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Supplemental Material to:

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Phospho-ΔNp63α/microRNA network modulates epigenetic regulatory enzymes in squamous cell carcinomas

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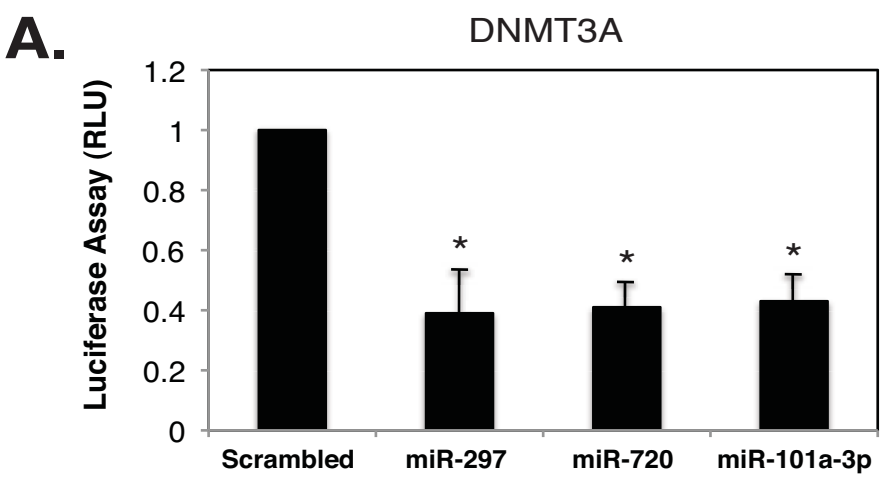
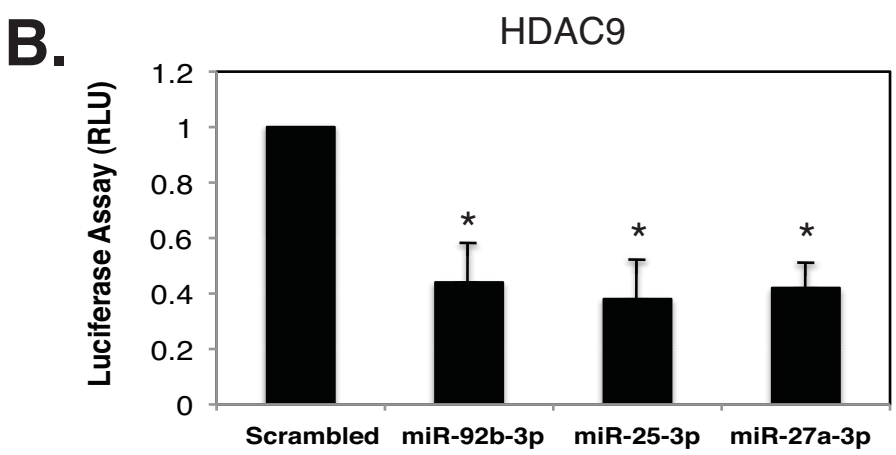
Figure S1


A.
DNMT3A
Luciferase Assay (RLU)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Scrambled
miR-297
miR-720
miR-101a-3p


B.
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Luciferase Assay (RLU)
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0.8
0.6
0.4
0.2
