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Supplemental information

TRIM8 modulates the EWS/FLI oncoprotein to promote survival in Ewing sarcoma

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Figure S1, Related to Figure 1

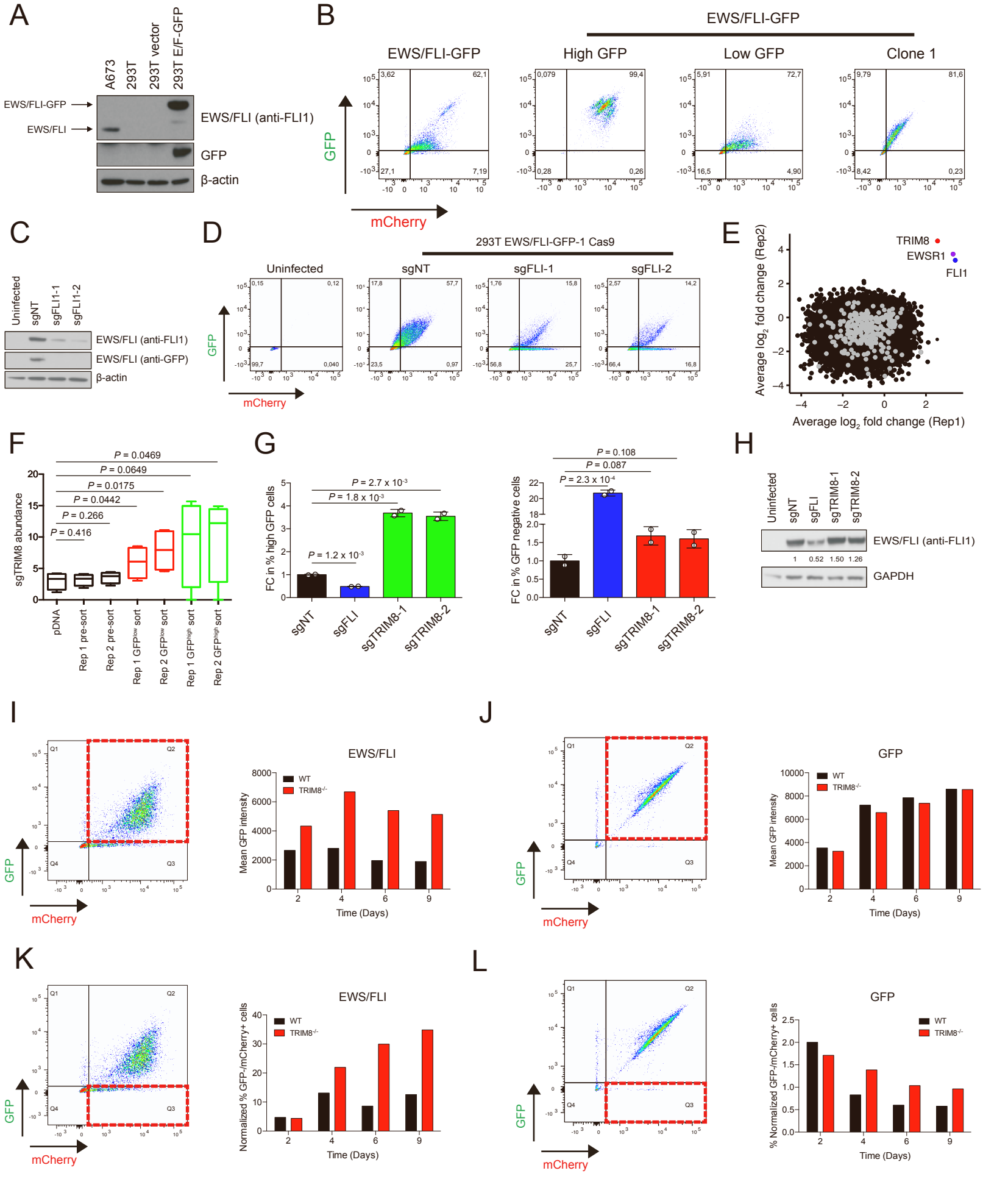

Figure S1. Generation and validation of the EWS/FLI-GFP reporter cell line and the flow-based CRISPR screen, related to Figure 1. (A) Immunoblot showing the expression of EWS/FLI-GFP in the reporter cell line. (B) Flow cytometry plot showing GFP expression of indicated subpopulations of the 293T EWS/FLI-GFP reporter cells. (C,D) 293T EWS/FLI-GFP cells were infected with non-targeting and *FLI1*-directed sgRNAs and underwent no selection. Cells were lysed and immunoblotted with indicated antibodies (C) and assessed for GFP and mCherry expression using flow cytometry (D). Data representative of three independent experiments. (E) Scatterplot showing average log2 fold changes in sgRNA expression in replicates in GFP^{low} sorted population. Negative control guides are highlighted in gray. sgRNAs targeting *EWSR1*, *FLI1*, and *TRIM8* are highlighted in purple, blue, and red, respectively. Each dot represents an average of log2 fold changes for 4 independent sgRNAs per gene. (F) Abundance of *TRIM8*-targeting sgRNAs in EWS/FLI protein stability CRISPR screen. Box plot showing the abundance of sgTRIM8 in different populations. Mean abundance of 4 independent sgRNA targeting TRIM8 ± S.D. are shown. Statistical significance using Student's t test is shown. (G,H) Low-throughput validation of TRIM8. GFP and mCherry expression in 293T EWS/FLI-GFP reporter cells infected with indicated sgRNAs were assessed by flow cytometry after 7 days. Fold change (FC) in %high GFP (left) and %GFP negative (right) cells were quantified (G). FC of 2 biological replicates ± S.D. are shown. Statistical significance using Student's t test is shown. EWS/FLI protein expression was assessed by immunoblot and quantified by densitometry (H). Data representative of two independent experiments. (I-L) 293T wild-type (WT) or *TRIM8* knockout ($TRIM8^{-/-}$) cells were infected with either EWS/FLI-GFP-IRES-mCherry (I,K) or control GFP-IRES-mCherry (J,L) and were assessed for GFP levels by flow-cytometry at indicated time-points. $GFP^{+}mCherry^{+}$ subpopulation are quantified and plotted in (I,J). Quantification of the normalized proportion of $GFP^{-}mCherry^{+}$ subpopulations is shown in (K,L). The proportion of GFP-mCherry+ subpopulation was normalized to the total population

