OMTO, Volume *26*

Supplemental information

Stabilization of DEPTOR sensitizes hypopharyngeal cancer to radiotherapy via targeting degradation

Xuecen Wang, Zhirui Cao, Xin Yue, Tingyu Liu, Gesi Wen, Dongmei Jiang, Weijian Wu, Liyuan Le, Yan Wang, Chengtao Wang, Ziyang Wang, Meng Jin, Meiyan Zhu, Shasha He, Xiaoyue Zhang, Xianzhang Bu, Ran-yi Liu, Zhenwei Peng, and Yong Chen

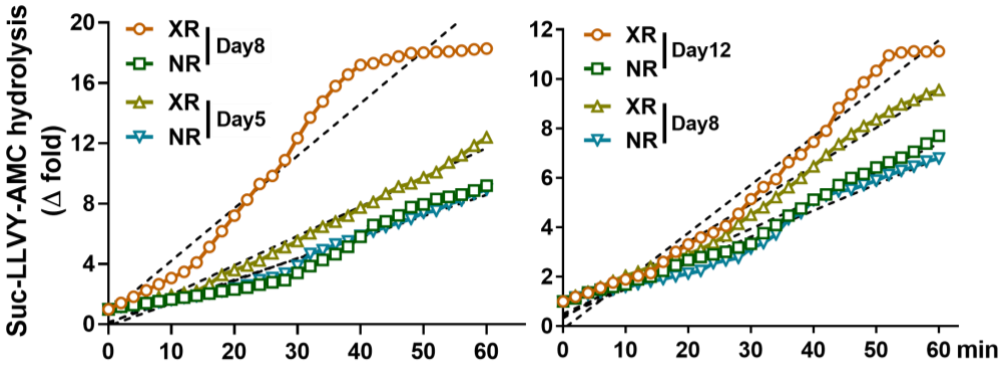


Fig. S1. XR induces the increase proteasome hydrolysis activities.

The proteasome hydrolysis activities in 2D adherent monolayers (Left) and 3D-spheroid suspensions (Right) FaDu cells lysis treated as in Figure 1A. (Related to Fig. 1B)

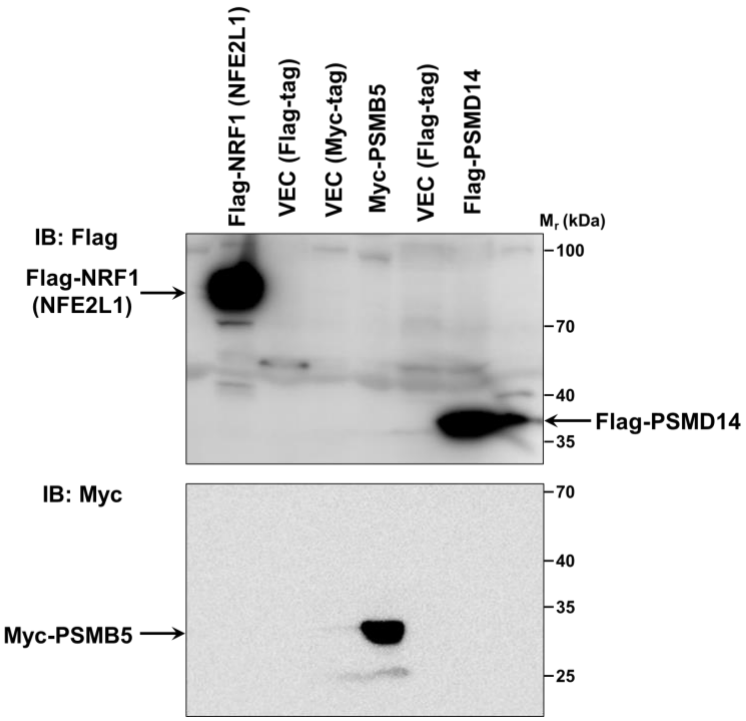
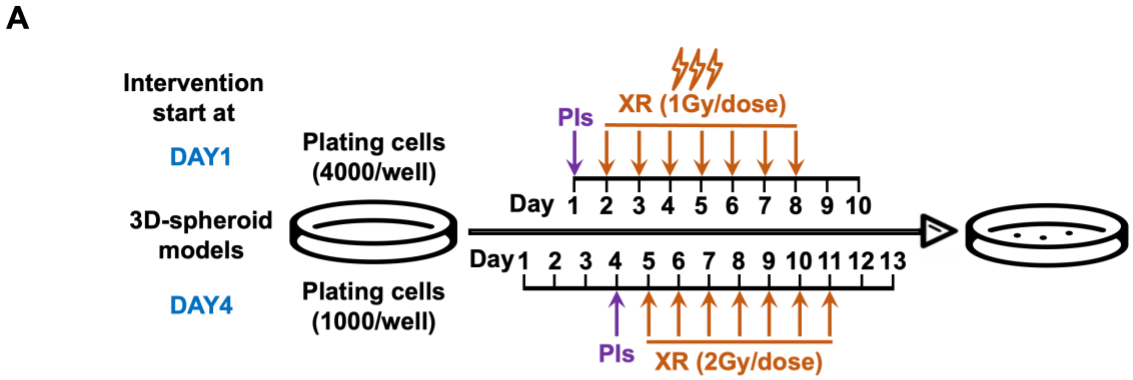
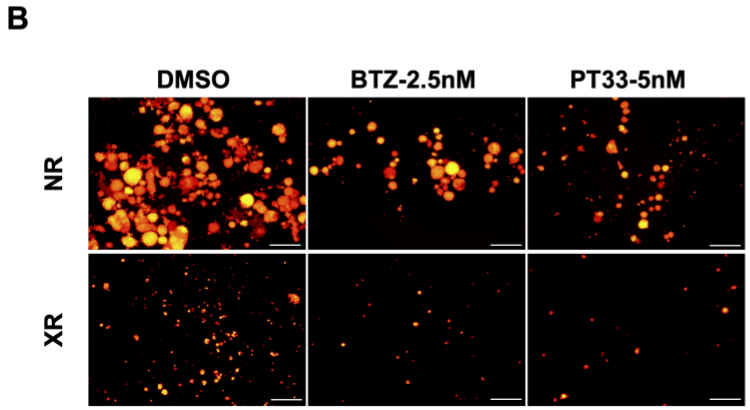
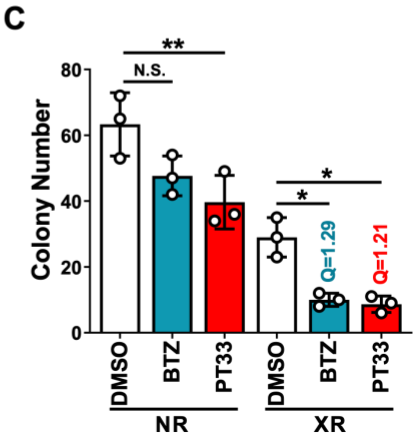


