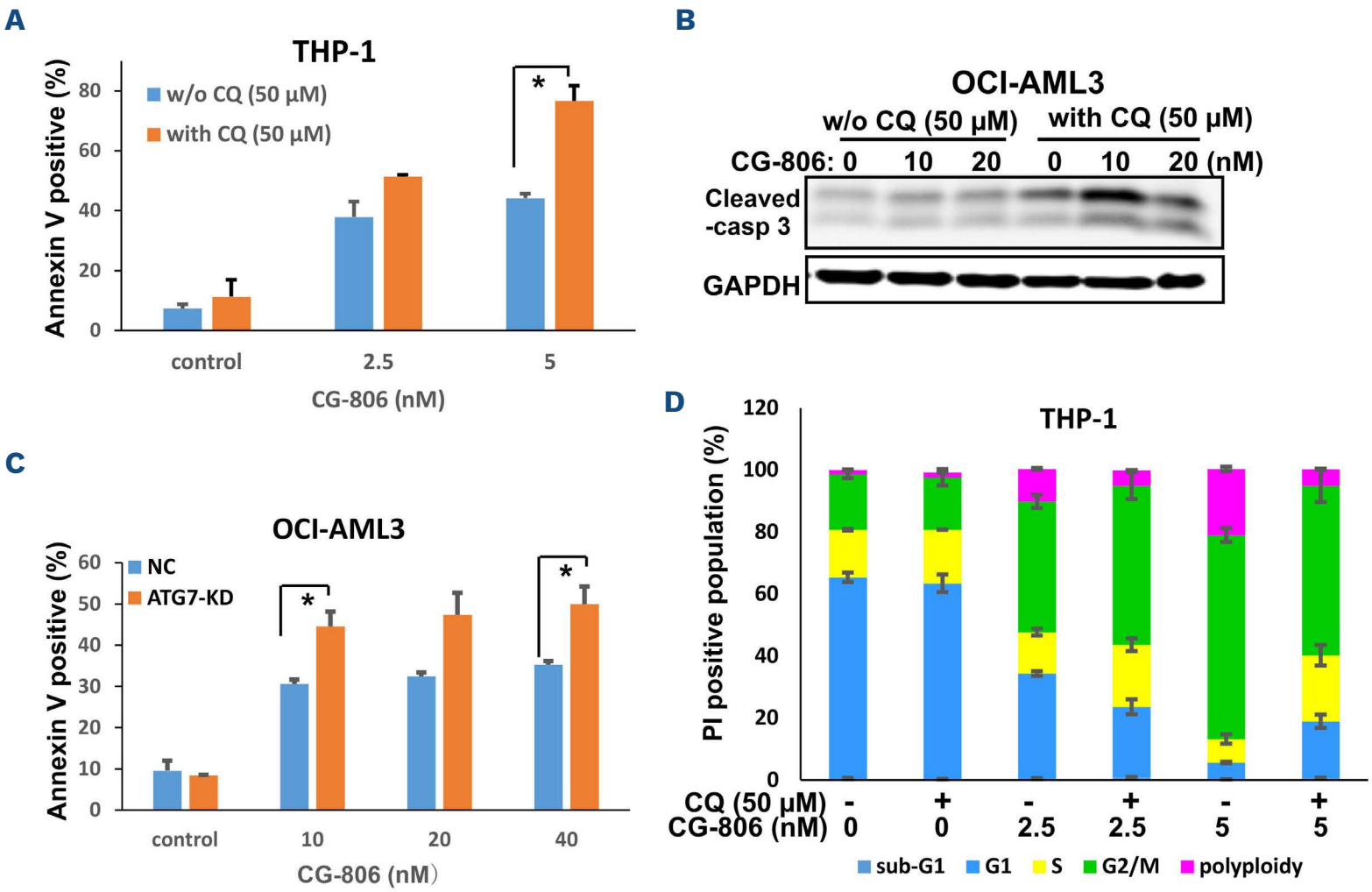

Figure 6. Inhibition of autophagy with chloroquine or with ATG7 knockdown re-sensitizes *FLT3* WT cells to CG-806-triggered pro-apoptotic effects. (A, B) Apoptosis induction was assessed in the *FLT3* WT leukemia cell lines THP-1 and OCI-AML3 by exposing the cells to the indicated concentrations of CG-806 for 24-48 h in the presence or absence of chloroquine and then measuring annexin V positivity by flow cytometry (A) and cleaved caspase-3 by immunoblotting (B). (C) ATG7 knockdown and control *FLT3* WT OCI-AML3 leukemia cells were exposed to the indicated concentrations of CG-806 for 48 h, and apoptosis induction was assessed by flow cytometric by measuring annexin V positivity. (D) THP-1 cells were exposed to different concentrations of CG-806 for 24 h in the presence (+) or absence (-) of chloroquine and the cell cycle phase distributions were determined by staining the cells with propidium iodide and assessing their DNA content using flow cytometry. Data in panels (A), (C) and (D) are presented as the means of three independent experiments ± standard deviations. w/o: without; CQ: chloroquine; casp: caspase; NC: normal control; KD: knockdown; PI: propidium iodide. *P<0.05.