0
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*
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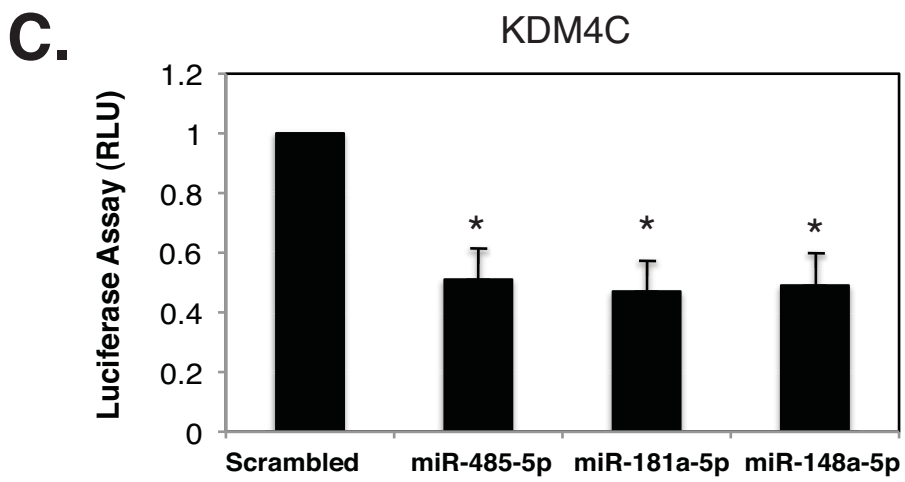
Scrambled
miR-92b-3p
miR-25-3p
miR-27a-3p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KDM4C
Luciferase Assay (RLU)
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1
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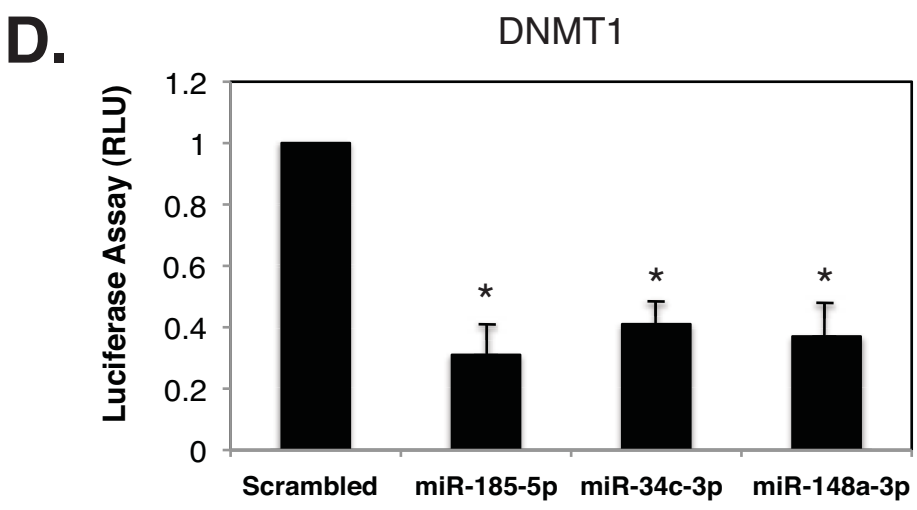
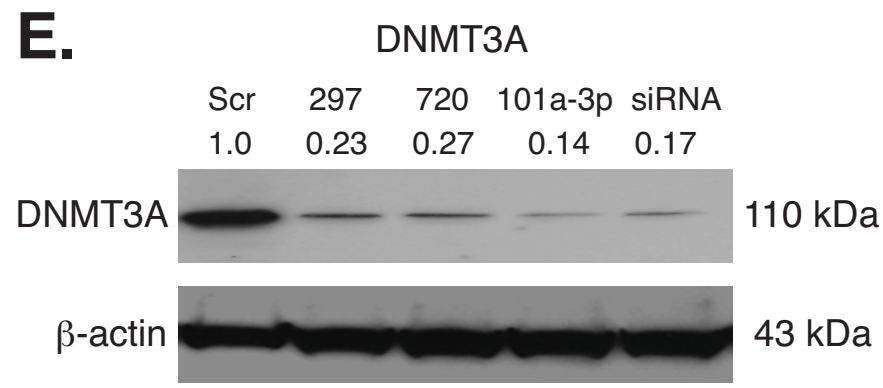
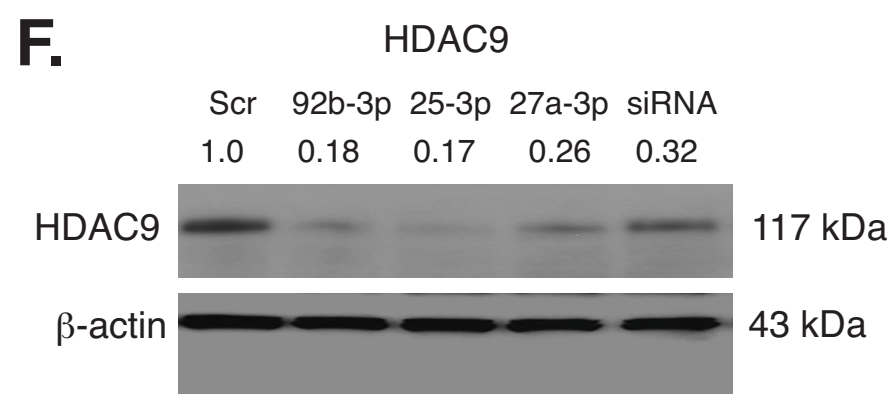
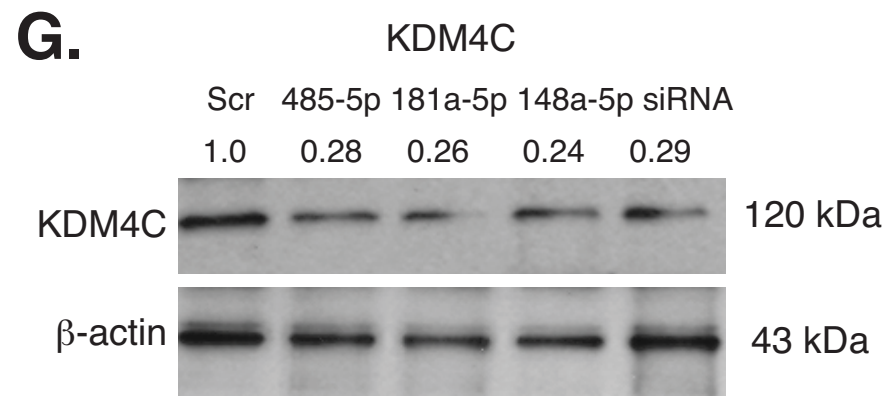
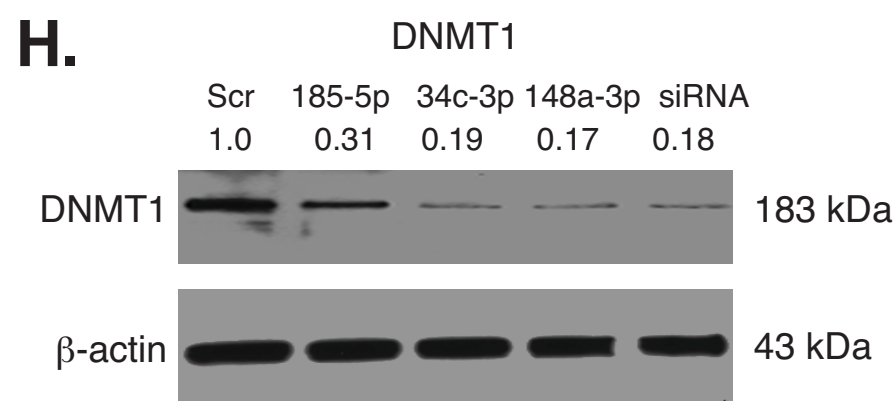
0.6
0.4
0.2
0
*
*
*
Scrambled
miR-485-5p
miR-181a-5p
miR-148a-5p


