

WT1 loss attenuates the TP53-induced DNA damage response in T-cell acute lymphoblastic leukemia

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Supplementary Methods

Cell viability assays and flow cytometric analysis

For cell viability assays and apoptosis analysis, T-ALL xenografts were treated *ex vivo* with γ -radiation or Etoposide and cultured *in vitro* with complete RPMI medium supplemented with 20% FCS (Gibco) in 24 well plates at a concentration of 2×10^6 cells/ml for 24h. For functional experiments that required longer incubations (>24h), for example for Embelin treatment (Selleck), PDX cells were maintained in complete RPMI medium supplemented with 20% FCS, cytokines (10 ng/ml IL-7, 20 ng/ml FLT3-L and 50 ng/ml SCF, all from Peprotech) and 20 nM insulin (Sigma Aldrich). For assays, T-ALL cell lines were cultured in complete RPMI medium supplemented with 10% FCS in 24 well at a concentration ranging from 3×10^5 to 5×10^5 cells /ml. Cell viability analysis of T-ALL cell lines treated either with γ -radiation or chemotherapeutic drugs was performed using the bioluminescent method Vialight plus (Lonza). The lethal dose 50 (LD50) of γ -radiation was calculated using COMPUSYN software and LD50 cut off value for sensitivity was arbitrarily established to be 1.5 Gray on the basis of dose-response curves obtained in T-ALL PDX. Apoptosis analysis was performed by flow cytometry (FACS) after staining with Annexin V-FITC and PI (Roche) or Sytox Red (Life Technologies). The specific apoptosis was calculated as previously described ¹. Analysis of proliferation combined with cell cycle profile was performed using the Click-iT™ EdU Flow Cytometry Assay Kit (Life Technologies). The samples were measured on a FACSCalibur (BD Biosciences) flow cytometer using Cell Quest software (BD Biosciences), and analyzed with FlowJo (Tree Star).

Plamids and constructs

pTripz shRNA construct targeting human *WT1* transcript (V2THS_202981) was used for knockdown experiments (Open Biosystem). pTripz non-silencing shRNA (sh-Scramble) was used as negative control (RHS4743, Open Biosystem). For knockdown experiments with pTRIPZ shRNA constructs, cells were cultured in completed RPMI supplemented 10% tetracycline-free serum (Clontech) and pre-treated for 72h with 0.5 μ M doxycycline (Sigma Aldrich). *BBC3* reporter construct was generated by cloning a short sequence containing the WT1-binding site into the pGL4.23[*luc2*/minP] vector (Promega) using the NheI and HindIII restriction sites (Genewiz). Migr1 vectors (generously provided by Prof. Warren Pear) were used

to clone full length and mutant *WT1* constructs using BglII and BglII-EcoRI restriction sites, respectively. *WT1* sequences were sub-cloned in pcDNA3.1 vector (Life Technologies) for transient *WT1* overexpression in U2OS cells.

Human Apoptosis pathway Arrays

We analyzed the expression profiles of 35 apoptosis-related proteins on cellular extracts using the Human Apoptosis Array (R&D Systems). The BioRad ChemiDoc XRS Imager was used to capture the signals from the arrays. The density of each spot was quantified by Quantity One software (BioRad). Raw signal intensities in each array were log₂-transformed and taken average across duplicate spots for each probe. Then processed data were normalized to negative control probe (PBS/neg) to be comparable between arrays.

Low Density Arrays and Quantitative PCR (qPCR)

Total RNA from T-ALL cells was extracted using Trizol reagent (Invitrogen). c-DNA was synthesized from 1 µg of RNA using the SuperScript® First-Strand Synthesis System (Life Technologies). Analysis of 96 genes related to DNA repair, apoptosis and cell cycle following DNA damaging conditions was performed using Custom TaqManArray Cards using the TaqMan Universal PCR Master Mix (Life Technologies) and ABI Prism 7900 Sequence Detection System (Applied Biosystems). Arrays were performed in triplicates. Regular qPCR experiments were performed using SensiMix™ SYBR® No-ROX Kit (Bioline) and ABI Prism 7900 Sequence Detection System. Every sample was analyzed in triplicate and relative expression levels were based on *Beta-2-Microglobulin* as a reference control.

Lentivirus production

pGipz (non-silencing shRNA control or shXIAP V2LSH_302105 (n.1) and V2LSH_94574) and pTripz (non-silencing shRNA control or *WT1* shRNA) and pLentiCRIPSR V2 were transfected in HEK293T cells (ATCC) using JetPEI transfection agent and the corresponding packaging plasmids. The viral supernatant was collected 48h after transfection, filtered and used to infect target cells. All the infections were performed by spinoculation of viral supernatants produced in HEK293T cells. After infection, MOLT4, PF382, CCRF-HSB2 and P12- Ichikawa cells were selected for 5-7 days in puromycin.