excluding the GFP$^-$mCherry$^-$ cells to exclude uninfected cells for quantification. Cell populations used for the quantification are highlighted in red. Flow plots represent 293T WT cells expressing EWS/FLI-GFP-IRES-mCherry Day 4 after infection. Data representative of two independent experiments.

Figure S2, Related to Figure 2

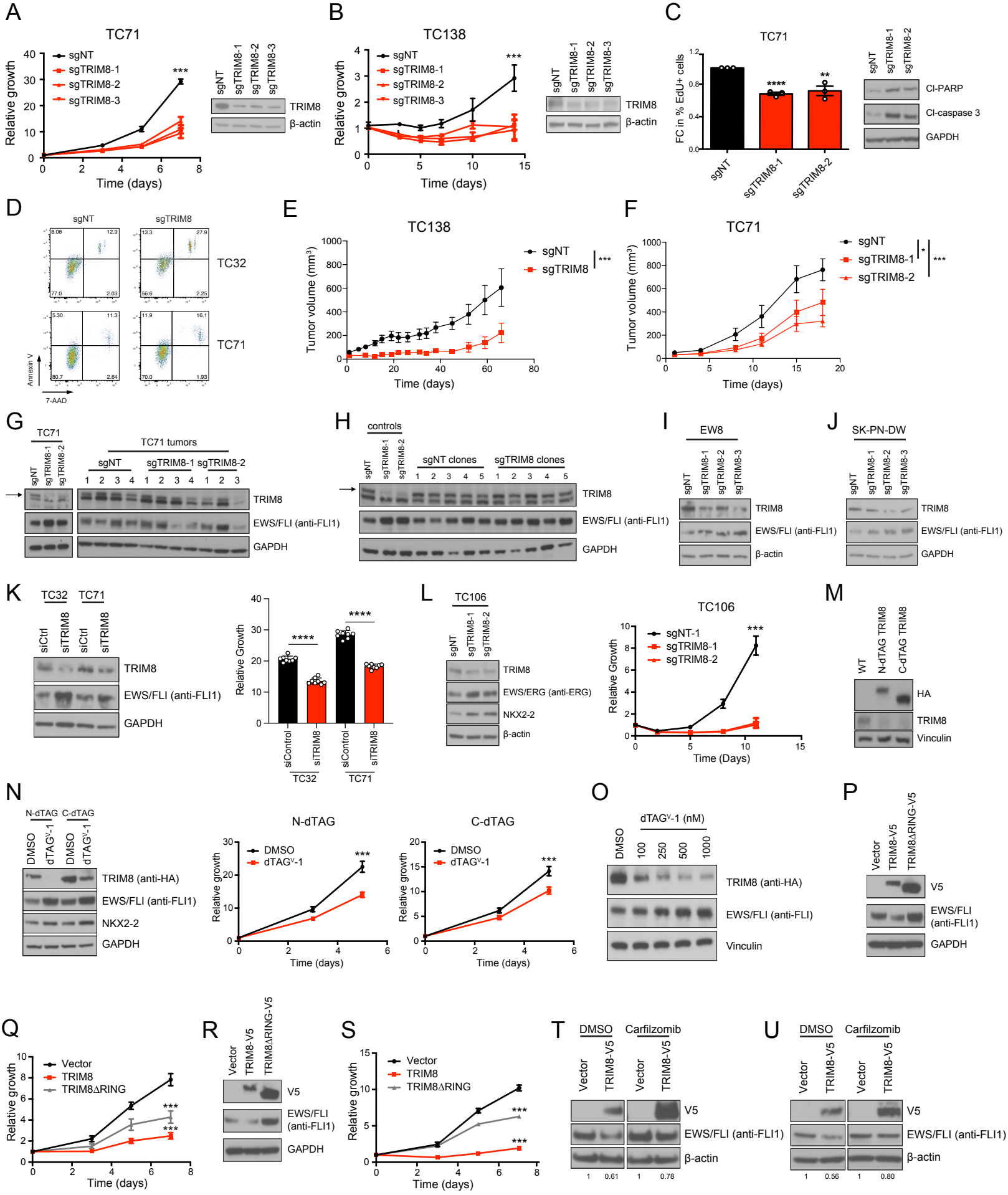

Figure S2. TRIM8 modulates EWS/ETS fusion oncoprotein expression and regulates Ewing sarcoma cell growth *in vitro* and *in vivo*, related to Figure 2. (A,B) TC71 (A), and TC138 (B) cells infected with non-targeting (NT) and *TRIM8*-directed sgRNAs were assessed for cell growth using CellTiter-Glo. Knockout was confirmed by immunoblot. Mean of 8 technical replicates ± S.D. of relative growth are shown. Statistical significance calculated using unpaired two-tailed student's t test. ***p<0.001. Data representative of three independent experiments. (C) Proliferation and induction of apoptosis in TC71 cells infected with non-targeting and *TRIM8*-directed sgRNAs were assessed by EdU incorporation (FC= fold change) and cleaved (Cl)-PARP and cleaved (Cl)-caspase 3 levels, respectively. Mean FC of 3 biological replicates ± S.E.M are shown. Statistical significance calculated using unpaired two-tailed student's t test. **p<0.01, ****p<0.0001. (D) Flow cytometry plot showing Annexin V staining in TC32 and TC71 cells infected with either non-targeting or sgRNA targeting *TRIM8*. (E) TC138 cells infected with non-targeting (NT) and two different *TRIM8*-directed sgRNAs were subcutaneously injected into nude mice. Mice from sgTRIM8-1 and sgTRIM8-2 groups were combined due to low engraftment rate. Mice for sgNT (n=10) and sgTRIM8 (n=10). Mean tumor volume ± S.E.M. are shown. Statistical significance calculated using unpaired two-tailed student's t test. ***p<0.001. (F) TC71 cells infected with non-targeting (NT) and two different *TRIM8*-directed sgRNAs were subcutaneously injected into NSG mice. Mice for sgNT (n=7), sgTRIM8-1 (n=8), and sgTRIM8-2 (n=8). Mean tumor volume ± S.E.M. are shown. Statistical significance calculated using unpaired two-tailed student's t test. *p<0.05, ***<0.001. (G) Tumors from TC71 *TRIM8* knockout experiment were harvested, lysed, and immunoblotted for TRIM8 and EWS/FLI. Cells injected into mice were also cultured *in vitro* in parallel and were harvested, lysed, and immunoblotted for TRIM8 and EWS/FLI as controls. (H) TC71 cells were infected with either non-targeting or *TRIM8*-targeting sgRNA and were plated at a limiting dilution density in 96-well plates for single-cell cloning. Selected clones from each group were grown and were immunoblotted for EWS/FLI

and TRIM8. (I,J) EW8 (I) and SK-PN-DW (J) cells infected with non-targeting and *TRIM8*-directed sgRNAs were harvested, lysed, and immunoblotted with indicated antibodies. Data representative of two independent experiments. (K) TC32 and TC71 cells were transfected with control (siCtrl) and TRIM8 (siTRIM8) siRNAs and immunoblotted with indicated antibodies (left) and assessed for cell growth (right). Mean of 8 technical replicates ± S.D. of relative growth are shown. Statistical significance calculated using unpaired two-tailed student's t test. ****p<0.0001. (L) TC106 cells infected with non-targeting and *TRIM8*-directed sgRNAs were harvested, lysed, and immunoblotted with indicated antibodies (left) and assessed for cell growth (right). Mean of 8 technical replicates ± S.D. of relative growth are shown. Statistical significance calculated using unpaired two-tailed student's t test. ***p<0.001. Data representative of two independent experiments. (M) Immunoblot showing expression of $FKBP12^{F36V}$-tagged TRIM8 (anti-HA) and endogenous TRIM8 (anti-TRIM8) in TC71 $FKBP12^{F36V}$-TRIM8 dTAG cells. (N) TC71 N-dTAG and C-dTAG TRIM8 cells were treated with $dTAG^{V}$-1 at 1 μM for 48 hours and immunoblotted with the indicated antibodies (left) and assessed for cell growth (middle, right). Mean of 8 technical replicates ± S.D. of relative growth are shown. Statistical significance calculated using unpaired two-tailed student's t test. ****p<0.0001. Data representative of three independent experiments. (O) TC32 C-dTAG TRIM8 cells were treated with indicated concentrations of $dTAG^{V}$-1 for 48 hours, harvested, lysed, and immunoblotted with indicated antibodies. (P-S) SK-PN-DW (P,Q) and EWS502 (R,S) cells infected with lentivirus encoding *TRIM8* and TRIM8ΔRING were immunoblotted with the indicated antibodies (P,R) and assessed for cell growth using CellTiter-Glo assay (Q,S). Mean of 8 technical replicates ± S.D. of relative growth are shown for (Q) and (S). Statistical significance calculated using unpaired two-tailed student's t test. ***p<0.001. Data representative of three independent experiments. (T,U) TC71 (T) and TC32 (U) cells infected with lentivirus encoding vector control or TRIM8 were treated with DMSO or carfilzomib at 500 nM for 6 hours. Cells were then harvested and immunoblotted with indicated antibodies. Data

representative of three independent experiments. Statistical significance calculated using unpaired two-tailed student's t test *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

