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Supplementary Materials for

YAP1 is a potent driver of the onset and progression of oral squamous cell carcinoma

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This PDF file includes:

Supplemental Data

Supplementary Materials and Methods

Cell proliferation, apoptosis, cell cycle, and immunofluorescence (IF) analysis using *iMob1*DKO cells

For the cell count curves in Fig. 2A, *iMob1*DKO cells were treated with or without 0.5 μM TAM for 3 days, replated in 48-well plates (3×10^4 cells/well), and cultured in the absence of TAM for up to 12 days before determination of total cell counts at various timepoints. For apoptosis, cell cycle, and IF assays, *iMob1*DKO cells were treated with or without 0.5 μM TAM for 3 days, replated in 6-well plates [1×10^6 cells/well (apoptosis) or 3×10^5 cells/well (cell cycle)] or 24-well plates on 15 mm coverslips [1×10^5 cells/well (multipolar mitotic spindle), 3×10^4 cells/well (micronuclei) or 1×10^6 cells/well (ZO-1 staining)], and cultured in the absence of TAM for 4 days. Apoptosis assays were performed using PI staining as previously described (*40*). Samples were analyzed using a BD FACSCalibur instrument with Cell Quest software. Cell cycle analysis was performed as described previously (*41*) using a BD FACSCalibur instrument and ModFit software. For IF staining, cells cultured on coverslips were fixed for 5 min at room temperature in cold 70% ethanol. For ZO-1 staining, cells were fixed in 4% paraformaldehyde/PBS for 15 min, followed by permeabilization in 0.5% Triton X-100/PBS for 1 min. To visualize centrosomes, microtubules, or ZO-1 expression, cells were incubated with primary anti-γ-tubulin (GTU-88; Sigma), anti-α-tubulin (B-5-1-2; Sigma), or anti-ZO-1 (61-7300; Invitrogen) antibodies, respectively, followed by incubation with secondary anti-rabbit/mouse IgG conjugated to Alexa Fluor 488 or

568 (Molecular Probes). Incubations were performed in PBS containing 2% normal goat serum as described previously (*42*). DNA was stained using DAPI. Over 100 mitotic cells (n = 9 /group) were counted to analyze the percentages of cells with two or more centrosomes or with micronuclei.

YAP1 nuclear localization

To visualize nuclear YAP1 localization, cells were incubated with primary anti-YAP1 antibody (WH0010413M1; Sigma) followed by incubation with secondary anti-mouse IgG conjugated to Alexa Fluor 568 (Molecular Probes). DNA was stained using DAPI (Dojindo) and cells were examined using a DM5000B microscope (Leica). The intensities of three points of nuclear YAP1 staining and three points of cytoplasmic YAP1 staining were measured in each cell by ImageJ software and the ratio of nuclear/cytoplasmic YAP1 staining intensity was calculated. At least 50 cells were evaluated for each culture.

Colony formation

For colony-forming assays, *iMob1*DKO cells were seeded in 6-well plates (0.4×10^4/well) and cultured with or without TAM for 3 days. After 1 week, half of the wells were stained with crystal violet and the number of primary colonies that formed more than 500 cells was counted. The *iMob1*DKO cells in the unstained wells were then trypsinized and reseeded in 6-well plates (0.4×10^4/well). Reseeded cells were cultured for 1 week to generate secondary colonies.

H1299-Luc YAP1-TEAD reporter cells

H1299 cells were engineered to express a reporter construct containing the TEAD-binding sequence from the *CTGF* gene, plus the firefly *luciferase* gene, plus the Renilla *luciferase* gene, all under the control of the CMV promoter (*43*). For use in experiments, reporter cells were seeded in 96-well microplates (0.5×10^4 cells per well) and cultured overnight in a CO_2 incubator with Dasatinib (Abcam), Simvastatin (TCI), Verteporfin (USP), or Y-27632 (Wako) at the concentrations indicated in Fig. S3B. Luminescence intensity was measured using an EnSpire Multimode Plate Reader (Perkin Elmer).

TUNEL assay

TUNEL assays were performed using the In Situ Cell Death Detection Kit, TMR red (Sigma-Aldrich). Stained sections were examined using a DM5000B microscope (Leica). At least 200 cells were evaluated for each section.

Cisplatin sensitivity

HSC4 cells were seeded at 5×10^4/well in 6-well plates and incubated for 24 hrs. Cells were then transfected with siSC#1 (scramble control), si*YAP1*#1 or si*YAP1*#2. At 3 days post-transfection, Cisplatin (10μM; Wako**)** or DMSO (vehicle control) was added to the cells as indicated in Fig. S4E. At 24 hr after Cisplatin administration, all cells (including floating cells) were suspended and stained with PI to assess viability. Percentages of PI positive apoptotic cells were calculated using a BD FACSCalibur instrument and ModFit software as previously described (*40*).

Quantitative RT-PCR

Total RNA from siRNA-transfected HSC4, WSU-HN30, or Cal27 cells was extracted using RNAiso (TAKARA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The primer sequences used for RT-PCR were as follows: *GAPDH*, GTGAAGGTCGGAGTCAACG/TGAGGTCAATGAAGGGGTC; *TP53*, GCCCAACAACACCAGCTCCT/CCTGGGCATCCTTGAGTTCC; *PTEN*, GAGTTCCCTCAGCCGTTACCT/GAGGTTTCCTCTGGTCCTGGTA; *FAT1*, TTCAAAATAGGTGAAGAGACAGGTG/TTGTGATGAGACCTGTTTTAGGATG; *NF2*, GGTGTCCTTGATCGTGTACTG/TCAATTGCGAGATGAAGTGGAA; *TP63* (*ΔNp63α)*, GAGCCAGAAGAAAGGACAGCAG/GAATCTGCTGGTCCATGCTGTTC.; *CTGF*, TGTGTGACGAGCCCAAGGA/TCTGGGCCAAACGTGTCTTC, *BIRC5*, GAGGCTGGCTTCATCCACTG/CTTTTTGCTTGTTGTTGGTCTCC; *TOP2A*, TGAAGAAGACAGCAGCAAAAAGTCAGT/AAAATTAGAGTCAGAATCATCAGAAGTGG. The mRNA levels in the mutant were expressed as the percent increase over control values.

