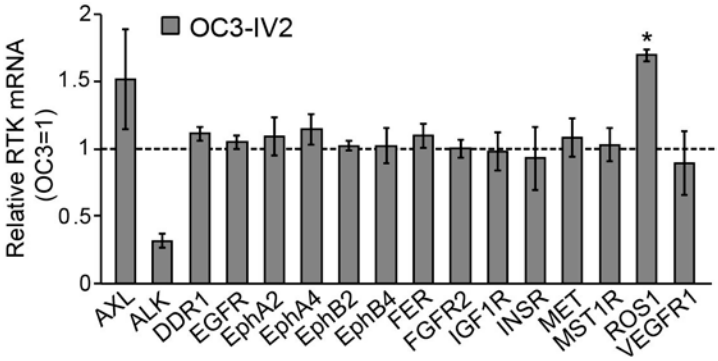
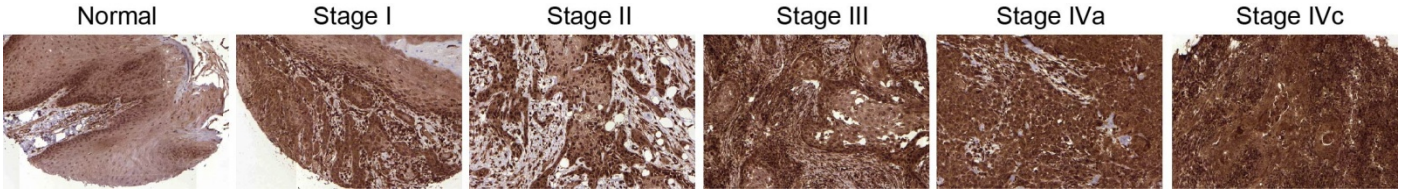
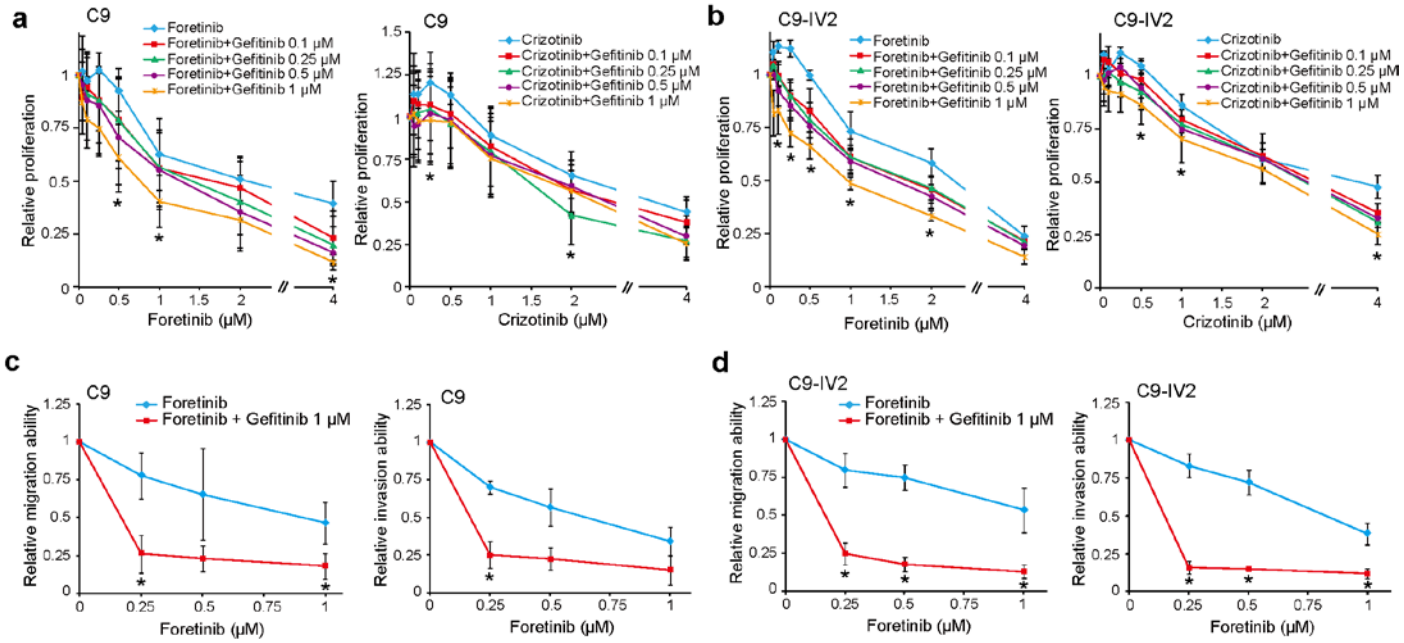
Supplemental Figures and Tables



