

Rewiring the Fusion Oncoprotein EWSR1::FLI1 in Ewing Sarcoma with Bivalent Small Molecules

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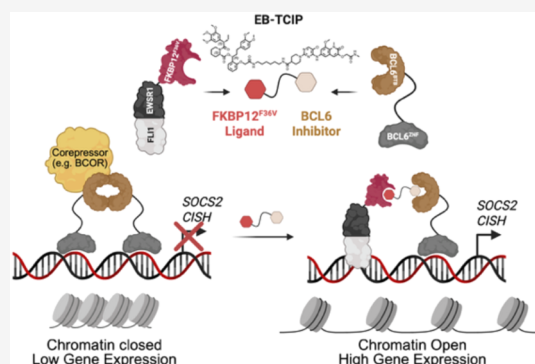


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ABSTRACT: Dysregulated transcription is a defining hallmark of cancer. Recently, novel chemically induced proximity approaches have enabled the rewiring of transcriptional machinery to drive expression of pro-apoptotic genes using bivalent small molecules. In this work, we demonstrate that this strategy is amenable to relocating DNA bound transcriptional machinery, such as fusion transcription factors that commonly drive pediatric malignancies. Targeting fusion transcription factors, such as EWSR1::FLI1 in Ewing sarcoma, with these bivalent compounds may open new therapeutic avenues. Here, we develop a small molecule, EB-TCIP, that recruits FKBP12^{F36V}-tagged EWSR1::FLI1 to DNA sites bound by the transcriptional regulator BCL6, leading to rapid chromatin remodeling and expression of BCL6 target genes. This proof-of-concept study demonstrates that DNA binding proteins with pioneering transcription factor activity, such as EWSR1::FLI1, can be relocated on chromatin to induce expression of repressed genes. Insights herein will guide the development of future bivalent molecules that rewire DNA binding transcriptional machinery.



INTRODUCTION

Over the past two decades, chemical biologists have reshaped how scientists interrogate biological systems by developing tool molecules that hijack and rewire numerous enzyme classes.^{1–7} Most recently, transcriptional machinery has been rewired to drive expression of pro-apoptotic genes using Transcriptional/epigenetic Chemical Inducers of Proximity (TCIPs).⁸ Binders of known transcriptional regulators BRD4, CDK9, or p300/CBP were linked to a B cell lymphoma 6 (BCL6) inhibitor to induce expression of pro-death genes leading to apoptosis in a lineage-specific fashion.^{8–10} These studies established TCIPs as a promising new therapeutic modality for cancers whose survival is dependent on suppression of apoptosis.¹¹ TCIPs, however, have yet to be shown to recruit DNA binding transcriptional machinery, such as fusion transcription factors (TFs). Hijacking fusion TFs expressed only in tumor cells presents another exciting application of this technology that could be leveraged toward tumor specific therapeutic benefit.

Many cancers, but particularly pediatric malignancies, are driven by fusion TFs that are expressed solely in tumor cells, and these cancers have otherwise relatively simple genomes with few additional genetic abnormalities.^{12–14} Therefore, directly targeting the fusion TF could yield potent therapeutic activity with a favorable toxicity profile. Ewing sarcoma (ES) is a solid tumor involving the bone that is driven by a

chromosomal translocation, which results in the expression of a fusion TF comprised of the N-terminal transactivation domain of a FUS, EWSR1, TAF15 (FET) family RNA binding protein fused to the DNA binding domain of an E26 Transformation Specific (ETS) family TF.¹⁵ ETS TFs contain a N-terminal regulatory domain and control expression of genes important for cell growth and survival.¹⁶ In the FET::ETS fusion proteins that arise in ES, the fusion TF retains the ability to bind to canonical ETS target genes but acquires the strong transactivation domain of the FET protein. Moreover, the fusion TF gains the ability to bind long GGAA microsatellite repeats, where it acts as a pioneering TF, opening chromatin and establishing *de novo* enhancers that interact with promoters and boost gene expression.^{17,18} The most common FET::ETS fusion results from the (11;22)-(q24;q12) translocation, which fuses the Ewing sarcoma breakpoint region 1 (EWSR1) protein to the Friend leukemia integration 1 (FLI1) TF, forming the EWSR1::FLI1 fusion TF.¹⁵

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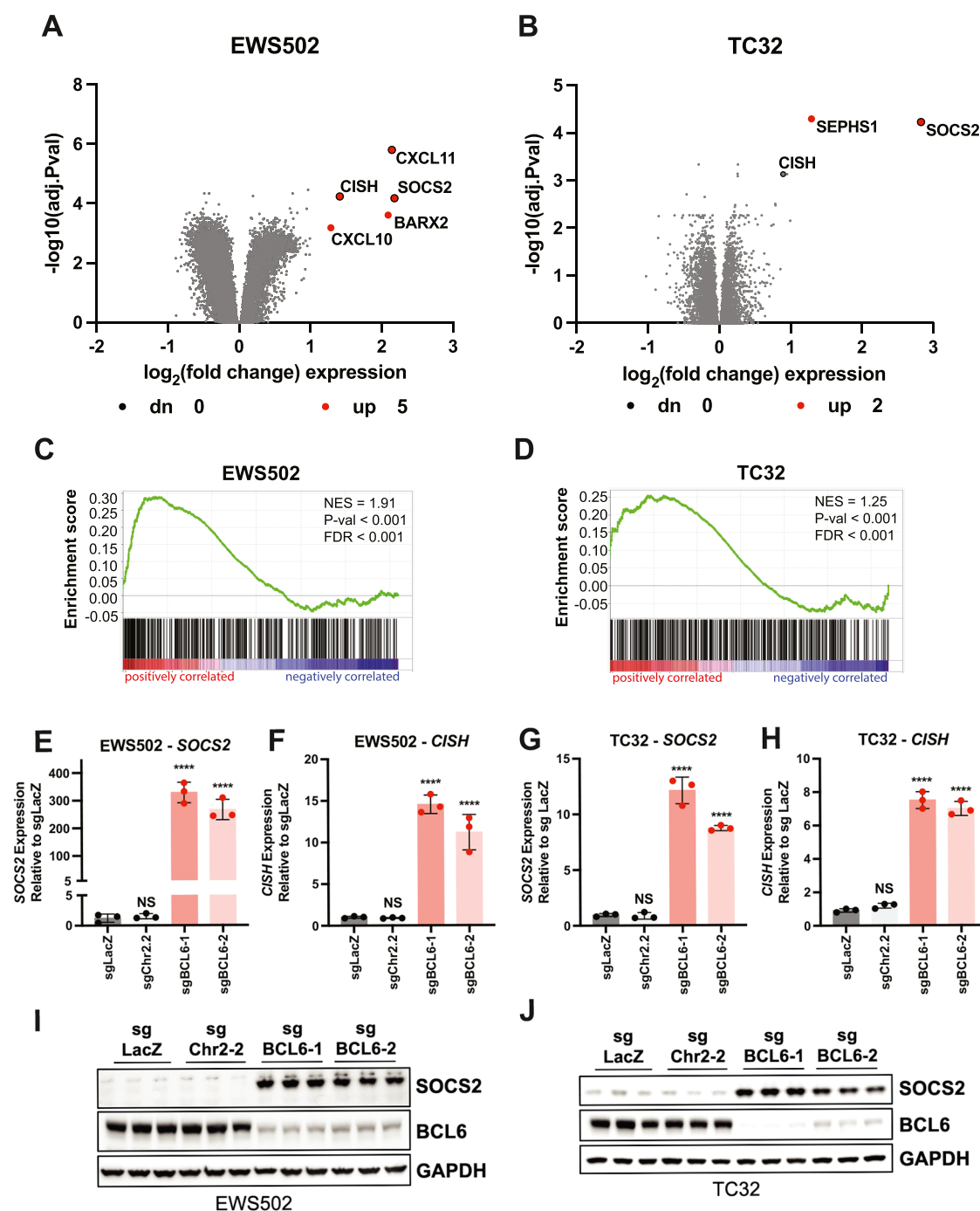


Figure 1. Determining BCL6 target genes in ES. Volcano plots of differentially expressed RNA species in *BCL6* KO (two guides averaged over three replicates per guide) EWS502 cells (A) or TC32 cells (B) vs Chr2.2 control cells. GSEA in EWS502 (C) or TC32 (D); *BCL6* KO cells shows a positive correlation with a published *BCL6* gene signature derived from *BCL6* promoter binding data.²⁵ SOCS2 and *CISH* transcripts have increased expression by RT-qPCR in *BCL6* KO EWS502 cells (E and F) or TC32 cells (G and H) vs control guides. Average expression from three independent cell transductions (performed in technical triplicate) is shown. RT-qPCR data was compared by one-way ANOVA; NS = not significant, **** $p < 0.001$. All error bars in the figure show mean $\pm$ SD. Immunoblotting shows *BCL6* KO and corresponding increase in SOCS2 protein levels in EWS502 (I) and TC32 (J) cells. Each lane is from an independent transduction of cells. GAPDH serves as a loading control.

EWSR1::FLI1 accounts for 85% of all ES cases.¹⁵ As a specific and strong dependency in ES based on CRISPR, RNAi, and degradation-based approaches, EWSR1::FLI1 should be a prime candidate for drug discovery.^{14,15} Unfortunately, the disordered nature of the fusion TF has made it difficult to identify small molecule binders. Due to the dearth of EWSR1::FLI1-specific ligands, we have used a N-FKBP12^{F36V}-EWSR1::FLI1 (FKBP-E::F) model system to test

whether EWSR1::FLI1 can be relocated to new sites on chromatin. The FKBP12^{F36V} domain of the FKBP-E::F fusion protein binds specifically and with high affinity to *ortho*-AP1867 (OAP),¹⁹ which can be used as a small molecule handle to hijack FKBP-E::F activity. Given that TCIPs have successfully targeted *BCL6* as a transcriptional repressor of interest (i.e., known chemical matter, validated exit vector, and assay availability) and that ES cells express this protein at

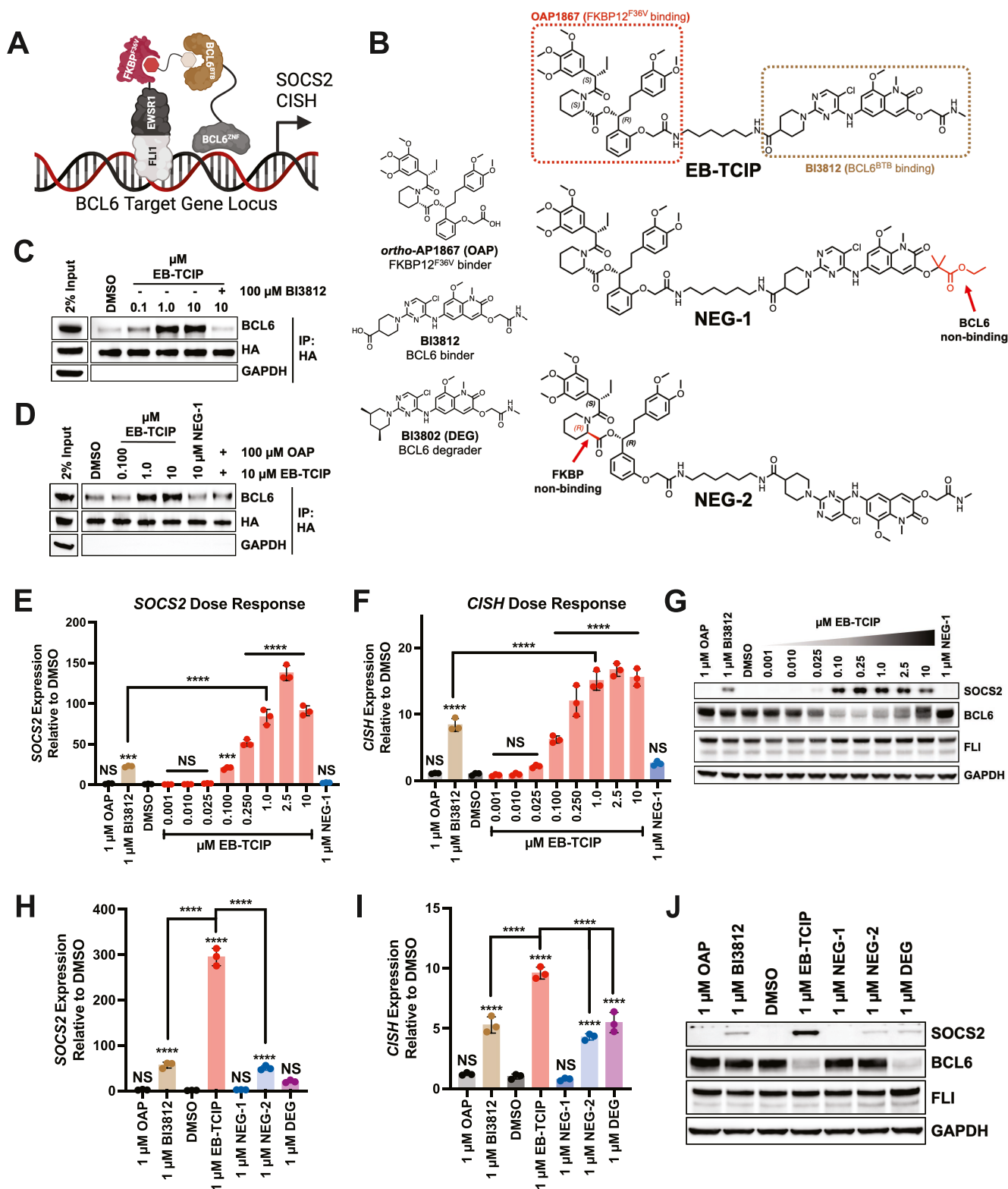


Figure 2. EB-TCIP increases expression of BCL6 target genes in ES cells at nanomolar concentrations. (A) Schematic of EB-TCIP mechanism of action. EB-TCIP induces a ternary complex between FKBP^{F36V} tagged EWSR1::FLI1 and BCL6, which leads to activation of BCL6 target gene transcription. Image made with Biorender. (B) Structures of compounds used in this work. (C) EB-TCIP increases the association of BCL6 with FKBP-E::F in a dose-dependent manner in EWS502 FKBP-E::F cell lysates while NEG-1 (D) does not induce a ternary complex. The association is reversible as excess BI3812 (C) and excess OAP (free acid) (D) abrogate ternary complex formation. GAPDH was probed to confirm that unbound proteins were removed by washing. EB-TCIP dose-dependently increases SOCS2 (E) and CISH (F) expression by RT-qPCR. (G) SOCS2 protein levels dose-dependently increase while BCL6 protein levels dose-dependently decrease after EB-TCIP treatment. EB-TCIP induces higher SOCS2 (H) and CISH (I) transcript levels than chemical inhibition with BI3812 or chemically induced degradation with BI3802 (DEG). (J) SOCS2 protein levels are highest in EB-TCIP treated cells compared to BI3812, BI3802 (DEG), or negative control compounds that do not form ternary complexes. Immunoblotting is representative of three biological replicates and GAPDH serves as a loading control. All

