Supplementary Materials for

DTX3L-mediated TIRR nuclear export and degradation regulates DNA repair pathway choice and PARP inhibitor sensitivity

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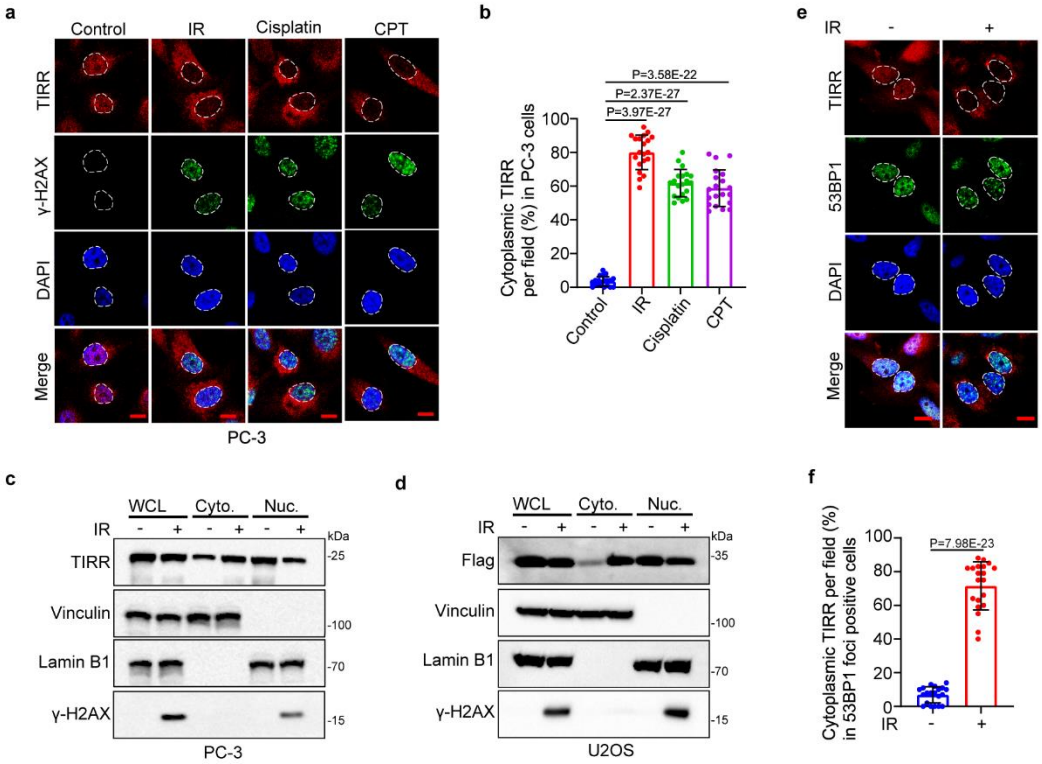
Supplementary Figure 1



Supplementary Fig. 1 TIRR is translocated to the cytoplasm after DNA damage. Related to Fig 1.

a IFC of TIRR and γ-H2AX in PC-3 cells treated with IR (8 Gy), Cisplatin (10 μM, 24 hours) or CPT (2 μM, 3 hours). Scale bar, 10 μm.

b Quantification of cells with cytoplasmic translocation of endogenous TIRR as shown in (**a**). Data were shown as the mean ± SD from 20 fields (>200 cells, n = 20) from three biological replicates. Two-tailed unpaired Student's *t*-test; *P* values based on the order of appearance: 3.97E-27, 2.37E-27, 3.58E-22.

