Inventory of Supplemental Data:

Supplemental Figures 1-7

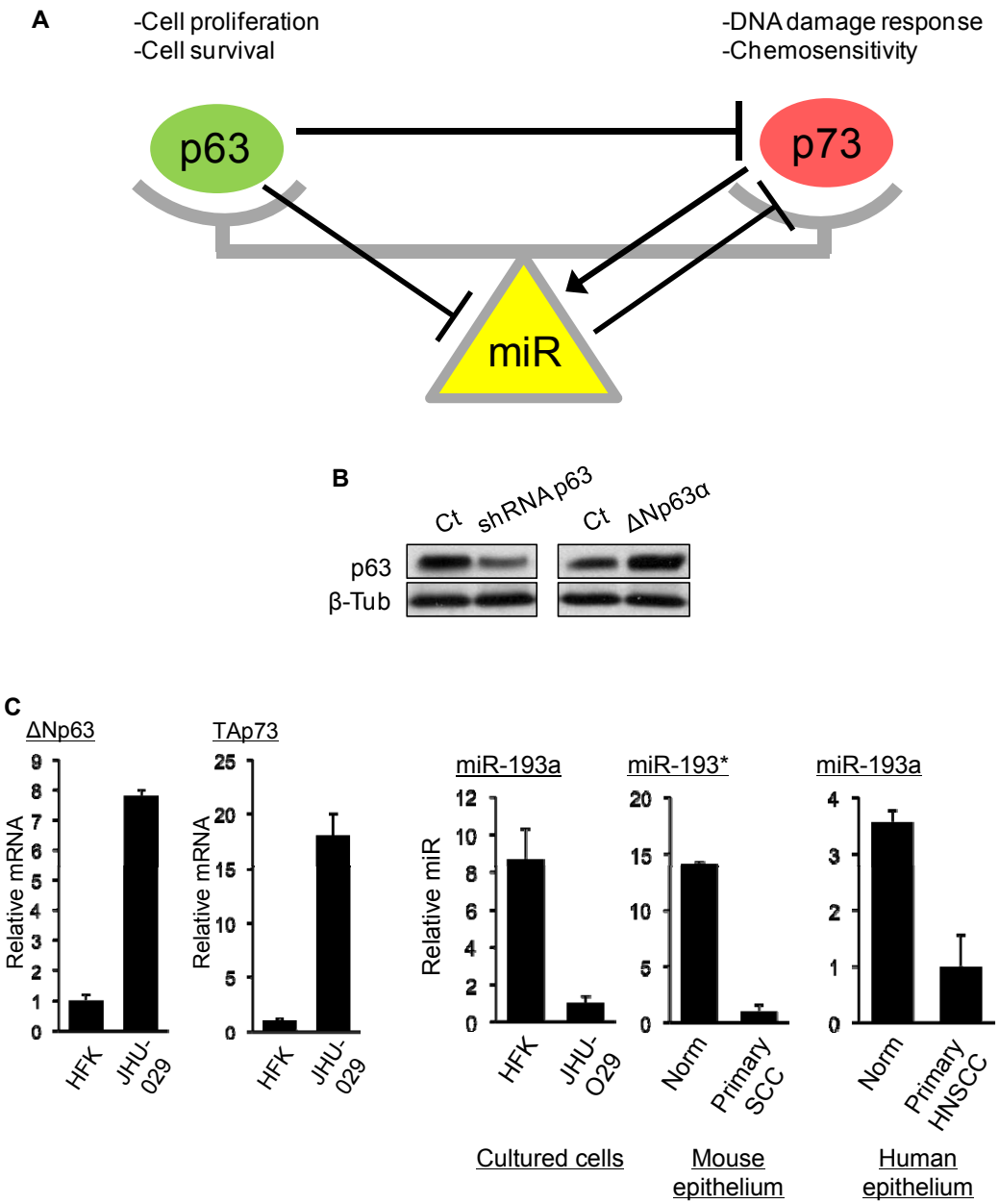
Supplemental Tables 1-8

Supplemental References

Supplemental Video:

