Supplementary Materials and Methods

Chromosome aberration analysis Chromosome aberrations were analyzed using the standard metaphase spread assay. Cells at exponential growth were plated one day before treatments. At 48 hr post radiation, colcemid (Sigma) were added for 2 hours to impede mitosis; mitotic cells were then collected, washed, and suspended in warm hypotonic (75 mM KCl + 10% FBS) solution for 20 min at 37 ºC. Cells were fixed in 3:1 methanol: glacial acetic acid at 4°C overnight. Cell suspensions were dropped onto warm slides at 50 ºC, and air dried for 24 hours afterwards. Chromosomes were stained with DAPI in Vectashield (Sigma). Metaphases were imaged using Zeiss AxioImager 2 at 63x magnification. Chromosome aberrations were scored manually by three blinded experimenters from at least 100 metaphases in each treatment conditions of three independent experiments.

Supplemental Data

Figure S1. TGFβ signaling is truncated in HPV-positive HNSCC. **A,** TGFβ gene signature contains TGFβ target genes (n = 50) that were up-regulated by TGFβ and down-regulated by TGFβ inhibitor on MCF10A cells. **B,** Histology and HPV status of HNSCC cell lines used in this study. **C,** Protein bands of canonical TGFβ components in five HNSCC cells. **D,** Representative images of PDX tumor specimens with hematoxylin and eosin staining. **E,** Patient characteristics of the tissue array used in Fig. 1F. **F,** Representative images to show scored p-SMAD2 intensity levels in patient tissue array (upper panel). P-SMAD2 intensity levels in epithelium were shown according to the HPV status in the respectivetumors. Mann-Whitney U test: NS, not significant.

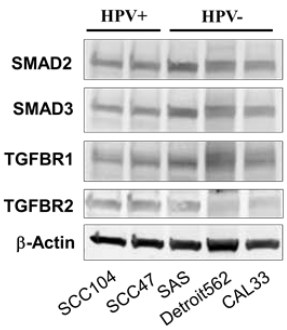
A

Genes upregulated by TGFβ (50)	
ABCG1	*LARP6*
AMIGO2	*LIPG*
CA12	*MAFF*
CCDC99	*MMD*
CCL20	*PDGFC*
CHRNA9	*PLEK2*
COL4A2	*PLXNA2*
CTGF	*POSTN*
DLC1	*PSCD1*
DNAJB9	*RICS*
DSC2	*RNF24*
ENC1	*RUNX1*
F3	*SAMSN1*
FAP	*SERPINE1*
FGF2	*SERPINE2*
FN1	*SH2D2A*
HEY1	*SH2D4A*
HMGA2	*SLC20A1*
ID1	*SLC22A4*
IGF2BP3	*TGIF1*
IGFBP3	*THBS1*
JAG1	*TMEPAI*
KLF4	*TNC*
LAMB3	*TNFRSF12A*
LAMC2	*VACN*

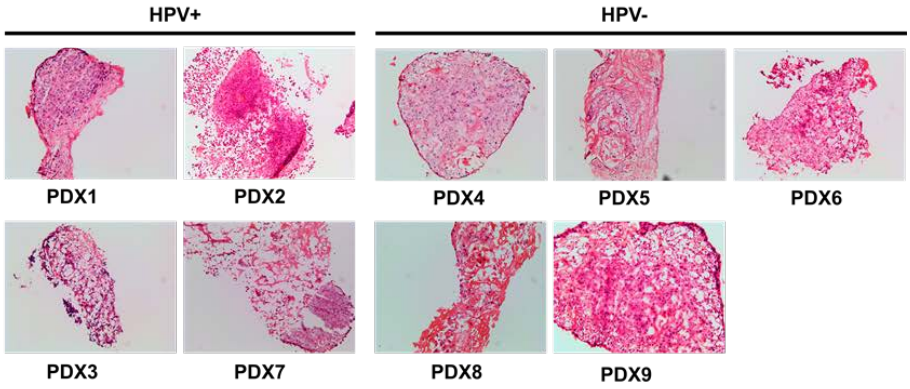
B

Cell line	Site	HPV
CAL-33	Tongue	negative
HSC-2	Oral cavity	negative
Detroit562	Pharynx	negative
FADU	Hypopharynx	negative
SAS	Tongue	negative
UPCI:SCC154	Base of tongue	HPV16
UM:SCC47	Tongue	HPV16
UM:SCC104	Oral cavity	HPV16
UPCI:SCC90	Base of tongue	HPV16

