Supplemental Figures and Legends

Figure S1. The relations of REGγ with Hippo-YAP signal pathway in colon cancer. A. The protein levels of Lats2 and p-Lats1 were unchanged in HCT116 and HT29 human colon cancer cells with REGγ knockdown. The expression of REGγ, Lats2 and p-Lats1 were measured by Western Blot. B. REGγ knockdown did not change the levels of p-YAP (S397) in HCT116 and HT29 human colon cancer cells. The expression of REGγ and p-YAP (S397) were measured by Western Blot. C. The levels of p-YAP (S397) were simialr in normal colon tissues or colon cancer tissues of *REGγ*$^{+/+}$ and *REGγ*$^{-/-}$ mice by Western Blot analysis. D. Stable knockdown of REGγ failed to change the level of Mst1 in HCT116 and HT29 human colon cancer cells. Mst1 and REGγ protein levels were detected by Western Blot. E. Western Blot showed that the levels of Lats1 and p-YAP were elevated in REGγ sh-R Hela cells but not in REGγ sh-R MEF cells compared with their sh-N control cells.

Figure S1

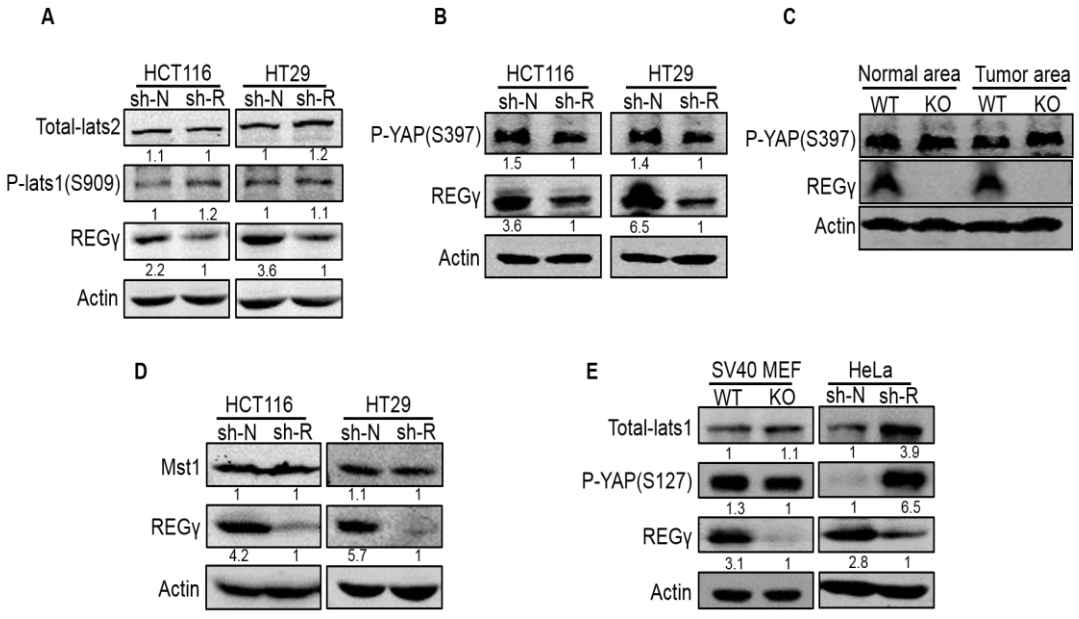


Figure S2. REGγ promotes degradation of Lats1. A. Silencing REGγ slowed down degradation of endogenous Lats1. REGγ sh-N or sh-R HCT116 cells were treated with cycloheximide (100μg/ml) for indicated time followed by Western Blotting. Quantitated results were plotted to indicate dynamic changes (Analysis of Variance, n=3, *p<0.05, **p<0.01, ***p<0.001). B. Ectopic expression of wild-type (WT), but not inactive mutant N151Y REGγ promoted the degradation of endogenous Lats1 in HEK293 cells. REGγ or a mutant form of REGγ was induced in HEK293 cells treated with 1μg/ml of doxycycline for 48 and followed by Western Blot analysis. C&D. REGγ knockdown did not changed the stability of p-YAP (S397) in both HCT116 (C) and HT29 (D) cells. Cells were treated with cycloheximide (100μg/ml) for indicated time followed by Western Blotting. All experiments were repeated three times.