Figure 2. continued

experiments were performed in FKBP-E::F expressing EWS502 cells. RT-qPCR experiments show one experiment with technical triplicate that is representative of three biological replicates. Means of *SOCS2* and *CISH* expression were compared using one-way ANOVA with multiple comparisons; NS = not significant, *** $p < 0.005$, **** $p < 0.001$. All error bars in the figure indicate mean $\pm$ SD. Unless indicated with brackets, significance above each condition indicates comparison of that mean to the mean of DMSO.

moderate to high levels (Figure SI-1A), we synthesized and tested a tool compound which linked OAP to BI3812, an inhibitor of BCL6. Although BCL6 is well studied in the maturation of B cells and the tumorigenesis of B cell lymphomas, its role in ES biology is less well understood. We first used genomic approaches to identify relevant BCL6 target genes in ES cells. We then used biochemical and omics approaches to characterize the ability of our tool compound, termed EB-TCIP, to induce expression of ES relevant BCL6 targets and compared its activity to the effect of small molecule inhibition and/or degradation of BCL6. Our study demonstrates that DNA binding transcription factor fusions, like EWSR1::FLI1, can be relocalized on chromatin to induce expression of neo-target genes, representing the first steps in understanding how the transcriptional machinery of EWSR1::FLI1 can be reprogrammed. Lessons learned from this study may inform future development of TCIPs that relocalize DNA binding transcriptional regulators.

RESULTS

Identifying BCL6 Target Genes in ES Cells. BCL6 is well-known for its oncogenic role in diffuse large B-cell lymphoma (DLBCL), where it acts as a repressor of *TP53* and associated DNA damage/pro-apoptotic genes, as well as cell cycle checkpoint genes such as *CDKN1A*.²⁰ However, little is known about the role of BCL6 in ES tumorigenesis, and while BCL6 corepressor (BCOR) fusions are common in Ewing-like sarcomas, not much is known about the role BCL6 plays in these tumors either.²¹ Nonetheless, DepMap expression data shows that ES cells express BCL6 at higher levels than in many other cancer types, DLBCL being an exception (Figure SI-1A).²² BCL6, however, is not a dependency in ES, while it is in DLBCL (Figure SI-1B).

To identify BCL6 target genes in ES cells, we used two BCL6 targeting guides from the Avana CRISPR guide library²³ to knockout (KO) BCL6 in two distinct ES models. Many cultured ES cell lines are *TP53* mutant, even though most patient tumors are *TP53* wild type.¹⁴ Since BCL6 represses *TP53* and related transcripts, we used RNA-seq to profile transcriptional changes in EWS502 (*TP53* mutant) and TC32 (*TP53* wild type) cells. KO of BCL6 led to few, but consistent changes in RNA transcripts in both models using strict criteria of $\log_2$ fold change ≥ 1.0 and P -value < 0.05 (Figure 1A,B). To verify that the observed signature was related to BCL6, we performed Gene Set Enrichment Analysis (GSEA)²⁴ against a gene set derived from BCL6 promoter binding data generated in primary B cells and DLBCL.²⁵ We observed a significant, positive correlation between both ES BCL6 KO models and the published BCL6 repressed gene set (Figure 1C,D). The two transcripts that were upregulated in both cell lines were *SOCS2* and *CISH*, although the latter was outside of our significance cutoff in TC32 cells in the RNA-seq data. These transcripts encode for E3 ligase subunit paralogs involved in the degradation of growth hormone receptor and other cytokine receptors within the JAK/STAT signaling pathway.^{26,27} We verified that *SOCS2* and *CISH* transcripts

significantly increase with BCL6 KO by RT-qPCR (Figure 1E–H). Further, *SOCS2* protein levels were enhanced upon BCL6 KO (Figure 1I,J). With *SOCS2* and *CISH* identified as *bona fide* BCL6 repressed targets, we set out to determine if a TCIP molecule could hijack EWSR1::FLI1 and enhance their expression.

EB-TCIP Induces BCL6 Target Gene Expression More Effectively than Chemical Inhibition or Degradation of BCL6. Although EWSR1::FLI1 has been a priority target for ES drug discovery, there has been limited success identifying EWSR1::FLI1 ligands. EWSR1::FLI1, like many TFs, is highly disordered and difficult to drug. To overcome this issue, we took advantage of N-terminal tagged FKBP12^{F36V}-EWSR1::FLI1 (FKBP-E::F) cell lines that were previously developed to study EWSR1::FLI1 degradation using the dTAG system.^{28,29} We envisioned that BAK-04-212, a bivalent molecule comprised of OAP and BI3812, which we call EB-TCIP, could redirect FKBP-E::F to BCL6 loci, thereby driving expression of BCL6 repressed transcripts such as *SOCS2* and *CISH* (Figure 2A,B).

We first wanted to demonstrate that EB-TCIP can induce a ternary complex between FKBP-E::F and BCL6 in a cell free system using time-resolved fluorescence energy transfer (TR-FRET) between the BTB domain of BCL6 (BCL6^{BTB}) labeled with fluorescein isothiocyanate (FITC) and a His-tagged FKBP^{F36V}, which was recognized by an anti-His-tag terbium-conjugated antibody.³⁰ EB-TCIP dose dependently increased TR-FRET signal with an EC₅₀ of $0.14 \pm 0.03 \mu\text{M}$, while the negative control bifunctional compound RPG-02-089, referred to as NEG-1, did not increase TR-FRET signal (Figure SI-2A). The addition of two vicinal methyl groups in NEG-1 sterically occludes binding to the BCL6^{BTB}. At concentrations of EB-TCIP above $0.31 \mu\text{M}$ a hook effect was observed. This is a characteristic property of bivalent molecules where at high concentrations, binary complexes between the compound and one target predominate over the ternary complex.³¹ Next, we investigated the formation of a ternary complex between FKBP-E::F and native BCL6. To this end, we treated EWS502 FKBP-E::F cell lysates with increasing concentrations of EB-TCIP. Since the FKBP-E::F construct contains an HA tag, we then used magnetic HA beads to immunoprecipitate FKBP-E::F and associated proteins. We observed a dose-dependent increase in the amount of BCL6 pulled down in the presence of EB-TCIP (Figure 2C,D). Further, NEG-1 was unable to pulldown BCL6. By pretreating lysates with either excess BI3812 or OAP before EB-TCIP treatment, the ternary complex was disrupted and little BCL6 was pulled down compared to treatment with 1 or $10 \mu\text{M}$ EB-TCIP alone (Figure 2C,D). These data demonstrate that EB-TCIP can form a reversible ternary complex between FKBP^{F36V} and BCL6^{BTB} *in vitro* and in cell lysates.

After confirmation of ternary complex formation, we next tested if EB-TCIP could enhance expression of BCL6 repressed targets. Previous TCIP studies monitored compound activity using a BCL6 repressed GFP reporter (Figure SI-2B).^{8,9} Using this same vector, we engineered an EWS502

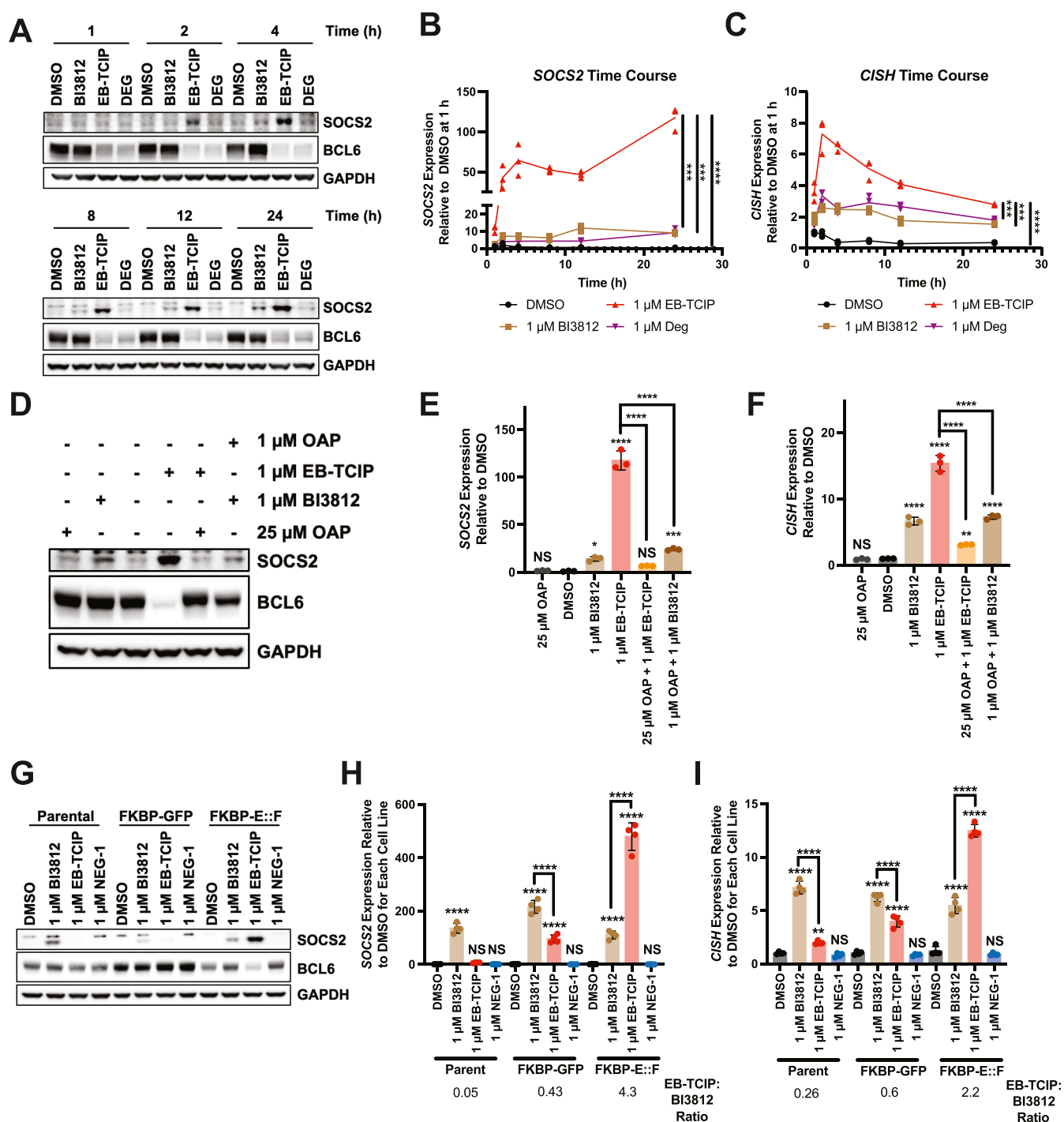


Figure 3. EB-TCIP activity is rapid, ternary complex dependent, and specific to cells expressing FKBP-E::F. (A) Time course of SOCS2 and BCL6 protein levels. BCL6 degradation occurs within 1 h for both EB-TCIP and BI3812 (DEG). EB-TCIP induces SOCS2 expression by 2 h and maintains higher expression levels than BI3812 or BI3812 (DEG) throughout the time course. SOCS2 (B) and CISH (C) transcripts reach a maximum between 2 and 4 h by RT-qPCR. (D) EB-TCIP induced SOCS2 protein expression can be reversed with 25-fold excess OAP (free acid). Co-treatment of 1 μ M BI3812 and OAP do not increase SOCS2 protein expression more than 1 μ M BI3812 alone. EB-TCIP induced SOCS2 (E) and CISH (F) transcript expression is reversed with excess OAP. BI3812 and OAP must be chemically linked to induce maximum transcript expression. (G) EB-TCIP induces the highest expression of SOCS2 protein in EWSS02 FKBP-E::F cells compared to EWSS02 parental cells or EWSS02 cells expressing FKBP-GFP. Only treatment with EB-TCIP induces more expression of SOCS2 (H) and CISH (I) than BI3812 in EWSS02 FKBP-E::F cells. EB-TCIP:BI3812 ratio was calculated by dividing the average expression of each transcript in EB-TCIP treated cells by the average expression of each transcript in BI3812 treated cells. Immunoblotting is representative of three biological replicates. RT-qPCR experiments show one experiment with technical triplicate or quadruplicate that is representative of three biological replicates. The means of SOCS2 and CISH expression were compared using one-way ANOVA with multiple comparisons; NS = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$. All error bars in the figure represent mean $\pm$ SD. Unless indicated with brackets, significance above each condition indicates comparison of that mean to the mean of DMSO. In (H) and (I), unless indicated with brackets, the means of BI3812, EB-TCIP, and NEG-1 were compared to the DMSO sample for the corresponding cell line.