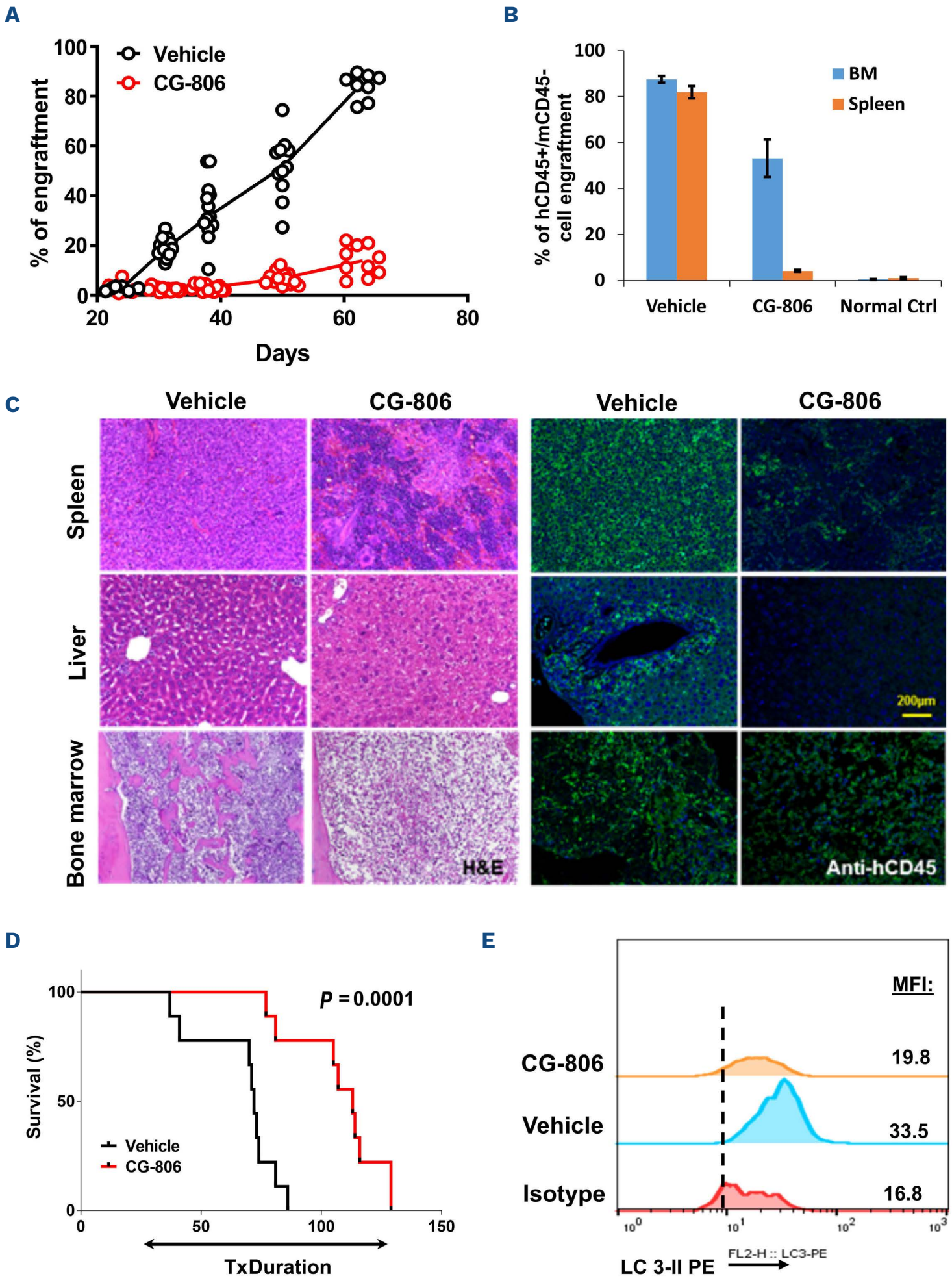

Figure 7. CG-806 has efficient anti-leukemia activity in FLT3 inhibitor-resistant acute myeloid leukemia, represses autophagy induction, and prolongs mouse survival in an *FLT3*-ITD/D835 bearing patient-derived xenograft leukemia model. (A) Leukemia cell engraftment in mouse peripheral blood was assessed by measuring the percentage of the hCD45$^+$/mCD45$^-$ population in vehicle- and CG-806-treated mice in a patient-derived xenograft model. CG-806 treatment started from day 27 after the injection of leukemia cells. (B) The percentage of leukemia cell engraftment was assessed by measuring the hCD45$^+$/mCD45$^-$ population in bone marrow and spleen 25 days after CG-806 treatment. (C) Leukemia cell engraftment was assessed by hematoxylin and eosin staining and anti-hCD45 (green) immunostaining of mouse organs. The scale bar represents 200 μm. (D) Mouse survival was estimated by the Kaplan-Meier method and log-rank statistics were used to test for differences in survival. The arrow indicates the duration of treatment. (E) Autophagy levels were assessed by flow cytometry after staining with anti-LC3II-PE and gated on the hCD45$^+$ population. BM: bone marrow; Ctrl: control; H&E: hematoxylin and eosin; Tx: treatment; MFI: mean fluorescence intensity; PE: phosphatidylethanolamine.

phagy and the increase in phospho-FLT3, phospho-BTK, and ATF4 in the resistant AML cells/blasts (Figure 1A, B, *Online Supplementary Figure S16*). A recent study also indicated that FLT3 activation drove autophagy in *FLT3*-ITD AML cells through upregulation of ATF4 protein in a manner independent of eukaryotic initiation factor 2α (eIF2α), an upstream protein of ATF4.[14] An increase of ATF4 was also associated with sorafenib resistance in hepatocellular carcinoma.[19] Of note, the increase of autophagy was also observed in *FLT3*-ITD-mutated AML cells in a BM-mimicking, co-culture system (Figure 1C), which implies that BM-mediated resistance may result from the upregulation of autophagy in addition to CXCL12-CXCR4 axis-mediated leukemia cell homing and HIF1α-induced modulation of cell survival signaling.