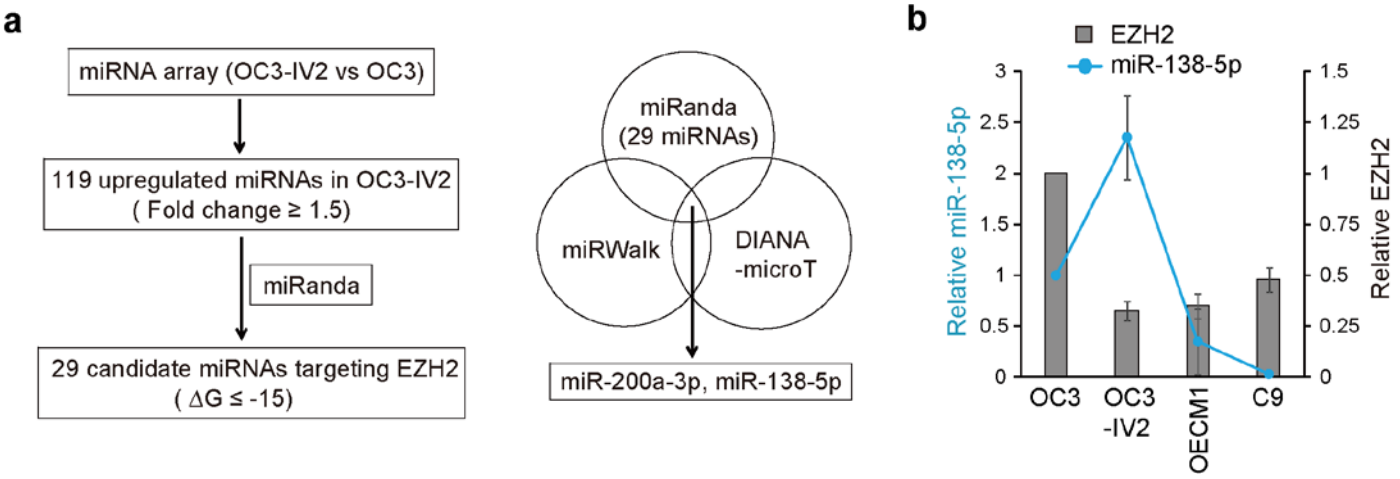
Supplementary Figure 1. ROS1 expression level is increased in the more invasive OSCC cells. mRNA levels of 16 RTKs in OC3 and OC3-IV2 cells were determined using reverse transcription-PCR. The mRNA levels in OC3-IV2 cells were normalized to those in OC3 cells. Data from at least three independent experiments are presented as mean ± SEM (*$P < 0.05$).