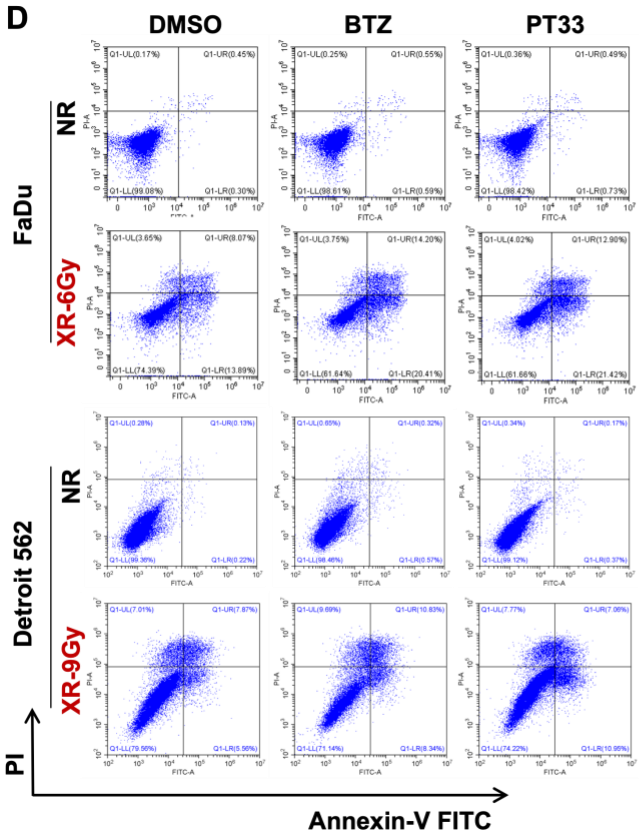
Fig. S2. Validation of plasmid overexpression.

Western blot analysis showing the validation of the exogenous overexpression of FLAG-NRF, FLAG-PSMD14 and Myc-PSMB5 in FaDu cells. (Related to Fig. 1D-H)


A
Intervention start at
DAY1
Plating cells (4000/well)
3D-spheroid models
DAY4
Plating cells (1000/well)
PIs
XR (1Gy/dose)
Day 1 2 3 4 5 6 7 8 9 10
Day 1 2 3 4 5 6 7 8 9 10 11 12 13
PIs
XR (2Gy/dose)


B
DMSO
BTZ-2.5nM
PT33-5nM
NR
XR
Scale Bars_500μm


C
Colony Number
80
60
40
20
0
**
N.S.
*
*
Q=1.29
Q=1.21
DMSO
BTZ
PT33
DMSO
BTZ
PT33
NR
XR


D
DMSO
BTZ
PT33
FaDu
NR
XR-6Gy
Detroit 562
NR
XR-9Gy
PI
Annexin-V FITC

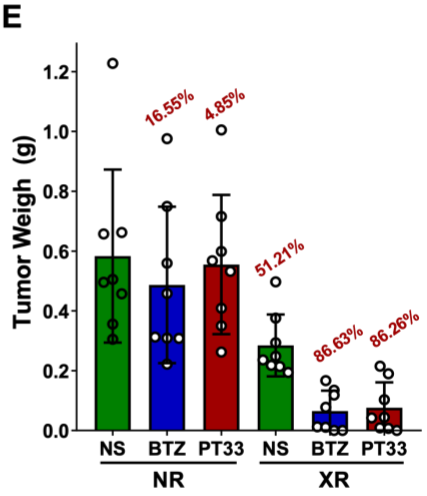

E
Tumor Weigh (g)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
16.55%
4.85%
51.21%
86.63%
86.26%
NS
BTZ
PT33
NS
BTZ
PT33
NR
XR

Fig. S3. Proteasome inhibitors sensitize HC cells to radiation.

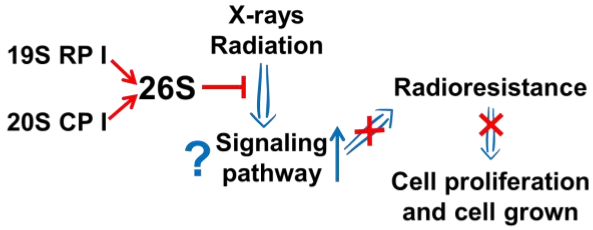
(A) Schematic illustration showing the treatment of XR plus PIs in two different modes in conditional three-dimensional spheroid culture models: cells were treated starting on day 1 (before forming spheroid) or day 4 (forming spheroid) after planting FaDu cells expressing mCherry. (related to Fig. 2C, 2E & S3B and S3C)

(B-C) Conditional 3D spheroid formation assays for FaDu cells treated with PIs (DMSO as vehicle control) plus XR (NR as control) starting on day 1 as in S3A. (B) Representative images. (C) Numbers of spheres (diameter ≥ 75 μm) from 3 independent experiments, shown as mean ± S.D (1-way ANOVA). Q value indicated the combination effect of XR treatment and PIs, referring to the legend for panels Fig. 2E.

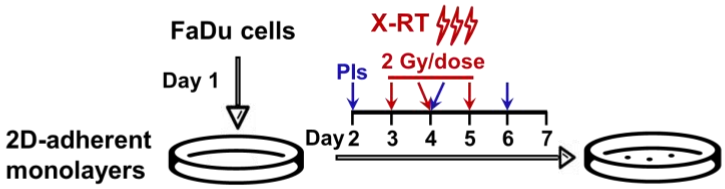
(D) Representative images of Annexin V/propidine iodide double-staining assays with flow cytometry in FaDu and Detroit 562 cells at 72 h after XR (6-Gy) treatment. Cells were pretreated with PIs (BTZ: 5 nM, PT33: 10 nM; DMSO as vehicle control) 12 h before XR. (related to Fig. 2F)

(E) The xenograft tumor weights were measured and the inhibitory rates were calculated. (Related to Fig. 2H) *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; N.S., not significant.