FKBP-E::F line expressing the reporter and found that EB-TCIP dose dependently increased the percentage of GFP positive cells to a greater extent than negative control compounds (Figure SI-2C). Next, we treated EWS502 FKBP-E::F cells with increasing concentrations of EB-TCIP and monitored expression of identified BCL6 targets by RT-qPCR and immunoblotting. EB-TCIP dose dependently increased expression of *SOCS2* and *CISH*, with an EC_{50} of 0.17 ± 0.05 and 0.11 ± 0.04 μ M respectively (Figure 2E,F). Maximal induction of both transcripts was reached at a concentration of 2.5 μ M with a hook effect evident at 10 μ M. EB-TCIP induced higher levels of expression of both transcripts compared to BI3812 at 1 μ M. Additionally, 1 μ M of NEG-1 did not increase *SOCS2* or *CISH* expression. A dose-dependent increase in *SOCS2* protein level was also observed, with a hook effect being observed above 2.5 μ M (Figure 2G). EB-TCIP induced higher *SOCS2* protein expression than BI3812 and NEG-1. Similar trends in transcript and protein expression were observed for TC32 FKBP-E::F cells (Figure SI-2D-F and SI Table 1) demonstrating that the activity of EB-TCIP is not unique to EWS502 FKBP-E::F cells. EB-TCIP dose dependently decreased proliferation of EWS502 FKBP-E::F cells over 72 h (Figure SI-2G). However, we also observed similar antiproliferative activity in parental EWS502 cells that do not express exogenous FKBP-E::F (Figure SI-2H). Our viability data suggests EB-TCIP induces off-mechanism cytotoxicity. Nonetheless, at shorter time points EB-TCIP is a useful tool molecule to study relocalization of FKBP-E::F on chromatin.

We observed a dose-dependent decrease in BCL6 protein levels in both EWS502 and TC32 FKBP-E::F cells at concentrations where *SOCS2* levels increase and ternary complex between FKBP-E::F and BCL6 is formed. A hook effect for BCL6 degradation was also observed above 250 nM. The decrease in BCL6 protein was not due to a decrease in BCL6 transcript levels as treatment with EB-TCIP increased BCL6 mRNA (Figure SI-2I), which is consistent with previous TCIP studies.⁸ We wondered if BCL6 induced degradation was enough to increase *SOCS2* and *CISH* transcript/protein levels to the same extent as EB-TCIP. Therefore, we treated cells with BI3802³² (referred to as DEG in the figures; Figure 2B), which induces polymerization and subsequent proteasome dependent degradation of BCL6.³³ BI3802 induced BCL6 degradation to a similar extent as EB-TCIP; however, EB-TCIP induced significantly higher levels of *SOCS2* and *CISH* transcripts, as well as *SOCS2* protein, compared to BI3802 (Figure 2H–J).

During our characterization of EB-TCIP, the synthesis of an OAP derivative that does not bind to FKBP12^{F36V} was described.³⁴ Using this synthesis, we generated a second negative control compound, RPG-02–205, referred to as NEG-2 (Figure 2B), that does not form a ternary complex but retains the ability to engage and inhibit BCL6. NEG-2 did not form a ternary complex by TR-FRET (Figure SI-2J). Further, NEG-2 was less active than EB-TCIP in the GFP reporter assay, albeit modestly so (Figure SI-2C). Moreover, NEG-2 did not increase BCL6 target gene/protein expression to the same extent as EB-TCIP (Figure 2H–J). The activity that was observed in some of these assays can be attributed to NEG-2's retained ability to inhibit BCL6. NEG-2 also did not induce BCL6 degradation, providing evidence that EB-TCIP decreases BCL6 protein levels in a ternary complex dependent manner.

Our observations above led us to hypothesize that EB-TCIP induces proteasome dependent degradation of BCL6. We tested this hypothesis by pretreating EWS502 FKBP-E::F cells for 1 h with the proteasome inhibitor MG132³⁵ or the neddylation inhibitor MLN4924³⁶ before treatment with BI3802 or EB-TCIP for 4 h. MG132 rescued BCL6 levels to a greater extent than MLN4924, which was seen previously for BI3802³³ (Figure SI-3A). As a control we also pretreated cells with the transcriptional inhibitor Actinomycin D³⁷ (ActD). ActD treatment abrogated EB-TCIP activity as expected (Figure SI-3A–C). These data suggest that EB-TCIP activity is dependent on both active transcriptional and degradation machinery.

EB-TCIP Induces Rapid, Ternary Complex Dependent Induction of BCL6 Targets That Is Specific to Cells Expressing FKBP-E::F. To determine the kinetics of BCL6 degradation and target induction we treated EWS502 FKBP-E::F cells with DMSO, BI3812, EB-TCIP, or BI3802 (1 μ M) over a 24 h time course. EB-TCIP and BI3802 induced rapid degradation of BCL6 with near maximal degradation observed within 1 h (Figure 3A). Despite similar degradation kinetics, EB-TCIP enhanced *SOCS2* protein levels to a greater extent than BI3802 at all time points beyond 1 h. EB-TCIP also enhanced *SOCS2* protein levels more than BI3812 at these time points. Protein expression lagged transcript expression, which at 1 h was significantly higher in EB-TCIP treated cells compared to DMSO or the other molecules (Figure 3B,C). EB-TCIP-induced expression of *SOCS2* and *CISH* showed a peak between 2 and 4 h. *SOCS2* expression leveled off before increasing at 24 h, whereas *CISH* expression continued to decrease until the end of the experiment. Increases in these transcripts in BI3812 and BI3802 treated cells were relatively stable after 2 h, suggesting that EB-TCIP may have a different mechanism of transcript induction than these compounds. Further evidence of this claim is that NEG-2, which can bind BCL6 but not relocalize FKBP-E::F, did not increase *SOCS2* to the same degree as EB-TCIP and showed a similar pattern of transcript induction as BI3812 and BI3802 over a similar time course (Figure SI-4A). These data show that EB-TCIP rapidly and more effectively induces expression of BCL6 targets compared to chemical inhibition or degradation of BCL6.

Next, we tested if EB-TCIP activity was via a ternary complex mechanism. To do this, we pretreated EWS502 FKBP-E::F cells with a 25-fold excess of OAP for 1 h before treating cells with EB-TCIP for 4 h. The excess OAP competed away EB-TCIP and abolished its ability to induce BCL6 target expression (Figure 3D–F). Further, cotreatment with 1 μ M of BI3812 and OAP did not increase BCL6 target expression as much as EB-TCIP alone (Figure 3D–F). These data show that BI3812 and OAP must be chemically linked to induce a ternary complex and maximally drive BCL6 target gene expression. To further validate the importance of ternary complex formation and show compound specificity, we tested the ability of EB-TCIP to increase BCL6 targets in parental, FKBP-GFP, and FKBP-E::F expressing EWS502 lines. BI3812 induced *SOCS2* protein expression in all cell lines as expected; however, EB-TCIP induced *SOCS2* protein expression only in FKBP-E::F expressing cells (Figure 3G and Figure SI-4B). Further, BCL6 target transcript levels were highest in FKBP-E::F expressing cells treated with EB-TCIP (Figure 3H–I). As a measure of EB-TCIP activity we compared the ratio of induction of transcript expression in samples treated with EB-TCIP or BI3812 for each cell line. We observed a positive

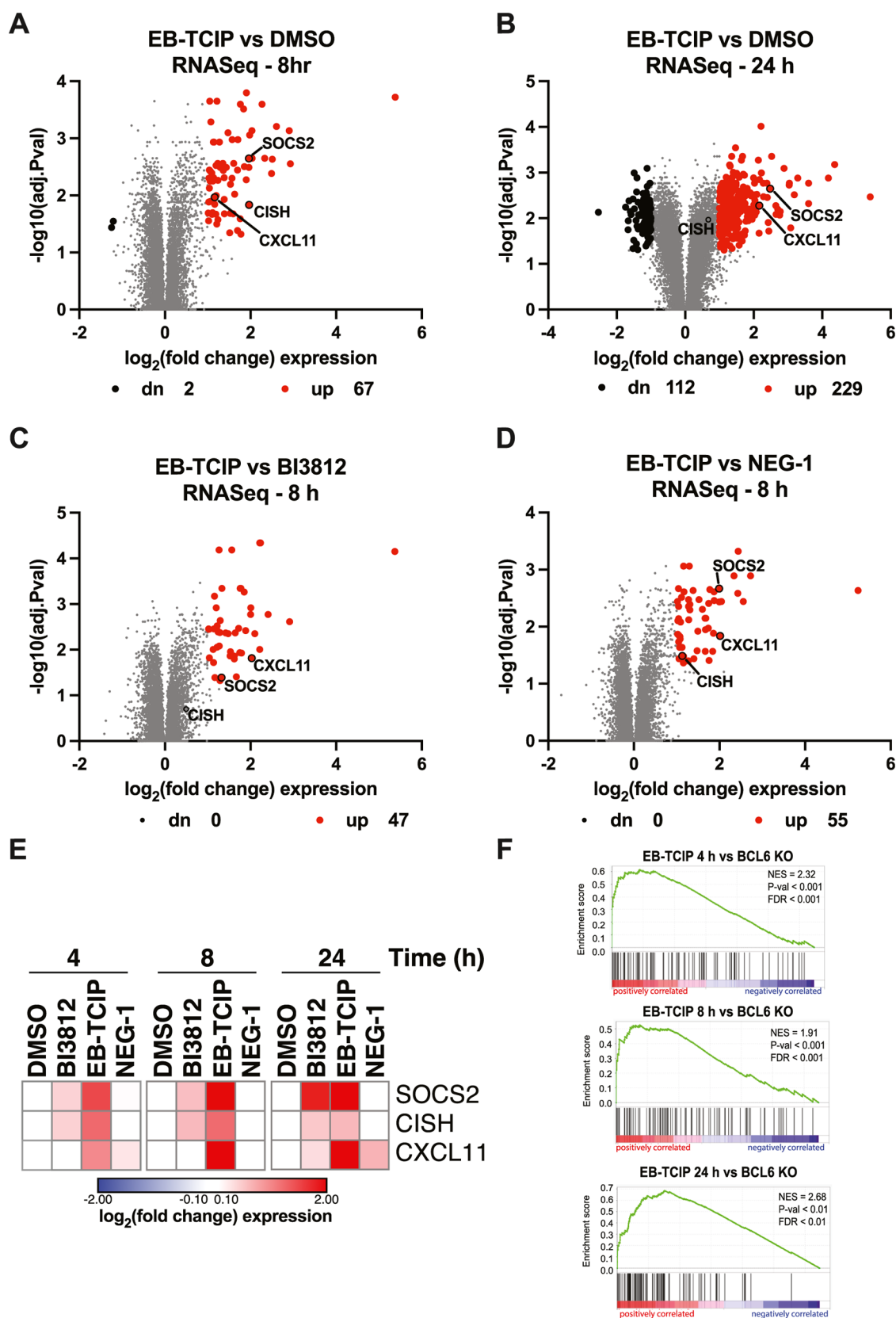


Figure 4. Global RNA changes induced by EB-TCIP are similar to genetic KO of *BCL6* in EWS502 cells. Volcano plots portraying $\log_2$ changes of gene expression from cells treated with $2.5 \mu\text{M}$ EB-TCIP versus DMSO at 8 (A) and 24 (B) h with a $-\log_{10}$ adjusted *P*-value cut off of 1. EB-TCIP treatment predominantly increases expression of transcripts at both time points. Volcano plots portraying $\log_2$ fold changes of cells treated with $2.5 \mu\text{M}$ EB-TCIP versus $2.5 \mu\text{M}$ BI3812 (C) or $2.5 \mu\text{M}$ NEG-1 (D) at 8 h with a $-\log_{10}$ *P*-value cut off of 1.3. EB-TCIP induces higher expression of *BCL6* transcripts than BI3812 or NEG-1 at this early time point. Dots corresponding to *SOCS2*, *CISH*, and *CXCL11* are labeled with black borders. (E) Heatmaps of changes in *BCL6* target gene expression at 4, 8, and 24 h show that EB-TCIP induces faster and/or

Figure 4. continued

higher expression of these select genes. (F) GSEA comparing EB-TCIP treated EWS502 FKBP-E::F cells to *BCL6* KO EWS502 parental cells (LFC ≥ 1.65) at 4 (top), 8 (middle) and 24 h (bottom) show significant positive correlation between the two gene sets. RNA-seq data is shown as the average of three independent replicates.

ratio, indicative of higher activity of EB-TCIP than BI3812, only in FKBP-E::F expressing cells. Together, these data show that the enhanced ability of EB-TCIP to induce *BCL6* target expression is dependent on ternary complex formation and FKBP-E::F expression.

EB-TCIP Induces Rapid, Dynamic Changes in Global Transcription. To profile how ternary complex formation between FKBP-E::F and *BCL6* affected transcription in an unbiased manner, we treated EWS502 FKBP-E::F cells with DMSO, BI3812, EB-TCIP, or NEG-1 (2.5 μ M) for 8 or 24 h and studied transcriptomic changes by RNA-seq. Given EWSR1::FLI1's ability to activate transcription, at 8 h we observed many more upregulated genes (67) than downregulated genes (2) (Figure 4A). Highly upregulated genes that we observed after *BCL6* KO, such as *SOCS2*, *CISH*, and *CXCL11*, were significantly upregulated at 8 h. The number of both upregulated (229) and downregulated genes (112) increased at 24 h with many increasing in magnitude (Figure 4B). However, some genes that were significantly upregulated at 8 h, such as *CISH*, had decreased expression at 24 h compared to 8 h, consistent with our earlier time course data.

Global transcriptomic changes were more robust with EB-TCIP in comparison to DMSO, BI3812, and NEG-1 (Figure 4C,D and Figure SI-5A-F). EB-TCIP induced the expression of more genes than BI3812 or NEG-1 at 8 h. The known *BCL6* targets *SOCS2* and *CXCL11* were more upregulated by EB-TCIP than BI3812 or NEG-1 at 8 h. *CISH* was significantly upregulated by EB-TCIP compared to NEG-1, but not BI3812 at 8 h, although expression did trend upward (Figure 4C-E). To further assess *BCL6* programming induced by EB-TCIP, we performed GSEA comparing up-regulated genes induced by EB-TCIP and the transcriptional changes that result from *BCL6* KO (LFC ≥ 1.65). At both 8 and 24 h, we observed a significant positive correlation between EB-TCIP induced gene expression and *BCL6* KO, with *SOCS2*, *CISH*, and *CXCL11* being leading-edge genes within the enriched signature (Figure 4F). Further, at 8 h we observed a trend of positive correlation between EB-TCIP induced gene expression and the previously published *BCL6* target gene set²⁵ (Figure SI-5G). At 24 h we observed a borderline significant positive correlation between these two gene signatures (Figure SI-5H).