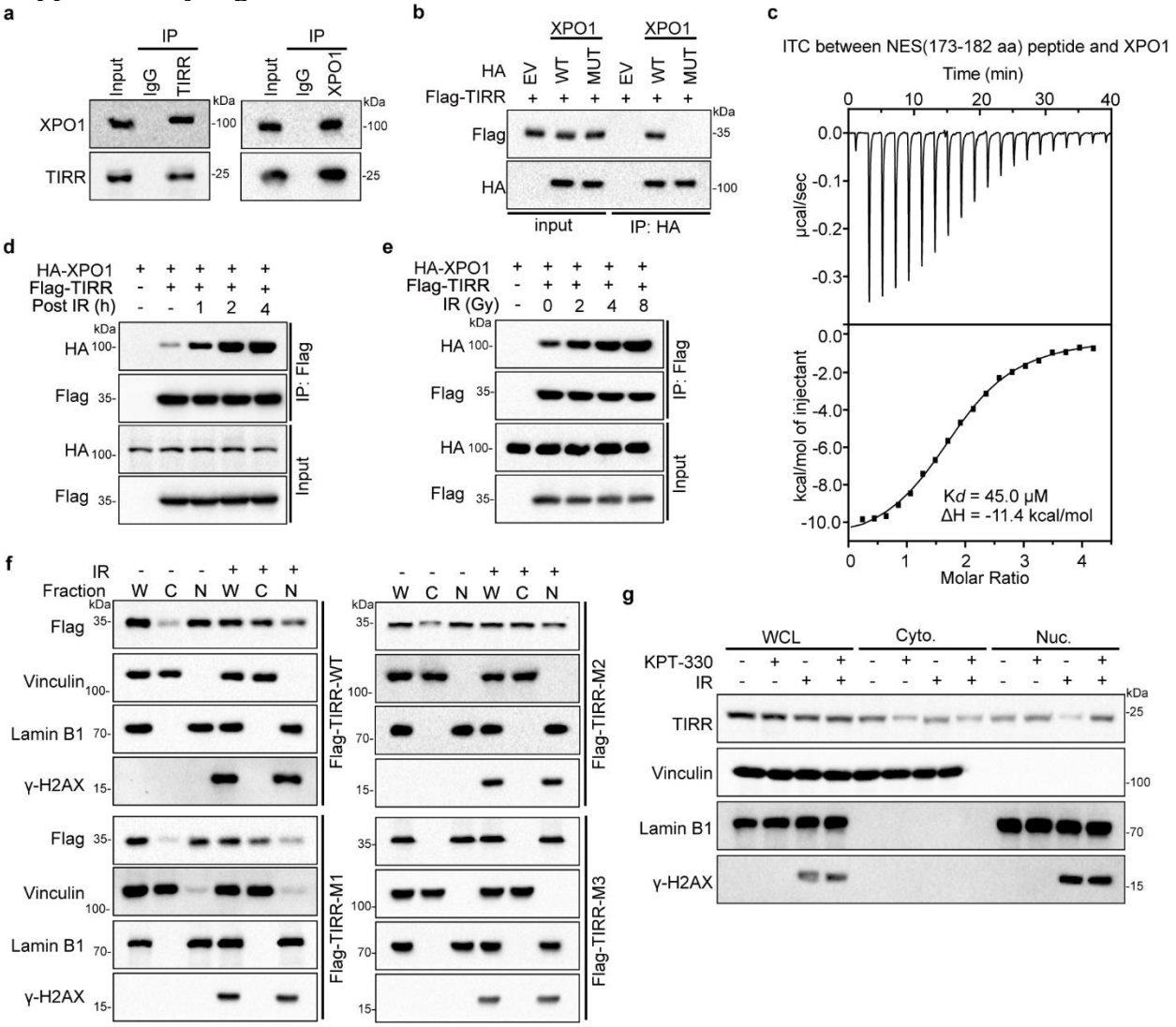
c WB analysis of whole-cell (WCL), cytosolic (Cyto.) and nuclear (Nuc.) fractions from PC-3 cells with or without IR (8 Gy).

d WB analysis of whole-cell (WCL), cytosolic (Cyto.) and nuclear (Nuc.) fractions from U2OS cells transfected with Flag-TIRR, and treated with or without IR (8 Gy).

e IFC of TIRR and 53BP1 in PC-3 cells treated with IR (8 Gy). Scale bar, 10 μm.

f Quantification of cells with positive 53BP1 foci and endogenous TIRR cytoplasmic translocation (**e**). Data were shown as the mean ± SD from 20 fields (>200 cells, n = 20) from three biological replicates. Two-tailed unpaired Student's *t*-test; *P* = 7.98E-23. Source data are provided as a Source Data file. Similar results for (**c, d**) panels were obtained in three independent experiments.