C



D



E

Parameter		N of 194	Fraction (%)
Gender	Male	155	79.9
	Female	39	20.1
UICC stage	II	8	4.1
	III	32	16.5
	IVa, b	154	79.4
Tumor localization	Oral cavity	53	27.3
	Oropharynx	114	58.8
	Hypopharynx	27	13.9
R status	Negative	108	55.7
	Positive	85	43.8
	Missing	1	0.5
ECE status	Negative	91	46.9
	Positive	103	53.1
HPV16 DNA	Negative	129	66.5
	Positive	65	33.5
Parameter			**Median (range)**
Age (years)			57.0 (29.0-75.7)
Dose (Gy)			64.0 (56.0-68.4)
Treatment time (days)			44.0 (32.0-57.0)

F

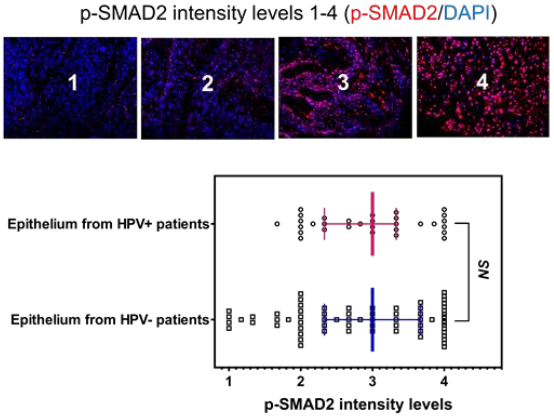


Figure S2. TGFβ signaling determines radiosensitivity in HNSCC. **A,** Correlation of percent nuclear p-SMAD2 positive cell following TGFβ treatment (from Fig. 1C) with SRF_{2Gy} of LY364947 (from Fig. 2B). Linear regression analysis was used to calculate *P* value and R^2. **B,** SRF_{2Gy} measured by a 5 day cell viability assay with TGFβ neutralizing antibody, 1D11, or receptor kinase inhibitors, LY364947 and LY2157299, on HPV- SAS or **C**,HPV+ SCC47 cell line. **D**, Relative cell number after combination treatments with cisplatin (5 μM) for 1 hr and 2 Gy, with or without LY364947. Two-tailed Student's t-test; *, $P < 0.05$.