To determine the more acute transcriptional changes we also conducted 4 h RNA-seq experiments with the compounds listed above in addition to NEG-2 (1 μ M; Figure SI-6A-D). Transcriptional dynamics were overall lower than at the longer time points (except for NEG-1). However, we observed similar trends in expression of *BCL6* target genes for EB-TCIP, BI3812, and NEG-1 as described above. NEG-2 did not show a significant increase in any *BCL6* target genes, although they trended upward. We also conducted a 4 h RNA-seq experiment comparing EB-TCIP (1 μ M) to DMSO in parental EWS502 cells in an attempt to determine acute transcriptional changes that might account for the compound's toxicity. However, we observed no significantly up or down regulated genes with EB-TCIP treatment in parental cells (Figure SI-6E). In EWS502 FKBP-E::F cells treated with EB-

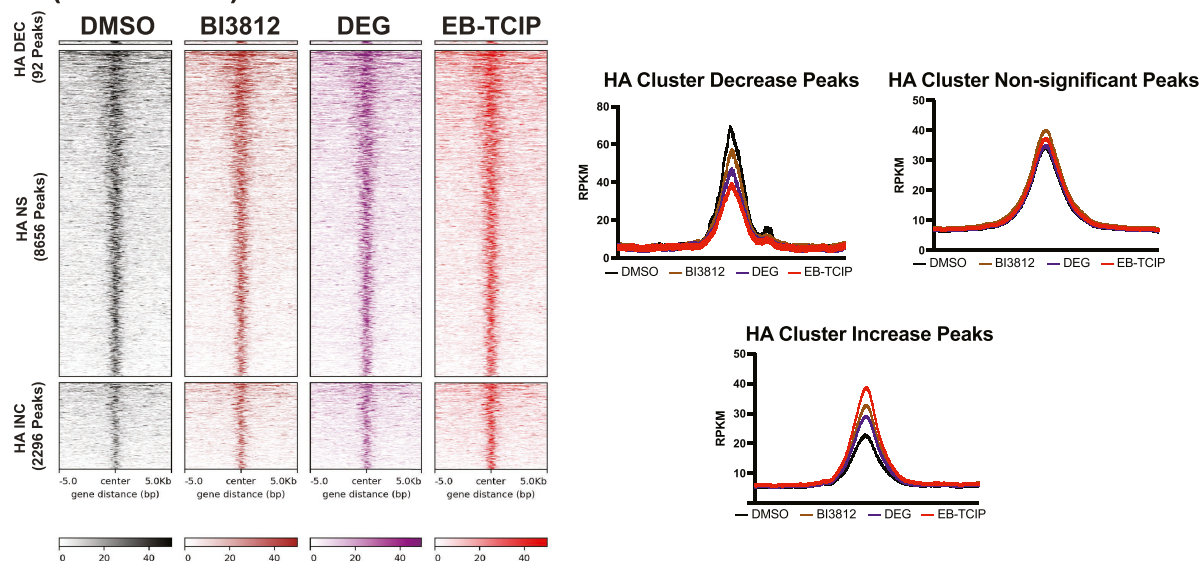
TCIP, we observed a trend of positive enrichment of the previously published *BCL6* target gene set²⁵ at this early time point (Figure SI-6F). For all RNA-seq experiments dendrograms are presented in the Supporting Information to show the distribution of samples. Taken together, these data further support that EB-TCIP rapidly enhances *BCL6* target genes compared to chemical inhibition.

EB-TCIP Relocalizes FKBP-E::F to *BCL6* Sites on Chromatin. To better understand the gene expression changes induced by EB-TCIP, we used chromatin immunoprecipitation with sequencing (ChIP-seq) to determine how EB-TCIP treatment changes FKBP-E::F and *BCL6* localization on chromatin. EWS502 FKBP-E::F cells were treated with DMSO, BI3812, BI3802 or EB-TCIP (1 μ M) for 24 h and then subjected to ChIP-seq, using antibodies for HA or *BCL6*. An HA antibody was used instead of a FLI1 antibody to ensure only FKBP-E::F, and not endogenous EWSR1::FLI1, was immunoprecipitated. Globally, treatment with all compounds modestly increased FKBP-E::F on chromatin to varying degrees (Figure SI-7A). As expected, degradation of *BCL6* induced by BI3802 and EB-TCIP decreased *BCL6* binding globally to chromatin, while inhibition with BI3812 had minimal effect (Figure SI-7B). We observed ~50% overlap between FKBP-E::F and *BCL6* binding sites in DMSO treated HA and *BCL6* samples (Figure SI-7C-E), and accordingly, similarities in the binding motifs of EWSR1::FLI1 and *BCL6* (Figure SI-7F). EWSR1::FLI1 binds DNA at "GGAA" repeats, and this sequence is present within the recognition motif of *BCL6*. Further, the sequence similarity may enable FKBP-E::F to bind *BCL6* target genes with greater affinity when brought into proximity by EB-TCIP.

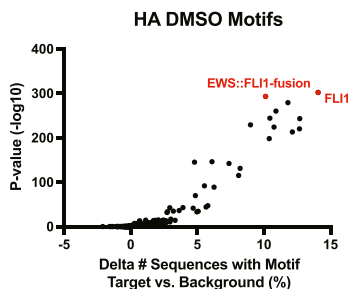
To determine chromatin changes specific to EB-TCIP treatment, we clustered peaks in all treatments based on decreasing, unchanged, and increasing peak intensity between EB-TCIP and DMSO for both antibodies. This clustered analysis revealed that EB-TCIP induced an increase in a subset of both HA and *BCL6* peaks, to a greater extent than that observed in BI3812 or BI3802 treated samples (Figure 5A and Figure SI-8A). We performed motif analysis to determine what DNA sequences were associated with the EB-TCIP treated HA increased peaks and compared this to motif analysis from global HA binding peaks in DMSO treated samples (SI Tables 2–4). In DMSO treated samples, the top two motifs were EWSR1::FLI1 related, as expected (Figure 5B) and no *BCL6* motif was observed. However, the *BCL6* motif was enriched in HA increased peaks with EB-TCIP treatment and ranked 29th (Figure 5C), while ETS-related motifs were enriched at sites where HA decreased with EB-TCIP treatment (Figure 5D). Since EB-TCIP induces a ternary complex between FKBP-E::F and *BCL6*, we explored if the *BCL6* increased peaks enriched for EWSR1::FLI1 signatures. *BCL6* increased peaks showed enrichment in EWSR1::FLI1 motifs and a decrease in the rank of the *BCL6* motif compared to DMSO peaks (Figure SI-8B-D and SI Tables 5–7).

We next visualized changes in FKBP-E::F and *BCL6* binding at both *BCL6* and EWSR1::FLI1 target gene sites. Robust *BCL6* peaks were observed at *SOCS2*, *CISH*, and *CXCL11* loci

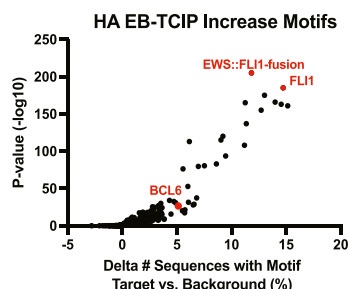
A HA (FKBP-E::F) EB-TCIP ChIP Clusters



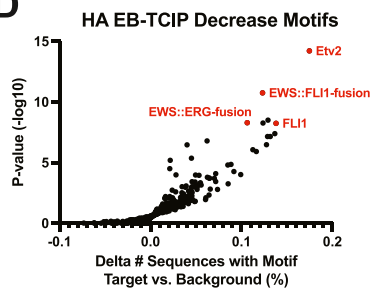
B



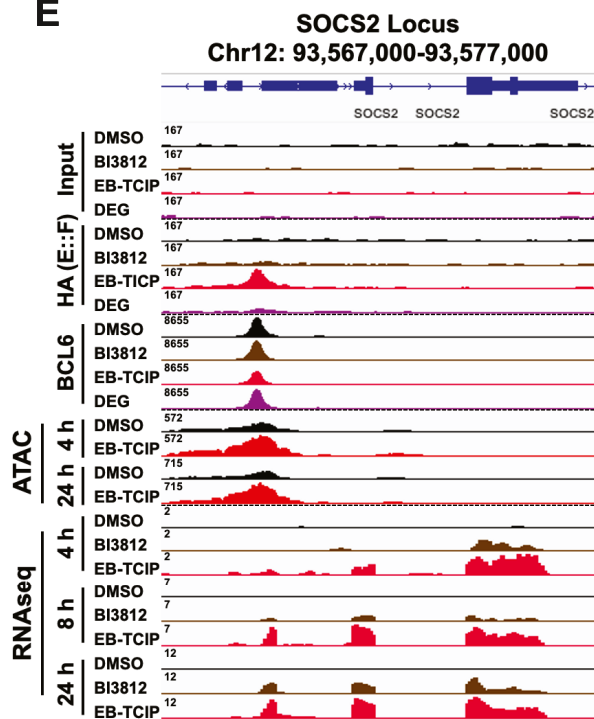
C



D



E



F

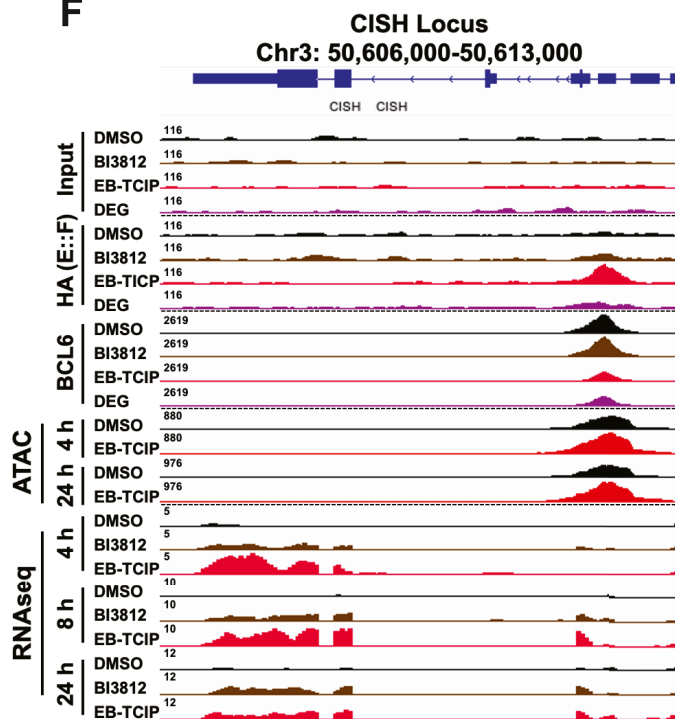


Figure 5. EB-TCIP changes the localization of FKBP-EWSR1::FLI1 on chromatin. (A) ChIP-seq tornado plots of HA (FKBP-E::F) binding signal of EB-TCIP (red) versus DMSO (black) peaks that are decreasing (DEC; 92), nonsignificantly changing (NS; 8656), and increasing (INC; 2296) at 24 h. Differential peaks between EB-TCIP and DMSO are shown for all compounds. Compared to BI3812 (brown) and BI3802 (DEG,

Figure 5. continued

purple), **EB-TCIP** increases FKBP-E::F binding at a subset of genes. Line plots for all compound treatments in each cluster are shown to the right. (B) Scatter plot portraying top enriched motifs of HA binding sites in DMSO treated cells. (C) Scatter plot portraying top motifs of HA increased peaks enriched in **EB-TCIP** treated cells. The BCL6 motif scores 29th. (D) Scatter plot portraying top motifs of HA decreased peaks enriched in **EB-TCIP** treated cells. IGV visualization of input, HA (FKBP-E::F), BCL6, ATAC-seq signal, and RNA-seq signal at the *SOCS2* (E) and *CISH* (F) with treatments DMSO (black), 1 μ M **BI3812** (brown), 1 μ M **BI3802** (DEG, purple), and 1 μ M **EB-TCIP** (red). All ChIP-seq and ATAC-seq is portrayed as the average of two independent replicates. RNA-seq is portrayed as the average of three independent replicates.

in all treatments (Figure 5E,F and Figure SI-9A). FKBP-E::F binding was not observed in DMSO treated cells and only **EB-TCIP** was able to induce binding. BCL6 degradation alone does not explain increased FKBP-E::F binding as **BI3802** and **EB-TCIP** induce similar levels of BCL6 loss at *CISH*, but FKBP-E::F binding is only induced by **EB-TCIP**. Moreover, **EB-TCIP** induced FKBP-E::F binding was enriched at BCL6 target loci as no FKBP-E::F was observed at the *GAPDH* genomic locus (Figure S9-B). A 4 h ChIP experiment showed a similar pattern of FKBP-E::F relocalization with more pronounced BCL6 loss at BCL6 target loci (Figure SI-9C-E and SI Tables 8–11). For visualization of changes at EWSR1::FLI1 target sites we focused on *NR0B1* and *VRK1*, where EWSR1::FLI1 canonically binds at a proximal and distal enhancer respectively (Figure SI-10A-B). We observed strong FKBP-E::F binding at both enhancer sites with all treatments. However, only **EB-TCIP**-treated samples showed increases in BCL6 binding, which mirrored the distinct pattern of FKBP-E::F at each site. Despite BCL6 levels increasing at the *NR0B1* and *VRK1* regulatory regions at 24 h, no change in expression of these genes was observed by RNA-seq. Together, our ChIP-seq data shows that **EB-TCIP**, but not **BI3812** or **BI3802**, can relocalize both FKBP-E::F and BCL6 on chromatin.