D.
DNMT1
Luciferase Assay (RLU)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Scrambled
miR-185-5p
miR-34c-3p
miR-148a-3p


E.
DNMT3A
Scr
297
720
101a-3p
siRNA
1.0
0.23
0.27
0.14
0.17
DNMT3A
110 kDa
β-actin
43 kDa


F.
HDAC9
Scr
92b-3p
25-3p
27a-3p
siRNA
1.0
0.18
0.17
0.26
0.32
HDAC9
117 kDa
β-actin
43 kDa


G.
KDM4C
Scr
485-5p
181a-5p
148a-5p
siRNA
1.0
0.28
0.26
0.24
0.29
KDM4C
120 kDa
β-actin
43 kDa


H.
DNMT1
Scr
185-5p
34c-3p
148a-3p
siRNA
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0.31
0.19
0.17
0.18
DNMT1
183 kDa
β-actin
43 kDa

Figure S2. DAPK1 promoter

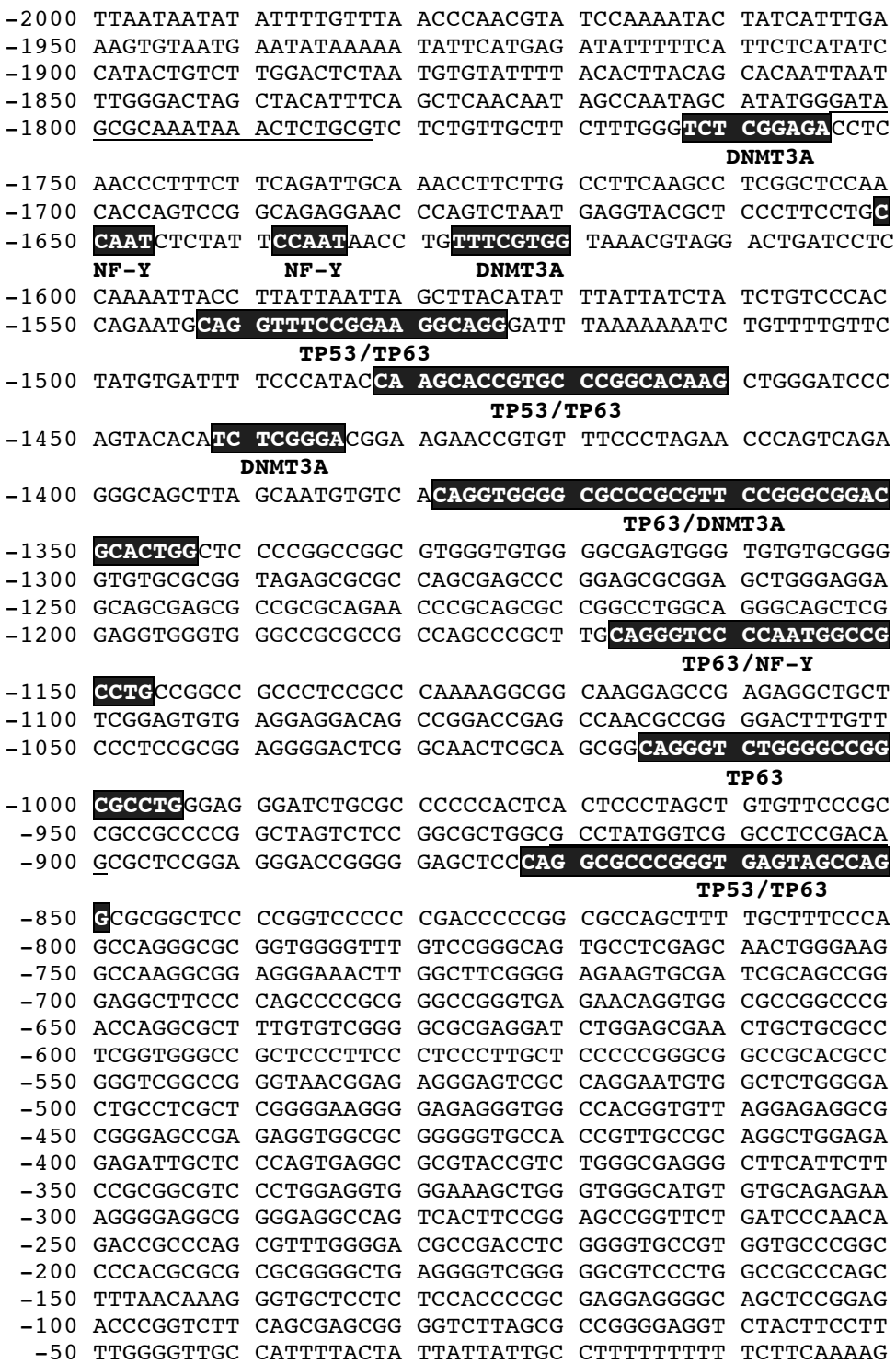

Figure S3. SMARCA2 (BRM) promoter

```
-2000 TATCTGGCTA TATTATTAGT TGCGACTTAT TAGGCAGTTA GAAGTTAGTA
-1950 TTTATGTTAC ACTTTTACTA TACTACTAGT CTTGTTTAAA ATTTCTCCCT
-1900 ATATATTTCT CAGTTAATAG TTATACCGGC ATATCCTCAG GCAGAAAAAC
-1850 AAGTTCTTAC AATGGATCCT TCAGGGCACA CTATATTCTA TTTCTCTTAG
-1800 CTGTGTTATC TTTCGTTGTC TTGAGGTTCA CTTCAAGATA GACAGAAAAT
-1750 GATGAAGCTA AAGTTAACAA GCAAGTAGCA AGACCTTAGA AAGCTATAGG
-1700 GTGGGTGATC ACCAACACTC CCAGCCATCA TGAAAATGCT GCCCTAGTGG
-1650 GCTCTGCTTC AGCCAGCAGT GGACCAGTGA TATAAAGAAT TCTTGCTTCT
-1600 TAATGACTAC ATGGCAGTAT TTGGCGTTCT TTCCATTACT TCTTGTCTTC
-1550 AACTTATCCA GCCTGAAGTT TTAAGCATCC TAGAACTGCA TTTCAGACCC
-1500 AGTTGCTCAA ATGGAGTCAA TTTTGCAACA GTAAAATGGT CTTAAAATAG
-1450 GATTTTACTC TAGGGGGAAG AATCCTCAAC CAGATAGTCA CAGTGCACTT
-1400 TGAATGACTA ATTTAAGCCA TTACATTACG AAATAAGGGA TAAATCATAC
-1350 TTTTCATAAC ACTACTGCAT AGGAACAGTT TTAAGAGTCA AGCATCTACA
-1300 TTAATCTGAG TCTAAAATAA TAGGCCCCTG ATTTAAAGTC TCCTAACATT
-1250 CTTTCACACT TCATAAAACT AAAAATAAAA TTGTCTCACC AAACACCGAC
-1200 CTATTGGTCC GGCCAAAGTA CTGTATTTCA AGGAAAATTG GAGCAAAGAG
-1150 GTCTCAAAAC CGCGACTTGC CAGCAAATTA CAATTTGCAG CCTTAATTGG
-1100 TCTCAAATGC GGGGTGAGAG GGAAGAGACT ACGGTCTGTA GAAAATAAGA
-1050 GATTTATTCC CCTTTTTAAA TCAAAACCAG ATTCCATGCT TCAGCCGCCC
-1000 CCTAACCCGC CAGCCCCGCC CCCAGGGTTT GTTTTTCCAT ATTTTTGTAT
 -950 ATATTTTAGC GAAGATGGCA GGTGAGGGAA GGTTATAGTG CTGTACCTAG
 -900 TCCACGAAGT AAACAGAGAG GTTAGGGTGG GTTTACTTAT TTATAAGGCG
 -850 TTCAGCCTCT CAGCTGTTTC TCCCTCGTTG GCATTTGGAA GCTTGCAGTC
 -800 CTTCAGGGAA GAGACAGATT TGGCAGGAAC GTTCTTTGTG CCCGCCTCCC
                  TP53/TP63
 -750 TTTTCTATTT TTTATTTTTT TACCTGGAAT AGGGGGCAGA TTTATAATGA
 -700 CAGCCTTAGG GAAGGGGGAG AAAAAGTTTC AGCCGGCACG ACAATGCCCG
 -650 TTTTTTCCAC AGTCCACACT GTGCCACAAA CAGCTTTGGT GCCACTCGGA
 -600 GCCCGTCCCC CGTCCCCTCC CTCTCTCTCT GCAGGCTCGC ACTGGCAGGC
                                      TP53/TP63
 -550 GGAGGCACAG TTAAATTCCA GCACCTTCTC CACATACCCC CGAACTACTA
 -500 CGCGCTATTA CTACGGCTGC CCTCCGTTTT CGCTTCGCCT CCTCCCCTTC
 -450 CGCAGTCTCC CTGGAGGAGC CCCGCGGCGC CCGAGGAAGA GGACTGCCAG
 -400 GGAAGGGACA GCGGGCGCCC AGCTCCAGCA GGGCTTGGGG CTTTCTGCAT
 -350 CCCGCGCAGT TTCTCTGCTC CAGGCACAAA CGCGGCCCGA GAGCCGGCGC
 -300 CTTGCAGTCA CACACGGATC CACGCATACA GTAGAGCTGT CTAGATCCAC
 -250 ATTCTTGCAC ACCGCCCCCT CCTCCCCCCG CGCTCCCGGA GTCGCTGAGC
 -200 TGAGCGAGTG ACAGGCGCGT CCCGCCAACC CGCGCCCGGA CGGGCAGGGA
 -150 GGAGCGGCGC GCGGGGCCAA CTGCGGCGCG TCTTCCGGCG CCCGCGGAGG
 -100 AGGCGAGGGT GGGACGCTGG GCGGAGCCCG AGTTTAGGAA GAGGAGGGGA
  -50 CGGCTGTCAT CAATGAAGTC ATATTCAT AATCTAGTCCTC TCTCCCTCTG
```

