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Targeting BCL-W and BCL-XL as a therapeutic strategy for Hodgkin lymphoma

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To the Editor:

Hodgkin lymphoma (HL), accounting for ~10% of lymphomas diagnosed in the United States (1), have a largely favorable outcome (5-year survival 85%) that may be improved with anti-CD30-drug conjugates or checkpoint inhibitors (2, 3). However, relapsed/refractory HL has poor outcomes and necessitates intensive salvage chemotherapy and/or autologous hematopoietic cell transplantation (4). Factors classifying HL relapse/refractory risk are needed for determining optimal treatment strategies (5). Therefore, identifying key factors underlying the mechanisms of HL cell survival and maintenance are necessary to uncover new therapeutic targets and/or develop innovative strategies to increase cure rates and long-term disease control while limiting toxicities in HL patients.

Apoptotic signals triggered by oncogenic stress or chemotherapies are countered by increased expression of anti-apoptotic BCL-2 family members (6). Consequently, specific inhibitors to anti-apoptotic family members (BCL-2, BCL-XL, MCL-1) have been developed, such as venetoclax that inhibits BCL-2 (7). Unfortunately, most B-cell lymphomas have proven resistant to venetoclax, likely due to their dependency on other anti-apoptotic BCL-2 family proteins (8). Our analysis of ~2300 B-cell lymphoma samples revealed the understudied anti-apoptotic BCL-2 family member *BCL-W* was elevated in six

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Author Contributions

CMA and CME designed the experiments; RM performed the bioinformatic analyses; JZG identified the patient samples; JZG, JL, and ANV performed the FISH and IHC analysis; CMA, RM, JZG, and CME analyzed and interpreted the data; CMA, RM, and CME wrote the manuscript; all authors edited and approved the manuscript.

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different types of B-cell lymphoma, including HL (9). We report here a comprehensive investigation into the contribution of BCL-W in HL cell survival, a mechanism of its overexpression, and its potential as a diagnostic and prognostic biomarker for HL.

To gain insight into the contribution of BCL-W to HL, we first performed a bioinformatic analysis of ~250 patient samples (Supplementary Tables S1–2), which showed *BCL-W* mRNA, but not *BCL-2* (negative control), was significantly overexpressed in HL compared to normal B-cells (Fig. 1A). *BCL-XL* mRNA was also significantly increased in HL (Fig. 1A), consistent with published BCL-XL immunohistochemistry data (10, 11). Analogous results were obtained from data of laser-microdissected HL cells compared to purified normal germinal center centrocytes and centroblasts (Supplementary Fig. S1). Further evaluation showed a significant inverse association of *BCL-W* and *BCL-2* expression, whereas *BCL-W* and *BCL-XL* expression had a significant positive association (Supplementary Fig. S2A). *BCL-2* and *BCL-XL* expression had no significant associations. Additionally, the majority (67.3%) of cases had increased *BCL-W* expression above the mean of the normal control samples, and increased *BCL-XL* expression, but not *BCL-2*, was also a frequent event (Fig. 1B). Notably, 94.9% of the samples had elevated levels of *BCL-W*, *BCL-XL*, or both above the mean of the normal B-cells (Supplementary Fig. S2B) with the majority overexpressing both *BCL-W* and *BCL-XL* (Spearman's correlation coefficient ρ=0.18, P=5.45×10^{-3}; Fig. 1C), suggesting co-overexpression of *BCL-W* and *BCL-XL* may be important in HL pathogenesis.