Recently, small molecules have been used to redirect the pioneering TF activity of FOXA1 on chromatin.³⁸ Since **EB-TCIP** relocalizes FKBP-E::F, which has pioneering TF activity, we used assay for transposase-accessible chromatin with sequencing (ATAC-seq) to determine if chromatin accessibility is changed at genomic loci where FKBP-E::F is gained. Globally, chromatin accessibility is not significantly changed with **EB-TCIP** treatment (Figure SI-11A). However, at BCL6 target sites where FKBP-E::F is gained, such as *SOCS2*, *CISH*, and *CXCL11*, open chromatin is increased, leading to increased RNA-seq peaks (Figure 5E,F and SI-9A). We also investigated changes in chromatin accessibility at BCL6 gained sites *NR0B1* and *VRK1*. EWSR1::FLI1 binding is relatively unaffected at these sites, as is chromatin accessibility and gene expression (Figure SI-10A-B). We also performed a 4 h ATAC-seq experiment that also included **NEG-2**. Again, global chromatin accessibility was not significantly changed, but **EB-TCIP** increased chromatin accessibility at BCL6 target genes (Figure SI-11B-E). **NEG-2**, which can inhibit BCL6, also increased chromatin accessibility at these sites but to a lesser extent than **EB-TCIP** (Figure SI-11E). Our ATAC-seq data suggests that relocalized FKBP-E::F increases gene expression by opening chromatin while relocalization of BCL6 is generally not sufficient to repress EWSR1::FLI1 target genes.

DISCUSSION

In our proof-of-concept study, we demonstrate that the pioneering TF activity of EWSR1::FLI1 can be redirected to genes typically inactivated by the repressor BCL6. Our tool compound **EB-TCIP**, which links **OAP** to **BI3812**, relocalized FKBP-E::F to chromatin sites bound by BCL6, thereby driving

expression of genes ordinarily repressed by BCL6. The compound is potent and induces rapid transcript and protein expression of *SOCS2* and *CISH*. ChIP-seq and ATAC-seq showed that **EB-TCIP** increases open chromatin at FKBP-E::F gained sites. We foresee **EB-TCIP** as being a useful tool compound to further probe the biology of ES cells in the context of relocalizing FKBP-EWSR1::FLI1. For example, future studies could investigate how **EB-TCIP** impacts transcriptional condensate formation, which is an important mechanism for gene activation by EWSR1::FLI1 and other TFs.^{39,40} Potentially, **EB-TCIP** may be forming new transcriptional condensates that contain both EWSR1::FLI1 and BCL6. Although the utility of **EB-TCIP** may be limited for phenotypic measurements, as we observed off-mechanism cytotoxicity, we have also gained insights that may help inform the next generation of EWSR1::FLI1 TCIPs, such as their proteasome dependent activity.

Our study is important because it demonstrates that TCIP molecules can bring together two DNA binding proteins. An unforeseen activity of **EB-TCIP** was its ability to induce the degradation of BCL6. We show that proteasome inhibition negates the degradation of BCL6 and attenuates the activity of **EB-TCIP**. With global protein degradation inhibited, BCL6 and FKBP-E::F levels increase, which we hypothesized would increase **EB-TCIP** induced ternary complex and BCL6 target expression. However, we observed the opposite and since proteasome inhibition should not limit transcription, these data suggest that BCL6 degradation is important for maximal activity of **EB-TCIP**. These observations are similar to pioneering studies in the transcription factor field that linked ubiquitin proteasome system function to the activity of transcription factors like Myc, androgen receptor, and estrogen receptor.^{41,42} The importance of BCL6 degradation to the **EB-TCIP** mechanism of action is also corroborated by our ChIP-seq data, as we generally observed decreases in BCL6 binding at sites where FKBP-E::F binding is gained and chromatin is opened. However, BCL6 degradation alone with **BI3802** treatment is not enough to activate transcription to the same level as **EB-TCIP**. The degradation of BCL6 induced by **EB-TCIP** was competed away with excess **OAP** and not observed when cells were treated with **NEG-2**, which retains the ability to bind BCL6, suggesting that FKBP-E::F or a protein associated with FKBP-E::F, may induce BCL6 degradation. Therefore, future EWSR1::FLI1 relocalizing TCIP molecules may also induce degradation of the targeted repressor.

Although our study focused on hijacking EWSR1::FLI1 TF activity, another interesting avenue of investigation would be recruiting genetic repressors to EWSR1::FLI1 to decrease oncogenic gene expression. Our chromatin data shows that BCL6 moves to FKBP-E::F loci in an **EB-TCIP** dependent manner. Even though BCL6 is redirected to FKBP-E::F sites, some sites show increases in gene expression rather than a decrease. The inability of BCL6 to consistently repress EWSR1::FLI1 activated genes could be for several reasons.

First, FKBP-E::F binding does not change at these sites and the transcriptional activation activity of FKBP-E::F may out compete the repressor activity of BCL6. Second, the magnitude of BCL6 gained at FKBP-E::F sites is lower (>10 fold) than the BCL6 present at SOCS2 or CISH. Therefore, there may not be enough BCL6 gained at these sites to repress gene expression. Finally, **EB-TCIP** uses an inhibitor of BCL6, and the BCL6 that is recruited to EWSR1::FLI1 may not be functional (e.g., the corepressor complex is disrupted). TCIPs containing repressor ligands that do not inhibit protein function may result in bivalent molecules that can repress expression of EWSR1::FLI1 activated genes. Alternatively, repressors with stronger repressive function may need to be recruited.

Next generation EWSR1::FLI1 TCIP molecules will need to address the limitations of **EB-TCIP**. First, endogenous EWSR1::FLI1 will need to be recruited. Although there is a lack of ligands for EWSR1::FLI1, future TCIPs could incorporate **MS0621**,⁴³ a recently described molecule that is reported to interact with EWSR1, EWSR1::FLI1, and SWI/SNF complex members. Although other molecules, such as **YK-4-279** and its clinical derivative **TK-216**,⁴⁴ are reported EWSR1::FLI1 inhibitors, these molecules may not be ideal ligands for TCIP development as they are also reported to destabilize microtubules at therapeutically relevant concentrations.⁴⁵ Considering these shortcomings, our study should encourage EWSR1::FLI1 ligand discovery as even functionally agnostic compounds could be used to relocalize EWSR1::FLI1.

Future TCIPs will need to direct EWSR1::FLI1 to repressors that are ES dependencies. TCIPs targeting BCL6 are antiproliferative in DLBCL because B cell lymphoma cells depend on BCL6 to evade apoptosis. BCL11B, another C2H2 zinc finger repressor, would be a good candidate for next-generation EWSR1::FLI1 TCIPs since it is a known ES dependency.⁴⁶ Although there is no reported BCL11B ligand, there is precedence for targeting these proteins with regulatory domain inhibitors (i.e., **BI3812** for BCL6) or iMIDs that bind the C2H2 zinc finger in a cereblon-dependent manner.⁴⁷ An interesting repressor candidate in which chemical tools may already exist is ZEB2. Like BCL11B, ZEB2 is a known ES dependency.⁴⁸ Recently, it was shown that ZEB2 forms a complex with the lysine demethylase KDM1A in T-ALL cells,⁴⁹ which are also dependent on ZEB2. Presumably, KDM1A interacts with ZEB2 at genomic loci where ZEB2 acts as a repressor. There are several classes of known KDM1A inhibitors,^{50,51} some with reported antiproliferative activity in ES,⁵² that could be used to recruit EWSR1::FLI1 to these genomic loci to enhance expression of ZEB2 repressed genes that may be more relevant to ES tumor survival.

The TCIP platform is a promising therapeutic modality for ES and other fusion TF driven cancers. Solid and hematological pediatric malignancies are driven by fusion TFs, such as PAX3::FOXO1⁵³ in rhabdomyosarcoma and CBFA2T3::GLIS2⁵⁴ in an aggressive subtype of pediatric acute myeloid leukemia (AML). FKBP tagged fusion TF systems could help determine if TCIPs can be used to relocalize fusion TFs beyond EWSR1::FLI1. ES, rhabdomyosarcoma, and CBFA2T2::GLIS2 AML express fusions that are unique to the tumor cell and are not expressed in healthy cells. Therefore, TCIPs hijacking the fusion TF may have an improved therapeutic window compared to standard chemotherapies/targeted therapies as TCIPs may exhibit reduced toxicity in noncancerous cells. Building on our proof-of-

concept study, future EWSR1::FLI1 relocalizing TCIPs could serve as novel targeted ES therapies with improved efficacy and safety profiles for patients.

CONCLUSIONS

Previous efforts to induce transcription with bivalent molecules have focused on recruiting transcriptional modulators that do not bind DNA, such as BRD4, p300/CBP, and CDK9/12. In this study, we show that a small molecule can induce a ternary complex between two DNA binding proteins and drive transcription. Our molecule, **EB-TCIP** relocalizes FKBP-E::F to BCL6 sites on chromatin. Redistribution of FKBP-E::F leads to rapid increases in chromatin accessibility and gene expression of BCL6 repressed target genes. An unanticipated function of **EB-TCIP** is its ability to induce BCL6 degradation, which may be important for opening sites on chromatin for FKBP-E::F to bind and drive transcription. Our study lays the foundation for the development of future EWSR1::FLI1 recruiting TCIPs that could serve as potential Ewing sarcoma therapies.

MATERIALS AND METHODS

Data Availability. All genome-scale dependency and expression data are available at the DepMap portal Web site: <https://depmap.org>. Graph Pad Prism 10 was used to calculate differences between BCL6 expression in DLBCL vs ES (unpaired *t* test with Welch's correction) and differences in BCL6 dependency between all other cancers vs DLBCL and all other cancers vs ES (one-way ANOVA). All functional transcriptomics and genomics have been made publicly available at the Gene Expression Omnibus (GEO) repository (<https://www.ncbi.nlm.nih.gov/geo/>) as GSE290895 (RNA-seq), GSE290894 (ChIP-seq), and GSE290893 (ATAC-seq). Under GEO GSE290895 (RNA-seq), GSE290894 (ChIP-seq), and GSE290893 (ATAC-seq) 8 and 24 h samples **ortho-AP1867** is labeled as **OAP**, **BI3812** is labeled as **BI**, **EB-TCIP** is labeled as **BAK212**, and **NEG-1** is labeled as **O89**.

Cell Lines and Reagents. All cell lines used were subject to short tandem repeat (STR) analysis for genotyping and tested for Mycoplasma using the MycoAlert test kit (Lonza, LT07-318). HEK293TF cells used to generate lentivirus were purchased from Thermo Fisher Scientific (#R70007) and grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, MT10013CM) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, F2442) and 1% penicillin-streptomycin (Life Technologies, 15140163). The EWS502 cell line (originally derived in Dr. J. Fletcher's Lab at Harvard University) was generously provided by Dr. Stephen L. Lessnick of Nationwide Children's Hospital and all EWS502 lines were grown in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, 11875119) supplemented with 15% FBS and 1% penicillin-streptomycin. The TC32 cell line (originally derived by Dr. T. Triche at UCLA School of Medicine) was generously provided by Dr. Todd Golub of the Broad Institute and Dana-Farber Cancer Institute (DFCI), and all TC32 lines were grown in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. To passage cells for maintenance and experiments, cells were washed with sterile phosphate buffered saline (PBS; Life Technologies, 10010023) and detached with 0.05% trypsin-EDTA (Life Technologies, 25300062). Puromycin (Life Technologies, A1113803) and blasticidin S HCl (Life Technologies, A1113903) were used to select cells as indicated below. Compounds used in this work were acquired from the following sources: **BI3812** (S8735) and **MLN4924** (S7109) were purchased from Selleck Chem. **BI3802** (HY-108705) and **ortho-AP1867** (HY-114434) were purchased from Med Chem Express. **MG132** (474790) and **Actinomycin D** (A4262) were purchased from Sigma-Aldrich.

Lentiviral CRISPR/Cas9 Plasmid Construction. Parent plasmids used for guide cloning include lentiCRISPR v2-Blast (Addgene

#83480) and lentiCRISPR v2-Puro (Addgene #98290). FastDigest Esp3I (Bsmbl; Thermo Scientific, FD0454) was used to digest each backbone, which was then purified by gel extraction (Qiagen, 28704). Synthetic oligonucleotides encoding gene-targeting single guide RNA (sgRNA) sequences (provided below) were purchased from Integrated DNA Technologies (IDT). sgRNAs were annealed and end-phosphorylated using T4 polynucleotide kinase (New England Biolabs, M0201S) in T4 DNA Ligase Reaction Buffer containing 10 mM ATP (New England Biolabs, B0202S). Ligated vectors were transformed into One Shot Stbl3 *Escherichia coli* (Life Technologies, C737303), shaken at 37 °C for 1 h, spread onto 100 µg/mL ampicillin Luria broth (LB) plates (Teknova, L1004) with a L-shaped cell spreader (Fisher Scientific, 14665230) and then grown overnight at 37 °C. Selected colonies were grown overnight in 5 mL of LB (Invitrogen, 12795-027) supplemented with 100 µg/mL ampicillin (Sigma-Aldrich, A9393). Plasmids were DNA-extracted (Qiagen, 27104) and submitted for Sanger sequencing validation at Genewiz (Azenta Life Sciences). Validated clones were cultured overnight in 250 mL volumes, and plasmids were extracted (Zymogen, D4203).