Western blot and antibodies

Total cell lysates were prepared using RIPA lysis buffer supplemented with phosphatase inhibitor cocktail set I and II (Sigma-Aldrich) and protease inhibitor cocktail tablets (Roche) and normalized for protein concentration using the BCA method (Pierce). For Western blot, protein samples were separated on 4-12% gradient Tris- Glycine or 3-8% Tris-Acetate SDS-PAGE (Invitrogen) and transferred to PVDF membrane (Millipore, Watford, U.K.). The antibody against TP53 (DO-1) was from Santa Cruz Biotechnology; antibodies recognizing Acetylated-TP53 (K382), β -Actin, BAX, cleaved-Caspase3, PARP, CDKN1A, BBC3, phospho-TP53 (S15), phospho-Histone H2A.X (S139), phospho-ATR (S428), phospho-ATM (S1981), phospho-CHK2 (Thr68), XIAP, and Survivin were from Cell Signaling Technology; antibody against the N-terminus of WT1 (6F-H2) was from Millipore; anti-HO-2/HMOX2 Antibody was from Novus.

WT1, TP53, NOTCH1 and FBXW7 sequencing

WT1 mutations were analyzed in Exon 1 C-terminus, Exon 7 and Exon 9, as previously described ², (Reference sequence: ENST00000332351). For *TP53* locus we sequenced all the coding gene (exon 2 to 11) and exon/intron junctions (IARC protocol). Briefly, DNA isolated from T-ALL cells was subjected to polymerase chain reaction (PCR) using primer pairs specific for each exon by IARC protocol. The amplified products were then sequenced by fluorescent capillary electrophoresis (ABI PRISM 310 genetic analyzer, Applied Biosystems) and sequences compared with NCBI Reference Sequences (NC_000017.10, NM_000546.4). *NOTCH1* and *FBXW7* mutations were analyzed as previously described ³.

Luciferase assay

U2OS cells (ATCC) were transfected with increasing concentrations of pcDNA3 plasmids overexpressing wild type full length WT1 isoforms or mutant WT1 isoforms together with pGL4.23 [luc2/minP] vector carrying the insertion of a specific response element upstream of a minimal promoter and *luc2* gene (Promega). For internal normalization, cells were also co-transfected with a plasmid which allowed Renilla expression (pGL4.74 [hRluc/TK]; Promega). We measured luciferase activity 48h after transfection with the Dual-Luciferase Reporter assay kit (Promega). Transfected cells were gently rinsed with PBS from culture medium, homogeneously lysated for 20 minutes in 150 μ l of passive lysis buffer and finally harvested. Cell

lysates (5 µl per well) were added to wells in a 96 well plate and luciferase assay reagent (50 µl) was added to each well. Firefly luciferase activity was measured with VICTOR™ X5 Multilabel Plate Reader (Perkin Elmer). *Renilla* luciferase activity was then measured after adding Stop & Glo® reagent (50 µl) to each well.

CRISPR-Cas9 construct

Inactivation of *WT1* gene in MOLT4 cells using the CRISPR-Cas9 technology was achieved as previously described^{4,5}. LentiCRISPRV2 vector was obtained from Addgene. We cloned the target sequence (single guide RNA, sgRNA) into the LentiCRISPRV2 plasmid that allows the co-expression of a mammalian Cas9 nuclease together with a sgRNA. For target sequence design, we used the following link: <http://www.e-crispr.org/E-CRIPR/deaigncrispr.html>. LentiCRISPRV2 expressed a sgRNA sequence that targets a *WT1* genetic site very close to the hot spot mutations that occurs in *WT1* locus in T-ALL (sgRNA: GTATTGGGCTCCGCAGAGGA).

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Table S1. Immunophenotype of T-ALL cells at the time of injection. *NOTCH1*, *FBXW7* genetic status and PTEN expression (mut: mutated; wt: wild-type; HD: hetero dimerization domain; PEST: proline (P), glutamic acid (E), serine (S), and threonine (T) rich domain; nd: not determined).

T-ALL PDX	Phenotype	<i>NOTCH1</i> status	<i>FBXW7</i> status	PTEN status
8	Thymic	mut (HD+PEST)	wt	positive
9	Early T	wt	wt	negative
10	Thymic	mut (HD)	mut	positive
11	Early T	mut (HD)	wt	negative
12	Early T	mut (PEST)	wt	positive
15	T mature	wt	wt	positive
16	T mature	wt	wt	negative
46R	Early T	mut (HD)	mut	positive
47R	Early T	mut (HD)	mut	positive
48	Thymic	wt	wt	negative
51	Early T	wt	wt	nd
51R	Early T/ETP like	wt	wt	positive

Table S2. *TP53* genetic status in selected T-ALL cell lines. HGVS-nomenclature was used for the description of sequence variants ⁶.

