Supplementary

Table S1 Vectors, insertion sites, and primer sequences

Vectors	Enzyme sites	Forward primers	Reverse primers
pCDNA3-Flag-EYA3	BamHI + XhoI	CGGGATCCATGGAAGAAGAGCAAGATTTAC	CCGCTCGAGTTAGAGAAAATCAAGCTCTAAAGCCT
pCDNA3-Myc-EYA3	KpnI + XhoI	GGGGTACCATGGAAGAAGAGCAAGATTTAC	CCGCTCGAGTTAGAGAAAATCAAGCTCTAAAGCCT
pCDNA3-Myc-p300	KpnI + XhoI	GGGGTACCATGGCCGAGAATGTGGTGGAACCG	CCGCTCGAGCTAGTGTATGTCTAGTGTACTCTG
pCDNA3-Myc-SIX5	KpnI + XhoI	GGGGTACCATGGCTACCTTGCCTGCGGAG	CCGCTCGAGTCACAGTTCCAAGGGCTCCTCCA
pCDNA3-Flag-p300	BamHI + XhoI	CGGGATCCATGGCCGAGAATGTGGTGGAACCG	CCGCTCGAGCTAGTGTATGTCTAGTGTACTCTG

Table S2 shRNA information (ordered from Sigma-Aldrich)

Genes	shRNA target sequences	Catalog numbers
EYA3	CCCTTCTACAAGTCCATCTTT	TRCN0000051603
	CACATTATTCTTATCCCATT	TRCN0000051606
SIX5	GCGCCAGCTCTTGCAGACTTT	TRCN0000015773
	CCCTGCCAATGTGCACCTCAT	TRCN0000015775
p300	CAATTCCGAGACATCTTGAGA	TRCN0000009883
	GCCTTCACAATTCCGAGACAT	TRCN0000039885
HIF1α	CCGCTGGAGACACAATCATAT	TRCN0000003808
	TGCTCTTTGTGGTTGGATCTA	TRCN0000010819
HIF2α	AGGTGGAGCTAACAGGACATA	TRCN0000003803
	CAGTACCCAGACGGATTTCAA	TRCN0000003806

shRNA, short hairpin RNA.

https://dx.doi.org/10.21037/atm-22-2663

Table S3 Primers used for RT-qPCR analysis to measure gene expression

Genes	Forward primers	Reverse primers
EYA3	GAGGCAAGACTCCTTCCAATGC	GAGCACTGACTTCAGCTACTCAG
HIF1α	TACTGCTAATGCCACCACTACCA	TGGTGATGATGTGGCACTAGTAG
HIF2α	AGACTTGTCCAGTGCTCCCACG	GCTGAATGACTCCACTGCTCGGAT
SIX5	TGCCAATGTGCACCTCATCAACTC	ATGGGGCTGCCAGACACAGGGTTG
p300	TGGCTTAGATGATGAGAGCAACA	ATTCCGACACTGGCAAGCATGGA
EGFR	AGCGTTCAATTCATCCTCACCAG	CTCACAAAGGAGGGAAGAGACTGG
VEGFD	GATGTTGTACGTCCAGCTGGT	CCAAACTAGAAGCAGCCCTGAT
MMP3	GAGGTGACTCCACTCACATTC	GGCATAGGCATGGGCCAAAACAT
MMP7	GTGGTCACCTACAGGATCGTATC	AGCAGTTCCCCATACAACTTTCC
MMP8	ATGAATGTGAGCTTACCAGGGT	CCTAGGTGACTATGCCTCTCTTC
MMP13	ATCCAGTCTCTCTATGGTCCAG	TCATTGTTTCTCCTCGGAGACT
MMP21	ACAATAGGACACGCTATGGGGA	GTCACTGTCATATCTCCAGTA
MMP26	GACATGCAGATGCATGCTCTGCT	CCTGTAAGTTAGAGTGTGC
RNF5	ATGTCTTCATCAGTGGCTGGA	TTAATCTGGGATCCTGGGGCT
TAP2	TGAACACTGCTACCTGCACAG	CATCACCTTATCATCTTCGCAG
STMN1	ATCCAAAGACTGTACTGGCCAG	CAGTTTCTCCCCTTTAGCCCCTA
BRD2	AGCTGCAATACCTACACAAGGT	AGTACCCATGTCCATAGGCTG
b-Actin	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT

RT-qPCR, real-time quantitative polymerase chain reaction.

Table S4 Primers used for ChIP RT-qPCR analysis

Genes	Forward primers	Reverse primers
EGFR	TACTGCAGGAGAAGGAACAGT	GTCCCACTGCCCCTGTAGCT
VEGFD	TGAACATTTGAGTCAGTTCTTA	GCACAACCTTCATGGAAGCTTG
MMP3	ATGTTCTATTCTGCCCATGAG	CTATATACAATTATACTC
MMP7	CGCATCACCATGTTTGGCTA	ATGCAAAGACACATCCATGG
MMP8	ACAAAGAATGGGTTGCTACA	ACAGCAGTGGTGTGGAGGGAGT
MMP13	TACCTCTGTCTGAATCTGT	TGGAGGTGCTACGGCACAAC
MMP21	CCGCAGTATATCTCCATAG	TGGTGTGAGAACTCCTTCTC
MMP26	ACTCTGGCTCTATGCAAAGTT	GTCTATCTTCACTCTTTCTTCCC

ChIP, chromatin immunoprecipitation; RT-qPCR, real-time quantitative polymerase chain reaction.