A


19S RP I
20S CP I
26S
X-rays
Radiation
?
Signaling
pathway
Radioresistance
Cell proliferation
and cell grown

B


FaDu cells
Day 1
2D-adherent
monolayers
X-RT
2 Gy/dose
PIs
Day 2 3 4 5 6 7

C

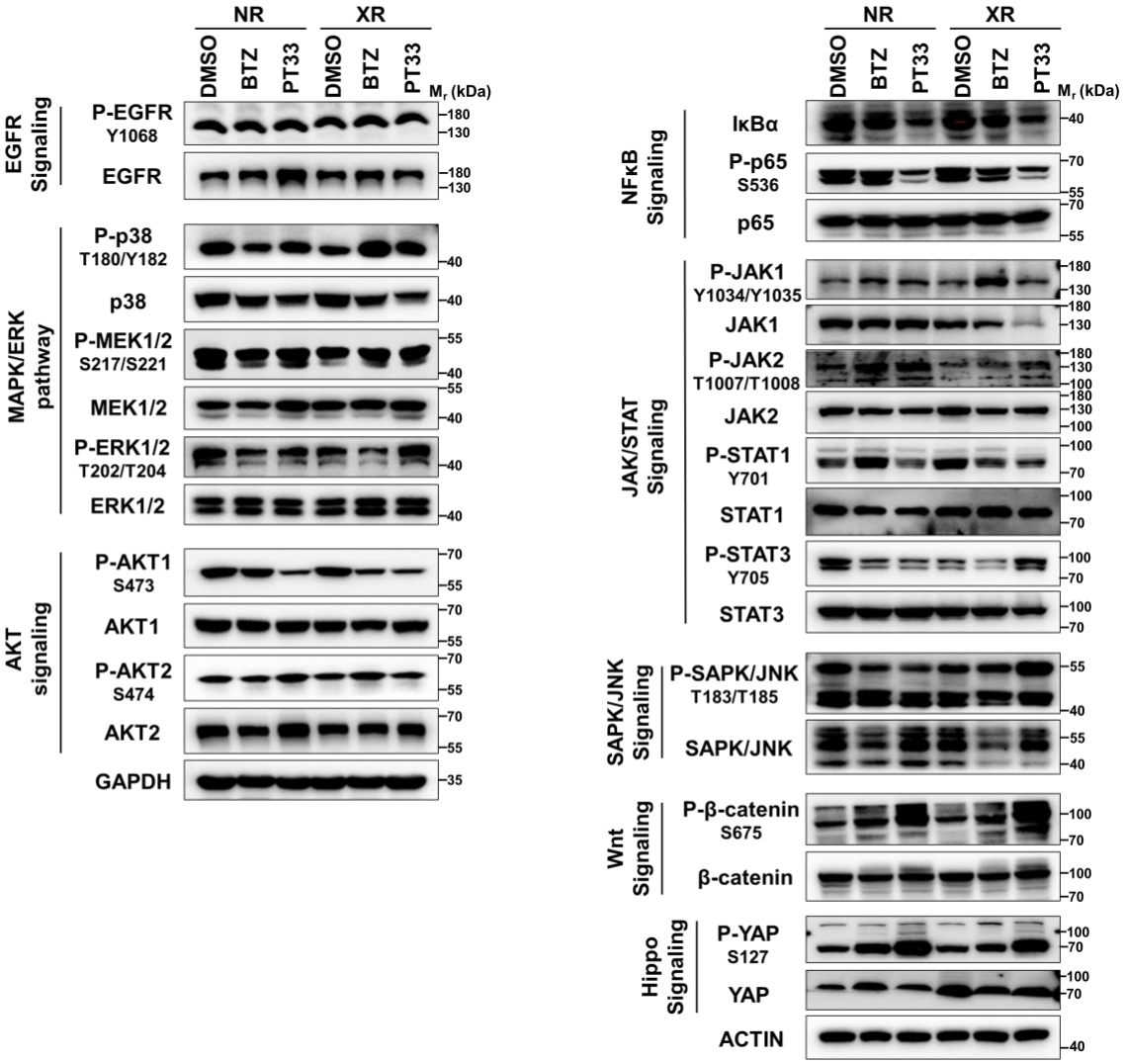

NR
XR
DMSO
BTZ
PT33
M, (kDa)
EGFR Signaling
P-EGFR Y1068
EGFR
MAPK/ERK pathway
P-p38 T180/Y182
p38
P-MEK1/2 S217/S221
MEK1/2
P-ERK1/2 T202/T204
ERK1/2
AKT signaling
P-AKT1 S473
AKT1
P-AKT2 S474
AKT2
GAPDH
NFκB Signaling
IκBα
P-p65 S536
p65
JAK/STAT Signaling
P-JAK1 Y1034/Y1035
JAK1
P-JAK2 T1007/T1008
JAK2
P-STAT1 Y701
STAT1
P-STAT3 Y705
STAT3
SAPK/JNK Signaling
P-SAPK/JNK T183/T185
SAPK/JNK
Wnt Signaling
P-β-catenin S675
β-catenin
Hippo Signaling
P-YAP S127
YAP
ACTIN

Fig. S4. Inhibition of proteasome suppresses XR-induced elevation in mTORC1 signaling.

(A) Schematic illustration showing the design of the signaling pathway screening.

(B) Schematic illustration showing the treatment of the combination of XR and PIs (BTZ: 10 nM, PT33: 20 nM) in FaDu cells. After plating the cells on Day 1, cells were treated with PIs on day 2, 4, 6, meanwhile, XR treatment of 2-Gy/day was carried out on day 3-5. (Related to Fig. 3A and Fig. S4C)

(C) Western blot assays of the activities of multiple vital signal pathways (including EGFR, MAPK/ERK, AKT signaling, NF-κB, JAK/STAT, SAPK/JNK, Wnt, Hippo signaling) in FaDu cells treated with PT33/BTZ (DMSO as vehicle control) plus XR (NR as control) as in **(B)**.

