Figures and figure supplements

The oncoprotein BCL6 enables solid tumor cells to evade genotoxic stress

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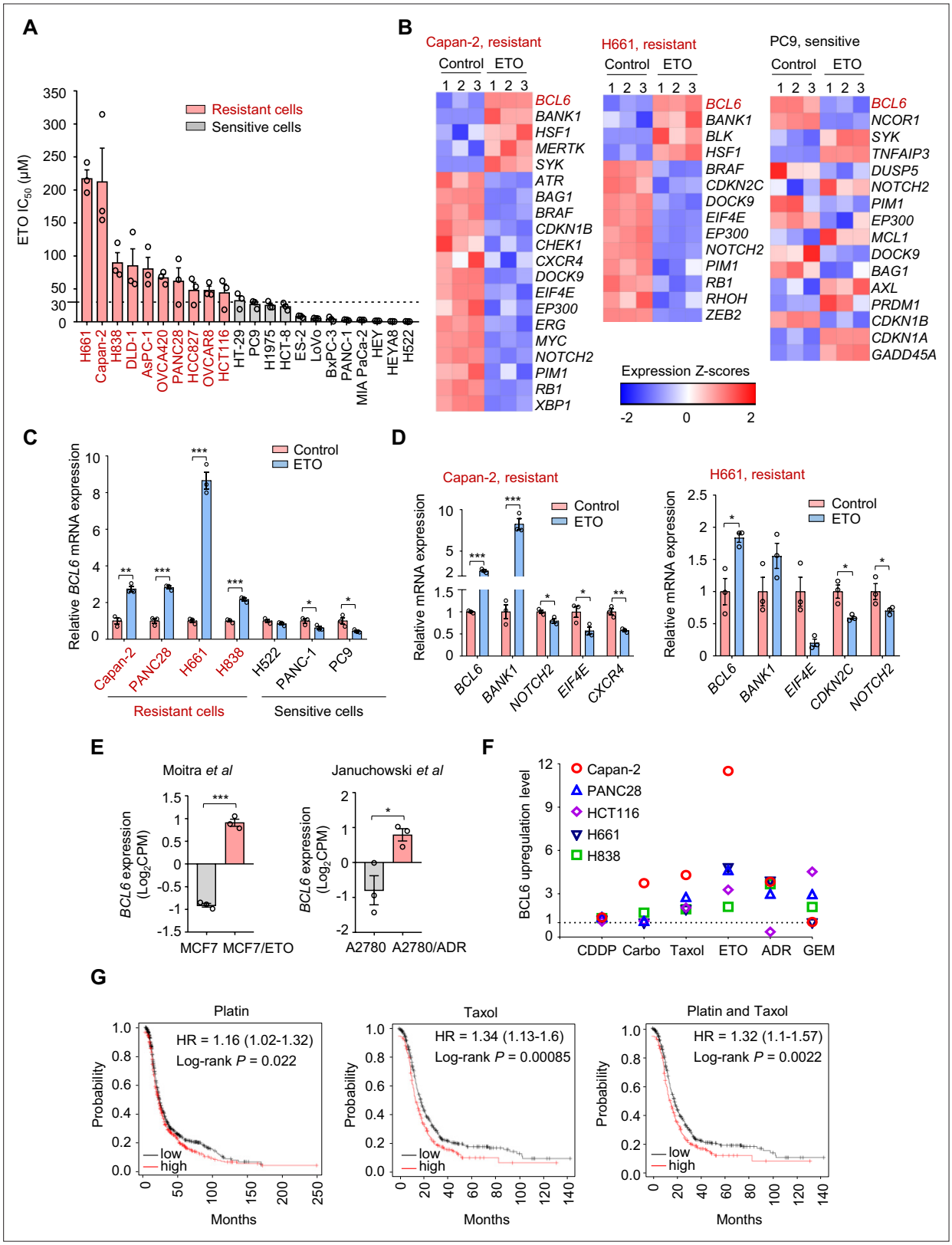

Figure 1. Genotoxic agents promote B cell lymphoma 6 (BCL6) expression. (**A**) Cell sensitivity to etoposide (ETO). Cancer cells were treated with etoposide at gradient concentrations for 48 hr. IC_{50}s were measured using sulforhodamine B (SRB) assays. Values are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results. ETO-resistant cell lines are marked in red. Cell sensitivity to doxorubicin (ADR) was also examined (see ***Figure 1—figure supplement 1A***). (**B**) Heat map illustrating expression of BCL6 target genes in Capan-2,

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H661, and PC9 cells. Cells were treated with etoposide at their respective 1/2 IC_{50}s for 24 hr. mRNA was isolated from treated cells and sequenced. Z-scores were calculated based on counts of exon model per million mapped reads. BCL6 target genes were identified by a cutoff of p< 0.05, n=3. (**C**) *BCL6* mRNA expression in ETO-resistant and -sensitive cells. Cells were treated with etoposide at their respective 1/2 IC_{50}s for 24 hr. QPCR assays were subsequently performed. ETO-resistant cell lines are marked in red. (**D**) Validation of differentially expressed target genes of BCL6 in Capan-2 and H661 cells using qPCR analysis. Values are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results. *p<0.05, **p<0.01 and ***p<0.001, unpaired, two tailed *t*-test. (**E**) Normalized *BCL6* mRNA expression in cells with acquired chemoresistance from published datasets. MCF7/ETO, required ETO-resistant MCF7; A2780/ADR, required ADR-resistant A2780. *p<0.05, ***p<0.001, unpaired, two tailed *t*-test. (**F**) BCL6 protein expression levels in different cancer cell lines in response to various genotoxic agents. Cells were treated with indicated genotoxic agents for 24 hr. BCL6 protein expression levels were detected and normalized to GAPDH expression using immunoblotting analysis. Representative images are shown in ***Figure 1—figure supplement 1B***. The ratio of genotoxic agent-treated group to the control group was calculated. CDDP, cisplatin; Carbo, carboplatin; GEM, gemcitabine. (**G**) Kaplan-Meier curves of ovarian cancer patients treated with cisplatin, taxol or both drugs. The curves were stratified by *BCL6* (215990_s_at) expression. The following source data, ***Supplementary file 1*** and figure supplements are available for ***Figure 1***.

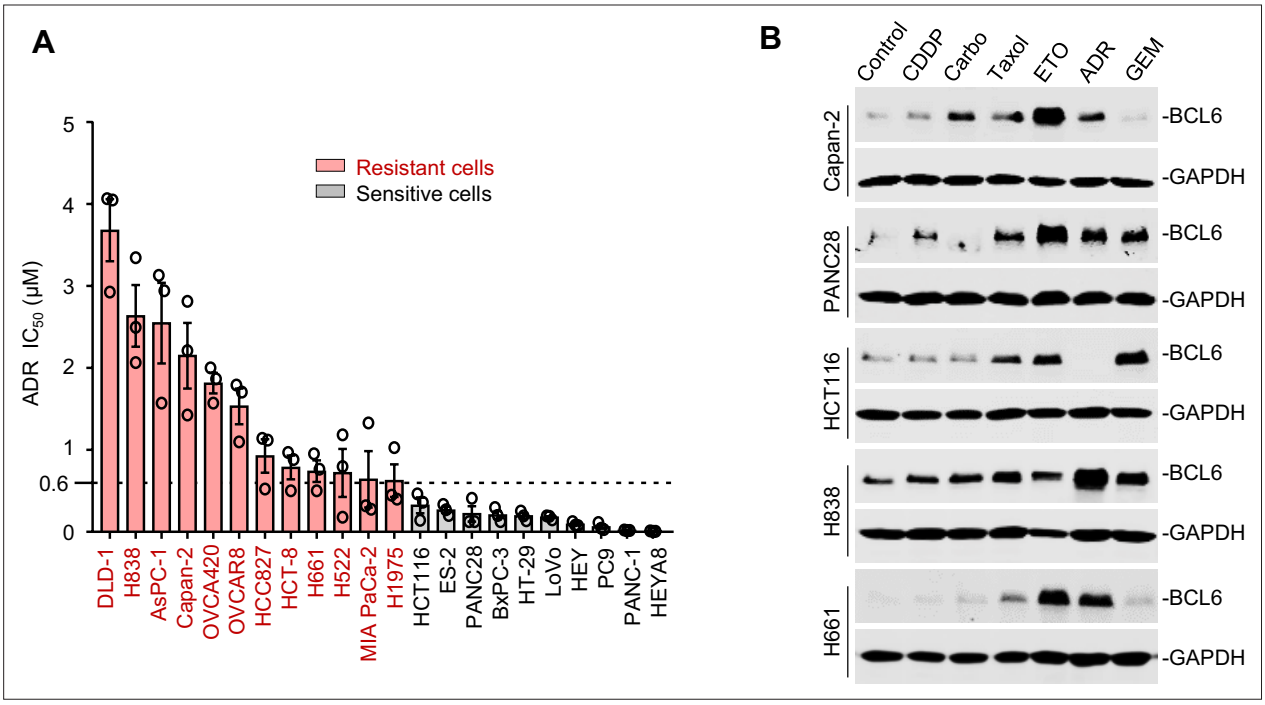

Figure 1—figure supplement 1. Genotoxic agents promote B cell lymphoma 6 (BCL6) expression. (**A**) Cell sensitivity to doxorubicin (ADR). Various cancer cell lines were treated with ADR at gradient concentrations for 48 hr. IC_{50}s were measured using SRB assays. Values are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results. ADR-resistant cell lines are marked in red. (**B**) BCL6 protein expression levels in different cancer cell lines in response to genotoxic agents. Cells were treated with indicated genotoxic agents at their respective IC_{50}s for 24 hr. Proteins lysates from each cell line were blotted individually. CDDP, cisplatin; Carbo, carboplatin; ETO, etoposide; GEM, gemcitabine.