Figure S3, Related to Figure 3

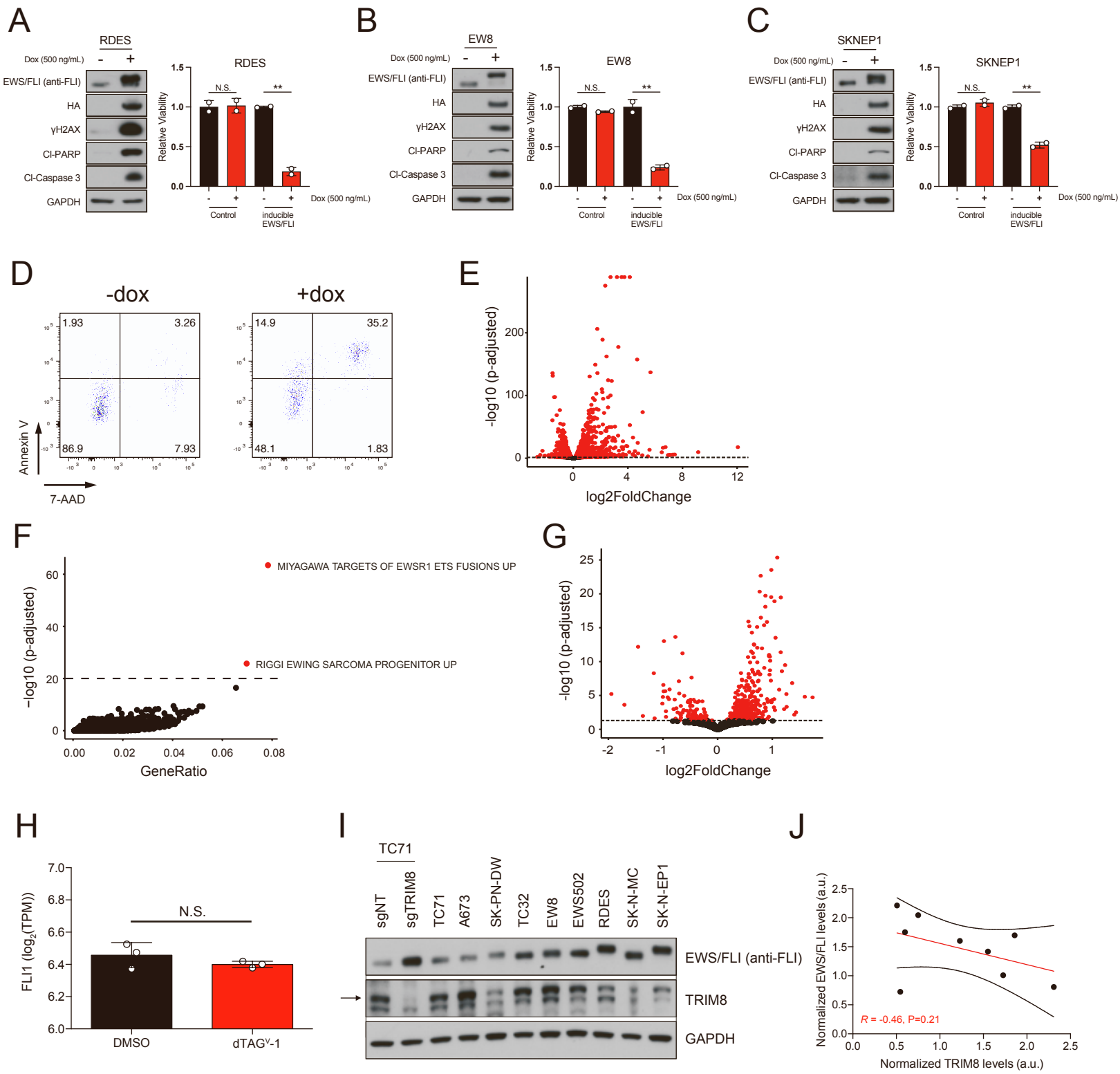

Figure S3. Increased level of EWS/FLI is lethal to Ewing sarcoma cells, related to Figure 3. (A-C) RDES, EW8, and SKNEP1 cells expressing dox-inducible EWS/FLI-HA were treated with doxycycline (500 ng/mL) for 24 (A) or 48 hours (B,C). Cells were immunoblotted with indicated antibodies (left panel) and assessed for viability using manual cell counting (right panel). Mean of 2 technical replicates ± S.D. of relative viability are shown. Statistical significance calculated using unpaired two-tailed student's t test **p<0.01. (D) Representative flow cytometry plot showing Annexin V and 7-AAD staining of RDES inducible EWS/FLI cells after dox-induction shown in Figure 3C. (E,F) A volcano plot showing differentially regulated (p<0.05) genes (E) and a scatter plot of the GSEA performed with significantly up-regulated genes (p<0.05) after EWS/FLI over-expression (8h) (F). (G) A volcano plot showing differentially regulated (p<0.05) genes after TRIM8 degradation (24h). (H) EWS/FLI transcript levels in RNA-seq from TC71 C-dTAG TRIM8 cells treated with 1 μM $dTAG^V$-1 molecule for 24 hours. TC71 cells do not express wild-type FLI1, thus, FLI1 transcript was used to quantify the EWS/FLI transcript levels. Mean TPM of 3 biological replicates ± S.D. are shown. Statistical significance calculated using unpaired two-tailed student's t test. ****p<0.0001. (I) EWS/FLI and TRIM8 protein expression was assessed in Ewing sarcoma cell line panel using immunoblotting. (J) A scatter plot showing the correlation between EWS/FLI and TRIM8 protein expression levels. Linear regression shown in red line and 95% confidence intervals shown in black lines. EWS/FLI and TRIM8 protein levels were quantified by using ImageJ and normalized to GAPDH loading control.