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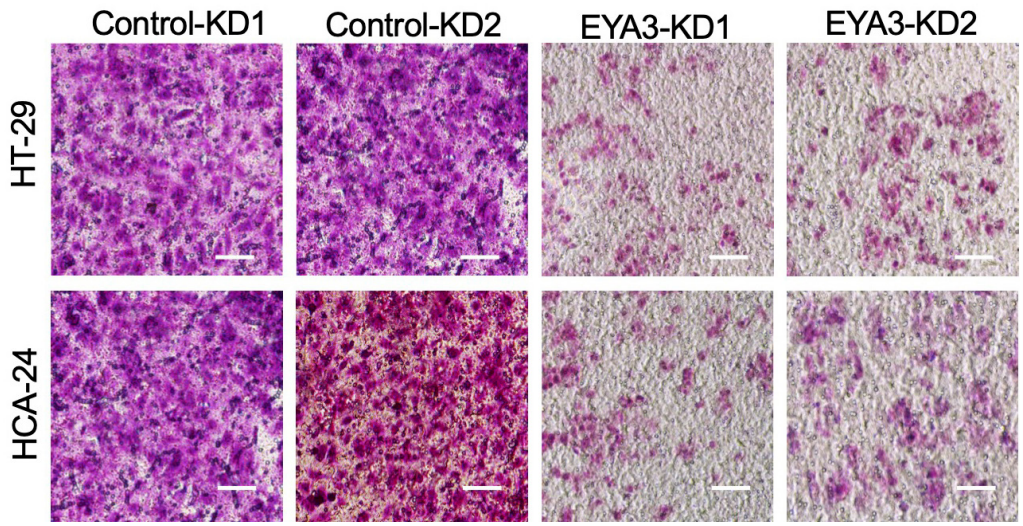

Figure S1 Cell invasion assay using control-KD and EYA3-KD cells. The same numbers of control-KD and EYA3-KD cells under both HT-29 and HCA-24 backgrounds were seeded into the upper chamber of Boyden chambers. After culturing for 24 h, the invaded cells in the lower chambers were fixed in methanol and stained with 0.2% crystal violet. Cells were photographed using a microscope with the magnification of 20-fold. Bars =100 μm. KD, knockdown.

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Table S5 EYA3-interacting proteins by MS analysis

Proteins	Protein description	Percolator score	Molecular weight (kD)	Matched queries	Matched peptides
EYA3	Eyes Absent Homolog 3	7123	63	57	43
SIX5	Sine Oculis Homeobox Homolog 5	6877	75	35	27
HMGN2	High Mobility Group Nucleosomal Binding Domain 2	6536	9	11	9
WDR1	WD Repeat Domain 1	6233	66	18	6
TRNP1	TMF1 Regulated Nuclear Protein 1	6094	23	8	5
MAPK8	Mitogen-Activated Protein Kinase 8	5887	48	20	10
p300	Histone Acetyltransferase P300	5649	264	33	18
KPNA2	Karyopherin Subunit Alpha 2	5369	58	32	11
TDP1	Tyrosyl-DNA Phosphodiesterase 1	5332	68	26	14
MDC1	Mediator Of DNA Damage Checkpoint 1	5128	227	44	27
UIMC1	Ubiquitin Interaction Motif Containing 1	4890	80	36	29
SMAD4	Mothers Against Decapentaplegic Homolog 4	4776	60	42	20
PITX1	Paired Like Homeodomain 1	4654	34	15	11
STIP1	Stress Induced Phosphoprotein 1	4454	63	25	12
CRY1	Cryptochrome Circadian Regulator 1	4092	66	30	25
MTMR3	Myotubularin Related Protein 3	3854	134	44	31
DLX2	Distal-Less Homeobox 2	3667	34	21	10
CHD9	Chromodomain Helicase DNA Binding Protein 9	3452	326	33	24
DIDO1	Death Inducer-Obliterator 1	3255	244	35	18
DDB2	Damage Specific DNA Binding Protein 2	3095	48	20	17
CDK1	Cyclin Dependent Kinase 1	3011	34	17	11
OGG1	8-Oxoguanine DNA Glycosylase	2943	39	18	10
COPS5	COP9 Signalosome Subunit 5	2901	38	24	20
RPA3	Replication Protein A3	2883	14	10	5
DNA2	DNA Replication Helicase/Nuclease 2	2654	120	25	17
HUS1	HUS1 Checkpoint Clamp Component	2412	32	16	10
GPS1	G Protein Pathway Suppressor 1	2267	56	25	21
RMI1	RecQ Mediated Genome Instability 1	2198	70	33	13
POLD4	DNA Polymerase Delta 4	2006	12	9	8
MCRS1	Microspherule Protein 1	1966	52	19	10
MCPH1	Microcephalin 1	1934	93	24	17
BAG6	BAG Cochaperone 6	1835	119	27	22
MYOD1	Myogenic Differentiation 1	1802	35	17	10
CNTN2	Contactin 2	1771	113	33	29
IRF3	Interferon Regulatory Factor 3	1643	47	27	20
HIPK2	Homeodomain Interacting Protein Kinase 2	1512	131	44	25
PTGS2	Prostaglandin-Endoperoxide Synthase 2	1442	69	33	27
SKP2	S-Phase Kinase Associated Protein 2	1023	48	19	10
BRD4	Bromodomain Containing 4	1009	152	44	18
NAP1L1	Nucleosome Assembly Protein 1 Like 1	954	45	24	11
NPM1	Nucleophosmin 1	903	33	15	10
DDX5	DEAD-Box Helicase 5	884	69	26	18
FEN1	Flap Structure-Specific Endonuclease 1	825	43	17	11
PIN1	Peptidylprolyl Cis/Trans Isomerase, NIMA-Interacting 1	775	18	10	7
NRG1	Neuregulin 1	701	70	32	23
NUDT21	Nudix Hydrolase 21	665	26	22	11
NOS2	Nitric Oxide Synthase 2	632	131	42	32
USP1	Ubiquitin Specific Peptidase 1	607	88	34	27
DUX4	Double Homeobox 4	587	45	25	20
IPO7	Importin 7	543	120	44	19
PLK1	Polo Like Kinase 1	513	68	35	22
DCAF7	DDB1 And CUL4 Associated Factor 7	486	39	20	19
SSRP1	Structure Specific Recognition Protein 1	447	81	34	20
CBX1	Chromobox 1	406	21	18	6
EEF2	Eukaryotic Translation Elongation Factor 2	367	95	17	8
SNTB2	Syntrophin Beta 2	332	58	15	4
RBM25	RNA Binding Motif Protein 25	302	100	25	7
CCN2	Cellular Communication Network Factor 2	285	38	21	17
CDR2	Cerebellar Degeneration Related Protein 2	265	52	16	10
CTNND2	Catenin Delta 2	234	133	22	11
HK2	Hexokinase 2	226	102	16	9
DDX1	DEAD-Box Helicase 1	213	82	25	19