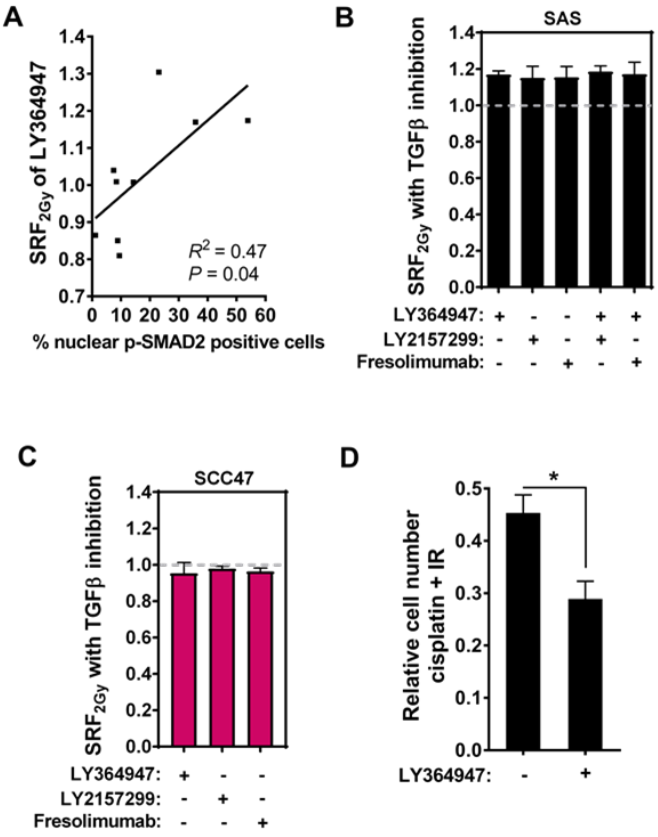
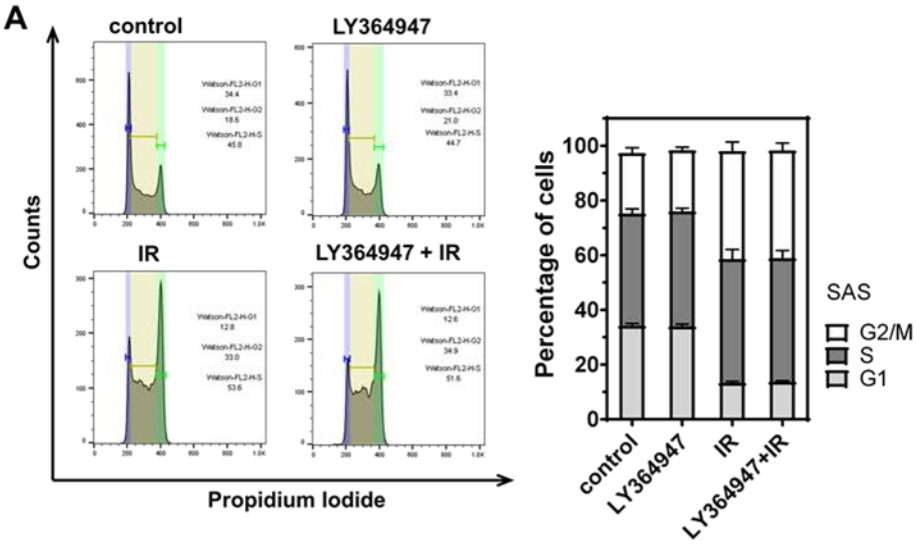
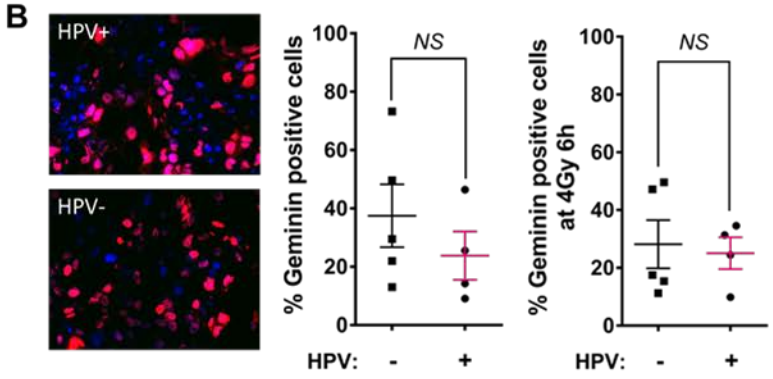
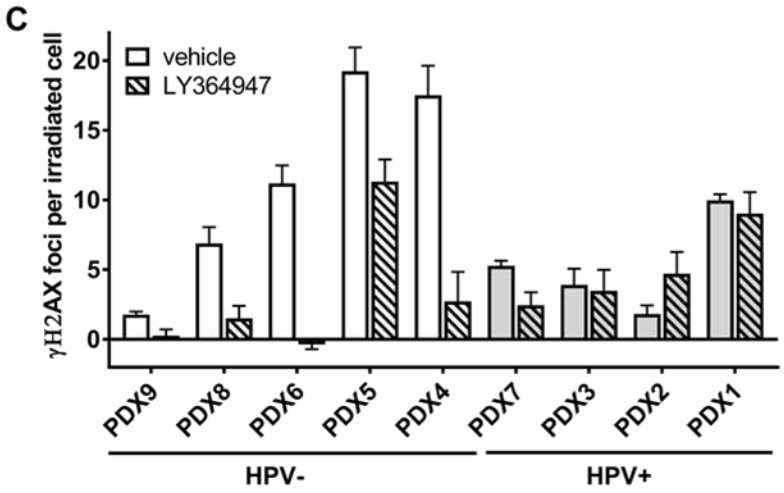

A
SRF2Gy of LY364947
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
R2 = 0.47
P = 0.04
0 10 20 30 40 50 60
% nuclear p-SMAD2 positive cells
B
SAS
SRF2Gy with TGFβ inhibition
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LY364947: + - - + +
LY2157299: - + - + -
Fresolimumab: - - + - +
C
SCC47
SRF2Gy with TGFβ inhibition
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
LY364947: + - -
LY2157299: - + -
Fresolimumab: - - +
D
Relative cell number cisplatin + IR
0.5
0.4
0.3
0.2
0.1
0.0
*
LY364947: - +

Figure S3. TGFβ inhibition effect on DDR is not due to cell cycle changes. **A,** Cell cycle distribution analyses after treatments with TβRI inhibitor LY364947, irradiation (4 Gy, 5 hr) or combinations. Percentage of cells in G1, S, or G2/M are shown on the right panel. **B,** Representative image from geminin (a cell proliferation maker) staining of PDX specimen; percentage of geminin positive cells were shown according to HPV status (right panel). Mann-Whitney U test; NS, not significant. **C,** Number of γH2AX foci per cell in irradiated (2 Gy) HPV- (white) and HPV+ (grey) PDX pre-treated with (hatched) or without (open) LY364947. **D,** IR induced p-ATM on SAS cells treated with or without LY364947.