All guides used in this work were from the Broad Institute's Avana CRISPR-Cas9 library (<https://depmap.org>). The following guides sequences were used: sgFLI-2 (5'-GATCGTTTGTGCCCCCTCCAA-3'), sgBCL6-1 (5'-AGATCCTGAGATCAACCCTG-3'), and sgBCL6-2 (5'-GATCCTGAGATCAACCCTGA-3'). The genomic location of the cut induced by sgFLI-2 is chr11:128,807,186 (FLI Exon 7). As previously described,⁵⁵ sgChr2.2 (5'-GGTGTGCGTATGAAGCAGTG-3') served as a cutting control and targets a gene desert on chromosome 2. sgLacZ (5'-AACGGCGGATTGACCGTAAT-3') served as a nontargeting, transduction control and targets a nonhuman gene. For ligation into the lentiCRISPRv2 (either Blast or Puro) plasmid, the additional bases 5'-CACCG-3' were added to the 5' end of the forward sequence. 5'-AAAC-3' and 5'-C-3' were added at the 5' and 3' ends of the reverse sequence, respectively. sgFLI-2 was cloned into the lentiCRISPR v2-Blast plasmid. sgLacZ, sgChr2.2, sgBCL6-1, and sgBCL6-2 were cloned into the lentiCRISPR v2-Puro vector.

Generation of Polyclonal FKBP-EWSR1::FLI1 and FKBP-GFP Expressing Cells. EWS502 cells expressing FKBP-EWSR1::FLI1 concurrent with knock out of endogenous EWSR1::FLI1 (sgFLI-Ex9:5'-GCCTCACGGCGTGCAGGAAG-3') as well as EWS502 cells expressing FKBP-GFP were generated as described previously.²⁹ The genomic location of the cut induced by sgFLI-Ex9 is chr11:128810443 (FLI Exon 9). TC32 cells expressing FKBP-EWSR1::FLI1 were generated in a similar manner, except cells were cotransduced with viral supernatants containing pLEX_305-dTAG-EWS/FLI and lentiCRISPR v2-Blast-sgFLI-2. Cells were then selected and maintained in 1 µg/mL puromycin and 10 µg/mL blasticidin. EWS502-FKBP-EWSR1::FLI1 cells were also maintained in 1 µg/mL puromycin and 10 µg/mL blasticidin. EWS502-FKBP-GFP cells were maintained in 1 µg/mL puromycin.

Lentivirus Production and Polyclonal CRISPR Cas9 KO of BCL6. CRISPR-Cas9 constructs were packed into lentiviral particles via transduction of HEK293TF cells in Falcon 6 well tissue culture treated plates (Corning, 353046). HEK293TF cells were seeded at a density of 400,000 cells/mL per well. The next day each well was cotransfected with 1250 ng of lentiCRISPR v2-Puro-sgRNA or FgH1tUTG-sgRNA construct plasmid, 250 ng of pVSVG plasmid (Addgene #8454), and 1250 ng of pPAX2 plasmid (Addgene #19319) using Lipofectamine 2000 (Life Technologies, 11668027) according to the manufacturer's recommended protocol. Plasmids and Lipofectamine 2000 were diluted and mixed in Opti-MEM (Life Technologies, 1058021). Mixtures of DNA and lipofectamine were added dropwise to each well followed by incubation for 8–16 h at 37 °C, after which media was aspirated and replaced with 3 mL of fresh DMEM. Forty-eight h after the media change, virus-containing media was collected in 10 mL Luer-Lok syringes (BD, 302995) and sterile-filtered through 0.45 µm syringe filters (Corning, 431225). All infections were performed with freshly produced virus.

For BCL6 KO experiments, 2×10^6 EWS502 or TC32 cells were seeded into 6-well plates in a volume of 1 mL of RPMI media

supplemented with 8 or 4 µg/mL of Polybrene (Santa Cruz Biotechnology, SC-134220), respectively. One mL of virus containing media was then added dropwise (final Polybrene concentration of 4 or 2 µg/mL) and cells were spin-infected at 30 °C at 2000 rpm for 2 h in a Sorvall Legend XTR centrifuge (Thermo Fisher Scientific). Cells were then incubated at 37 °C overnight. The next day, cells were lifted with trypsin from the 6-well plate and selected with 1 µg/mL of puromycin in a T75 flask (Thermo Fischer Scientific, 156753) for 72 h. Separate samples of noninfected cells subject to the same conditions were treated with puromycin to confirm cell death.

RNA Sequencing (RNA-seq). For all BCL6 KO experiments, three separate wells of cells were transduced and selected as described above. Approximately two million cells transduced with control or BCL6 sgRNAs from each well were washed with PBS and then detached from the plate using trypsin. Half of the cells were aliquoted into a 1.5 mL Eppendorf tube and were set aside for protein purification to confirm KO. The other half of cells were pelleted by centrifugation at 2500g for 3 min in a tabletop centrifuge (Eppendorf, 5425). Media was aspirated and total RNA was extracted using the RNeasy Plus kit (Qiagen, 74134). Preparation of RNA-seq libraries from total RNA and sequencing was performed by Novogene (<https://en.novogene.com>). Sequencing was done at ~20 million reads per sample. Per Novogene correspondence, RNA integrity was assessed using a Bioanalyzer 2100 System (Agilent Technologies). Libraries were then prepared by purifying mRNA from total RNA samples using poly-T oligo-attached magnetic beads. Purified mRNA was fragmented and library prep completed using Fast RNA seq Lib Prep Kit V2 (AbClonal Technology, RK20306). Library quality and concentration were assessed using real-time PCR and Qubit fluorometric quantitation (Thermo Fisher Scientific). Libraries were then pooled based on concentration and sequenced in 150-bp paired-end fashion on a Novaseq6000 instrument (Illumina).

For RNA sequencing experiments of EB-TCIP treated cells, 800,000 EWS502 FKBP-E::F cells were seeded into a 6 well plate. The next day, cells were treated in sextuplicate with DMSO, 2.5 µM B13812, 2.5 µM EB-TCIP, or 2.5 µM NEG-1. One set of triplicates was collected as described above 8 h post treatment and the second set of triplicates was collected as described above 24 h post treatment. At both time points cells collected for RNA harvesting were frozen in 350 µL of RLT plus buffer (Qiagen) at -80 °C. All samples were thawed at the same time and total RNA purified using the RNeasy Plus kit. Total RNA was then subjected to library prep and RNA-seq by Novogene as described above. Two 4 h experiments were conducted as described above except in one experiment cells were treated with DMSO, 1 µM B13812, 1 µM EB-TCIP, or 1 µM NEG-2. In the other experiment cells were treated with DMSO or 1 µM NEG-1. For parental EWS502 RNA-seq experiments 1×10^6 cells were seeded and treated the next day with DMSO or 1 µM EB-TCIP.

Quantitative-Real Time PCR (qPCR). Total RNA from 400,000 to 800,000 cells was extracted using the RNeasy Plus kit. If cells were split for protein and RNA isolation, trypsin was used to detach cells from the plate as described earlier. For experiments where only RNA was harvested, cells were lysed in RLT plus buffer on the plate. Between 1 and 1.5 µg of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4368814) and then diluted 1:5 with UltraPure DNase/RNase-Free Distilled Water (Invitrogen, 10977015). All qPCR reactions were performed using the TaqMan system (Thermo Fisher Scientific) with technical triplicate or quadruplicate. Probes used in this study include: SOCS2: Hs00919620_m1, CISH: Hs00367082_G1, BCL6: Hs00153368_m1, and GAPDH: Hs02786624_G1 (60x primer limiting). In each qPCR reaction, the gene of interest was measured using FAM dye while the GAPDH control was measured using VIC dye. Samples were analyzed in 384-well plate format using 5 µL of either TaqMan Universal Master Mix (Thermo Fisher Scientific, 4304437) or Fast Advanced Master Mix for qPCR (Thermo Fisher Scientific, 4444557), 0.5 µL of FAM-emitting probe, 0.17 µL of VIC-emitting GAPDH probe (60x stock), 2 µL of diluted cDNA and 2.33 µL of UltraPure water for a total of 10 µL per reaction. From the 10 µL reaction volume, 8 µL were pipetted

into a MicroAmp Optical 384-well plate (Thermo Fisher Scientific, 4309849) using a 0.5–12.5 μ L E1-ClipTip electronic pipet (Thermo Scientific). The plate was spun briefly and then sealed with an optical adhesive cover (Thermo Fisher Scientific, 4360954). The QuantStudio 6 Flex Real-Time PCR machine and the accompanying QuantStudio Real-Time PCR software v.1.7 (Thermo Fisher Scientific) was used to produce and analyze data. The delta-threshold cycle number (Δ Ct) was calculated as the difference in threshold cycle number (Ct) between the gene of interest and *GAPDH*. The $\Delta\Delta$ Ct was calculated as the difference between the Δ Ct of a particular sample and the average Δ Ct of the DMSO-treated or sgLacZ control samples. The fold change in gene expression (after *BCL6* KO or compound treatment) was calculated using the following exponential equation: fold change in expression of experimental vs control sample = $2^{-\Delta\Delta\text{Ct}}$. Microsoft Excel was used to calculate Δ Ct, $\Delta\Delta$ Ct, and fold change in gene expression.

One-way ANOVAs were used to compare changes in gene expression between control conditions (sgLacZ or DMSO). For time course experiments, one-way ANOVAs were used to compare the mean of EB-TCIP to all other conditions. For experiments comparing parental, FKBP-GFP, and FKBP-E::F cells, the ratio of BI3812 induced expression compared to DMSO vs EB-TCIP induced expression compared to DMSO was calculated in Microsoft Excel. One-way ANOVA statistics were also used to compare differences between treatments for each cell type. All ANOVA statistics were calculated with Graph Pad Prism 10 using technical replicates.

Generation of EWS502 FKBP-EWSR1::FLI1 BCL6 GFP Reporter and Flow Cytometry. The BCL6 GFP reporter plasmid used in previous TCIP publications^{8,9} was graciously provided by the lab of Dr. Jerry Crabtree of Stanford University. Lentiviral particles containing the construct were produced as described above. EWS502-FKBP-EWSR1::FLI1 cells were infected with the lentiviral particles. Cells were selected for 72 h with 1 and 10 μ g/mL of puromycin and blasticidin, respectively. After selection, cells were sorted on a BD Symphony S6 UV Cell Sorter at the DFCI Flow Cytometry Core, which yielded a polyclonal cell population with uniform GFP signal. From this population, single clones were selected by plating 0.5 cells/well into 96 well plates. After 4 weeks, single colonies were harvested and expanded. The clone that displayed the brightest GFP fluorescence by flow cytometry after 1 μ M EB-TCIP treatment for 24 h was selected for further experiments.

Fifty-thousand reporter cells were plated per well in a Falcon 24 well-plate (Corning, 353047). The next day cells were treated with a dose response of EB-TCIP, NEG-1, NEG-2, or DMSO. Twenty-four h later, cells were collected, filtered through Falcon Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap (Fisher Scientific, 0877123) and GFP intensity was measured by flow cytometry at 10,000 cells per sample on a BD FACSCelesta instrument. Live cells were gated using FSC-A and SSC-A. Data was analyzed using FlowJo v.10.4 software. Ratios of the number of cells with GFP intensity $>10^3$ in bivalent compound treated cells vs DMSO treated cells were calculated in Microsoft Excel and are reported. Differences in the mean of the ratios at each concentration were compared using multiple *t* tests in Graph Pad Prism 10.

Time Resolved Fluorescence Energy Transfer (TR-FRET). Each reaction contained 25 nM His6-TEV-FLAG-FKBP12-F36V, 200 nM BCL6^{BTB}-Avi-Biot, 20 nM Streptavidin-FITC (Thermo #SA1001), and 1:400 anti-6xHis terbium antibody (PerkinElmer #61HI2TLF) in 10 μ L of buffer containing 20 mM HEPES, 150 mM NaCl, 0.1% BSA, 0.1% NP40, and 1 mM TCEP in a 384-well plate. Protein was incubated with drug digitally dispensed (Tecan D300e) for 1 h in the dark room at room temperature before excitation at 337 nm and measurement of emission at 520 nm (FITC) and 490 nm (terbium) with a PHERAstar FS plate reader (BMG Labtech). The ratio of signal at 520 to 490 nm was calculated in Microsoft Excel and normalized to DMSO-treated conditions and plotted.

Protein Constructs and Purification for TR-FRET. Biotinylated BCL6^{BTB}-AviTag protein used for TR-FRET assays included BCL6 amino acids 5–129 with the following mutations: C8Q, C67R,

C84N.⁵⁶ These enhance stability but do not affect the affinity for BI3812. Preparation of this protein has been described previously.⁸

The construct used for FKBP12^{F36V} was pNSG317 (His6-TEV-FLAG-FKBP12-F36V). Rosetta 2(DE3) (Sigma #71400) *E. coli* cells were transformed with plasmid and inoculated as a starter culture in 50 mL Luria Broth supplemented with chloramphenicol and carbenicillin overnight. Saturated culture was divided into 2L 2XYT medium supplemented with appropriate antibiotics and grown to OD800 = 0.8 at 37 °C. Protein expression was induced by addition of 400 μ M IPTG (final concentration, Sigma #1678) and the temperature was adjusted from 37 to 18 °C for overnight incubation. After incubation overnight, cells were harvested by centrifugation. Cell pellets were resuspended in $\sim$ 2 mL/L D800 buffer (20 mM HEPES, pH 7.5; 800 mM NaCl; 10 mM imidazole, pH 8.0; 10% glycerol, 2 mM β mercaptoethanol) supplemented with protease inhibitors (1 mM PMSF, 1 mM benzamidine, $\sim$ 20 μ g/mL pepstatin, aprotinin, and leupeptin) and frozen at -80 °C. Cell pellets were thawed briefly in warm water and lysed by sonication and addition of solid egg white lysozyme (Goldbio, L-040–10) before centrifugation at 16,233g for 1 h at 12 °C. Clarified lysate was mixed with $\sim$ 0.5 mL/L of growth cobalt resin (Goldbio) for 1h before centrifugation at low speed to separate the beads, which were then washed by gravity flow with $\sim$ 25 column volumes ice cold D800 buffer before a final wash with B50 (D800 with 50 mM NaCl) and elution with C50 (B50 with 400 mM imidazole, pH 8.0). Cobalt eluate was applied to a 5 mL anion exchange column (Q HP, Cytiva) and eluted with an 8-column volume gradient from B50 to D800. After concentration in a 3000 MWCO Amicon filter (Millipore #UFC9003), the sample was applied to a 24 mL gel filtration column (S200 increase, Cytiva) primed with GF150 buffer (20 mM Tris-HCl, pH 8.5, 150 mM NaCl, 1 mM TCEP). S200 peak fractions were again concentrated by ultrafiltration, supplemented with 5% glycerol (*v:v*, final), and aliquoted and frozen at -80 °C. A fresh aliquot was thawed for each assay.