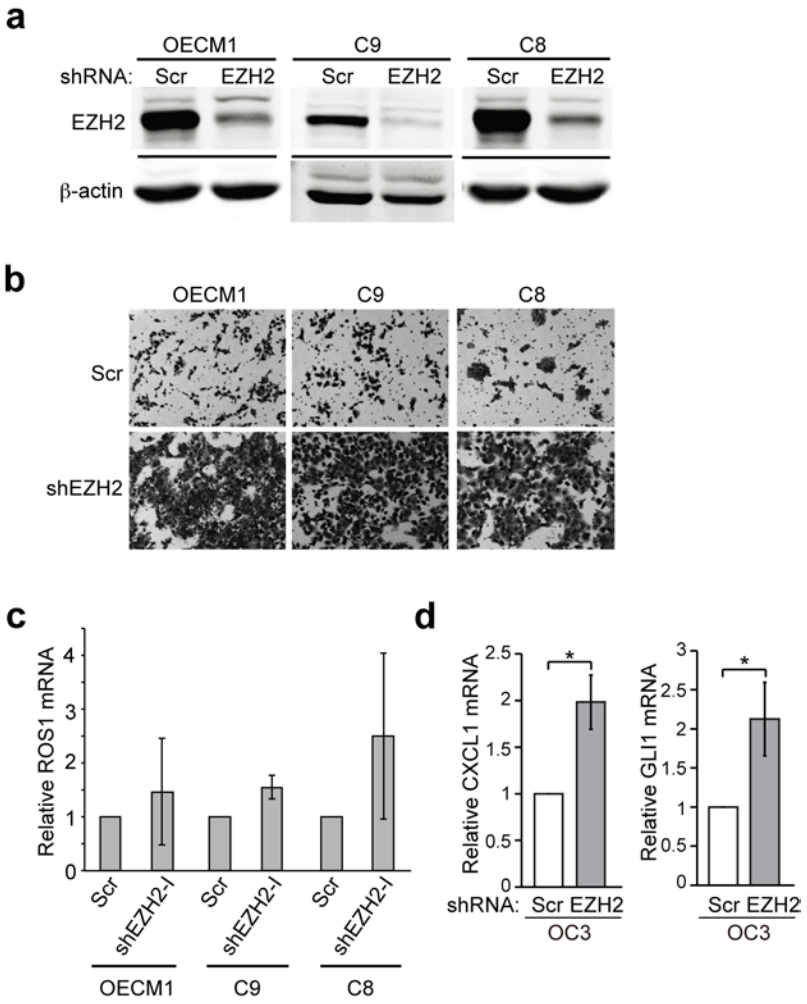
Supplementary Figure 2. Images of the oral cancer tissue array. Stage I: primary tumor is less than 2 cm in diameter. Stage II: primary tumor is more than 2 cm in diameter, but less than 4 cm. Stage III: primary tumor is more than 4 cm in diameter or cervical lymph node metastasis is detected (metastatic tumor is less than or equal to 3 cm in diameter). Stage IVa: primary tumor invades adjacent tissues and/or cervical lymph node metastasis is detected (metastatic tumor is more than 3 cm in diameter, but less than or equal to 6 cm). Stage IVc: distant metastasis has occurred.