Transfection of cDNA

Transfection of pSLIK-*Flag*-*Myc*-*YAP15SA*, pTRIPZ-sh*YAP1*, or pSLIK-*ΔNp63α* plasmid DNAs into SCC9, HSC4 or WSU-HN30 cells was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. At 48 hr post-transfection, cells were transferred to growth medium containing G418 to select for stable transfectants. Stable transfectants were treated with or without Dox (1 μg/ml) for 4 days before use in experiments.

MTT Proliferation Assay

The cell proliferation determinations shown in Fig. S8B were conducted using the MTT assay according to the manufacturer's recommendations (Roche). SCC9 cells harboring Dox-inducible YAP(5SA) expression construct were seeded at 1.5×10^4/well in 96-well plates and incubated for 24 hrs with or without Dox. Cells were then transfected with siSC#1 (scramble control), si*TP63*#1 or si*TP63*#2. At 3 days post-transfection, MTT assay was done as indicated in Fig. S8B.

Microarray analysis

*iMob1*DKO cells treated with or without TAM (0.5 μM) for 3 days were further cultured without TAM for 4 days. Total RNA was isolated using TRIzol reagent (Invitrogen) and purified using the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. cRNA was amplified, labeled, and hybridized to a 60K Agilent 60-mer oligomicroarray according to the manufacturer's instructions. All hybridized microarray slides were scanned using an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (version 9.5.1.1). Raw signal intensities and Flags for each probe were calculated from hybridization intensities (gProcessedSignal) and spot information (gIsSaturated, etc.) according to the procedures recommended by Agilent. Raw signal intensities of two samples were $\log_2$-transformed and normalized by a quantile algorithm using the "preprocessCore" library package and Bioconductor software.

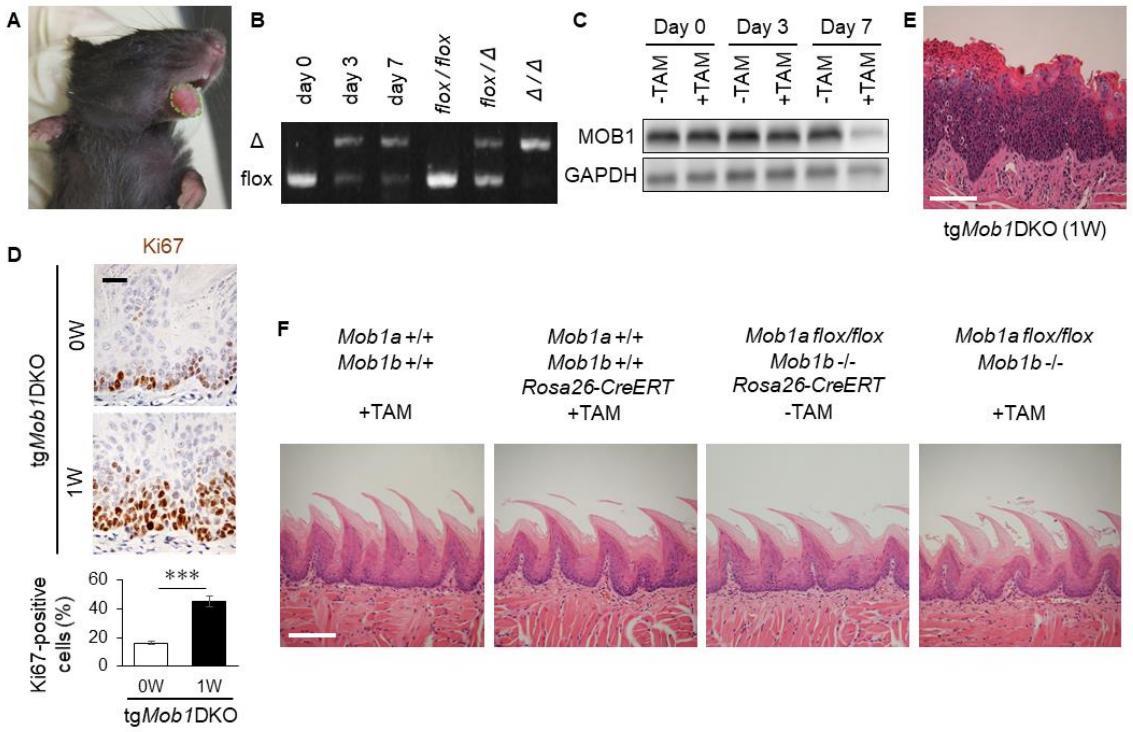
Supplemental Table 1

Table S1. Clinicopathological features of 86 cases of human tongue squamous cell carcinoma.

	All (*n*=86)		YAP status				P value
			Low (*n*=72)		High (*n*=14)		
	number	%	number	%	number	%	
Age (y)							
≧60	29	33.7%	25	34.7%	4	28.6%	0.6560
＜60	57	66.3%	47	65.3%	10	71.4%	
Sex							
Male	62	72.1%	53	73.6%	9	64.3%	0.4766
Female	24	27.9%	19	26.4%	5	35.7%	
History of smoking							
Brinkman Index <1000	70	82.6%	57	79.2%	13	92.9%	0.2284
Brinkman Index ≧1000	16	17.4%	15	20.8%	1	7.1%	
History of alcohol							
No (never/social)	34	46.5%	29	38.9%	5	28.6%	0.7493
Yes (current/former)	52	53.5%	43	61.1%	9	71.4%	
T stage (primary tumor size and site)							
T1/T2	66	75.6%	57	79.2%	9	64.3%	0.2278
T3/T4	20	24.4%	15	20.8%	5	35.7%	