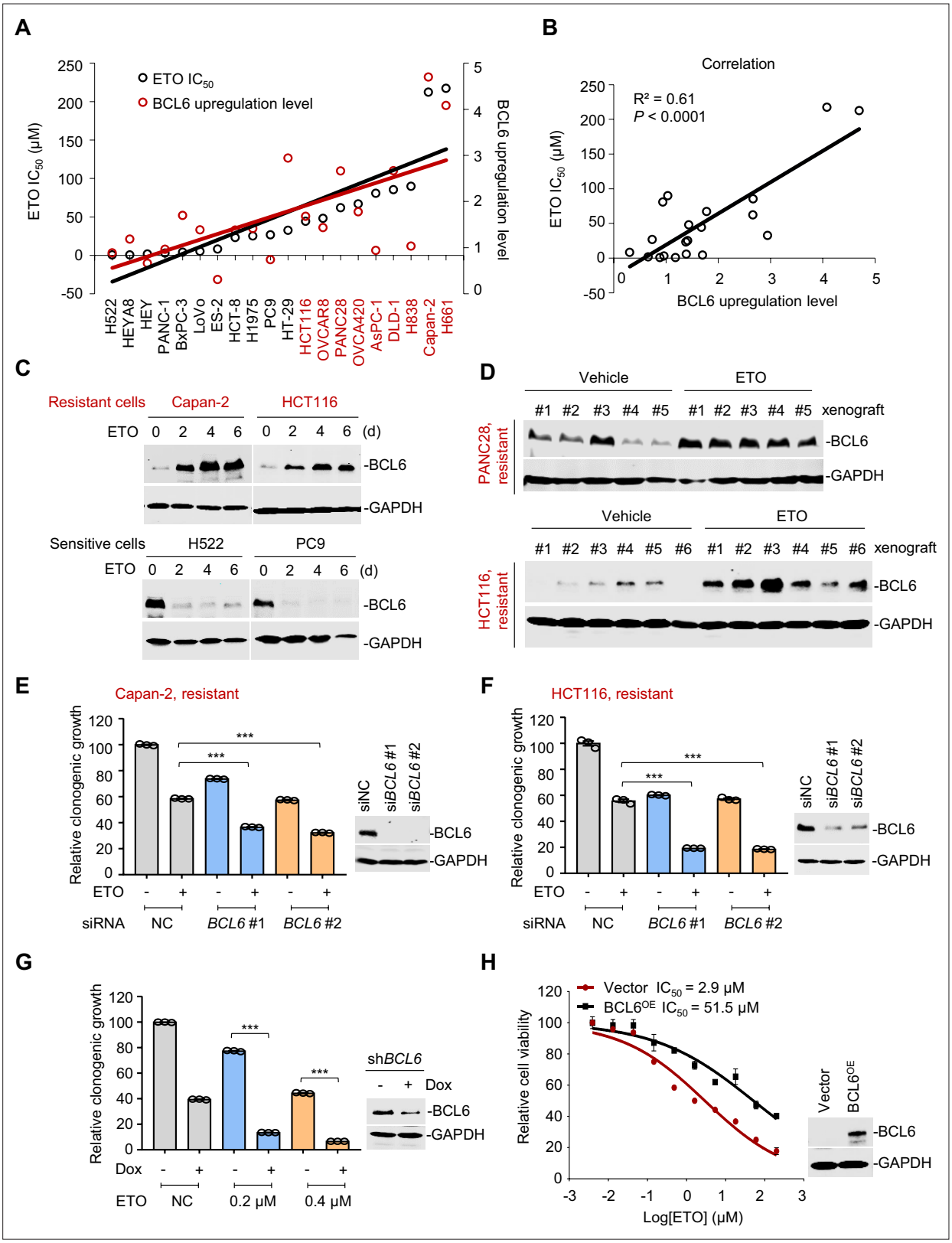

Figure 2. B cell lymphoma 6 (BCL6) transactivation is correlated with therapy resistance. (**A**) Association between BCL6 upregulation with ETO sensitivity in various cancer cell lines. Representative images are shown in ***Figure 2—figure supplement 1A***. Left vertical axis, IC_{50}s of etoposide in different cancer cell lines; right vertical axis, relative BCL6 protein levels compared with that of the control group; horizontal axis, cancer cell lines. ETO-resistant cell lines are marked in red. (**B**) Correlation analysis. Correlation between BCL6 upregulation levels and ETO IC_{50}s or ADR IC_{50}s (see ***Figure 2—figure***

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supplement 1B). (**C**) Etoposide induced BCL6 protein expression in a time-dependent manner. ETO-resistant or -sensitive cells were treated with etoposide at their respective 1/4 IC_{50}s for 2, 4, or 6 days. Cell lysates were collected and probed with specific antibodies using Western blotting assays. (**D**) Etoposide increased BCL6 expression in PANC28 and HCT116 xenografts treated with 10 mg/kg etoposide for 14 days. At the end of the experiment, tumor tissues were isolated and subjected to immunoblotting analysis. Biologically independent samples of each group are shown. Tumor volume curves and tumor weight are shown in ***Figure 2—figure supplement 1C-D***. (**E and F**) Clonogenic growth of ETO-resistant cells. Capan-2 (**E**) or HCT116 cells (**F**) were transfected with *BCL6* siRNAs or the control siRNA, followed by the treatment of 0.2 μM etoposide for 7 days. The expression of BCL6 was detected by immunoblotting analysis (*right*). Values are expressed by setting the control group as 100%. (**G**) Clonogenic growth of ETO-resistant cells. HCT116 cells stably transfected with shRNA targeting *BCL6* were exposed to etoposide (0.2 or 0.4 μM) with or without doxycycline (Dox) for 7 days. The clonogenic growth were examined. The BCL6 expression levels were detected by an immunoblotting assay (*right*). (**H**) BCL6 overexpression decreased the sensitivity of H522 cells to etoposide (*left*). ETO-sensitive H522 cells were transfected with pcDNA3.1-BCL6 or control plasmid, and then treated with etoposide at gradient concentrations for 48 hr. The etoposide IC_{50}s were detected by SRB assays. BCL6 overexpression efficiency was examined by an immunoblotting assay (*right*). Values are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results. ***p<0.001, unpaired, two tailed *t*-test. The following source data, ***Supplementary file 1*** and figure supplements are available for ***Figure 2***.