[28,29] HIF1α may also be involved in the upregulation of autophagy, as shown in Figure 1C. Indeed, HIF1α or HIF2α/BNIP3 triggered induction of autophagy during hypoxia in prostate carcinoma cells,[30] and recently the HIF 1α/miR 224 3p/ATG5 axis was reported to be involved in autophagy regulation and induced drug resistance in glioblastoma and astrocytoma cells.[31] Hypoxia can activate autophagy through the endoplasmic reticulum stress-related PERK/eIF2α/ATF4 signaling pathway as well.[32]

Interestingly, we observed a positive association between the upregulation of phospho-BTK and autophagy in the resistant cells, especially in the presence of BM-mimicking hypoxia or MSC, implying a pivotal role for BTK level in the modulation of autophagy. In addition, BTK could be activated by FLT3 activation and sensitizes AML to BTK inhibition,[33] supporting the putative association of BTK and FLT3 activation, which may also result in the upregulation of BTK in *FLT3*-mutated AML cells along with resistance to FLT3 inhibitors (sorafenib in this case). In terms of BM-mediated resistance, BTK could also be upregulated by CXCL12 in chronic lymphocytic leukemia,[34] and its high level is associated with the upregulation of CXCR4 in myeloma as well.[35] AML cells with *FLT3* mutations demonstrate a high level of CXCR4,[36] which could be further upregulated with FLT3-targeted treatment (*Online Supplementary Figure S17*). A clinical trial with a combinatorial regime co-targeting FLT3 and CXCR4 with sorafenib and plerixafor led to substantial response rates in relapsed/refractory *FLT3*-mutated AML.[37] Therefore, suppression of BTK might provide additional benefit for overcoming FLT3 inhibitor resistance of the FLT3/CXCR4 combinatorial regime in a BM microenvironment scenario by repressing BTK-mediated upregulation of autophagy. It has been observed that suppression of BTK with ibrutinib, BTK knockdown or CG-806 profoundly abrogated the resistance and sensitized AML cells to FLT3-targeted therapy, accompanied by a decrease of phospho-BTK, ATF4 (or beclin-1) and autophagy in our *in vitro* and *in vivo* experiments, suggesting a potential of targeting BTK in overcoming FLT3-inhibitor resistance in *FLT3*-mutated AML.