Lysate Preparation and Immunoblotting. Cells were lysed in Radio Immunoprecipitation Assay (RIPA) lysis and extraction buffer (Thermo Scientific, 89900) supplemented with Halt protease inhibitor (Thermo Scientific, 87786) and Halt phosphatase inhibitor (Thermo Scientific, 78420). For on plate lysis, plates with attached cells were placed on ice for 3 min, media aspirated, and then cells were washed with ice-cold PBS. PBS was aspirated and RIPA buffer was added for 15 min with plates on ice. Cells were scraped into chilled 1.5 mL Eppendorf tubes, vortexed for 20 s and then placed on ice for 15 min, after which the lysate was vortexed for another 20 s. Lysates were then clarified at 21,100g for 20 min at 4 °C in a Sorvall Legend Micro 21R centrifuge (Thermo Scientific). For experiments in which protein and RNA were isolated, cells were harvested as described above. Suspended cells were placed on ice for 3 min and then pelleted at 2500g at 4 °C. Media was aspirated and the pellets were washed with 1 mL of ice-cold PBS followed by another centrifugation at 2500g at 4 °C. PBS was aspirated and pelleted cells were then resuspended in RIPA buffer, vortexed for 20 s every 15 min over a 30 min period and then clarified as described above.

Lysates were prepared for gel electrophoresis by adding 4X NuPAGE LDS loading buffer (Life Technologies, NP0007) supplemented with 10% β -mercaptoethanol (BME, Sigma-Aldrich, M6250). Before addition of loading buffer, protein was quantified by colorimetric Pierce BCA assay (Thermo Scientific, 23227). One microliter of lysate was mixed with 100 μ L of BCA:4% copper(IV) sulfate pentahydrate (50:1) in a Falcon 96 well plate (Corning, 353072). The plate was incubated at 37 °C for 30 min and then absorbance read at 562 nm on a Benchmark Plus microplate spectrophotometer (BioRad). The linear correlation from a standard curve of 0, 1, and 5 μ g/ μ L was used to calculate protein concentrations in Microsoft Excel. Thirty to 45 μ g of protein was run on 4–15% 1.5 mm NuPAGE Bis-Tris mini precast gels (Thermo Fisher Scientific, NP0336) using NuPAGE MOPS SDS Running Buffer (Thermo Fisher Scientific, NP0001). Protein was run at 80–90 V for $\sim$ 20 min and then run at 130–145 V for an additional $\sim$ 90 min. Once electrophoresis was complete, protein was transferred to a 0.2 μ m nitrocellulose membrane (BioRad, A30741963) using the Trans-

Blot Turbo System (BioRad) at 1.3 A and 25 V for 10 min. Membranes were then incubated in 1X Tris Buffered Saline (TBST; Boston BioProducts, IBB-181) for 3 min with agitation. Next, membranes were blocked for 15 min at room temperature in EveryBlot Blocking Buffer (EBB; BioRad, 12010020). Membranes were then cut at 25 and 50 kDa and incubated in primary antibody diluted in EBB supplemented with 0.02% sodium azide (Sigma-Aldrich, S2002) overnight (12–16 h) at 4 °C with agitation. The next morning membranes were washed three times with 5 mL of TBST for 5 min per wash at room temperature. Membranes were then incubated in anti-rabbit IgG HRP-linked secondary antibody (Cell Signaling Technologies (CST), 7074S) diluted 1:10,000 in TBST for 1 h at room temperature. Next, membranes were washed three times with 5 mL of TBST for 5 min each at room temperature. Protein signal was then visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, 34096). Stable peroxide buffer was mixed 1:1 with the luminol/enhancer for 30 s after which the blot was incubated in the mixture for 1 min before visualizing on a ChemiDoc MP Imaging System (BioRad, 10000062126) using 2×2 binning with rapid or optimal automated exposure. When probing for EWSR1::FLI1 after BCL6, blots were stripped using Restore Western Stripping Buffer (Life Technologies, 21059) for 1 h at room temperature. Blots were washed three times with TBST for 5 min each at room temperature and then reblocked for 15 min with EBB. EWSR1::FLI1 primary antibody diluted in EBB was then added, incubated overnight at 4 °C and imaged as described above. Image Lab Version 6.1.0 build 7 was used to export image files for figures.

The following primary antibodies were used at the following dilutions: rabbit monoclonal anti-SOCS2 (Abcam, ab109245) at 1:1000, rabbit monoclonal anti-BCL6 (CST, 14895) at 1:1000, rabbit monoclonal anti-FLI1 (Abcam, ab133485) at 1:1000, rabbit monoclonal anti-HA (CST, 3724) at 1:1000, and rabbit monoclonal anti-GAPDH (CST, 5174) at 1:2000.

Ternary Complex Pulldowns. EWS502 FKBP-EWSR1::FLI1 cells growing on 15 cm² dishes (Thermo Scientific, 150350) were washed with PBS, lifted with trypsin, trypsin neutralized with RPMI media, and then pelleted at 1400 rpm for 3 min in an Eppendorf 5910 R centrifuge. Trypsin/media was aspirated, and the cells were washed with 5 mL of PBS and then counted using a Countess 3 cell counter (Invitrogen). Cells were then pelleted again at 1400 rpm for 3 min, PBS was aspirated and the cells placed on ice for 5 min. Next, cells were resuspended in IP lysis buffer (20 mM Tris pH 7.5 (diluted from 1 M Tris pH 8.0, Invitrogen, AM9856), 150 mM NaCl (diluted from 5 M, Invitrogen, AM9759), and 1% NP-40 (diluted from 10%, Abcam, ab142227)) supplemented with Halt protease and phosphatase inhibitors at a concentration of 10×10^6 cells per 250 μ L of lysis buffer. Lysate was kept on ice and vortexed for 20 s every 15 min for 1 h. Lysate was transferred to a chilled 1.5 mL Eppendorf tubes and cleared at 21,100g for 20 min at 4 °C. Lysate was pooled into one chilled 15 mL Falcon tube and then split into 250 μ L aliquots in separate, chilled tubes. Twenty-one microliters of lysate were saved as the input sample and mixed with 7 μ L of 4X LDS buffer supplemented with 10% BME. Each tube of lysate was then treated with either 0.25 μ L of DMSO or 1000 $\times$ stock of the indicated compound. Lysate was incubated with compounds for 1 h at 4 °C with agitation. For competition experiments, lysates were pretreated with 1000 $\times$ stocks of BI3812 or OAP or 0.25 μ L DMSO for 1 h before addition of EB-TCIP. While lysates incubated with compound, 25 μ L of Pierce Anti-HA Magnetic Beads (Thermo Scientific, 88837) per pulldown was aliquoted into a 1.5 mL Eppendorf tube. One milliliter of IP lysis buffer was added and then the tube was placed into a DynaMag-2 magnetic rack (Invitrogen, 12321D) until the solution was clear. Buffer was removed and the beads were washed twice more with 1 mL of IP lysis buffer. After the final wash, the beads were resuspended in 26 μ L of IP lysis buffer per pulldown and placed on ice. After the incubation with compounds, 25 μ L of washed beads was added to each tube. The beads were incubated with treated lysates overnight (16–24 h) at 4 °C. The next day, samples were quickly spun in a microcentrifuge and then beads separated using the magnetic rack. Beads were washed three times with ice-cold IP wash

buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.01% NP-40), with quick spins in between each wash to remove liquid from the cap of the tube. After the third wash the beads were resuspended in 1.5X LDS Buffer supplemented with 2.5% BME and boiled for 10 min at 95 °C. Boiled samples were spun at max speed in a tabletop centrifuge for 1 min to collect condensate and then placed on a magnetic rack. Supernatant was loaded into a 4–15% 1.5 mm NuPAGE Bis-Tris mini precast gel and subject to electrophoresis and immunoblotting as described above.

Time Courses. Seven-hundred thousand EWS502 FKBP-EWSR1::FLI1 cells were plated into each well of four 6 well tissue culture plates. The next day, wells were treated in sextuplicate with either DMSO, 1 μ M BI3812, 1 μ M EB-TCIP, or 1 μ M BI3802. Cells were then harvested at each time point by trypsinization as described above. At each time point half the cells were collected for RNA extraction and the other half used for protein isolation. RNA samples were frozen at –80 °C in RLT plus buffer while protein samples were frozen at –80 °C in RIPA buffer. All RNA or protein samples were thawed at the same time and processed together in a single batch. Purified RNA was subject to RT-qPCR as described above. Lysates were subject to immunoblotting as described above. For time courses with DMSO, 1 μ M EB-TCIP, and 1 μ M NEG-2, 400,000 cells were seeded into 12 well plates. The next day cells were treated in triplicate and then harvested at the designated time points by trypsinization. Cells were lysed on the plate with RLT plus buffer and RNA extract as described above.

Competition Assay. One million, two hundred thousand EWS502 FKBP-EWS/FLI1 cells were plated into each well of two 6 well tissue culture plates. The next day, cells were treated with either DMSO or 25 μ M OAP (free acid) for 1 h at 37 °C. After this pretreatment, media was aspirated and cells were treated with either DMSO, 25 μ M OAP, 1 μ M BI3812, 1 μ M EB-TCIP, 25 μ M OAP plus 1 μ M EB-TCIP, or 1 μ M BI3812 plus 1 μ M OAP for an additional 4 h at 37 °C. Cells were then harvested by trypsinization as described above. Half the cells were collected for RNA extraction and the other half used for protein isolation. RNA samples were frozen at –80 °C in RLT plus buffer while protein samples were frozen at –80 °C in RIPA buffer. All RNA or protein samples were thawed at the same time and processed together. Purified RNA was subject to RT-qPCR as described above. Lysates were subject to immunoblotting as described above.

Ubiquitin/Proteasome & Transcription Inhibitor Treatment. One million, two hundred thousand EWS502 FKBP-EWS/FLI1 cells were plated into each well of three 6 well tissue culture plates. The next day, cells were treated with DMSO, 1 μ M MG132, 1 μ M MLN4924, or 1 μ M Actinomycin D for 1 h at 37 °C. After pretreatment, media was aspirated and cells were treated with either DMSO, 1 μ M EB-TCIP, or 1 μ M BI3802 plus and minus each inhibitor for an additional 4 h at 37 °C. Cells were then harvested by trypsinization. Half the cells were collected for RNA extraction and the other half used for protein isolation. RNA samples were frozen at –80 °C in RLT plus buffer while protein samples were frozen at –80 °C in RIPA buffer. All RNA or protein samples were thawed at the same time and processed together. Purified RNA was subject to RT-qPCR as described above. Lysates were subject to immunoblotting as described above.

Chromatin-Immunoprecipitation Sequencing (ChIP-seq). Eleven million EWS502 FKBP-EWSR1::FLI1 cells were plated into 15 cm² dishes. The next day cells were treated with DMSO, 1 μ M BI3812, 1 μ M EB-TCIP, or 1 μ M BI3802 in quadruplicate. After 24 h, media was aspirated, and cells were harvested by trypsinization as described above. Cells from two 15 cm² plates treated with the same condition were pooled and counted. Forty million EWS502 FKBP-EWSR1::FLI1 cells per condition (20 million cells per ChIP reaction) were collected in a 50 mL Falcon tube. Cells were pelleted at 300g for 5 min and then washed twice in 5 mL PBS. Cells were then cross-linked by resuspension in 10 mL PBS containing 1% methanol-free formaldehyde (Thermo Fisher Scientific, 28906) and rotated slowly by hand for 10 min at room temperature. The reaction was quenched by the addition of 1 mL of 2.5 M glycine (Sigma-Aldrich, G7126).

Cells were pelleted at 800g for 5 min at 4 °C pellets and then washed twice with 10 mL PBS at room temperature supplemented with 1 mM PMSF. After resuspending in the second wash, the cell suspension was split into two chilled 50 mL Falcon tubes (5 mL each). After spinning at 800g for 5 min and removing the second PBS wash, cell pellets were flash frozen in liquid nitrogen. When processing samples one set of tubes for all conditions was thawed on ice and a pulldown for either HA or BCL6 was performed as described below. For 4 h experiments cells were processed as described above, 20 million cells were treated with DMSO or 1 μ M EB-TCIP.

For each immunoprecipitation (IP), 100 μ L of protein A Dynabeads (Thermo Fisher Scientific, 10002D) was washed three times in 1 mL BSA blocking solution (0.5% w/v sterile-filtered BSA in UltraPure H₂O) and resuspended in 250 μ L BAS blocking solution. Beads were pooled and then 10 μ g of either anti-HA (Cell Signaling Technologies, 86124SF) or anti-BCL6 antibody (Thermo Fischer Scientific, PA5-27390) per IP was added. Two micrograms of spike-in antibody recognizing a *Drosophila*-specific histone variant was added (Active Motif, 61686) to normalize samples. The following morning, the antibody-conjugated beads were washed four times in 1 mL BSA blocking solution and then resuspended in 100 μ L of the solution per IP and stored at 4 °C.