A
control
LY364947
IR
LY364947 + IR
Counts
Propidium Iodide
Percentage of cells
100
80
60
40
20
0
SAS
G2/M
S
G1
control
LY364947
IR
LY364947+IR


B
HPV+
HPV-
% Geminin positive cells
NS
HPV: - +
% Geminin positive cells at 4Gy 6h
NS
HPV: - +


C
γH2AX foci per irradiated cell
vehicle
LY364947
PDX9
PDX8
PDX6
PDX5
PDX4
PDX7
PDX3
PDX2
PDX1
HPV-
HPV+

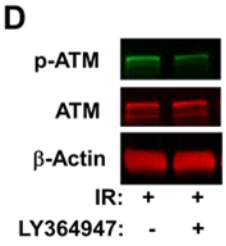

D
p-ATM
ATM
β-Actin
IR: + +
LY364947: - +

Figure S4. TGFβ controls DDR through miR-182. **A,** qRT-PCR of *BRCA1* expression in SAS or SCC47 treated with or without TGFβ. **B,** qRT-PCR of *BRCA1* expression in SAS cells treated with TGFβ or its inhibitor, LY364947. **C,** Protein bands of FOXO3 and β-actin measured on SAS cells, which were transfected with *FOXO3*-expressing plasmids or scramble control, and then treated with or without LY364947. Protein expression was quantified from western blots. **D,** Cell cycle distribution analyses after irradiation (4 Gy, 24 hr) on SAS cells transfected with mimic miR-182, anti-miR-182, or scramble RNA for two days. **E,** Nuclear p-SMAD2 intensity at two days after transfection with mimic miR-182, anti-miR-182, or scramble RNA. **F,** qRT-PCR of *BRCA1* expression in SAS cells transfected with scrambles, mimic miR-182, or anti-miR-182. **G,** Representative protein bands of FOXO3, BRCA1, and β-actin in transfected cells with scramble or anti-miR-182 and treated with or without LY364947. Quantified protein expression of BRCA1 from western blots; SAS cells transfected with scramble anti-miR (scr-anti-miR) or anti-miR-182 were treated with or without LY364947; difference in β-actin levels were corrected by normalization. Two-tailed Student's t-test; *, $P < 0.05$; **, $P < 0.01$; NS, not significant.