Figure S4. MDM2 promoter

-2000 TGCAAGTCAA ACGGATTTAT TTATTGTATC TCAAACAGTC CTCTTATATT
-1950 TTCCTTTCTG TATTTTTTGA TAATTTTCTT GTGCCTGGAA TATTCTCTGC
-1900 TTTTATTTAA GTCTCACCAT CTTTCAAGAC ACAAAATATG TGTGATCTCT
-1850 GAGGCCTTTG TGAACTGCCT CCAGCATTTA CTAAGCAATT AATTCTGTCT
-1800 CTGAACTTTT ATAGCACAAA CCCATTCGTT CACTTGATAT TTAACAAATA
-1750 TCAACCTCTA TCACTTATCT CTTTTTTTTT TTTTTTCTTC TGAGATGGAG
-1700 TCTTGCTCTG TCACCCAGGC TGGACTGCAG TGGCGCGATC TCCGCTCACT
-1650 GCAACCTCTG CCTCCCGAGA TCAAGCGATT CTTCTGCCTC AGCCTCCCAA
-1600 GTGGCTAGGA TTACAGGCGC CCGCCACGGG GCCCGGCTAA TTTTTGTATT
-1550 TTTAGTAGAG ACAAGGTTTC ACCATCTTGG CCAGGCTGGT CTCGAACTCC
-1500 TAACCTCGTG ATCCACCCAC CTCAGCCTCC CAAAGTGCTG GGATTACAAG
-1450 CGTGAGCCAC CGTGCGCAGC CTATCACTTA TCTTTTCATG CTTACATTTT
-1400 CTCACTGCTT GAGATTTTCT TTCATCCAGG GTCAAGCACT GAGTCTATTA
-1350 GAAACCCCAC TGTATATGAT CTGATGAAGA TGAGATTATC TTTTTAAAAG
-1300 CCACATATTA AATCTCTTAA GTTAATAATA TTGGCAATCA ACTTAAATCA
-1250 ACTGAGTGAC CACTATGTTT AAGGAAGTTT CCTTTCTGGT AGGCTGGGAA
-1200 GCGGGAGAAG GGAAGGATAT AACTTTATAA AAAAAAAAAA GCTGCAGAAG
-1150 GGAAGGATAT AACTTTATAA AAAAAGGTCC TTGTTCACAA GGAGCTAATC
-1100 ACTTACTGGC CAGAGAAGAA ACGTGTTCCT TCTTTGCAAT GGCTGTGTTT
-1050 TTGGGTTTAG CTAACTCAAT TCTTTATGTC CTCAATTTAT GAGACCGCAT
-1000 CAAAAACGTT TTTGCCACAT CTCTGTGGGA AAGTAGGTGA GTCAGAATGA
 -950 GTGCATAATA TATAAGTATA TATTTTAATG ATAATGAATG TGTAGATTAA
 -900 CAAACCCAAA CTTTGTTTTT GTTTTTTTGA GAGTCTTGCC CTGTCGCCCA
 -850 GGCTGGAGTG CAATGGCATG ATCTTGGCTC ACTGCAGCCT CTGCCTCCCG
 -800 GGTTCACAGG ATTGTCCTGC CTCAGCCTCC GGAGTAGAGT AGCTGGGATT
 -750 ACAGGTGACC GCCACCACGC CCGACTAATT TTTGTAGTTT TTAGTAGAGA
 -700 CGGCGTTTCC CATGTTGGCC AGGTTGGTCT TGAACTCCTG ACCTCAAGTG
 -650 ATCCACCCGC CTCGGCCTAC CCAAAGTGAT GGGATTACAA GCGTGAGCCA
 -600 CCGCGCCCAG CCAAACCCAA ACATTCTAAA AGCGCAGAGT AACCGCTCCC
 -550 CTCCCCGCCA ACGGTAACCA GCATCTTCGT TCTCCAACCA GAACAGCTGT
 -500 ATGCACCGGT GTATAAGCCT GCCTTAAGTG CTATTTTTAA TCTTTTTTAA
 -450 AAACAGGCCT TAAAATAACT CATTTGGGTA CAACTCCAGC GGCTGGTGGA
 -400 GTGGGCTTAA TATATTAAAC AGCTGTTAAT TTTGGTTTCT TTTTGGTAAC
 -350 AGCGACACGG AGATCCGCAT TTGTCGCAGT TTCCACCGCG GGCGGGAAGT
 -300 GTAAACACAA GAAATACAAA CATAGCGCAA CGGCTAAAGG AGTGTCACAG
 -250 CGCCAAACCT TGCTGGCTCC GCGCCAGGCC GAGCCCCACC CTCTCAGCTC
 -200 GCGGCCAACA CCCCCACCCC GCCTCACAGC CCGCCGCGCC CGCGGGGCGA
 -150 CACCCCCCAC TCCATCATCC CGGAGGTGGT GCGGCCGAGC CCCGGACCCA
 -100 ATTGGCGGAA GCGGGGCCGG TTGTGTGCGC GCGCACAAAT GCCCGGATGC
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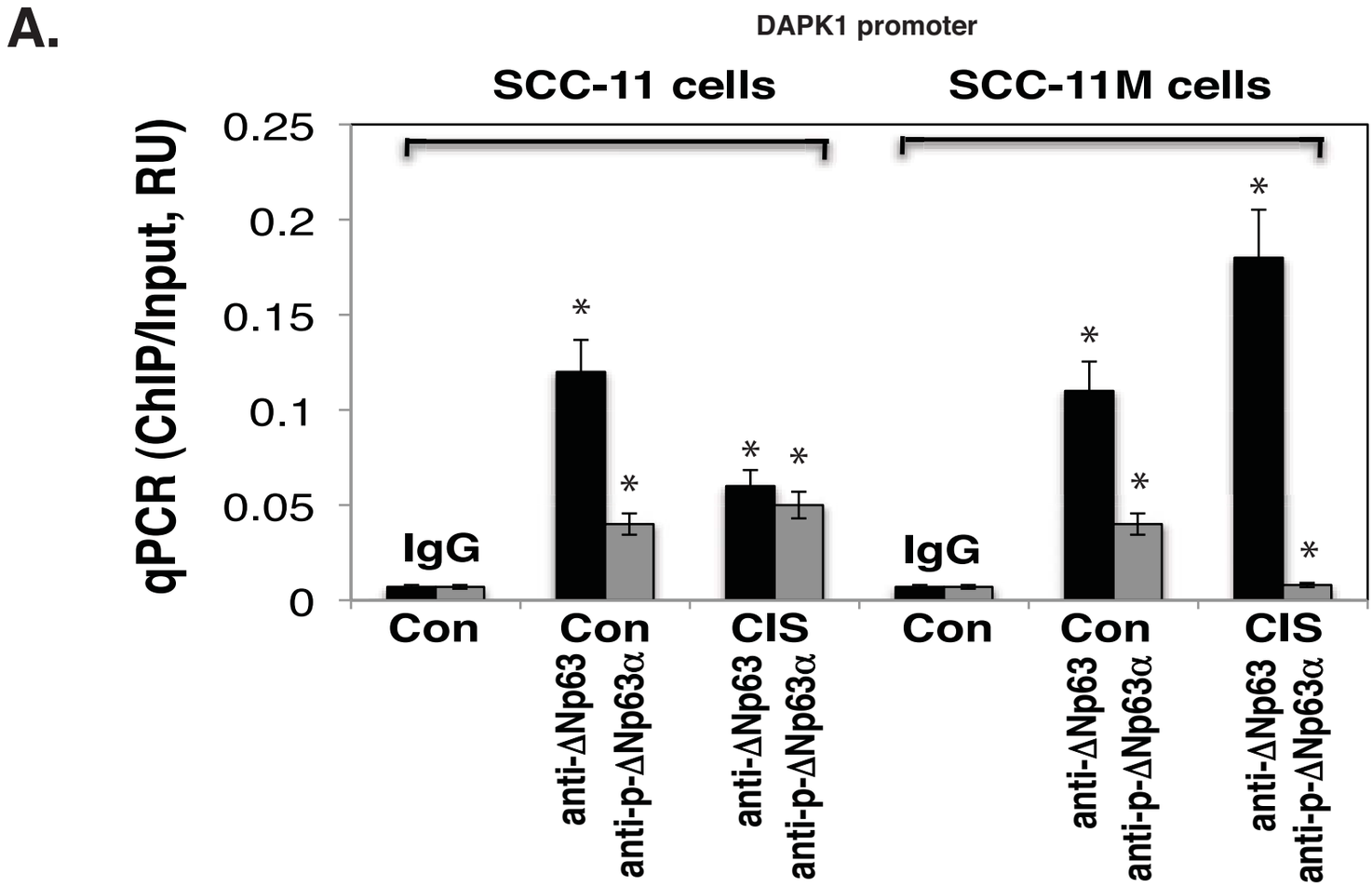
TP53
TP63

Figure S5


A.
DAPK1 promoter
SCC-11 cells
SCC-11M cells
qPCR (ChIP/Input, RU)
0.25
0.2
0.15
0.1
0.05
0
IgG
IgG
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α

