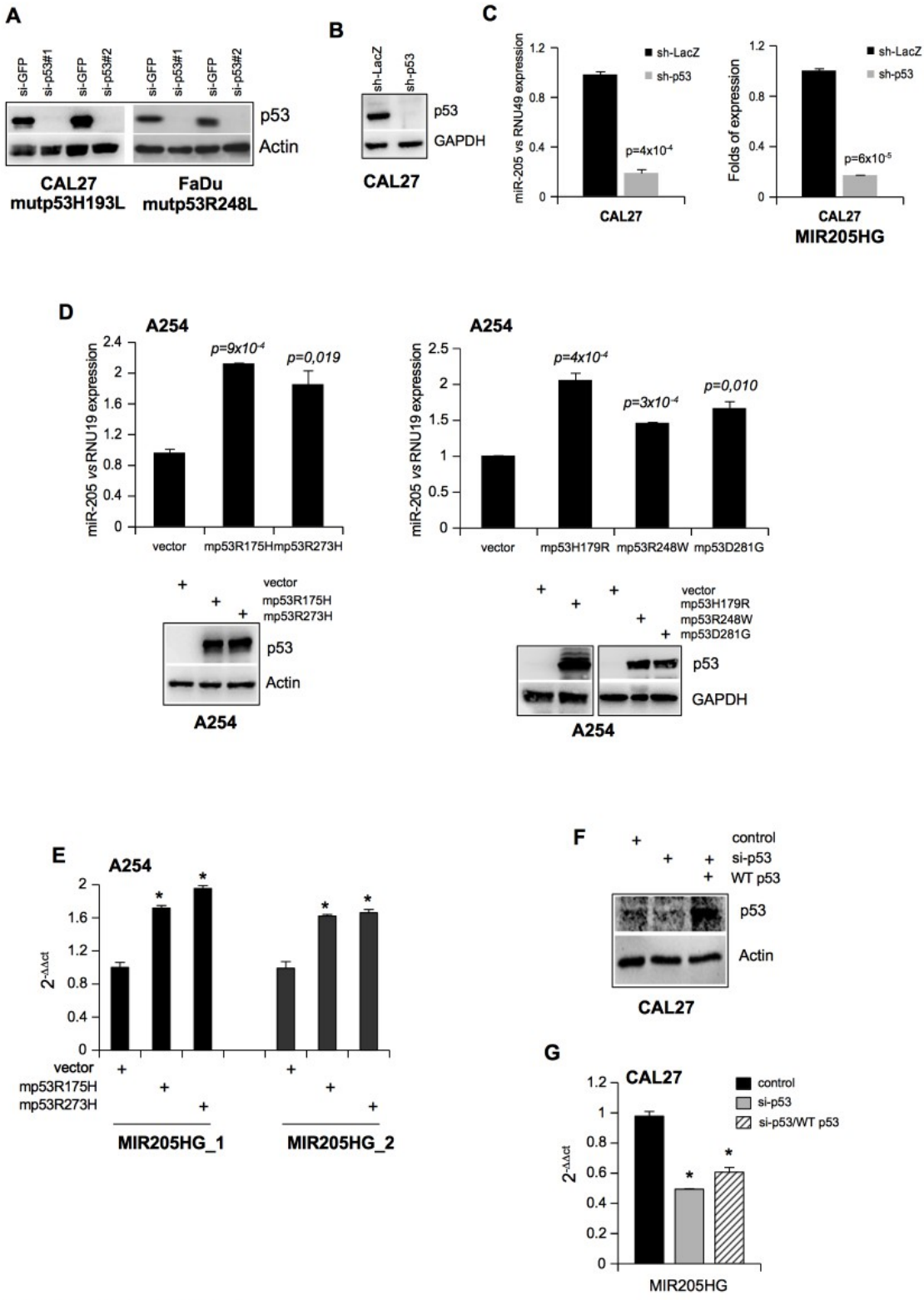
Supplementary Figures

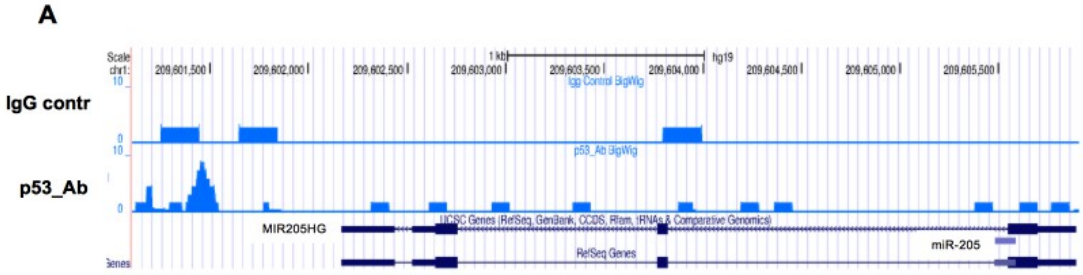


Supplementary Figure 1

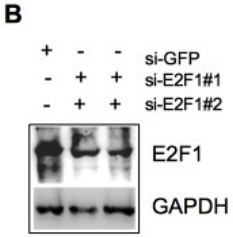
Supplementary Figure S1:

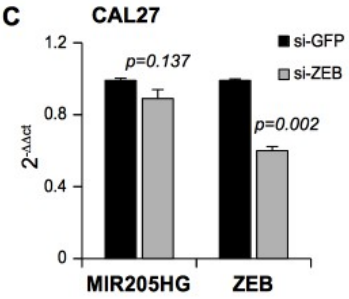
(A) Immunoblotting to show p53 and Actin (loading control) protein expression from whole protein lysates of CAL27 and FaDu cells transfected with si-GFP and two different si-RNAs against p53 transcript, si-p53#1 and si-p53#2 oligos. Purified RNAs by these cells were used in the experiments showed in Figs. 1B and C. Representative images are shown. (B) Immunoblotting to check p53 and GAPDH (loading control) protein expression from whole protein lysates of CAL27 cell line transfected with pRSuper-p53 (sh-p53) and pRSuper-LacZ (sh-LacZ) as control. We produced sh-p53 and sh-LacZ stable lines after selection with puromycin incubation (details are described in Supplementary Materials and Methods). (C) Left side: hsa-miR-205-5p expression from sh-p53 and sh-LacZ -CAL27 cells was analyzed by Taq-Man assay with a specific probe. The expression was normalized versus RNU49. Right side: MIR205HG expression was analyzed by RT-qPCR in cDNA samples derived from the same cells. (D) Tumoral head and neck A254 cells were transiently transfected with the indicated overexpressing mutp53 pCDNA vectors. The empty vector was used as negative control. Immunoblotting for p53 and Actin (loading control) has shown. Hsa- miR-205-5p expression was analyzed by Taq-Man assay with a specific probe. The expression was normalized versus RNU19. (E) MIR205HG expression was analyzed by RT-qPCR in cDNA samples derived from A254 described in (D). (F and G) CAL27 cells were depleted of mutp53 protein by si-RNA transfection and subsequently they were transfected with wt-p53 expressing vector. The expression of p53 protein was checked by western analysis (F) and MIR205HG expression were analyzed by RT-qPCR (G).

