Additional files

Circ-HuR suppresses HuR expression and gastric cancer progression by inhibiting CNBP transactivation

Yang, et al.

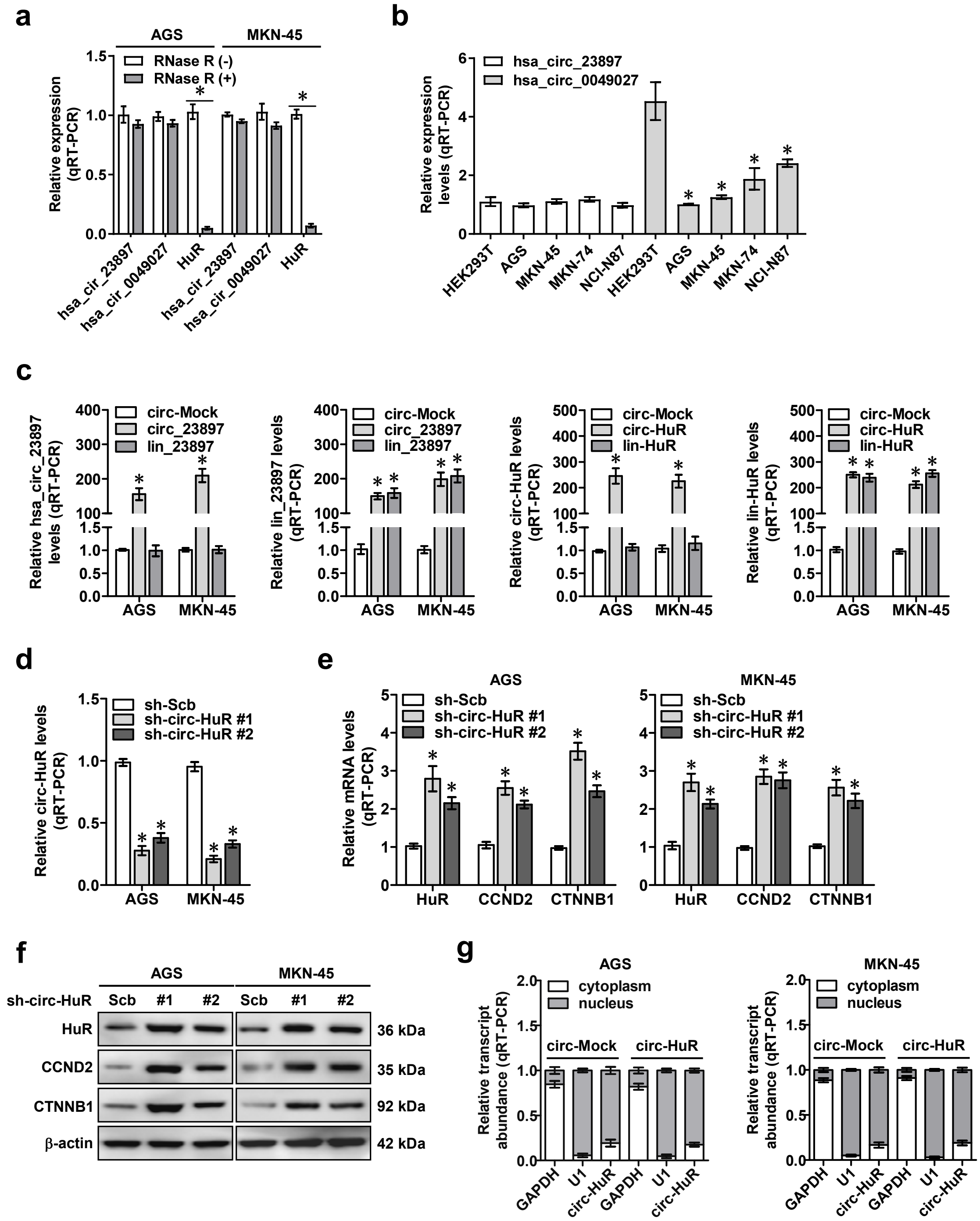

Figure S1. Expression of *HuR*-derived circRNAs and their effects on *HuR* expression in gastric cancer cells. a Real-time qRT-PCR assay indicating the levels (normalized to β-actin, *n*=5) of *hsa_circ_23897* or *hsa_circ_0049027* after RNase R treatment (3 U/mg) in AGS and MKN-45 cells. **b** Real-time qRT-PCR assay revealing the relative levels of *circ-HuR* (normalized to β-actin, *n*=5) in HEK293T and gastric cancer cell lines. **c** Real-time qRT-PCR assay showing the levels (normalized to β-actin, *n*=5) of *hsa_circ-23897*, linear *hsa_circ-23897* (*lin_23897*), *circ-HuR*, and linear *circ-HuR* (*lin-HuR*) in AGS and MKN-45 cells stably transfected with empty vector (*circ-Mock*), *hsa_circ-23897* (*circ_23897*), *lin_23897*, *circ-HuR*, or *lin-HuR*. **d** Real-time qRT-PCR assay indicating the levels (normalized to β-actin, *n*=5) of *HuR* in AGS and MKN-45 cells stably transfected with scramble shRNA (sh-Scb), sh-circ-HuR #1, or sh-circ-HuR #2. **e** and **f** Real-time qRT-PCR (**e**, normalized to β-actin, *n*=5) and western blot (**f**) assays revealing the transcript and protein levels of *HuR* and its downstream target genes in AGS and MKN-45 cells stably transfected with sh-Scb, sh-circ-HuR #1, or sh-circ-HuR #2. **g** Real-time qRT-PCR indicating the distribution of *GAPDH*, *U1*, and *circ-HuR* in the cytoplasmic and nuclear fractions of AGS and MKN-45 cells stably transfected with *circ-Mock* or *circ-HuR*. Student's *t*-test or ANOVA analyzed the difference in **a**-**e**. * P<0.01 vs. RNase R (-), HEK293T, circ-Mock, or sh-Scb. Data are shown as mean ±SEM (error bars) and representative of three independent experiments in **a**-**g**.