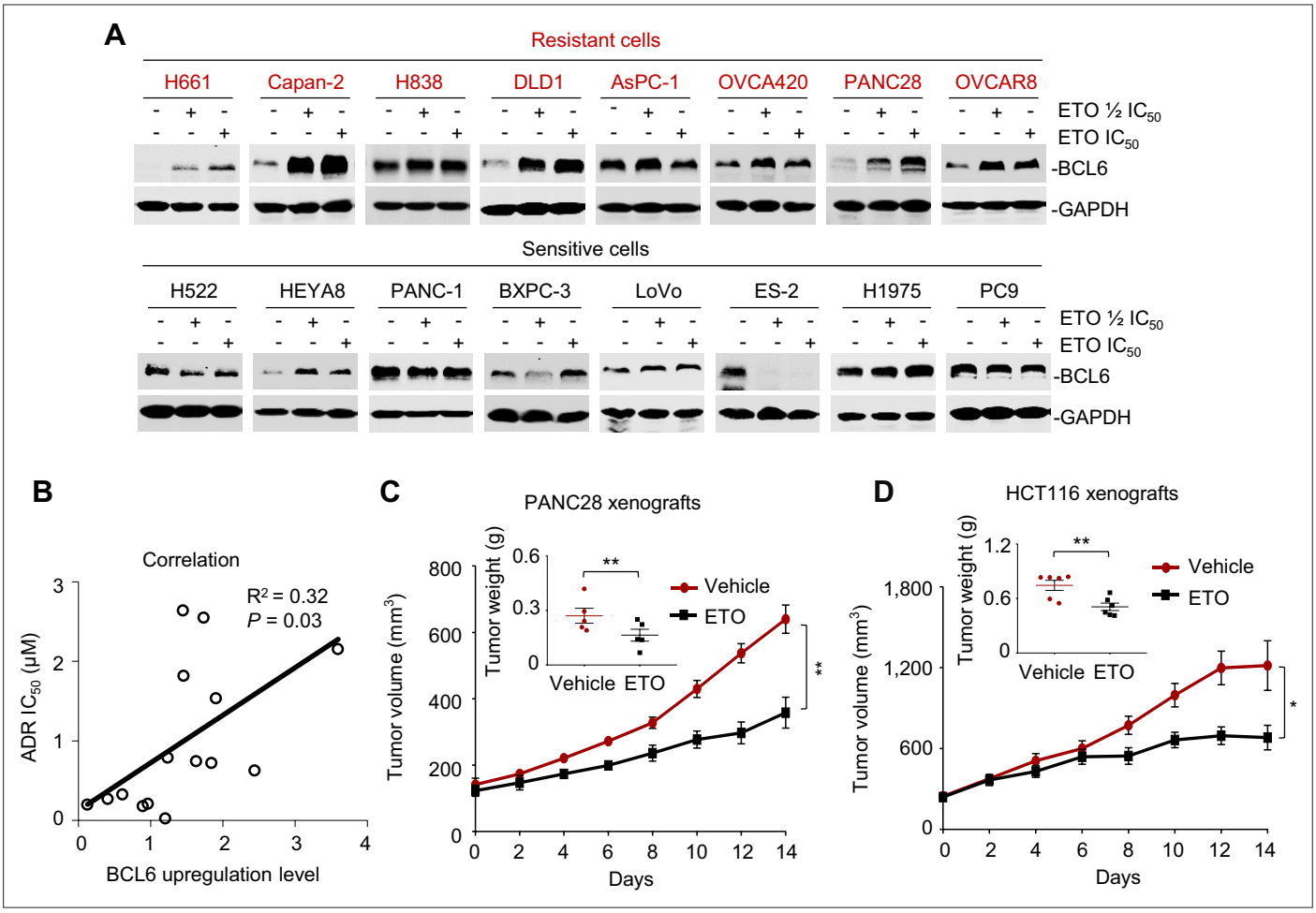

Figure 2—figure supplement 1. B cell lymphoma 6 (BCL6) upregulation is associated with therapy resistance. (**A**) ETO induced BCL6 protein expression. ETO-resistant or -sensitive cancer cells were treated with etoposide at their 1/2 IC_{50}s or IC_{50}s for 24 hr, respectively. Proteins lysates from each cell line were blotted individually. ETO-resistant cell lines are marked in red. (**B**) Correlation between BCL6 upregulation levels and ADR IC_{50}s in various cancer cell lines. (**C and D**) Tumor volume and tumor weight of PANC28 (**C**) and HCT116 xenografts (**D**) on day 14. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, unpaired, two tailed *t*-test. Five to six mice each group.

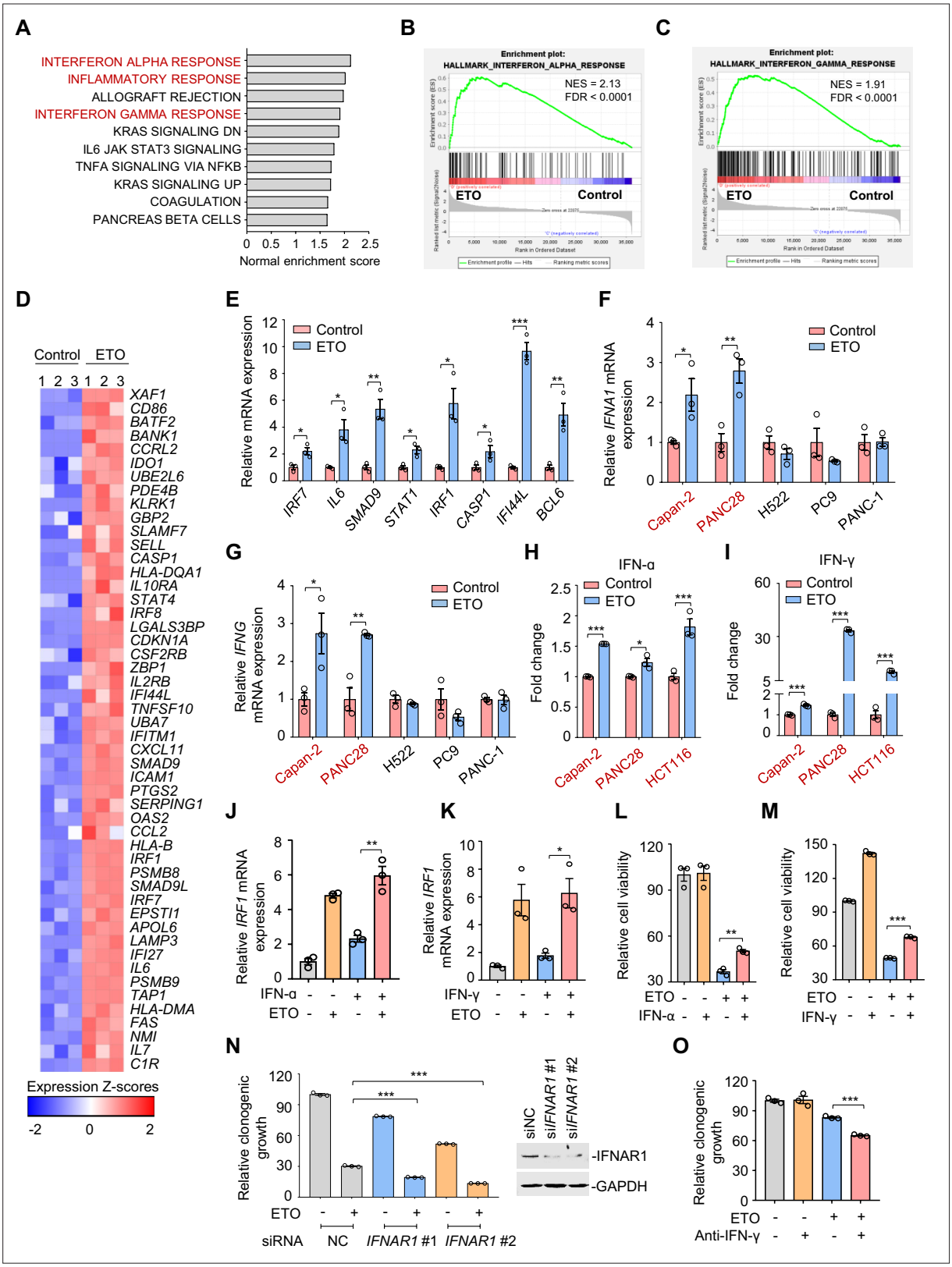

Figure 3. Genotoxic stress activates interferon responses. (**A**) Gene set enrichment analysis of pathways significantly upregulated in Capan-2 cells treated with 50 μM etoposide for 24 hr (n=3). GO analysis of PC9, Capan-2, and H661 cells are shown in ***Figure 3—figure supplement 1A***. (**B and C**) Enrichment plots for genes associated with interferon α (IFN-α), (**B**) and interferon γ (IFN-γ), (**C**) responses in etoposide-treated Capan-2 cells or H661 cells (see ***Figure 3—figure supplement 1B, C***). (**D**) Heat map illustrating of representative gene expression of IFN-α and IFN-γ responses in