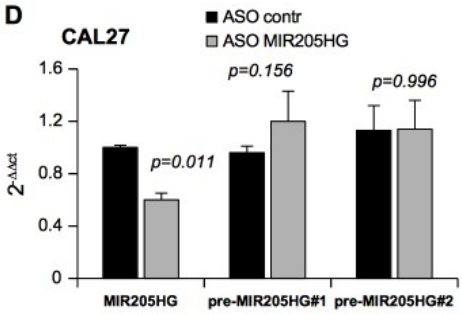
For all Real Time assays, bars represent mean ± s.d. from three biological replicates. Statistically significant results were referred with P- value <0.05.



ChIP seq by H1299 cells-mutp53R273H (Vaughan et al., 2014)





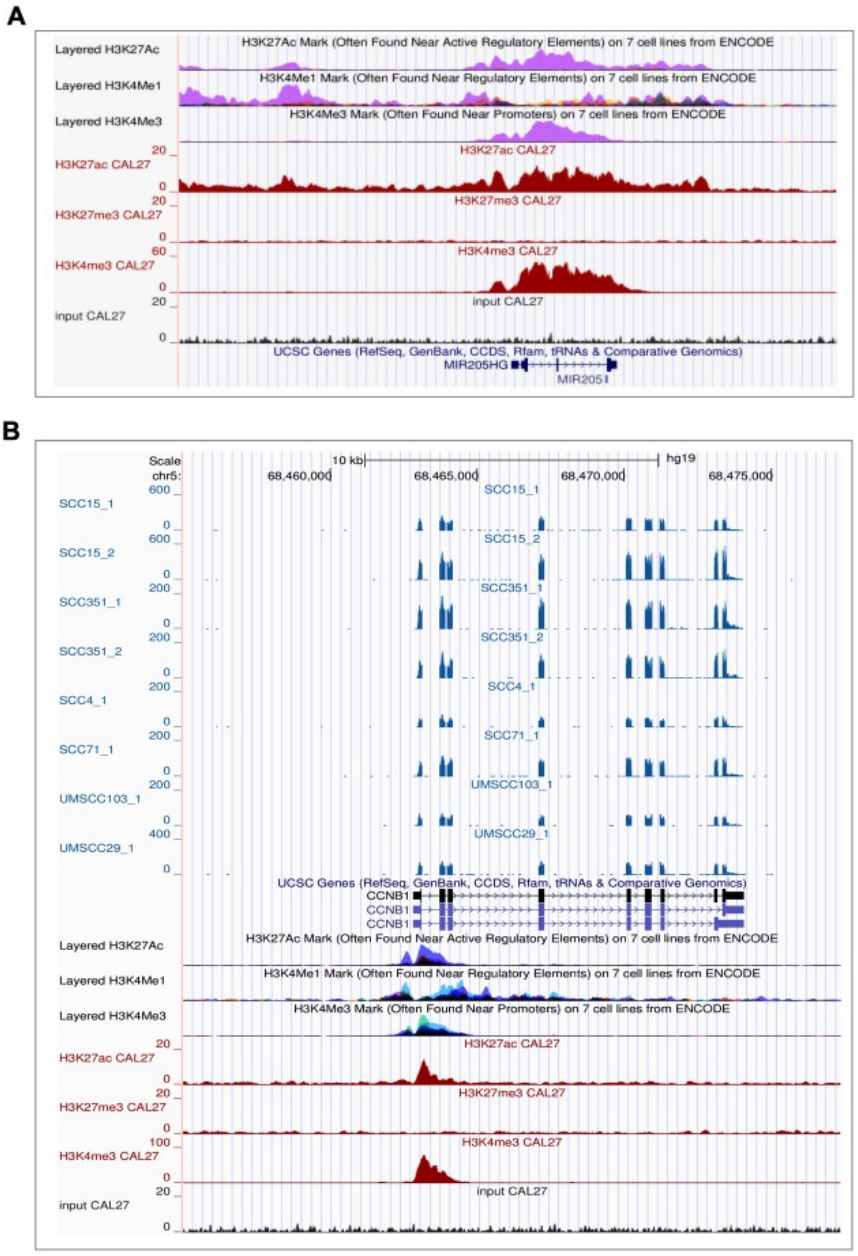


Supplementary Figure S2

Supplementary Figure S2:

(A) Analysis of mutp53 binding on MIR205HG gene using the ChIP-seq data previously published by Vaughan et al. [40]. Chromatin from H1299 stably overexpressing mutp53R273H protein was immunoprecipitated with anti-p53 antibody and relative IgG as negative control. (B) A representive immunoblotting analysis of E2F1 and GAPDH (loading control) in CAL27 cells transfected with either si-GFP and two different si-E2F1 oligos. (C) CAL27 cells were transfected with the commercial smart pool to knock-down ZEB expression. *MIR205HG* and *zeb* expression was analyzed by RT-qPCR in cDNAs. (D) CAL27 cells were transfected with ASO-control or ASO-MIR205HG oligos. Spliced *MIR205HG* mRNA and *pre-MIR205HG* RNA expression were evaluated with the indicated oligonucleotides.