Supplementary Figure 2



Supplementary Fig. 2 XPO1 mediates TIRR nuclear exporting in response to DNA damage. Related to Fig 2.

a Co-IP of endogenous TIRR and XPO1 in whole-cell lysates from PC-3 cells.

b Co-IP of whole-cell lysates from 293T cells transfected with Flag-TIRR, HA-XPO1-WT or HA-XPO1 mutant (I521A/L525A/F561A/F572A).

c Binding affinity measured by isothermal titration calorimetry (ITC) between XPO1 and the synthesized NES peptide (173-182 aa of TIRR).

d Co-IP of whole-cell lysates from 293T cells transfected with Flag-TIRR and HA-XPO1, and harvested at indicated time points post-IR (8 Gy).

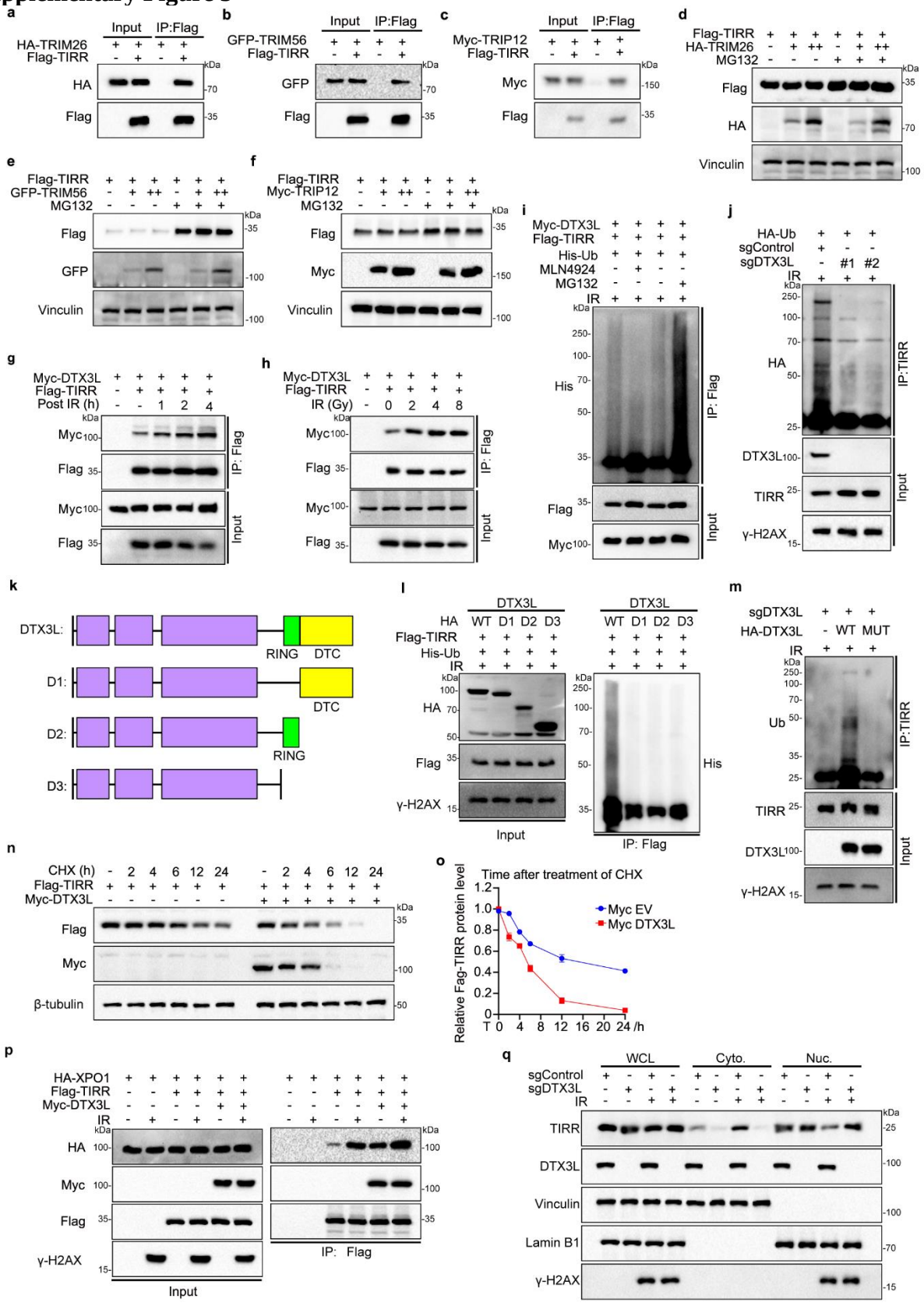
e Co-IP of whole-cell lysates from 293T cells transfected with Flag-TIRR and HA-XPO1, treated with varying doses of IR from 0 to 8 Gy.