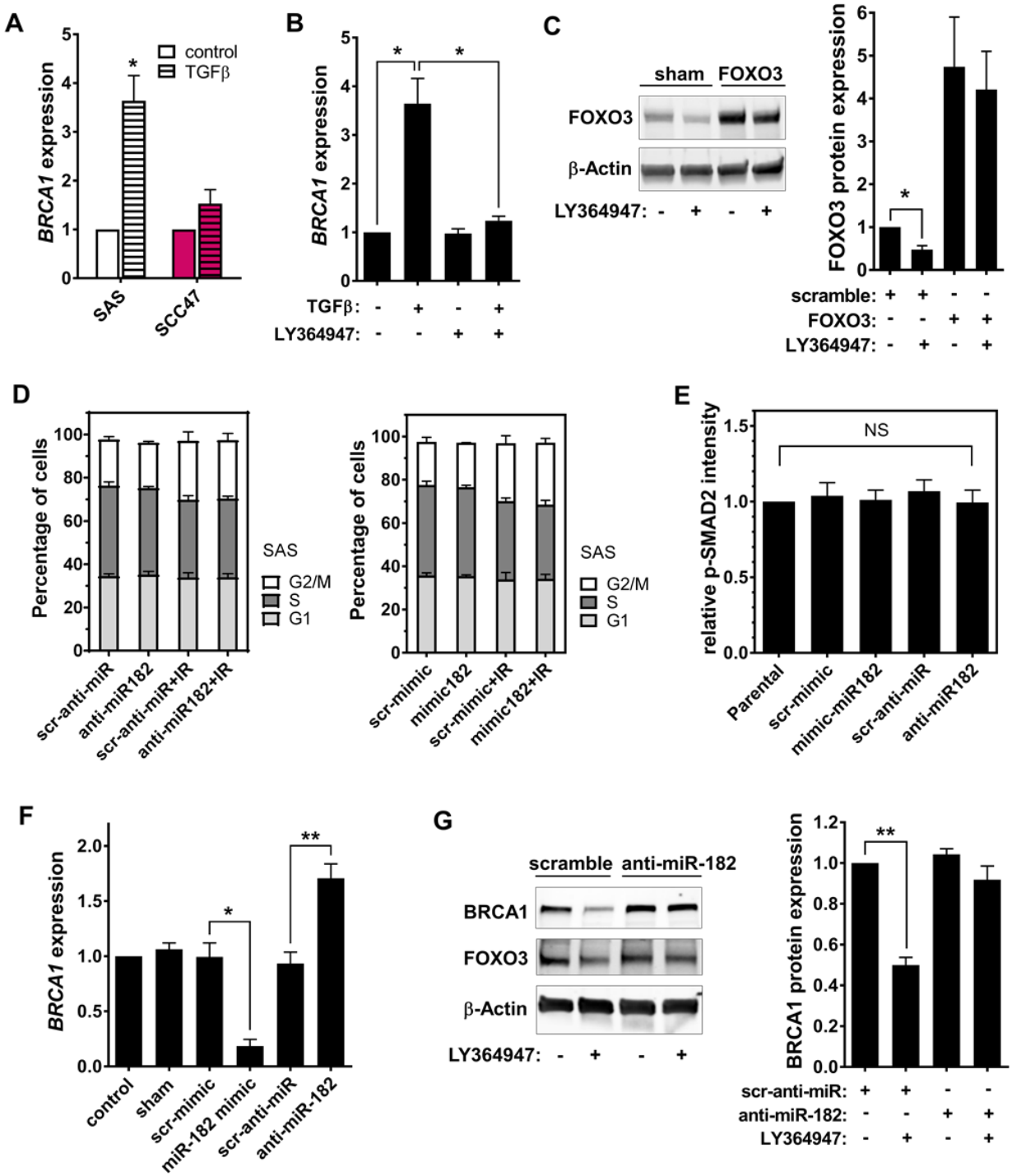

A
BRCA1 expression
control
TGFβ
SAS
SCC47
B
BRCA1 expression
TGFβ: - + - +
LY364947: - - + +
C
sham FOXO3
FOXO3
β-Actin
LY364947: - + - +
FOXO3 protein expression
scramble: + + - -
FOXO3: - - + +
LY364947: - + - +
D
Percentage of cells
SAS
G2/M
S
G1
scr-anti-miR
anti-miR182
scr-anti-miR+IR
anti-miR182+IR
scr-mimic
mimic182
scr-mimic+IR
mimic182+IR
E
relative p-SMAD2 intensity
NS
Parental
scr-mimic
mimic-miR182
scr-anti-miR
anti-miR182
F
BRCA1 expression
control
sham
scr-mimic
miR-182 mimic
scr-anti-miR
anti-miR-182
G
scramble anti-miR-182
BRCA1
FOXO3
β-Actin
LY364947: - + - +
BRCA1 protein expression
scr-anti-miR: + + - -
anti-miR-182: - - + +
LY364947: - + - +

Figure S5. Effects of TGFβ signaling in DDR is mediated through miR-182 and SMAD4. **A,** Representative western blot of p-ATM, ATM and β-actin on SAS cells which were transfected with scramble RNA or anti-miR-182 and irradiated (5 Gy) with or without LY364947. **B,** Clonogenic cell surviving fractions of SAS cells transfected with anti-miR scramble or anti-miR-182, and treated with or without TβRI inhibitor LY364947. Statistical difference between survival curves was from F-test; ***, $P < 0.005$. **C,** Surviving fractions and fitted curves from clonogenic assay on CAL33 cells treated with or without TβRI inhibitor LY364947 prior to irradiation. **D,** Protein bands of SMAD4 in five HNSCC cell lines. **E,** qRT-PCR analyses of miR182 expression in CAL33 cells treated with or without TGFβ. **F,** Protein bands of SMAD4 in SAS cells transducted with scramble or shRNA-SMAD4 lentivirus. **G,** Relative expression of miR182 in SMAD4 isogenic cells. **H,** SRF_{2Gy} of LY364947 on SAS isogenic cells from lentiviral transduction with scramble or shRNA against SMAD4. **I,** IC50 of cisplatin on HNSCC cell lines with wild type (WT) or mutant (Mut) *SMAD4* genes obtained from Sanger database [37]. Two-tailed Student's t-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