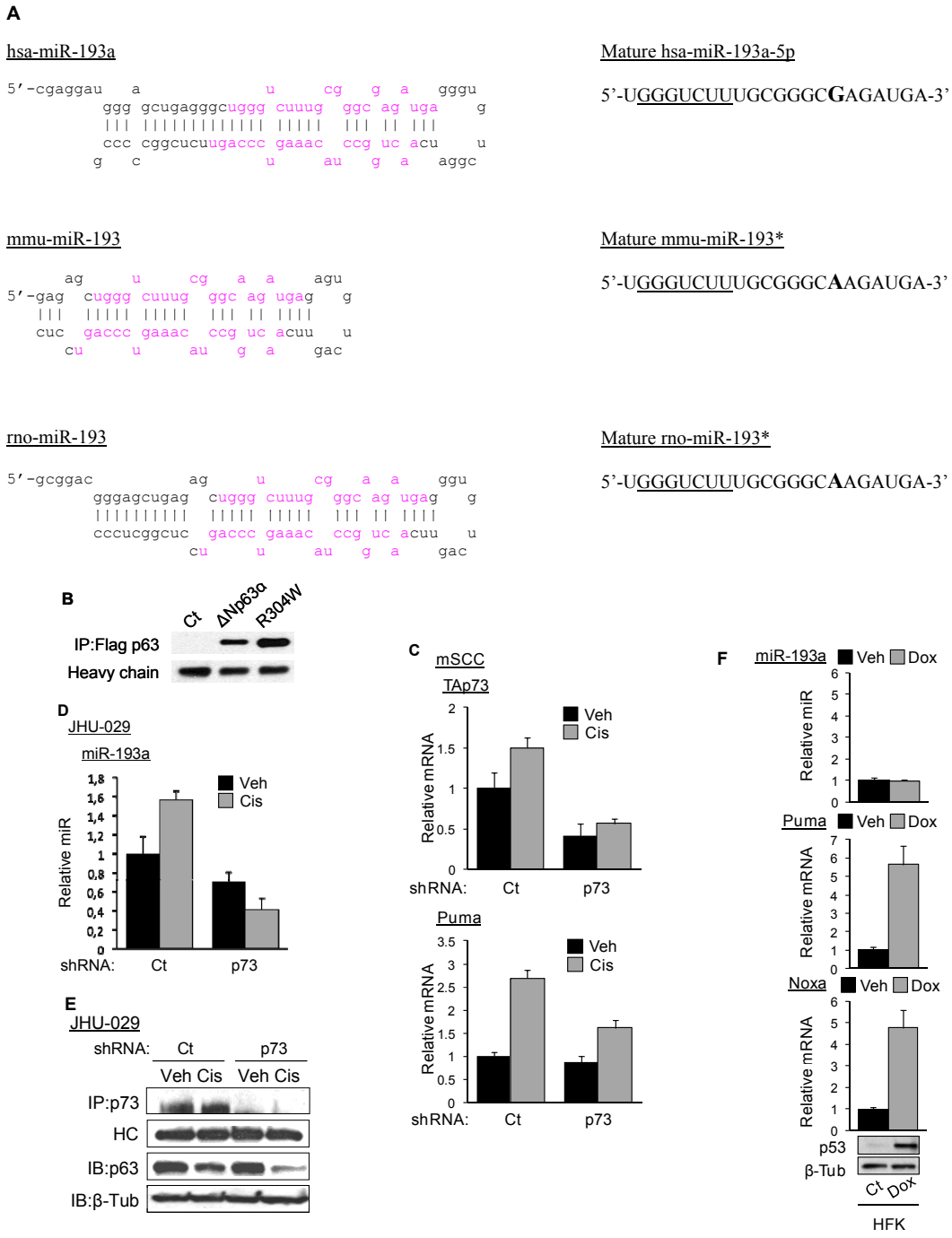
Note that the video shows JHU-029 cells transfected with miR-193a antagomir or control antagomir, then 24h later treated with Cisplatin (0.5 μM, 1hr), followed 3hr later by application of the Ethidium Homodimer (red cell death indicator, 16nM, as supplied in the LIVE⁄DEAD® Viability Kit (Invitrogen)) , at which point the video begins. Images were captured every 10 min for 69h. End point results are summarized in Figure S6.

Supplemental Figure 1



A miR-dependent regulatory circuit controls p63/p73 cross-talk, cell survival and chemosensitivity. (A) A new mechanism for cross-talk within the p53 family through direct transcriptional co-regulation of miRs. This mechanism serves to mediate inducible chemoresistance in squamous cell carcinoma. P63 is a direct transcriptional repressor of miRs, including miR-193a, that target p73 for inhibition. P63 also inhibits p73 function by direct physical interaction and by binding to shared promoter elements. Cisplatin chemotherapy activates p73, a direct transcriptional regulator of miR-193a, leading to feedback inhibition of p73 itself and chemoresistance. Disruption of this network by miR inhibition increases p73 activity, leading to impaired cellular viability and enhanced chemosensitivity. (B) Knockdown and overexpression of p63 in cells co-transfected with the p73 3'UTR (Fig. 1F). Representative immunoblot of lysates from JHU-029 cells transfected with a ΔNp63α cDNA or control (Ct) vector (left); or lentiviral p63 shRNA or GFP control shRNA (right). In both cases cells were pre-infected with Drosha lentiviral shRNA or control. (C) Levels of miR-193a/193* correlate inversely with those of p63 in human and murine epithelium and SCC. Left, analysis of ΔNp63 and TAp73 RNA by QRT-PCR in cultured HFK and JHU-029 cells. Right, QRT-PCR analysis of miR levels in normal cells relative to tumor cells.