Figure S4, Related Figure 4

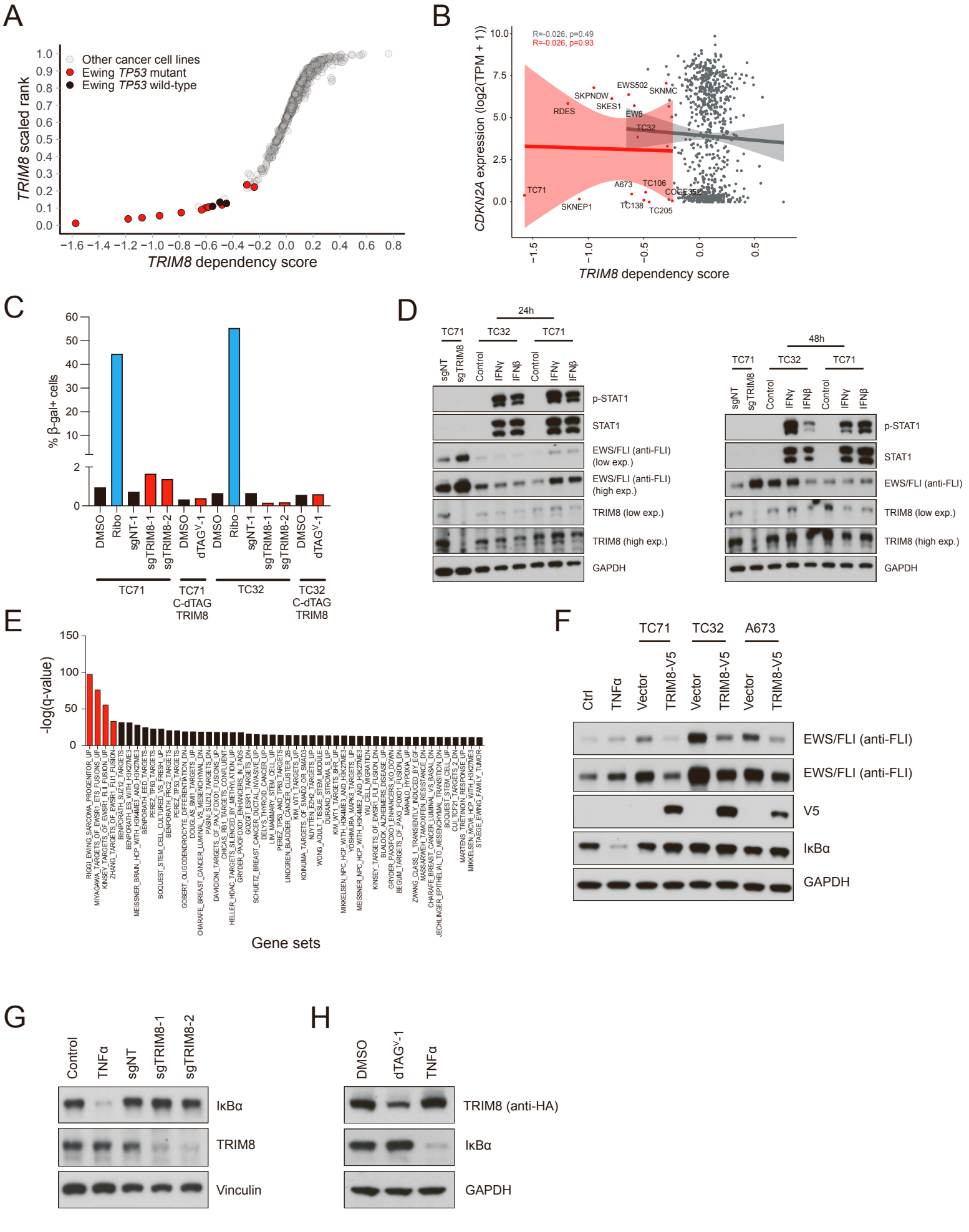

Figure S4. TRIM8 dependency is not associated with *TP53* mutational status, *CDKN2A* loss, or senescence phenotype, related to Figure 4. (A) Scatter plot showing relative TRIM8 dependency between *TP53* mutant vs wild-type. X-axis shows the gene's dependency score in each cell line. Y-axis shows the gene's dependency rank in an individual cell line. (B) Scatter plot showing the correlation of *CDKN2A* (p19 ARF) expression with *TRIM8* dependency in Ewing sarcoma cell lines (red) and all other cancer cell lines (gray). X-axis shows the gene's dependency score in each cell line. Y-axis shows the *CDKN2A* expression in an individual cell line. (C) TC71 and TC32 wild-type cells infected with either non-targeting or sgRNA targeting *TRIM8* for 7 days and TC71 and TC32 C-dTAG TRIM8 cells treated with 1 μM $dTAG^V$-1 for 5 days were used to measure β-Galactosidase activity to assess senescence. Ribociclib, a CDK4/6 inhibitor previously shown to induce senescence in Ewing sarcoma, was used as a control. (D) Ewing sarcoma cells were treated with IFNγ (100 ng/mL) or IFNβ (100 ng/mL) for either 24 (left) or 48 hours (right), harvested and immunoblotted with indicated antibodies. Activation of STAT1 signaling was used to assess the downstream pathway activation of IFNs. (E) Bar plot showing GSEA on significantly differentially expressed genes (FDR q-value <0.05) with TRIM8 degradation (24h). EWS/FLI UP signatures are highlighted in red. (F) Assessment of *TRIM8* over-expression on NFκB pathway activation in Ewing sarcoma cell lines. Ewing sarcoma cells were infected with TRIM8-V5 construct, harvested, and immunoblotted with indicated antibodies. TC71 cells treated with TNFα (100 ng/mL) for 24 hours were used as a control, and the degradation of IκBα was used to assess the activation of the NFκB pathway. (G) TC32 cells infected with either *TRIM8*-targeting or non-targeting sgRNA were harvested, lysed, and immunoblotted with indicated antibodies. Treatment with TNFα (100 ng/mL) for 24 hours was used as a control. (H) TC32 C-dTAG TRIM8 cells were treated with either dTAGV-1 (1 μM) or TNFα (100 ng/mL) for 24 hours, harvested, lysed, and immunoblotted with indicated antibodies.