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treated Capan-2 cells or H661 cells (see ***Figure 3—figure supplement 1D***). Z-scores were calculated based on counts of exon model per million mapped reads. Upregulated and downregulated genes were identified by a cutoff of $p < 0.05$. (**E**) Validation of inflammation-related gene expression in (**D**). Capan-2 cells were treated with 50 μM etoposide for 24 hr. QPCR assays were subsequently performed. (**F and G**) IFN-α (**F**) and IFN-γ (**G**) mRNA expression levels in treated cells. ETO-sensitive and -resistant cells were treated with etoposide at their respective 1/2 IC_{50}s for 24 hr, and qPCR analysis was further performed. ETO-resistant cell lines are marked in red. (**H and I**) IFN-α (**H**) and IFN-γ (**I**) production in ETO-resistant cells. Cells were treated with etoposide at their respective 1/2 IC_{50}s for 48 hr. The concentrations of IFN-α and IFN-γ in cell lysates were measured using an ELISA assay. (**J and K**) Relative *IRF1* mRNA levels in Capan-2 cells. Capan-2 cells were treated with 50 ng/mL IFN-α (**J**) or 10 ng/mL IFN-γ (**K**) in the presence or absence of 50 μM etoposide. *IRF1* mRNA levels were detected by qPCR assays. (**L and M**) Relative cell viability. ETO-sensitive H522 cells were treated with etoposide alone, 50 ng/mL IFN-α (**L**), 10 ng/mL IFN-γ (**M**) or their combinations. Cell viability were examined by SRB assays. Values are expressed as mean ± SEM by setting the control group as 100%. (**N**) Clonogenic growth of Capan-2 cells treated with si*IFNAR1*, 0.4 μM etoposide, or their combinations (*left*). *IFNAR1* silencing efficiency was examined using immunoblotting analysis (*right*). Cell viability curves are shown in ***Figure 3—figure supplement 1E***. (**O**) Clonogenic growth showing the relative survival of Capan-2 cells treated with 0.2 μM etoposide, 10 μg/mL anti-IFN-γ or both. Cell viability curves are shown in ***Figure 3—figure supplement 1F***. Results in this panel (**E–O**) are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results. *p<0.05, **p<0.01 and ***p<0.001, unpaired, two tailed *t*-test. The following source data, ***Supplementary file 1*** and figure supplements are available for ***Figure 3***.

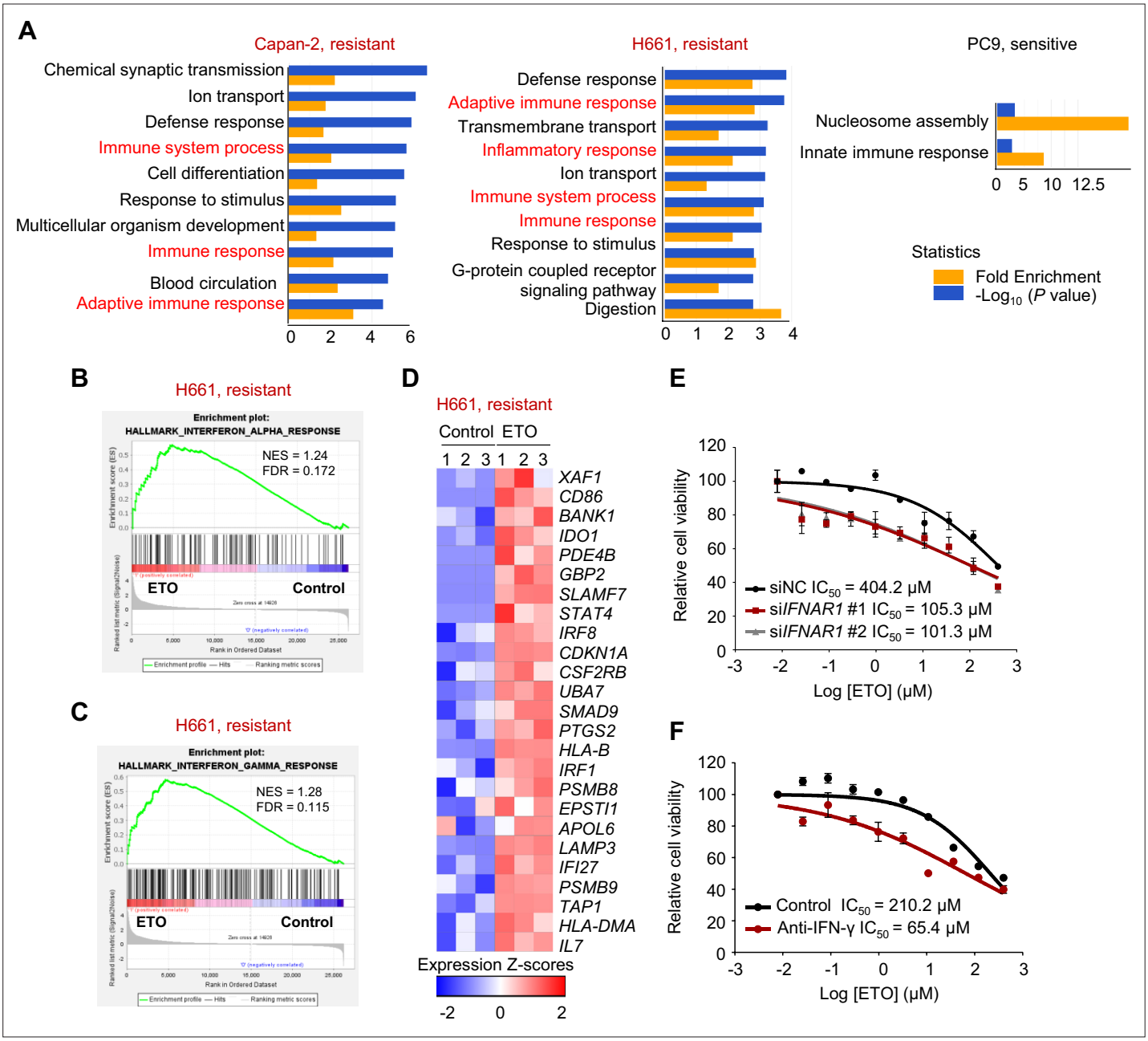

Figure 3—figure supplement 1. Genotoxic stress activates interferon responses. (**A**) GO analysis of RNA-seq data (ETO treatment group versus the control group). The upregulated pathways in Capan-2, H661, and PC9 cells are shown, n=3. Graph displays category scores as $\log_{10}$ (p value) from Fisher's exact test. (**B and C**) Enrichment plots for genes associated with interferon α (IFN-α) (**B**) and interferon γ (IFN-γ) (**C**) responses in etoposide-treated H661 cells. (**D**) Heap map illustrating of representative gene expression of IFN-α and IFN-γ responses in treated H661 cells. Z-scores were calculated based on counts of exon model per million mapped reads. Upregulated and downregulated genes were identified by a cutoff of $p<0.05$. (**E**) Silencing of *IFNAR1* enhanced Capan-2 cells sensitivity to etoposide. Capan-2 cells were transfected with *IFNAR1* siRNAs or the control siRNA for 48 hr. Transfected cells were then exposed to etoposide at gradient concentrations for 48 hr. Cell viability was detected using SRB assays. (**F**) Anti-IFN-γ antibody increased etoposide cytotoxicity. Capan-2 cells were treated with etoposide at gradient concentrations for 48 hr in the presence or absence of 10 μg/mL anti-IFN-γ antibody. Cell viability was detected using SRB assays. Values in (**E**) and (**F**) are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results.