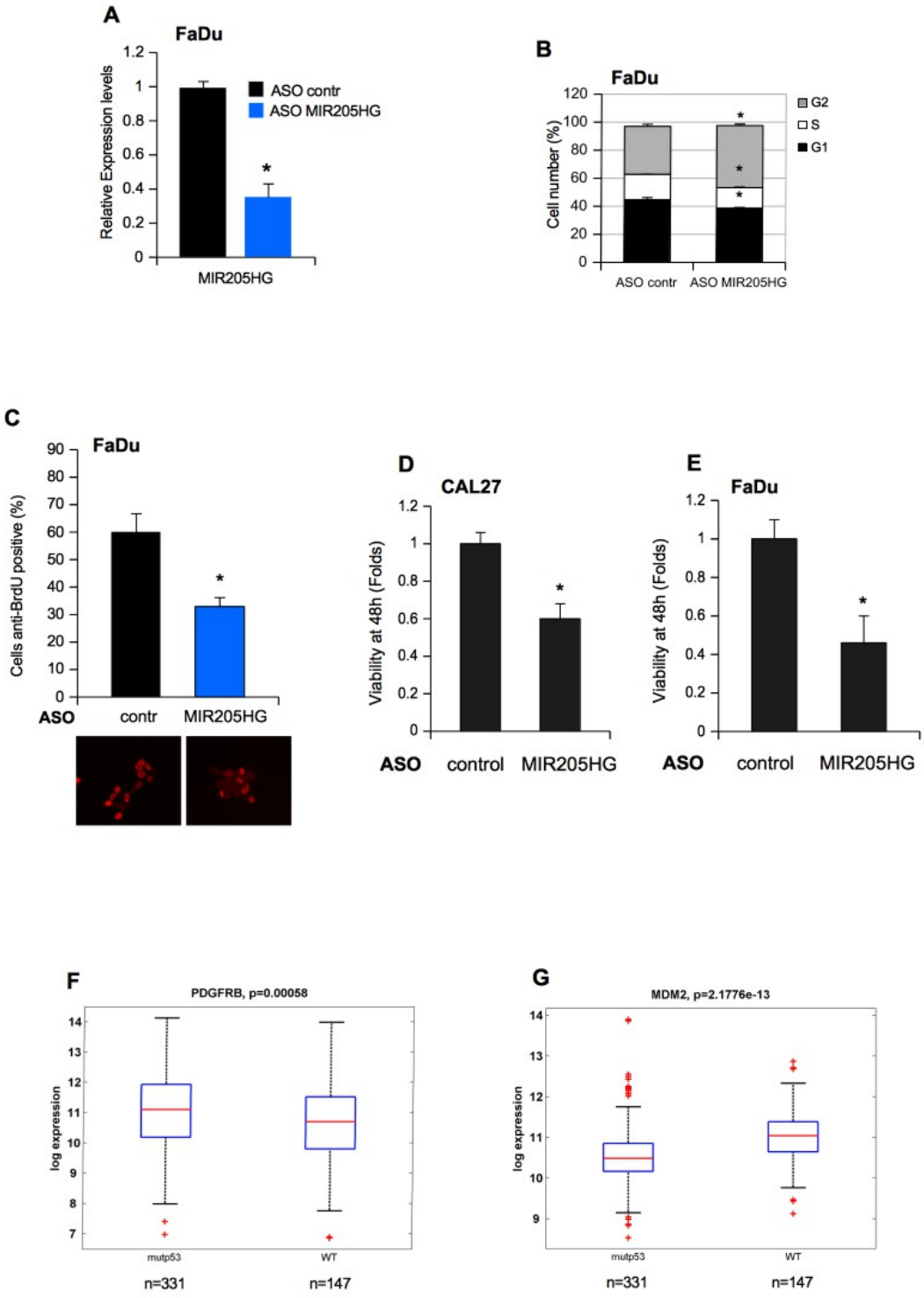
In all RT-qPCR analysis, bars represent mean ± s.d. from three biological replicates. Statistically significant results were referred with P- value <0.05.



Supplementary Figure 3

Supplementary Figure S3:

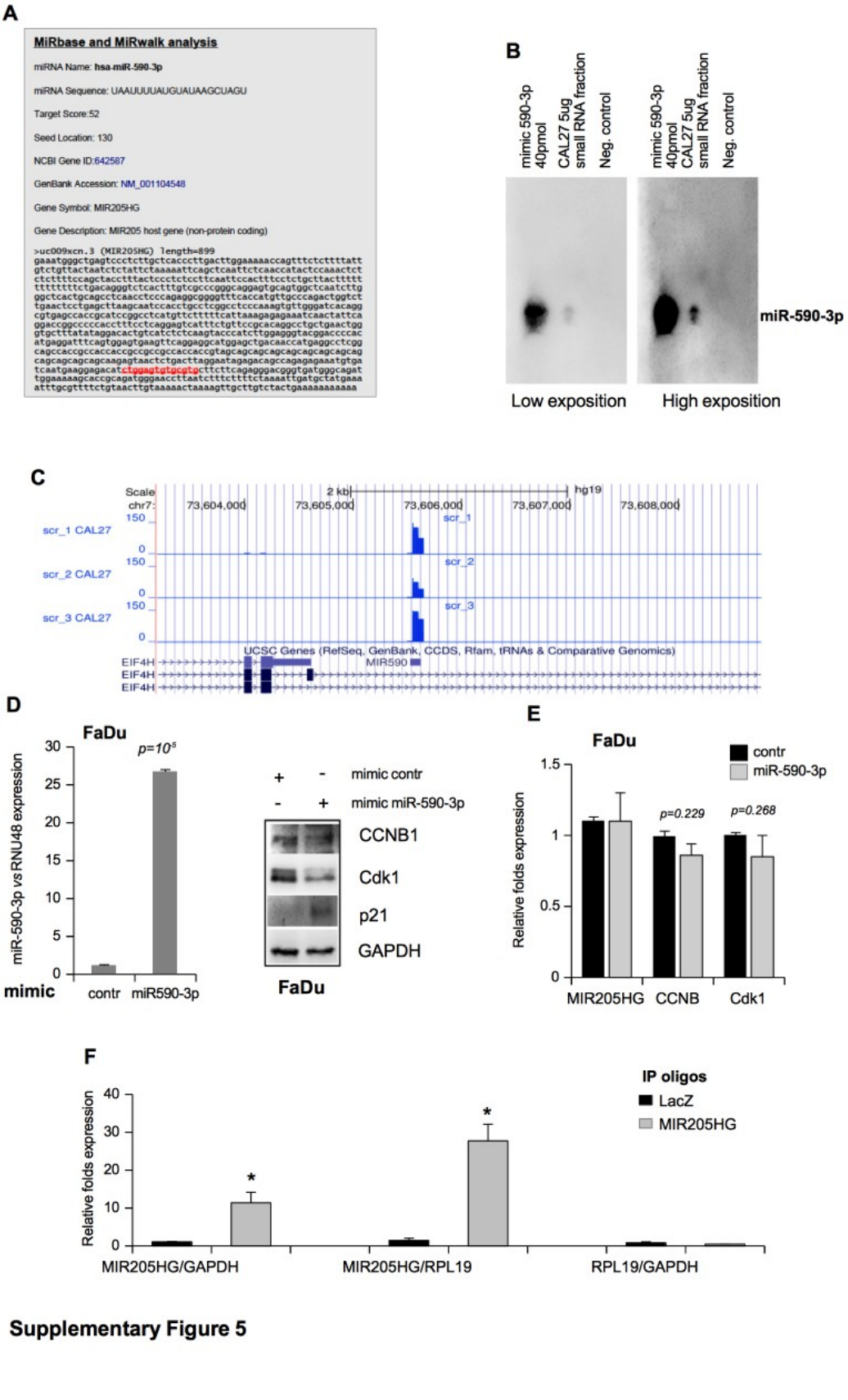
(A) and (B) UCSC Genome Browser screenshots for MIR205HG and CCNB1 loci, respectively. Displayed tracks include, from bottom to the top, UCSC genes, ChIP-seq enrichment for H3K27ac, H3K27me3 and H3K4me3 plus input on CAL27 cells, histone modifications for 7 ENCODE cell lines (different tissues) and regarding CCNB1 the RNA-seq expression level on 8 different HNSCC cell lines (GSE68872). (C) MIR205HG expression was analyzed by RT-qPCR in cDNA derived from CAL27 and FaDu cells. The amplification has led in the same experimental PCR plate to compare the lncRNA expression level between the two cell lines.