MS, mass spectrometry.

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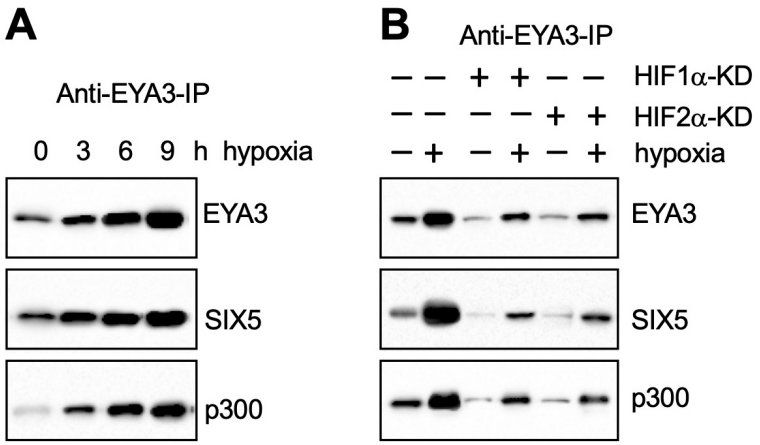

Figure S2 Hypoxia promoted the assembly of EYA3-SIX5-p300 complex. (A) The effect of hypoxia on the assembly of EYA3-SIX5-p300 complex. HCEC-1CT cells were treated with hypoxia for 0, 3, 6, and 9 h, followed by immunoprecipitation with anti-EYA3-conjugated agarose. The outputs were probed using anti-EYA3, anti-SIX5, and anti-p300. (B) The effect of HIF1α/2α depletion on the assembly of EYA3-SIX5-p300 complex. The Control-KD, HIF1α-KD1, and HIF2α-KD1 cells were treated with or without hypoxia for 9 h, followed by immunoprecipitation with anti-EYA3-conjugated agarose. The outputs were probed using anti-EYA3, anti-SIX5, and anti-p300.