Figure S2

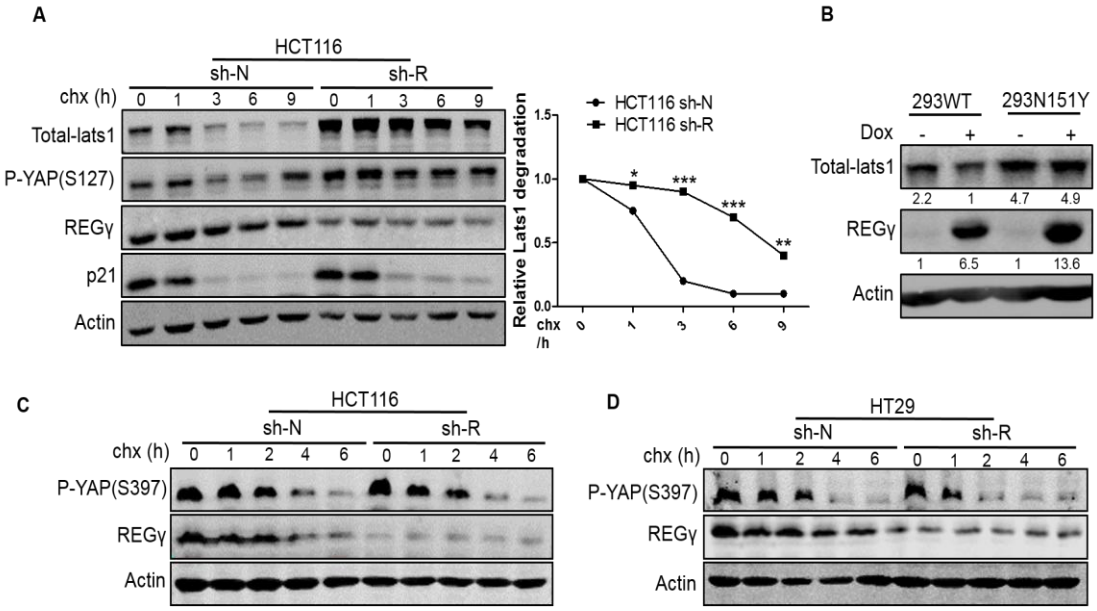
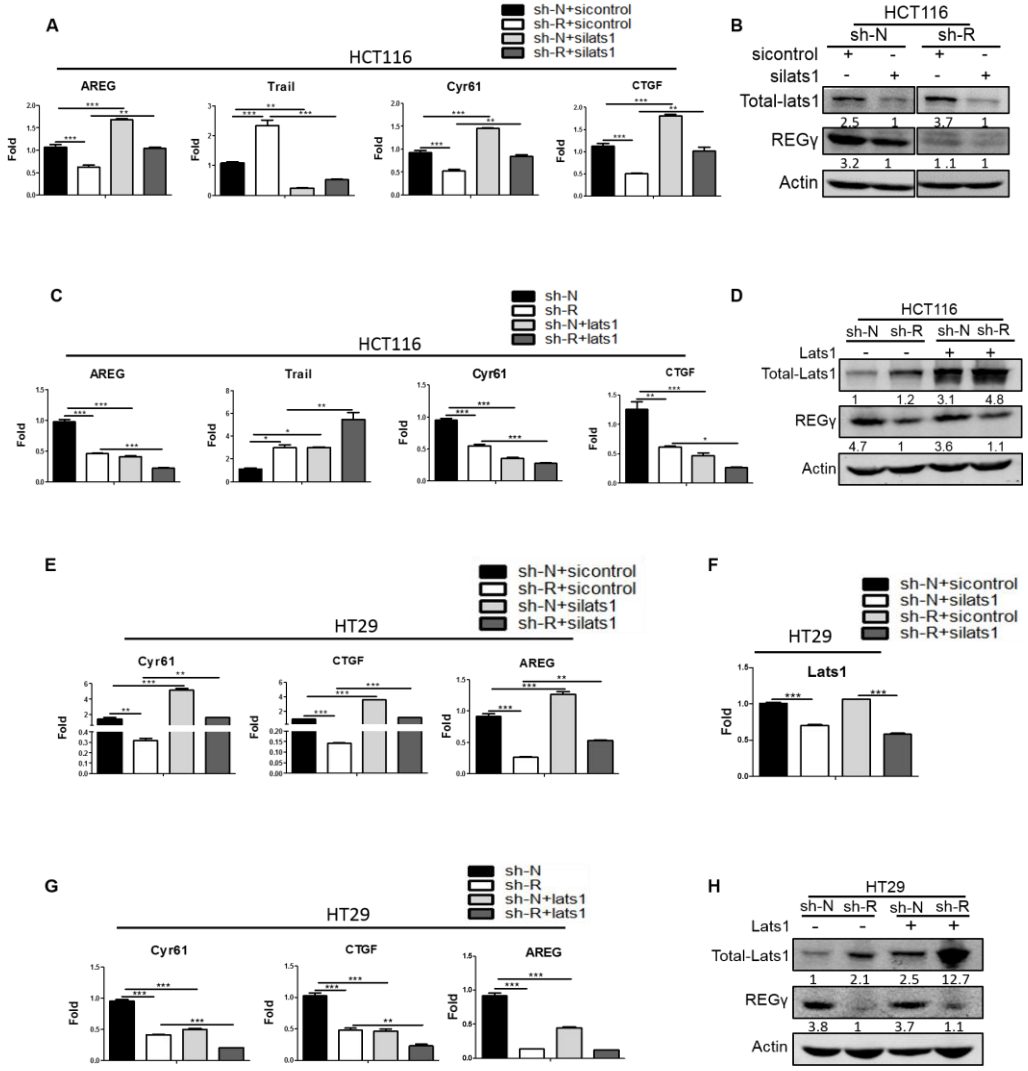