TALL cell lines	Exons	<i>TP53</i>
CCRF-CEM	5 and 7	Mut: c.524G>A, p.R175H; c.743G>A, p.R248Q
RPMI-8402	8	Mut: c.817C>T, p.R273C
P12-Ichikawa	2 and 7	Mut: c.31G>C, p.E11Q; c.743G>C, p.R248P and c.743G>A, p.R248Q
PF382	8	Mut: c.817C>T, p.R273C
DND41	7	Mut: c.743G>A, p.R248Q
Jurkat	6	Mut:c.586C>T, p.R196*
HPB-ALL	4	Mut: c.375G>A, p.T125T ^{7,8}
CCRF-HSB2	2-11	wt
MOLT4	2-11	Mut: c.916C>T, p.R306* ⁹

Figure S1.

WT1-knockout protects from DNA damage MOLT4 cells.

Cell viability assay and apoptosis analysis in MOLT4 cells infected either with lentiCRIPSR V2 Empty (Ctrl) or lentiCRPSR V2-*WT1* (*WT1*-KO) after 24h-treatment with increasing doses of γ -radiation and Etoposide (A and B, respectively). Quantitative data are shown as mean \pm SD; assays were performed in triplicates and reproduced at least twice. (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$). Western blot analysis of WT1 protein showed complete knockout.

Figure S2.

Quantification of consistently modulated proteins detected by protein peptide arrays and Western blot.

(A) Apoptotic proteins that were consistently modulated in the peptide arrays (Cleaved Caspase3, Survivin, HO-2, XIAP, phospho-TP53; indicated in red, Figure 3A) were quantified using the Quantity One software (BioRad) and processed data was normalized to negative control probe (PBS) to be comparable between arrays. The plots represent the relative intensity signal calculated respect to untreated Ctrl cells (fixed at 1). (B) Western blot quantification of XIAP, Survivin and HO-2 proteins in additional independent experiments ($n=2$). The plots represent the relative intensity signal calculated respect to untreated Ctrl cells (fixed at 1). (C) Western blot analysis of cleaved-Caspase3, PARP and XIAP at 24h from 6 Gray of γ -radiation in *WT1*-knockout (*WT1*-KO or CRISPR-Cas9-*WT1*) respect to control (Ctrl or CRIPSR-Cas9-empty) MOLT4 cells. Relative expression of XIAP bands are shown compared to untreated Ctrl cells (fixed at 1).

Figure S3.

Impaired TP53 DNA damage response in *WT1*-knockout MOLT4 cells and quantification of γ H2AX positive cells upon DNA damage in MOLT4 cells.

(A) Immunoblot of cleaved-Caspase3, TP53, XIAP and BBC3 following 1, 3, 6 and 12h from 6 Gray of γ -radiation. Relative expression of TP53, XIAP and BBC3 bands are shown relative to untreated Ctrl cells (E= infected with lentiCRIPSR V2 empty). β -Actin is shown as loading control. Western blot quantification of critical proteins is reported for each band. The numbers represent the relative intensity signal calculated respect to untreated Ctrl cells (fixed at 1). (B) Histograms representation of γ H2AX positive cells following

10 and 30 minutes following 6 Gray from γ -radiation. Plots represent data obtained from 3 independent experiments.

Figure S4.

WT1 directly regulates pro-apoptotic factor BBC3.

Luciferase reporter activity of the *BBC3* reporter construct in U2OS cells in response to increasing doses (250 and 500 ng) of full length WT1 or *WT1*-mutant isoforms. Western blot analysis of full length WT1 or *WT1*-mutant isoform expression is shown. β -Actin is shown as a loading control.

Figure S5.

***WT1*-knockout protects CCRF-HSB2 from γ -radiation and Etoposide treatments**

(A) Cell viability assay and apoptosis analysis in CCRF-HSB2 cells infected either with sh-Scramble (Ctrl) or Sh-*WT1* (*WT1*-KD) after 24h-treatment with increasing doses of γ -radiation (bottom panels). Quantitative data are shown as mean \pm SD; assays were performed in triplicates. (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$). Western blot analysis of WT1 protein showed knockdown of protein levels. Western blot quantification of WT1 protein is reported on top of the gel (top panel). Values are normalized respect to the loading control. The numbers represent the relative intensity signal calculated respect to Ctrl cells (fixed at 1). (B) Apoptosis analysis of CCRF-HSB2 cells infected either with sh-Scramble (Ctrl) or Sh-*WT1* (*WT1*-KD) after 24h-treatment with increasing doses of Etoposide (left panel). Immunoblot of PARP and XIAP after 24h-treatment with 0.5 or 1 μ M Etoposide. Western blot quantification of XIAP protein is reported on top of the gel. Values are normalized respect to the loading control. The numbers represent the relative intensity signal calculated respect to untreated Ctrl cells (fixed at 1).