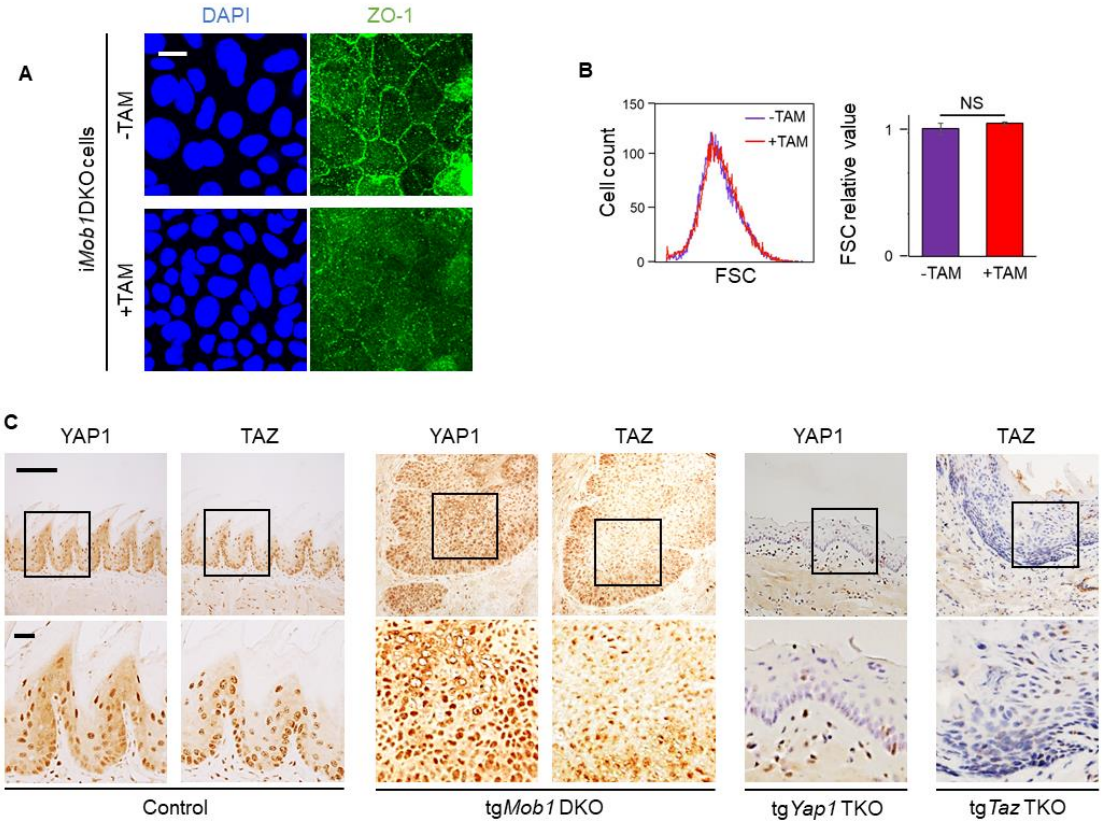
N Stage (regional lymph node involvement)							
N0 (N-)	71	80.2%	62	86.1%	9	64.3%	0.0489
N1,2,3 (N+)	15	19.8%	10	13.9%	5	35.7%	
Clinical Stage							
I/II	62	75.6%	54	75.0%	8	57.1%	0.1729
III/IV	24	24.4%	18	25.0%	6	42.9%	
Recurrence							
(-)	57	66.3%	52	72.2%	5	35.7%	0.0082
(+)	29	33.7%	20	27.8%	9	64.3%	
Degree of Tumor Differentiation							
Well/Moderate	80	93.0%	68	94.4%	12	85.7%	0.2407
Poor	6	7.0%	4	5.6%	2	14.3%	
Multiple Cancers							
(-)	72	83.7%	59	81.9%	13	92.9%	0.3115
(+)	14	16.3%	13	18.1%	1	7.1%	
TP53 status							
TP53 wild type	23	26.7%	23	100%	0	0%	0.0135
TP53 mutation	63	73.3%	49	77.8%	14	22.2%	