Supplementary Figure 4

Supplementary Figure S4:

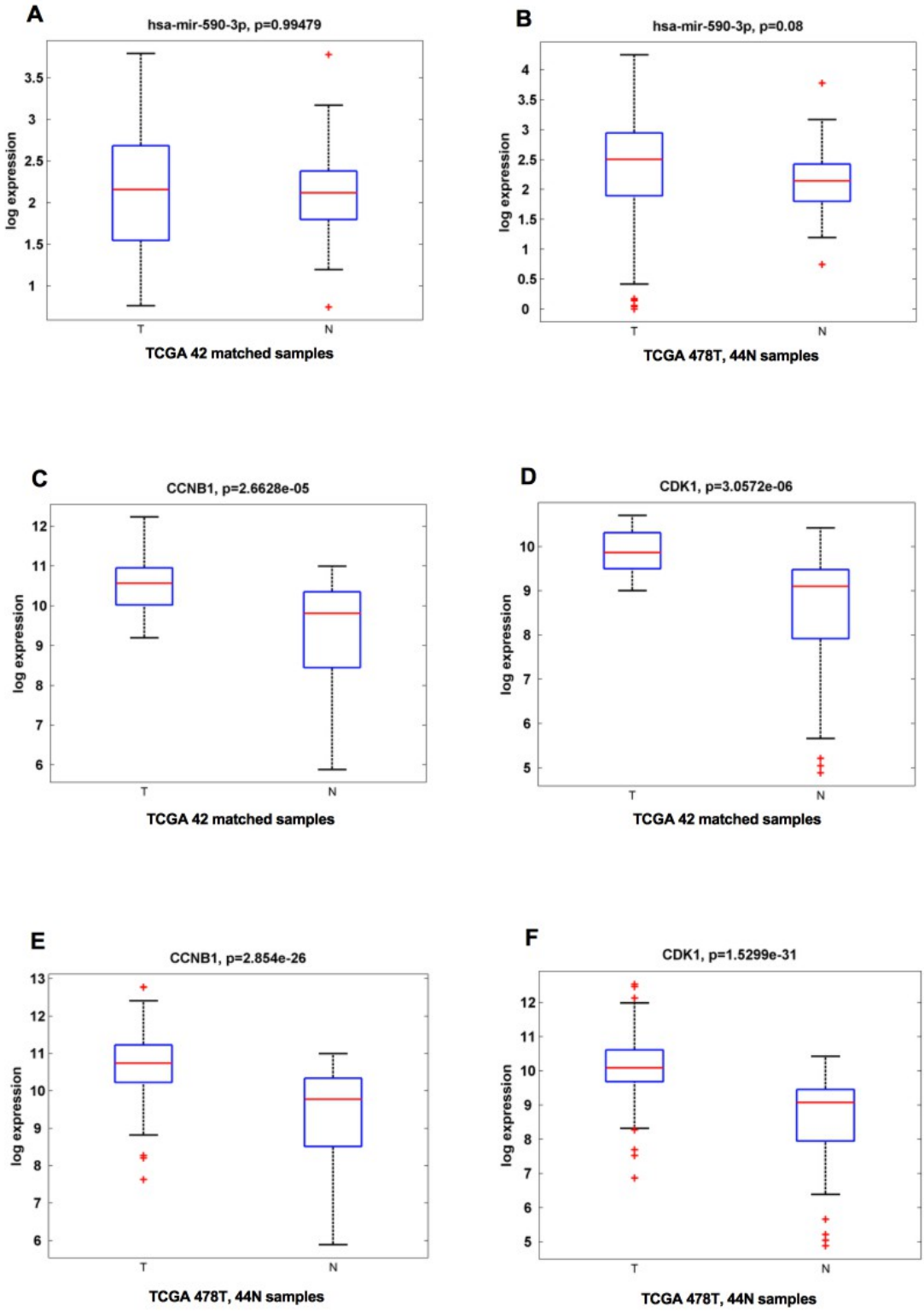
(A) MIR205HG expression was analyzed by RT-qPCR from FaDu cells transfected with ASO-control and ASO-MIR205HG oligos for 48h. (B) Northern blot analysis of MIR205HG expression in total RNA extracts from CAL27 cells transfected with ASO-control and ASO-MIR205HG oligos for 48h. (C) FaDu cells transfected as described in (A) were labeled with PI staining solution and analyzed by flow cytometry (pre-G1, G0/G1, S, and G2/M). Experiments performed in triplicate yielded similar results. Statistical analysis of the number of cells (%) in G1, S and G2 phases has shown. Data are presented as means ± SD of three independent tests. *P- value < 0.05. (D) Cells treated as in (A) were incubated with BrdU for 20min. The BrdU-positive cells were then identified by immunofluorescence staining and observed under a fluorescence microscope. The proportion of BrdU-positive cells. Data are expressed as the means of the percentage of BrdU-positive cells ± SD. *P<0.05. (E) and (F) Viability at 48h of CAL27 and FaDu cell lines transfected with ASO-control or ASO-MIR205HG oligos was obtained by MTT assay. Bars represent mean ± s.d. from six replicates of three independent experiments. P-value were calculated with two- tailed t-test. Significant P- value was indicated in the graph. (G) and (H) Box-plot analysis representing the expression of PDGFRB (G) and MDM2 (H) in mutp53 and wtp53 tumoral tissue subgroups from HNSCC casuistry by TCGA.



Supplementary Figure 5

Supplementary Figure S5:

