PNAS
www.pnas.org

Supplementary Information for

Reciprocal modulation of long noncoding RNA EMS and p53 regulates tumorigenesis

Chenfeng Wang[1,#], Yang Yang[1,#], Xianning Wu[2,#], Jingxin Li[1], Kaiyue Liu[1], Debao Fang[1], Bingyan Li[1], Ge Shan[1], Xinyu Mei[2,*], Fang Wang[1,*] and Yide Mei[1,2*]

*Correspondence: Yide Mei (Email: meiyide@ustc.edu.cn), Fang Wang (Email: wmfang@ustc.edu.cn), and Xinyu Mei (Email: mxyahslyy@163.com)

This PDF file includes:

Supplementary Figures

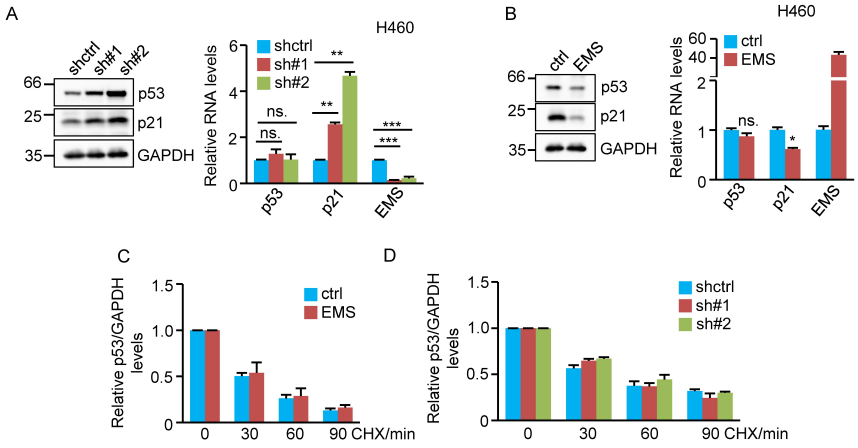

Fig. S1. EMS down-regulates p53 expression. (A) H460 cells were infected with lentiviruses expressing control shRNA, EMS shRNA#1, or EMS shRNA#2. Forty-eight hours later, cell lysates and total RNA were analyzed by Western blotting and real-time RT-PCR, respectively. Data shown are mean ± SD (n=3). **, $p < 0.01$; ***, $p < 0.001$; ns., no significance. (B) H460 cells were infected with lentiviruses expressing either control (pSin) or EMS (pSin-EMS). Forty-eight hours later, cell lysates and total RNA were analyzed by Western blotting and real-time RT-PCR, respectively. Data shown are mean ± SD (n=3). *, $p < 0.05$; ns., no significance. (C and D) The intensities of the bands shown in Fig. 3E (C) and 3F (D) were quantified by using ImageJ software. The ratio of p53 to GAPDH is presented as bar graph. Data shown are mean ± SD (n=3).