Next, we investigated whether *BCL-W* levels would be differentially expressed depending on the clinical features of HL patients. *BCL-W* was significantly elevated in the common nodular sclerosis subtype (Supplementary Fig. S3A), whereas due to sample size there was only a trend toward elevated *BCL-W* expression in the mixed cellularity subtype (Supplementary Fig. S3A). Significantly increased *BCL-W* levels were detected in early- and late-stage HL (Supplementary Fig. S3B). However, when divided into individual stages, *BCL-W* expression increased as stage increased (Fig. 1D), suggesting *BCL-W* expression may correlate with disease severity. In support of this, *BCL-W* levels were significantly elevated in relapsed/refractory samples, and were significantly higher in those that relapsed early (5 years) versus those that relapsed later (>5 years) (Fig. 1E, Supplementary Fig. S3C). *BCL-W* levels were also significantly higher in patients with a high international prognostic score (IPS) (>3–7) versus those with lower IPS (0–3) (Fig. 1F). Therefore, increased *BCL-W* expression correlates with aggressive, relapsed/refractory HL.

Although Epstein-Barr virus (EBV) contributes to HL pathogenesis (12), EBV status did not alter the increased *BCL-W* levels in HL samples above the normal control B-cells (Supplementary Fig. S3D). Also, there was no significant difference in *BCL-W* expression between younger (<50 years-old) and older (50 years-old) patients or between genders (Supplementary Fig. S3E–F). *BCL-2* expression (negative control) was not significantly different from that in normal B-cells for any characteristics (Fig. 1D–F, Supplementary Fig. S3), whereas *BCL-XL* expression was similar to the results obtained for *BCL-W* for each of the characteristics (Supplementary Fig. S4). Collectively, our data demonstrate that *BCL-W* and *BCL-XL* expression correlate with HL severity and treatment response, which could serve as novel, much-needed biomarkers of risk for early relapse, treatment-resistant HL.

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Subsequently, we performed IHC on 48 HL patient samples (Supplementary Table S3; 15 previously evaluated (9)), which showed increased BCL-W protein is a very frequent event in HL. All but one of the 48 patient samples had positive staining (1 pathologist score) for BCL-W (Fig. 1G) with 94% (45 of 48) having high BCL-W levels (scoring 2+ or 3+). Only 14 cases, with the majority being scored 1+, showed any BCL-2 staining (Fig. 1G, Supplementary Fig. S5A). To investigate whether copy number gains in chromosome 14 or *BCL-W*'s genetic locus could explain how BCL-W is overexpressed in HL, we performed dual-colored fluorescence *in situ* hybridization (FISH) on touch-preparation HL samples using two probes: one *BCL-W*-specific (red) and one chromosome 14 centromere-specific (green) as a control. Eight of 14 samples had significantly more *BCL-W* signals than centromeric signals in the Hodgkin Reed-Sternberg (HRS) cells (Fig. 1H, Supplementary Table S4). Twelve of 14 had more *BCL-W* signals compared to background lymphocytes (Fig. 1H). To assess the relationship between *BCL-W* copy number and RNA expression, *BCL-W* FISH, using a second independent probe (13) binding next to *BCL-W*, and qRT-PCR were performed on 10 HRS cell-rich FFPE HL samples. All samples had elevated *BCL-W* mRNA levels (Supplementary Fig. S5B) and all had increased *BCL-W* FISH signals in lymphoma cells compared to background lymphocytes (Supplementary Fig. S5C, Supplementary Table S5). There was a significant positive correlation between the number of *BCL-W* FISH signals and *BCL-W* mRNA levels (Spearman's correlation coefficient ρ=0.97, P=5.44×10^{-6}; Fig. 1I). Thus, amplification of the region containing *BCL-W* and/or copy number gains of chromosome 14 is one mechanism of BCL-W overexpression in HL.

Next, we investigated whether *BCL-W* and/or *BCL-XL* contributed to HL cell survival. mRNA (Fig. 2A) and protein (Fig. 2B) levels of all five anti-apoptotic BCL-2 family members in three HL cell lines compared to normal controls (B-cells, lymph node, spleen) showed only BCL-W and BCL-XL were elevated. To pharmacologically inhibit BCL-W and BCL-XL, we used navitoclax (ABT-263), which inhibits BCL-W, BCL-XL, and BCL-2. All HL cell lines were sensitive to navitoclax, even at low concentrations (Fig. 2C). Decreased cell growth following navitoclax was due to loss of viability (Supplementary Fig. S6) from apoptosis (Annexin-V-positive cells; Fig. 2D).