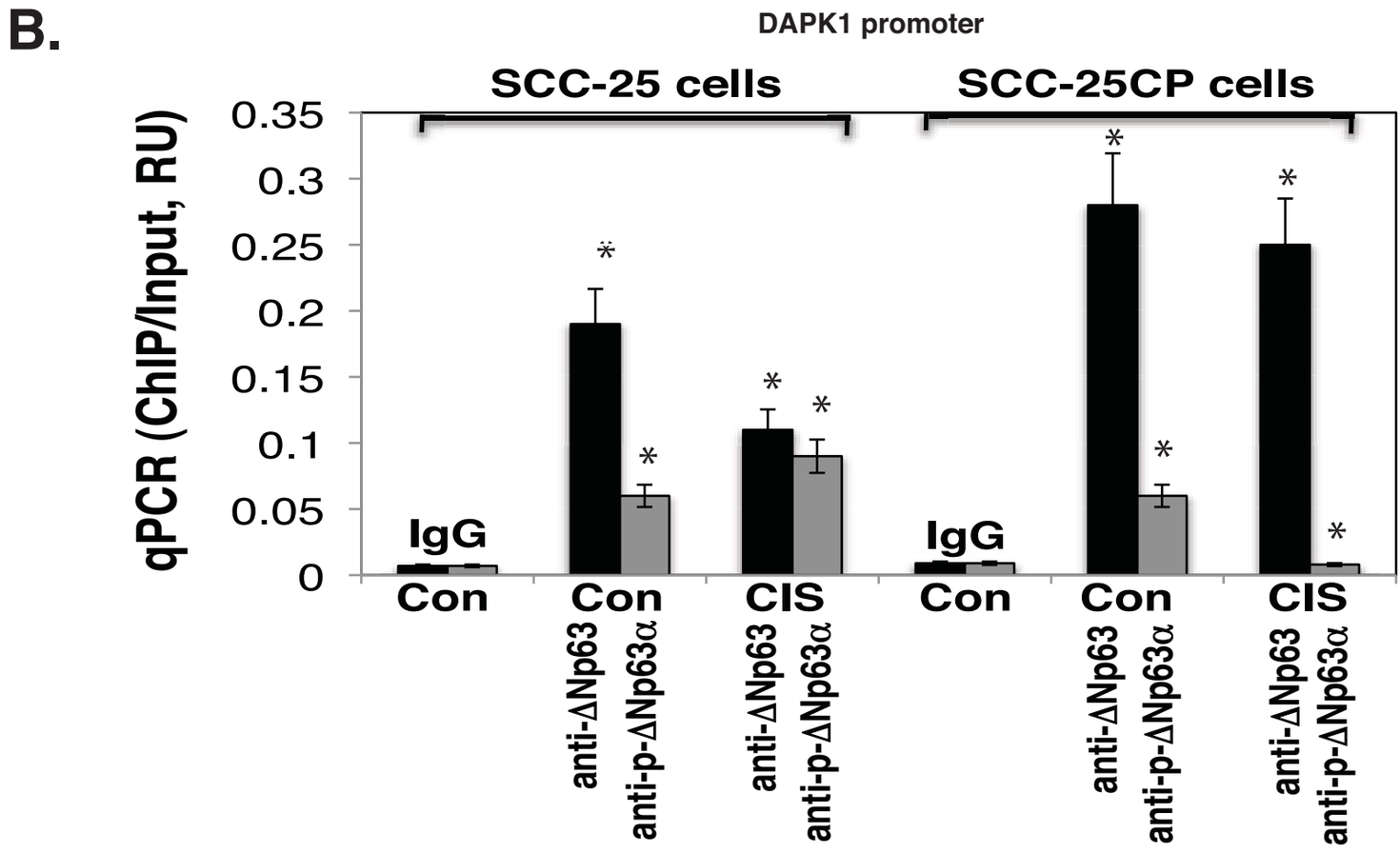
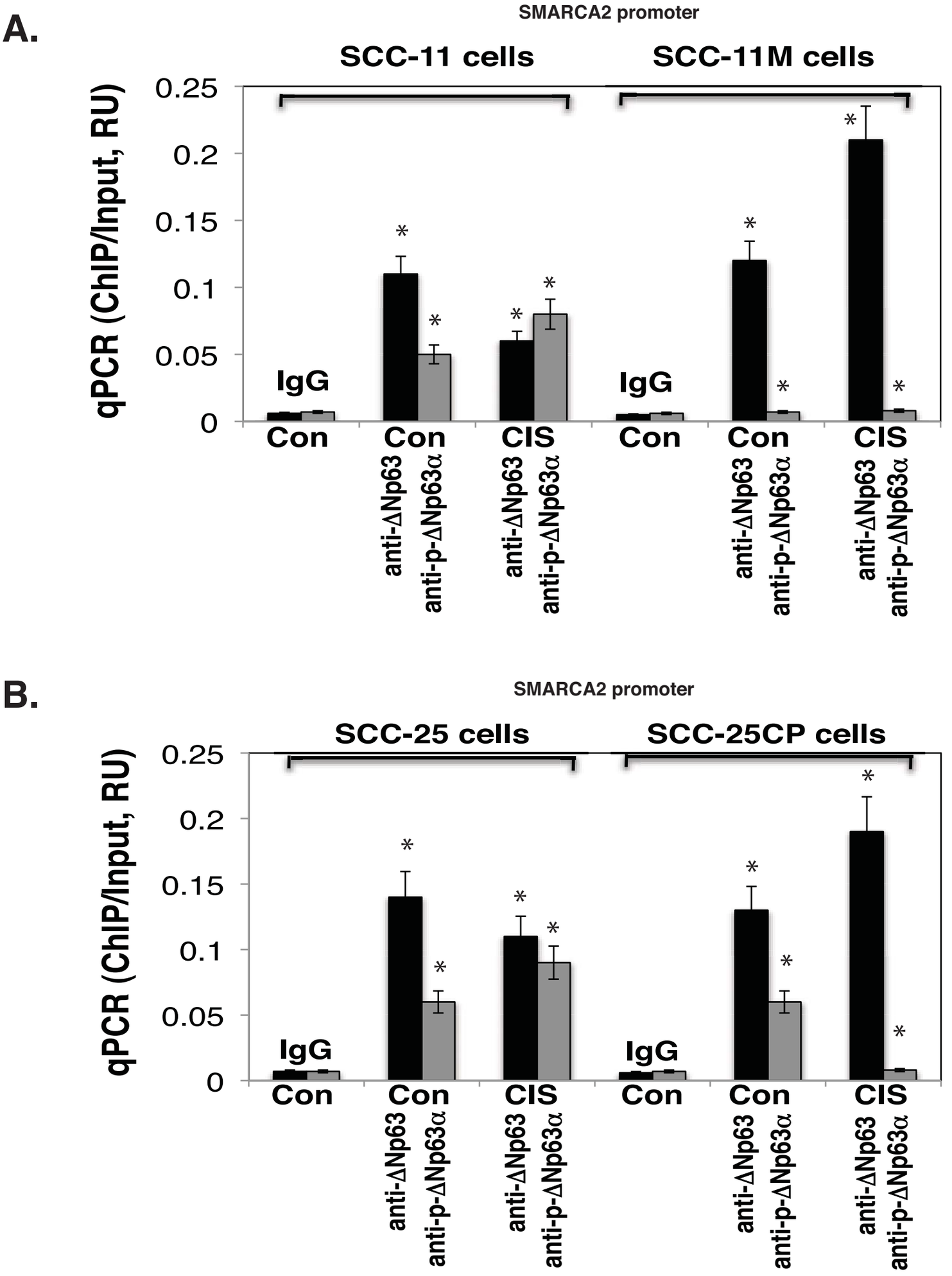

B.
DAPK1 promoter
SCC-25 cells
SCC-25CP cells
qPCR (ChIP/Input, RU)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
IgG
IgG
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α