Supplemental Figures and Figure Legends



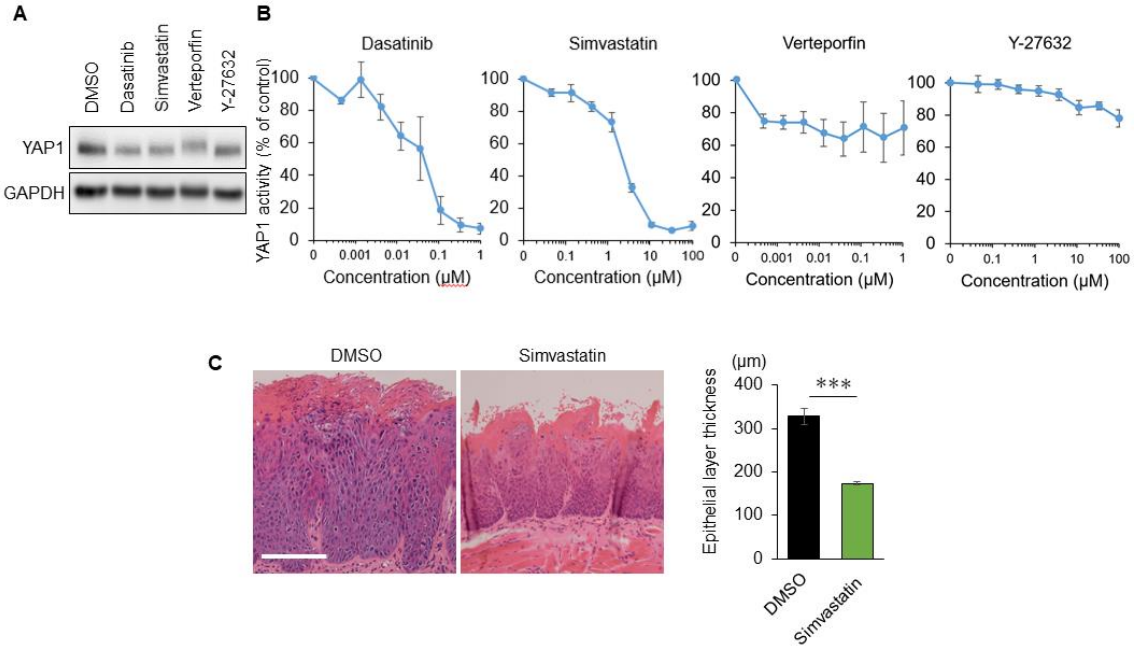
Omori et al. Supplemental Figure 1

Fig. S1. Induction of *Mob1a/b* deletion in tg*Mob1*DKO mice by postnatal application of TAM. (**A**) TAM (10 mg/ml) was directly applied to the indicated area of the mouse tongue (green dashed line) using a small paint brush. Application was usually performed daily for 5 days. Photo credit: Hirofumi Omori, Kobe University. (**B**) PCR detection of *Mob1a* deletion in tg*Mob1*DKO mouse tongue epithelium on days 0, 3, and 7 after the initiation of TAM application. (**C**) Immunoblots confirming MOB1 protein depletion in tg*Mob1*DKO mouse tongue epithelium on the indicated days post-TAM initiation. GAPDH, loading control. (**D**) Top: Representative images of Ki67 immunostaining of tongue epithelium from tg*Mob1*DKO mice at 0 week (W) (n=10) or 1W (n=10) after TAM initiation. Scale bar, 20 μm. Bottom: Quantitation of Ki67-positive cells as a percentage of the total cell number in the tongue sections of the mice in the top panels. Data are the mean ± SEM. ***$p < 0.001$, *t* test. (**E**) Representative H&E-stained section of a tg*Mob1*DKO mouse tongue exhibiting carcinoma *in situ* (CIS) at 1 wk post-TAM initiation. Scale bar, 100 μm. (**F**) H&E-stained sections of tongue epithelium from mice of the indicated genotypes at 4 wks after starting TAM application (+TAM) or not (-TAM). Scale bar, 100 μm. Results shown are representative of at least three independent trials.



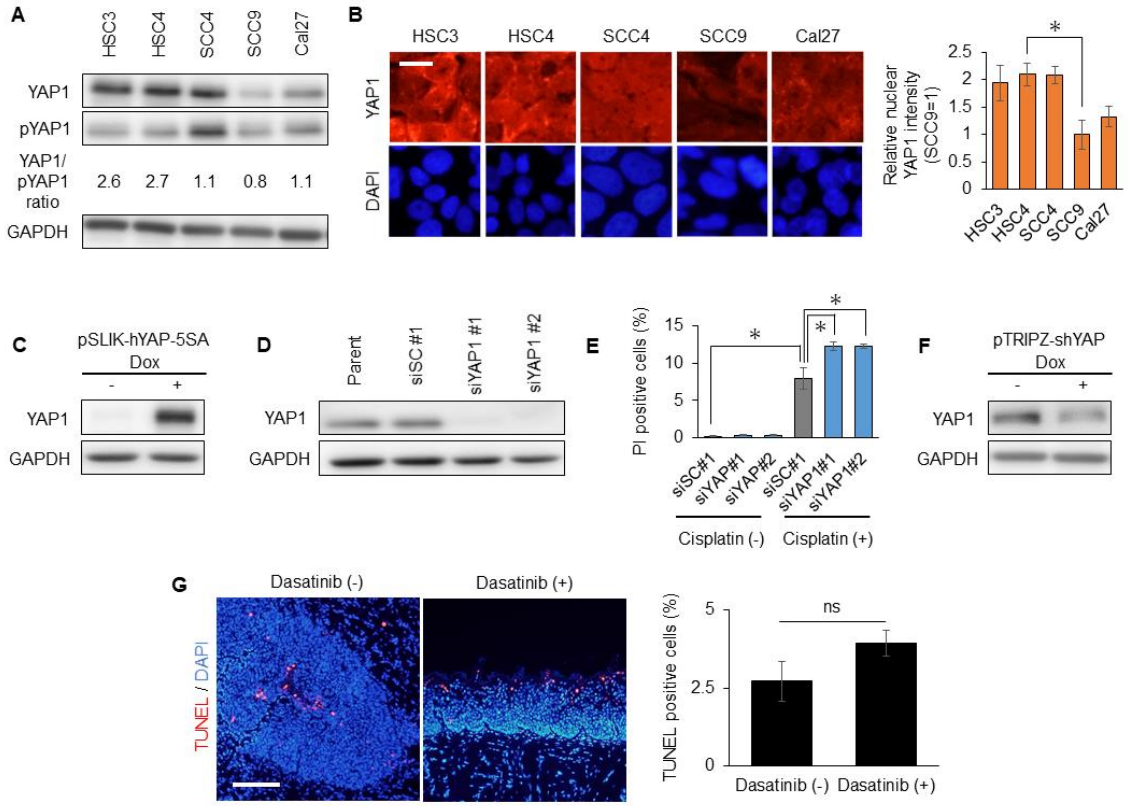
Omori et al. Supplemental Figure 2

Fig. S2. Cell-cell junction collapse and retained cell size of *iMob1*DKO cells and YAP1/TAZ expression and localization in tongue epithelium of tg*Mob1*DKO, tg*Yap1*TKO, and tg*Taz*TKO mice. (**A**) TAM-treated (+TAM, 0.5μM TAM for 3 days) or untreated (-TAM) *iMob1*DKO cells (1.5×10^5) were cultured on 15-mm coverslips for 5 days, then the cell junction protein ZO-1 was stained. Confocal images of ZO-1 staining and nuclear staining (DAPI) are represented. Scale bar, 10 μm. (**B**) Left: Flow cytometric determination of cell size (FSC) of *iMob1*DKO tongue epithelial cells that were treated with 0.5 μM TAM for 3 days followed by an additional 4 days of culture without TAM (*iMob1*DKO+TAM cells). *iMob1*DKO cells cultured for the entire period without TAM (*iMob1*DKO-TAM cells) served as the control. Right: Quantitation of FSC relative values in the top panels**.** Data are the mean ± SEM (n = 3). n.s., not significant. *t* test. (**C**) Immunostaining of YAP1 and TAZ confirming their deletion and intracellular localization in tongue epithelium of the Control, tg*Mob1*DKO, tg*Yap1* TKO, or tg*Taz* TKO mice in Fig. 3C. For tg*Yap1* TKO and tg*Taz* TKO mice, the nuclei were counterstained with hematoxylin to clearly show basal cell layer. Scale bars in top panels (low magnification), 100 μm; in bottom panels (high magnification), 20 μm.