To differentiate the effects of navitoclax inhibiting BCL-W from that of inhibiting BCL-XL and/or BCL-2, we targeted only BCL-XL (A-1155463), only BCL-2 (venetoclax, ABT-199), or both. Currently, no BCL-W-specific inhibitors exist. Two HL lines (U-HO1, L-428) with the highest levels of BCL-XL protein showed sensitivity to BCL-XL inhibition, whereas KM-H2 with lower BCL-XL levels had little response (Fig. 2C–D, Supplementary Fig. S6). Only L-428 responded to BCL-2 inhibition at high concentrations (Fig. 2C). Combination treatment with both BCL-XL- and BCL-2-specific inhibitors yielded no effects beyond that with BCL-XL inhibition alone (Fig. 2C–D, Supplementary Fig. S6). Likely due to a lack of MCL-1 detected in HL (Fig. 2A–B), there was no change in cell growth, viability, or apoptosis following MCL-1 inhibition (A-1210477; Supplementary Fig. S7). Although no BCL2-A1/BFL-1-specfic inhibitor exists, we predict little effect from its inhibition since HL cells have low BCL2-A1/BFL-1 levels (Fig. 2A–B). BCL-XL inhibition accounted for only a portion of the apoptosis from navitoclax treatment; therefore, BCL-XL contributes to HL cell survival, but BCL-W must have a significant role.

To determine the dependence of HL on BCL-W specifically, we utilized doxycycline-inducible *BCL-W* shRNA in U-HO1 and KM-H2 HL lines, representing the most common HL subtypes, nodular sclerosing and mixed cellularity, respectively. Following induction of *BCL-W* shRNA, BCL-W protein levels decreased (Fig. 2E). BCL-XL levels remained unchanged. *BCL-W* knockdown significantly reduced cell growth (Fig. 2F), live cell numbers (Supplementary Fig. S8), and viability (Fig. 2G) due to increased apoptosis (Annexin-V positivity, Fig. 2H; cleaved Caspase 3, Fig. 2E). Therefore, BCL-W is essential for HL cell survival.