Figure S3. REGγ promotes YAP signaling via degradation of Lats1. A Silencing Lats1 restored the expressions of YAP target genes in HCT116 sh-R cells similar to those in HCT116 cells by RT-PCR analysis. Data are presented as the means ± SEM (Analysis of Variance, n=3, **p<0.01, ***p<0.001). B. RNAi efficiently depleted Lats1 in HCT116 cells for experiments in A. HCT116 cells were harvested 72h after transient transfection of a control (Ctrl) or Lats1 small interfering RNA (siRNA) followed by Western blot analysis. C. Overexpression of Lats1 in HCT116 sh-N cells changed the expression of YAP target genes similar to the levels in HCT116 sh-R cells by RT-PCR analysis. Data are presented as the means ± SEM (Analysis of Variance, n=3, *p<0.05, **p<0.01, ***p<0.001). D. Western blot analysis validated successful expression of exogenous Lats1 in experiments shown in C. E. The decrease of *AREG, CTGF, Cyr61* mRNA expression induced by REGγ knockdown was reversed by Si-lats1 in HT29 cells. Data are presented as the means ± SEM (Analysis of Variance, n=3, **p<0.01, ***p<0.001). F. Transient siRNA expression resulted in marked silencing of Lats1 expression in HT29 cells by real-time PCR analysis (Analysis of Variance, n=3, ***p<0.001). G. Transfection of Lats1 in HT29 sh-N cells effectively decreased AREG *CTGF, Cyr61* mRNA expression of YAP downstream (Analysis of Variance, n=3, **p<0.01, ***p<0.001). H. Western Blot analysis demonstrating obvious overexpression of Lats1 in HT29 cells after transient transfection with the pCDNA3.1-lats1- vector. All experiments were repeated three times.

Figure S3


A
sh-N+sicontrol
sh-R+sicontrol
sh-N+silats1
sh-R+silats1
HCT116
AREG
Trail
Cyr61
CTGF
Fold
B
HCT116
sh-N
sh-R
sicontrol
silats1
Total-lats1
REGγ
Actin
2.5
1
3.7
1
3.2
1
1 .1
1
C
sh-N
sh-R
sh-N+lats1
sh-R+lats1
HCT116
AREG
Trail
Cyr61
CTGF
Fold
D
HCT116
sh-N
sh-R
sh-N
sh-R
Lats1
Total-Lats1
REGγ
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1
1.2
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3.6
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E
sh-N+sicontrol
sh-R+sicontrol
sh-N+silats1
sh-R+silats1
HT29
Cyr61
CTGF
AREG
Fold
F
sh-N+sicontrol
sh-N+silats1
sh-R+sicontrol
sh-R+silats1
HT29
Lats1
Fold
G
sh-N
sh-R
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sh-R+lats1
HT29
Cyr61
CTGF
AREG
Fold
H
HT29
sh-N
sh-R
sh-N
sh-R
Lats1
Total-Lats1
REGγ
Actin
1
2.1
2.5
12.7
3.8
1
3.7
1.1

Figure S4. REGγ promotes proliferation of human colon cancer cells. A. REGγ deletion and YAP silencing inhibited cell proliferation of HCT116 human colon cancer cells to a similar extent. After 72 h of transfection with a control- siRNA or *YAP*-siRNA, cell viability was measured on days 1, 2, 3, 4 and 5 by using MTT assays. Western Blot analysis showing marked silencing of YAP expression in HCT116 cells after 72h transfection with control (Ctrl) or YAP small interfering RNA (siRNA) oligos. B. Silencing REGγ or YAP alone or in combination inhibited cell proliferation of HT29 human colon cancer cells. Cell viability was measured on days 1, 2, 3, 4 and 5 by MTT assays following RNAi for 72h. Western blot analysis demonstrated the knockdown efficiency. C. Lats1 silencing increased cell viability of HCT116 and HT29 human colon cancer cells. After 72 h of transfection with a control- siRNA or *Lats1*-siRNA, cell viability was measured on days 1, 2, 3, 4 and 5 by using MTT assays. D. The mRNA expression of PCNA was decreased in the tumor of REGγ knockout mice. PCNA mRNA levels were quantified using real-time PCR (Analysis of Variance, n=3, **p<0.01). E. The protein expression of PCNA was also decreased in the tumor of REGγ knockout mice. Expression of PCNA and REGγ in the tumors from REGγ+/+ and REGγ-/- mice was detected by Western Blot. F. IHC analysis showed weak positive staining of PCNA in the tumors of REGγ-/- mice compared with the REGγ+/+ mice. Scale bar, 50μm (magnification, ×20). All results are representatives of three repeats.