f WB analysis of whole-cell (W), cytosolic (C) and nuclear (N) fractions from 293T cells transfected with Flag-TIRR-WT or its NES mutants, followed by IR (8 Gy).

g WB analysis of whole-cell (WCL), cytosolic (Cyto.) and nuclear (Nuc.) fractions from PC-3 cells pretreated with or without KPT-330 (1 μM, 12 hours), followed by IR (8 Gy). Source data

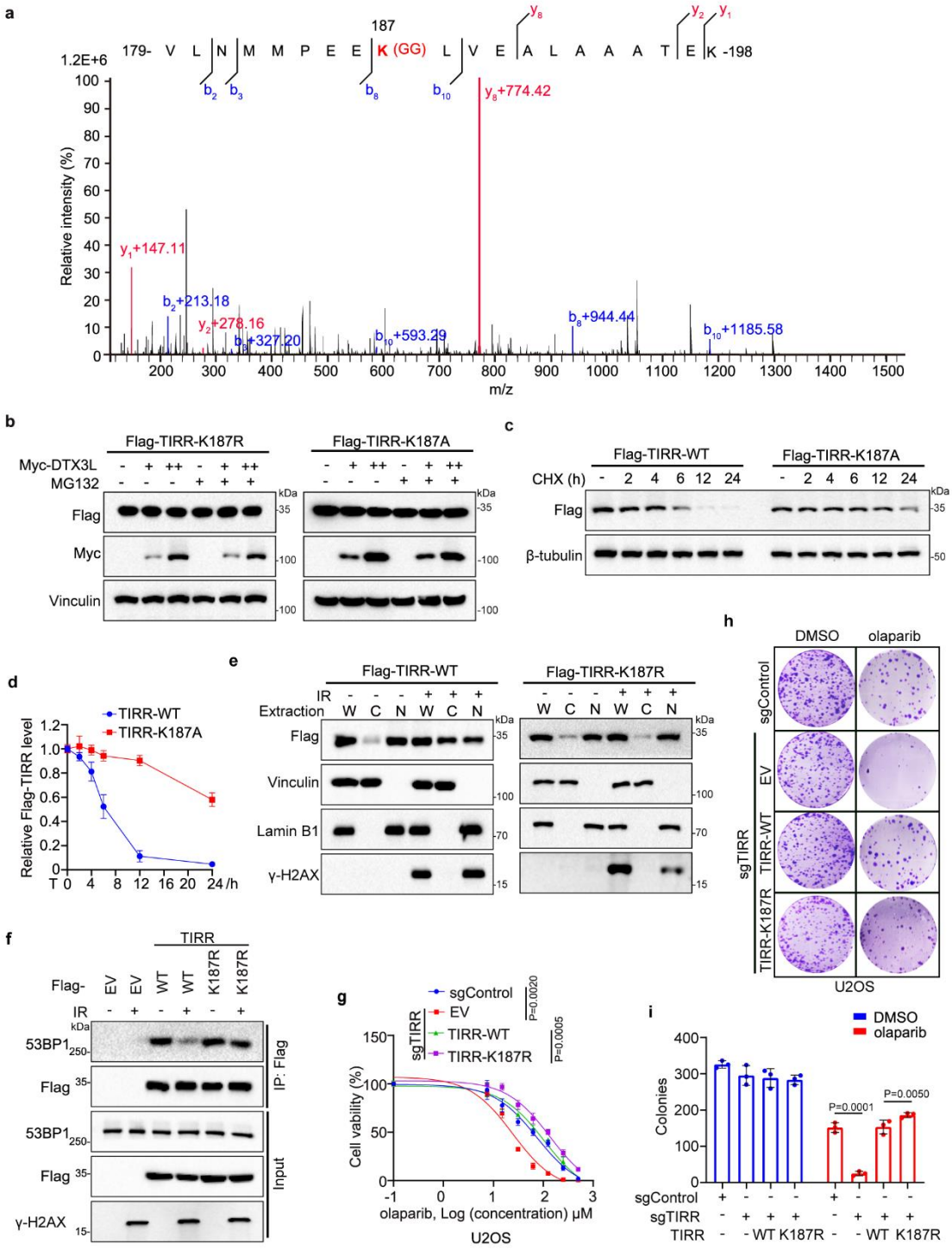
are provided as a Source Data file. Similar results for (**a, b, d-g**) panels were obtained in three independent experiments.