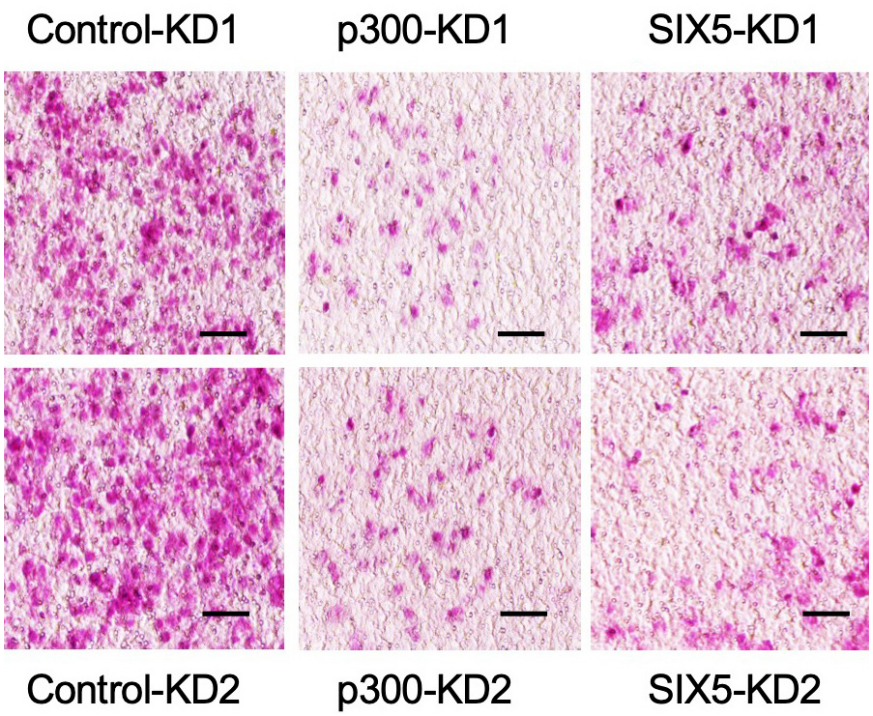

Figure S3 Cell invasion assay using control-KD, p300-KD, and SIX5-KD cells. The same numbers of control-KD, p300-KD, and SIX5-KD cells under HT-29 background were seeded into the upper chamber of Boyden chambers. After culturing for 24 h, the invaded cells in the lower chambers were fixed in methanol and stained with 0.2% crystal violet. Cells were photographed using a microscope with the magnification of 20-fold. Bars =100 μm. KD, knockdown.

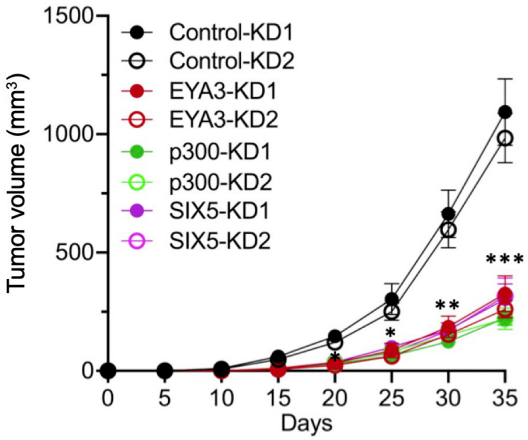

Figure S4 Tumor volumes in mice administrated with control-KD, EYA3-KD, p300-KD, and SIX5-KD cells. The control-KD, EYA3-KD, p300-KD, and SIX5-KD cells were injected into nude mice (n=10 for each cell line). Tumor volumes were determined at 5-day intervals. * P<0.05, ** P<0.01, and *** P<0.001.

Table S6 Differentially expressed genes by microarray analysis

Genes	Gene description	Control-KD1	EYA3–KD1	p300–KD1
EGFR	Epidermal Growth Factor Receptor	2	–12.1	–11.4
VEGFD	Vascular Endothelial Growth Factor D	2	–9.4	–8.5
MMP8	Matrix Metallopeptidase 8	2	–10.2	–9.6
MMP3	Matrix Metallopeptidase 3	2	–8.6	–8.9
MMP7	Matrix Metallopeptidase 7	2	–8.1	–10.3
MMP26	Matrix Metallopeptidase 26	2	–6.7	–8.5
MMP21	Matrix Metallopeptidase 21	2	–6.5	–6.8
LEMD2	LEM Domain Nuclear Envelope Protein 2	2	–5.7	–7.4
HCG25	HLA Complex Group 25	2	–5.2	–6.7
DDX58	DExD/H-Box Helicase 58	2	–4.6	–5.8
CDK3	Cyclin Dependent Kinase 3	2	–4.2	–6.2
HMGN4	High Mobility Group Nucleosomal Binding Domain 4	2	–4	–5.5
GPN2	GPN-Loop GTPase 2	2	–3.5	–3.7
HOXB2	Homeobox B2	2	–3.2	–3.1
MXD1	MAX Dimerization Protein 1	2	–2.2	–3.4
NUP85	Nucleoporin 85	2	–2.2	–3.1
NFX1	Nuclear Transcription Factor, X-Box Binding 1	2	13.2	11.1
STMN1	Stathmin 1	2	10.9	11.9
BRD2	Bromodomain Containing 2	2	9.9	10.6
GBP2	Guanylate Binding Protein 2	2	9.4	8.7
NOP56	NOP56 Ribonucleoprotein	2	9.1	8.2
CDKN1A	Cyclin Dependent Kinase Inhibitor 1A	2	8.4	9.4
MYOD1	Myogenic Differentiation 1	2	8.2	6.7
TDP1	Tyrosyl-DNA Phosphodiesterase 1	2	7.5	7.2
BRCC3	BRCA1/BRCA2-Containing Complex Subunit 3	2	7	5.4
MDC1	Mediator Of DNA Damage Checkpoint 1	2	6.5	7.8
TBK1	TANK Binding Kinase 1	2	6.2	4.3
CHD7	Chromodomain Helicase DNA Binding Protein 7	2	5.4	5.6
DLX3	Distal-Less Homeobox 3	2	4.3	3.4