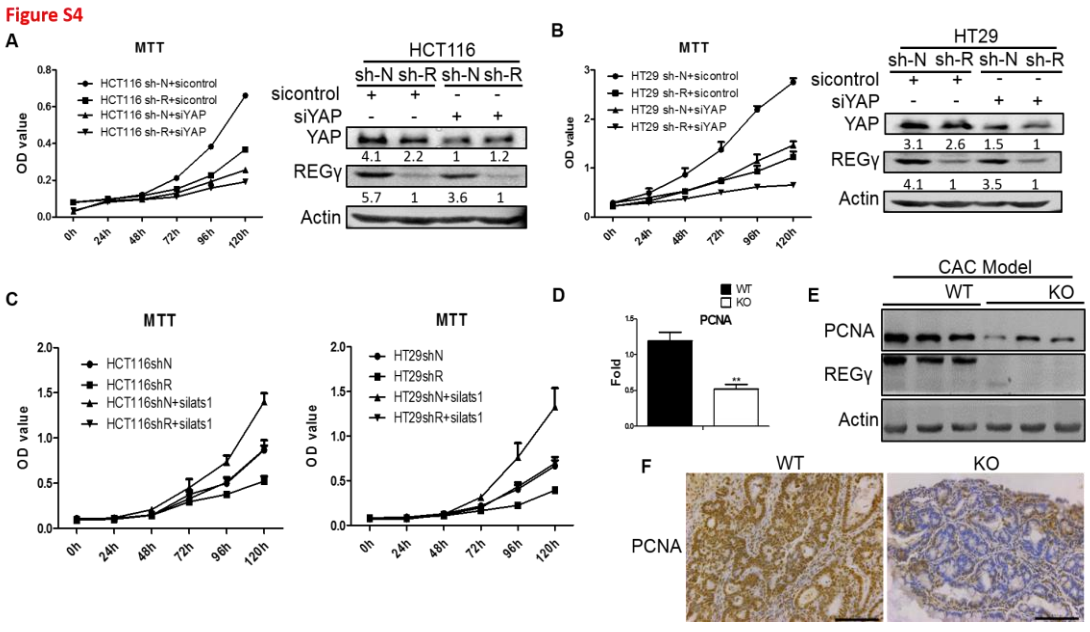


Figure S5. Constitutive YAP fully reversed the retardation of tumor growth induced by REGγ depletion. A. Xenograft tumors were generated by injecting HCT116 sh-N, HCT116 sh-N+YAP (S127A), HCT116 sh-R and HCT116 sh-R+YAP (S127A) cells into dorsal flanking sites of nude mice. B Tumors were dissected and volumes were measured. Values were presented as the means ± SEM (two-tailed Analysis of Variance, n=3, *p < 0.05, **p < 0.01). C. The xenografts were harvested 30 days post-injection and analyzed by Western blotting with antibodies against YAP or REGγ. Data in this figure are representatives of three independent repeats.