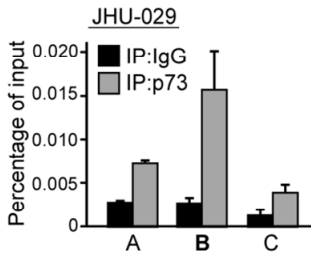
Supplemental Figure 2



miR-193a is a phylogenetically conserved miR regulated by p63 and p73. (A) Alignment of human (hsa), mouse (mmu) and rat (rno) pre-mirs (left) and mature miRs (right). Note the mature miRs differ by only one base (bold). The miR "seed" sequence is underlined. (B) Immunoblot showing retroviral expression of wild-type ΔNp63α or the point mutant R304W in JHU-029 cells. (C) (Top) P73 knockdown by lentiviral shRNA following cisplatin treatment of murine SCC cells (Cis, 4M, 24h) demonstrates p73-dependent induction of Puma mRNA (Bottom), assessed by QRT-PCR. (D) P73-dependent induction of miR-193a by cisplatin treatment of human JHU-029 cells, treated and analyzed as in (C). (E) IP/western (top panels) and western (bottom panels) showing p73 knockdown and p63 degradation, respectively, for JHU-029 cells treated as in (C). Ig heavy chain (HC) serves as a loading control for IP. (F) P53-mediated induction of Puma and Noxa mRNA but not miR-193a in primary human foreskin keratinocytes (HFK) following doxorubicin treatment (Dox, 1.5M, 13h). Immunoblot below shows p53 protein induction. All error bars show s.e.m. for triplicate measurements from representative experiments.

Supplemental Figure 3

A



B

Human Mar. 2006 chr17:26901123-26901312 (+)
Mouse Jul. 2007 chr11:79518077-79518243 (+)

```
026901123  GGGCGGGACCGGGGGCGGGGCCGAGGCCGCAGCTCCCAGCCGGTACCACCTCCCCTCCAC
           ||||||| |  |                 |  |||||  || | ||||||||||||||||
079518077  GGGCGGGGCTTG---------------TCCGCCTCCCCACCCGCACCACCTCCCCTCCAC
```

Site 1

```
026901183  TGAGAGATTCCACCGGTCCCTGGGCTGTCAGGCGCGGTGGCCAAGTGGTAAGGCGTCGGT
           ||||||  ||||||| | | || ||||||||||||||||||||||||||||||||||||
079518122  TGAGAGCTTCCACCAGCCGCTCGGCTGTCAGGCGCGGTGGCCAAGTGGTAAGGCGTCGGT
```

Site 2

```
026901243  CTCGTAAACCGAAGATCGCGGGTTCGAACCCCGTCCGTGCCTGAGACCCGAGGTAGGGCT
           |||||||||||||||||||||||||||||||||||||||||| |    || |      |
079518182  CTCGTAAACCGAAGATCGCGGGTTCGAACCCCGTCCGTGCCTGCGTTTGGAAGAGATATT
```

Human Mar. 2006 chr17:26901123-26901312 (+)
Rat Nov. 2004 chr10:65893966-65894136 (+)

```
026901123  GGGCGGGACCGGGGGCGGGGCCGAGGCCGCAGCTCCCAGCCGGTACCACCTCCCCTCCAC
           ||||||| || |               | || ||||| ||| | |||| ||||||||||
065893966  GGGCGGGGCCTG---------------CCCACCTCCCCGCCCGCACCA-CTCCCCTCCAC
```

Site 1

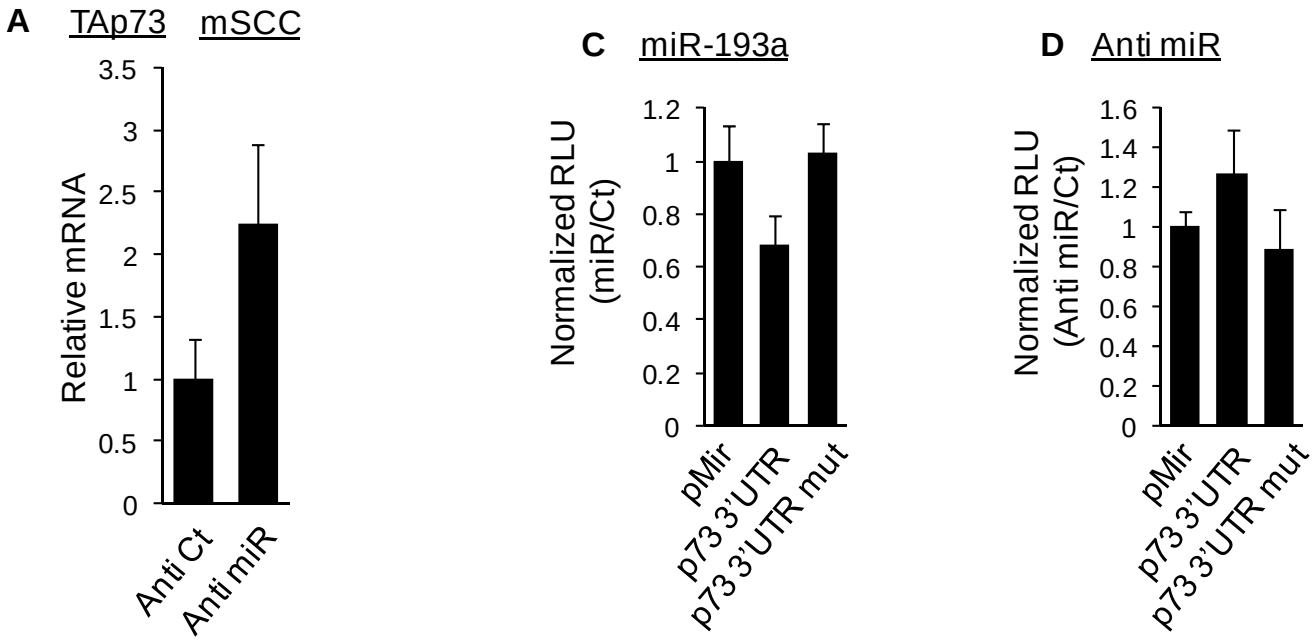
```
026901183  TGAGAGATTCCACCGGTCCCTGGGCTGTCAGGCGCGGTGGCCAAGTGGTAAGGCGTCGGT
           ||||||  ||||||||    || ||||||||||||||||||||||||||||||||||||
065894010  TGAGAGCTTCCACCGGCAGCTCGGCTGTCAGGCGCGGTGGCCAAGTGGTAAGGCGTCGGT
```