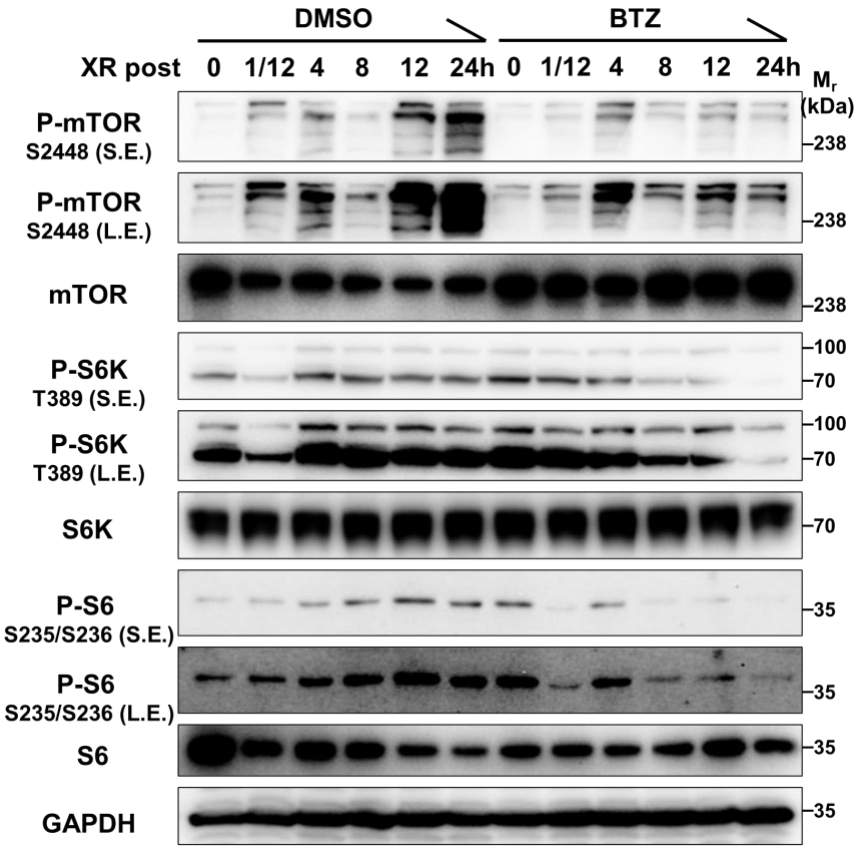


Fig. S5. BTZ suppresses XR-induced elevation in mTORC1 signaling.

Western blot analysis of the process of the mTORC1 signaling activation in FaDu cells treated with BTZ (10 nM, DMSO as vehicle control) followed by XR (6-Gy) at indicated times.

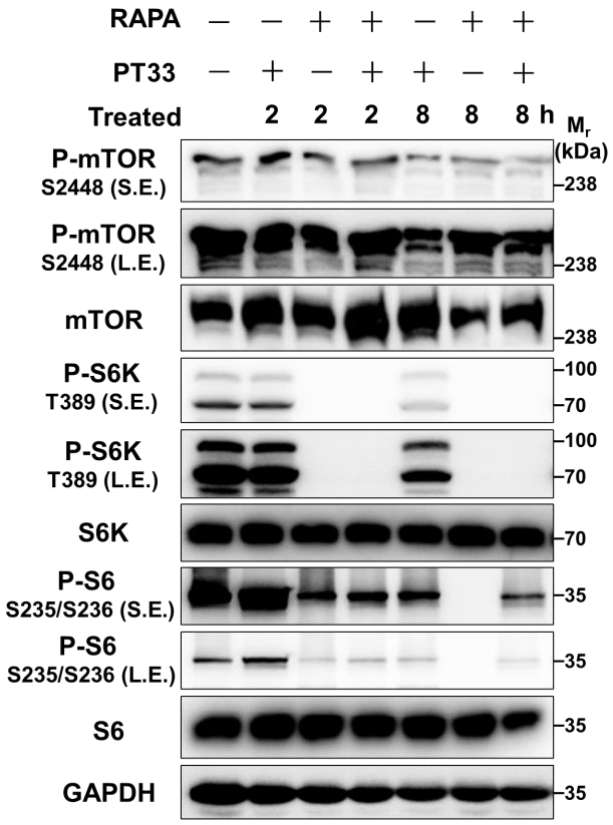


Fig. S6. Effects of RAPA/PT33 on mTORC1 signaling induced by XR.

Western blot analysis of the levels of mTORC1 signaling pathway components in FaDu cells treated with rapamycin (RAPA, 100 nM) or PT33(20 nM) for 2 or 8 h, DMSO as vehicle control. (Related to Fig. 3E)