Supplementary Figure 3



Supplementary Fig. 3 DTX3L-mediated TIRR ubiquitination triggers TIRR nuclear translocation and degradation after DNA damage. Related to Fig. 4.
a-c Co-IP of whole-cell lysates from 293T cells transfected with Flag-TIRR, HA-TRIM26, GFP-TRIM56 or Myc-TRIP12.
d-f WB analysis of whole-cell lysates from 293T cells transfected with Flag-TIRR, HA-TRIM26, GFP-TRIM56 or Myc-TRIP12, pretreated with or without MG132, and harvested 2 hours post-IR (8 Gy).
g Co-IP of whole-cell lysates from 293T cells transfected with Flag-TIRR and Myc-DTX3L at indicated time points post-IR (8 Gy).
h Co-IP of whole-cell lysates from 293T cells transfected with Flag-TIRR and Myc-DTX3L, treated with varying doses of IR (0 ~ 8 Gy).
i In vivo polyubiquitination assay of whole-cell lysates from 293T cells transfected with HA-Ub, Myc-DTX3L, Flag-TIRR, pretreated with DMSO, MG132 or MLN4924, and harvested after IR (8 Gy).
j In vivo polyubiquitination assay of whole-cell lysates from control or DTX3L knockout PC-3 cells transfected with HA-Ub, pretreated with MG132 and exposed to IR (4 Gy).
k Diagram showing wild-type DTX3L and mutant constructs with deletions in the RING (green) or DTC (yellow) domain.
l In vivo polyubiquitination assay of whole-cell lysates from 293T cells transfected with Flag-TIRR, His-Ub, HA-DTX3L WT or D1, D2, D3 mutant, pretreated with MG132 and exposed to IR (8 Gy).
m In vivo polyubiquitination assay of whole-cell lysates from DTX3L depleted PC-3 cells transfected with HA-DTX3L WT or catalytic inactive mutant (C561S/C564S), pretreated with MG132 and exposed to IR (8 Gy).
n, o WB analysis (**n**) of whole-cell Flag-TIRR protein after CHX treatment and quantification (**o**) in 293T cells transfected with Flag-TIRR and Myc-DTX3L. Data were shown as the mean ± SD from three biological replicates (n = 3).
p Co-IP of whole-cell lysates from 293T cells transfected with Flag-TIRR, Myc-DTX3L and HA-XPO1, with or without IR (8 Gy).
q WB analysis of whole-cell (WCL), cytosolic (Cyto.) and nuclear (Nuc.) fractions from control or DTX3L knockout PC-3 cells after IR (8 Gy). Source data are provided as a Source Data file. Similar results for (**a-j, l-n, p, q**) panels were obtained in three independent experiments.