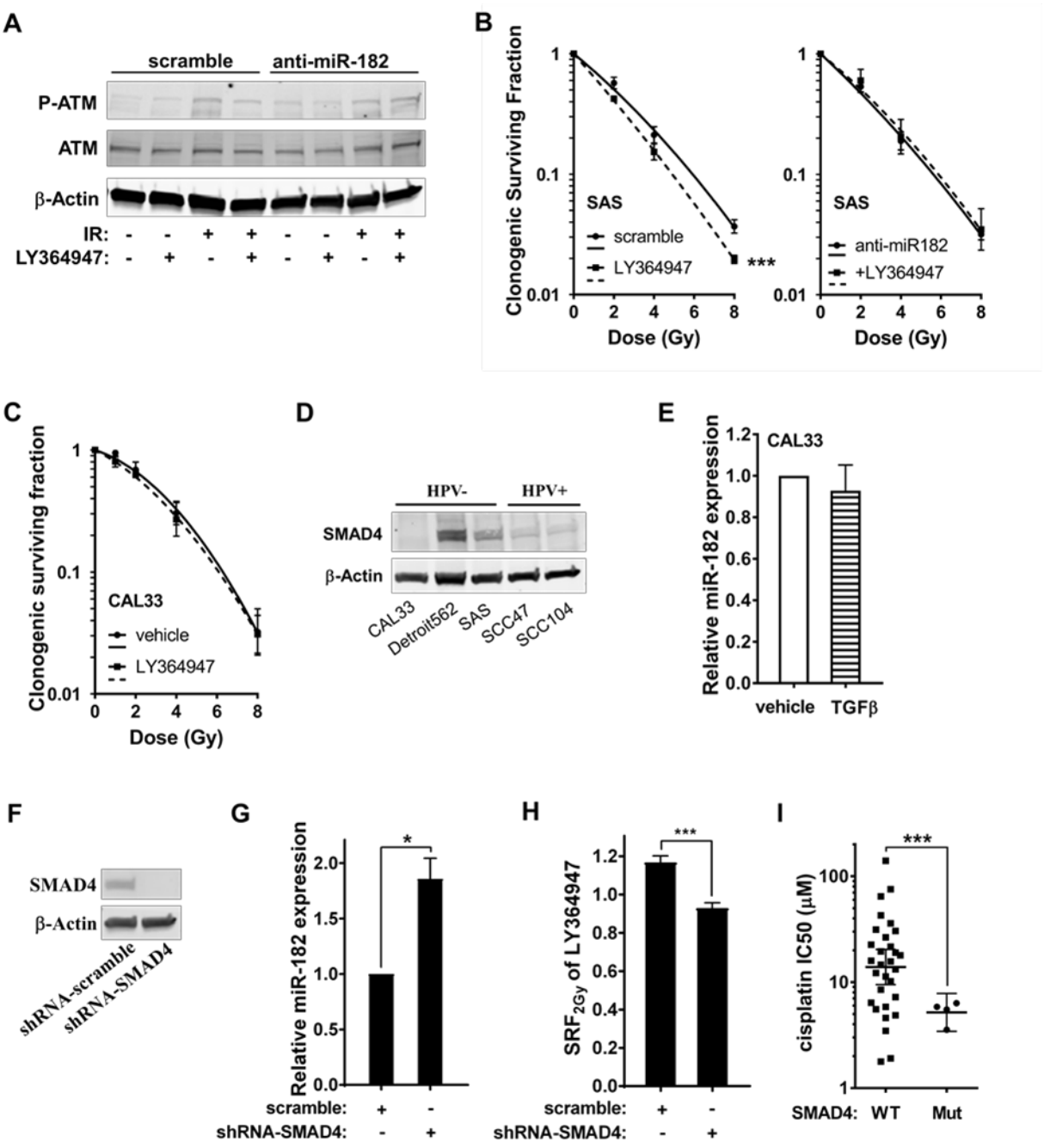

A
scramble
anti-miR-182
P-ATM
ATM
β-Actin
IR: - - + + - - + +
LY364947: - + - + - + - +
B
Clonogenic Surviving Fraction
SAS
scramble
LY364947
***
Dose (Gy)
anti-miR182
+LY364947
C
Clonogenic surviving fraction
CAL33
vehicle
LY364947
Dose (Gy)
D
HPV-
HPV+
SMAD4
β-Actin
CAL33
Detroit562
SAS
SCC47
SCC104
E
Relative miR-182 expression
CAL33
vehicle
TGFβ
F
SMAD4
β-Actin
shRNA-scramble
shRNA-SMAD4
G
Relative miR-182 expression
*
scramble: + -
shRNA-SMAD4: - +
H
SRF2Gy of LY364947
***
scramble: + -
shRNA-SMAD4: - +
I
cisplatin IC50 (μM)
***
SMAD4: WT Mut