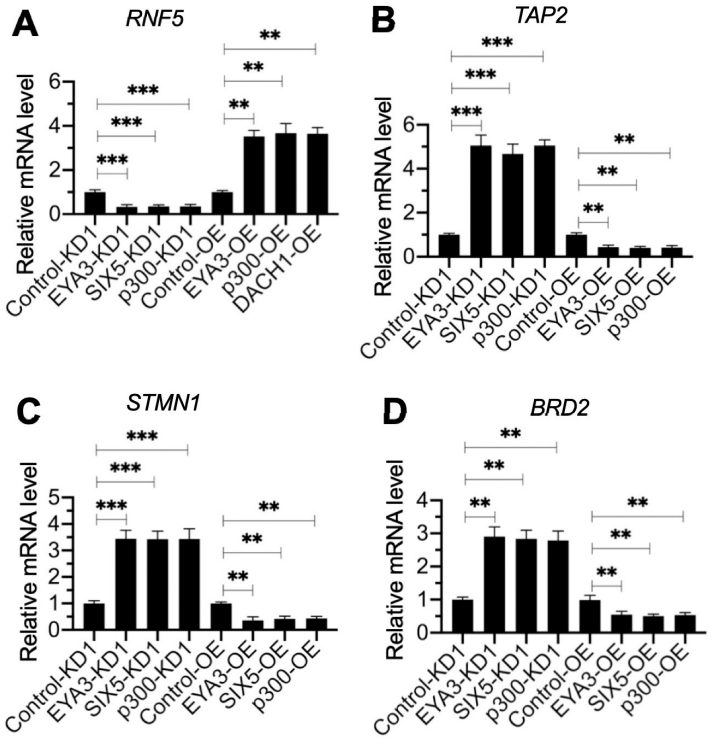

Figure S5 The mRNA levels of RNF5, TAP2, STMN1, and BRD2 in the KD and OE cell lines of EYA3-p300-SIX5 members. The mRNA levels of *RNF5* (A), *TAP2* (B), *STMN1* (C), and *BRD2* (D) in the KD and OE cell lines of EYA3, p300, and SIX5. ** P<0.01, and *** P<0.001. mRNA, messenger RNA; KD, knockdown; OE, overexpression.

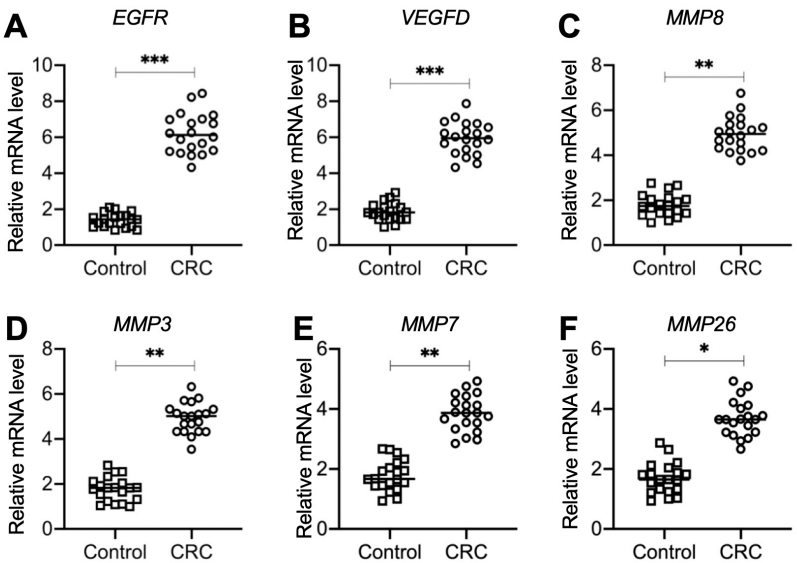

Figure S6 The mRNA levels of EYA3-p300-SIX5 targets in CRC biopsies. The same RNA samples as in Figure 1A were used for qRT-PCR analyses to measure mRNA levels of *EGFR* (A), *VEGFD* (B), *MMP8* (C), *MMP3* (D), *MMP*7 (E), and *MMP26* (F). * P<0.05, ** P<0.01, and *** P<0.001. mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; EGFR, epidermal growth factor receptor; VEGFD, vascular endothelial growth factor receptor; MMP, matrix metalloproteinase.