Figure S6.

BAX, HO-2 and Survivin quantification upon DNA damage in *WT1* wild type and in *WT1*-mutated T-ALL PDX samples.

Histogram plots representing Western blot quantification of BAX, HO-2 and Survivin proteins in T-ALL PDX samples after 3h from 6 Gray of γ -radiation. *WT1*-mutated T-ALL PDX samples are indicated in red. Values

are normalized respect to the loading control. Plots represent the relative intensity signal calculated respect to untreated Ctrl cells (fixed at 1).

Figure S7.

T-ALL PDX sample sensitivity to increasing doses of Embelin.

Cell viability assay and apoptosis analysis of T-ALLPDX samples exposed for 72h to increasing doses of Embelin. *WT1*-mutated T-ALL PDX samples are indicated in red.

Figure S8.

***XIAP*-knockdown significantly rescues resistance to Etoposide treatment in *WT1*-deficient MOLT4 cells.**

(A-B) Apoptosis assay in *WT1*-KD MOLT4 cells infected with 2 specific hairpins for XIAP, pGipz shXIAP V2LSH_302105 (n.1) and pGipz V2LSH_94574 (n.2), and exposed to increasing doses of Etoposide for 48h (0.25 Gray and 0.5 μ M). Western blot analysis of XIAP in sh-Scramble and *XIAP*-knockdown MOLT4 cells showed an efficient knockdown. Western blot analysis of XIAP protein is reported on top of the gel. Values are normalized respect to the loading control. The numbers represent the relative intensity signal calculated respect to Ctrl cells (fixed at 1). (C) A representative flow cytometry analysis is shown. (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$).

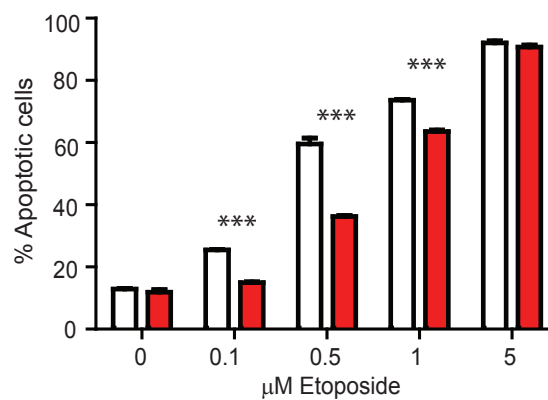
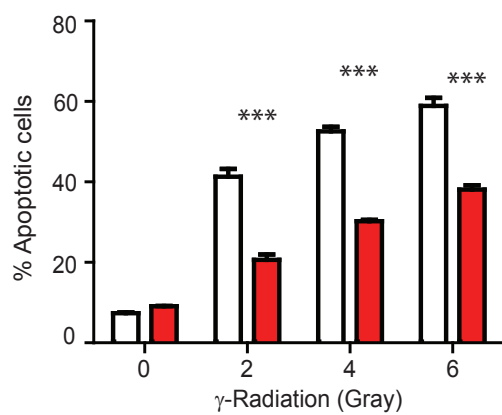
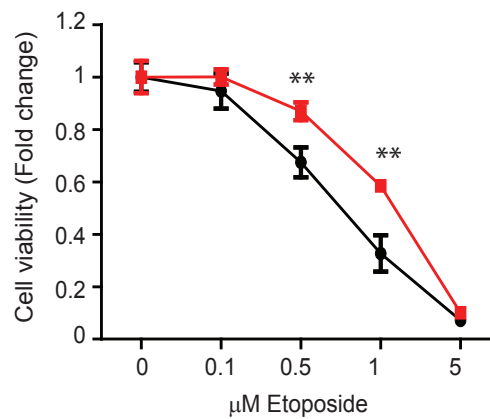
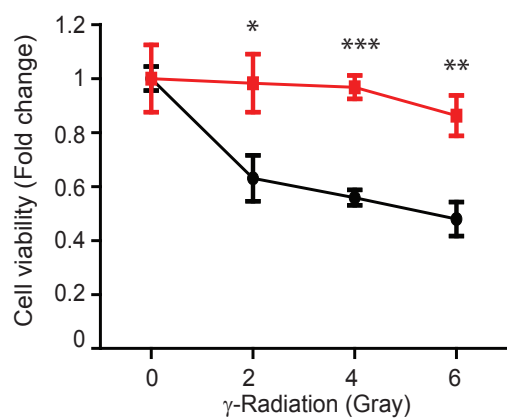
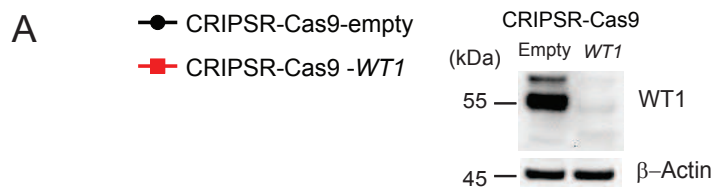