Figure S6. Loss of TGFβ signaling increases sensitivity to PARP inhibition. **A,** RAD51 foci per cell from irradiated (4 Gy) SAS cells that were transfected with scrambled or anti-miR-182 with or without TβRI inhibitor LY364947. **B,** Relative cell number after olaparib treatments with or without LY364947. Data were fitted with 4th polynomial sigmoidal curve for calculations of half maximal inhibitory (IC50) concentrations, which was 5.6 μM for olaparib alone and 1.4 μM for olaparib in combination with LY364947, respectively. **C,** Relative cell number for isogenic SMAD4 SAS cells treated with PARP1 inhibitor AG14361 (i.e. PARP1i). **D,** Percentage of annexin V positive cells induced by AG14361 on the SMAD4 cell pair. **E,** IC50 of olaparib on HNSCC cell lines with wild type (WT) or mutant (Mut) *SMAD4* genes obtained from Sanger database [37]. Two-tailed Student's t-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

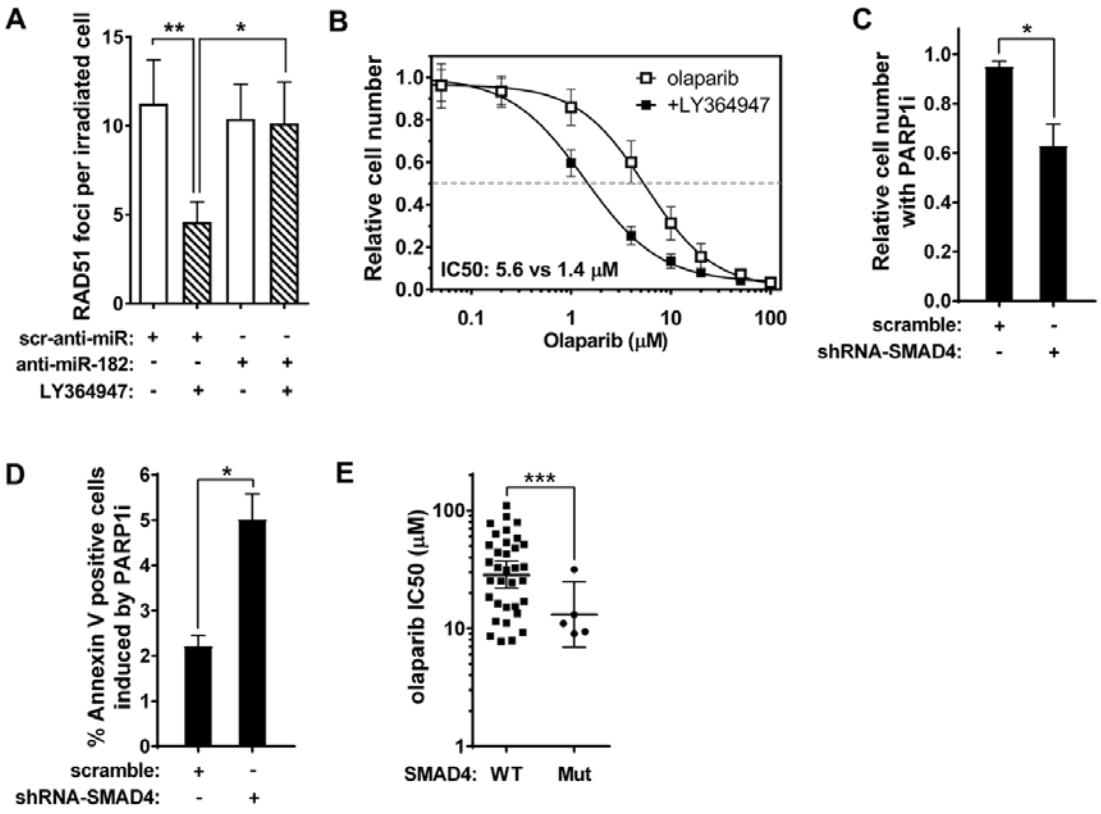
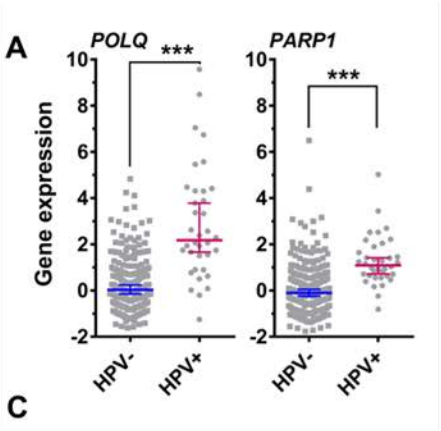
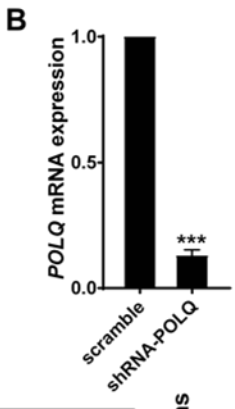
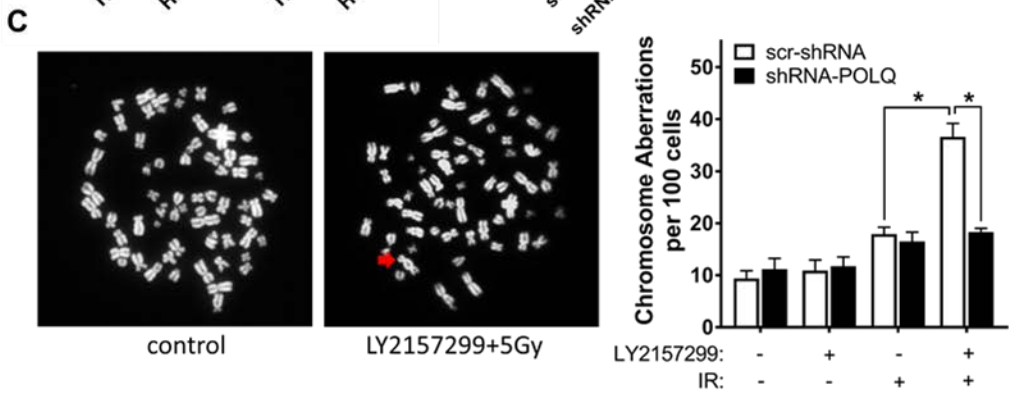