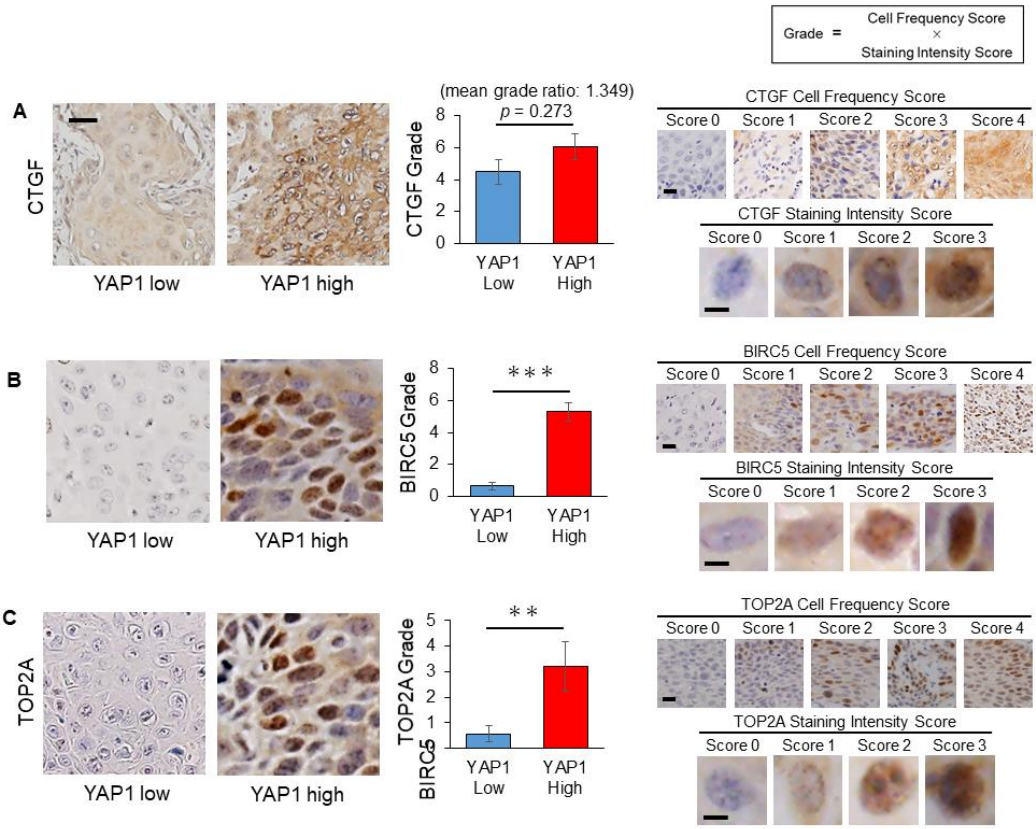
Omori et al. Supplemental Figure 3

Fig. S3. Effects of dasatinib, simvastatin, verteporfin, and Y-27632 on YAP1 protein expression and activation and tumor-suppressive effect of simvastatin. (**A**) Immunoblot to detect YAP1 protein in HSC4 cells that were treated (separately) with 5 μM of the indicated compounds for 24 hr. DMSO, vehicle control. GAPDH, loading control. (**B**) H1299-Luc cells were treated with indicated compounds at the indicated concentrations for 24 hr. Firefly luciferase activity (YAP1-TEAD transcriptional activity) and Renilla luciferase activity (internal control) were then determined. Firefly luciferase activity was normalized to Renilla luciferase activity and represented as a percentage of the DMSO vehicle control value (set to 100%). Data are the mean ± SEM (n = 6). (**C**) Left: Representative images of H&E-stained sections of tongues from tg*Mob1*DKO mice that were pretreated with DMSO (*n* = 6) or 50 mg/kg Simvastatin (*n* = 6) and then subjected to 5 days of TAM administration. Simvastatin was administered using the same procedure used for Dasatinib treatment in Fig. 4A. Scale bar, 100 μm. Right: Quantitation of the thickness of the epithelial layers in the tongues of the mice examined in the left panels. Data are the mean ± SEM (n = 6). ***$p < 0.001$, *t* test.