Site 2

```
026901243  CTCGTAAACCGAAGATCGCGGGTTCGAACCCCGTCCGTGCCTGAGACCCGAGGTAGGGCT
           |||||||||||||||||||||||||||||||||||||||| |    |       | |
065894070  CTCGTAAACCGAAGATCGCGGGTTCGAACCCCGTCCGTGCCTGCGTTTGGGAACGATGTT
```

Conserved sequence for p63/p73 binding and miR-193a regulation. (A) ChIP showing binding of endogenous p73 to region B indicated in Fig. 3A after Cisplatin treatment (24 h, 4 μM) in JHU-029 cells. (B) Genomic region of peak homology 10kb 5' of the miR-193a, indentified by Vista analysis (see Figure 3 and text). This region includes the p63 and p73 ChIP binding region and the essential p63/p73 consensus regulatory motif (Site 1). Pink shows most highly conserved sequences. Putative p63/p73 regulatory sequences are shown in yellow with key bases in bold. Only motif Site 1 is conserved and required for miR-193a regulation (see Figure 3).

Supplemental Figure 4



B

Site 1

miR-193a-5p 3' aguaGAGCGGGCGUUUCUGGGu 5'

5' ccugCCCGACUGC-**AAGGCCC**g 3' p73 3'UTR

Site 2

miR-193a-5p 3' aguagagcGGGCGUUUCUGGGU 5'

5' tgcgccacCGCCCAG**AGACCCA** 3' p73 3'UTR

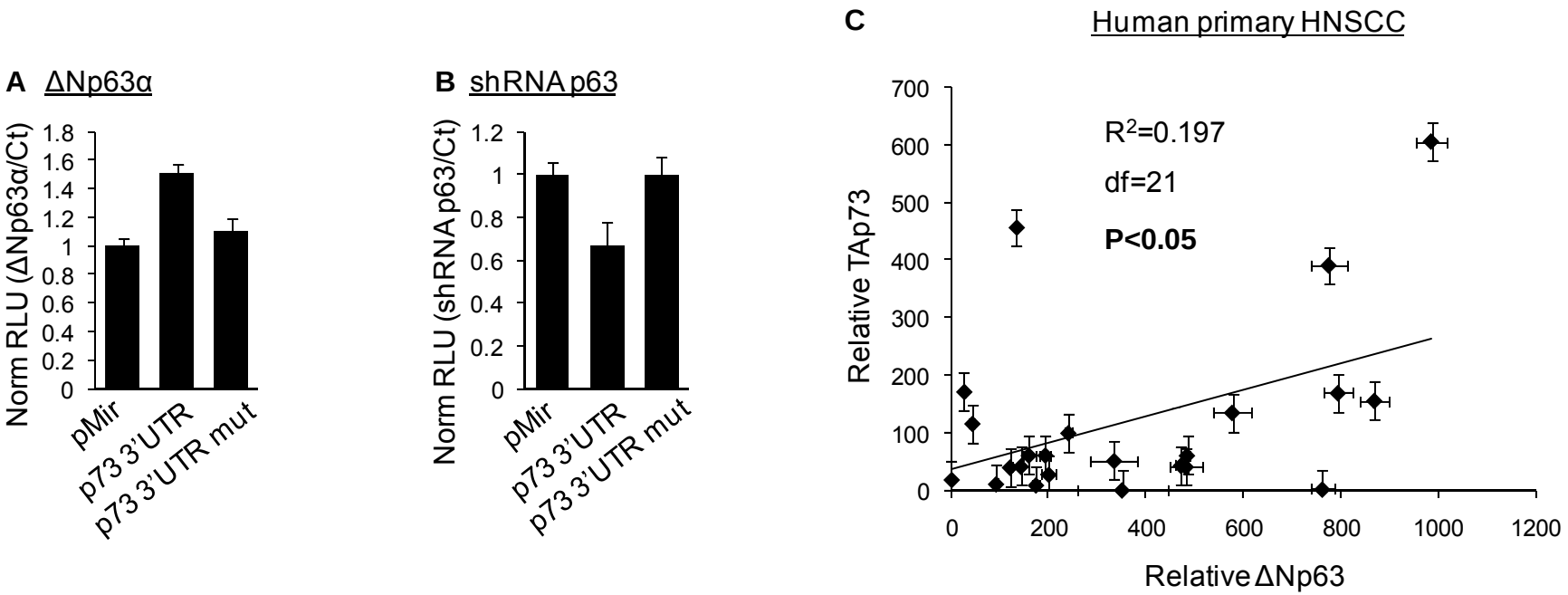
Site 3

miR-193a-5p 3' aguagaGCGGGCGUUUCUGGGU 5'