Figure S5, Related to Figure 5

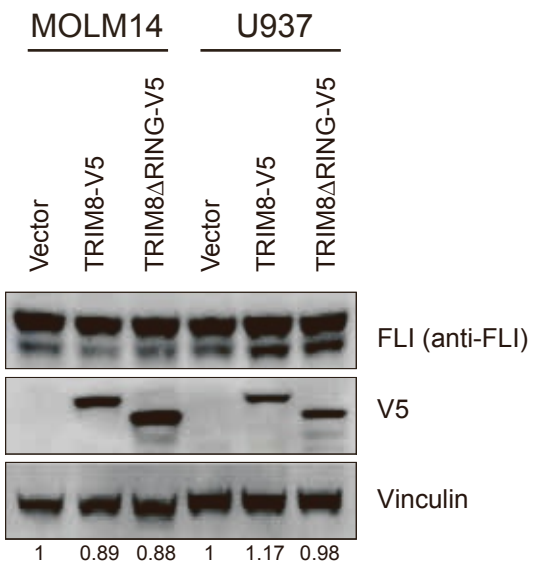

Figure S5. TRIM8 does not regulate wild-type FLI in AML cells., related to Figure 5. The AML cell lines MOLM14 and U937 were infected with lentivirus encoding wild-type *TRIM8* and an E3 ligase domain deletion mutant (TRIM8ΔRING). Lysates were harvested and immunoblotted with the indicated antibodies. AML cell lines were chosen because they express wild-type FLI1 while Ewing sarcoma cells generally do not typically express wild-type *FLI1*. Data representative of two independent experiments.