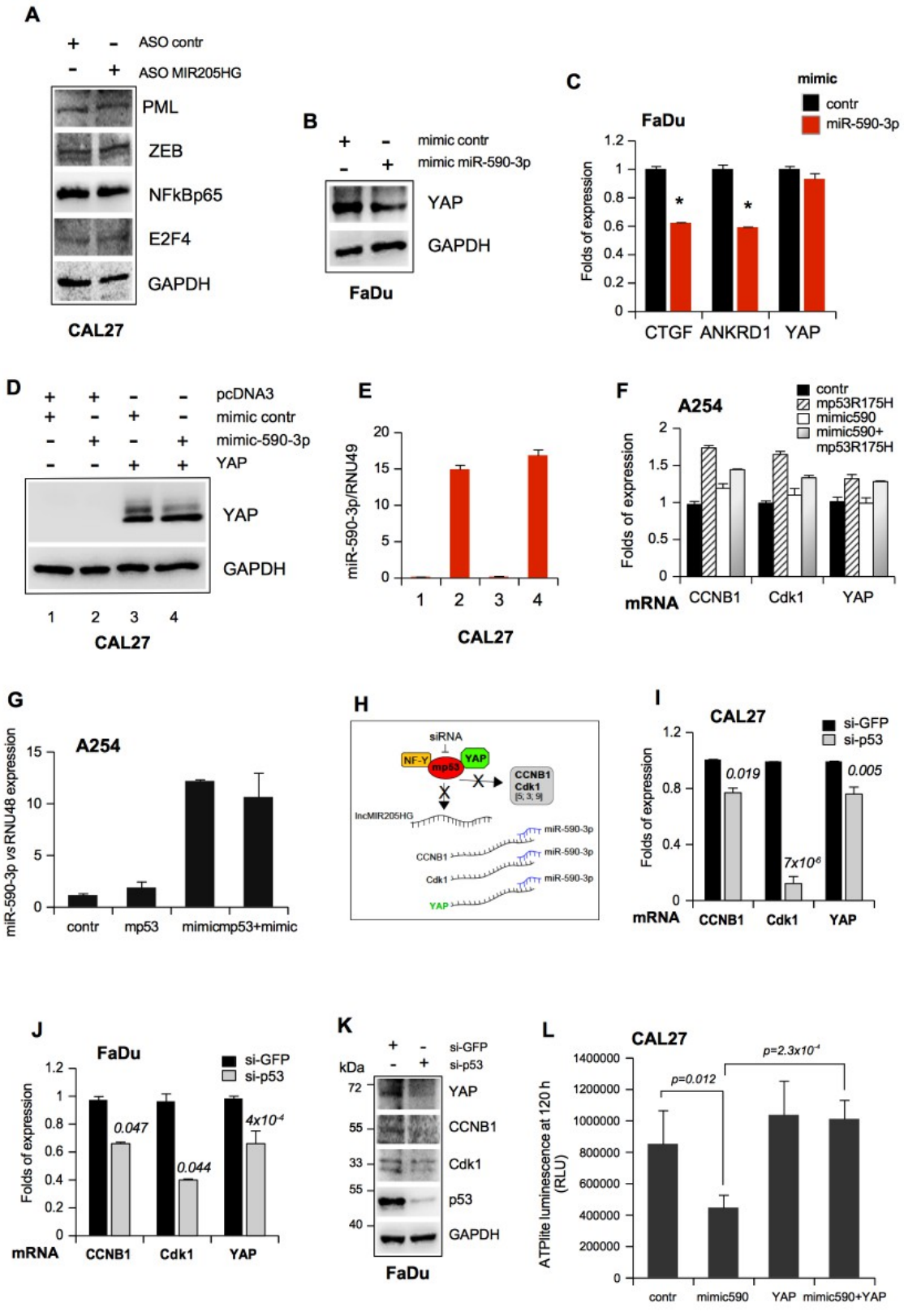
(A) Prediction by MiRbase and MiRwalk software of MIR205HG RNA sequence responsible for the miR-590-3p binding. (B) Northern Blot analysis led in small RNA purified fraction from CAL27 cells. 40pmol of mimic miR-590-3p and mimic miR-205-5p are loaded in the gel were used as positive control and negative control, respectively. (C) Bigwig enrichment or miR-590 locus of three miRNA-seq replicates on CAL27 cell line. MicroRNA sequencing was performed using NGS technology within our institute's projects. (D) Left side: miR-590-3p expression was analyzed by Taq-Man assay with a specific probe in RNA derived from FaDu cells transfected with mimic control and mimic miR-590-3p oligonucleotides. The expression was normalized versus RNU48. Bars represent mean ± s.d. From three biological replicates. P- values were calculated with two-tailed t- test. Right side: a representive immunoblotting analysis of CCNB, Cdk1, p21 and GAPDH (loading control) in FaDu cells transfected with either mimic control and mimic miR-590-3p oligonucleotides. (E) *MIR205HG*, *ccnb1* and *cdk1* expression was analyzed by RT-qPCR in CAL27 cells transfected with either mimic control and mimic miR-590-3p oligos. Bars represent mean ± s.d. from three biological replicates. P- values were calculated with two- tailed t- test. (F) We considered two control mRNAs, *gapdh* and *rpl19* to validate the specific precipitations of MIR205HG in the samples. The normalization of ChIRP experiment was carried out considering the background produced by not specific GAPDH and RPL19 RNA binding to the streptavidin resin used in the ChIRP assay (Figure 5F). We have further checked that GAPDH and RPL19 did not have unspecific enrichment comparing the expression of one versus the other in the ChIRP experiments. Bars represent mean ± s.d. from three biological replicates. Significant P- values were referred *P- value <0.05.



Supplementary Figure 6

Supplementary Figure S6:

(A and B) Box-plot analysis representing the expression of miR-590-3p in 42 tumoural and normal matched tissues (A) and in 478 tumoural and 44 normal tissues (B) from HNSCC by TCGA. (C and D) Box-plot analysis representing the expression of *ccnb1* (C) and *cdk1* (D) in 42 tumoural and normal matched tissues from HNSCC casuistry by TCGA. (E and F) Box-plot analysis representing *ccnb1* (E) and *cdk1* (F) in 478 tumoural and 44 normal tissues from HNSCC by TCGA.



Supplementary Figure 7

Supplementary Figure S7:

(A) From the same protein extract samples shown in the Figure 6A, western blot analysis was performed to check the expression of other unrelated transcriptional factors, as control of the specificity of ASO-MIR205HG oligos transfection on decrease of YAP protein expression. (B) Immunoblotting to detect YAP and GAPDH (loading control) proteins in whole protein extracts from FaDu cells transfected with mimic control and mimic miR-590-3p. Representative image is shown. (C) *ctgf*, *ankrd1* and *yap1* expression was analyzed by RT-qPCR in FaDu cells treated as in (B). (D) Immunoblotting to detect YAP and GAPDH (loading control) proteins in whole protein extracts from CAL27 transfected as indicated. Representative image is shown. These cells were used in Luciferase reporter assay (Figure 6G) and in ATPlite viability assay (Figure 6J). (E) TaqMan-assay to detect the miR-590-3p expression in CAL27 cells previously described and used in Luciferase reporter assay (Figure 6G) and in ATPlite viability assay (Figure 6J). (F) *ccnb1*, *cdk1* and *yap1* expression was analyzed by RT-qPCR in A254 cells transfected as indicated in the figure. (G) TaqMan-assay to detect the miR-590-3p expression in A254 cells previously described in (F). (H) Schematic representation of two levels of cell cyle -related gene expression regulation by mutant p53. (I) and (J) RT-qPCR of CCNB1 Cdk1 and YAP mRNA levels in CAL27 (I) and FaDu (J) cells upon transduction with si-GFP and si-p53 oligos. Values are means $\pm$ s.d. of three replicates from three independent experiments. (K) Western blot analysis for YAP, CCNB, Cdk1 and GAPDH (loading control) protein expression in lysates from control (si-GFP) and p53-depleted (si-p53) FaDu cell line. Representative images are shown. (H) Viability of CAL27 cell line at 120 h, transfected as reported in the graph, was determined by ATPlite luminescence analysis. ATP amount was expressed as relative light units (RLU). All the values are means ± s.d. of six replicates from three independent experiments. P- values were calculated with two-tailed t-test.

Table S1

Primer sequences for amplification of cDNAs in Real Time PCR experiments.