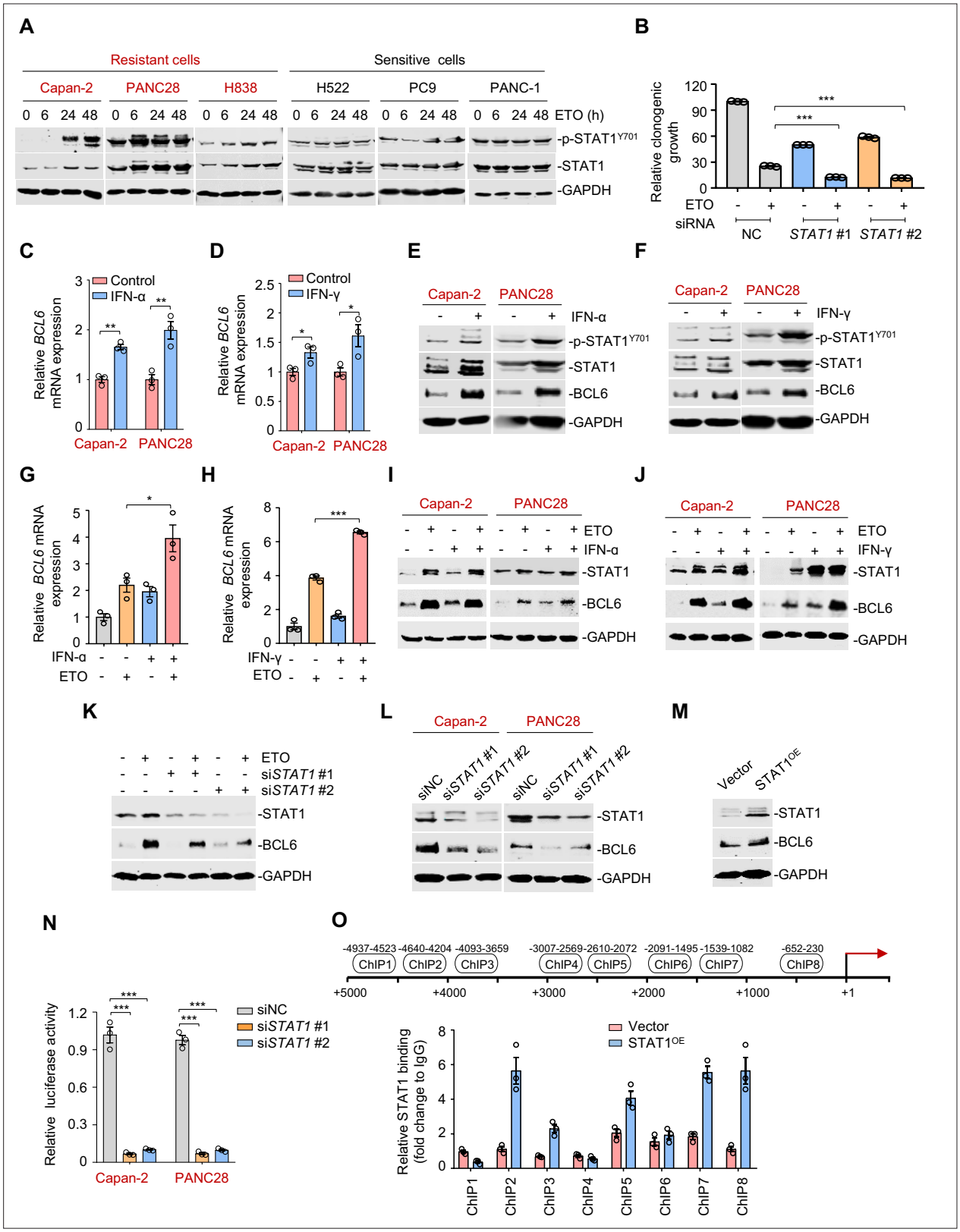

Figure 4. The interferon/STAT1 axis directly regulates B cell lymphoma 6 (BCL6) expression. (**A**) STAT1 protein and its phosphorylation levels by immunoblotting analysis. ETO-resistant and -sensitive cells were treated with etoposide at their respective 1/2 IC_{50}s for indicated time points. Cell lysates were collected and subjected to immunoblotting analysis. (**B**) Clonogenic growth of Capan-2 cells treated with siRNAs targeting *STAT1*, 0.2 μM etoposide, or their combinations. (**C and D**) Relative *BCL6* mRNA expression. Capan-2 or PANC28 cells were treated with 50 ng/mL IFN-α (**C**) or 10 ng/

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mL IFN-γ (**D**) for 24 hr. *BCL6* mRNA levels were detected by qPCR assays. (**E and F**) IFN-α and IFN-γ increased BCL6 and STAT1 protein levels. Capan-2 or PANC28 cells were treated with 50 ng/mL IFN-α (**E**) or 10 ng/mL IFN-γ (**F**) for 24 hr. Cell lysates were subjected to immunoblot analysis with indicated antibodies. (**G and H**) Relative *BCL6* mRNA expression. Capan-2 cells were treated with 50 ng/mL IFN-α (**G**) or 10 ng/mL IFN-γ (**H**) in the presence or absence of 50 μM etoposide. *BCL6* mRNA levels were detected. The same experiments were also repeated in PANC28 cells (see ***Figure 4—figure supplement 1A, B***). (**I and J**) Immunoblotting analysis for BCL6 and STAT1 protein expression. Capan-2 or PANC28 cells were treated with 50 ng/mL IFN-α (**I**) or 10 ng/mL IFN-γ (**J**) in the presence or absence of etoposide for 48 hr. Cell lysates were subjected to immunoblotting analysis with specific antibodies against BCL6, STAT1, and GAPDH. (**K**) *STAT1* knockdown impaired etoposide-induced BCL6 activation. *STAT1* silencing was performed by RNA interference in Capan-2 cells. Transfected cells were treated with 50 μM etoposide for 24 hr, and cell lysates were subjected to immunoblotting analysis. (**L**) Silencing of *STAT1* decreased BCL6 expression in ETO-resistant Capan-2 and PANC28 cells. (**M**) Overexpression of STAT1 increased BCL6 expression. Capan-2 cells were transfected with pcDNA3.1-STAT1 or the control plasmid for 48 hr. Cell lysates were subjected to immunoblotting. (**N**) Relative luciferase activity. siRNAs targeting *STAT1* and BCL6n-luc vector were transiently co-transfected into ETO-resistant Capan-2 and PANC28 cells. Luciferase activity was measured 48 hr post-transfection. (**O**) ChIP-qPCR data showing the enrichment of STAT1 binding to the *BCL6* promoter region in Capan-2 cells. Capan-2 cells were transfected with pcDNA3.1-STAT1 or the control plasmid for 48 hr, and ChIP-qPCR analysis was then performed. Results are expressed as mean ± SEM of three technical replicates, representative of two or three independent experiments with similar results. * $p<0.05$, ** $p<0.01$, *** $p<0.01$, unpaired, two tailed *t*-test. The following source data, ***Supplementary file 1*** and figure supplements are available for ***Figure 4***.

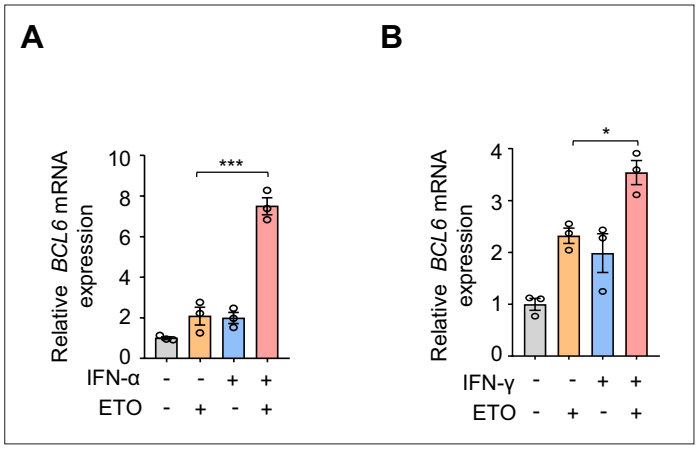

Figure 4—figure supplement 1. The interferon/STAT1 axis directly regulates B cell lymphoma 6 (BCL6) expression. (**A and B**) Relative *BCL6* mRNA expression in PANC28 cells. Cells were treated with 50 ng/mL IFN-α (**A**) or 10 ng/mL IFN-γ (**B**) in the presence or absence of 50 μM etoposide. *BCL6* mRNA levels were detected by qPCR assays. All values are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results. *p<0.05, ***p<0.001, unpaired, two tailed *t*-test.