Unexpectedly, targeting BTK with CG-806 did not inhibit autophagy in *FLT3* WT AML cells. Conversely, we observed upregulation of the pro-autophagic proteins Beclin-1, ATG7 and phospho-ULK1 (Figure 5A). Our data documented the profound suppression of aurora kinases B and C, rather than BTK inhibition, in these cells (*Online Supplementary Figure S10*). Several groups have reported that targeting aurora kinase triggers pro-autophagic effects in an AMPK-ULK1-dependent manner in cancer cells.[38-40] The multi-kinase inhibitor CG-806 exerts potent inhibition of FLT3, BTK, and aurora kinases at relatively low concentrations (e.g., IC_{50} values less than 5 nM) (*Online Supplementary Table S3*). However, it mainly triggers aurora kinase inhibition in *FLT3* WT cells. In fact, *FLT3* WT cells demonstrated higher activation of aurora kinase (i.e., a high basal level of phospho-aurora kinase) compared to phospho-FLT3 and phospho-BTK, implying that aurora kinase, but not FLT3 or BTK, might be a driver of survival signaling in *FLT3* WT AML cells (*data not shown*). Aurora kinase signaling has recently been identified by Druker's group as an important mechanism of early resistance to the FLT3 inhibitor gilteritinib, and pharmacological inhibition of aurora kinase re-sensitized gilteritinib-induced anti-leukemia effects in *FLT3*-mutant AML.[41] Of note, CG-806 led to impressive suppression of aurora kinase not only in *FLT3* WT cells but also in *FLT3*-mutant AML cells (*data not shown*), implying that targeting aurora kinase in addition to FLT3 can overcome resistance to FLT3 inhibitors in treating both *FLT3* WT and *FLT3*-mutant AML patients.

Our data confirm that pharmacological inhibition of autophagy using chloroquine, or knockdown of ATG7, notably enhanced sensitivity of the *FLT3* WT AML cells to CG-806 (Figure 6A-C), suggesting a protective effect of autophagy in *FLT3* WT AML cells as well. Of note, CG-806 showed much more pronounced cytostatic effects than cytotoxic effects in *FLT3* WT leukemia, even at extremely low concentrations (the IC_{50} values were 3.88 and 11.81 nM in THP-1 and OCI/AML3 cells, respectively), but no apoptogenic effect was observed even at micromolar concentrations (*data not shown)*. Thus, CG-806 predominantly inhibits aurora kinase activity in *FLT3* WT cells and further downregulates the phospho-histone-H3 and CDC25c-CDK2-cyclin B1 axis, which triggers G2/M arrest, but not apoptosis, resulting in preferential inhibition of proliferation, which may be one of main anti-leukemia mechanisms of CG-806 in *FLT3* WT AML.

Interestingly, we observed marked G2/M arrest in the *FLT3* WT cells OCI/AML3 and THP-1 (Figure 5B), but only G1 arrest in the *FLT3*-mutated cells MOLM14 and MV4;11 (*Online Supplementary Figure S18*), after being exposed to low IC_{50} doses (5 to 20 nM) of CG-806. The increase in DNA content of more than 4N within a single nucleus of the *FLT3* WT cells may have resulted from abrogation of mitosis as well as S-phase perturbation.

The formation of polyploidy is frequently observed when cancer cells are exposed to DNA-damaging agents at doses that are insufficient to induce apoptosis, such as those used in chemotherapy or radiotherapy. DNA-damaged cancer cells stop mitotic procession while undergoing repeated cycles of DNA synthesis, leading first to an increase in cells in the G2 phase of the cell cycle, which can then lead to polyploidy. Although the impact of polyploidy on leukemia cell fate is still largely unclear, inhibition of aurora kinase has been reported to induce a mitotic block resulting in apoptosis-resistant polyploidy, which can be enhanced by cyclin G1 in breast cancer cells.[42] Activation of aurora kinase is important for mitotic procession.[43] Selective inhibition of aurora kinase B triggered inhibition of phosphorylated-histone H3, inducing polyploidy formation. Further inhibition of proliferation of a variety of leukemia, lymphoma, and solid tumor cell lines was eventually followed by cell death after 48 h.[44-46] Although we have no direct evidence to address whether polyploidization plays a role in leukemia cell killing in *FLT3* WT cells after exposure to CG-806, polyploid AML cells that transition through mitosis would be expected to trigger lethal multipolar cell division in the next interphase.[47] Our data have indeed shown a marked inhibition of proliferation in *FLT3* WT cells exposed to low doses of CG-806, which was closely associated with G2/M arrest, polyploidy formation, and DNA damage. The last effect was shown with immunoblotting by an increase of γH2AX (Figure 5E). γH2AX is a central player in DNA damage repair.[48] In fact, it has been reported that polyploid cancer cells enter aberrant mitosis leading to cell death.[49] PLK1 is a key intermediate in triggering mitotic exit through activation of cyclin B1 and cdc25c,[50] which have been observed following exposure of *FLT3* WT cells to low doses of CG-806. It has been reported that inhibition of PLK1 resulted in G2/M cell cycle arrest and polyploidy, which mediated anti-tumor activity in a PDX model of colorectal cancer.[51]