MIR205HG_1 FW	5’- GGAGTGCAGTGGCTCAATCT-3’
MIR205HG_1 RV	5’- TGGATTGCTTAAGCTCAGGA -3’
MIR205HG_2FW	5’- CAGCAGCAGCAGCAAGAGTA -3’
MIR205HG_2 RV	5’- CTCTGAAGAAGCACGCACAC -3’
Pre-miR-205-5p FW	5’- TCATTCCACCGGAGTCTGT -3’
Pre-miR-205-5p RV	5’- AGCTCCATGCCTCCTGAACT -3’
Pre-MIR205HG#1 FW	5'- AGCTTTGCTGAGAGGTGCAT -3'
Pre-MIR205HG#1 RV	5'- GCTGGAAAAGAGAGAGTTTGGA -3'
Pre-MIR205HG#2 FW	5'- CCACCTTTCCTCAGGAGTCA -3'
Pre-MIR205HG#2 RV	5'- CAGCCATGCAGATGAGAAGA -3'
CTGF FW (PMID: 17525740)	5′- CAAGGGCCTCTTCTGTGACT -3’
CTGF RV (PMID: 17525740)	5’- ACGTGCACTGGTACTTGCAG -3’
ANKRD1 FW (PMID: 24976009)	5’- CTGTGAGGCTGAACCGCTAT -3’
ANKRD1 RV (PMID: 24976009)	5’- CTGTGAGGCTGAACCGCTAT -3’
YAP1 FW (PMID: 24976009)	5’- AAGGAGAGACTGCGGTTGAA -3’
YAP1 RV (PMID: 24976009)	5’- CCTGAGACATCCCAGGAGAA -3’
GAPDH FW (PMID: 23125021)	5'- GAGTCAACGGATTTGGTCGT -3'
GAPDH RV RV (PMID: 23125021)	5'- GAGTCAACGGATTTGGTCGT -3'
Actin FW	5'- GAGGCCCAGAGCAAGAGAG -3'
Actin RV	5'- AGGTGTGGTGCCAGATTTTC -3'
Cdk1 FW (PMID: 26691213)	5'- GGAAGGGGTTCCTAGTACTGC -3'
Cdk1 RV (PMID: 26691213)	5'- TGGAATCCTGCATAAGCACA -3'
CCNB1 FW (PMID: 26691213)	5'- GTTCCTACGGCCCCTGCT -3'
CCNB1 RV (PMID: 26691213)	5'- ATTTTGGCCTGCAGTTGTTC -3

RPL19 FW (PMID: 28652379)	5'- CGGAAGGGCAGGCACAT -3'
RPL19 RV(PMID: 28652379)	5'- GGCGCAAAATCCTCATTCTC -3'

Table S2

Primers used for the amplification of different regulatory regions of MIR205HG promoter.

MIR205HG promoter_NF-Y seq FW	5'- AGCCTTCCCTGAATCTCACA-3'
MIR205HG promoter_NF-Y seq RV	5- CATCTACAAGAAGAGGCAAAAACA-3'
MIR205HG promoter_E2F1 seq FW	5'- TTCCCGAAGTGCATTGTGTA-3'
MIR205HG promoter_E2F1 seq RV	5'- TTTGTGTGTGTGTGTGTGTGTG-3'
MIR205HG third intron FW	5'- TGAATCATTTTCCAGGCACA-3'
MIR205HG third intron RV	5'- CACTGTCCAAAGAGCACGAA-3'

Table S3

Schematic representation of the primers used in ChIRP experiments onto MIR205HG tanscript sequence and the nucleotide sequences. LacZ oligos are used as negative control of chromatin precipitation. The primers have 3' modified by adding a biotinylated group.

MIR205HG transcript

1 gaaatgggct gagtccctct tgctcaccct tgacttggaa aaaccagttt ctcttttatt

61 gtctgttact aatctctatt ctaaaaattc agctcaattc tcaaccatac tccaaactct

121 ctcttttcca gctaccttta ctccctctcc ttcaattcca ctttcctctg cttacttttt

181 tttttttct gacagggtct cactttgtcg cccgggcagg agtgcagtgg ctcaatcttg

241 ggctcactgc agcctcaacc tcccagaggc ggggtttcac catgttgccc agactggtct

301 tgaactcctg agcttaagca atccacctgc ctcggcctcc caaagtgttg ggatcacagg

361 cgtgagccac cgcatccggc ctcatgttct ttttcattaa agagagaaat caactattca

421 ggaccggccc ccacctttcc tcaggagtca tttctgttcc gcacaggcct gctgaactgg

481 gtgctttata taggacactg tcatctctca agtacccatc ttggagggta cggaccccac

541 atgaggattt cagtggagtg aagttcagga ggcatggagc tgacaaccat gaggcctcgg

601 cagccaccgc caccaccgcc gccgccacca ccgtagcagc agcagcagca gcagcagcag

661 cagcagcagc agcaagagta actctgactt aggaatagag acagccagag agaaatgtga

721 tcaatgaagg agacatc***tgg agtgtgcgtg c***ttcttcaga gggacgggtg atgggcagat

781 tggaaaaagc accgcagatg ggaaccttaa tctttctttt ctaaaattga tgctatgaaa

841 atttgcgttt tctgtaactt gtaaaaacta aaagttgctt gtctactgaa aaaaaaaaa

hsa-miR590-3p pairment

ChIRP oligos

CTA_1: 5'- AAGAGGGACTCAGCCCATTT -3'

CTA_2: 5'- ACAGACAATAAAAGAGAAAC -3'

CTA_3: 5'- ATTGAGCCACTGCACTCCTG -3'

CTA_4: 5'- TTGAGGCTGCAGTGAGCCCA -3'

CTA_5: 5'- CAGGTGGATTGCTTAAGCTC -3'

CTA_6: 5'- CTGTGATCCCAACACTTTGG -3'

CTA_7: 5'- ACAGAAATGACTCCTGAGGA -3'

CTA_8: 5'- CTCATGGTTGTCAGCTCCAT -3'

CTA_9: 5'- TCCTAAGTCAGAGTTACTCT -3'

CTA_10: 5'- AGACAAGCAACTTTTAGTTT -3'

LacZ_1: 5'- GTCATATGCATAAAGCGTTG -3'

LacZ_2: 5'- TTAACGCCGC AGTGGTAGAA -3'

Table S4

27 oligonucleotides that form the specific probe for the MIR205HG FISH analysis (Stellaris; Biosearch Technologies, Inc.). They are designed using the software "https://www.biosearchtech.com/stellarisdesigner/".

Probe Sequence (5' to 3')
aagagggactcagcccattt
tttttccaagtcaagggtga
acagacaataaaagagaaac
gtgagaccctgtcagaaaaa
attgagccactgcactcctg
ttgaggctgcagtgagccca
gagttcaagaccagtctggg
caggtggattgcttaagctc
ctgtgatcccaacactttgg
tgaaaaagaacatgaggccg
ccggtcctgaatagttgatt
acagaaatgactcctgagga
cagttcagcaggcctgtgcg
acagtgtcctatataaagca
tccaagatgggtacttgaga
cactgaaatcctcatgtggg
ctcatggttgtcagctccat
ctgctgctgctgctacggtg
ctgctgctgctgctgctgct
tcctaagtcagagttactct
atttctctctggctgtctct
cagatgtctccttcattgat
ctctgaagaagcacgcacac
gtgctttttccaatctgccc
aagattaaggttcccatctg
acaagttacagaaaacgcaa
agacaagcaacttttagttt