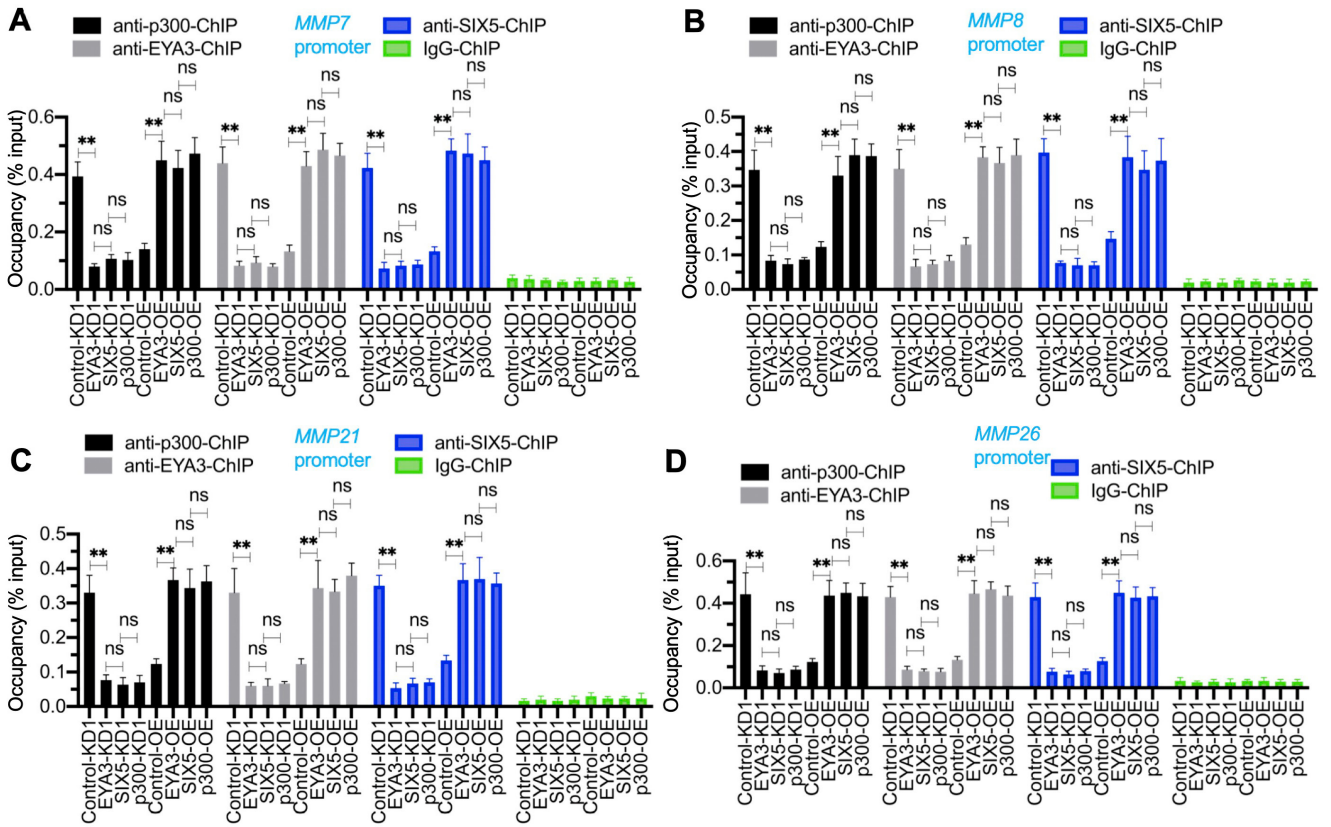

Figure S7 The EYA3-p300-SIX5 members bound to the promoters of MMP7, MMP8, MMP21, and MMP26. The same input and output DNA as used in Figure 6B were subjected to qRT-PCR analyses to measure the enrichment of EYA3-p300-SIX5 members on the promoters of *MMP7* (A), *MMP8* (B), *MMP21* (C), and *MMP26* (D). ns: no significant difference; ** P<0.01. qRT-PCR, quantitative real-time polymerase chain reaction; MMP, matrix metalloproteinase.

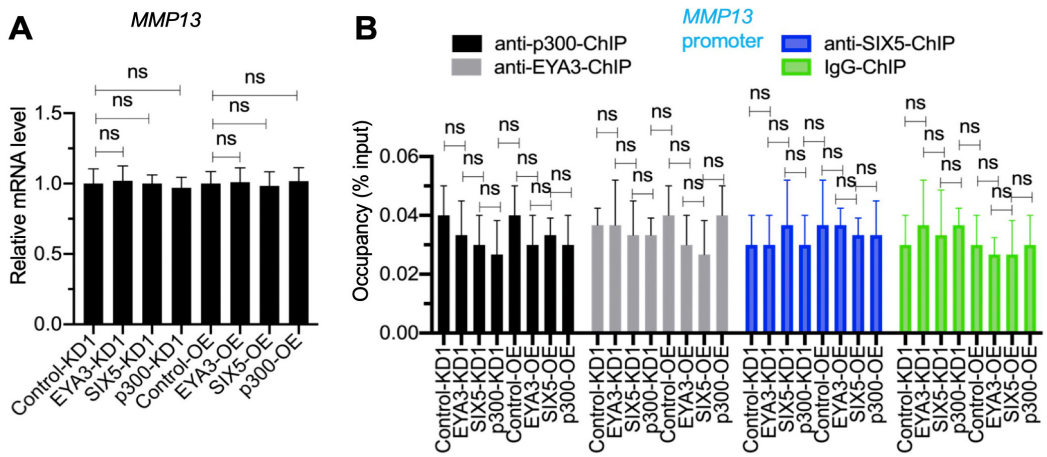

Figure S8 MMP13 was not a target of EYA3-p300-SIX5 complex. (A) *MMP13* mRNA levels in the KD and OE cell lines of EYA3-p300-SIX5 members. ns: no significant difference. (B) ChIP results. The same input and output DNA as used in *Figure 6B* were subjected to qRT-PCR analyses to measure the enrichment of EYA3-p300-SIX5 members on the promoters of *MMP13*. ns, no significant difference; MMP, matrix metalloproteinase; KD, knockdown; OE, overexpression; qRT-PCR, quantitative real-time polymerase chain reaction.