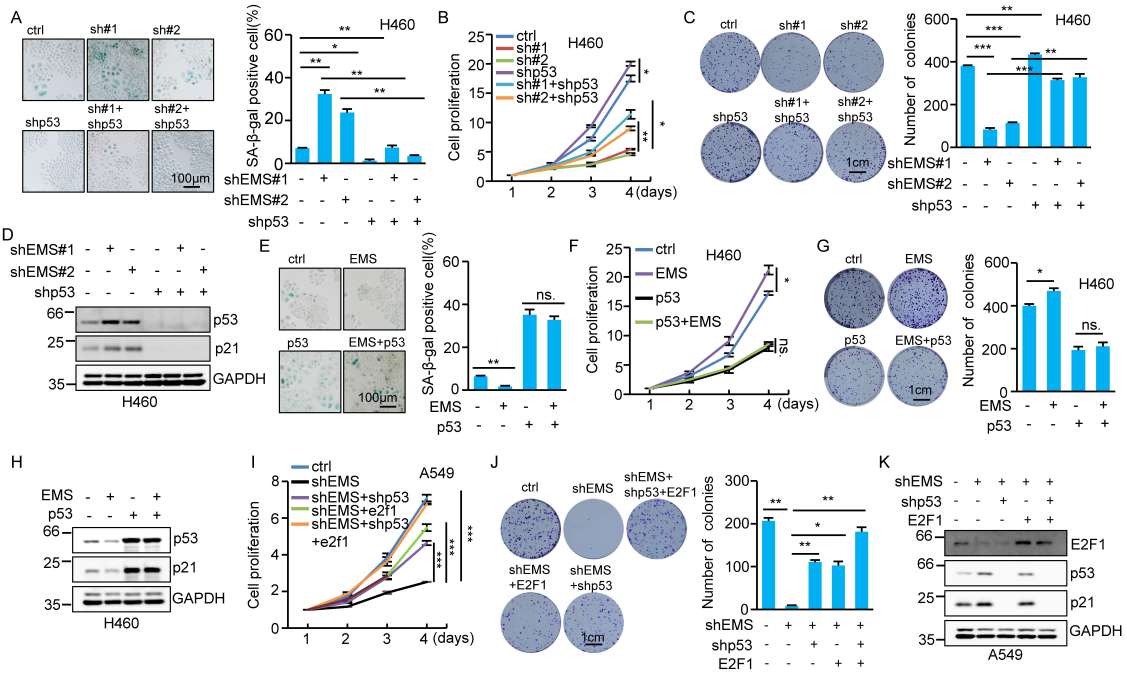

Fig. S2. The EMS-p53 axis regulates cellular senescence and cell proliferation. (A) Senescence-associated (SA) β-galactosidase staining in H460 cells expressing control shRNA, EMS shRNA#1, EMS shRNA#2, p53 shRNA, EMS shRNA#1 plus p53 shRNA, or EMS shRNA#2 plus p53 shRNA. The shown images are representative of three independent experiments. Data shown are mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$. (B) Shown are the growth curves of H460 cells expressing control shRNA, EMS shRNA#1, EMS shRNA#2, p53 shRNA, EMS shRNA#1 plus p53 shRNA, or EMS shRNA#2 plus p53 shRNA. Data shown are mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$. (C) Colonies of H460 cells expressing control shRNA, EMS shRNA#1, EMS shRNA#2, p53 shRNA, EMS shRNA#1 plus p53 shRNA, or EMS shRNA#2 plus p53 shRNA were stained with crystal violet after 10 days of incubation. The shown images are representative of three independent experiments. Data shown are mean ± SD (n=3). **, $p < 0.01$; ***, $p < 0.001$. (D) Western blot analysis of lysates from H460 cells expressing control shRNA, EMS shRNA#1, EMS shRNA#2, p53 shRNA, EMS shRNA#1 plus p53 shRNA, or EMS shRNA#2 plus p53 shRNA. (E) Senescence-associated (SA) β-galactosidase staining in H460 cells expressing control, EMS, p53, or both EMS and p53. The shown images are representative of three independent experiments. Data shown are mean ± SD (n=3). **, $p < 0.01$; ns., no significance. (F) Shown are the growth curves of H460 cells expressing control, EMS, p53, or both EMS and p53. Data shown are mean ± SD (n=3). *, $p < 0.05$; ns., no significance. (G) Colonies of H460 cells expressing control, EMS, p53, or both EMS and p53 were stained with crystal violet after 10 days of incubation. The shown images are representative of three independent experiments. Data shown are mean ± SD (n=3). *, $p < 0.05$; ns., no significance. (H) Western blot analysis of lysates from H460 cells expressing control, EMS, p53, or both EMS and p53. (I) A549 cells were infected with lentiviruses expressing control, EMS shRNA, p53 shRNA, and E2F1 in the indicated combination. Forty-eight hours later, the cell growth curves were measured. Data shown are mean ± SD (n=3). ***, $p < 0.001$. (J) Colonies of A549 cells expressing control, EMS shRNA, p53 shRNA, and E2F1 in the indicated combination were stained with crystal violet after 10 days of incubation. The shown images are representative of three independent experiments. Data shown are mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$. (K) Western blot analysis of lysates from A549 cells expressing control, EMS shRNA, p53 shRNA, and E2F1 in the indicated combination.