Supplementary Figure 4

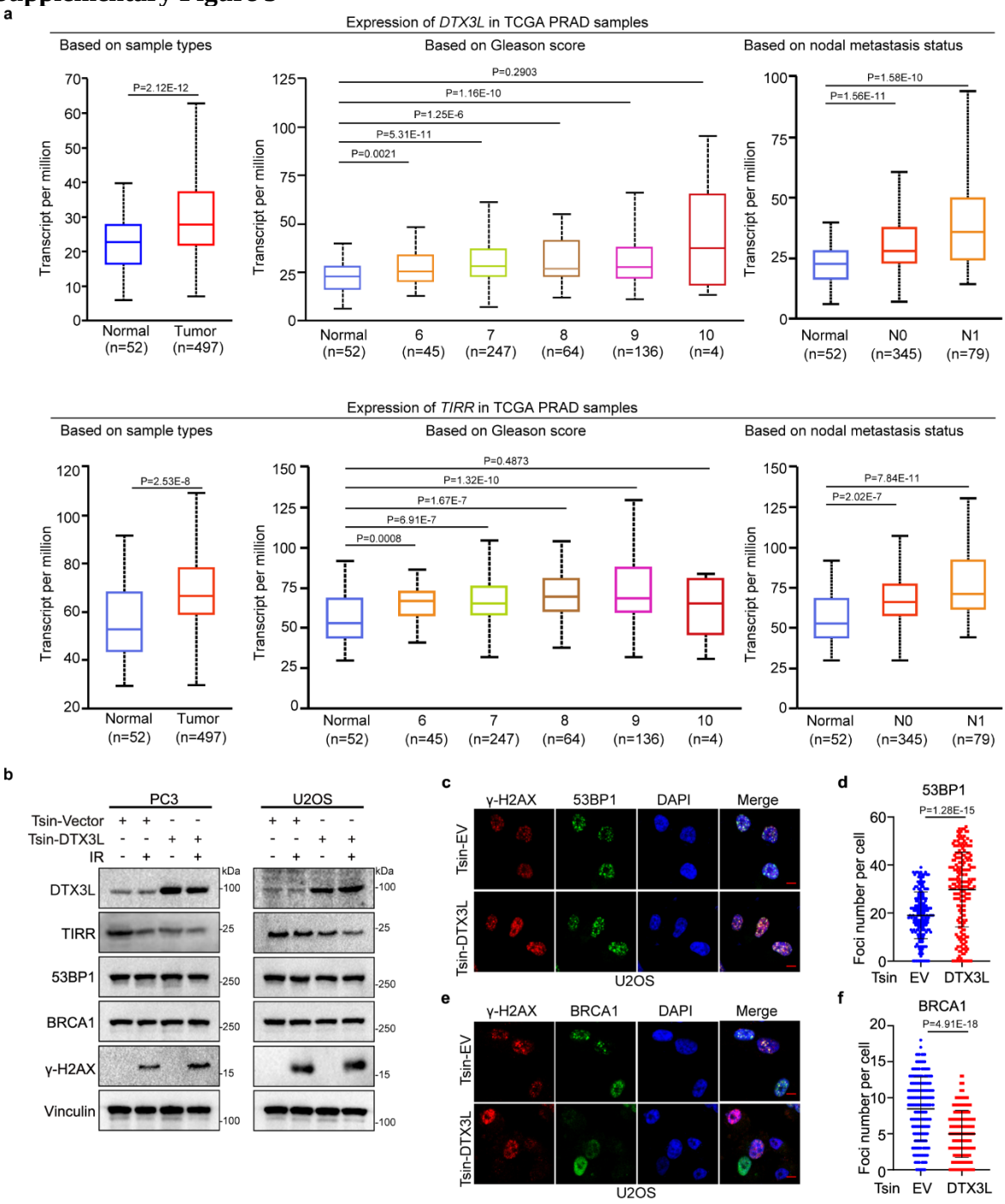


Supplementary Fig. 4 Ubiquitination of TIRR at lysine-187 by DTX3L dictates NHEJ pathway activity and PARP inhibitor sensitivity. Related to Fig. 5.

a Representative annotated LC-MS/MS spectra of TIRR peptide 179-VLNMMPEEKLVEALAAATEK-198, containing a Gly-Gly modified (ubiquitinated) lysine-187.

b WB analysis of Flag-TIRR in whole-cell lysates from 293T cells transfected with Flag-TIRR K187R or K187A mutant, and pretreated with or without MG132 treatment, 2 hours post-IR (8 Gy).
c, d WB analysis of whole-cell Flag-TIRR WT or K187A mutant protein from 293T cells at indicated time points of CHX treatment (**c**), with corresponding quantification analysis (**d**). Data were shown as the mean ± SD from three biological replicates (n = 3).
e WB analysis of whole-cell (W), cytosolic (C) and nuclear (N) fractions from 293T cells transfected with Flag-TIRR-WT or K187R mutant, and treated with or without IR (8 Gy).
f Co-IP of whole-cell lysates from 293T cells transfected with Flag-TIRR-WT or K187R mutant, and treated with or without IR (8 Gy).
g Dose-response survival curves of control or TIRR knockout U2OS cells transfected with EV, Flag-TIRR-WT or K187R mutant exposed to increasing concentrations of olaparib. Data were shown as the mean ± SD of three independent experiments (n = 3). Two-way ANOVA; *P* values based on the order of appearance, $P = 0.0020$, $F = 51.98$; $P = 0.0005$, $F = 108.8$.
h, i Colony formation assays in control or TIRR knockout U2OS cells transfected with EV, Flag-TIRR-WT or K187R mutant. The number of colonies was counted. Representative colonies are shown in (**h**), with quantification data shown in (**i**). Data were presented as the mean ± SD of three independent experiments (n = 3). Two-tailed unpaired Student's *t*-test. *P* values based on the order of appearance: 0.0001,0.0050. Source data are provided as a Source Data file. Similar results for (**b, c, e, f**) panels were obtained in three independent experiments.