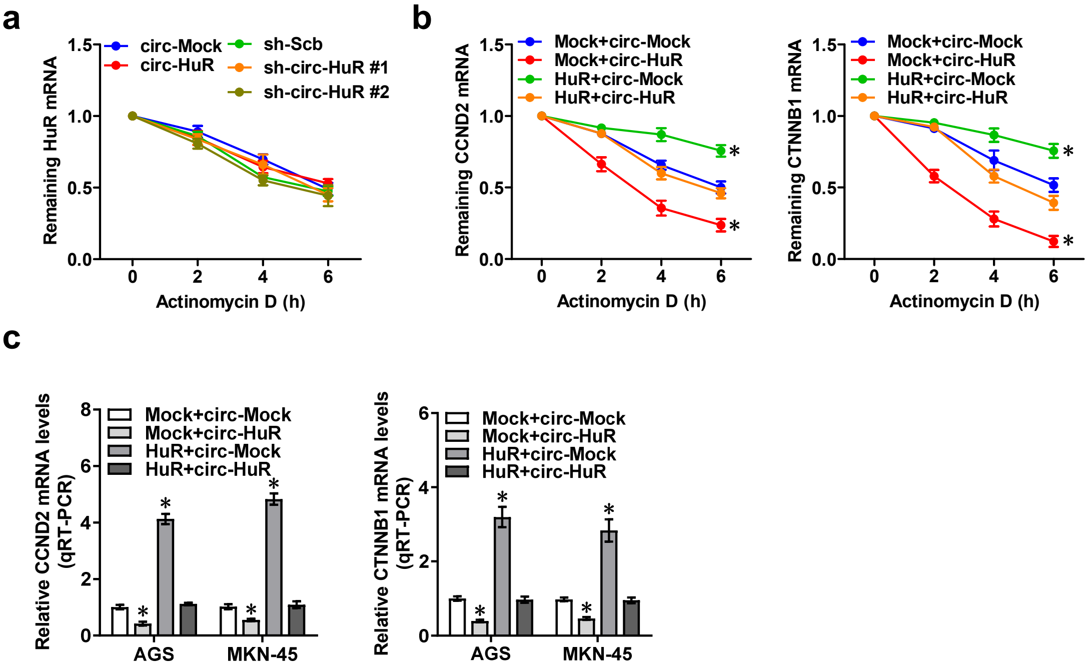

Figure S2. Effects of *circ-HuR* on expression of *HuR* and downstream genes in gastric cancer cells. a Real-time qRT-PCR assay indicating the half-life of *HuR* mRNA (normalized to β-actin, *n*=5) in AGS cells stably transfected with empty vector (*circ-Mock*), *circ-HuR*, scramble shRNA (sh-Scb), sh-circ-HuR #1, or sh-circ-HuR #2, and those treated with actinomycin D (1 μg/mL) for indicated periods of time. **b** and **c** Real-time qRT-PCR assay showing the half-life and levels of *CCND2* and *CTNNB1* mRNA (normalized to β-actin, *n*=5) in AGS cells stably transfected with empty vector (mock), *HuR*, *circ-Mock*, or *circ-HuR*, and those treated with actinomycin D (1 μg/mL) for indicated periods of time. Student's *t*-test or ANOVA analyzed the difference in **a**-**c**. * $P<0.01$ vs. mock+circ-Mock. Data are shown as mean ±SEM (error bars) and representative of three independent experiments in **a**-**c**.