A
RAD51 foci per irradiated cell
15
10
5
0
**
*
scr-anti-miR: + + - -
anti-miR-182: - - + +
LY364947: - + - +
B
Relative cell number
1.0
0.8
0.6
0.4
0.2
0.0
olaparib
+LY364947
IC50: 5.6 vs 1.4 μM
0.1
1
10
100
Olaparib (μM)
C
Relative cell number with PARP1i
1.0
0.8
0.6
0.4
0.2
0.0
*
scramble: + -
shRNA-SMAD4: - +
D
% Annexin V positive cells induced by PARP1i
6
5
4
3
2
1
0
*
scramble: + -
shRNA-SMAD4: - +
E
olaparib IC50 (μM)
100
10
1
***
SMAD4: WT Mut

Figure S7. Loss of TGFβ signaling increases reliance on alt-EJ. **A,** mRNA expression levels of POLQ and PARP1 based on z-scored TCGA data from cbioportal (www.cbioportal.org). Mann-Whitney U test; ***, P < 0.005. **B,** *POLQ* mRNA expression of isogenic SAS cell pair transfected with shRNA against POLQ or scramble. **C,** Representative images showing chromosome spreads and one dicentric aberration (red arrow; left panel); scored chromosome aberrations per one hundred cells in each condition (right panel); SAS cells with POLQ knock-down or scramble control were fixed 48hr after treatment +/- LY2157299 and +/- irradiated with 5 Gy. **D,** Patient characteristics of the primary HNSCC tissues. **E,** Percentage of p-SMAD2 positive cells in primary HNSCC. **F,** Percentage of p-SMAD2 positive cells were grouped according to their HPV status. Two-tailed Student's t-test; *, $P < 0.05$; ***, $P < 0.005$.







D

HNSCC #	AGE	Gender	Pathology	p16	HPV16	stage	histology
A	49	M	SCC	negative	negative	cT3N0 AJCC stage III	oral tongue
B	57	M	SCC	positive	positive	T1N2bM0	Carcinoma of tonsil
C	55	M	SCC	positive	positive	rpT4aNx	Malignant neoplasm of tonsil
D	85	F	SCC	N/A	negative	pT2NXR1	Pharynx cancer
E	69	F	Keratinzing SCC	negative	negative	T4N2c	Carcinoma of palate
F	63	F	SCC	N/A	negative	pT4aN0	glottic/subglottic cancer
G	70	M	SCC	negative	negative	Stage II T2N0M0	supraglottis
H	75	F	SCC	N/A	positive	pT3(m)N2b	Floor Of Mouth
I	70	M	Invasive SCC	negative	negative	pT2N1	Floor Of Mouth
J	39	F	Invasive SCC	negative	negative	pT3N2c	left anterior tongue