Figure S6, Related to Figure 6

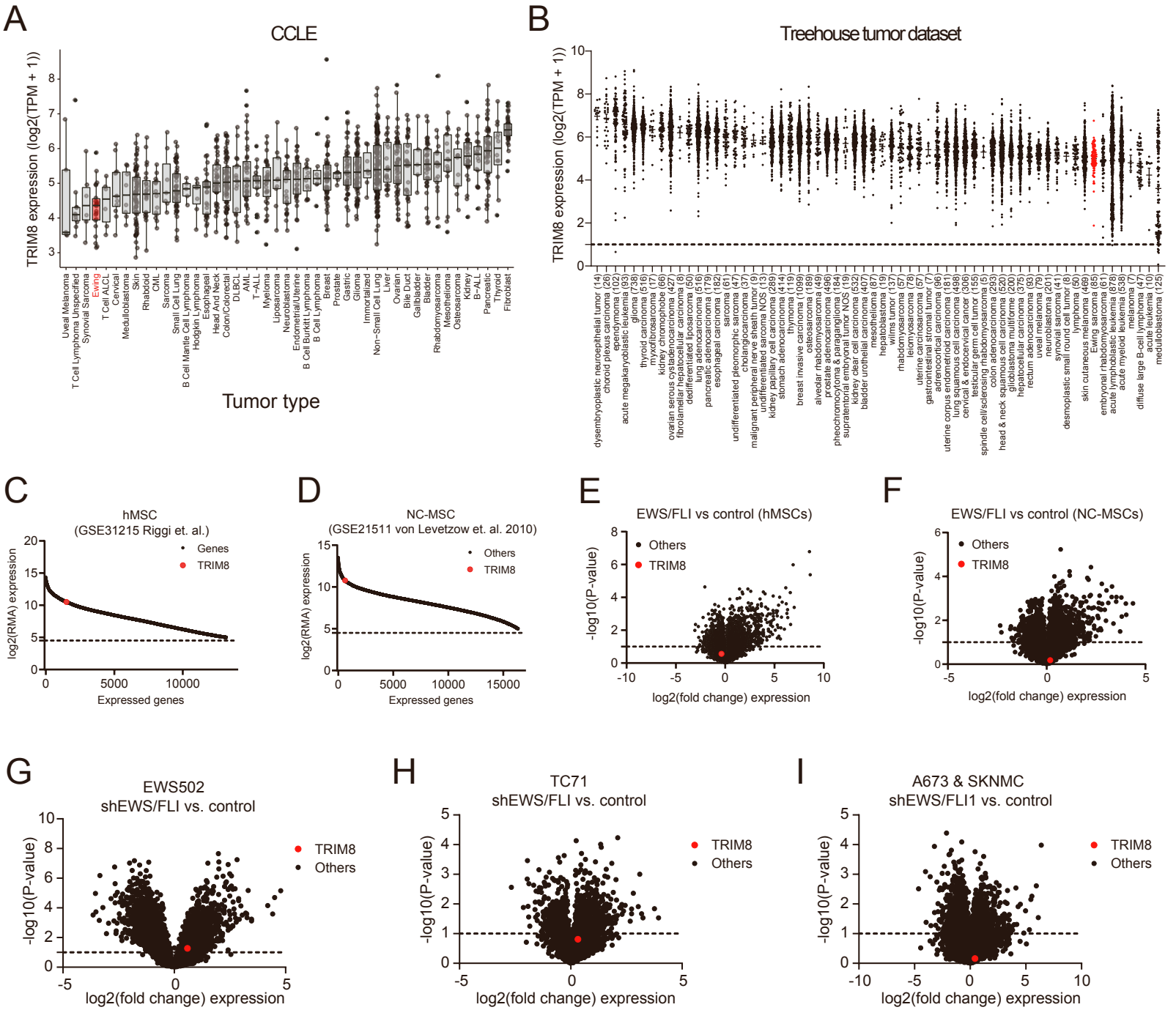

Figure S6. *TRIM8* expression across cancer cell lines, tumors, and mesenchymal stem cells, related to Figure 6. (A) Box plot showing *TRIM8* RNA expression across cancer cell lines from different tumor type in the CCLE. (B) TRIM8 log2(TPM+1) expression across the ~12,000 tumor samples (85 Ewing sarcoma) in the Treehouse v11 RNA-Seq data. Mean+S.E.M. dotplots per tumor lineage with expression cut-off of log2(TPM+1)>1, one-sample t-test per lineage, **** P < 0.0001. (C, D) Hockey stick plots depicting the genome-wide avg log2(RMA) expression in hMSC control cells, Riggi et al. GSE31215 (C) and neural crest (NC)-MSC control cells, Levetzow et al. GSE21511 (D). *TRIM8* is highlighted in red and log2(RMA)>4.5 was used as a cut-off for expression status. (E) Volcano plot depicting the transcriptional changes between hMSC with induced EWS/FLI1 vs. hMSC control cells, Riggi et al., 2010 Affymetrix arrays data GSE31215. TRIM8 is highlighted in red. Significance cutoffs: limma eBayes |log2(fold change)| ≥ 1.5, P-value ≤ 0.10. ns = not significant. (F) Volcano plot depicting the transcriptional changes between NC-MSC with induced EWS/FLI1 vs. NC-MSC control cells, Levetzow et al., 2011 Affymetrix arrays data GSE21511. TRIM8 is highlighted in red. Significance cutoffs: limma eBayes |log2(fold change)| ≥ 1.5, P-value ≤ 0.10. ns = not significant. (G-I) EWS/FLI knockdown does not change *TRIM8* gene expression. Volcano plot depicting the transcriptional changes induced by EWS/FLI1 knock-down in EWS502 (G) and TC71 (H) in Kinsey et al. 2006 and A673 & SKNMC cells (I) in Riggi et al. 2014. TRIM8 is highlighted in red. Significance cutoffs: limma eBayes |log2(fold change)| ≥ 1.5, P-value ≤ 0.10. ns = not significant.