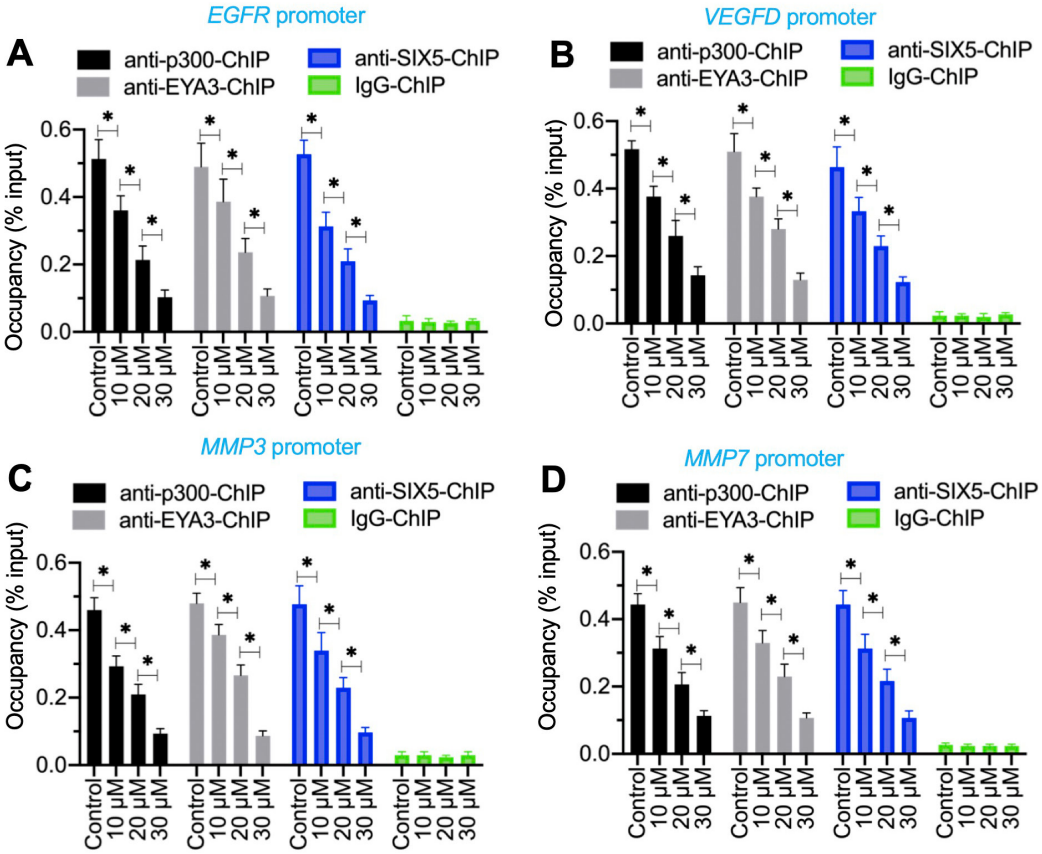

Figure S9 Benzarone attenuated the occupancies of EYA3-p300-SIX5 components on the promoters of *EGFR*, *VEGFD*, *MMP3*, *and MMP7*. HCEC-1CT cells were treated with different doses of benzarone (0, 10, 20, and 30 μM) for 12 h, followed by ChIP assays using anti-p300, anti-EYA3, anti-SIX5, and IgG (negative control). The input and output DNA were subjected to qRT-PCR analyses to measure the enrichment of EYA3-p300-SIX5 components on the promoters of *EGFR* (A), *VEGFD* (B), *MMP3* (C), and *MMP7* (D). * P<0.05. EGFR, epidermal growth factor receptor; VEGFD, vascular endothelial growth factor receptor; MMP, matrix metalloproteinase; ChIP, chromatin immunoprecipitation; qRT-PCR, quantitative real-time polymerase chain reaction.