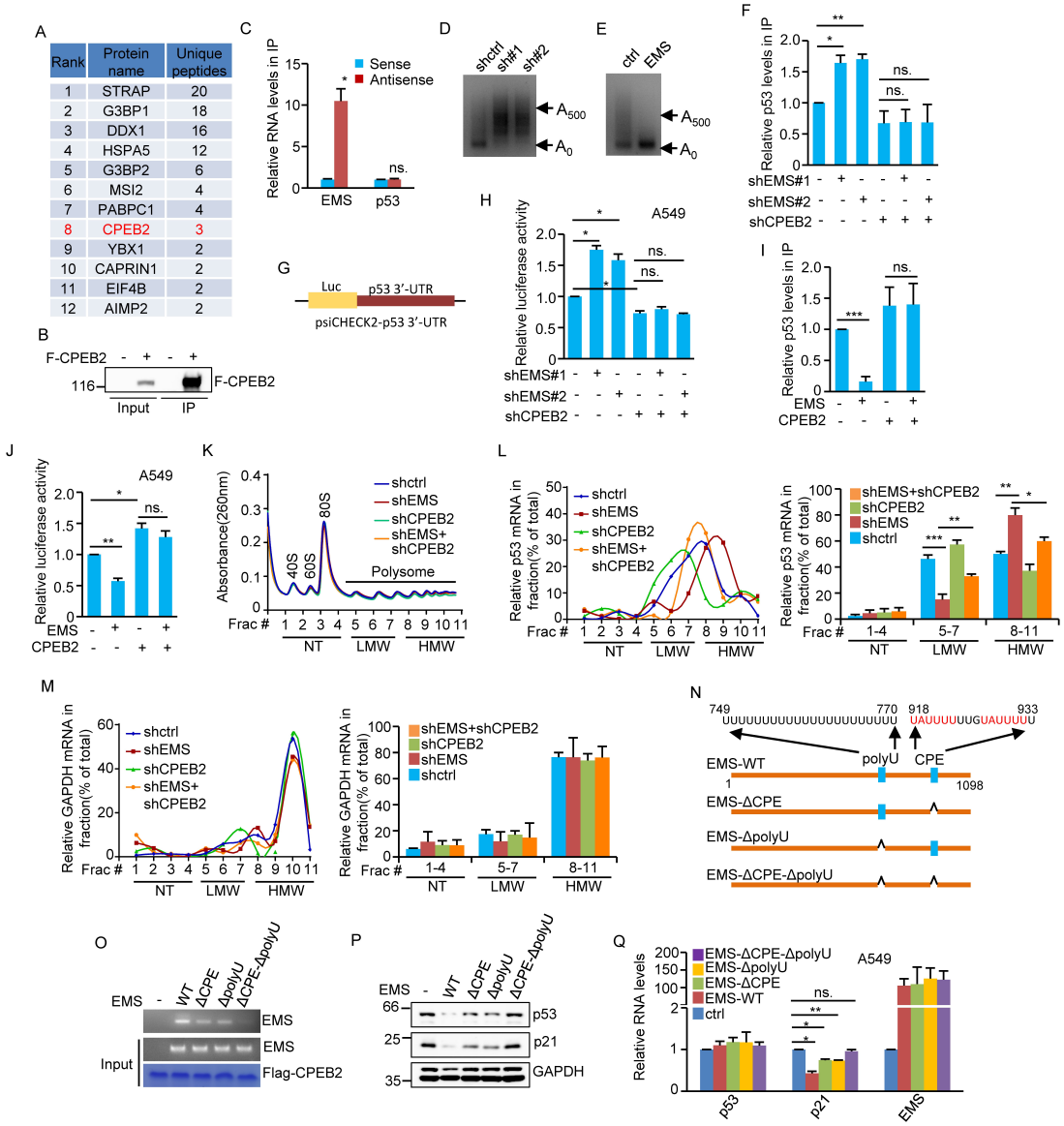

Fig. S3. EMS inhibits p53 expression through interaction with CPEB2. (A) Lysates from A549 cells were incubated with antisense biotin-labeled DNA oligomers against EMS, followed by the pull-down experiments using streptavidin-coated beads. The pull-downed proteins were eluted and analyzed by mass spectrometry. Listed are top 12 candidate binding proteins for EMS. (B) The input and immunoprecipitates from Fig. 5A were analyzed by Western blotting with anti-Flag antibody. (C) The efficient enrichment of endogenous EMS by its antisense DNA oligomers (Fig. 5B) was confirmed by real-time RT-PCR analysis. (D) A549 cells were infected with lentiviruses expressing control shRNA, EMS shRNA#1, or EMS shRNA#2. Forty-eight hours later, the poly(A) tail length of p53 mRNA was examined using the ligation-mediated poly(A) test (LM-PAT) assay. (E) A549 cells were infected with lentiviruses expressing control or EMS. Forty-eight hours later, the poly(A) tail length of p53 mRNA was examined using the ligation-mediated poly(A) test (LM-PAT) assay. (F) The intensities of the p53 bands in IP shown in Fig. 5H were quantified by using ImageJ software. Data shown are mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$; ns., no significance. (G) Shown is the psiCHECK2-based p53 3'-UTR reporter construct used for luciferase assay. (H) A549 cells expressing control shRNA, EMS shRNA#1, EMS shRNA#2, CPEB2 shRNA, EMS shRNA#1 plus CPEB2 shRNA, or EMS shRNA#2 plus CPEB2 shRNA were transfected with psi-

p53-3'UTR luciferase reporter plasmid. Twenty-four hours after transfection, the reporter activity was measured. Data shown are mean ± SD (n=3). *, $p < 0.05$; ns., no significance. (I) The intensities of the p53 bands in IP shown in Fig. 5I were quantified by using ImageJ software. Data