Table S5

patient number	codon	Type of mutation
5	H193Y	missense
18	V157I, R158H	missense
69	N247 in frame	missense
84	F106 in frame	frameshift
105	H179R	missense
93	del 4bp introne 8	frameshift
3	w t	
87	w t	
90	w t	
15	w t	
45	del 3bp exon 9	frameshift
12	w t	
115	w t	
109	C275R	missense
97	w t	
120	w t	
71	w t	
101	R213X	nonsense
86	I255F	missense
104	H179R	missense
99	E198X	nonsense
28	P278T	missense
29	R156H; C275S; R282Q	missense
49	E198X	nonsense
17	R196X	nonsense
61	V157T	missense
82	del 2 bp P152-E153 not in frame	frameshift
30	del. T155-M169 (nt 12453-12492),not in frame; 242 tgc(C)>tac(Y)	frameshift; missense
70	S183X	nonsense
74	slice site exon 9	splice site
42	w t	
8	w t	
7	G266R	missense
66	del 1bp exon 4 not in frame	frameshift
113	R196X	nonsense
63	P273C	missense
88	del 4 bp exon 8	framshift
54	w t	
48	w t	
14	P278S	missense
37	W146X	nonsense
22	R248Q	missense
59	H193R	missense
58	ins 1 bp not in frame	frameshift
51	Y234C	missense
55	Y205C	missense
36	w t	
107	w t	
57	w t	
102	w t	
10	w t	
1	w t	
2	w t	
40	w t	
44	w t	
41	w t	
112	w t	
73	w t	
32	w t	
65	w t	
60	w t	
114	w t	
67	w t	
33	w t	

Mutational status of *TP53* gene of HNSCC tumors used for RT-qPCR expression profiles.

Supplementary Materials and Methods

Cells and transfections

pRetroSuper-p53 and pRetroSuper-LacZ constructs [5, 8] were transfected in CAL27 cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. After 48 h from the transfection, the selection started using 1ug/ml puromycin for 2 weeks. Experiments were performed using stable, pooled clones.

CAL27 cells were transfected with 20 μM of si-ZEB smart pool composed by four oligos from Dharmacon (L-006564-01), using RNAiMax (Invitrogen) following the manufacturer's instructions. The cells were plated in 35mm plates in biological triplicates. After 24 h of incubation, the transfection were stopped by recovering the cells.

Plasmids and RNA Transfection

Mutant p53 and YAP exogenous expression was performed using pcDNA3-p53-R175H, pcDNA3-p53-R273H, and pcDNA3-YAP vectors and empty pcDNA3 was used as control [9]. Cells were transfected with Lipofectamine 2000 following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA).

The following sequences are employed for si-RNA oligonucleotides transfection: si-p53#1 and si-p53#2 oligos previously described [5, 9], si-GFP employed as nonsilencing control was 5'-GGCTACGTCCAGGAGCGCACC-3' [5, 9], si-NF-YB smart pool of three target specific oligos (sc-29945 Santa Cruz Biotechnology) [9]; si-E2F1#1 and si-E2F1#2 oligos previously described [6].

To knock-down the hsa-miR-205 expression, we used miRCURY LNA™ miRNA Inhibithor purchased from Exiqon. These miRCURY LNA™ miRNA Inhibithors contains phosphorothioate bonds. The sequences were: "LNA-miR-205": 5'-AGACTCCGGTGGAATGAAGG-3' (n°410131-00); "LNA-miR-control": 5'-GTGTAACACGTCTATACGCCCA-3' (n° 199004-00).

To down-regulate lncMIR205HG expression, we used Exiqon LNA™ GapmeR antisense technology. The LNA™-containing flanking regions confer nuclease resistance to the antisense

oligo while at the same time increase target binding affinity regardless of the GC content. The central DNA “gap” activates RNase H cleavage of the target RNA upon binding. Antisense oligonucleotide (ASO) is 16 nucleotides long containing LNA™ in the flanking regions and DNA in a LNA™ free central gap. We used the Exiqon online design tool to produce the following antisense oligo at the junction of two exons: ASO-MIR205HG: 5'-GTGTCCTATATAAAGC-3'; ASO-negative control A supplied by Exiqon n°300610.

RNAs were transfected with RNAiMax reagent following the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA).

Fluorescence In Situ Hybridization (FISH)

Detection of lncMIR205HG transcript on CAL27 cell line was conducted by FISH using Custom Stellaris™ probes (Biosearch Technologies, Inc.). Stellaris FISH probe sets consist of multiple singly labeled oligonucleotides designed to hybridize along targeted RNA transcripts. The potential for false positives is low because the signal is only detectable when tens of probes are bound [70]. The entire MIR205HG nucleotide sequence was used to design the probe. The 27 oligonucleotides forming the probe were designed using the specific software "https://www.biosearchtech.com/stellarisdesigner/" and they are are listed in Supplementary File D. Quasar® 570 was the reporter dye used in the FISH experiments. DNA was stained with TO-PRO-3 (ThermoFisher Scientific). Images were obtained using CLSM Zeiss 700 confocal laser scanning microscopes with a 100× oil objective and ZEN 2003 software.

Cell viability and colony formation assays

Cell Viability was carried out using the MTT assay kit supplied by Abnova (KA1606) following the manufacturer’s instructions. The fluorometry was recorded to 570 nm with a plate reader (Thermo Scientific). Cell viability was assayed with ATPlite (Perkin Elmer) according to the manufacturer's instructions using the EnSpireMultilabel Reader (Perkin Elmer). Cells (10^4 per well) were plated in triplicate in 96-well plates and treated as indicated.

With regard to colony-forming assays, cells are transfected as indicated in the figure accordingly

with the procedures described in Materials and Methods section. After 24 h of transfection, 800 cells from each point were seeded in 12-multiwell plates and grown for 10 days. Cells were stained with crystal violet and colonies evaluated by using ImageJ software (https://imagej.nih.gov/ij/).

Boyden chamber migration assay

Cells are transfected as indicated in the figure accordingly with the procedures described in Materials and Methods section. After 24 h of transfection, 50000 cells were suspended in 500 µl serum free medium in transwells (PET membrane of 8 µm pore size , Falcon n. 353097) and placed in 24-well plates containing medium with 10% FBS. After 18 h, chambers were washed and cells were removed from the upper side of the chamber with a cotton swab. Migrated cells were fixed and stained using DAPI cell stain solution (Chemicon International). Images were captured using the 20x objective of the Axiovert 200 microscope (Carl Zeiss) and analyzed using AxioVision software (Carl Zeiss). Representative images are shown. The average number of migrated cells from 10 representative fields (six replicates per condition) was counted under a phase contrast microscope. P-values were calculated with two-tailed Student's t-test. Statistically significant results were referred with a p-value < 0.05.

Flow cytometric analysis of cell cycle

Cells were seeded in triplicate in 6-well plates in a number of 2×10^5 cells per well and transfected as indicated. After 48 h of transfection, cells were washed in PBS, fixed in ethanol solution and stained in a PI solution (50µg PI in 0.1% sodium citrate, 0.1% NP40, pH7.4) for 30 min at 4°C in the dark. Flow cytometry analysis was performed using a Guava® easyCyte flow cytometer (Merck-Millipore). Cell cycle was evaluated by InCyte Guava Cell Cycle Software (Merck-Millipore). Each point were analyzed in at least three different experiments. P-values were calculated with two-tailed Student's t-test. Statistically significant results were referred with a p-value < 0.05.