Figure S1

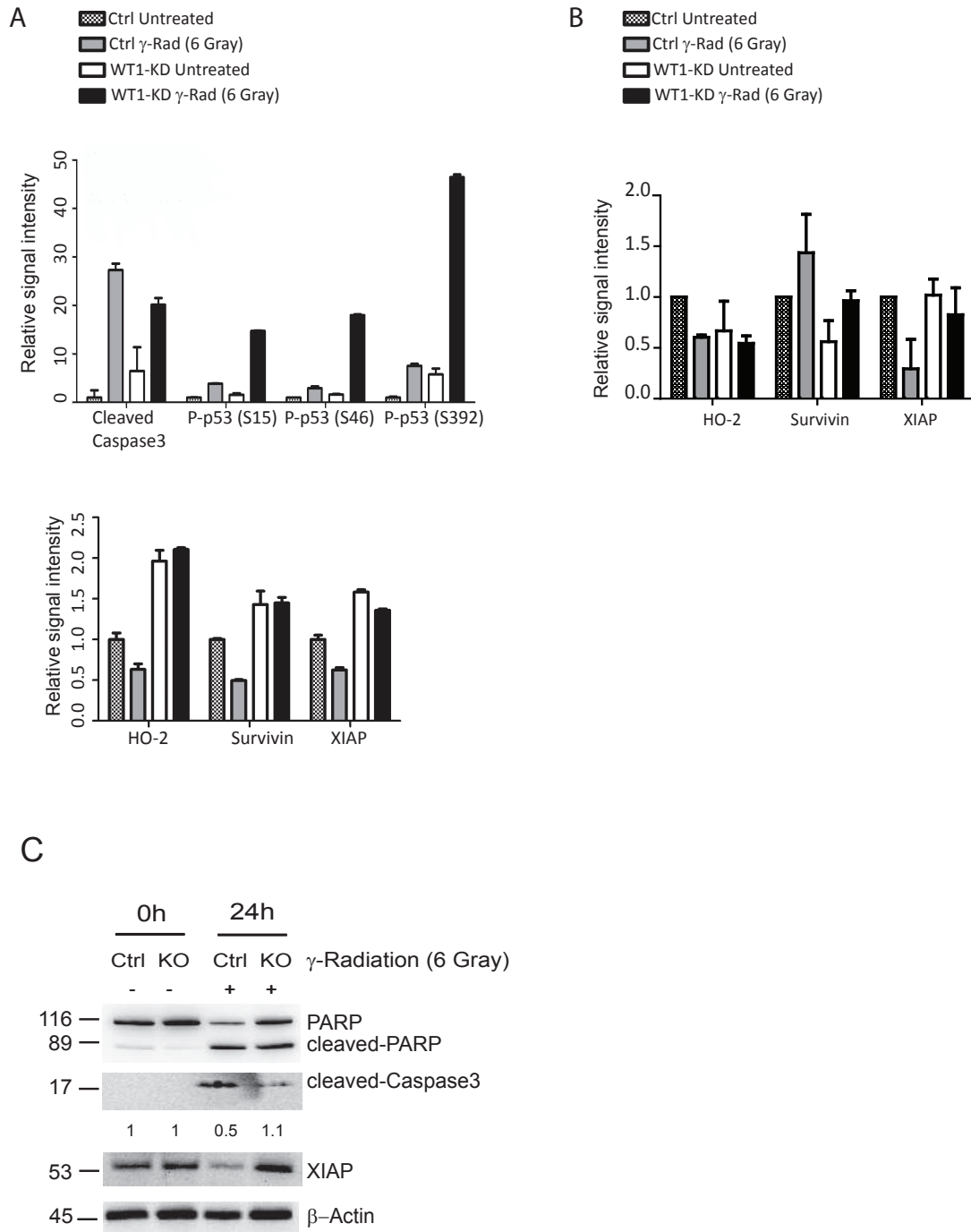
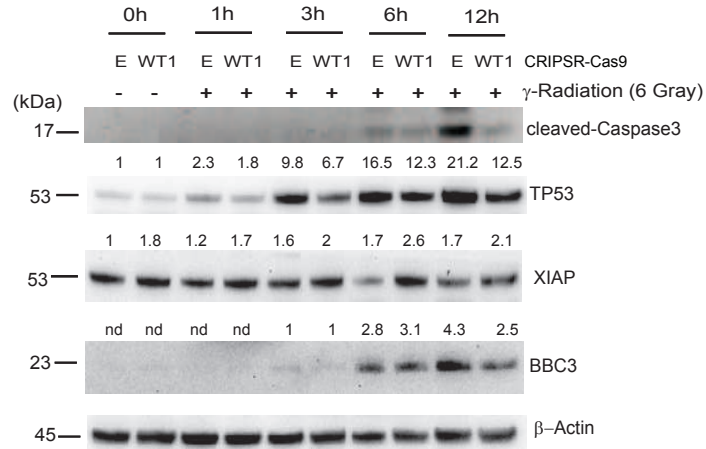