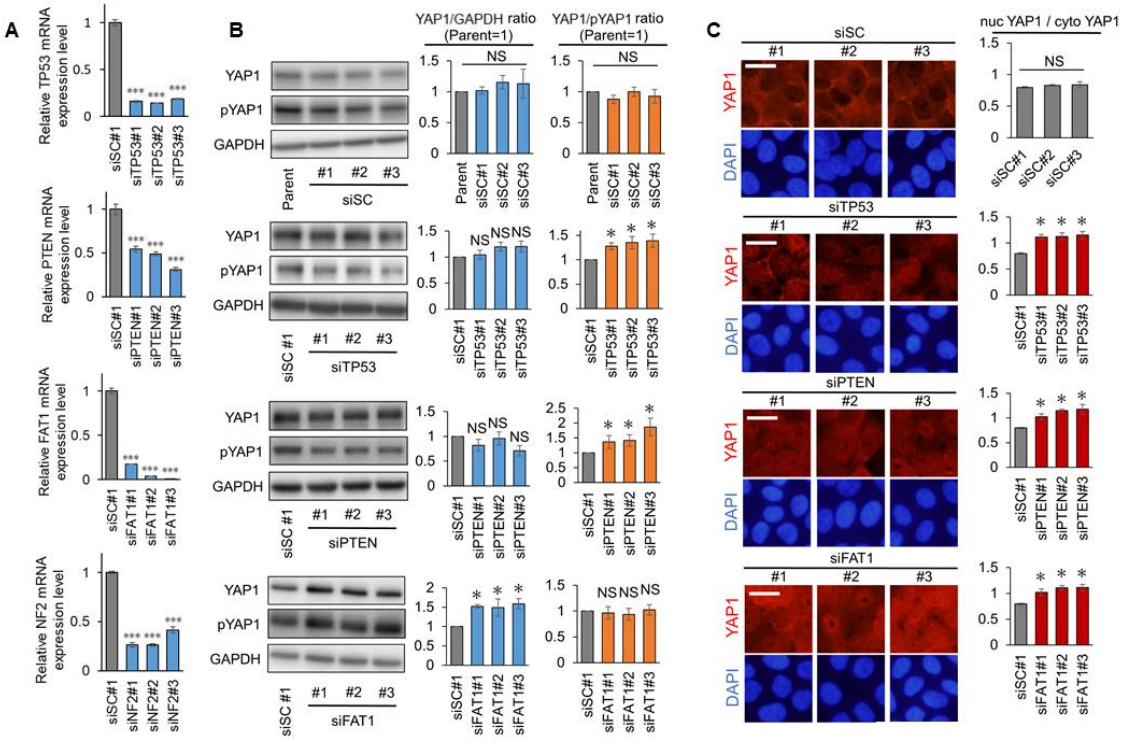
Omori et al. Supplemental Figure 4

Fig. S4. YAP1 expression in OSCC cell lines, the effect on an OSCC cell line of YAP1 depletion combined with cisplatin, and the effect of dasatinib on cell death in tg*Mob1*DKO mice. (**A**) Immunoblot to detect YAP1 and pYAP1(S127) in the indicated HNSCC cell lines. The ratio of YAP1/pYAP1 signal intensity for each sample was determined by densitometry of the blot. (**B**) Left: Immunocytochemical detection of YAP1 (red) and DAPI-stained nuclei (blue) in the indicated HNSCC cell lines. Scale bar, 10 μm. Right: Quantitation of the fold increase in nuclear YAP1 in the cells in the left panel relative to SCC9 cells set to 1. At least 100 cells were analyzed for each cell line. (**C**) Immunoblot to detect YAP1 overexpression in SCC9 cells with inducible ectopic expression of YAP1(5SA) that were treated (or not) for 3 days with Dox (1 μg/ml). (**D**) Immunoblot to confirm the knockdown of YAP1 protein in HSC4 cells that were left untreated (Parent) or transfected with control si-scramble siRNA (siSC#1) or one of two independent siRNAs targeting *YAP1* (si*YAP1* #1, #2). YAP1 expression was analyzed at 4 days after siRNA transfection. (**E**) Quantitation of flow cytometric determination of the percentage of PI-positive cells among total HSC4 cells that were treated with the indicated siRNAs for 3 days, and then treated with DMSO vehicle control [Cisplatin (-)] or 10 μM Cisplatin (Wako) for 24 hr. Data are the mean ± SEM (n=3). (**F**) Immunoblot confirming the knockdown of YAP1 in HSC4 cells with inducible expression of sh*YAP1* that were treated with Dox (1 μg /ml) for 3 days. (**G**) Left: TUNEL assay of epithelial cells in the tongues of the tg*Mob1*DKO mice in Fig. 4J, which were treated with or without 5 mg/kg Dasatinib for 2 wks (n=6 / group). Right: Quantitation of TUNEL-positive cells in the tongues in the left panel. Data are presented as the percentage of TUNEL-positive cells among total DAPI-positive cells, and are the mean ± SEM (n=3). ns, not significant, $*p < 0.05$, $***p < 0.001$, *t* test



Omori et al. Supplemental Figure 5

Fig. S5. YAP1 target gene expression correlates with YAP1 nuclear expression in human clinical OSCC specimens. (**A-C**) Left: Representative images of IHC staining to detect the YAP1 target genes CTGF, BIRC5 or TOP2A in human OSCC specimens showing high (YAP1-high) or low (YAP1-low) levels of nuclear YAP1. Scale bar, 10 μm. Center: Grades of CTGF, BIRC5 or TOP2A expression in the YAP1-high and YAP1-low groups of specimens in the left panels. The grade for each YAP1 target was defined by multiplying the "Frequency Score" by the "Intensity Score" for each, as was performed to determine the YAP1 grade. $^{**}p < 0.01$, $^{***}p < 0.001$, *t* test. n.s., not significant. Right: Representative sections from the left panels were scored for the frequency and intensity of indicated target protein. The Grade for each target protein was the product of these scores (see Materials and Methods). Scale bar: 10 μm (frequency score) and 3 μm (intensity score).



Omori et al. Supplemental Figure 6

Fig. S6. Evaluation of gene knockdown and ectopic gene expression in the WSU-HN30 HNSCC cell line and activation of YAP1 by knockdown of *TP53*, *PTEN*, or *FAT1*. (**A**) Quantitative RT-qPCR determination of knockdown efficiency of the indicated siRNAs targeting *TP53*, *PTEN*, *FAT1*, or *NF2* in WSU-HN30 cells. Cells were analyzed at 4 days after transfection. Results are expressed as the mRNA level relative to that in the siSC-transfected control. Data are shown as the mean ± SEM of at least triplicate samples. ***$p < 0.001$, *t* test. (**B**) Left: Immunoblots to detect YAP1 and pYAP1 proteins in WSU-HN30 cells that were transfected for 4 days with control siSC#1 or with the indicated siRNAs targeting *TP53*, *PTEN*, or *FAT1*. Right: Ratios of YAP1/GAPDH (protein) and YAP1/pYAP1 (activation) were calculated from the blots in the left panel (see Materials and Methods). Data are the mean ± SEM of 7 independent siRNA-transfected samples for each gene. *$p < 0.05$, *t* test. n.s., not significant. A summary of these results appears in Fig. 6A. (**C**) WSU-HN30 cells were transfected for 4 days with control siSC#1 or with the indicated siRNAs targeting *TP53*, *PTEN*, or *FAT1*. Left: Representative images of YAP1 nuclear localization analyzed as described in Supplementary Methods. Scale bar, 10 μm. Right. Quantitation of the ratio of nuclear YAP1 to cytoplasmic YAP1 for the cells in the left panel. Data are the mean ± SEM of three independent experiments. *$p < 0.05$, *t* test. n.s., not significant. Over 50 cells were counted for each sample. A summary of these results appears in Fig. 6C.