BrdU incorporation assay

CAL27 cells were seeded onto 22-mm diameter coverglasses placed in 6-well plates (3×10^5

cells/coverglass) and transfected with ASO control and ASO MIR205HG oligos for 48 h. Before to fixing the cells, 10 μM BrdU (Sigma Chemicals) were added to the cultures for 20 min. The cells were rinsed and fixed in 4% phosphate-buffered paraformaldehyde for 10 min. Following aspiration, the cells were rinsed 3 times in PBS for 5 min and 0.5% Triton X-100/2N Hcl solution was added to the specimens for 20 min. After neutralization using PBS, the specimens were blocked in mouse serum for 60 min. The blocking solution was aspirated and the specimens were incubated in diluted primary mouse-monoclonal antibody to BrdU (1:25; BD Biosciences n°347580) overnight at 4°C. After rinsing 3 times in PBS for 5 min, the specimens were incubated in fluorochrome-conjugated secondary antibody diluted in PBS at room temperature in the dark and observed under fluorescent microscope. At least 1,000 cells/treatment using at least 2 cover-glasses/treatment were counted, and the number of positive cells was recorded. Labeling indexes were calculated as the number of positively stained cells divided by the number of total cells. The total cells for each field was counted using phase contrast light.

Cell extracts and western blotting

Cells were homogenized in a lysis buffer composed by 50 mM hepes pH 7.5, 5 mM EDTA pH 8.0, 10 mM MgCl2, 150 mM NaCl, 50 mM NaF, 0.5% NP40, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 mM DTT and protease and phosphatase inhibitors. Total cell lysates were sonicated for 20 sec and clarified by centrifugation to remove cell debris. Protein concentrations were determined by colorimetric assay (Bio-Rad, Hercules, CA, USA). Nuclei and citosol extracts were prepared as previously described (5). Western blotting was performed using following primary antibodies: mouse monoclonal p53 (DO1) (Santa Cruz Biotech.), GAPDH (Santa Cruz Biotech.), Actin (Sigma), Nucleolin (Sigma); rabbit polyclonal NF-YB (Rockland), CCNA (Santa Cruz Biotech.), CCNB1 (Santa Cruz Biotech.), CCNE (Santa Cruz Biotech.), Cdk1 (Santa Cruz Biotech.), YAP1 (Santa Cruz Biotech.), p21 (Santa Cruz Biotech.), E2F1 (Santa Cruz Biotech.), E2F4 (Santa Cruz Biotech.), NfkBp65 (Santa Cruz Biotech.), ZEB (Cell Signaling), PML (Santa Cruz Biotech.). The immunodetection was performed with the aid of the enhanced chemiluminescence system (Thermo

Fisher Scientific, Rockford, IL, USA). The acquisition of the chemiluminescence has performed by using Alliance 4.7 by UVITEC (Eppendorf).

Luciferase reporter gene assays

Cells ($2x10^5$) were seeded into 6- or 12-well culture plates and transiently transfected with 100ng of pCCAAT-B2LUC or pmutCCAAT-B2LUC reporter constructs [5, 9], 500 ng of pcDNA3-YAP1 and with mimic miR-590-3p or mimic control. 1/10 of CMV-*Renilla* plasmid as an internal control was also co-transfected for transfection efficiency.

For luciferase reporter experiments, a region of 285 bp of the YAP1 3′ untranslated region (UTR) including the binding site for miR-590-3p was amplified from CAL27 cells. The PCR product was then digested with XhoI and NotI and cloned into the reporter plasmid pGL3 (Promega, Madison, WI) downstream of the luciferase gene. 100 ng of 3'UTR-YAP-pGL3 reporter vector carrying the miR-590-3p binding site (or pGL3 empty vector) and 50 nM mimic miR-590-3p (or mimic control) were co-transfected into H1299 cells in 24-well plates. Firefly luciferase activity was measured with a Dual Luciferase Assay Kit (Promega) 24 h after transfection and normalized with a Renilla luciferase reference plasmid. Reporter assays were carried out in quadruplicate and the mean ± S.D. was reported. Statistical significance was analyzed by the unpaired Student *t*-test**.**

Formaldehyde cross-linking and chromatin immunoprecipitation (ChIP)

MatInspector software (www.genomatix.de) was used to perform an in silico promoter analysis to search DNA consensus sequences specific to be bound by transcriptional factors.

Formaldehyde cross-linking and chromatin immunoprecipitations were performed as described [9]. The chromatin solution was immunoprecipitated with sheep polyclonal p53 Ab7 (Millipore), rabbit polyclonal H4Ac (Cell Signaling), H4AcK5 (Cell Signaling), mouse monoclonal RNA-Polimerase II (Millipore) or IgG antibodies as negative control. The immunoprecipitations were performed using Pierce ChIP-grade Protein A/G magnetic beads (Thermo Fisher Scientific, Rockford, IL, USA). Primers used for amplification of different regulatory regions are listed in Supplementary File B. The ChIP samples were further analysed by qPCR for a region resulted negative for the

recruitment of mutant [71]. The promoter occupancy was analyzed by RT-qPCR using the SYBR Green assay (Applied Biosystems, Carlsbad, CA, USA) and the 7500 Fast Real-Time PCR System (Applied Biosystems). P-values were calculated with two-tailed t-test. Normalization was performed to the amount of input chromatin. Statistically significant results were referred with p-value < 0.05.

Microarray data analysis

Head and Neck microarray experiments on Agilent platform for microRNA profiling were performed as previously described [11].

The signals were verified for quality control and extracted by Agilent Feauture Extraction 10.7.3.1 software and entirely processed by MATLAB (The MathWorks Inc.) in house-built routines. All values lower than 1 were considered below detection and thresholded to 1. The arrays were normalized by dividing by the mean intensity only using the 25^{th} and 75^{th} percentile range of the data, preventing large outliers from skewing the normalization. Intensity level of each miRNA was scaled by the median of the distribution of the miRNA for samples processed in the first chip version and by the median of the distribution of the miRNA for samples processed in the second chip version, and all values multiply for the mean value between the two medians. Data were log2-trasformed.

Local recurrence-free survival (RFS) were evaluated by using Kaplan-Meier analysis and Cox proportional hazard regression model.

We sorted the samples by the intensity level. We divided the set in three subgroups (high intensity level, medium intensity level, low intensity level) approximately counting the same amount of samples and we used for comparison just the two groups where the difference in the signal's levels was wider (high intensity versus low intensity). The log-rank test was used to evaluate differences between curves.

As validation set we used TCGA Head and Neck squamous cell carcinoma (TCGA Research Network: http://cancergenome.nih.gov/): 522 total samples, 44 of which are non-tumoral and

tumoral matched tissues.

MiRNA and gene deregulation was assessed by paired or unpaired Student's t-test.

A Pearson's coefficient was calculated to establish positive or negative correlation for miRNA\trascript pairs.

Significance was defined at the $p<0.05$ level if not differently specified.