Figure S2

A



B

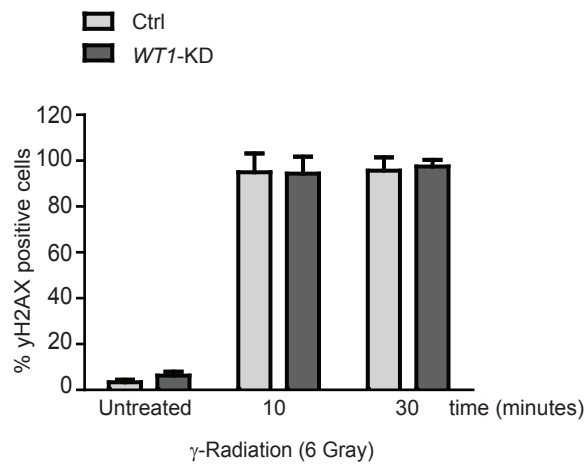


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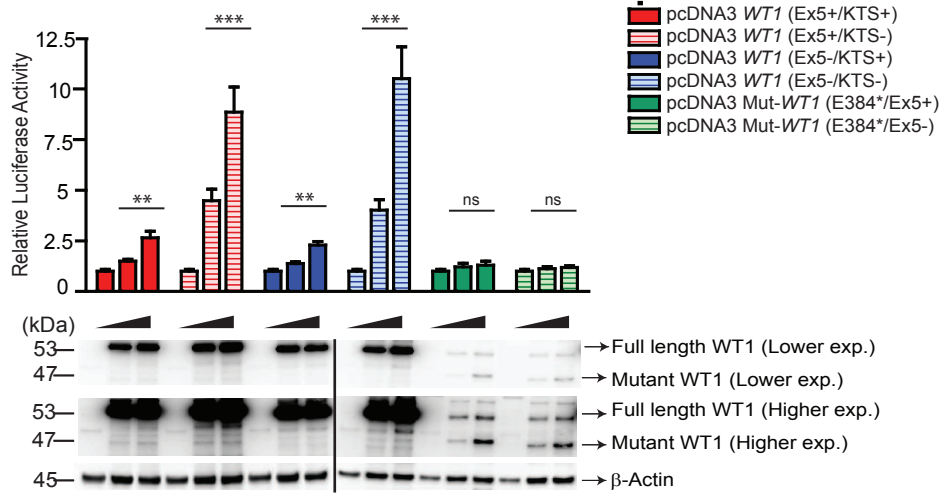
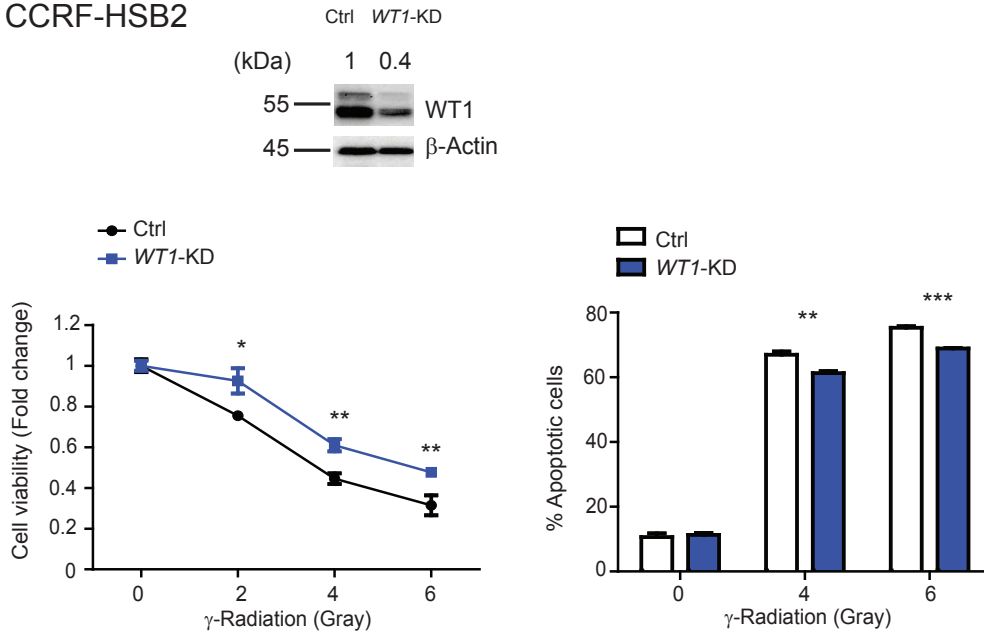