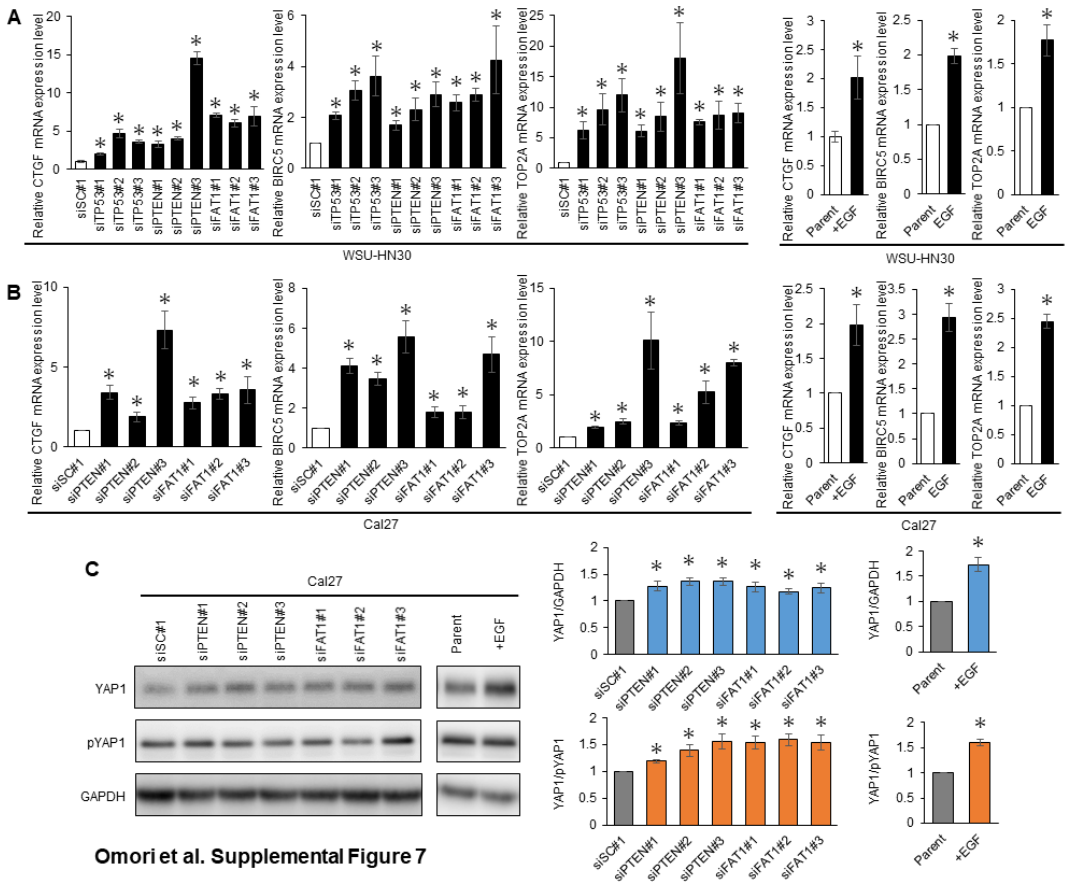
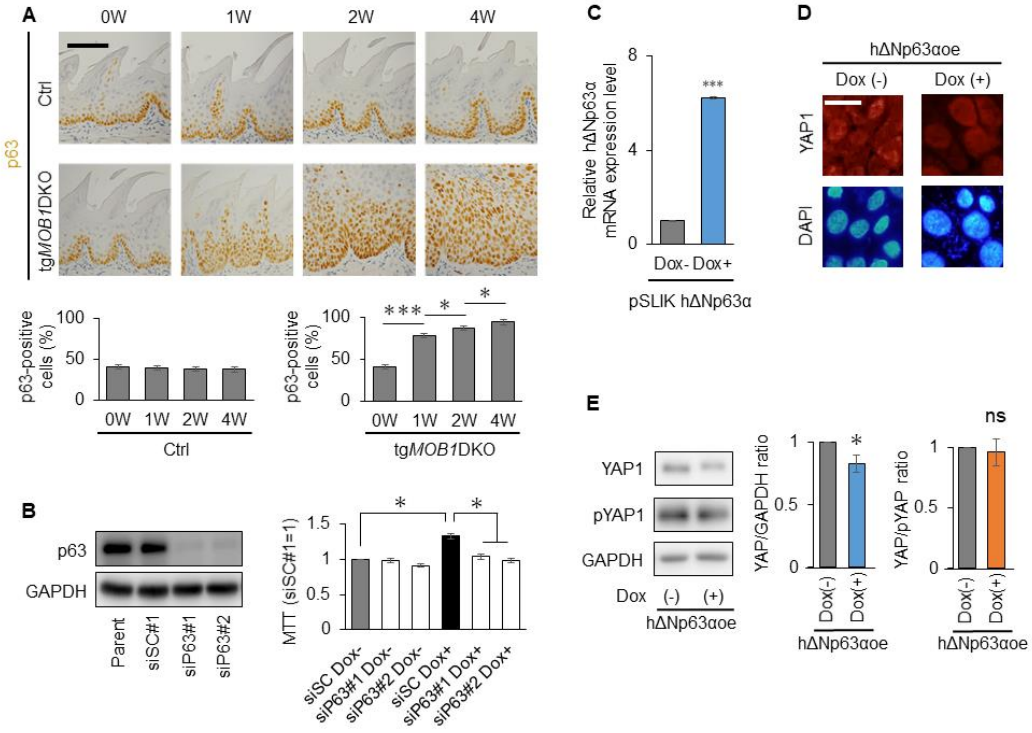
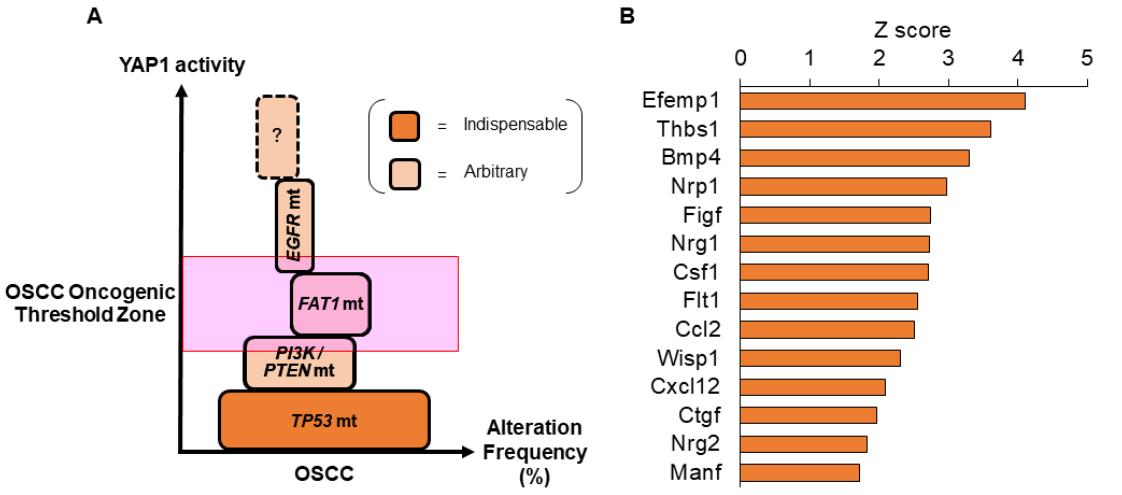