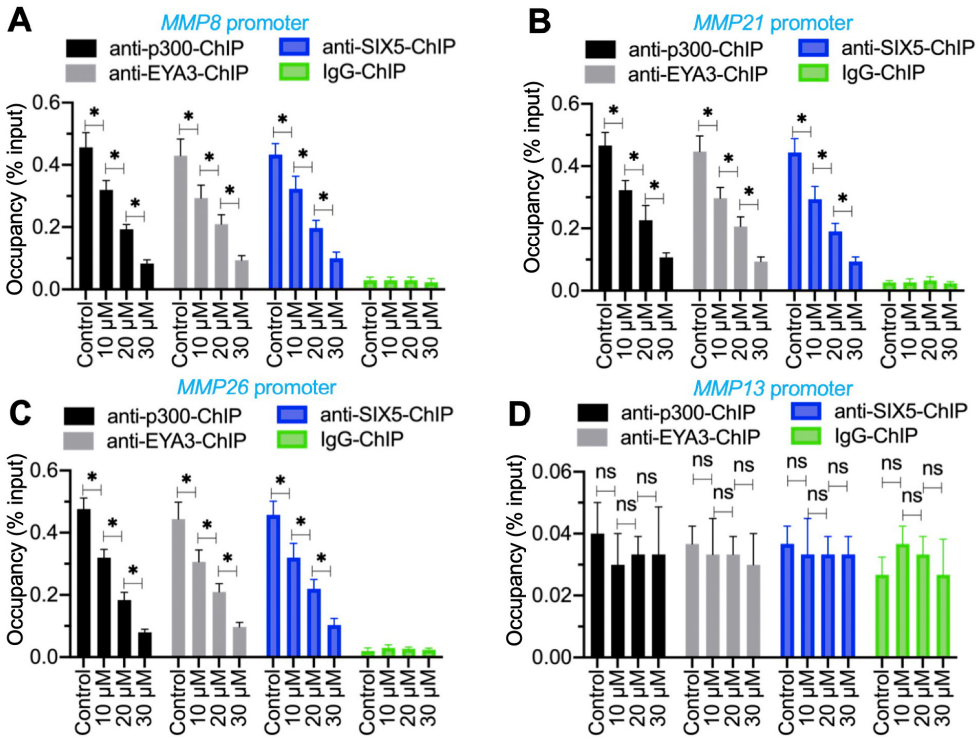

Figure S10 Benzarone attenuated the occupancies of EYA3-p300-SIX5 components on the promoters of *MMP8*, *MMP21*, *MMP26*, and *MMP13*. The same input and output DNA as used in Figure S9 were subjected to qRT-PCR analyses to measure the enrichment of EYA3-p300-SIX5 components on the promoters of *MMP8* (A), *MMP21* (B), *MMP26* (C), and *MMP13* (D). * P<0.05. ns, no significant difference; MMP, matrix metalloproteinase; qRT-PCR, quantitative real-time polymerase chain reaction.

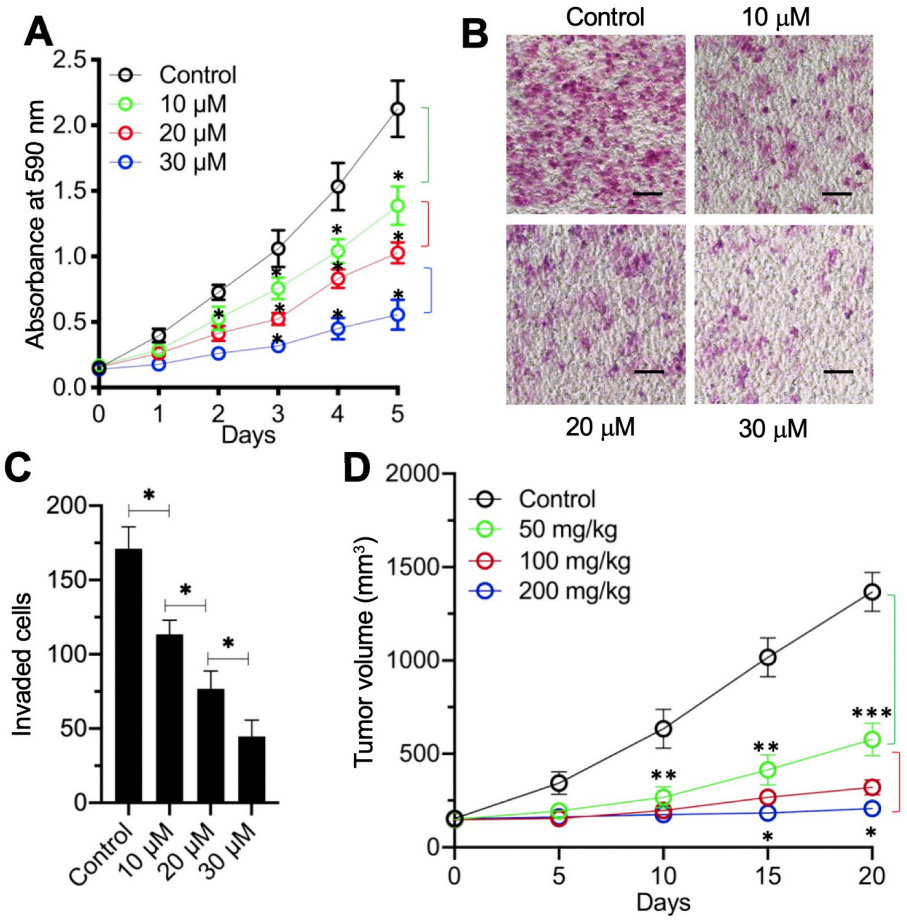

Figure S11 Benzarone inhibited cell proliferation, invasion, and tumor growth. (A) Cell viability in benzarone-treated HT-29 cells (0, 10, 20, and 30 μM) at different time points (days 0, 1, 2, 3, 4, and 5). * P<0.05. (B,C) Cell invasion results. The benzarone-treated HT-29 cells (0, 10, 20, and 30 μM) were seeded into the upper chamber of Boyden chambers. After culturing for 24 h, the invaded cells in the lower chambers were fixed in methanol and stained with 0.2% crystal violet. Cells were photographed using a microscope with the magnification of 20-fold. Bars =100 μm (B). (C) Quantified cell numbers in (B). * P<0.05. (D) Tumor volumes. HT-29 cells were injected into nude mice to generate tumors. After tumor volumes reached approximately 150 mm^3, mice were randomly grouped and administrated with PBS (Control) and different doses of benzarone (50, 100, and 200 mg/kg) at 5-day intervals (n=10 for each concentration). Tumor volumes were determined at 5-day intervals. * P<0.05, ** P<0.01, ***, P<0.001. PBS, phosphate-buffered saline.