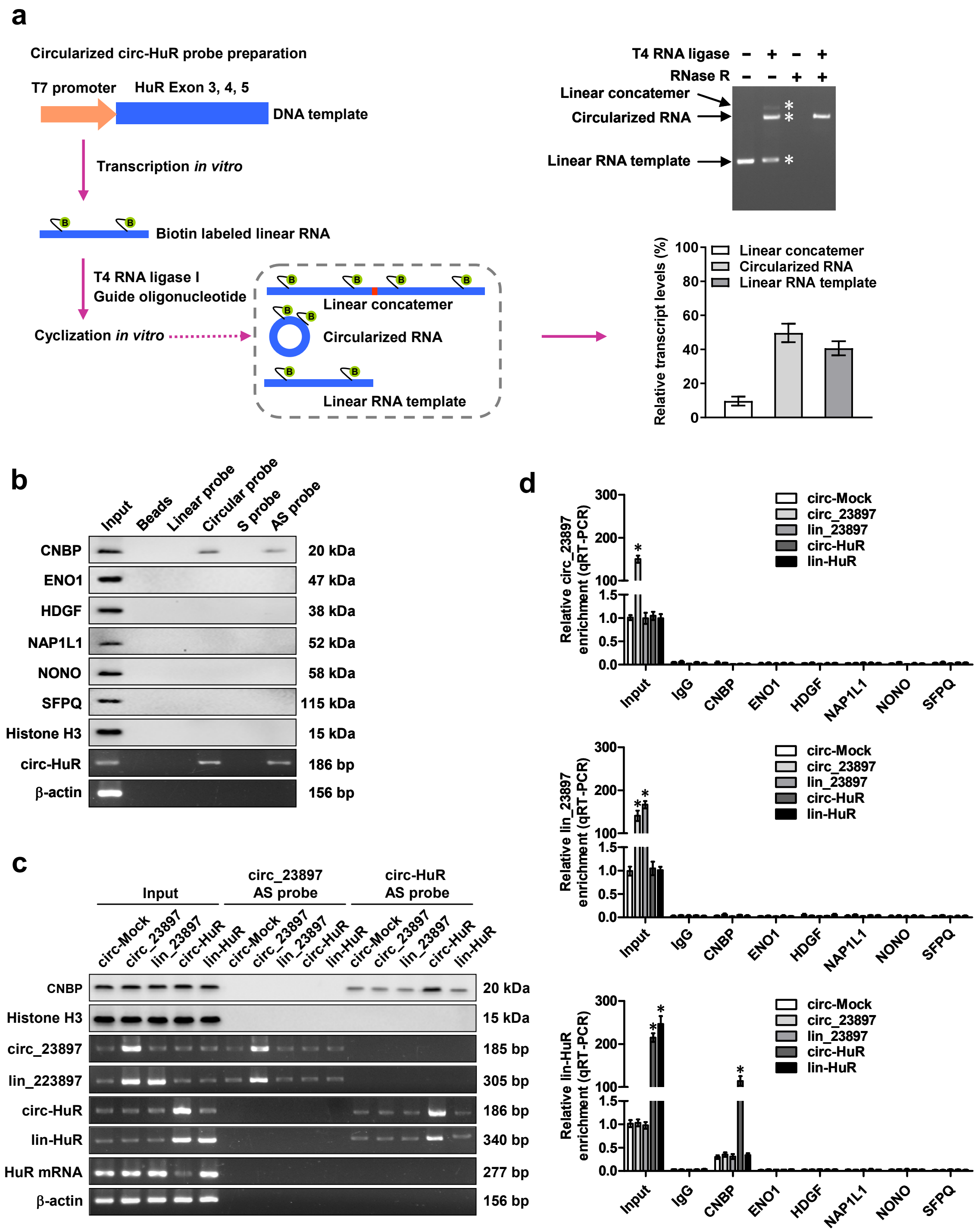

Figure S3. Interaction between *circ-HuR* and CNBP protein in gastric cancer cells. **a** Schematic diagram showing the process to generate biotin-labeled *circ-HuR* probe by ligation of *in vitro* transcribed linear transcript (left panel), and validation by denaturing PAGE, SYBR Green II staining, and RNase R (3 U/mg) treatment (right upper panel), with circularization efficiency as indicated (right lower panel). **b** Western blot (upper panel) and RT-PCR (lower panel) assays indicating the proteins and transcripts pulled down by biotin-labeled linear or circular exogenous *circ-HUR*, sense (S) or antisense (AS) probe targeting junction site of endogenous *circ-HuR* from AGS cell lysates. **c** Western blot (upper panel) and RT-PCR (lower panel) assays revealing the proteins and transcripts pulled down by biotin-labeled antisense (AS) probe targeting junction site of *hsa_circ-23897* (*circ_23897*) or *circ-HuR* from lysates of AGS cells stably transfected with empty vector (*circ-Mock*), *circ_23897*, linear *circ_23897* (*lin_23897*), *circ-HuR*, or linear *circ-HuR* (*lin-HuR*). **d** RIP and real-time qRT-PCR assays showing the interaction of *circ_23897*, *lin_23897*, or *lin-HuR* with six proteins in AGS cells stably transfected with *circ-Mock*, *circ_23897*, *lin_23897*, *circ-HuR*, or *lin-HuR*. ANOVA analyzed the difference in **d**. * $P<0.01$ vs. circ-Mock. Data are shown as mean±SEM (error bars) and representative of three independent experiments in **b**-**d**.