Fig. S7. Activation of YAP1 target gene expression by molecules that are frequently altered in human OSCC. (**A, B**) RT-qPCR determination of relative *CTGF*, *BIRC5*, and *TOP2A* mRNA levels in WSU-HN30 cells (A) and Cal27 cells (B) that were transfected for 24 hr with siSC#1 or one of three independent siRNAs (#1–3) targeting *TP53*, *PTEN* or *FAT1* (left), or treated (or not) with 1 μg/ml EGF for 96 hr (right). Data are the mean ± SEM of three independent experiments. *$p < 0.05$, *t* test. (**C**) Left: Immunoblots to detect YAP1 and pYAP1 in Cal27 cells that were transfected with the indicated siRNAs, or treated (or not) with EGF as in (B). Right: Ratios of YAP1/GAPDH and YAP1/pYAP1 were calculated from the blot in the left panel as for Fig. S6B. Data are the mean ± SEM of either three independent siRNA-transfected samples for each gene, or three independent EGF-treated cultures. *$p < 0.05$, *t* test.



Omori et al. Supplemental Figure 8

Fig. S8. Positive correlation of ΔNp63α protein expression with YAP1 protein expression. (**A**) Top: Representative images of IHC detection of TP63 protein (p63) in the tongue epithelium of control and tg*Mob1*DKO mice at 0, 1, 2, or 4 wks after TAM initiation. Scale bar, 100 μm. Bottom: Percentages of p63-positive cells in the tongues of the control (Ctrl) and tg*Mob1*DKO mice in the top panels. Data are the mean ± SEM (n = 6 mice/group). (**B**) Left: Immunoblot to confirm the knockdown of TP63 protein (p63) in SCC9 cells that were left untreated (Parent) or transfected for 4 days with either siSC#1 or one of two independent siRNAs targeting *TP63* (si*TP63* #1 or si*TP63* #2). Right: MTT proliferation assay of SCC9 cells that were transfected with Dox-inducible YAP1-5SA and treated for 4 days with Dox (1 μg/ml) and/or the indicated siRNAs. Data are the mean ± SEM (n = 3). *$p < 0.05$, t test. (**C**) Quantitative RT-PCR determination of ectopic expression of h*ΔNp63α* transcripts in WSU-HN30 cells that were transfected with pSLIK h*ΔNp63α* and treated (or not) with Dox (1 μg/ml) for 3 days. Data are the mean ± SEM (n = 3). (**D**) Representative images of IF detection of YAP1 (red) in nuclei (blue) in the cells in (C). Scale bar, 10 μm. (**E**) Left: Immunoblot to detect YAP1 and pYAP1 in WSU-HN30 cells that were transfected with pSLIK h*ΔNp63α* (h*ΔNp63α*oe), followed by Dox treatment (1 μg/ml, 3 days) or not. Middle: Quantitation of the ratio of YAP1/GAPDH signal intensity (protein) in the blots in the left panel**.** Right: Quantitation of the ratio of YAP1/pYAP1 signal intensity (activation) in the blots in the top panel**.** For the middle and bottom panels, data are the mean ± SEM (n = 3). *$p < 0.05$, ***$p < 0.001$, t test. n.s., not significant.



Omori et al. Supplemental Figure 9

Fig. S9. Graphical abstract and microarray analysis of growth factors and receptors whose mRNAs are up-regulated in tg*Mob1*DKO tongue epithelial cells. (A) Proposed model of OSCC genesis. OSCCs may be caused by an accumulation of YAP1 activity that is usually driven by the mutation of TP53 plus one or more of the indicated genes. When the oncogenic threshold of YAP1 activity is surpassed, transformation may ensue. For each rounded rectangle representing a mutated gene contributing to OSCC, the height of the rectangle shows the YAP1 activation intensity, and the width of the rectangle shows the relative frequency of that mutation. 'YAP1 Activity' on the y-axis was defined by multiplying the 'ratio of YAP1/GAPDH band intensities' by the 'ratio of 'YAP1/pYAP1 band intensities', which were determined by immunoblot analysis. '?' indicates that other unknown factor(s) can upregulate YAP1. Please see the Discussion in the main text for details. (**B**) DNA microarray analysis of mRNAs from Control and tg*Mob1*DKO tongue epithelial cells conducted as described in Supplementary Methods. *Z* scores for the genes encoding the top 14 soluble growth factors or their receptors that were upregulated in tg*Mob1*DKO cells over control cells are presented.