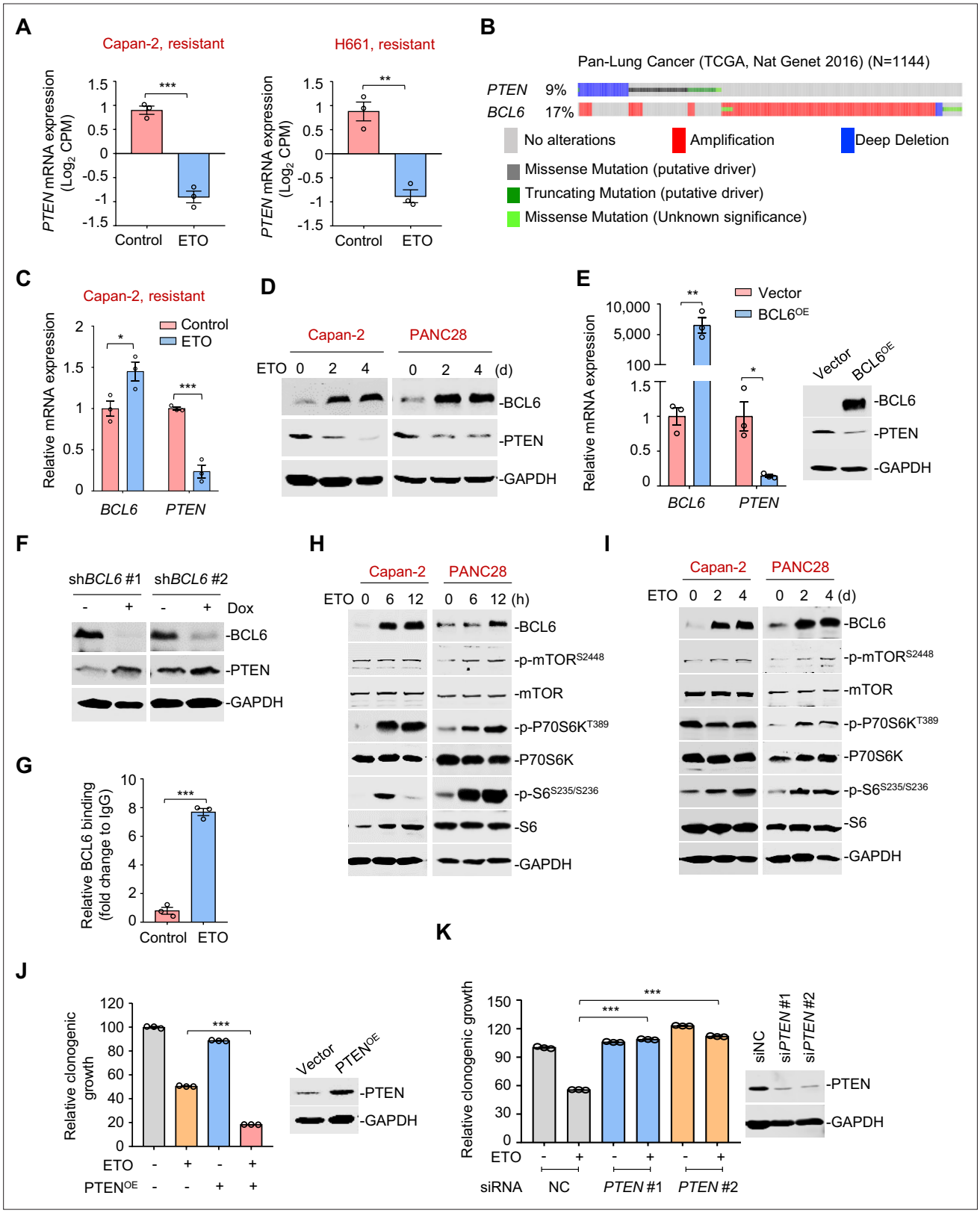

Figure 5. The tumor suppressor PTEN is a functional target of B cell lymphoma 6 (BCL6). (**A**) Normalized *PTEN* expression levels in etoposide-resistant Capan-2 and H661 cells treated with etoposide at their respective IC_{50}s for 24 hr. RNA-seq tag count at exons was plotted as counts of exon model per million mapped reads. (**B**) Genomic alteration of *BCL6* and *PTEN* according to TCGA database (n = 1144). The percentage of gene alteration is shown. (**C**) Relative mRNA expression of *BCL6* and *PTEN*. Capan-2 cells were exposed to etoposide at their respective 1/2 IC_{50}s for 24 hr. QPCR analysis was

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further carried out. (**D**) BCL6 and PTEN protein levels in Capan-2 and PANC28 cells. Cells were treated with etoposide at their respective 1/4 IC_{50}s for 2 or 4 days. Cell lysates are subjected to immunoblotting analysis. (**E**) BCL6 overexpression decreased *PTEN* mRNA and protein levels in HCT116 cells. Cells were transfected with pcDNA3.1-BCL6 or the control plasmid. Total mRNA and protein were extracted and subjected to qPCR analysis (*left*) and immunoblotting analysis (*right*). (**F**) *BCL6* inducible knockdown increased PTEN expression. Immunoblotting analysis of PTEN in HCT116 cells treated with or without doxycycline. (**G**) BCL6 binding levels at the promoter region of *PTEN* examined by ChIP-qPCR assays. (**H**) Etoposide activated mTOR signaling components in etoposide-resistant Capan-2 and PANC28 cells. Cells were treated with etoposide at their respective 1/2 IC_{50}s for 6 or 12 hr. Whole-cell lysates were prepared and subjected to immunoblotting analysis. (**I**) A long-term treatment with etoposide activated mTOR signaling components in ETO-resistant cells. Capan-2 and PANC28 cells were treated with 10 μM etoposide for 2 or 4 days. Cell lysates were subjected to immunoblotting analysis. (**J**) PTEN overexpression increased the sensitivity of etoposide-resistant cells to etoposide. PANC28 cells were transfected with pCDH-PTEN or the control plasmid. PTEN overexpression efficiency was measured immunoblotting analysis (*up*). Quantification of clonogenic growth after 7 days treatment with 0.2 μM etoposide (*down*). (**K**) Clonogenic growth of ETO-sensitive cells. PC9 cells were transfected with *PTEN* siRNAs or the control siRNA, followed by the treatment of 0.2 μM etoposide for 7 days. The expression of PTEN was detected by immunoblotting analysis (*right*). Values are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results. *p<0.05, **p<0.01, ***p<0.001, unpaired, two tailed *t*-test. The following source data and ***Supplementary file 1*** are available for ***Figure 5***.

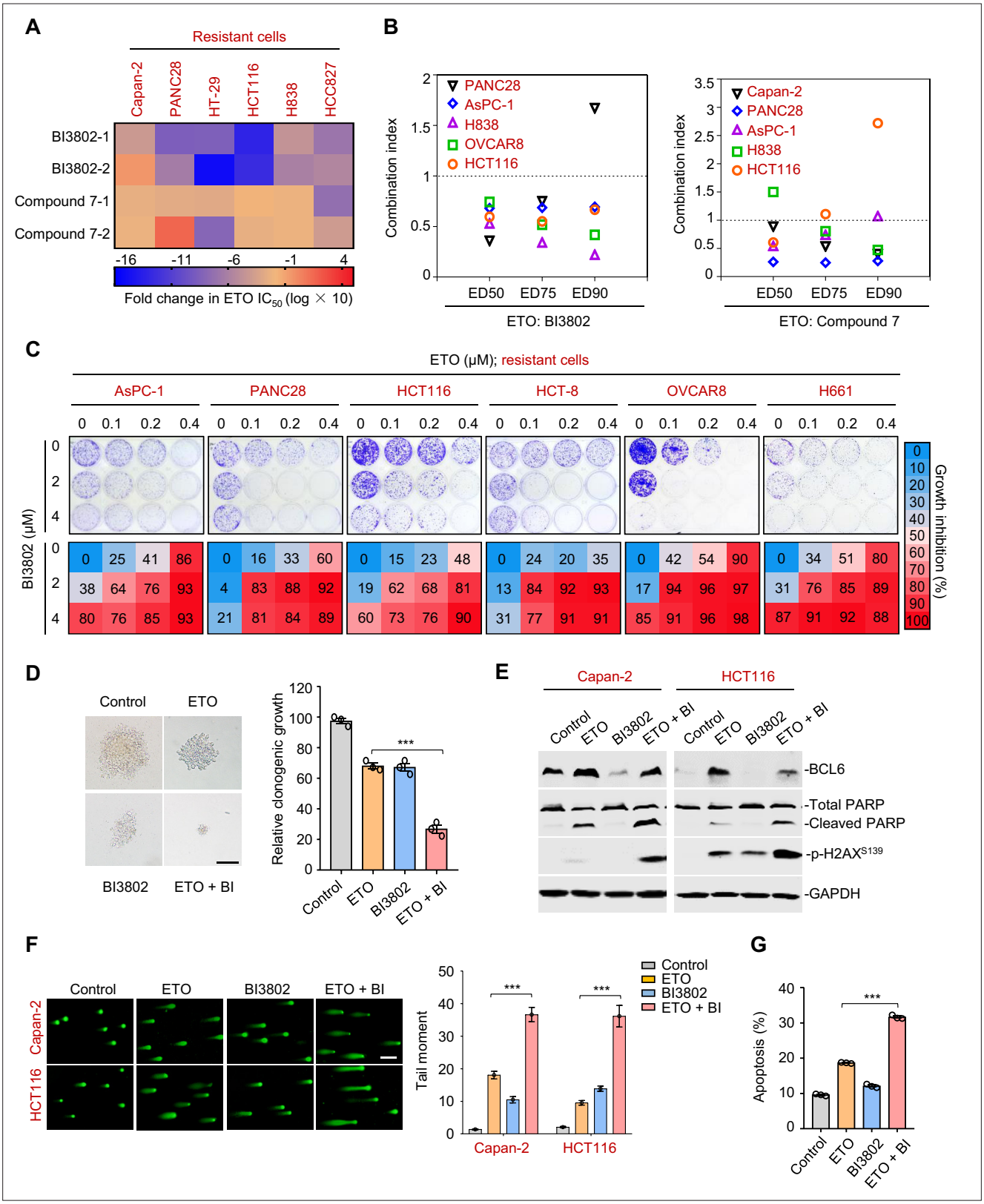

Figure 6. Therapeutic suppression of B cell lymphoma 6 (BCL6) sensitizes genotoxic agents. (**A**) Pharmacological inhibition of BCL6 increased ETO sensitivity. Various types of cancer cells were treated with etoposide at gradient concentrations for 48 hr in the presence of 10 μM BI3802 or 20 μM Compound 7 (n=2 biological replicates). IC_{50}s were measured using SRB assays. For graphs, log(IC_{50}) of control cells was subtracted from log(IC_{50}) of BI3802 or Compound 7-treated cells and multiplied by ten to be depicted as log fold change × 10. Targeted inhibition of BCL6 also increased ADR