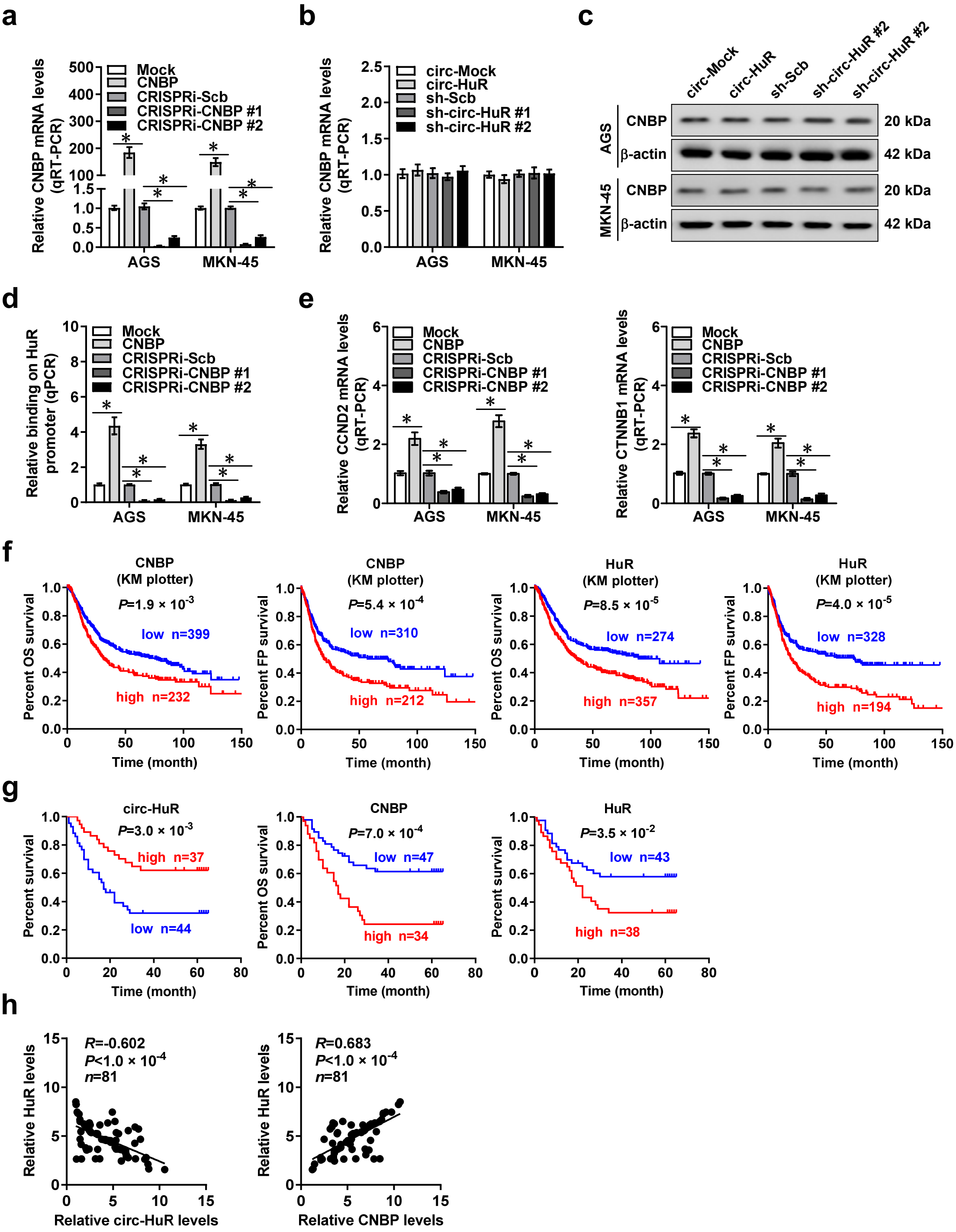

Figure S4. Roles of *circ-HuR* and CNBP in regulating transcription of *HuR* and downstream genes. a Real-time qRT-PCR assay indicating the *CNBP* transcript levels (normalized to β-actin, *n*=5) in AGS and MKN-45 cells stably transfected with empty vector (mock), *CNBP*, CRISPRi-Scb, CRISPRi-CNBP #1, or CRISPRi-CNBP #2. **b** and **c** Real-time qRT-PCR assay (**b**, normalized to β-actin, *n*=5) and western blot (**c**) assays showing the transcript and protein levels of *CNBP* in AGS and MKN-45 cells stably transfected with empty vector (*circ-Mock*), *circ-HuR*, sh-Scb, sh-circ-HuR #1, or sh-circ-HuR #2. **d** ChIP and qPCR assays indicating the changes in the binding of CNBP (normalized to input) to *HuR* promoter in AGS and MKN-45 cells stably transfected with mock, *CNBP*, CRISPRi-Scb, CRISPRi-CNBP #1, or CRISPRi-CNBP #2. **e** Real-time qRT-PCR assay revealing the transcript levels (normalized to β-actin, *n*=5) of *CCND2* and *CTNNB1* in AGS and MKN-45 cells stably transfected with mock, *CNBP*, CRISPRi-Scb, CRISPRi-CNBP #1, or CRISPRi-CNBP #2. **f** Overall (OS) and first progression (FP) survival of gastric cancer samples derived from Kaplan-Meier Plotter database (http://kmplot.com/analysis), with high or low expression of *CNBP* (cutoff values=487.0 and 482.0) or *HuR* (cutoff values=363.5 and 419.5). **g** Kaplan–Meier curves showing the survival of 81 gastric cancer patients with low or high expression of *circ-HuR* (cutoff value=4.1), *CNBP* (cutoff value=5.5), or *HuR* (cutoff value=4.7). **h** The expression correlation of *HuR* with *circ-HuR* or *CNBP* in 81 gastric cancer tissues. Student's *t*-test or ANOVA analyzed the difference in **a**, **b**, **d**, and **e**. Log-rank test for survival comparison in **f** and **g**. Pearson's correlation coefficient analysis for gene expression in **h**. *$P<0.01$ vs. mock, CRISPRi-Scb, circ-Mock, or sh-Scb. Data are shown as mean ±SEM (error bars) and representative of three independent experiments in **a**-**e**.