Figure S6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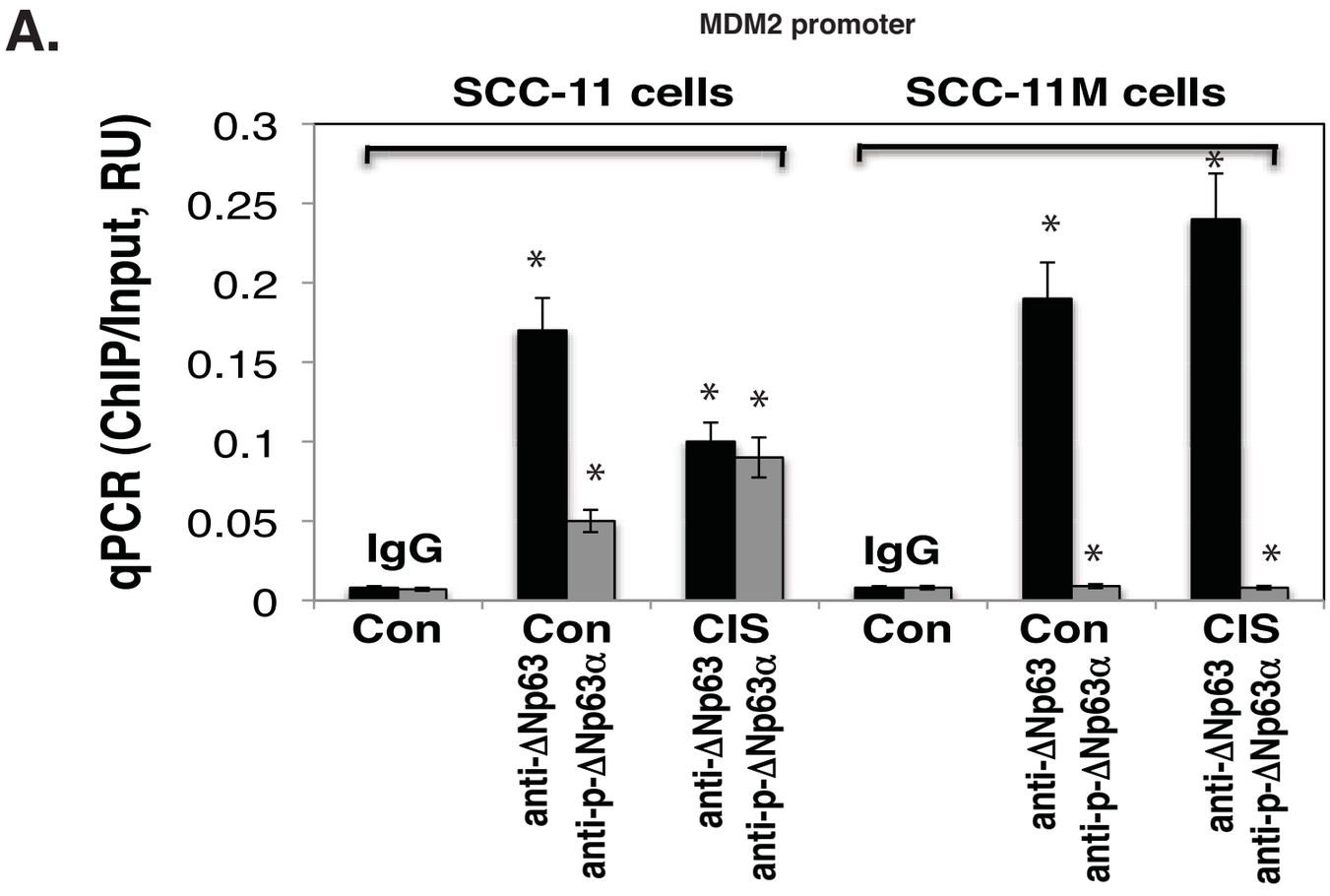
SMARCA2 promoter
SCC-11 cells
SCC-11M cells
qPCR (ChIP/Input, RU)
0.25
0.2
0.15
0.1
0.05
0
IgG
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α
B.
SMARCA2 promoter
SCC-25 cells
SCC-25CP cells
qPCR (ChIP/Input, RU)

Figure S7


A.
MDM2 promoter
SCC-11 cells
SCC-11M cells
qPCR (ChIP/Input, RU)
0.3
0.25
0.2
0.15
0.1
0.05
0
IgG
IgG
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α

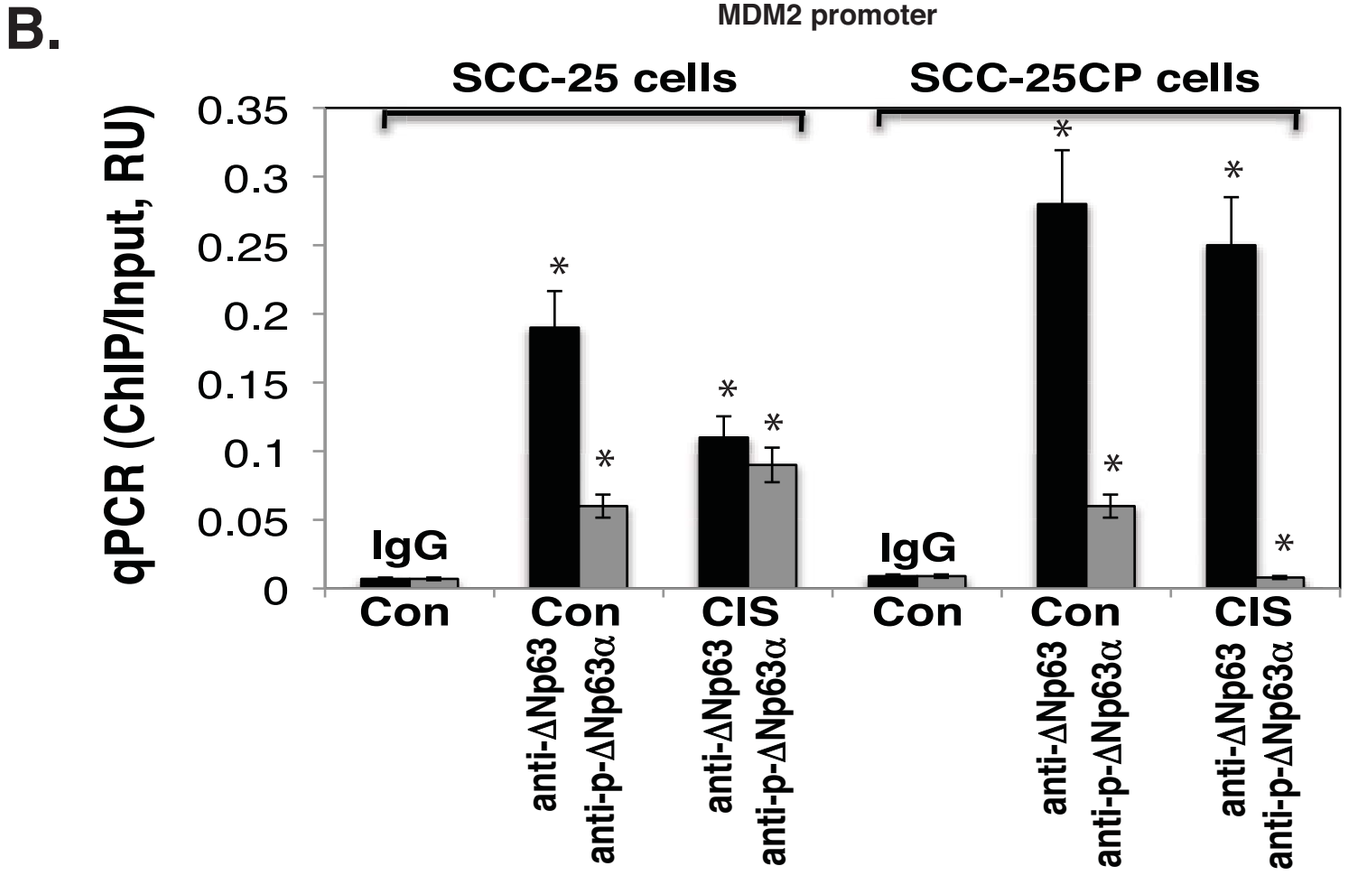

B.
MDM2 promoter
SCC-25 cells
SCC-25CP cells
qPCR (ChIP/Input, RU)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
IgG
IgG
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α
Con
Con
anti-ΔNp63
anti-p-ΔNp63α
CIS
anti-ΔNp63
anti-p-ΔNp63α