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sensitivity (see ***Figure 6—figure supplement 1A***). (**B**) Synergistic interaction between BCL6 inhibitors (BI3802 or Compound 7) and ETO. Growth inhibition was averaged and input into CalcuSyn software to extrapolate combinational index values (CI) at 50% effective dose (ED50), 75% effective dose (ED75), and 90% effective dose (ED90). CI values < 1 represent synergism. The synergy between BI3802 and ADR was also detected in H838, Capan-2, and AsPC-1 cells (see ***Figure 6—figure supplement 1B***). (**C**) Inhibition of clonogenic growth by the combined regimen. Representative long-term clonogenic images (*up*) and quantified clonogenic growth inhibition results (*down*) for cells treated with ETO, BI3802, or their combinations. The same experiments were also conducted for ADR (see ***Figure 6—figure supplement 1C***). (**D**) Inhibition of soft-agar colony growth by the combined regimen. HCT116 cells were exposed to 0.2 μM etoposide, 2 μM BI3802, or their combinations. Representative images of soft-agar colonies (*left*) and the quantified clonogenic growth (*right*) are shown. Scale bar, 100 μm. (**E**) Immunoblotting analysis showing the protein expression of BCL6, p-H2AX^{S139}, and cleaved-PARP in Capan-2 and HCT116 cells treated with 15 μM etoposide, 10 μM BI3802 or their combinations for 48 hr. Cell lysates were subjected to immunoblotting analysis. (**F**) Comet assays. Capan-2 and HCT116 cells were treated with etoposide, BI3802, or their combinations for 48 hr. The tail moment was quantified for 50 cells for each experimental condition (*right*). Scale bar, 100 μm. (**G**) Quantification of apoptotic cells in Capan-2 cells analyzed by flow cytometry. Cells were exposed to 15 μM etoposide, 10 μM BI3802 or their combinations for 48 hr. Percentage of positive cells was counted. Values are expressed as the mean of three replicates ± SEM, representative of three independent experiments with similar results. ***p <0.001, unpaired, two tailed *t*-test. The following source data and figure supplements are available for ***Figure 6***.

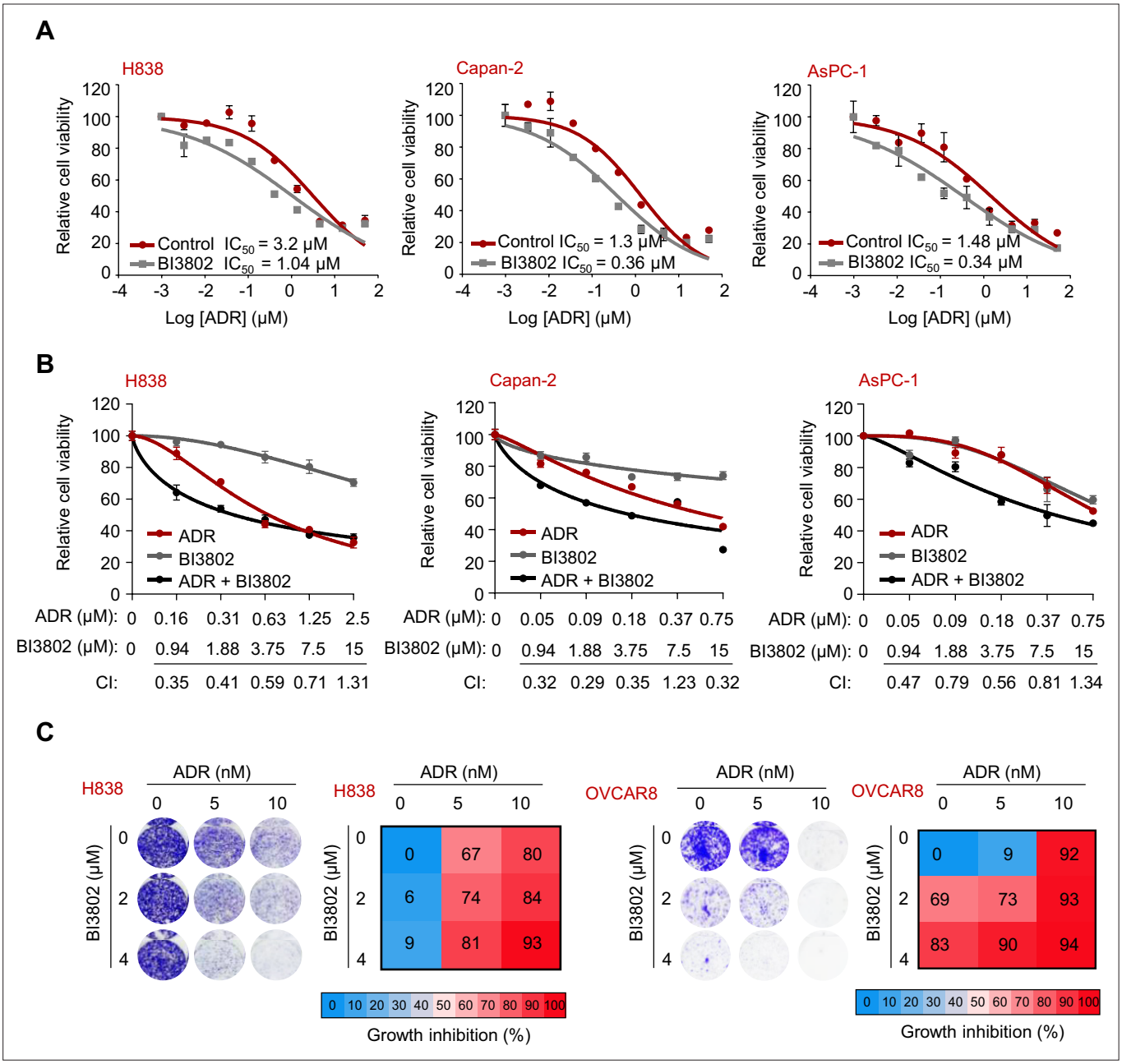

Figure 6—figure supplement 1. B cell lymphoma 6 (BCL6) inhibition sensitizes cancer cells to doxorubicin. (**A**) Increased sensitivity of cancer cells to doxorubicin. ADR-resistant cancer cells were treated with doxorubicin at gradient concentrations for 48 hr in the presence of 10 μM BI3802. IC_{50}s were measured using SRB assays. (**B**) Cell viability of ADR-resistant cancer cells treated with different concentrations of doxorubicin in the combination with BI3802. Growth inhibition for three independent biological replicate experiments was averaged and input into CalcuSyn software to extrapolate CI values. CI values < 1 represent synergism. Values are expressed as the mean of three replicates ± SEM, representative of three independent experiments with similar results. (**C**) Representative long-term clonogenic assays (*left*) and quantified clonogenic growth inhibition data (*right*) for H838 and OVCAR8 cells treated with ADR, BI3802, or their combinations. ADR-resistant cell lines are marked in red.