shown are mean ± SD (n=3). ***, $p < 0.001$; ns., no significance. (J) A549 cells expressing EMS, Flag-CPEB2, or both Flag-CPEB2 and EMS were transfected with psi-p53-3'UTR luciferase reporter plasmid. Twenty-four hours after transfection, the reporter activity was measured. Data shown are mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$; ns., no significance. (K-M) A549 cells were infected with lentiviruses expressing control shRNA, EMS shRNA, CPEB2 shRNA, or both EMS shRNA and CPEB2 shRNA. Forty-eight hours later, polysomes in cytoplasmic extracts were fractionated by sucrose gradients (K). The levels of p53 (L) and GAPDH (M) mRNAs in each fraction were measured by real-time RT-PCR analysis and plotted as a percentage of the total p53 or GAPDH mRNA levels in that sample. NT, not translated; LMW, low-molecular-weight polysomes; HMW, high-molecular-weight polysomes. Data shown are mean ± SD from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (N) Shown are the sequences of poly(U) and putative CPE (cytoplasmic polyadenylation element) existing in EMS. EMS deletion mutants used in this study are also illustrated. (O) Purified recombinant Flag-CPEB2 bound with M2 beads was incubated with *in vitro* synthesized wild-type EMS, EMS-ΔCPE, EMS-ΔpolyU, or EMS-ΔCPE-ΔpolyU. The bead-bound RNAs were then eluted as templates for RT-PCR analysis. (P) A549 cells were infected with lentiviruses expressing wild-type EMS, EMS-ΔCPE, EMS-ΔpolyU, or EMS-ΔCPE-ΔpolyU. Forty-eight hours later, cell lysates were analyzed by Western blotting. (Q) A549 cells were infected with lentiviruses expressing wild-type EMS, EMS-ΔCPE, EMS-ΔpolyU, or EMS-ΔCPE-ΔpolyU. Forty-eight hours later, total RNA was analyzed by real-time RT-PCR. Data shown are mean ± SD (n=3). *, $p < 0.05$; **, $p < 0.01$; ns., no significance.