Frozen, cross-linked cells were thawed briefly on ice and then resuspended in 1 mL of SDS lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM Tris-HCl pH 8.0, 100 mM NaCl, and 0.02% sodium azide) supplemented with Halt protease inhibitor and incubated at room temperature for 2 min with gentle agitation. Lysates were transferred to microfuge tubes and centrifuged at 15,000g for 10 min at 4 °C. The nuclear pellet was resuspended in 950 μ L of ChIP IP buffer (2 parts SDS lysis buffer and 1 part Triton dilution buffer, which was composed of 100 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM EDTA, 0.2% NaN₃ and 5% Triton X-100) supplemented with Halt protease inhibitor. Nine-hundred microliters were then transferred to a milliTUBE (Covaris, 520130). Sonication was performed on an E220 Focus Ultra sonicator (Covaris) at 5% duty cycle, 140 W peak power, 200 cycles per burst, at 4 °C for 25 min per milliTUBE. Sheared chromatin was transferred to a 1.5 mL tube and centrifuged at 15,000g for 10 min at 4 °C. The supernatant of sheared chromatin was transferred to a new reaction tube. To prepare the ChIP DNA input sample, 5 μ L of sheared chromatin was transferred to a PCR strip-tube (USA Scientific, 1402-4700) and mixed with 40 μ L de-cross-linking buffer (100 mM NaHCO₃ and 1% SDS buffer), 1 μ L RNase A (Thermo Fisher Scientific, 12091021) and 1 μ L proteinase K (Thermo Fisher Scientific, AM2546). The tube was incubated for 2 h at 65 °C in a ProFlex PCR thermal cycler (Applied Biosystems) to de-cross-link DNA–protein covalent bonds. DNA was isolated using Agencourt AMPure XP bead-based purification at a 1.2 times ratio (Beckman Coulter, A63881). Briefly, beads were mixed with the sample in the PCR tube and incubated for 10 min at room temperature. Tubes were then placed in a magnetic separation rack (EpiCypher, 10-0008) and washed twice with 500 μ L of 80% ethanol. DNA was then eluted in 50 μ L Tris-EDTA (SigmaAldrich, 93283) and stored at -20 °C. To the remainder of sheared chromatin was added 100 μ L of conjugated bead–antibody solution was. Before addition of the antibody bound beads, 40 ng per reaction of *Drosophila* spike-in chromatin (ActiveMotif, 53083) was added to the pooled antibody bound beads. IP reactions were rotated overnight at 4 °C.

The following day, ChIP reactions were washed twice in 1 mL low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, and 500 mM NaCl), lithium chloride buffer (0.25 M LiCl, 1% IGEPAL-CH 630, 1% sodium deoxycholate, 10 mM Tris-HCl pH 8.0, and 1 mM EDTA) and then once in 700 μ L ice-cold Tris-EDTA buffer (Sigma-Aldrich, 93283). Chromatin was eluted using 100 μ L fresh ChIP elution buffer (1% SDS and 0.1 M NaHCO₃) and rotated at room temperature for 15 min. Eluate was transferred to PCR tubes and mixed with 8 μ L 2.5 M NaCl, 1 μ L RNase A and 1 μ L proteinase K. Samples were de-cross-linked for 12–16 h at 65 °C in a thermal cycler. ChIP DNA was extracted from the de-cross-linked samples

using AMPure XP beads at a 1.2 $\times$ ratio as described above and eluted in 20 μ L of Tris-EDTA. DNA was quantified using a Qubit dsDNA high sensitivity assay (Q32851). DNA fragment sizes were measured with a TapeStation 2200 instrument (Agilent, ScreenTape, 5067-5584; reagents, 5067-5585).

ChIP-seq libraries were prepared using a NEBNext Ultra II DNA Library Kit for Illumina sequencing (NEB, E7645S) and NEBNext Multiplex Oligos for Illumina sequencing (NEB, E6440S). HA and BCL6 samples were PCR-amplified for 12 cycles. Library pooling and indexing was evaluated with shallow sequencing on an Illumina MiSeq. Subsequently, libraries were sequenced on an Illumina NovaSeq X Plus targeting roughly 40 million, 150bp read pairs per sample by the Molecular Biology Core Facilities at Dana-Farber Cancer Institute.

Assay for Transposase-Accessible Chromatin with Sequencing (ATAC-seq). Three hundred thousand EWS502 FKBP-EWSR1::FLI1 cells were plated into each well of a 12-well tissue culture plate. The next day cells were treated with DMSO or 1 μ M EB-TCIP in duplicate. After 24 h, cells were harvested by trypsinization as described above and counted. Next, 100,000 cells from each sample were used to prepare libraries for ATAC-seq using a commercially available kit (ActiveMotif, 53150). The molarity of each library was calculated using a Qubit dsDNA Broad Range Assay kit (Thermo Fisher Scientific, Q32850) and an Agilent TapeStation 2200. Library pooling and indexing was evaluated with shallow sequencing on an Illumina MiSeq. Subsequently, libraries were sequenced on an Illumina NovaSeq X Plus targeting roughly 20 million, 150bp read pairs per sample by the Molecular Biology Core Facilities at Dana-Farber Cancer Institute. For 4 h ATAC-seq experiments samples were processed as described above except 600,000 cells were plated into each well of a 12-well tissue culture dish and treated the next day with DMSO, 1 μ M EB-TCIP or 1 μ M NEG-2.

Cell Viability. Fifty microliters of a 10,000 cell/mL suspension of EWS502 or EWS502 FKBP-EWSR1::FLI1 were seeded into each well of a white polystyrene 384 well cell culture plates (Corning, 3570). The next day cells were treated with DMSO, OAP, BI3812, EB-TCIP, NEG-1, or NEG-2 using a HP D300e Digital Dispenser. Cells were treated with 8-point dose responses starting at 10 μ M with 1:2 dilutions. Treated cells were incubated for 72 h at 37 °C, after which 10 μ L of Cell-Titer-Glo (Promega, G7573) was added to each well using a 2–125 μ L E1-ClipTip electronic pipet (Thermo Scientific). The plate was then incubated at room temperature for 15 min with 350 rpm rotation in an Eppendorf MixMate. Luminescence was determined using a CLARIOStar Plus plate reader (BMG Labtech). The ratio of the luminescence of compound treated samples to DMSO treated samples was calculated in Microsoft Excel. Dose response curves were then generated by fitting the data to an [inhibitor] vs dose response nonlinear regression using GraphPad Prism 10.

RNA-seq Data Analysis. RNA-seq data analysis was performed according to the ENCODE standards (<https://www.encodeproject.org/data-standards/rna-seq/long-rnas/>). Quality check of unaligned reads was performed using FastQC v.0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC v.1.14⁵⁷ respectively. Using STAR v.2.7.11a⁵⁸ the paired end reads were aligned to hg38/genomev30 with standard parameters—outSAMtype BAM SortedByCoordinate --outSAMunmapped None--outSAMattributes NH HI NM MD AS XS --outReadsUnmapped FastX --outSAMstrandField intronMotif --quantMode TranscriptomeSAM GeneCounts --quantTranscriptomeBan IndelSoftclipSingleend --readFilesCommand zcat. Gene level reads were counted and summarized across hg38 exons by using featureCounts v.2.0.3 from the Subread v.2.0.0 package (<https://subread.sourceforge.net/>). Following alignment, quality control checks were performed using SARTools v.1.7.3⁵⁹ with DESeq2 v.1.44.0. Gene level expression was estimated as log₂(TPM + 1) normalized reads. Gene counts were normalized and used to quantify the differential gene expression between the experimental and control conditions using eBayes limma package v3.60.3 (80). Gene level differential expression was assessed based on log₂ fold change expression between conditions and

adjusted P -value. The cutoffs for gene differentiation were $|\text{fold change expression}| \geq 1.5$ and adjusted $P \leq 0.05$. Heatmaps displaying transcriptional changes were created using the Morpheus software platform (<https://software.broadinstitute.org/morpheus/>) based on $\log_2(\text{fold change})$ expression data.

Gene Set Enrichment Analysis. GSEA software v.4.2.2²⁴ was used to identify signature enrichment of experimental conditions in *BCL6* KO, compound treatment, and corresponding conditions. MSigDB v7.4 collections, a published *BCL6* target gene set,²⁵ and an in-house curated ES *BCL6* KO gene set were analyzed for enrichment against the data. A cut off of $\text{LFC} \geq 1.65$ was used to define the *BCL6* KO gene set in all GSEA. For each experimental condition, the expressed genes were ranked based on the expression fold change in sgBCL6 vs sgChr2.2 or compound treated versus DMSO control. Results were visualized with volcano plots with Normalized Enrichment Score (NES) versus $-\log_{10}(P)$ and GSEA plots. Significance cutoffs for GSEA enrichments: $|\text{NES}| \geq 1.3$, $P \leq 0.05$, $\text{FDR} \leq 0.05$ (although the GSEA authors recommend the FDR cutoff ≤ 0.25).

ChIP-Seq Data Analysis. The analysis of the spiked-in ChIP-Seq data was performed according to the ENCODE standards (<https://www.encodeproject.org/chip-seq/>). Quality control was performed on unmapped sequences using FastQC v.0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC v.1.14.⁵⁷ Adapters and low-quality reads were removed using Trimmomatic v.0.39.⁶⁰ Reads were mapped to the human genome (GRCh38/hg38) and to the spike-in *Drosophila melanogaster* (dm6) using bowtie2 v.2.5.1⁶¹ with the "local very_sensitive" parameters. Mapped reads were processed with SAMtools v.0.1.19⁶² and reads with low mapping quality ($\text{MAPQ} < 5$) were disregarded. Duplicate reads were removed using the Picard Mark Duplicates method implemented in the sambamba 0.7.1 tool.⁶³ Fragment size distributions were computed using the PEFragmentSize tool available in the deepTools v.3.5.1 package.⁶⁴

Human and *Drosophila* genome-wide counts across 2000 bp bins were computed with the bamSummary tool available in the deepTools package v.3.5.0.⁶⁴ Bins with at least 10 reads in less than 3 samples and bins overlapping ENCODE blacklisted regions were excluded. The Active Motif Spike-in Normalization protocol was then applied to compute the scaling factors per antibody samples as ratios between the average dm6 counts across antibody samples vs the dm6 counts for that sample. The normalization factor was set to 1 if the percentage of *Drosophila* reads was less than the 1% minimum cutoff.

The bamCoverage tool from the deepTools package v.3.5.0⁶⁴ was used to generate normalized reads per kilobase per million (RPKM) genome-wide coverage bigwig files with specified bin sizes of 20 bp and scaled with the precomputed spike-in scale factors.

Peak calling was performed using the model-based MACS2 v.2.1.1.20160309⁶⁵ software against experimental inputs with a significance cutoff $\text{FDR} \leq 0.01$. Bwtool software⁶⁶ was used to compute the area under the curve (AUC) for the RPKM normalized signal across genomic regions. MACS2 peaks were filtered by removing binding regions with low AUC coverage of $[\log_2(\text{AUC} + 1) < 14]$ and ENCODE hg38 black-list regions (<https://www.encodeproject.org/annotations/ENCSCR636HFF/>). Various mapping and genomic analyses including indexing, sorting, intersection, and merging were executed using SAMtools v.1.9 and Bedtools v.2.29.^{67,68} Next, quality control for peaks called was performed using ChIPQC⁶⁹ under the Bioconductor package v.3.9. Homer v.4.11⁷⁰ platform was used to annotate peaks called with the closest hg38 genes using the annotatePeaks function. Peak binding signals were visualized using the Integrative Genomic Viewer (IGV) v.2.12.3.⁷¹ Promoter regions were defined as the area of the genome ± 5.0 kb from gene transcription start sites (TSS).

Antibody binding sites identified by MACS2 were merged into a set of aggregated peaks for control and treatment across conditions. Utilizing deepTools multiBamSummary tool, peak by sample counts was generated. Counts were used to perform differential peak analysis. Changes between two conditions binding signal were identified as increase, decrease or not significant based on absolute cutoff of 1.5 for

delta area under curve. Significance of changes for binding reads was calculated by using DESeq2 v.1.44.0 with a cutoff of $P \leq 0.05$.

Heatmaps of normalized AUC signal were created using deepTools v.3.5.1 computeMatrix and plotHeatmap functions. Metaplots displaying average normalized scores across genomic regions were created using deepTools v.3.5.1 plotProfile function. Motif enrichment analysis was performed using Homer v.4.11.⁷⁰

ATAC-Seq Data Analysis. ATAC-Seq analysis was performed according to the ENCODE standards (<https://www.encodeproject.org/atac-seq/>). Quality control was performed on the unmapped paired end reads with FastQC v.0.11.9 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and MultiQC v.1.14.⁵⁷ Adapters were then trimmed and filtered using Trimmomatic v.0.36.⁶⁰ Using bowtie2 v.2.5.1, the trimmed paired end reads were aligned to the hg38 genome with the -local -very_sensitive -X 2000 parameters. Reads mapped to the hg38 genome to chromosomes 1 to 22 with a $\text{MAPQ} > 5$ were kept. Duplicates were removed using Picard Mark Duplicates method implemented in the sambamba 0.7.1 tool.⁶³ deepTools v.3.5.1⁶⁴ AlignmentSieve tool was used to shift reads 4bp on the positive strand and -5bp on the negative strand. Replicate correlations were calculated and visualized using multiBamsummary and bamCorrelate, along with fragment size distributions using PEFragmentSize within the deepTools v.3.5.1 package.⁶⁴ Replicates were merged and then peak calling was performed with MACS2 v.2.1.1.20160309.⁶⁵ Next, AUC binding signal was computed with the bwtool program.⁶⁶ The Homer v.4.11⁷⁰ program was employed to annotate called peaks to the closest hg38 genes using the annotatePeaks function. Promoter regions were defined as the area of the genome ± 3.0 kb from gene transcription start sites (TSS). Peak binding signal was visualized using the Integrative Genomic Viewer (IGV) v.2.12.3.⁷¹

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.Sc05634>.

Supporting Figures 1–11, Chemical Synthesis, Supporting Tables 1–11, RNA-seq dendrograms, and additional references (PDF)

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Notes

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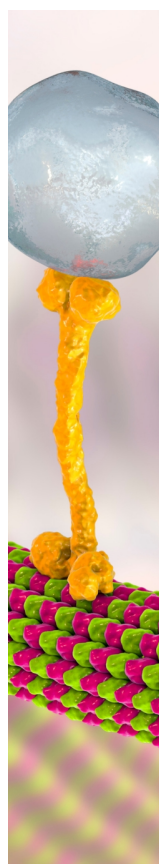
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