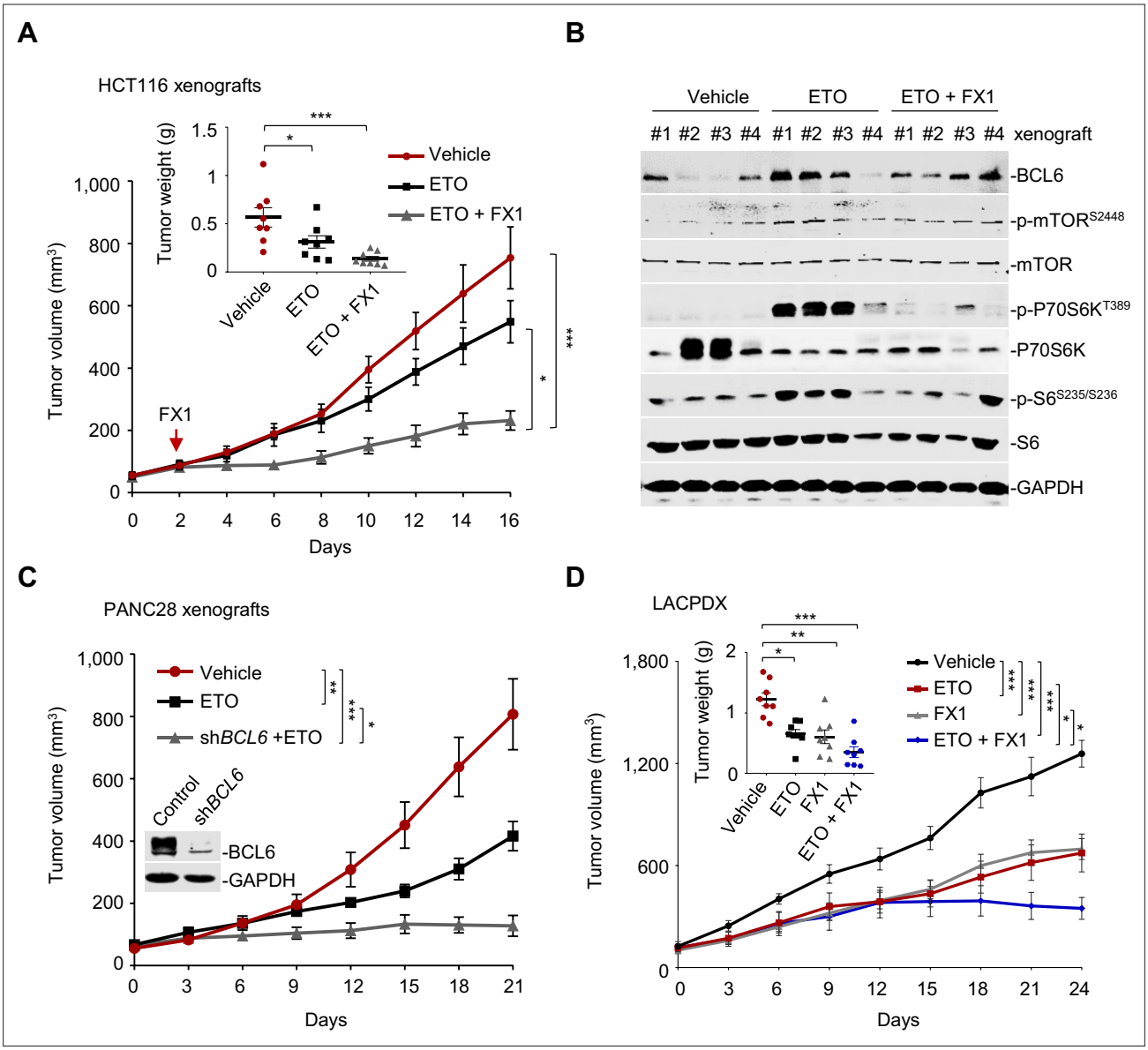

Figure 7. Pharmacological inhibition of B cell lymphoma 6 (BCL6) synergizes etoposide in vivo. (**A**) Tumor growth curves. Mice bearing HCT116 xenografts were treated with vehicle, etoposide (10 mg/kg body weight), and etoposide plus FX1 (5 mg/kg body weight) for indicated times. Average tumor weight on day 16 is shown in the inset. Values are expressed as mean ± SEM, n=8. *p<0.05, ***p<0.001, one-way ANOVA with Tukey's multiple-comparisons test. QPCR and immunoblotting analysis for BCL6 expression of tumors on day 2, 4, or 8 were conducted (see ***Figure 7—figure supplement 1A***). (**B**) Protein expression of BCL6 and mTOR signaling components in HCT116 xenografts. Tumors were harvested at the end of treatment and subjected to immunoblotting analysis. Four biologically independent samples per group are shown. Representative immunohistochemical images are shown in ***Figure 7—figure supplement 1B***. (**C**) Tumor growth curves. Mice were implanted with sh*BCL6* PANC28 or control cells and received etoposide treatment (10 mg/kg body weight). Values are expressed as mean ± SEM, n=6. *p<0.05, ***p<0.001, one-way ANOVA with Tukey's multiple-comparisons test. (**D**) Tumor growth curves. Mice bearing primary *KRAS*-mutant lung cancer xenografts (LACPDX) were treated with vehicle, etoposide (10 mg/kg body weight), FX1 (5 mg/kg body weight) or both drugs in combination for 24 days. Average tumor weight on day 24 is shown in the inset, n=8. Values are expressed as mean ± SEM. *p<0.05, **p<0.01, ***p <0.001, one-way ANOVA with Tukey's multiple-comparisons test. Representative immunohistochemical images are shown in ***Figure 7—figure supplement 1C***. The following source data, ***Supplementary file 1*** and figure supplements are available for ***Figure 7***.

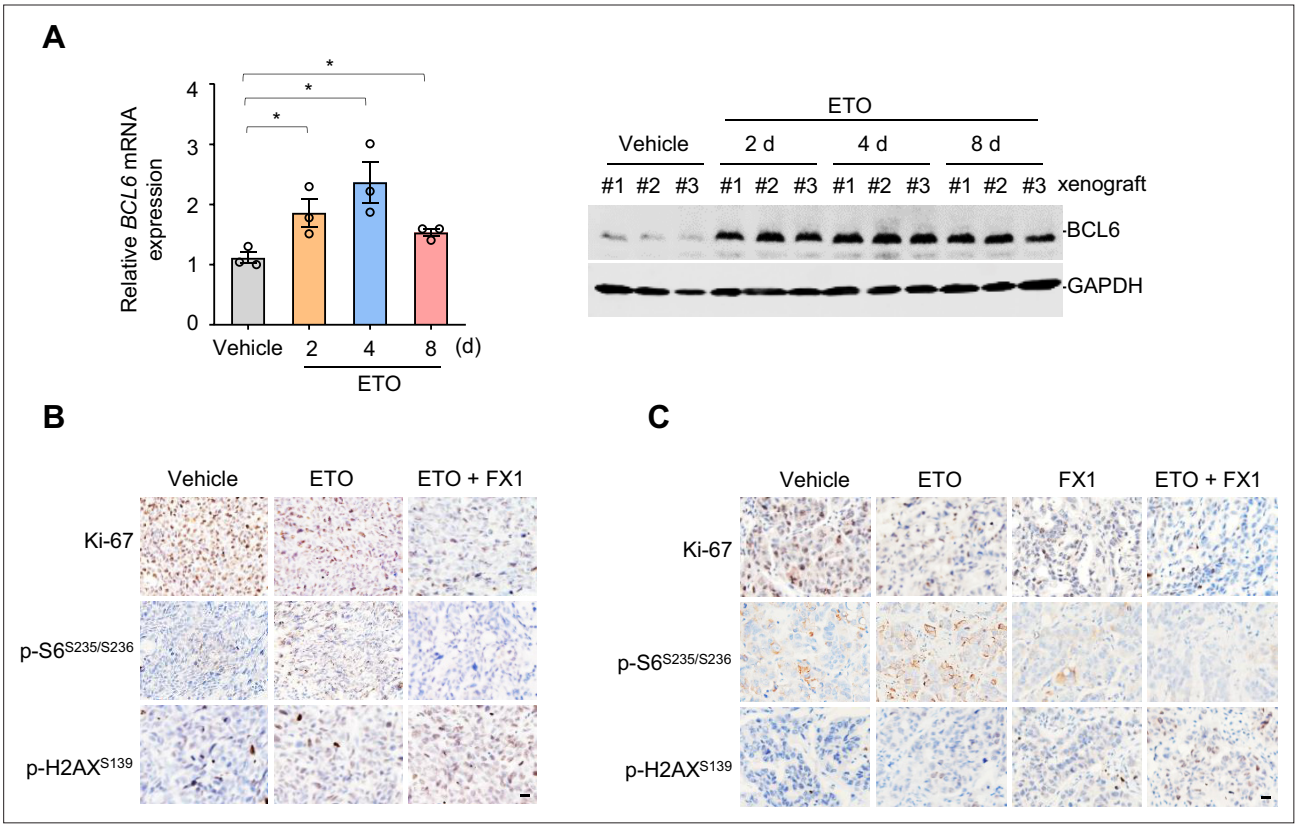

Figure 7—figure supplement 1. Pharmacological inhibition of B cell lymphoma 6 (BCL6) synergizes etoposide in vivo. (**A**) Etoposide increased *BCL6* mRNA (*left*) and protein (*right*) expression in HCT116 xenografts. Tumor tissues were isolated on day 2, 4, or 8 after etoposide treatment. QPCR and immunoblotting analysis for BCL6 expression were conducted. Values are expressed as mean ± SEM of three technical replicates, representative of three independent experiments with similar results. *p<0.05, unpaired, two tailed *t*-test. (**B**) Representative immunohistochemical staining of HCT116 xenografts. Xenografts on day 16 were examined for the expression of Ki-67, p-H2AX^{S139}, and p-S6$^{S235/S236}$. Scale bar, 50 μm. (**C**) Representative immunohistochemical staining images of LACPDX tumors. Xenografts on day 24 were evaluated for the expression of Ki-67, p-S6$^{S235/S236}$, and p-H2AX^{S139}. Scale bar, 50 μm.