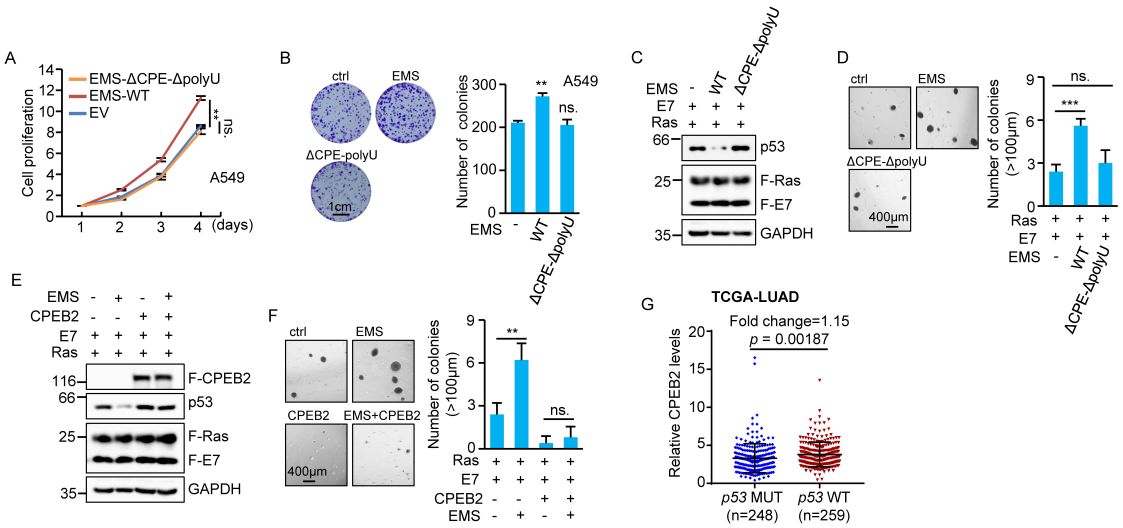

Fig. S4. Effects of EMS deletion mutants on cell proliferation and cellular transformation. (A) Shown are the growth curves of A549 cells expressing control, wild-type EMS, or EMS-ΔCPE-ΔpolyU. Data shown are mean ± SD (n=3). **, $p < 0.01$; ns., no significance. (B) Colonies of A549 cells expressing control, wild-type EMS, or EMS-ΔCPE-ΔpolyU were stained with crystal violet after 10 days of incubation. The shown images are representative of three independent experiments. Data shown are mean ± SD (n=3). **, $p < 0.01$; ns., no significance. (C and D) hTERT-immortalized BJ cells were sequentially infected with lentiviruses expressing EMS, EMS-ΔCPE-ΔpolyU, E7, and KRAS G12V in the indicated combination. (C) Forty-eight hours later, cell lysates were analyzed by Western blotting. (D) These cells were also subjected to soft agar colony formation assay. 20 days after seeding, colonies were stained with crystal violet and images were acquired. Numbers of colonies in six randomly selected areas (40 × magnification) were counted and averaged. Data shown are mean ± SD (n=3). ***, $p < 0.001$; ns., no significance. (E and F) hTERT-immortalized BJ cells were sequentially infected with lentiviruses expressing EMS, CPEB2, E7, and KRAS G12V in the indicated combination. (E) Forty-eight hours later, cell lysates were analyzed by Western blotting. (F) These cells were also subjected to soft agar colony formation assay. 20 days after seeding, colonies were stained with crystal violet and images were acquired. Numbers of colonies in six randomly selected areas (40 × magnification) were counted and averaged. Data shown are mean ± SD (n=3). **, $p < 0.01$; ns., no significance. (G) CPEB2 was expressed at relatively higher levels in lung adenocarcinoma (LUAD) with wild type *p53* than in those carrying mutated *p53*.