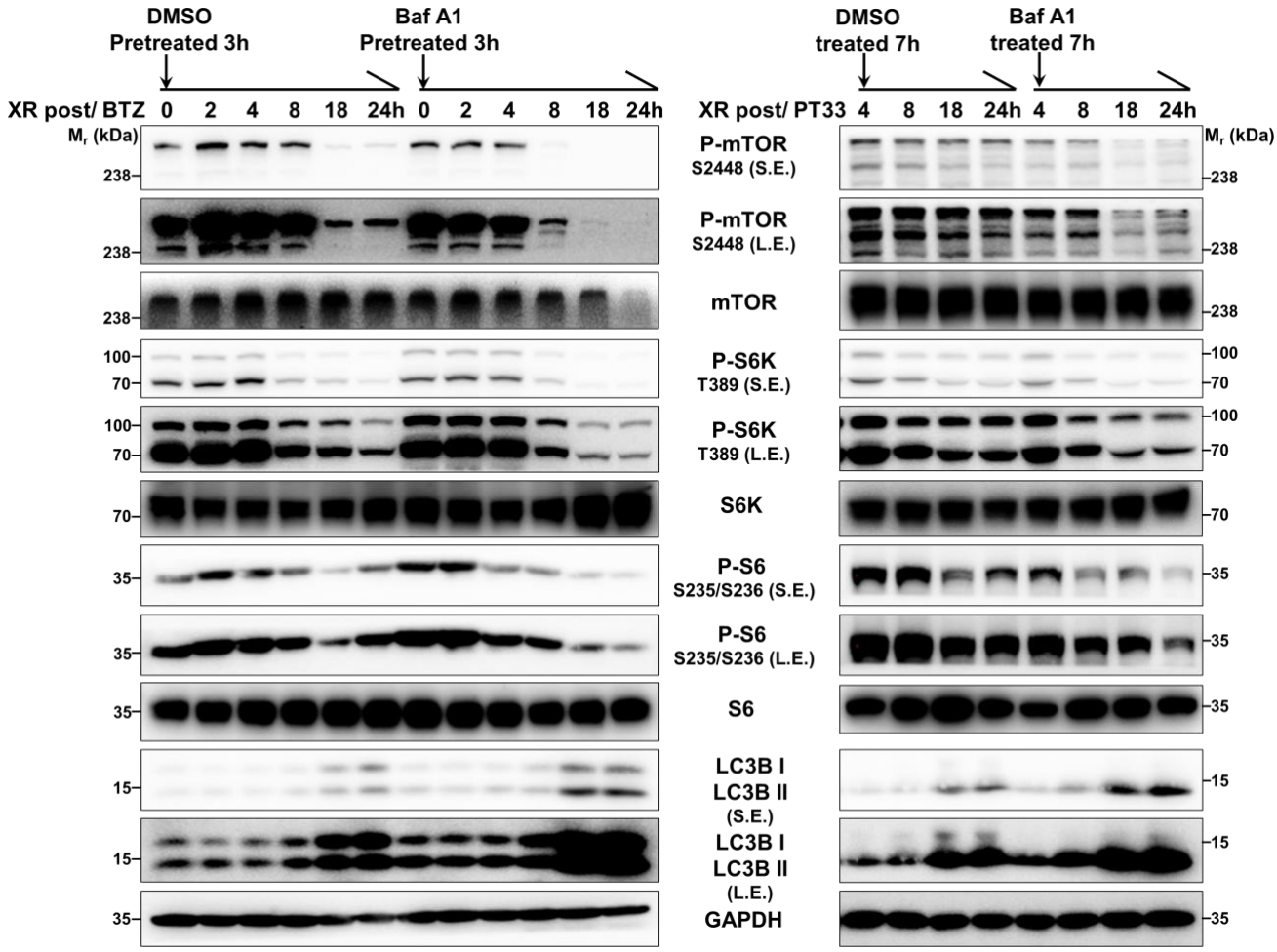


Fig. S7. Effects of Baf A1 on PI-mediated suppression of elevated mTORC1 signaling after XR. Western blot analysis of the influence on mTORC1 signaling in FaDu cells pretreated with Baf A1 (100 nM) for 3 h before BTZ (10 nM) plus XR treatment (Left) or for 7 h before PT33 (20 nM) plus XR treatment (Right) (6-Gy), DMSO as vehicle control.

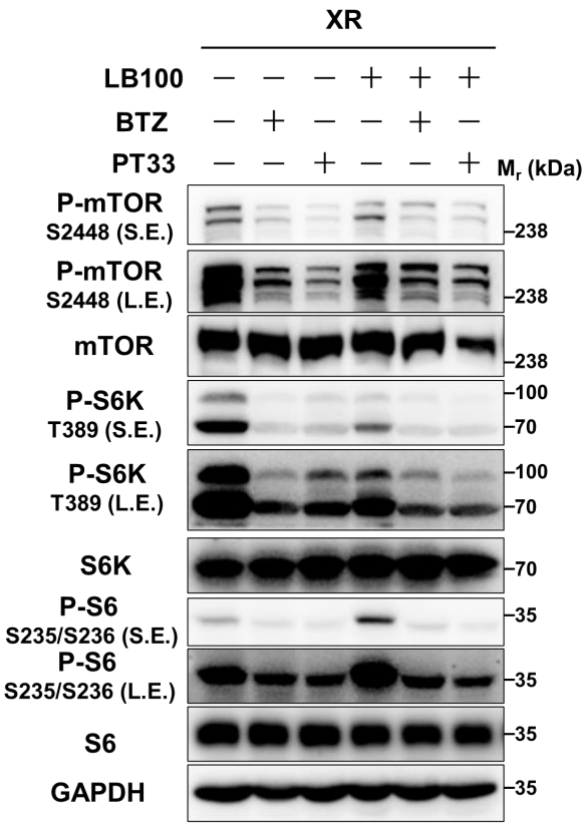


Fig. S8. Effects of LB100 on PI-mediated suppression of elevated mTORC1 signaling after XR. Western blot analysis of the influence on mTOR signaling by LB100 in FaDu cells, which after treated with 6-Gy XR plus PIs (10 nM BTZ or 20 nM PT33, DMSO as vehicle control).

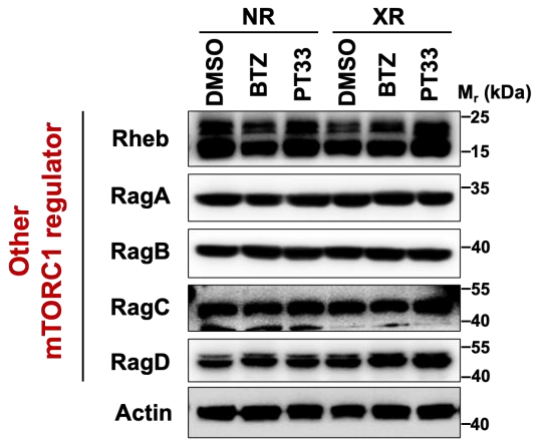


Fig. S9. The effects of BTZ/PT33 plus XR on other mTORC1 components/regulators. Western blot analysis of the levels of the other mTORC1 components/regulators in FaDu cell s treated with PIs (DMSO as vehicle control) plus XR (NR as control) treatment as in Fig. S4B. (Related to Fig. 4A)

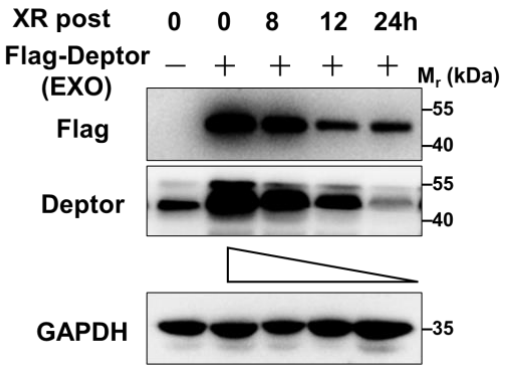


Fig. S10. Effects of XR on DEPTOR.

Western blot analysis of the levels of endogenous DEPTOR and exogenous FLAG-tagged DEPTOR after XR (6-Gy) treatment at the indicated time.