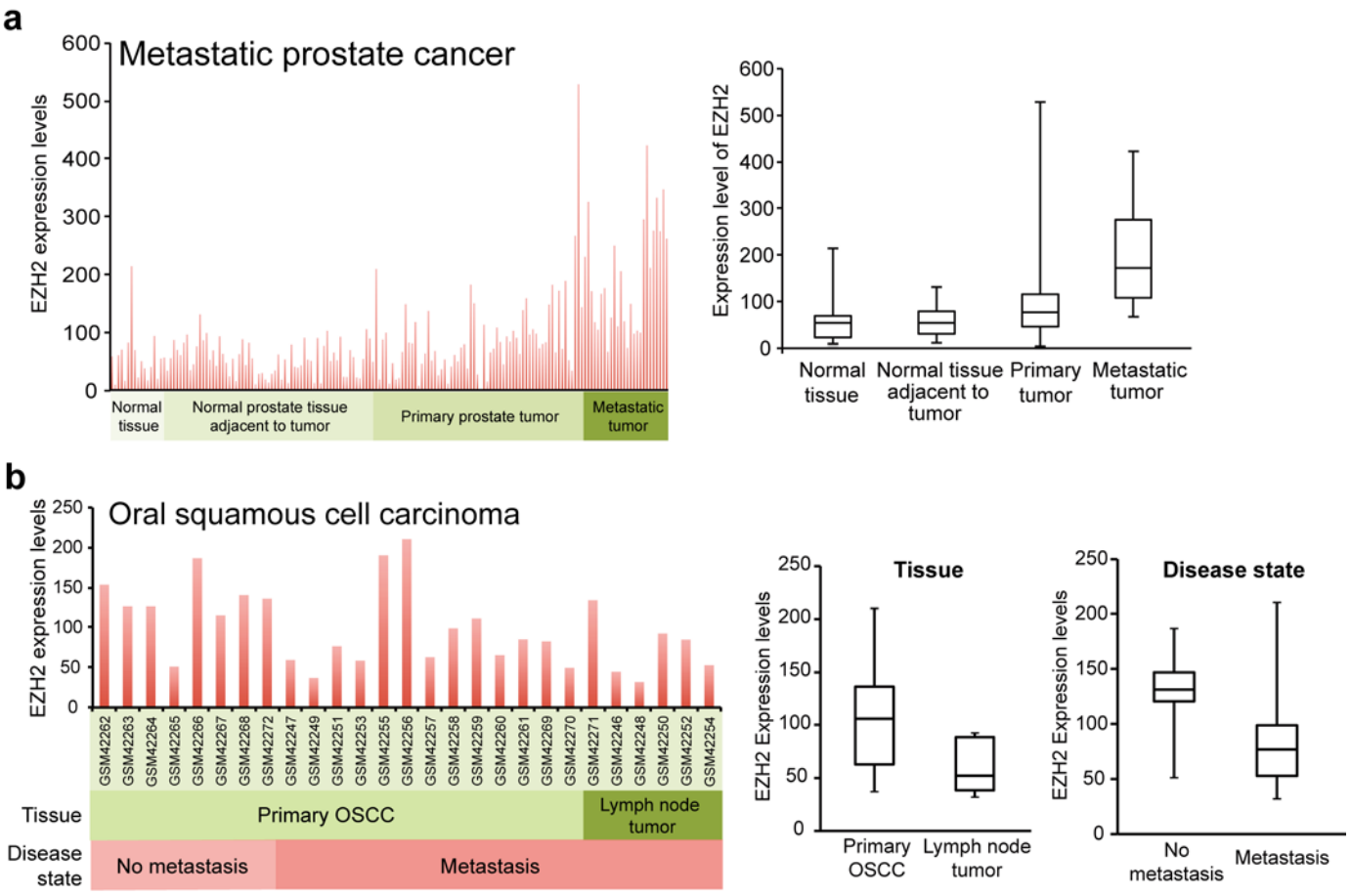
Supplementary Figure 3. Inhibitory effect of combined treatment with gefitinib and foretinib or crizotinib on C9 and C9-IV2 cell proliferation, migration, and invasion. (**a** and **b**) Proliferation of C9 and C9-IV2 cells treated with foretinib/crizotinib alone or in combination with the indicated concentrations of gefitinib for 72 h was measured with the MTT assay. (**c** and **d**) Migration and invasion of C9 and C9-IV2 cells treated with foretinib alone or in combination with 1 μM gefitinib were assessed with the Boyden chamber assay. Values for proliferation, migration, and invasion were normalized to those in C9 and C9-IV2 cells treated with control (DMSO). Data from at least three independent experiments are presented as mean ± SEM (*$P < 0.05$).