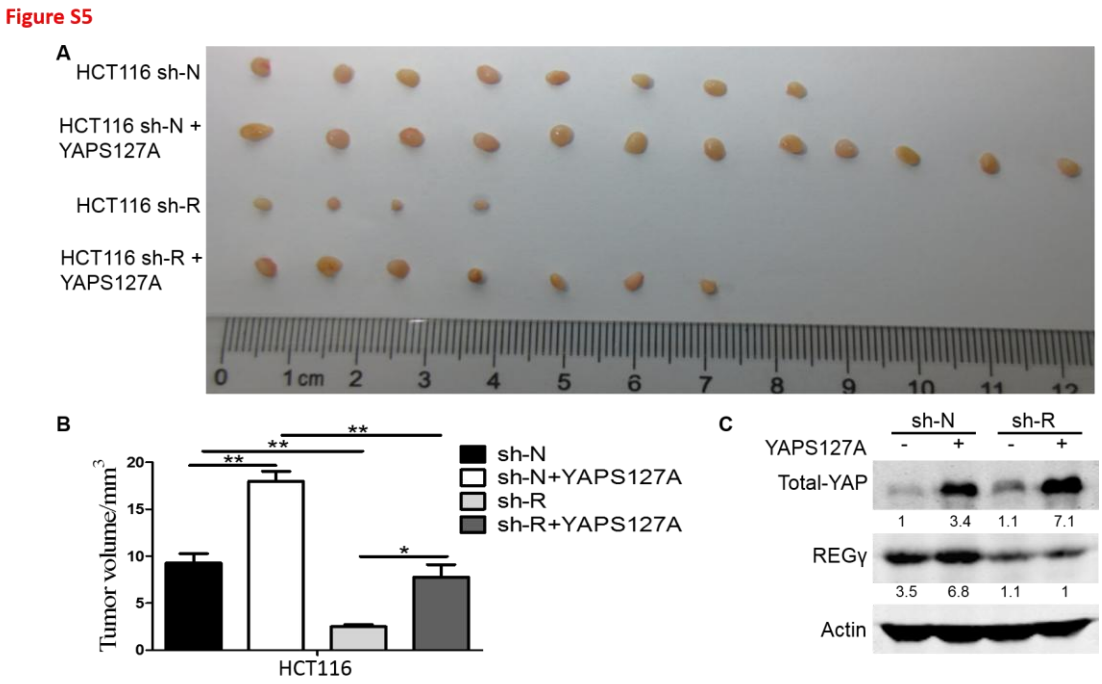


Figure S6. P65 strengths YAP transcription and its signal pathway

A. Quantitative PCR analysis of YAP expression in HT29 cells treated with TNFα (20 ng/ml) or IL-6 (20 ng/ml). Data are presented as the means ± SEM (Analysis of Variance, n=3, ***p<0.001). B. HCT116 cells were treated with or without TNFα (20 ng/ml) for 3 hours and processed for ChIP assay using anti-YAP antibodies. Immunoprecipitated chromatin was analyzed by RT-PCR using the specific primers for *Cyr61* promoter. C. and D. TNFα or IL-6 treatment enhanced the expression of YAP downstream positive regulatory genes (*Cyr61, AREG*) and decreased the expression of negative regulatory gene (*Trail, DDT4*) in HCT116 and HT29 cells. Cells were treated were treated with TNFα (20 ng/ml) or IL-6 (20 ng/ml) with or without Verteporfin (VP) for 3 hours and were analyzed by real-time PCR. Data are presented as the means ± SEM (Analysis of Variance, n=3, *p<0.05, **p<0.01, ***p<0.001). E-F. DSS treatments increased the expression of NF-κB downstream genes and YAP target gene (*Cyr61*) in the mouse colon tissues. *REGγ*$^{+/+}$ and *REGγ*$^{-/-}$ mice were treated with 2% DSS for five days and the mRNA levels of *IL-1β, IL-6* and *Cyr61*in the mouse colon tissues were quantified using real-time PCR analysis (Analysis of Variance, *p<0.05, ***p<0.001). All experiments were repeated three times.

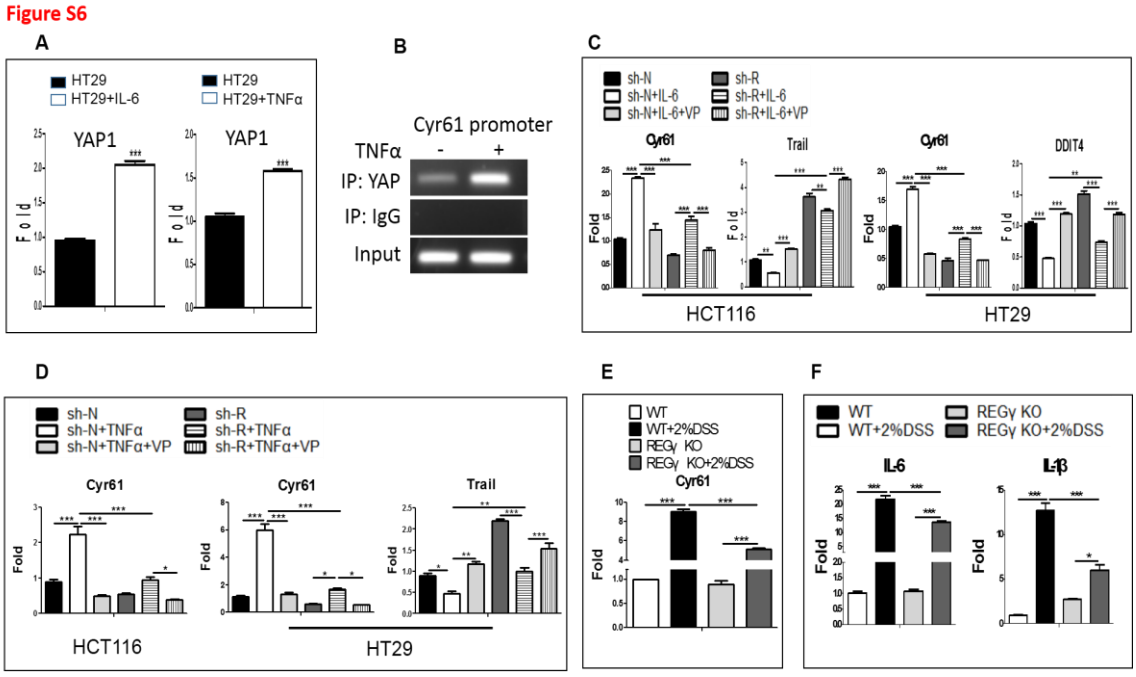


Figure S7. YAP enhances p65 transcription and its signal pathway

A. Quantitative PCR analysis of p65 expression in HT29 cells treated with/without Verteporfin (VP) or transfected with S127A mutant YAP (S127A-YAP) compared with the empty vector. Data are presented as the means ± SEM (Analysis of Variance, n=3, **p<0.01). B. YAP activation increased the transcriptional activity of NF-κB in HT29 human colon cancer cells. NF-κB luciferase reporter activities were measured in sh-N, sh-N+YAP (S127A), sh-R and sh-R+YAP (S127A) cells. Data represent the means ± SEM (Analysis of Variance, n=3, ***P<0.001). C. and D. The high expression of IL-6 in HCT116 and HT29 cells induced by TNF- α (C) or IL-6 (D) was attenuated by YAP inhibitor. HCT116 and HT29 cells were treated with with TNFα (20 ng/ml) or IL-6 (20 ng/ml) with or without Verteporfin (VP) for 3 hours and were analyzed by real-time PCR (Analysis of Variance, n=3, *p<0.05, **p<0.01, ***p<0.001). E. and F. YAP silencing also blocked the increase of IL-6 expression in HCT116 and HT29 cells treated with TNFα or IL-6. At 72 hours after transfection, cells were treated with or without TNFα or IL-6 for 3 hours. The mRNA levels of IL-6 were quantified using real-time PCR analysis (Analysis of Variance, n=3, *p<0.05, **p<0.01, ***p<0.001). Data in this figure are representatives of three repeats.