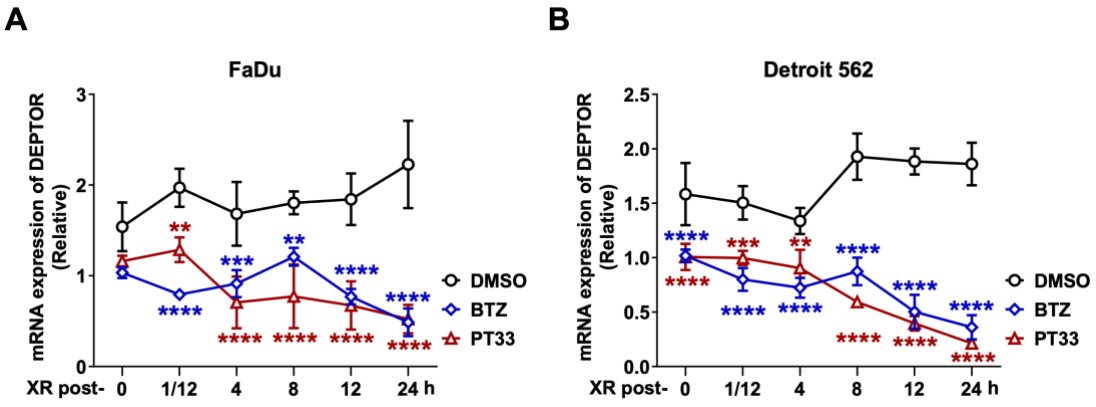


Fig. S11. PIs efficiently maintained the stability of DEPTOR after XR treatment at mRNA levels.

RT-qPCR assays of the DEPTOR levels in **(A)** FaDu and **(B)** Detroit 562 cells treated with BTZ (20 nM) or PT33 (10 nM) followed by XR for indicated times at the mRNA levels. Data are presented as mean ± S.D (n=3). All data are presented as mean ± S.D. (n = 3). 1 way ANOVA. **P < 0.01; ***P < 0.001; ****P < 0.0001.

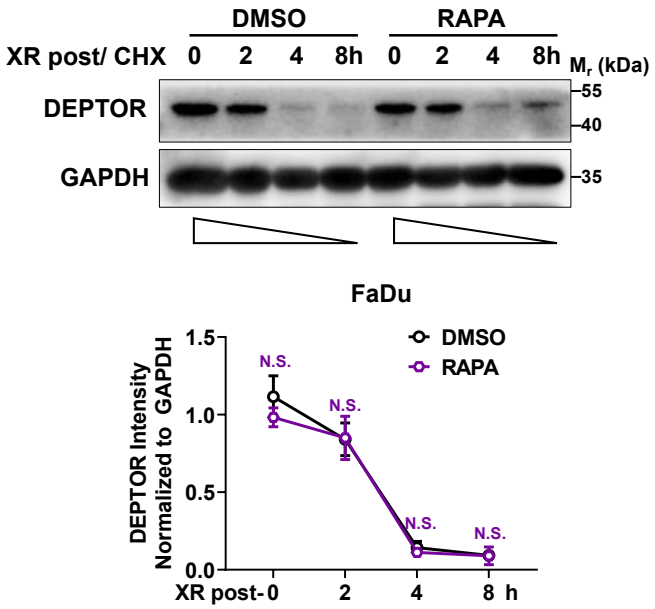


Fig. S12. Rapamycin hardly affected DEPTOR persistently after XR plus CHX treatment. Western blot analysis of the DEPTOR levels in FaDu cells treated with rapamycin (RAPA, 100 nM) for 12 h before CHX (50 μM) plus XR treatment for the indicated time. (Upper) Representative images; (Below) the relative DEPTOR levels, shown as the DEPTOR grayscale intensity normalized to GAPDH at indicated times after XR treatment. Data are presented as mean ± S.D (n=3). All data are presented as mean ± S.D. (n = 3). 2-sample t-test. N.S., not significant.

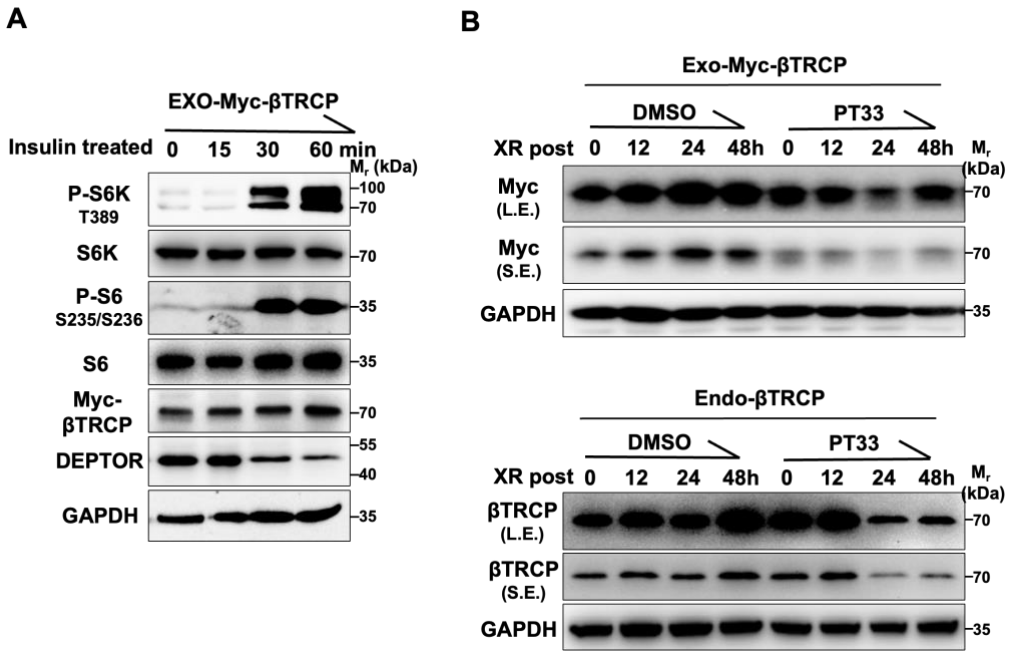


Fig. S13. The effects of Insulin or XR plus PT33 on β-TRCP.

(A) Western blot analysis of mTORC1 signaling and Myc-βTRCP levels in FaDu cells transfected exogenous Myc-βTRCP and treated with Insulin for indicated the time.

(B) Western blot analysis of the exogenous (Upper) and endogenous (Below) Myc-βTRCP levels in FaDu cells pretreated with PT33 (10 nM), and consequently exposed to XR treatment for the indicated time.