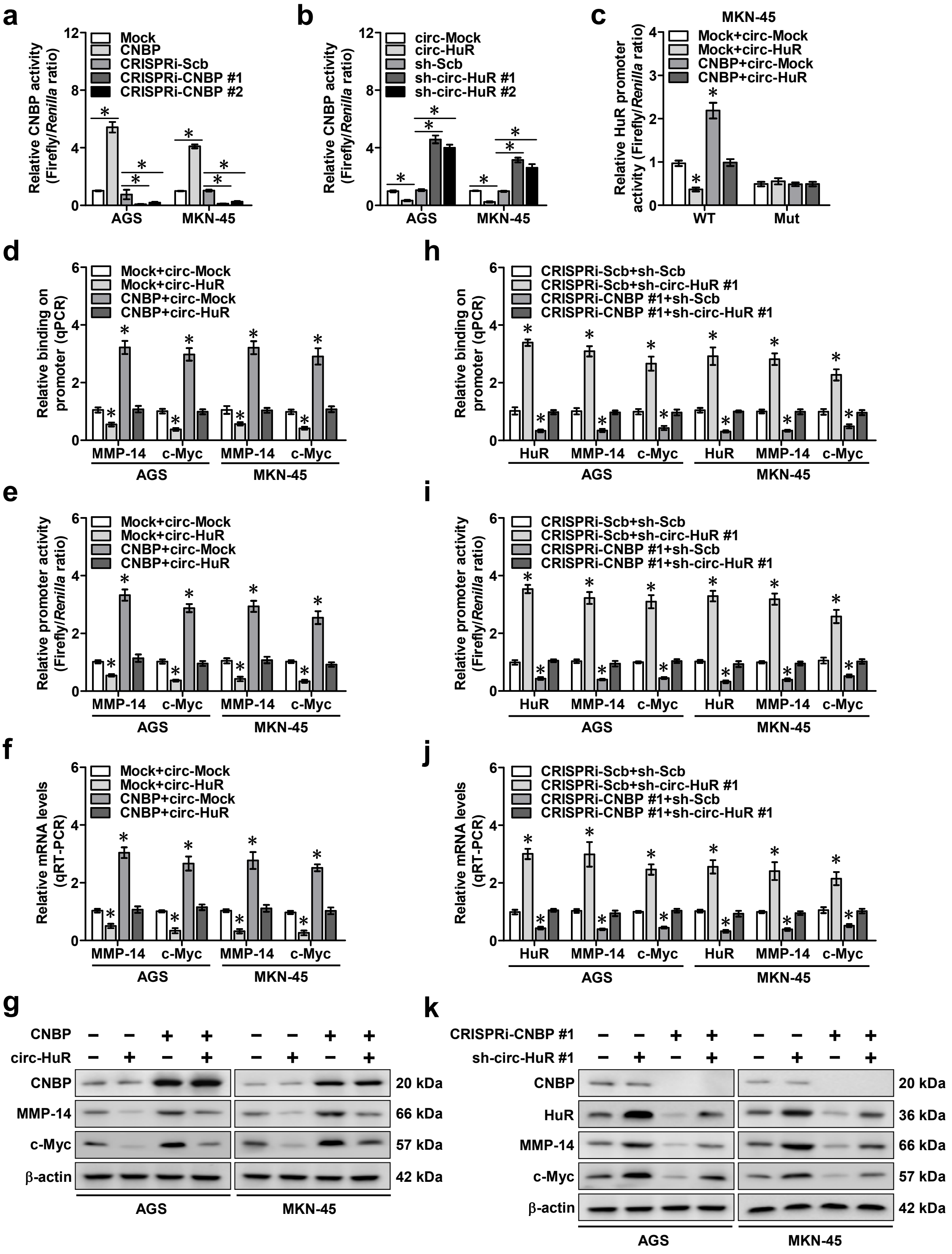

Figure S5. ***circ-HuR* regulates CNBP transactivation and target gene expression in gastric cancer cells. a** Dual-luciferase assay revealing the activity of CNBP in AGS and MKN-45 cells stably transfected with empty vector (mock), *CNBP*, CRISPRi-Scb, CRISPRi-CNBP #1, or CRISPRi-CNBP #2 (*n*=5). **b** Dual-luciferase assay indicating the activity of CNBP in AGS and MKN-45 cells stably transfected with empty vector (*circ-Mock*), *circ-HuR*, scramble shRNA (sh-Scb), sh-circ-HuR #1, or sh-circ-HuR #2 (*n*=6). **c** Dual-luciferase assay showing the activity of *HuR* promoter with wild type (WT) or mutant (Mut) CNBP binding site in AGS and MKN-45 cells stably transfected with mock or *CNBP*, and those co-transfected with *circ-HuR* (*n*=5). **d-g** ChIP and qPCR (d), dual-luciferase reporter (e), real-time qRT-PCR (f, normalized to β-actin, *n*=5), and western blot (g) assays showing the CNBP enrichment, promoter activity, transcript and protein levels of *MMP-14* and *c-Myc* in AGS and MKN-45 cells stably transfected with *circ-Mock* or *circ-HuR*, and those co-transfected with *CNBP*. **h-k** ChIP and qPCR (h), dual-luciferase reporter (i), real-time qRT-PCR (j, normalized to β-actin, *n*=5), and western blot (k) assays showing the CNBP enrichment, promoter activity, transcript and protein levels of *MMP-14* and *c-Myc* in AGS and MKN-45 cells stably transfected with sh-Scb or sh-circ-HuR #1, and those co-transfected with CRISPRi-Scb or CRISPRi-CNBP #1. Student's *t*-test or ANOVA analyzed the difference in **a-f** and **h-j**. * $P<0.01$ vs. mock, CRISPRi-Scb, circ-Mock, sh-Scb, mock+circ-Mock, or CRISPRi-Scb+sh-Scb. Data are shown as mean ±SEM (error bars) and representative of three independent experiments in **a-k**.