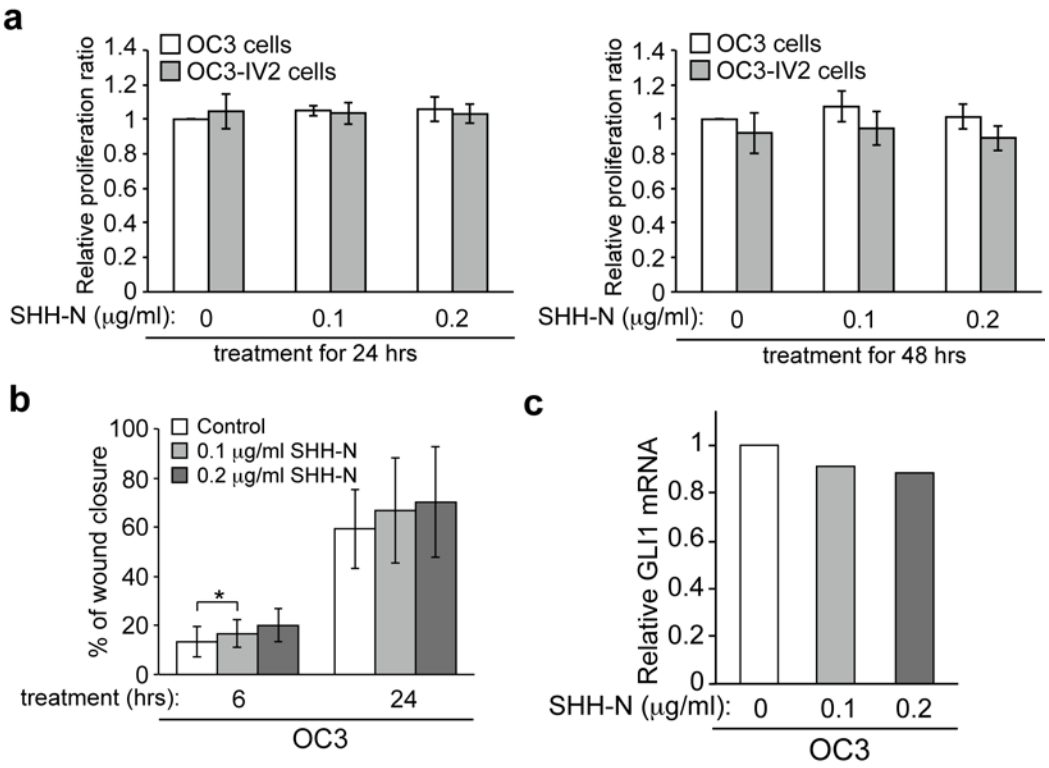
Supplementary Figure 4. Identification of miRNAs targeting EZH2 mRNA. (a) Schematics for the identification of candidate miRNAs targeting EZH2 mRNA. (**b**) Relative miR-138-5p and EZH2 protein levels in OSCC cells were determined using Q-PCR and Western blotting, respectively. Data from at least three independent experiments are presented as mean ± SEM.



Supplementary Figure 5. Effect of EZH2 knockdown on OSCC cell invasion and on expression of *ROS1*, *CXCL1*, and *GLI1*. (**a**) Western blotting for EZH2 level in OSCC cells stably expressing EZH2 shRNA (OSCC-shEZH2). (**b**) Invasion of OSCC-shEZH2 cells was determined with the Boyden chamber assay. (**c**) Western blotting for ROS1 in OSCC-shEZH2-I1 cells (in vitro selection of OSCC-shEZH2 cells). Data from two independent experiments are presented as mean ± SD. (**d**) The mRNA levels of *CXCL1* and *GLI1* in OC3-shEZH2 cells were normalized to those in OC3-Scr cells. Data from at least three independent experiments are presented as mean ± SEM (*$P < 0.05$).



Supplementary Figure 6. Expression of EZH2 mRNA in patients with prostate cancer or OSCC with metastasis. Gene expression of EZH2 in samples of prostate cancer and OSCC from Gene Expression Omnibus (GEO) profiles. (a: http://www.ncbi.nlm.nih.gov/geoprofiles/34860075; b: http://www.ncbi.nlm.nih.gov/geoprofiles/9656685).



Supplementary Figure 7. Proliferation, migration, and *GLI1* gene expression are not affected by treatment with Shh in OC3 and OC3-IV2 cells. (**a** and **b**) Cell proliferation and migration of OC3 and OC3-IV2 cells treated with different concentrations of the N-terminal Shh ligand (SHH-N) were assessed for different time intervals with the MTT assay and wound-healing assay. The quantified results are shown. (**c**) Relative mRNA expression of *GLI1* in OC3 cells treated with different concentrations of SHH-N for 48 h as measured with Q-PCR.

Supplementary Table 1. Sequences of primers used in ChIP assays.