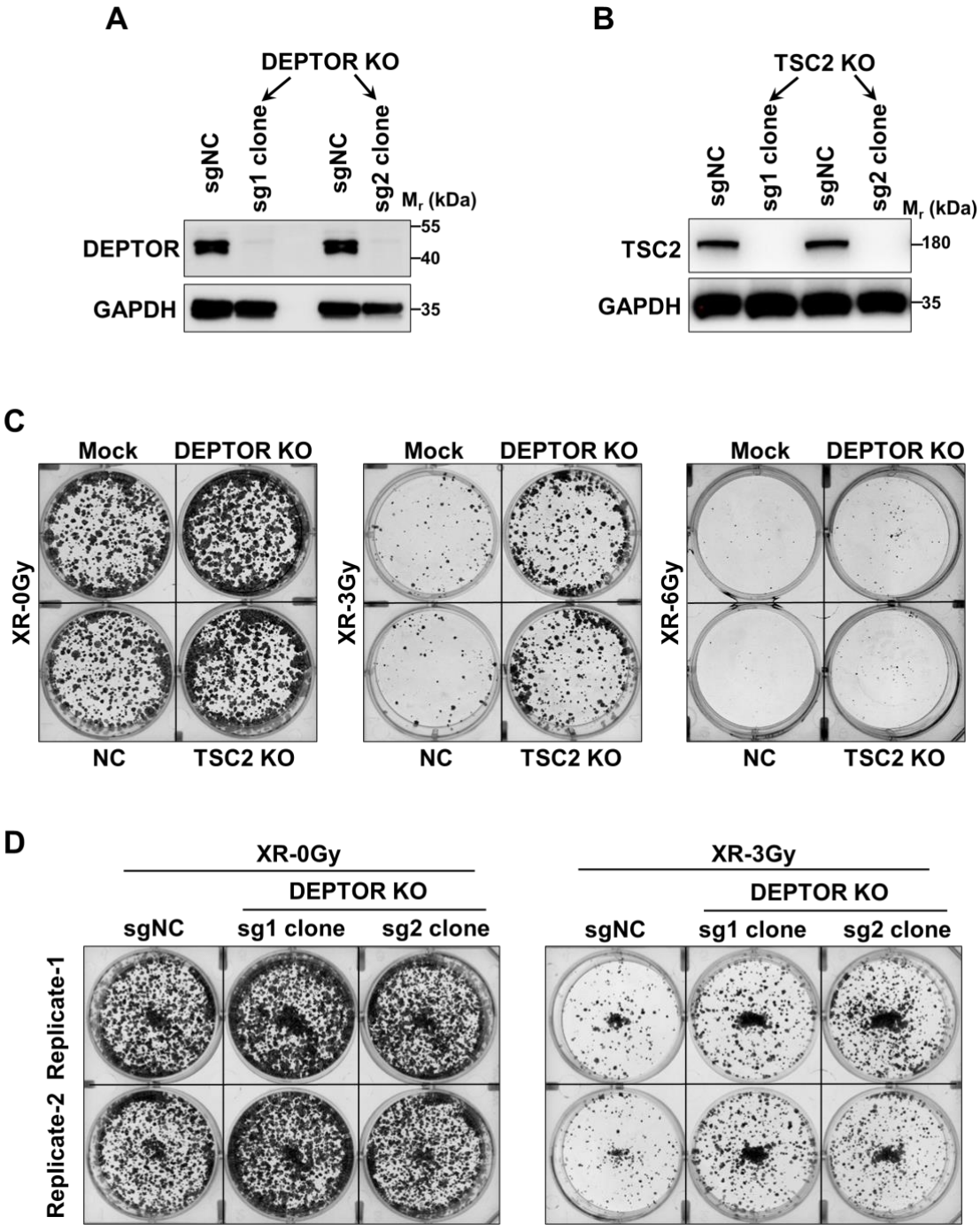


Fig. S14. Effects of DEOTOR or TSC2 on mTORC1-governed radioresistance.

DEPTOR- **(A)** and TSC2- **(B)** Knockout (KO) efficiencies by CRISPR-Cas9 gene editing were validated by western blot. **(C-D**), Representative images of 2D adherent colony formation assays in DEPTOR/TSC2 CRISPR KO FaDu cells treated with a single 3- or 6-Gy dose of XR. (Related to Fig. 4G)

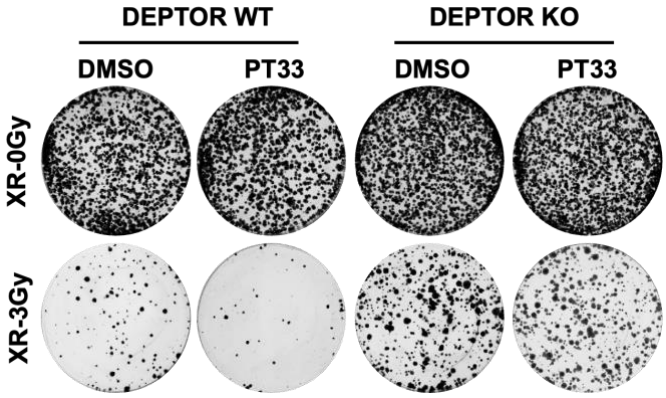


Fig. S15. Effects of DEOTOR on radioresistance in cells treated with PT33 plus XR.

Representative images of 2D adherent colony formation assays in DEPTOR-KO or WT FaDu cells pretreated with PT33 (10 nM), and consequently exposed to 3-Gy XR treatment. (Related to Fig. 4I)

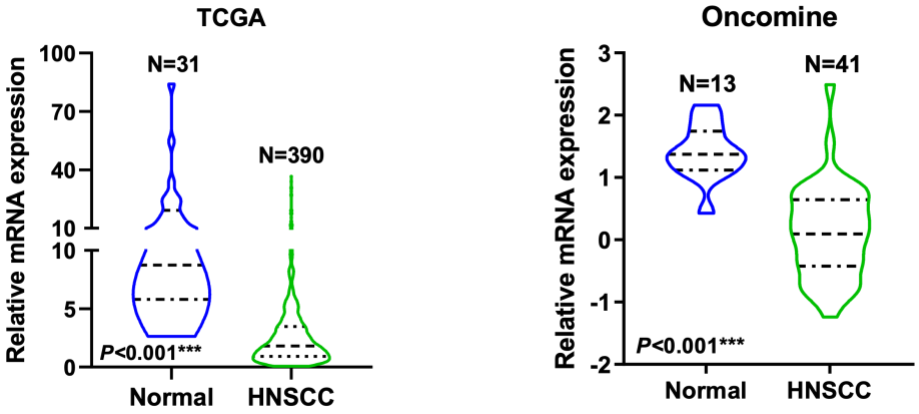


Fig. S16. DEPTOR levels in gene expression public databases.