Figure S4

A CCRF-HSB2



B CCRF-HSB2

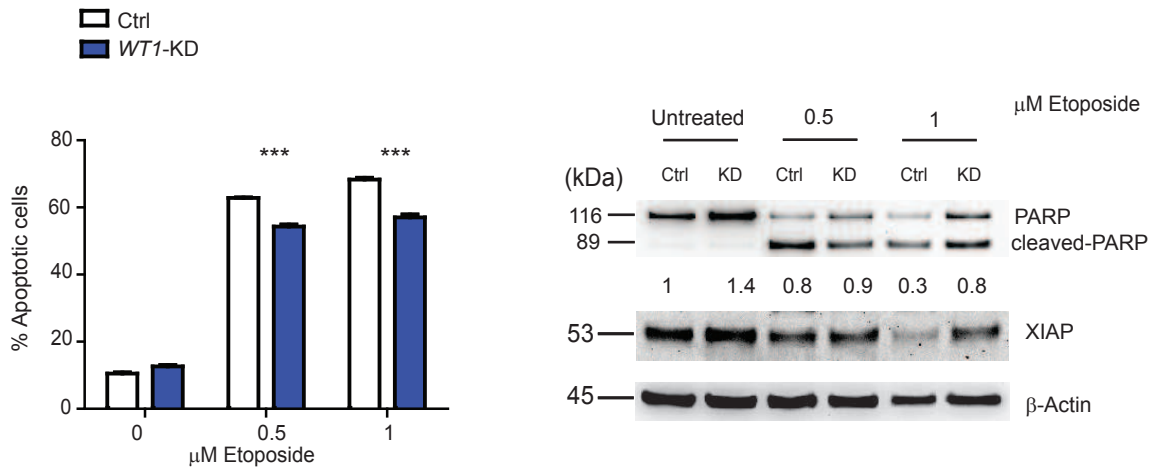


Figure S5

T-ALL PDX

Untrated
 γ -radiation treated

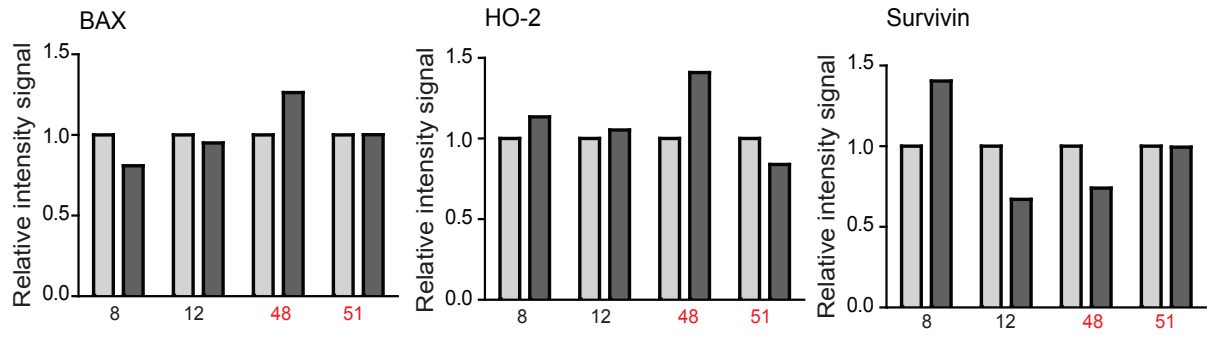


Figure S6

T-ALL PDX

- 48
- 51
- ▲ 8
- ▼ 12
- ◆ 15