Figure S7, Related to Figure 7

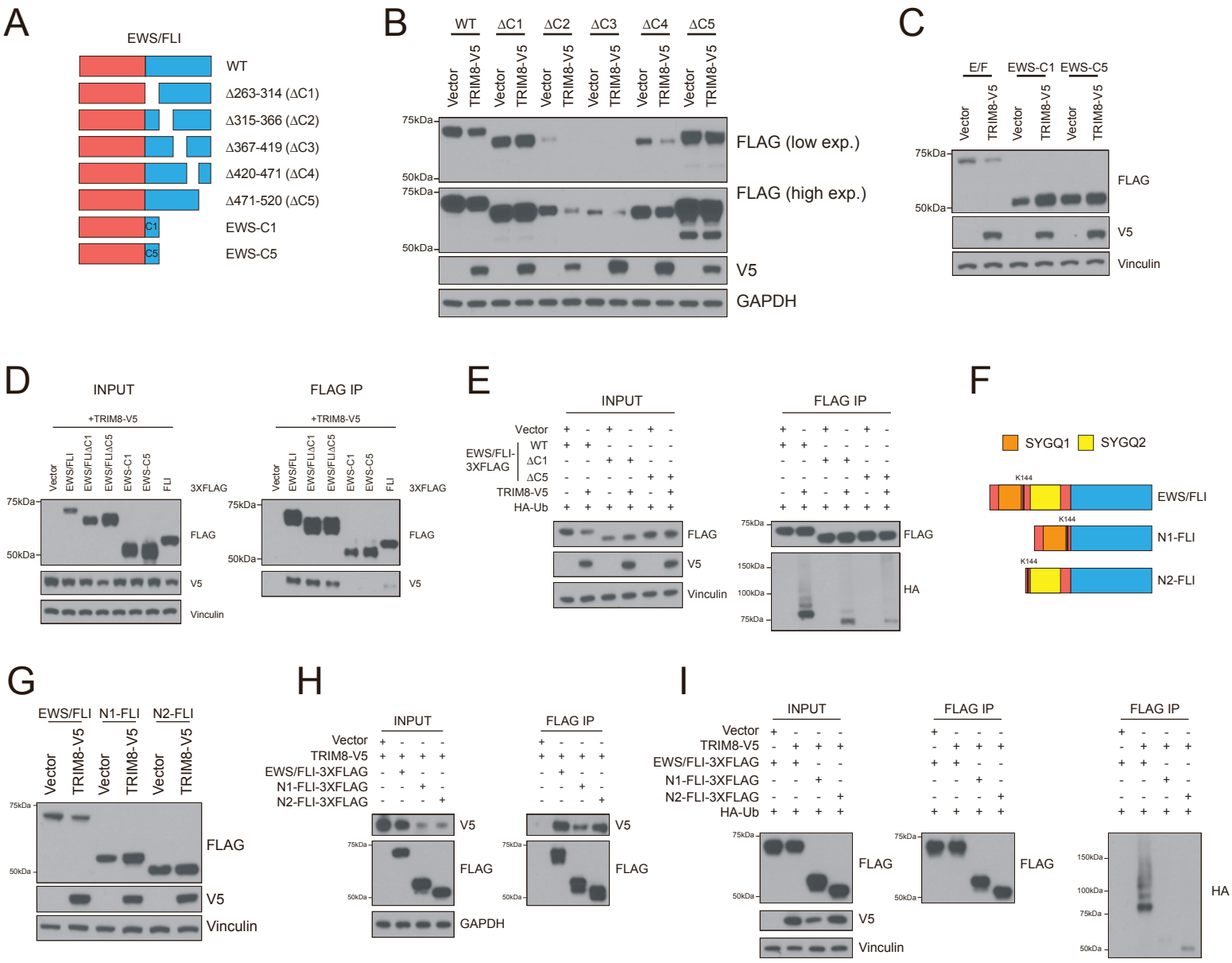

Figure S7. C1, C5, and SYGQ prion-like domains of EWS/FLI are critical for TRIM8-mediated ubiquitination and degradation, related to Figure 7. (A) Schematics of the EWS/FLI mutants. (B) EWS/FLI WT and ΔC1-5 mutants tagged with 3XFLAG were co-transfected with either TRIM8 or a vector control in 293T cells. After 48 hours, cells were harvested, lysed and immunoblotted with the indicated antibodies. (C) EWS/FLI, EWS-C1 and, EWS-C5 mutants tagged with 3XFLAG were co-transfected with either TRIM8 or a vector control in 293T cells. After 48 hours, cells were harvested, lysed and immunoblotted with the indicated antibodies. (D) 293T cells were transfected with indicated constructs for 48 hours, lysed, immunoprecipitated, and immunoblotted with indicated antibodies. (E) 293T cells were transfected with indicated constructs. After 48 hours, cells were treated carfilzomib at 500 nM for 6 hours and harvested. Cells were then lysed with ubiquitination lysis buffer, immunoprecipitated, and immunoblotted with indicated antibodies. All data representative of three independent experiments. (F) Schematics of N1-FLI and N2-FLI mutants. (G) EWS/FLI, N1-FLI, and N2-FLI mutants tagged with 3XFLAG were co-transfected with either TRIM8 or a vector control in 293T