Table S1 Univariate and multivariate analysis of prognostic factors in gastric cancer patients

Clinicopathologic factor	n	Univariate analysis		Multivariate analysis		
		Mean ± SEM (months)	*P*-value	Hazard ratio	95% CI	*P*-value
Age (years)						
≤60	42	39.1 ± 4.1	0.332	1.374	0.755-2.500	0.298
>60	39	34.3 ± 4.2				
Sex						
Male	57	33.9 ± 3.5	0.168	0.572	0.281-1.163	0.123
Female	24	43.7 ± 5.3				
Size (diameter)						
≤6 cm	40	33.6 ± 4.2	0.221	0.736	0.404-1.341	0.736
>6 cm	41	39.9 ± 4.1				
Laurén classification						
Intestinal type	47	36.3 ± 3.8	0.731	0.822	0.444-1.521	0.533
Diffuse type	34	37.5 ± 4.6				
Gastric wall invasion						
T1/T2	34	49.1 ± 4.1	<0.001	1.266	1.066-2.901	0.213
T3/T4	47	27.9 ± 3.6				
Lymph node metastasis						
Negative	22	51.6 ± 5.3	<0.001	1.368	1.052-2.894	0.135
Positive	59	31.2 ± 3.3				
Distant metastasis						
Negative	62	41.9 ± 3.2	<0.001	2.809	1.432-5.511	0.002
Positive	19	20.3 ± 5.4				
TNM stage						
I/II	27	48.3 ± 4.7	0.003	2.519	1.193-5.319	0.015
III/IV	54	31.1 ± 3.5				
circ-HuR expression						
Low	44	28.3 ± 3.8	0.003	0.616	0.440-0.861	0.012
High	37	46.9 ± 3.9				
CNBP expression						
Low	47	45.4 ± 3.7	<0.001	2.643	1.194-4.260	0.003
High	34	25.1 ± 4.0				
HuR expression						
Low	43	43.0 ± 4.1	0.035	1.411	1.030-1.932	0.232
High	38	29.9 ± 4.0				

HuR, human antigen R; circ-HuR, circular RNA derived from HuR; CNBP, CCHC-type zinc finger nucleic acid binding protein; TNM, tumor-node-metastasis. Log-rank test and Cox regression model were applied for univariate and multivariate analysis. *n*, number of patients; SEM, standard error of the mean; 95% CI, 95% confidence interval.