Figure S8

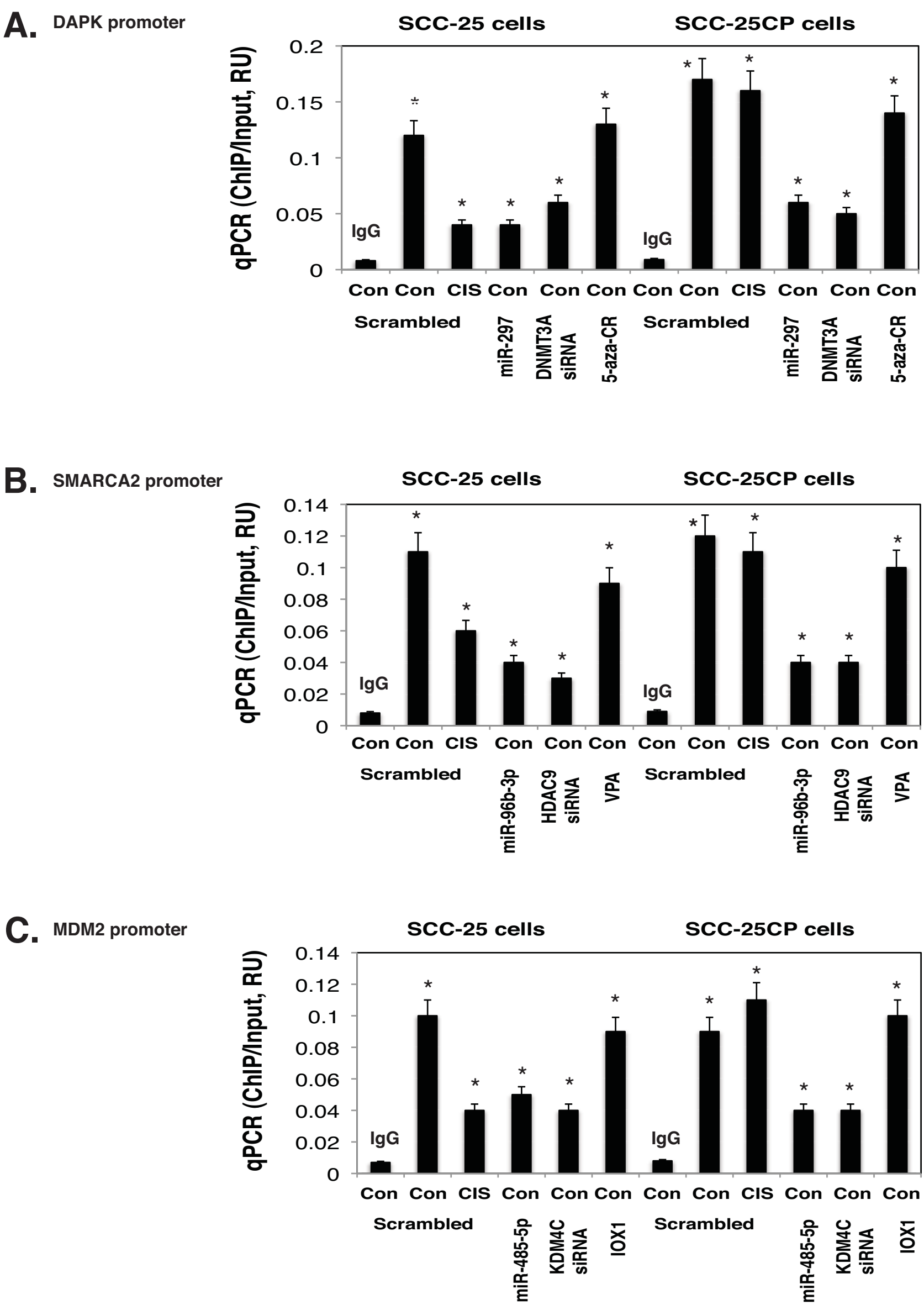
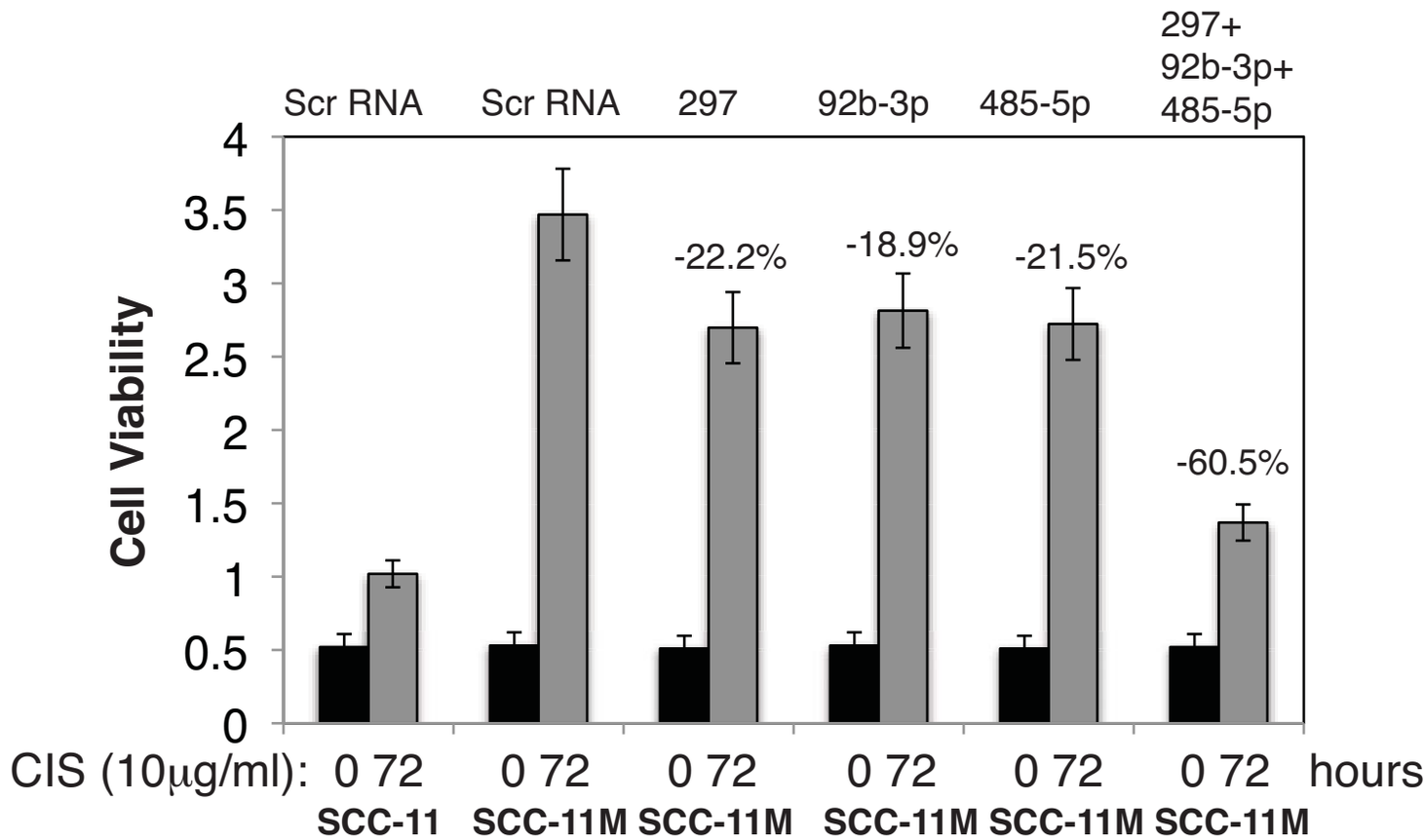