cells. After 48 hours, cells were harvested, lysed and immunoblotted with the indicated antibodies. Data representative of two independent experiments. (H) TRIM8 was co-transfected with either EWS/FLI, N1-FLI, N2-FLI, or a vector control in 293T cells. After 48 hours, cells were treated with carfilzomib at 500 nM for 6 hours. Cells were then harvested, lysed, immunoprecipitated, and immunoblotted with the indicated antibodies. Data representative of two independent experiments. (I) 293T cells were transfected with indicated constructs. After 48 hours, cells were treated carfilzomib at 500 nM for 6 hours and harvested. Cells were then lysed with ubiquitination lysis buffer, immunoprecipitated, and immunoblotted with indicated antibodies. Data representative of two independent experiments.

Figure S8, Related to Figure 8

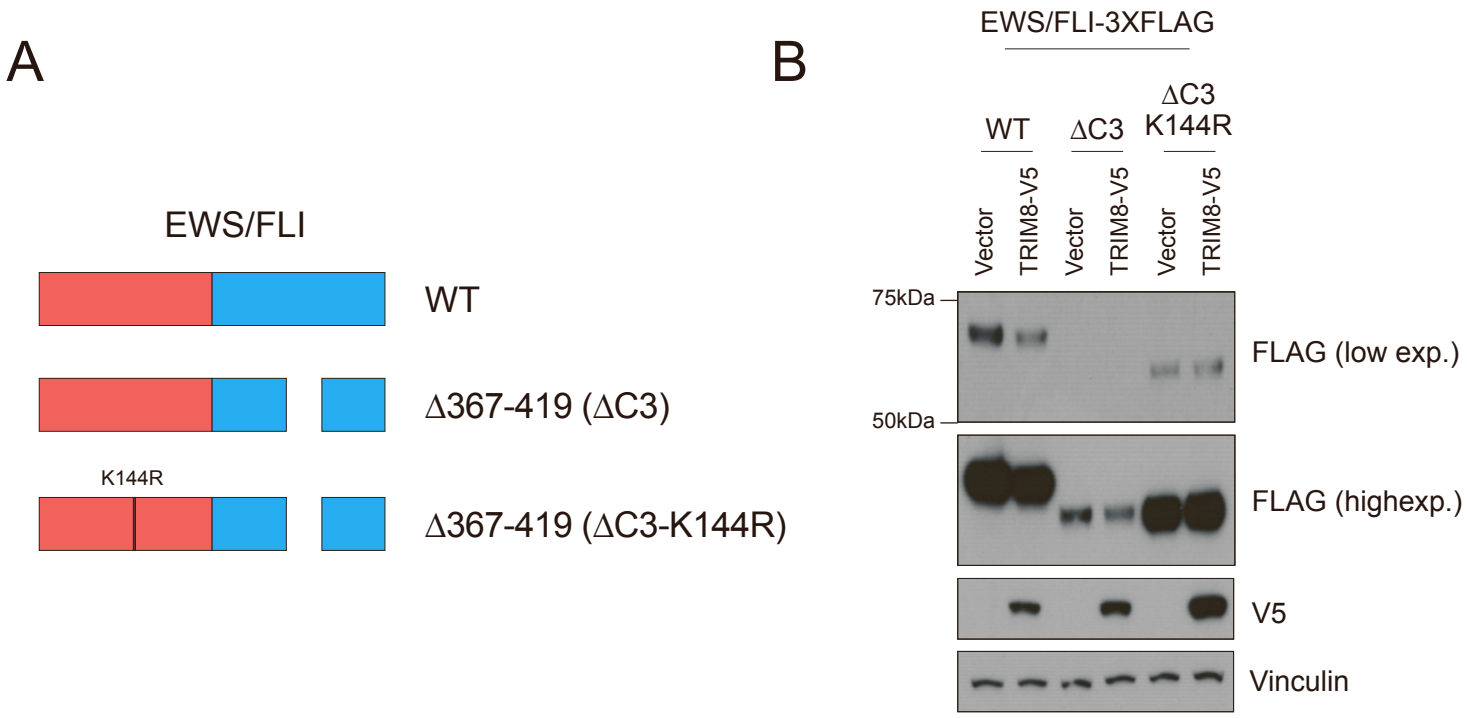

Figure S8. Mutating K144 rescues TRIM8-mediated degradation of EWS/FLI-ΔC3 mutant, related to Figure 8. (A) Schematics showing wild-type EWS/FLI, EWS/FLI-ΔC3, and EWS/FLI-ΔC3-K144R mutants. (B) 293T cells were transfected with wild-type EWS/FLI, EWS/FLI-ΔC3, and EWS/FLI-ΔC3-K14 with either control vector or TRIM8-V5 for 48 hours. Cells were then harvested, lysed, and immunoblotted with indicated antibodies. Data representative of two independent experiments