Table S2 Primer sets used for RT-PCR, qPCR, RIP, and ChIP

Primer set	Primers	Sequence	Product size (bp)	Application
circ-HuR (Divergent)	Forward	5’-ACAAAAACGTGGCACTCCTCT-3'	186	RT-PCR qPCR, RIP
	Reverse	5’-GGTTTTTGACTGGAGCCTCAA-3'		
lin-HuR (Convergent)	Forward	5’-TTGAGGCTCCAGTCAAAAACC-3'	340	RT-PCR qPCR, RIP
	Reverse	5’-AGAGGAGTGCCACGTTTTTGT-3'		
hsa_circ_23897 (Divergent)	Forward	5’-ATGACCCAGAAGGACGTAGAAG-3'	185	RT-PCR qPCR
	Reverse	5’-CTGAGGGAGGTAGTTGACGATC-3'		
lin_circ_23897 (Convergent)	Forward	5’-GATCGTCAACTACCTCCCTCAG-3’	305	RT-PCR qPCR, RIP
	Reverse	5’-CTTCTACGTCCTTCTGGGTCAT-3'		
hsa_circ_0049026 (Divergent)	Forward	5’-CAGGTTCGTGTTAAACGCTGT-3'	180	RT-PCR qPCR
	Reverse	5’-GGTTTTTGACTGGAGCCTCAA-3'		
β-actin (Divergent)	Forward	5'-AAATCGTGCGTGACATTAAGGAGA-3’	-	RT-PCR
	Reverse	5'-CATACCCCTCGTAGATGGGCA-3’		
β-actin (Convergent)	Forward	5'-TGCCCATCTACGAGGGGTATG-3'	156	RT-PCR, qPCR
	Reverse	5'-TCTCCTTAATGTCACGCACGATTT-3'		
GAPDH	Forward	5'-AGAAGGCTGGGGCTCATTTG-3'	258	qPCR
	Reverse	5'-AGGGGCCATCCACAGTCTTC-3'		
U1	Forward	5'-ACTTACCTGGCAGGGGAGATACC-3’	137	qPCR
	Reverse	5'-CCACTACCACAAATTATGCAGTCG-3’		
HuR	Forward	5’-GTCCTCGTGGATCAGACTAC-3'	277	qPCR
	Reverse	5’-TCATGTGATCGACGCCCATG-3’		
CNBP	Forward	5’-GCTACAACTGTGGCAAACC-3'	213	qPCR
	Reverse	5’-GTGCAAGGTGCCCTGACT-3’		
CCND2	Forward	5’-ACCTTCCGCAGTGCTCCTAC-3'	161	qPCR
	Reverse	5’-CCCAGCCAAGAAACGGTCCA-3'		
CTNNB1	Forward	5’-TGCTGAAGGTGCTATCTGTC-3'	198	qPCR
	Reverse	5’-GAAGCTGAACAAGAGTCCCA-3'		
MMP-14	Forward	5’-GCCTTCTGTTCCTGATAA-3'	225	qPCR
	Reverse	5’-CCATCCTTCCTCTCGTAG-3'		
c-Myc	Forward	5’-ATTCTCTGCTCTCCTCGACG-3'	224	qPCR
	Reverse	5’-CAGTGGGCTGTGAGGAGGTT-3'		
HuR ChIP (-679/-455)	Forward	5'-TGCATGTATTTCTACTAGGGCATT-3'	225	qPCR
	Reverse	5'-TGAGGAGGCGGGGCATGGAACG-3'		
MMP-14 ChIP (-850/-604)	Forward	5'-CTCCCTTCCCTACATACCTCCA-3'	247	qPCR
	Reverse	5'-GTCATTTGTAATGTGGCTGCAC-3'		
c-Myc ChIP (-1199/-961)	Forward	5'-CTCCCGTCTAGCACCTTTGATT-3'	239	qPCR
	Reverse	5'-GGCGTCTGTTTAGCCCTGAGAT-3'		

HuR, human antigen R; circ-HuR, circular RNA derived from HuR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; U1, U1 small nuclear 1; CNBP, CCHC-type zinc finger nucleic acid binding protein; CCND2, cyclin D2; CTNNB1, catenin beta 1; MMP-14, matrix metallopeptidase 14; c-Myc, MYC proto-oncogene, bHLH transcription factor; RT-PCR, reverse transcription PCR; qPCR, quantitative PCR; RIP, RNA immunoprecipitation; ChIP, chromatin immunoprecipitation.

Table S3 Primer sets used for constructs

Primer Set	Sequences
pLCDH-circ-HuR	5'-CCGGAATTCTGAAATATGCTATCTTACAGGACACAGCTTGGGCTATGGCTTT-3' (sense); 5'-CGCGGATCCTCAAGAAAAAATATATTCACCTGAATCTCTGCGCCTGGTGGTG-3' (antisense)
pLCDH-lin-HuR	5'-CCGGAATTCTGAAATATGCTATCTTACAGGACACAGCTTGGGCTATGGCTTT-3' (sense); 5'-CGCGGATCCGGCTGAATCTCTGCGCCTGGTGGTG-3' (antisense)
pLCDH-circ_23897	5'-CCGGAATTCTGAAATATGCTATCTTACAGATTTTTGAAAAATACAATGTCTA-3' (sense); 5'-CGCGGATCCTCAAGAAAAAATATATTCACCTGTAGTCTGATCCACGAGGACC-3' (antisense)
pLCDH-lin_23897	5'-CCGGAATTCTGAAATATGCTATCTTACAGATTTTTGAAAAATACAATGTCTA-3' (sense); 5'-CGCGGATCCGGCTGTAGTCTGATCCACGAGGACC-3' (antisense)
pCMV-3Tag-1A-CNBP-WT	5'-CGCGGATCCATGAGCAGCAATGAGTGCTT-3' (sense); 5'-CCGCTCGAGTTAGGCTGTAGCCTCAATTG-3' (antisense)
pCMV-3Tag-1A-CNBP-ΔRGG-1	5’-CGCGGATCCATGAGCAGCAATGAGTGCTT-3' (sense-1); 5’-CAAACTGGAAAGTAGGACATTCCCGGGCCC-3' (antisense-1)
pCMV-3Tag-1A-CNBP-ΔRGG-2	5’-ATGTCCTACTTTCCAGTTTGTTTCCTCGTC-3' (sense-2); 5’-CCGCTCGAGTTAGGCTGTAGCCTCAATTG-3' (antisense-2)
pCMV-3Tag-1A-CNBP-Δ1-RGG	5’-CGCGGATCCCCAGACATTTGTTATCGCTG-3' (sense); 5’-CCGCTCGAGTTAGGCTGTAGCCTCAATTG-3’' (antisense)
pCMV-3Tag-1A-CNBP-Δ5-7	5’-CGCGGATCCATGAGCAGCAATGAGTGCTT-3' (sense); 5’-CCGCTCGAGTGCATGGTCGCAGTCACGAG-3’ (antisense)
pGEX-6P-1-CNBP-WT	5’-CGCGGATCCATGAGCAGCAATGAGTGCTT-3’ (sense); 5’-CCGCTCGAGTTAGGCTGTAGCCTCAATTG-3’ (antisense)
pGEX-6P-1-CNBP-ΔRGG-1	5’-CGCGGATCCATGAGCAGCAATGAGTGCTT-3' (sense-1); 5’-CAAACTGGAAAGTAGGACATTCCCGGGCCC-3' (antisense-1)
pGEX-6P-1-CNBP-ΔRGG-2	5’-ATGTCCTACTTTCCAGTTTGTTTCCTCGTC-3' (sense-2); 5’-CCGCTCGAGTTAGGCTGTAGCCTCAATTG-3' (antisense-2)
pGEX-6P-1-CNBP-Δ1-RGG	5’-CGCGGATCCCCAGACATTTGTTATCGCTG-3' (sense); 5’-CCGCTCGAGTTAGGCTGTAGCCTCAATTG-3’' (antisense)
pGEX-6P-1-CNBP-Δ5-7	5’-CGCGGATCCATGAGCAGCAATGAGTGCTT-3' (sense); 5’-CCGCTCGAGTGCATGGTCGCAGTCACGAG-3’ (antisense)
pGL3-HuR promoter WT	5’-CGGGGTACCGGCACGAGAATCACTTGAATAGGG-3’ (sense); 5’-CCCAAGCTTCCGCCCGGTAAGGTAGGAAAGGT-3' (antisense)
pGL3-HuR promoter Mut	5'-TTTAATGAATCCGCACCCTGCTGAGCCTTCGGGAACTGGCTTGCTCAA-3’ (sense); 5'-AGGCTCAGCAGGGTGCGGATTCATTAAAATGTCATTGAGCGCCTACTG-3’ (antisense)
pGL3-CNBP reporter	5’-CAAATGAGAATAAAATGAGATATCTGAAAATATACTGAAAATA-3' (sense); 5'-AGCTTATTTTCAGTATATTTTCAGATATCTCATTTTATTCTCATTTGGTAC-3' (antisense)
pGL3-c-Myc promoter	5’-CGGAAGATCTGTTTGTCCGGGGAGGAAAGAGTT-3' (sense); 5'-CCCAAGCTTCGCTGGAATTACTACAGCGAGTT-3' (antisense)