Supplementary Table

Table S1. Oligonucleotides used in this study

Primers used in qRT-PCR assays	
β-Actin	Fw:5'GACCTGACTGACTACCTCATGAAGAT3'
	Rev:5'GTCACACTTCATGATGGAGTTGAAGG3'
GAPDH	Fw: 5'CCATGGGGAAGGTGAAGGTC3'
	Rev: 5'GAAGGGGTCATTGATGGCAAC3'
P53	Fw: 5' GCCATCTACAAGCAGTCACAG3'
	Rew: 5' TCATCCAAATACTCCACACGC3'
P21	Fw: 5' TGTCACTGTCTTGTACCCTTG3'
	Rev: 5'GGCGTTTGGAGTGGTAGAA3'
EMS	Fw: 5'TGCGCGGAGAAATTGGAT3'
	Rev: 5'CACATCCTGTGTAAATTGCTTCTT3'
CPEB2	Fw: 5' ATGTCACTTGCCTGCAGTATTA3'
	Rev: 5' GCGATCAGCACCTTCCTTTA 3'
Primers used for antisense oligomer affinity pull-down assays	
EMS sense oligo DNA	5' (biotin-)AAAGCAAGGAAGTGGGACGC3'
EMS antisense oligo DNA	5' (biotin-)GCGTCCCACTTCCTTGCTTT3'
Primers used in ChIP assays	
CHIP-RE1	Fw: 5' GCCTCCTAAGTAGCTGGGATTA 3'
	Rev: 5' GCCTCCTAAGTAGCTGGGATTA 3'
CHIP-RE2	Fw: 5' CGTCTGTAATCCCAGCACTTT 3'
	Rev: 5' TGCCCTGCCTAATTTCGTATT 3'
CHIP-RE3	Fw: 5' AGATCACGAGGTCAGGAGTT 3'
	Rev: 5' TCCCGAGTAGCTGGGTTTAT 3'
CHIP-RE4	Fw: 5' CGGAGCTTTCAGTCCAGTC '
	Rev: 5' GCCATCTTGAGCCTGTAGTC 3'
GAPDH promoter	Fw: 5'TACTAGCGGTTTTACGGGCG3'
	Rev: 5'TCGAACAGGAGGAGCAGAGAGCGA3'
Oligonucleotide sequence of shRNAs	
shEMS#1	5'GATAGACTAGATCAAGCAGAA3'
shEMS#2	5'ACTCTGGCCATTCAGGTGAAC3'
sh-p53	5'GACTCCAGTGGTAATCTAC3'
sh-CPEB2	5'AGCTTCAGCATGGTGATATTG3'
sh-control	5'CCTAAGGTTAAGTCGCCCTCG3'
Primers for in vitro transcription	
The T7 RNA polymerase sequence(T7) was 5'CCAAGCTTCTAATACGACTCACTATAGGGAGA 3'	
EMS(sense)	5' (T7)GTTTCCACCTAGGAC3'
	5' TTTCATTTCACCTTTAATG3'
EMS(antisense)	5'GTTTCCACCTAGGACTACAG3'
	5' (T7) TTTCATTTCACCTTTAATG3'
p53-3'UTR	5' (T7) CATTCTCCACTTCTTGTTCCC3'
	5'GGCAGTGACCCGGAAGGC3'
Primers for LM-PAT	
PAT-anchor-oligo d(T)	5'GCGAGCTCCGCGGCCGCGTTTTTTTTTTTTTT3'

PAT-p53-specific oligomers	5'CTGCATTTTCACCCCACCCTTCC3'
Primers for RT-PCR assays	
p53-3'UTR	Fw: 5'GGAGTAGGACATACCAGCTTAGA3'
	Rew: 5'CCTACCTAGAATGTGGCTGATTG3'
EMS and its antisense RNA	Fw: 5'TGCGCGGAGAAATTGGAT3'
	Rev: 5'CACATCCTGTGTAAATTGCTTCTT3'