5' cacaggCCCCAGGAA**AGGCCCA** 3' p73 3'UTR

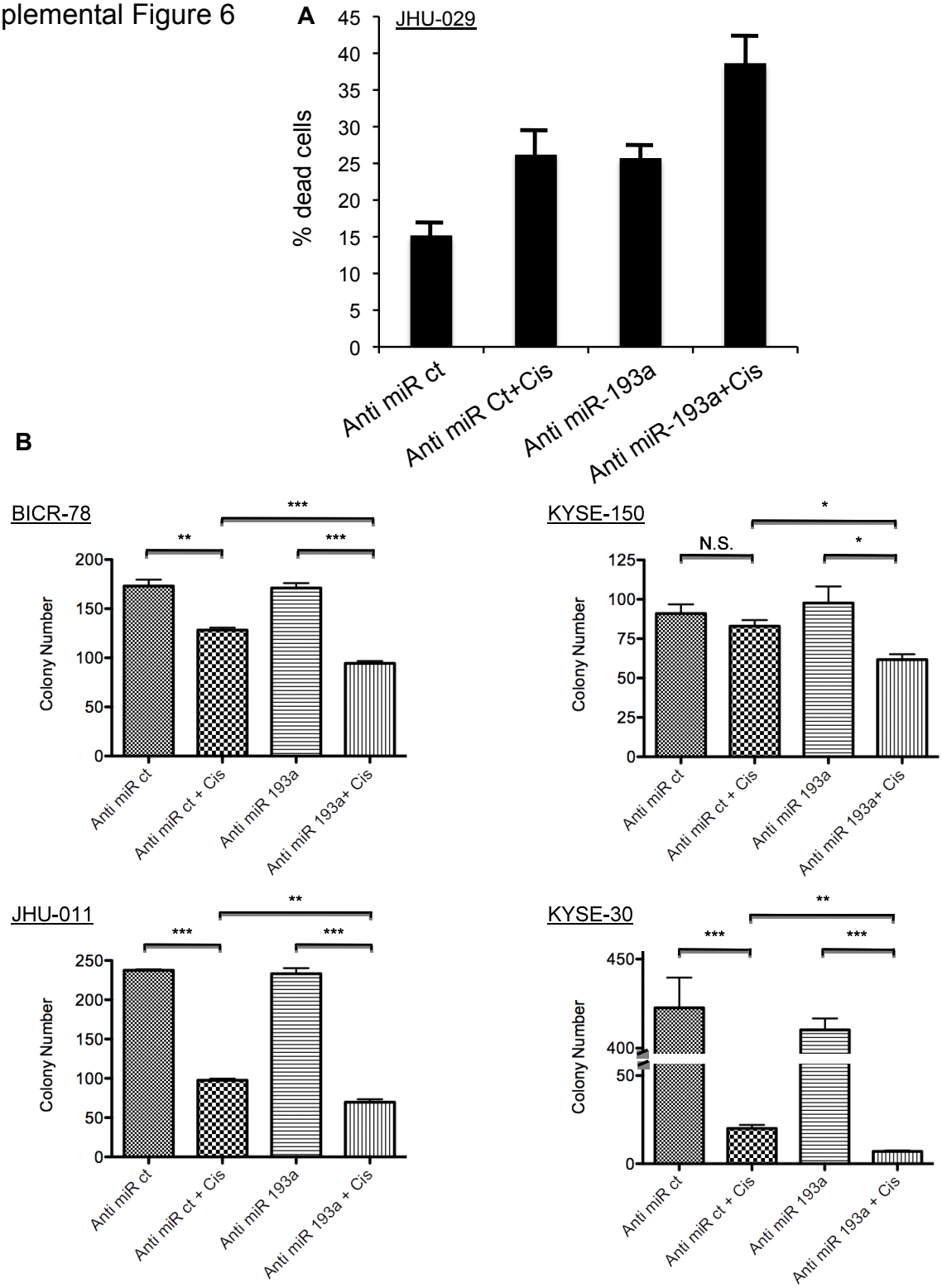
miR-193a is a direct, constitutive regulator of endogenous p73 in SCC. (A) Endogenous murine p73 mRNA is de-repressed 48h following transfection of murine SCC cells with a miR-193* antagomir (Anti miR) compared to scrambled control (Anti Ct) antagomir. (B) Three predicted miR-193a seed binding sequences within the p73 3'UTR. Alignment with the entire mature miR-193a is shown, with putative seed binding sequences in bold. (C, D) P73 UTR-dependent expression regulation by miR-193a requires the seed binding sequences. Co-transfection of the indicated reporter with a miR-193a mimic (C) or antagomir (D) or their respective controls (Ct) in JHU-029 cells. Note the pMIR reporter lacks p73 3'UTR.