Taken together, our results suggest that autophagy is accompanied by an increase in BTK activity as one of the potential mechanisms of resistance, which can be induced by culture conditions that mimic the BM microenvironment. Pharmacological inhibition of autophagy re-sensitized resistant leukemia cells to cell death and co-targeting BTK/FLT3 with CG-806 exerted a marked anti-leukemia effect in *FLT3*-ITD and *FLT3*-ITD/TKD double-mutated AML cells by suppressing survival signaling and autophagy. In addition, CG-806 had a robust anti-leukemia effect in *FLT3* WT AML by arresting cell cycle progression and suppressing DNA re-

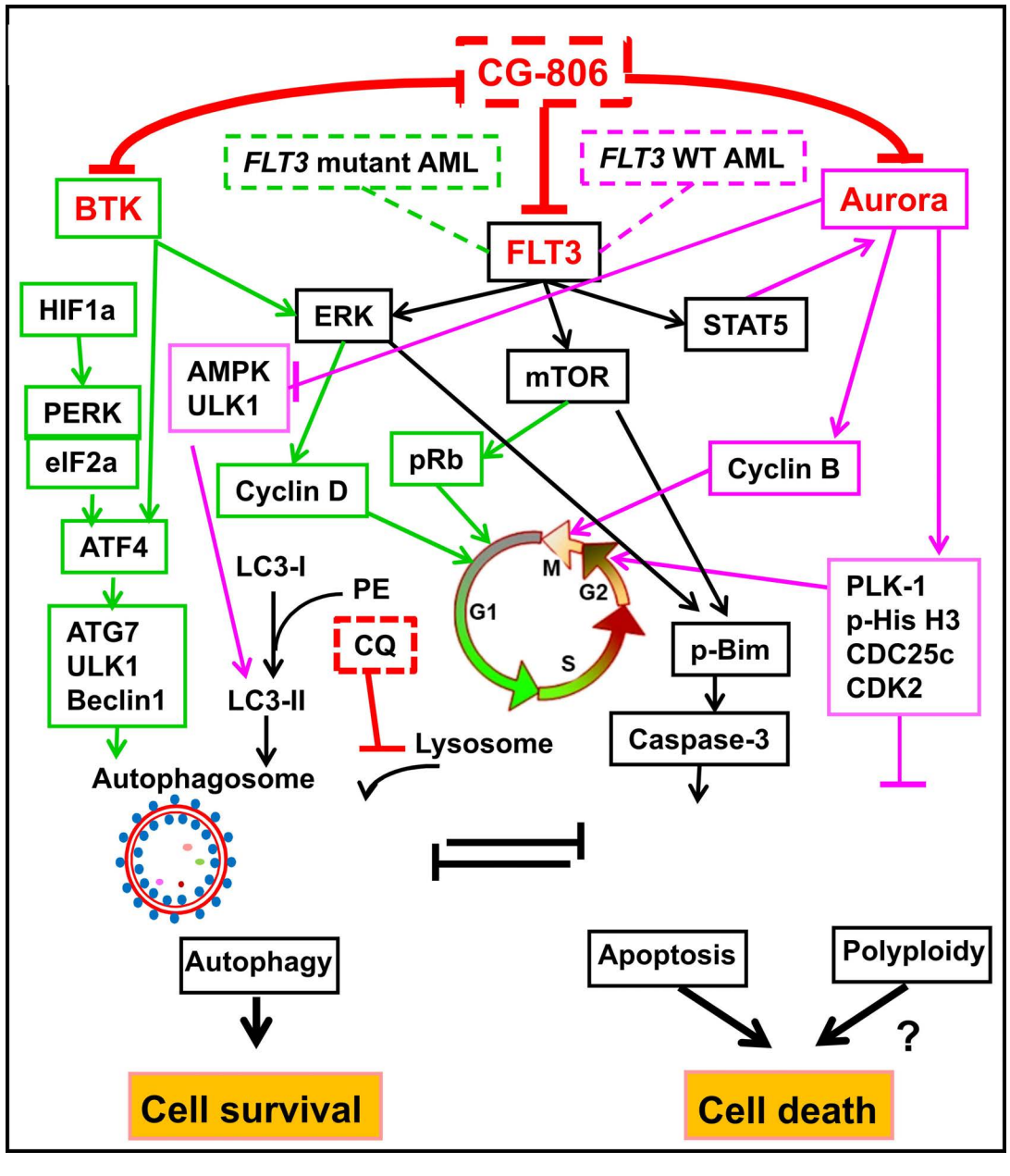

Figure 8. Summary of proposed effects of CG-806 in acute myeloid leukemia. *FLT3*-ITD mutations constitutively activate downstream proliferative signaling pathways, including the MEK/ERK, PI3K/Akt/mTOR and STAT5 pathways, and lead to aberrant growth of leukemia cells. Leukemia cells harboring TKD mutations (with or without internal tandem duplications) demonstrate higher basal levels of autophagy, which can be upregulated under FLT3-inhibitor stress. Phospho-FLT3, phospho-BTK and bone marrow microenvironment-mediated HIF1α also upregulate ATF4 and result in further upregulation of autophagy. The autophagy level is strongly associated with resistance to FLT3 inhibitors. Suppression of autophagy by knocking down BTK or impairing autophagic flux by chloroquine (CQ) through decreasing autophagosome-lysosome fusion re-sensitizes *FLT3*-mutated acute myeloid leukemia (AML) cells to FLT3 inhibition or CG-806-induced pro-apoptotic effects and G1 arrest, leading to the killing of leukemia cells. On the other hand, aurora kinase plays a critical role in driving survival signaling in *FLT3* WT AML cells. Targeting aurora kinase with either its specific inhibitor SNS-314 or CG-806 upregulates pro-autophagic proteins phospho-AMPK, -ULK1, -ATG7, and -Beclin-1. CG-806 exerts dominant inhibition of aurora kinase activity in *FLT3* WT cells and further downregulates PLK-1, phospho-Histone-H3 and the proteins of the CDC25c-CDK2-cyclin B1 axis, which trigger G2/M arrest, but not apoptosis. Inhibition of aurora kinase also impairs the procession through mitosis and results in polyploidization. Targeting *FLT3* WT AML cells with CG-806 triggers cell growth inhibition through G2/M arrest, polyploidy formation, and DNA damage by dominant suppression of aurora kinase.