Expression levels of DEPTOR in HC and normal tissues in **(Left)** TCGA and **(Right)** Oncomine databases.

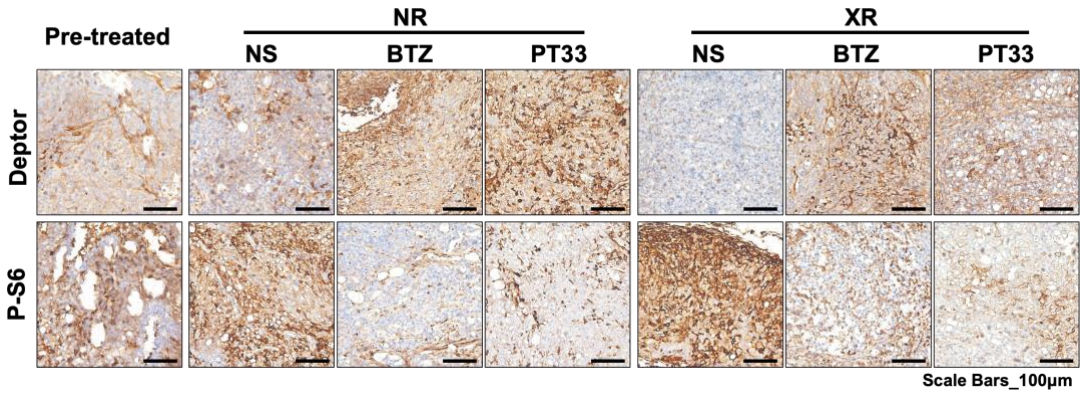


Fig. S17. Representative IHC images.

The expression of DEPTOR or P-S6 in nude mice bearing subcutaneous FaDu xenograft with the combined treatment of PIs (Normal Saline group as vehicle control) and XR (NR as control).

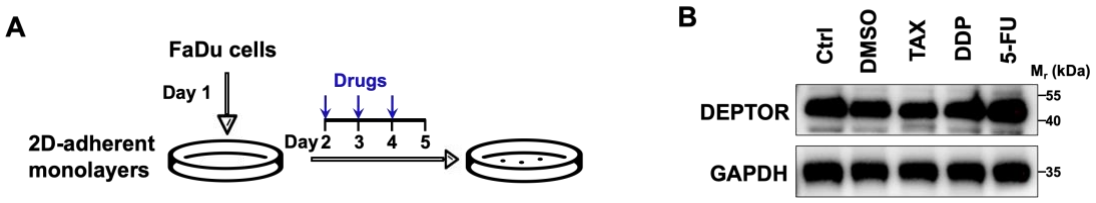


Fig. S18. Effects of chemotherapy drugs on DEPTOR.

Western blot analysis of DEPTOR levels in FaDu cells with various chemotherapy drugs treatment. **(A)** Schematic illustration showing the treatment method of drugs. **(B)** Representative images.

Table S1. Univariate and multivariate Cox regression analyses of prognostic factors for hypopharyngeal cancer patient

Variables	PFS				OS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Gender (Female vs. male)	0.042 (0.000-15.145)	0.292			0.04 (0-23.056)	0.321		
Age (y) (>58 vs. ≤58)	1.661 (0.760-3.629)	0.203			1.089 (0.438-2.704)	0.855		
Smoke (Yes vs. No)	1.416 (0.568-3.533)	0.456			1.971 (0.646-6.008)	0.233		
Drink (Yes vs. No)	1.560 (0.656-3.713)	0.315			2.810 (0.919-8.599)	0.07		
cT (III-IV vs. I-II)	1.101 (0.462-2.625)	0.829			2.788 (0.806-9.637)	0.105		
cN (2-3 vs.0-1)	1.184 (0.475-2.951)	0.717			2.084 (0.605-7.179)	0.245		
cTNM (IVA-IVB vs. II-III)	0.637 (0.283-1.431)	0.275			1.174 (0.422-3.268)	0.758		
Pathology classification					0.690 (0.264-1.801)	0.448		
(High-In situ vs. Low-Intermediate)	0.992 (0.420-2.342)	0.986						
Induced chemotherapy (Yes vs. No)	0.550 (0.128-2.356)	0.421			0.212 (0.047-0.960)	**0.044**		
Induced chemotherapy regimens					1.243 (0.764-2.021)	0.381		
(TPF vs. TP vs. PF vs. TPF/TP+PD-L1)	1.478 (0.971-2.249)	0.068						
Concurrent chemotherapy (Yes vs. No)	0.501 (0.171-1.473)	0.209			0.266 (0.085-0.830)	**0.022**		
tumour size (cm) (≥4 vs. > 2-≤4 vs. ≤2)	1.183 (0.684-2.047)	0.548			2.187 (1.079-4.430)	**0.03**		
RECIST group (SD/PD vs. CR/PR)	2.771 (1.192-6.438)	**0.018**			3.117 (1.131-8.588)	**0.028**		
Deptor (high vs. low)	0.102 (0.038-0.278)	**<0.001**	0.09 (0.030-0.271)	**<0.001**	0.203 (0.072-0.572)	**0.003**	0.180 (0.058-0.561)	**0.003**
p-S6 (high vs. low)	4.926 (1.959-12.384)	**0.001**			5.334 (1.754-16.215)	**0.003**		

Boldface values are statistically significant.

Table Footnotes: Abbreviations: HR, hazard ratio; CI, confidence interval. cT, clinical T stage; cN, clinical N stage

Table S2. Realtime q-PCR primers, related to method

Primers	5’ to 3’
DEPTOR-F	GCAGCAGGAATGAAGGTCTG
DEPTOR-R	GTATGTGCGGAGAAGACTCGTAT
GAPDH-F	GAAGGTGAAGGTCGGAGTC
GAPDH-R	GAAGATGGTGATGGGATTTC