Supplemental Figure 5



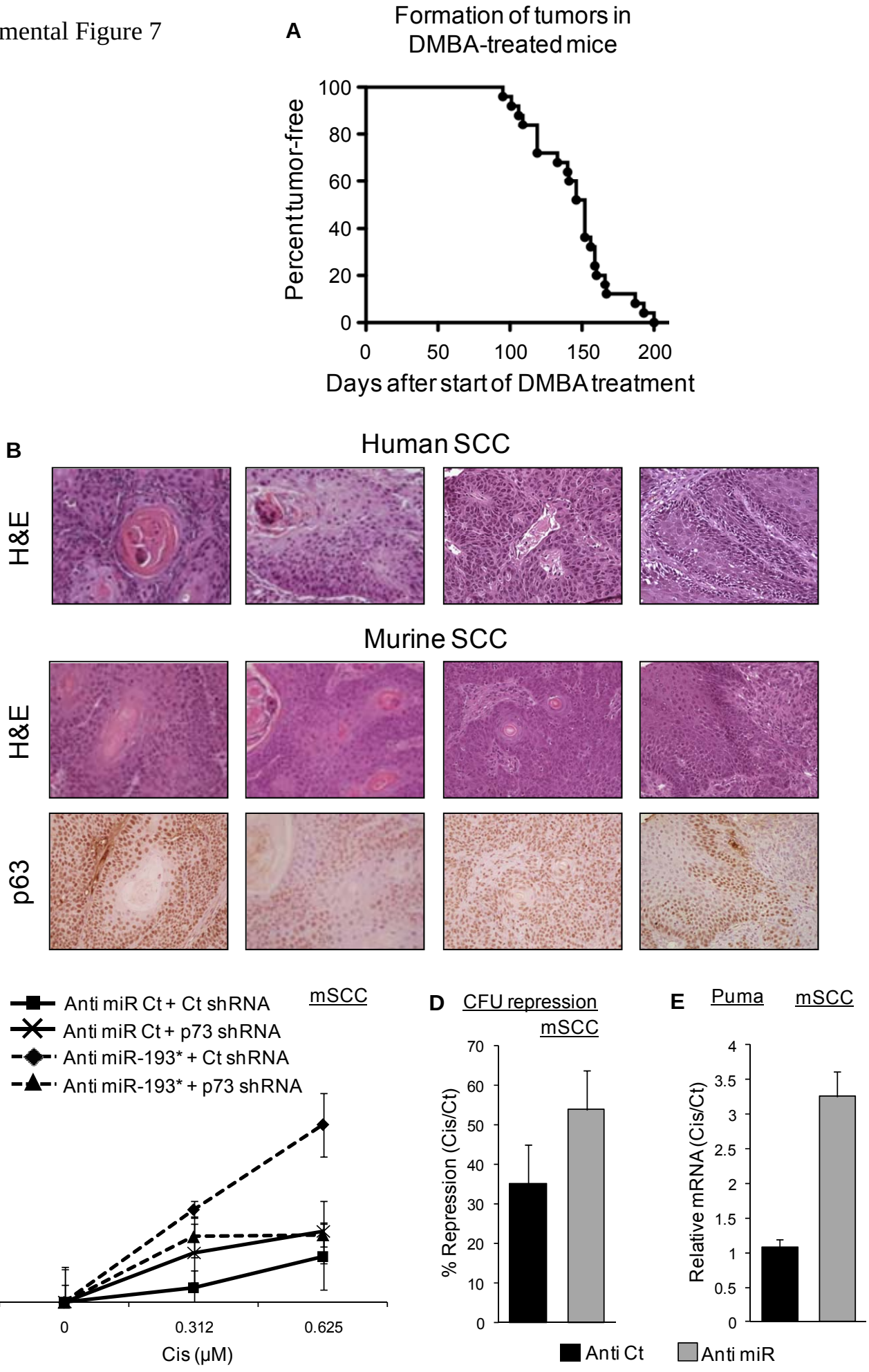
Coordinate regulation of p63 and p73 in human HNSCC. (A-B) UTR-dependent regulation of gene expression by p63 requires the miR-193a seed binding sites. JHU-029 cells were co-transfected with the indicated reporter, together with either ΔNp63α (A), p63 shRNA (B) or their respective controls. (C) Positive correlation between p63 and p73 expression in primary human HNSCC specimens, assessed by QRT-PCR. All error bars show s.e.m. for triplicate measurements.