Supplementary Figure 5

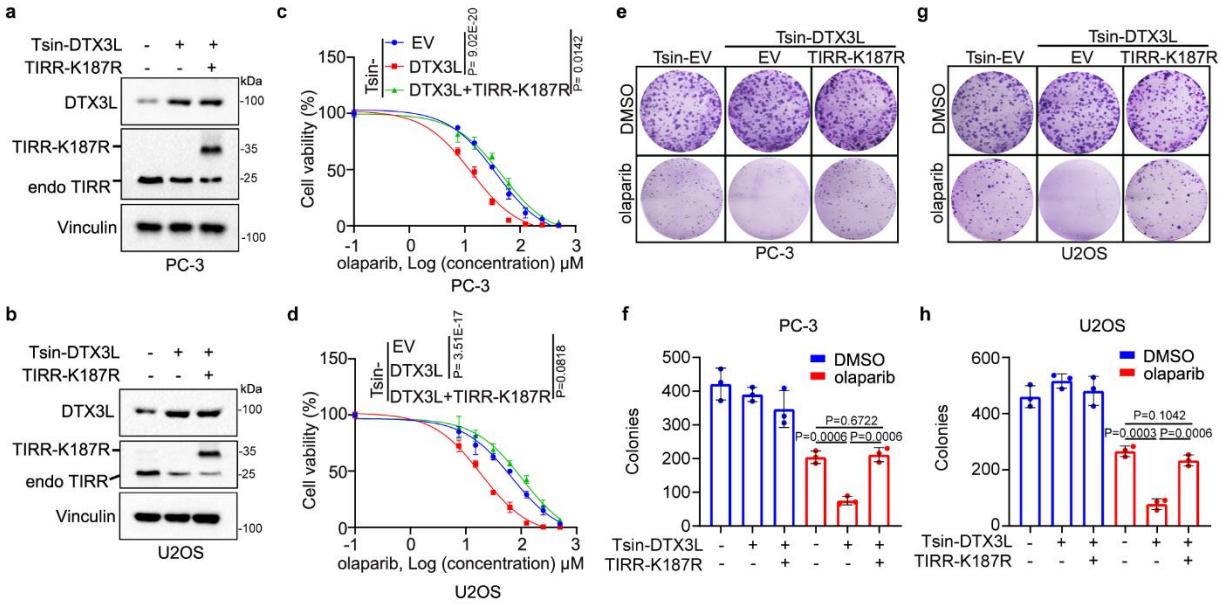


Supplementary Fig. 5 DTX3L overexpression impairs HR activity and promotes chromosomal instability in prostate cancer. Related to Fig. 6.

a Normalized expression of *DTX3L* or *TIRR* mRNA levels based on sample types, Gleason scores and metastasis status from TCGA prostate cancer dataset generated from UALCAN (https://ualcan.path.uab.edu/index.html). Means and SDs are represented. *P* values based on the order of appearance: 2.12E-12; 0.0021, 5.31E-11,1.25E-6, 1.16E-10, 0.2903; 1.56E-11, 1.58E-10; 2.53E-8; 0.0008; 6.91E-7, 1.67E-7, 1.32E-10, 0.4873; 2.02E-7, 7.84E-11.

b-f PC-3 and U2OS cells infected with lentivirus expressing EV or DTX3L were exposed to IR (8 Gy), followed by WB analysis of whole-cell lysates and IFC of 53BP1 (**c**) and BRCA1 (**e**). Scale bar, 10 μm. Similar results for (**b**) panel were obtained in three independent experiments. The average foci number (**d, f**) in each cell were quantified. Scale bar, 10 μm. Data were shown as the mean ± SD from three biological replicates (n = 200). Two-tailed unpaired Student's *t*-test; *P* values based on the order of appearance: 1.28E-15 (**d**); 4.91E-18(**f**). Source data are provided as a Source Data file.