A.
DAPK promoter
SCC-25 cells
SCC-25CP cells
qPCR (ChIP/Input, RU)
0.2
0.15
0.1
0.05
0
IgG
IgG
Con Con CIS Con Con Con Con Con CIS Con Con Con
Scrambled
miR-297
DNMT3A siRNA
5-aza-CR
Scrambled
miR-297
DNMT3A siRNA
5-aza-CR
B.
SMARCA2 promoter
SCC-25 cells
SCC-25CP cells
qPCR (ChIP/Input, RU)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
IgG
IgG
Con Con CIS Con Con Con Con Con CIS Con Con Con
Scrambled
miR-96b-3p
HDAC9 siRNA
VPA
Scrambled
miR-96b-3p
HDAC9 siRNA
VPA
C.
MDM2 promoter
SCC-25 cells
SCC-25CP cells
qPCR (ChIP/Input, RU)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
IgG
IgG
Con Con CIS Con Con Con Con Con CIS Con Con Con
Scrambled
miR-485-5p
KDM4C siRNA
IOX1
Scrambled
miR-485-5p
KDM4C siRNA
IOX1

Figure S9


Scr RNA
Scr RNA
297
92b-3p
485-5p
297+
92b-3p+
485-5p
Cell Viability
4
3.5
3
2.5
2
1.5
1
0.5
0
-22.2%
-18.9%
-21.5%
-60.5%
CIS (10μg/ml): 0 72 0 72 0 72 0 72 0 72 0 72 hours
SCC-11 SCC-11M SCC-11M SCC-11M SCC-11M SCC-11M

Supplemental Figure Legends:

Figure S1. Tested microRNAs and siRNAs modulate their protein targets. (A and E). SCC-11 cells were transfected with the scrambled RNA (#4464058), miR-297 (MC10176), miR-720 (MC13574) and miR-101a-3p (MC11414) mimics along with the pLight_Switch DNMT3A plasmid (#S808608, SwitchGear Genomics). (B and F). SCC-11 cells were transfected with the scrambled RNA (#4464058), miR-92b-3p (MC10102), miR-25-3p (MC10584) and miR-27a-3p (MC10939) mimics along with the pLight_Switch HDAC9 plasmid (#S811202, SwitchGear Genomics). (C and G). SCC-11 cells were transfected with the scrambled RNA (#4464058), miR-485-5p (MC10837), miR-181a-5p (MC10421) and miR-148a-5p (MC12683) mimics along with the pLight_Switch KDM4C plasmid (#S806873, SwitchGear Genomics). (D and H). SCC-11 cells were transfected with the scrambled RNA (#4464058), miR-185-5p (MC12486), miR-34c-3p (MC12342) and miR-148a-3p (MC10263) mimics along with the pLight_Switch DNMT1 plasmid (#S802002, SwitchGear Genomics). All microRNA mimics were obtained from Ambion/Life Technologies. $5x10^4$ cells/well in a 24-well plate were transfected with the control (empty) pLightSwitch_3UTR vector (S890005, SwitchGear Genomics), respectively using Fugene HD reagent (Roche). Cells were also transfected with the selected 3'-UTR plasmids (listed in A-D) along with 100ng of the tested microRNA mimics, as well as with siRNAs [against DNMT3A (sc-37757), HDAC9 (sc-35550), and KDM4C (sc-92765) all obtained from Santa Cruz Biotechnology] for 48h. Renilla luciferase activity was measured at 480nm using a luminometer. Data obtained from the control samples were presented in relative units (RU) and designated as 1. Data were expressed as means ± SD from three independent experiments in triplicate. (E-H). Immunoblotting analysis of expression of indicated proteins in SCC-11 cells. Antibodies used against β-actin (Sigma), DNMT1 (GTX30364m GenTex), DNMT3A (GTX30365, GenTex), HDAC9 (PA5-11246, Thermo Fisher Scientific), and KDM4C (A300-885A, Bethyl Laboratories). Blots were scanned and quantified by the Image Quant software version 3.3. Values

indicated above the blots were normalized for β-actin levels and expressed as a fold-change to a control sample defined as 1.

Figures S2-S4. Schematic representation of the *DAPK1, SMARCA1 and MDM2* promoters. Potential binding sites for the transcription factors (S2-S4) and DNMT3A (S2) are highlighted in bold, boxed and shadowed. Primer sequences for the ChIP specific regions are underlined.

Figure S5. ChIP enrichment of the *DAPK1* promoter DNA in SCC cells upon cisplatin exposure. Chromatin was precipitated with 5μg of anti-ΔNp63 antibody (black) or with 5μg of anti-p-ΔNp63α antibody (grey). (A). SCC-11 versus SCC-11M cells. (B). SCC-25 versus SCC-25CP cells. QPCR assay was performed using three independent experiments in triplicate (*, $p<0.05$). The amount of ChIP-enriched DNA (ChIP/Input) represented as a signal relative to the total amount of chromatin DNA (Input) using the same primers, as described in Materials and Methods. Normal IgG was used as a negative control.

Figure S6. ChIP enrichment of the *SMARCA2* promoter DNA in SCC cells upon cisplatin exposure. Chromatin was precipitated with 5μg of anti-ΔNp63 antibody (black) or with 5μg of anti-p-ΔNp63α antibody (grey). (A). SCC-11 versus SCC-11M cells. (B). SCC-25 versus SCC-25CP cells. QPCR assay was performed using three independent experiments in triplicate (*, $p<0.05$). The amount of ChIP-enriched DNA (ChIP/Input) represented as a signal relative to the total amount of chromatin DNA (Input) using the same primers, as described in Materials and Methods. Normal IgG was used as a negative control.

Figure S7. ChIP enrichment of the *MDM2* promoter DNA in SCC cells upon cisplatin exposure. Chromatin was precipitated with 10μg of anti-ΔNp63 antibody (black) or with 10μg of anti-p-

ΔNp63α antibody (grey). (A). SCC-11 versus SCC-11M cells. (B). SCC-25 versus SCC-25CP cells. QPCR assay was performed using three independent experiments in triplicate (*, $p<0.05$). The amount of ChIP-enriched DNA (ChIP/Input) represented as a signal relative to the total amount of chromatin DNA (Input) using the same primers, as described in Materials and Methods. Normal IgG was used as a negative control.

Figure S8. ChIP analysis of the *DAPK1, SMARCA1 and MDM2* promoters in SCC-25 and SCC-25CP cells. (A). ChIP-qPCR assay of the DNMT3A binding to the specific region of the *DAPK1* promoter. (B). ChIP-qPCR assay of the HDAC9 binding to the specific region of the *SMARCA2* promoter. (C). ChIP-qPCR assay of the KDM4C binding to the specific region of the *MDM2* promoter. QPCR assay was performed using three independent experiments in triplicate (* indicates $p<0.05$). The amount of ChIP-enriched DNA (ChIP/Input) represented as a signal relative to the total amount of chromatin DNA (Input) using the same primers. The normal rabbit IgG was used as a negative control.

Figure S9. microRNAs modulate the resistance of SCC-11M cells to cisplatin exposure. Cell viability assay. SCC-11 cells and SCC-11M cells were transfected with the scrambled RNA (Scr RNA) for 32h, and then exposed to 10μg/ml cisplatin (CIS) for 0 and 72h. Cells were also transfected with indicated microRNA mimics (miR-297, B, miR-92b-3p, and C, miR-485-5p alone or in a combination thereof) for 32h, and then exposed to 10μg/ml cisplatin (CIS) for 0 and 72h. Cell viability (MTT assay) was monitored in triplicate in three independent experiments. Black bars are representative of 0h, while grey bars – of 72h of cisplatin treatment.