plication upon aurora kinase inhibition. Mechanistically, low doses of CG-806 triggered the inhibition of proliferation in *FLT3* WT AML through induction of G2/M phase arrest and polyploidization, while high doses triggered apoptosis (Figure 8). This is the first report that the novel multi-kinase inhibitor CG-806 counteracts FLT3-inhibitor resistance in AML by suppressing autophagy through co-targeting BTK and FLT3. It was recently reported that CG-806 demonstrated greater potency against primary AML samples from 364 patients with relapsed or transformed AML.[52] Our work provides additional evidence to suggest that CG-806 could serve as a clinical drug for treating relapsed/refractory AML irrespective of *FLT3* mutational status. A clinical trial in AML (*https://clinicaltrials.gov/ct2/show/NCT04477291*) is ongoing with recently observed complete remission in a FLT3-inhibitor-resistant patient.[53] More detailed data will be reported at the 2022 ASH Annual Meeting (Goldberg AD, *et al.* ASH Abstract #2676).

Disclosures

HZ and WGR are employees of Aptose Biosciences; MA serves on the Aptose Biosciences Scientific Advisory Board.

Contributions

WZ contributed to the design and conduct of the experiments, animal studies, data analysis, and preparation of the manuscript; GY performed most of the in vitro experiments including transmission electron microscopy and analyzed the data; HZ and WGR provided the CG-806 and were involved in analyzing the data; MB and CL performed some of the in vitro experiments; BY and QZ assisted with conducting in vivo studies; VR contributed to vector construction and gene knockdown experiments; SP, SB, GB, VLB and MK contributed to discussions about the manuscript; and MA contributed to the experimental design, data analysis and interpretation, and reviewed and edited the manuscript.

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Data-sharing statement

The data that support the findings of this study are available from the corresponding author (MA), upon reasonable request.

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