Primers used for ChIP assay			
Promoter	**Site**	**Foreward primers (5' - 3')**	**Reverse primers (5' - 3')**
ROS1	Region#1	GTGTCACACCCTTGCTGAAATA	TACTCCCATAAAGTCACCCAGTG
	Region#2	GCTTGATTTGGGTGCCTCTAT	CTCTGCAGGGAATTGGGTTT
	Region#3	AGGCAGGAAGGAAGCTTGTAC	CAGGTCTATCGAGACAGCTGAA
	Region#4	CCTCATGCATGTGTTACTCATAGG	CAGGCCAACCTCCTTGTTT
CXCL1	NF-κB(p65)	GGCTGCATCAGTGGACCC	GAACCCCTTTTATGCATGGT
	STAT1#1	TAGGGCGAGTTTACCAGGTT	TCTTTCCTGTGGCATTGAAA
	STAT1#2	TCTTCTTGCCTTTTTGGTTTG	CTGGGATTTGATGGGAAACT
GLI1	Histone modification	AAAGCCTGGAAGAGCAGCTA	AAGCCCCTTTCCCTAGAACC
	NF-κB(p65)#1	GGGTAAGGGCTGTTGAGGTA	AAATGCTTGTCTCCCAGTGG
	NF-κB(p65)#2	GAGCTAGGATGTGGGAGGTC	TGAGAAACGGAGAGGCAGAG
	NF-κB(p65)#3	AGGGTCGGAATAAGTGTGGT	GTGTGTATGGGGAGGAGGAG
	NF-κB(p65)#4	ACGCCATGTTCAACTCGATG	GAGATCTGCCAAATCCTCAAGG
	NF-κB(p65)#5	GCCCAATCCTTCCTGAGACT	CGGGCAGAGTCATGGGGA
	STAT1	AGCCTGGGGTGAGACATTAG	TATATGGGAGAGGCGGAGAC

Supplemental Materials and Methods

Cell proliferation assays

The proliferative capacity of viable cells was assessed using MTT and colony formation assays. For the MTT assay, cells were seeded in 96-well plates at 20% confluency and incubated for different time intervals. The absorbance of dissolved MTT product formazan was measured at 565 nm with a spectrophotometer. For colony formation assays, equal numbers of cells (500 cells/well) were seeded onto 6-well plates. After incubation for 12 days, cells were fixed in 4% (v/v) paraformaldehyde and stained with crystal violet.

In vitro migration and invasion assays

For wound-healing assays, cells were seeded in a 6-well plate at 90% confluency and wounded by scraping cells with a P1000 pipette tip. After incubation for 6 or 24 h, live-cell images were taken using the Carl Zeiss Observer Z1 microscope. The width of the remaining wounded gaps was averaged from two wounded gaps and the average of the width of the remaining wounded gaps was divided by that of the initial wounded gaps. The percentage of wound closure was calculated as (100% – remaining wounded gaps%). For Boyden chamber assays, cell migration/invasion was assessed by SPLInsert with a polyethylene terephthalate membrane (pore size: 8.0 μm; SPL Lifesciences, Pocheon, South Korea). Cells were harvested and suspended in serum-free DMEM or RPMI-1640 containing 0.1% (w/v) BSA and then seeded in the upper chamber of each insert. The bottom well of the 24-well plate was filled with DMEM, DMEM/KSFM, or RPMI-1640 containing 10% (v/v) FBS (chemoattractant). For the invasion assay, the upper side of the polyethylene terephthalate membrane was coated with Matrigel (BD Biosciences, San Jose, CA, USA).

After incubation, cells on the membrane were fixed with paraformaldehyde and stained with crystal violet. Non-migrated or invasive cells on the upper side of the membrane were removed by gently scraping with a wet cotton swab. Photos of stained cells on the underside of the membrane were taken using a Zeiss Observer Z1 microscope. The number of cells was counted using Image J. Relative migration/invasion ability was calculated as cell number per area.

Knockdown of selective genes via RNAi

pLKO.1 lentiviral constructs that contain oligonucleotides targeting specific human gene sequences,

TRC2.Void [Scramble (Scr)]