In conclusion, although advances in treatments have substantially increased the possibility of a cure for HL patients, those with refractory HL or who relapse still have a poor prognosis (4). Our results identify novel therapeutic targets and predictive biomarkers for patients with HL, an unmet need. Here, we revealed an essential survival role of BCL-W in HL and that its overexpression was frequently due to *BCL-W* copy number gains from regional amplification of *BCL-W* or polyploidy, suggesting additional copies of *BCL-W* contribute to HL pathogenesis. Moreover, since HL samples frequently co-overexpressed *BCL-W* and *BCL-XL* and HL cells relied on them both and not on MCL-1 or BCL-2 for survival, there is un-tapped potential of targeting BCL-W and BCL-XL, possibly with navitoclax, in combination with other anti-cancer therapies in HL. In support of this idea, navitoclax increased sensitivity of HL lines to conventional chemotherapy, brentuximab-vedotin, and ruxolitinib (14, 15). Combination treatment strategies targeting BCL-W and BCL-XL may be especially beneficial for relapse/refractory HL, as levels of both increased with increasing disease severity and risk of poor outcome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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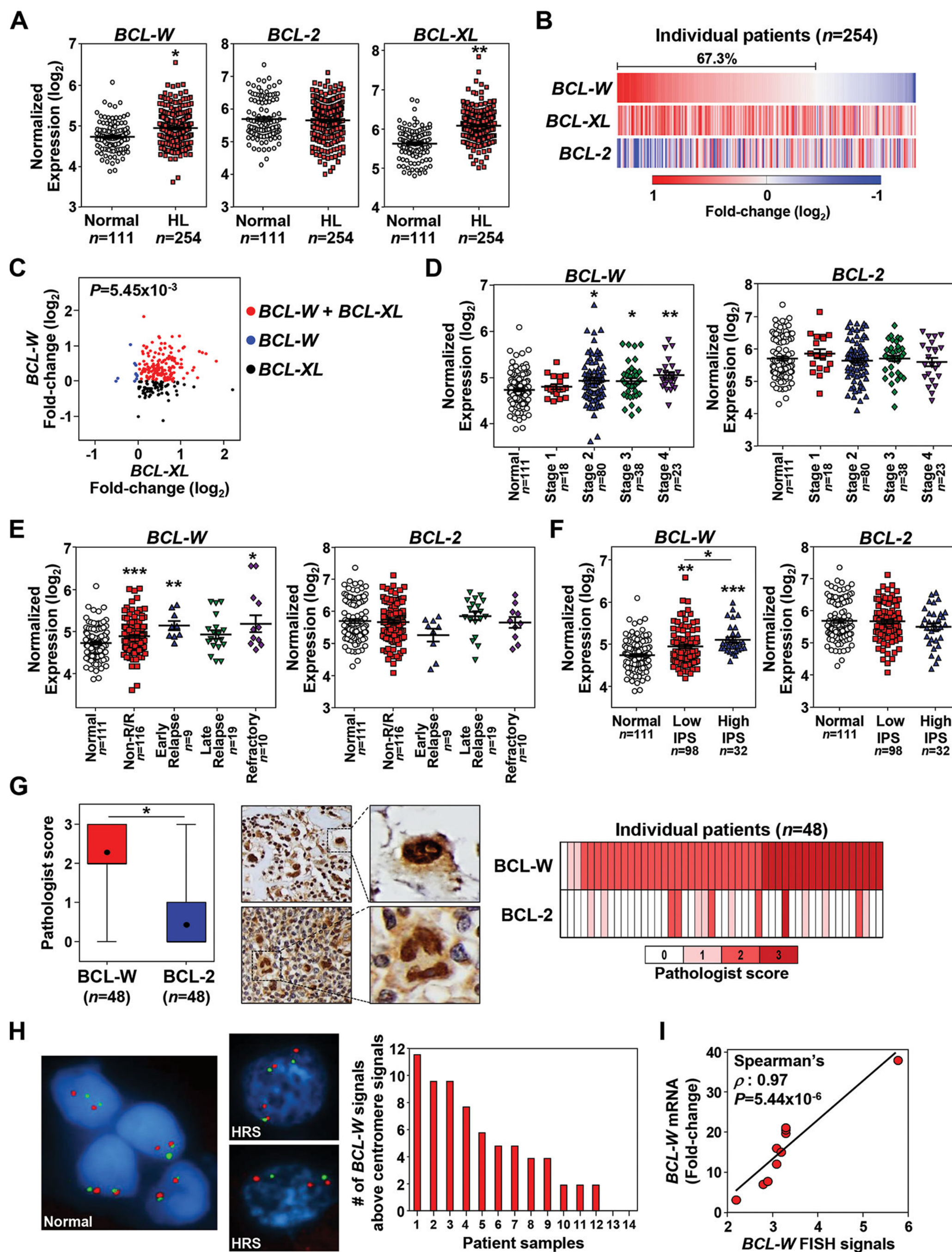

Figure 1. BCL-W overexpression in HL is a frequent event, associates with increased severity of disease, and occurs, in part, to increased copy number.

A, Gene expression data ($\log_2$) for *BCL-W*, *BCL-2*, and *BCL-XL* mRNA in HL (*n*=254) compared with normal human B-cells (*n*=111); $*P=1.76\times10^{-6}$ and $**P=2.14\times10^{-20}$, two-tailed *t*-tests. **B,** Heatmap illustrating the fold-change differences in gene expression above (red) or below (blue) the mean of the normal B-cell controls (white) for the three genes. The *BCL-W* fold-change was ranked highest to lowest for all 254 HL samples with the fold-changes for *BCL-XL* and *BCL-2* for each patient sample aligned under *BCL-W*. Each