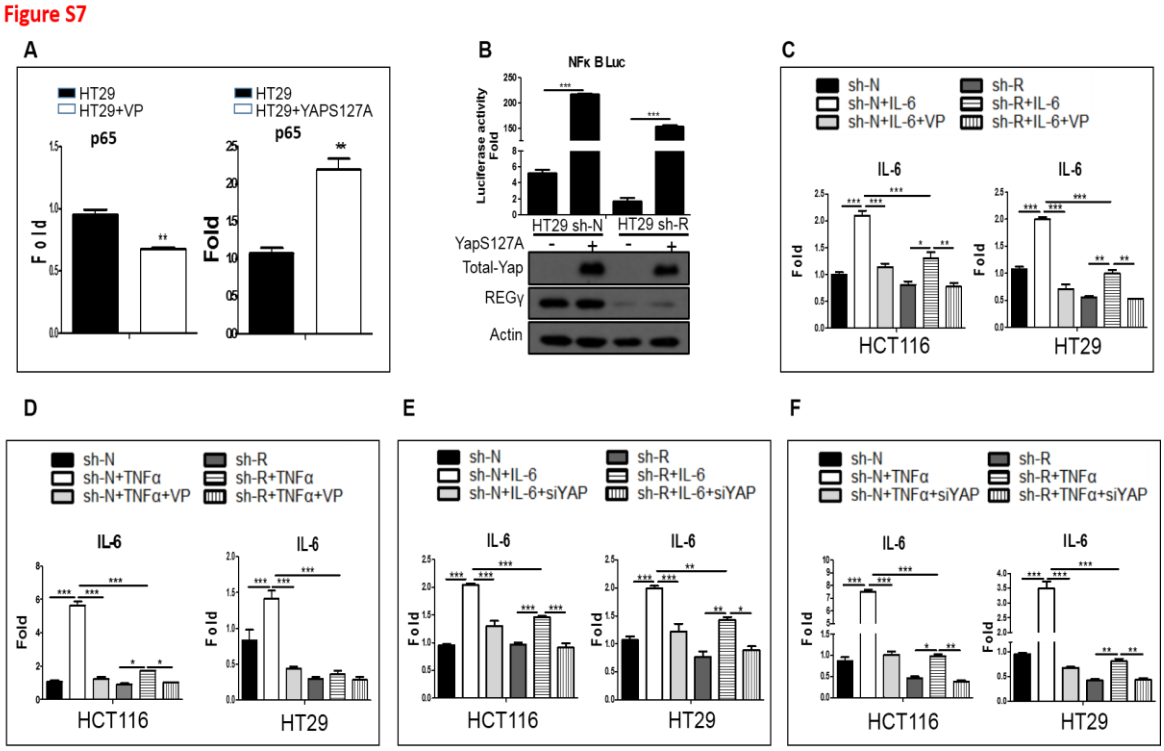
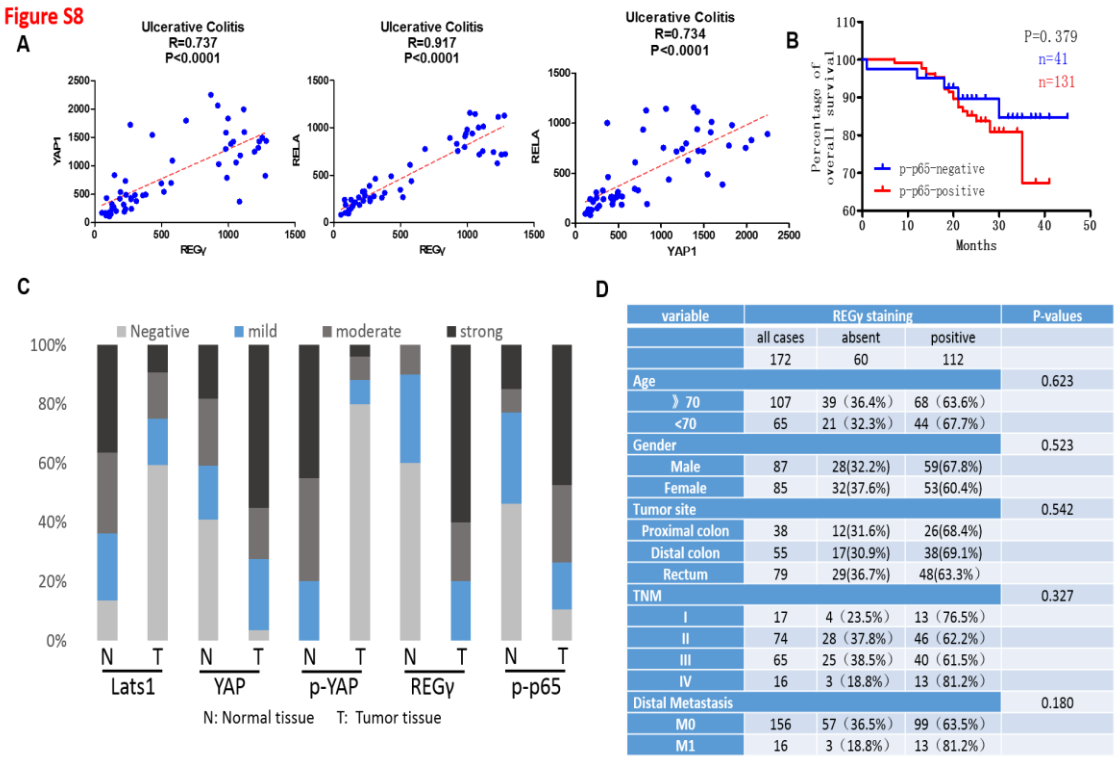