HuR, human antigen R; lin, linear; CNBP, CCHC-type zinc finger nucleic acid binding protein; RGG, arginineglycine-glycine-rich region; WT, wild type; Mut, mutation; c-Myc: MYC proto-oncogene, bHLH transcription factor.

Table S4 Oligonucleotide sets used for CRISPR-dCas9, short hairpin RNAs, or probe

Oligo Set		Sequences
sgRNA-CRISPRi-CNBP #1	Forward	5'-CACCGGCCGTGTGCAGACCCGCGTG-3'
	Reverse	5'-AAACCACGCGGGTCTGCACACGGCC-3'
sgRNA-CRISPRi-CNBP #2	Forward	5'-CACCGGCGTGTGGCGCAGGCAAGGA-3'
	Reverse	5'-AAACTCCTTGCCTGCGCCACACGCC-3'
sh-Scb	Antisense	5'-CCGGGCGAACGATCGAGTAAACGGACTCGAGTCCGTTTACTCGATCGTTCGCTTTTT-3'
	Sense	5'-AATTCAAAAAGCGAACGATCGAGTAAACGGACTCGAGTCCGTTTACTCGATCGTTCGC-3'
sh-circ-HuR #1	Antisense	5'-CCGGTCAGAGATTCAGGACACAGCCTCGAGGCTGTGTCCTGAATCTCTGTTTTTG-3'
	Sense	5'-GATCCAAAAACAGAGATTCAGGACACAGCCTCGAGGCTGTGTCCTGAATCTCTGA-3'
sh-circ-HuR #2	Antisense	5'-CCGGTAGATTCAGGACACAGCTTGCTCGAGCAAGCTGTGTCCTGAATCTTTTTTG-3'
	Sense	5'-GATCCAAAAAAGATTCAGGACACAGCTTGCTCGAGCAAGCTGTGTCCTGAATCTA-3'
circ-HuR probe	Antisense	5'-AGCCCAAGCTGTGTCCTGAATCTCTGCGCC-3'
	Sense	5'-GGCGCAGAGATTCAGGACACAGCTTGGGCT-3'
circ_23897 probe	Antisense	5'-GTATTTTTCAAAAATCTGTAGTCTGATCCACGAGG-3'
HuR mRNA probe	Antisense	5'-ATCGACGCCCATGGGGGAGAACCTGAATCT-3'
circ-HuR transcription *in vitro*	Forward	5'-TAATACGACTCACTATAGGGGACACAGCTTGGGCTATGGCTTT-3'
	Reverse	5'-CTGAATCTCTGCGCCTGGTGGTG-3'
circ-HuR circularization	Guide DNA	5'-AGCCCAAGCTGTGTCAATCTCTGCGCCTGG-3'

sgRNA, small guide RNA; CRISPRi, clustered regularly interspaced short palindromic repeats interference; dCas9, dead mutant of Cas9 endonuclease; Scb, scramble; shRNA, short hairpin RNA; HuR, human antigen R; circ-HuR, circular RNA derived from HuR.