Supplementary Figure 6



Supplementary Fig. 6 DTX3L expression sensitizes prostate cancer cells to synthetic lethality by PARP inhibitors. Related to Fig. 7.

a, b WB analysis of DTX3L and TIRR in whole-cell lysates from PC-3 (**a**) and U2OS (**b**) cells expressing either EV or DTX3L, and further transfected with EV or TIRR-K187R mutant. Similar results were obtained in three independent experiments.

c, d Dose-response survival curves of PC-3 (**c**) and U2OS (**d**) cells exposed to increasing concentrations of olaparib. Data were shown as the mean ± SD of three independent experiments (n = 3). Two-way ANOVA; *P* = 9.02E-20, F = 407.583, *P* = 0.0142, F = 6.727 (**c**); *P* = 3.51E-17, F = 271.129, *P* = 0.0818, F= 5.347 (**d**).

e-h Colony formation assays in PC-3 (**e, f**) and U2OS (**g, h**) cell lines treated with DMSO or olaparib. The number of colonies was counted. Representative colonies are shown in (**e**, **g**), with quantification data shown in (**f, h**). Data were presented as the mean ± SD of three independent experiments (n = 3). Two-tailed unpaired Student's *t*-test. *P* values based on the order of appearance: 0.0006, 0.6722, 0.0006 (**f**); 0.0003, 0.1042, 0.0006 (**h**). Source data are provided as a Source Data file.

Supplementary Table 1: Oligonucleotides. Related to Methods.

OLIGO NAME	SEQUENCE 5'-3'
TIRR-F	GAGCTGAAGCAGATCAGCCG
TIRR-R	CGTCGAAACGCATCTGCATC
18S-F	GGAGTATGGTTGCAAAGCTGA
18S-R	ATCTGTCAATCCTGTCCGTGT
FLAG-TIRR-M1-F	GCGGAGCAGGCGCACGCCGCGGAGGCCAGCGCGGTGCAC
FLAG-TIRR-M1-R	CGTCAGCTGCCGCGCG
FLAG-TIRR-M2-F	GCGGAGGTGGCGGGCCTCGCGCGGGCCCCGCTGTACACC
FLAG-TIRR-M2-R	GCCGTGGTCGCGCGAGT
FLAG-TIRR-M3-F	GCCCTCTTTGCCGCCAAGGTGGCCAACGCGATGCCCGAGGAG
FLAG-TIRR-M3-R	CTGGCACTTAGCCGTGCTCACG
HA-XPO1-I521A/L525A-F	GCAAAGGATCTAGCAGGATTATGTGAACAG
HA-XPO1-I521A/L525A-R	AACAGTAACAAGAAATCG
HA-XPO1-F561A-F	GCTCTGAAGACTGTAGTTAACAAGC
HA-XPO1-F561A-R	TTTCCAGTGAGCTCTC
HA-XPO1-F572A-F	GCCATGCATGAGACCCATGATG
HA-XPO1-F572A-R	TTCGAACAGCTTGTTAACTAC
HA-DTX3L-D1-F	ACTTCCTATGGTATTCAG
HA-DTX3L-D1-R	GATGCCCTTTTCCTTCTTGTC
HA-DTX3L-D2-F	TAAGCGGCCGCGGGGATC
HA-DTX3L-D2-R	CTGGCATGTGGGACAGATTG
HA-DTX3L-D3-F	TAAGCGGCCGCGGGGATC
HA-DTX3L-D3-R	CTTGTCCAGTTCTGAGGC
HA-DTX3L-C561S/C564S-F	AGTGTCATCAGTATGGACACCATTAGTAAC
HA-DTX3L-C561S/C564S-R	GATTGGCTTATATGACATGGC
DTX3L-PLVX3-ECORI-F	GAGGATCTATTTCCGGTGAATTATGGCCTCCCACCTGCGC
DTX3L-PLVX3-BAMHI-F	GGGAGAGGGGCGGATCTTACTCAATTCCTTTGGCTTTCAGCTCCT