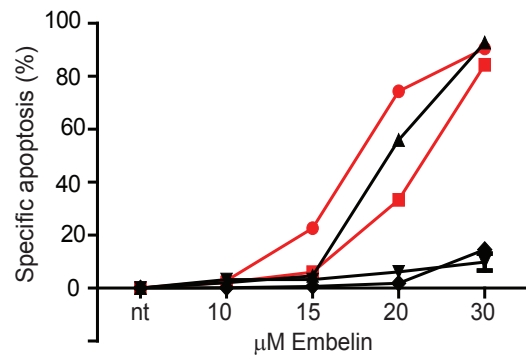
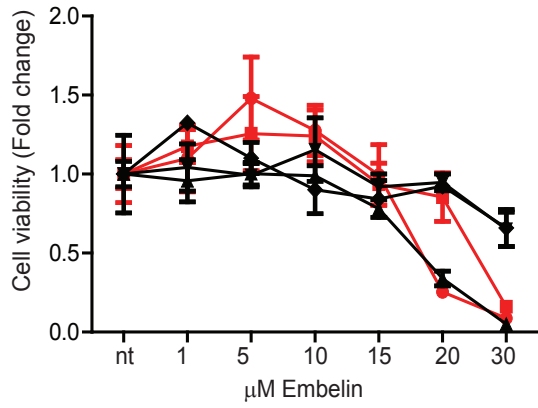
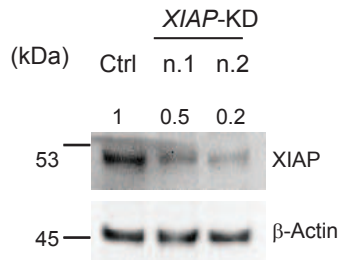
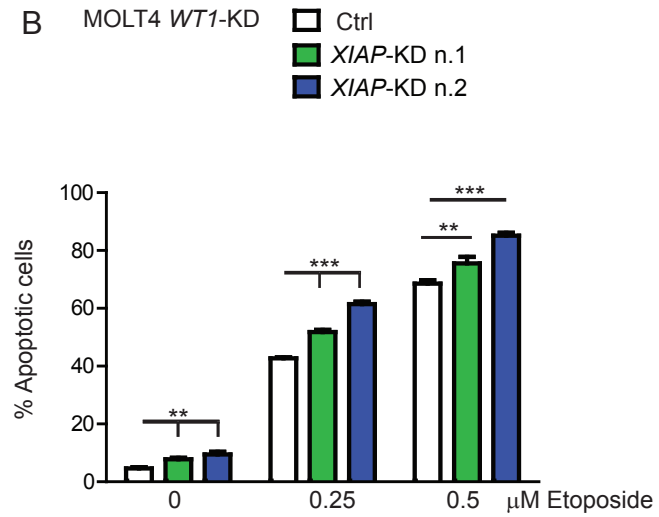


Figure S7

A MOLT4 WT1-KD



B MOLT4 WT1-KD



C

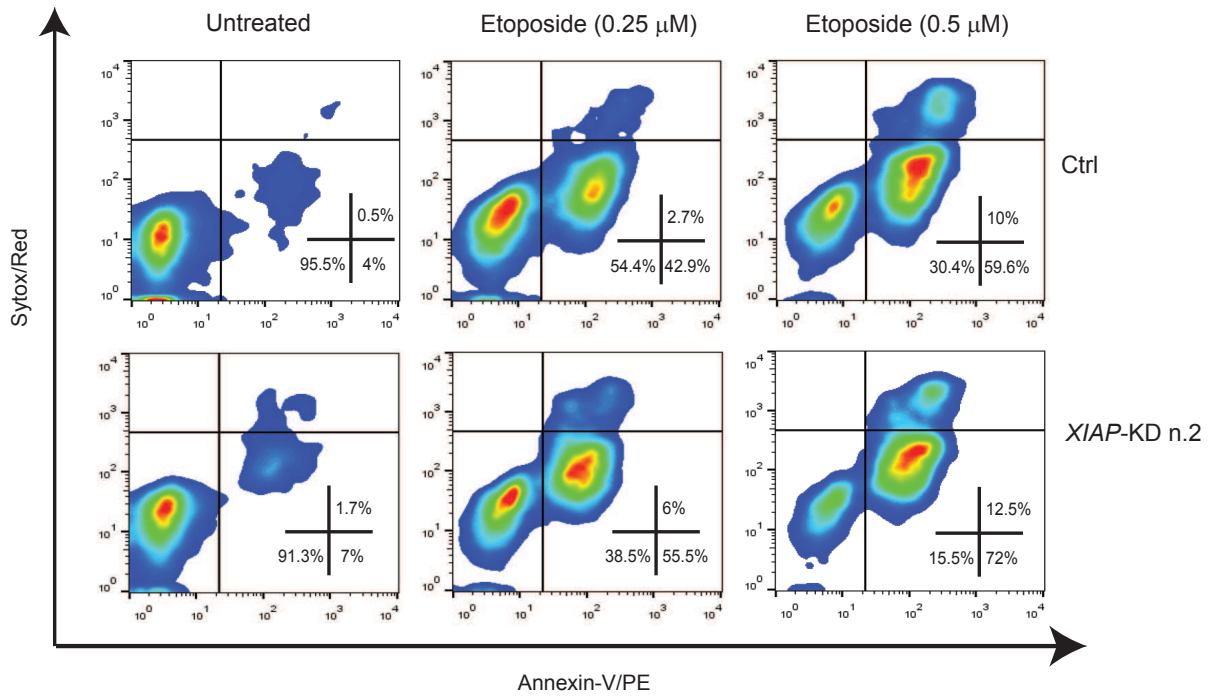


Figure S8