Figure S8. Clinical implication of REGγ in human colon cancer.

A. A significant correlation among REGγ, YAP1 and RELA mRNA expression in 53 patients with ulcerative colitis. Pearson correlation coefficient was 0.737, 0.917 and 0.734, respectively. P<0.0001. B. The correlation of survival rate with p-p65 overexpression in 172 CRC patients. p=0.379. C. Diagrams summarizing the percentage of positive staining for each marker examined. Results were evaluated in a double-blinded fashion. D. High expression of REGγ was not related with patient age, patient gender, tumor size, tumor grade and tumor metastasis (p>0.05).



D

variable	REGγ staining			P-values
	all cases	absent	positive	
	172	60	112	
Age				0.623
》70	107	39（36.4%）	68（63.6%）	
<70	65	21（32.3%）	44（67.7%）	
Gender				0.523
Male	87	28(32.2%)	59(67.8%)	
Female	85	32(37.6%)	53(60.4%)	
Tumor site				0.542
Proximal colon	38	12(31.6%)	26(68.4%)	
Distal colon	55	17(30.9%)	38(69.1%)	
Rectum	79	29(36.7%)	48(63.3%)	
TNM				0.327
I	17	4（23.5%）	13（76.5%）	
II	74	28（37.8%）	46（62.2%）	
III	65	25（38.5%）	40（61.5%）	
IV	16	3（18.8%）	13（81.2%）	
Distal Metastasis				0.180
M0	156	57（36.5%）	99（63.5%）	
M1	16	3（18.8%）	13（81.2%）	

Figure S9. A Model of crosstalk among REGγ, Hippo-Yap, and NF-κB. In human colon cancer cells, overexpression of REGγ promotes the degradation of Lats1, thus activating YAP signaling. YAP activation empowers a reciprocal positive regulation with NF-κB, leading to aberrant cell proliferation and development of inflammation-associated colon cancer. In contrast, cells with REGγ depletion are resistant to Lats1 inactivation, inhibiting YAP activation and its crosstalk to NF-κB to prevent tumor formation.

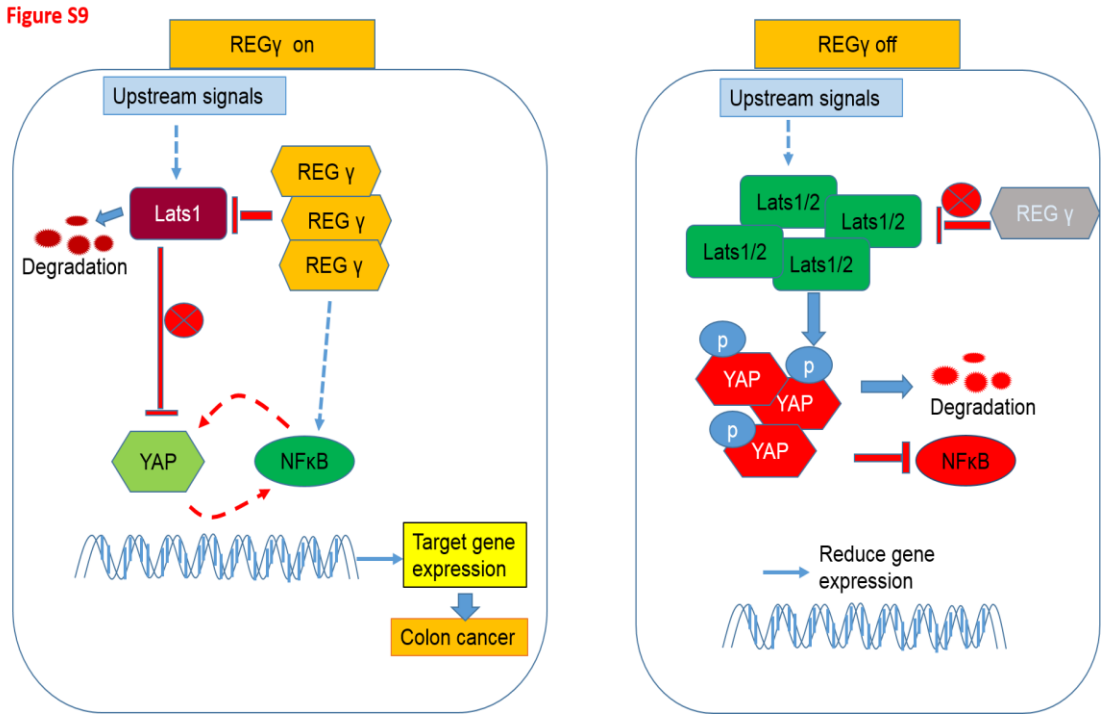
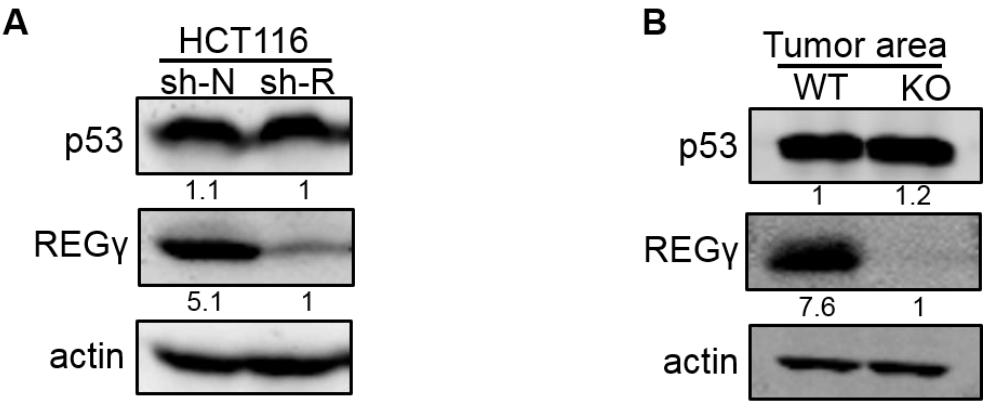