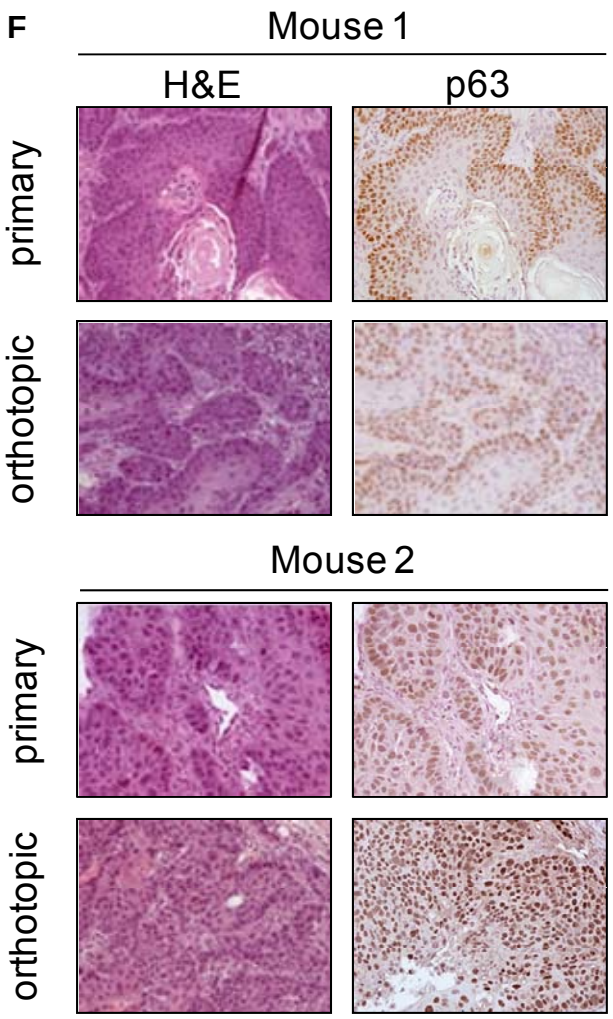
Supplemental Figure 6



miR-193 antagonism promotes cisplatin chemosensitivity. The indicated SCC cell lines were transfected with control antagomiR (Anti miR ct) or antagomiR directed against miR-193a (Anti miR-193). (A) After 24 hours, cells were treated with cisplatin (+Cis, 0.5μM) for one hour, then assayed for viability 72h later using the LIVE⁄DEAD® Viability Kit (Invitrogen). (B) Following one hour cisplatin treatment, cells were plated at clonal density and colonies were counted at indicated time: BICR-78 (5μM, 5 days) (ref. S1); KYSE-150 (2μM, 5 days) (ref. S2); JHU-011 (15μM, 6 days) (ref. S3), and D) KYSE-30 (15μM, 6 days) (ref. S2). Error bars indicate +/- SD * P<0.05, ** P< 0.01, *** P<0.001. Note that all lines lack wild-type p53 expression.

Supplemental Figure 7





miR-dependent regulation of cell viability and chemosensitivity in murine SCC. (A) Latency for tumor formation following DMBA treatment of C57/BL6 mice. (B) Murine SCC model recapitulates human SCC. Top, Hematoxylin and eosin (H&E) staining of primary human head and neck SCC specimens showing the typical varying degrees of squamous differentiation. Bottom, Murine SCC mimics the human disease as assessed by H&E staining and by high-level expression of nuclear p63, assessed by immunochemistry (IHC). (C) Effect of miR-193* on cisplatin sensitivity is p73-dependent. Murine SCC cells were infected with lentiviral p73 shRNA or control, then treated for 24h with a specific miR-193* antagomir or control (Ct) prior to 1h cisplatin treatment and cell counts 72h later. (D, E) Endogenous miR-193* controls cisplatin-mediated suppression of clonogenic growth and induction of p73 target gene. Murine SCC cells were treated for 24h with a miR-193* antagomir (Anti miR) or scrambled control (Anti Ct) prior to cisplatin treatment (2μM, 1h), then plated at clonal density for colony (CFU) counts (D), or harvested at 24h for RNA analysis by QRT-PCR (E). All error bars show s.e.m. for triplicate measurements. (F) Transplanted (orthotopic) tumors mimic primary SCCs assessed by H&E staining (left) and nuclear p63 staining by IHC (right).

Table S1. Significantly regulated miRs after p63 knock-down in JHU-029

microRNAs exhibiting a Fold Change > 1.50 in either replicate compared to control, following normalization using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm.

miR name	Max fold difference
hsa-miR-198	5.97
hsa-miR-30c-2*	3.31
hsa-miR-602	2.7
hsa-miR-193a-5p	2.57
hsa-miR-675	2.51
hsa-miR-371-5p	2.45
hsa-miR-765	2.31
hsa-miR-518a-5p/hsa-miR-527	1.95
hsa-miR-184	1.81
hsa-miR-30b*	1.76
hsa-miR-518c*	1.74
hsa-miR-665	1.59
hsa-miR-519e*	1.59
hsa-miR-516a-5p	1.55
hsa-miR-583	1.54
hsa-miR-185	1.51
hsa-miR-22	0.66
hsa-miR-19a	0.65
hsa-miR-27a	0.63
hsa-miR-887	0.63
hsa-miR-25*	0.63
hsa-miR-96	0.61
hsa-miR-298	0.6
hsa-miR-720	0.6
hsa-miR-200a	0.57
hsa-miR-519d	0.56
hsa-miR-487b	0.52
hsa-miR-381	0.49
hsa-miR-29b	0.37

Table S2. shRNA target sequences

shRNA	Target sequence
p63 all isoforms	5'-GGGTGAGCGTGTTATTGATGCT-3'
TAp73	5'-GGATTCCAGCATGGACGTCTT-3'
DROSHA	5'-GCCAGATGAGACTGAAGACAT-3'