(5’-CCGGAGTTCAGTTACGATATCATGTCTCGAGACATTCGCGAGTAACTGAACTTTTTT-3’),

pLKO.1-shROS1#1

(5’-CCGGCTGTGGATGGAGATCTTATATCTCGAGATATAAGATCTCCATCCACAGTTTTTG-3’),

pLKO.1-shROS1#2

(5’-CCGGTGATAATGAGATGGGATATTACTCGAGTAATATCCCATCTCATTATCATTTTTG-3’),

pLKO.1-shEZH2

(5’-CCGGCGGAAATCTTAAACCAAGAATCTCGAGATTCTTGGTTTAAGATTTCCGTTTTTG-3’),

pLKO.1-shCXCL1

(5’-CCGGCAAATGGCCAATGAGATCATTCTCGAGAATGATCTCATTGGCCATTTGTTTTTG-3’),

pLKO.1-shGli1#1

(5’-CCGGCATCCATCACAGATCGCATTTCTCGAGAAATGCGATCTGTGATGGATGTTTTT-3’), and

pLKO.1-shGli1#2

(5’-CCGGCATCCATCACAGATCGCATTTCTCGAGAAATGCGATCTGTGATGGATGTTTTTG-3’) were obtained from the National Core Facility at the Institute of Molecular Biology, Genomic Research Center, Academic Sinica, Taiwan. Lentivirus containing pLKO.1-shRNA was prepared from 293T cells co-transfected with pLKO.1-shRNA, pCMVΔR8.91, and pMD.G using Lipofectamine 2000 (Invitrogen). Medium containing lentivirus was harvested and added to OSCC cells and then subjected to puromycin selection for at least 2 weeks to establish stable cell lines.

Reverse Transcription-PCR and Q-RCR

TRIzol reagent (Invitrogen) was used to isolate total RNA from oral cancer cells. For reverse transcription, 2 μg of total RNA was converted to cDNA using reverse transcription kit (Applied Biosystems, Foster City, CA, USA), followed by PCR with specific primers and analysis by agarose gel electrophoresis. The mRNA level of each RTK gene from reverse transcription-PCR was normalized to that of GAPDH. Q-PCR was performed using specific primers. The relative amount of each gene was calculated using the equation $2^{-\Delta\Delta Ct}$. GAPDH served as an endogenous control for Q-PCR.

Western blotting

Cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EGTA, 1% (v/v) Triton X-100) or SDS-lysis buffer (240 mM Tris-acetate, pH 7.8, 5 mM EDTA, 1% (w/v) SDS, 0.5% (v/v) glycerol) containing protease inhibitors (10 ng/ml leupeptin and 10 ng/ml aprotinin; 1 mM PMSF) and phosphatase inhibitors (1 mM Sodium orthovanadate). The protein concentration of each sample was determined by BCA assay (Santa Cruz Biotechnology). Equal amount of proteins were resolved

by SDS-PAGE and then transferred to a nitrocellulose membrane for Western blotting. Immunoreactive bands were detected using IRDye-conjugated IgG and the Odyssey Infrared Imaging System (LI-COR Biosciences).

FISH

FISH was performed on cells seeded on slides. The ROS1 split FISH probes (FS0015, Abnova, Taipei, Taiwan) were used. Slides were fixed with 95% (v/v) ethanol for 10 min at room temperature, dehydrated, and air dry. The cells on the slides were then digested with Digest-all (Zymed, South San Francisco, CA, USA) at 37°C for 5 min. *ROS1* probe was applied to each slide, with denaturation at 94°C for 4 min; hybridization was then done overnight at 37°C in VYSIS HYBrite (Abbott Molecular Inc, Des Plaines, IL, USA). Post-hybridization wash was done with 2× standard saline citrate at 72°C for 5 min and then mounted with VECTASHIELD mounting medium containing DAPI, followed by evaluation with a Leica DMR fluorescence microscope.

Bisulfite sequencing

Genomic DNA from OSCC cells was analyzed using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA). The ROS1 promoter region was amplified from bisulfite-treated DNA by PCR using the following primers: forward primer, 5'-GTATGTGTTATTTATAGGGAGAATT-3'; reverse primer, 5'-CATTATACAAACTTCCTTCC-3'.[25] The PCR products were subsequently cloned into the pGEM-T vector (Promega), and independent clones were sequenced.

ChIP assays

Cells ware treated with 1% (v/v) formaldehyde to crosslink DNA-protein, then harvested, re-suspended in lysis buffer and sonicated (Bioruptor) to generate chromatin fragments. Sheared chromatin was incubated with an appropriate antibody or control IgG conjugated with protein A or protein G agarose beads. Immunoprecipitated DNA was quantified by Q-PCR with specific primers. Sequences of all primers used for ChIP assays are listed in Supplementary Table 1.