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vertical line represents one patient. **C,** Scatter plot of HL patients with fold-change (log_2) mRNA expression of *BCL-W*, *BCL-XL*, or both above the mean of normal B-cells. Each dot represents one sample (*BCL-W* + *BCL-XL*, n=159; *BCL-W*, n=12; *BCL-XL*, n=70); Spearman's correlation coefficient ρ=0.18, P=5.45×10^{-3}. **D-F**, Gene expression data (log_2) for *BCL-W* and *BCL-2* based on stratification into individual stages (**D**); non-relapse/refractory (Non-R/R), early relapse, late relapse, and refractory (**E**); and low (0–3) and high (>3–7) international prognostic score (**F**). HL samples were compared with normal B-cell controls (**D-F**) and between HL sample groups (**F**). For **D,** *P<0.012 and **P=2.73×10^{-4}; for **E,** *P=0.049, **P=4.61×10^{-3}, and ***P=1.80×10^{-3}; for **F,** *P=0.034, **P=2.74×10^{-4}, and ***P=2.13×10^{-6}; two-tailed t-tests; ns, no statistical significance. Number of samples (n) is indicated. **G,** Immunohistochemistry (IHC) of BCL-W and BCL-2 protein in HL patient samples (n=48). Box and whisker plot of pathologist scores (left) and representative images (middle, 40X objective, 3+) of BCL-W staining are shown (*P<0.0001, two-tailed t-test). Heatmap (right) showing the IHC pathologist score for BCL-W and BCL-2 within the same individual patient sample. **H,** Representative images (left, 100X objective) of FISH analysis for the genomic locus that includes *BCL-W* (red) and chromosome 14 centromere (green) as a control in HL patient samples. Normal lymphocytes (control) and two representative images of Hodgkin Reed-Sternberg (HRS) cells are shown. FISH signals were quantified in 25 HRS cells in 14 patient samples and the number of *BCL-W* signals above the number of centromere signals were graphed for each patient (right). **I,** Spearman's correlation comparing the number of *BCL-W* FISH signals to the fold-change in *BCL-W* mRNA (determined by comparing each HL sample to the mean of the normal samples) for the 10 HL patient samples evaluated (see Supplementary Fig. S5B–C). Spearman's correlation coefficient and P-value are indicated.