Figure S10. P53 was not affected by REGγ deficiency in human colon cancer cells. A. Western Blot analysis for REGγ and p53 in HCT116 sh-N and sh-R human colon cancer cells. β-actin served as a loading control. B. Western Blot analysis of p53 from mouse normal colon and tumor tissues of REGγ$^{+/+}$ and REGγ$^{-/-}$ mice showed no marked changes. All experiments were repeated three times.

Figure S10



Supplemental Tables

Table 1. Sequences of primers used for Q-PCR

Gene	Forward (5'→3')	Reverse (5'→3')
AREG(homo)	GAGCCGACTATGACTACTCAGA	TCACTTTCCGTCTTGTTTTGGG
CTGF(homo)	AAAAGTGCATCCGTACTCCCA	CCGTCGGTACATACTCCACAG
Cyr61(homo)	CTCGCCTTAGTCGTCACCC	CGCCGAAGTTGCATTCCAG
Trail(homo)	TGCGTGCTGATCGTGATCTTC	GCTCGTTGGTAAAGTACACGTA
DDIT4(homo)	TGGGCAAAGAACTACTGCG	AGAGTTGGCGGAGCTAAACAG
AREG(mus)	GGTCTTAGGCTCAGGCCATTA	CGCTTATGGTGGAAACCTCTC
CTGF(mus)	GGGCCTCTTCTGCGATTTC	ATCCAGGCAAGTGCATTGGTA
Cyr61(mus)	CTGCGCTAAACAACTCAACGA	GCAGATCCCTTTCAGAGCGG
Trail(mus)	ATGGTGATTTGCATAGTGCTCC	GCAAGCAGGGTCTGTTCAAGA
DDIT4(mus)	CAAGGCAAGAGCTGCCATAG	CCGGTACTTAGCGTCAGGG
Lats1(homo)	AATTTGGGACGCATCATAAAGCC	TCGTCGAGGATCTTGGTAACTC
Lats1(mus)	AAAGCCAGAAGGGTACAGACA	CCTCAGGGATTCTCGGATCTC
yap1(homo)	TAGCCCTGCGTAGCCAGTTA	TCATGCTTAGTCCACTGTCTGT
yap1(mus)	ACCCTCGTTTTGCCATGAAC	TGTGCTGGGATTGATATTCCGTA
PCNA(mus)	TTTGAGGCACGCCTGATCC	GGAGACGTGAGACGAGTCCAT
IL-6(homo)	AATTCGGTACATCCTCGACGG	TTGGAAGGTTCAGGTTGTTTTCT
IL-8(homo)	GCATAAAGACATACTCCAAACC	AAAACTTCTCCACAACCCTC
IL-6(mus)	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
IL-1β(mus)	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG

Table 2. Sequences of primers used for ChIP

Gene	Forward (5'→3')	Reverse (5'→3')
IL-8(homo)	GGGCCATCAGTTGCAAATC	TTCCTTCCGGTGGTTTCTTC
p65(homo)	TTCGGTGGTGGCTGTCTC	GGCAGTTTGGCTGTATCGT
YAP1(homo)	TAACTTTAGTTTTGGCGTTTGAG	CTCTTTATTTCCTTTTCTTGTTCCT
Cyr61(homo)	CTCGCCTTAGTCGTCACCCT	AAACGGCTACCCGCAAAC