Table S3. ChIP primers

Set	Position relative to miR-193a	Forward primer (5'-3')	Reverse primer (5'-3')
A	11 kb up	ACAGGCCAAACAAGAACAGG	GGAAAAACAGAGAGGGAGCC
B	10 kb up	TCCCCTCCACTGAGAGATTC	GCAGAATCCGAAAATGGAAA
C	8 kb up	TGCAAGGTACAGAAGAGGTCAG	AGATACCTGCTGGCCTTCAA
D	2.5 kb up	AAAAGGCCAGGACCTCGATC	TGAAAGGGCAGGGTTGTAATG
E	2 kb up	GACCTCGATCTGCCTTTCTG	GATGAAAGGGCAGGGTTGTA
F	1.2 kb up	TAATCTTTGCCACCAAAGCC	GGAGCCCAGTAATTTTCCCT
G	100 bp up	TGAGGGACACCCAGAGCTT	GCCGAGAACTGGGACTTTGT
H	1.7 kb down	AATCTCCAGAATATTGCGTGG	CGAAGCACCAAGTCATTTCC
I	2.5 kb down	CAGCTTGGCCCTTGATACTG	CACGGAAACCAAAATGATCC

Table S4. mRNA QRT-PCR primers

gene	Forward primer (5'-3')	Reverse primer (5'-3')
ΔNp63 (human)	GGAAAACAATGCCCAGACTC	GTGGAATACGTCCAGGTGGC
Tap73 (human)	GCACCACGTTTGAGCACCTCT	GCAGATTGAACTGGGCCATGA
PUMA (human)	ACGACCTCAACGCACAGTACGAG	AGGAGTCCGCATCTCCGTCAGTG
NOXA (human)	GAGATGCCTGGGAAGAAGG	ACGTGCACCTCCTGAGAAAA
GAPDH (human)	CACCCAGAAGACTGTGGATGG	GTCTACATGGCAACTGTGAGG
ΔNp63 (mouse)	GGAAAACAATGCCCAGACTC	GTGGAATACGTCCAGGTGGC
Tap73 (mouse)	TCGAGCACCTGTGGAGTTCTCT	CTGGTCCATGGCACTGCTGA
PUMA (mouse)	ATGGCCCGCGCACGCCAGG	CCGCCGCTCGTACTGCGCGTT
BAX (mouse)	CGTGGTTGCCCTCTTCTACT	CTCAGCCCATCTTCTTCCAG
NOXA (mouse)	GGGCAGAGCTACCACCTGAG	GCACACTCGTCCTTCAAGTCTG
β-Tub (mouse)	ATGAGCTGCCTGACGGCCAGGTCATC	TGGTACCACCAGACAGCACTGTGTTG

Table S5. TaqMan QRT-PCR primers

gene	Forward primer (5'-3')	Reverse primer (5'-3')
ΔNp63 (human)	GGAAAACAATGCCCAGACTCA	TGTTCAGGAGCCCCAGGTT
β-Actin (human)	CTTCCTGGGCATGGAGTCC	ACGTCACACTTCATGATGGAGTT

Table S6. TaqMan probes

gene	Vic-MGBNFQ TaqMan probe
ΔNp63 (human)	TTAGTGAGCCACAGTACAC
β-Actin (human)	ATCCACGAAACTAC

Table S7. microRNA Retro Transcription primers

miRNA	**Forward primer (5'-3')**	**Reverse primer (5'-3')**
miR-193a-5p	TATATGGGTCTTTGCGGGCG	GTGCAGGGTCCGAGGT
miR-602	TATAGACACGGGCGACAGCTG	GTGCAGGGTCCGAGGT
miR-765	GGCAGCTGGAGGAGAAGGAA	GTGCAGGGTCCGAGGT
miR-193*	TATATGGGTCTTTGCGGGC	GTGCAGGGTCCGAGGT
RNU6B	CGCAAGGATGACACGCAA	GTGCAGGGTCCGAGGT

Table S8. microRNA Q-PCR primers

miRNA	**Hairpin RT primer (5'-3')**
miR-193a-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**TCATCT**
miR-602	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**GGGCCG**
miR-765	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**CATCACC**
miR-193*	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**TCATCT**
RNU6B	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC**AAAAATA**

Supplemental References (For cell lines tested in Figure S6)

1. Edington, K.G., Loughran, O.P., Berry, I.J., and Parkinson, E.K. Cellular immortality: a late event in the progression of human squamous cell carcinoma of the head and neck associated with p53 alteration and a high frequency of allele loss. *Mol Carcinog*. 1995;13(4):254-265.
2. Shimada, Y., Imamura, M., Wagata, T., Yamaguchi, N., and Tobe, T. Characterization of 21 newly established esophageal cancer cell lines. *Cancer*. 1992;69(2):277-284.
3. DeYoung, M.P., Johannessen, C.M., Leong, C.O., Faquin, W., Rocco, J.W., and Ellisen, L.W. Tumor-specific p73 up-regulation mediates p63 dependence in squamous cell carcinoma. *Cancer Res*. 2006;66(19):9362-9368.