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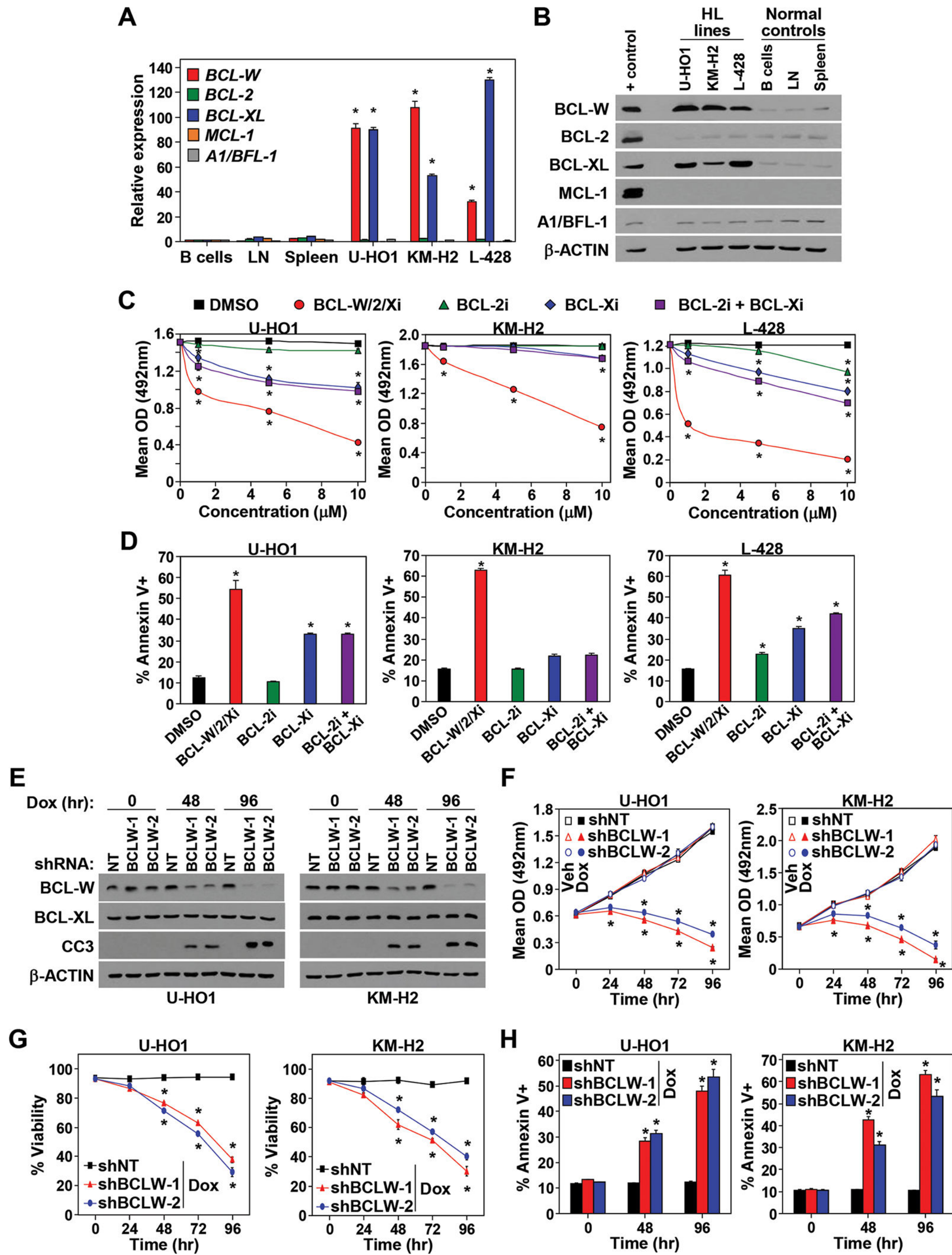

Figure 2. Hodgkin lymphoma cells undergo apoptosis following pharmacological inhibition of BCL-W and BCL-XL and after knockdown of *BCL-W*.

A and **B,** qRT-PCR analysis (**A,** in triplicate, *P<0.0001; one-way ANOVA) and Western blotting (**B**) for the anti-apoptotic BCL-2 family members in HL cell lines (U-HO1, KM-H2, and L-428) and normal human controls (purified B-cells, lymph node (LN), and spleen). **C-D,** HL cell lines were either administered ABT-263/navitoclax (BCL-W, BCL-2, BCL-XL inhibitor; BCL-W/2/Xi); ABT-199/venetoclax (BCL-2 inhibitor; BCL-2i); A-1155463 (BCL-XL inhibitor; BCL-Xi); or a combination of ABT-199 and A-1155463 at the indicated

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concentrations in C or at 10μM in D. DMSO served as vehicle control. Forty-eight hours following treatment, cell growth was measured by MTS assays (**C,** in quadruplicate) and apoptosis by Annexin-V staining (**D,** in triplicate). For C-D, *$P<2.9\times10^{-5}$, two-tailed *t*-tests. **E-H,** HL cell lines (U-HO1, KM-H2) were infected with lentivirus encoding two different doxycycline (Dox)-inducible shRNA against *BCL-W* (BCLW-1 or BCLW-2) or a non-targeting shRNA control (NT). Following addition of Dox to induce shRNA expression or PBS (vehicle control, Veh), Western blotting (**E**; CC3, cleaved Caspase 3) and MTS assays to measure cell growth (**F,** in quadruplicate) were performed. Viability (**G**, in triplicate) was determined by Trypan Blue Dye exclusion, and Annexin-V staining (**H,** in triplicate) was performed at the indicated intervals. For F-H, *$P<2.5\times10^{-2}$, two-tailed *t*-tests. A representative experiment of 2–4 independent experiments performed for each assay for each cell line is shown.