REGγ controls Hippo signaling and reciprocal NF-κB–YAP regulation to promote colon cancer

Qingwei Wang†*, Xiao Gao†*, Tong Yu², Lei Yuan¹, Jie Dai¹, Weicang Wang¹, Geng Chen¹, Chan Jiao¹, Wang Zhou³, Quan Huang³, Long Cui², Pei Zhang⁴, Robb E. Moses⁵, Jianhua Yang⁶, Fengyuan Chen⁷, Junjiang Fu⁸*, Jianru Xiao³, Lei Li¹‡, Yongyan Dang¹‡, Xiaotao Li¹.⁵‡

*Co-first authors; ‡Co-corresponding authors.

¹Shanghai Key Laboratory of Regulatory Biology, Institute of Biomedical Sciences, School of Life Sciences, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China.
²Xinhua Hospital Affiliated to Shanghai Jiaotong University, 1665 Kongjiang Road, 200092, Shanghai, China
³Department of Orthopedic Oncology, Changzheng Hospital, The Second Military Medical University, 415 Fengyang Road, Shanghai 200003, China.
⁴Department of Pathology, the Second Chengdu Municipal Hospital, Chengdu, 610017, China
⁵Department of Molecular and Cellular Biology, Dan L. Duncan Cancer Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
⁶Texas Children's Cancer Center, Department of Pediatrics, Dan L. Duncan Cancer Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030, USA
⁷The fifth hospital of Shanghai, Fudan University, Shanghai, 200240, China
⁸Key Laboratory of Epigenetics and Oncology, the Research Center for Preclinical Medicine, Southwest Medical University, Sichuan 646000, China

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Correspondence to:
Xiaotao Li
Department of Molecular and Cellular Biology
Baylor College of Medicine
One Baylor Plaza, Houston, TX, USA 77030
Tel: 713-7983817
Fax: 713-7901275
Email: fujunjiang@hotmail.com, lllkj@163.com, yydang@bio.ecnu.edu.cn,
xiaotaol@bcm.edu

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Statement of translational relevance

REGγ is an ATP- and ubiquitination-independent proteasome activator that has been valued in recent years. With bioinformatics and experimental analysis, REGγ and Hippo pathways are found closely related in human colon cancer tissues. Molecular mechanism studies have shown that REGγ can directly degrade Lats1 (large tumor suppressor kinase 1), thus affecting YAP (Yes-associated protein) function, and then control Hippo pathway on the occurrence and development of mouse colon cancer. Importantly, REGγ is defined as a crucial factor in the regulation of Hippo pathway and NF-κB pathway in human colon cancer cells and mouse tumors. Clinical and experimental study of colorectal cancers indicate that high expression of REGγ is associated with poor prognosis. These results indicate REGγ is a new marker for the prognosis of CRC patients.
Abstract

Background and aims: Colorectal cancer (CRC) is one of the most commonly diagnosed cancers closely associated with inflammation and hyperactive growth. We have previously demonstrated a regulatory circuit between the proteasome activator REGγ and NF-kappaB (NF-κB) during colon inflammation, known to be important in the development of colitis-associated cancer as well as sporadic CRC. How the inflammatory microenvironment affects the Hippo pathway during CRC development is largely unknown.

Methods: Here, we used REGγ deficient colon cancer cell lines, REGγ knockout mice and human CRC samples to identify the novel molecular mechanism by which REGγ functions as an oncoprotein in the development of colorectal cancer.

Results: REGγ can directly interact with Lats1 and promote its degradation, which facilitates Yes-associated protein (YAP) activation in colon cancer cells. REGγ deficiency significantly attenuated colon cancer growth, associated with decreased YAP activity. Suppression of tumor growth due to REGγ depletion was overcome by constitutively active YAP. Surprisingly, reciprocal activation of YAP and NF-κB pathways was observed in human colon cancer cells. REGγ Overexpression was found in over 60% of 172 CRC specimens, highly correlating with the elevation of YAP and p65. Post-operative follow-up revealed a significantly lower survival rate in patients with concomitantly high expression of REGγ, YAP and p-p65.

Conclusion: REGγ could be a master regulator during CRC development to promote YAP signaling and reinforce cross-talks between inflammation and growth pathways, and that REGγ could be a new marker for prognosis of CRC patients.
**Introduction**

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers in both men and women. Multiple signaling pathways and predisposing factors are involved in the development of CRC, including colonic inflammation and YAP activation (1,2). Patients with severe ulcerative colitis with hyperactive NF-κB have an increased risk of developing colorectal cancer (3). Aberrant regulation of NF-κB along with other signals are involved in colitis-associated carcinogenesis (4,5). How inflammatory signals interact with the YAP pathway to promote CRC remains poorly understood. Our previous finding that the proteasome activator REGγ promotes experimental colitis encouraged us to explore REGγ functions further.

REGγ (also known as PA28γ), a 11s proteasome activator, promotes the degradation of multiple target proteins in an ubiquitin-independent manner in many cellular processes (6-10), including tumorigenesis and angiogenesis (11-13). In our recent study, REGγ was demonstrated to promote bowel inflammation by degrading IκBε and activating the NF-κB signal pathway (14). The strong correlation between YAP signaling and REGγ led us to investigate whether REGγ could be a driver for CRC development.

The Hippo signal pathway was first discovered in Drosophila fruit fly by mosaic genetic screens. The highly conserved mammalian Ste20-like kinases (Mst)1/2 and the effector, large tumor suppressor kinase (Lats)1/2, represent a core component of the Hippo pathway with the transcription coactivator, Yes-associated protein (YAP) as a major downstream effector of the Hippo pathway. The Hippo-YAP signal pathway plays pivotal roles in tissue homeostasis, organ size, regeneration and tumorigenesis (15,16). Inactivation of YAP severely impairs dextran sulfate sodium (DSS)-induced intestinal regeneration, while hyperactivation of YAP results in polyp formation following DSS treatment (17). Deletion of Mst1/2 genes in the intestinal epithelium induces YAP hyperactivation, a phenomenon frequently found in human colonic cancer specimens (18). Despite this evidence of YAP as an oncogene (19), inactivation of YAP causes no obvious intestinal and colon defects under normal homeostasis, except...
alterations in colonic inflammation (17). YAP phosphorylation leads to its ubiquitination-dependent degradation (20).

In this study, we found that REGγ could activate YAP by degrading Lats1 to promote cell proliferation and colitis-associated CRC in mice. Stable silencing of REGγ in colon cancer cells retarded tumor growth, which was effectively reversed by activation of YAP. Overexpression of REGγ was found in human colon cancer samples, correlating with a poor prognosis for colon cancer patients. Prominently, reciprocal activation of YAP and NF-κB pathways was found in human colon cancer cells with prominent level of REGγ. Thus, REGγ plays a key role in colon cancer development via suppression of Lats1 to promote the action of YAP on NF-κB activity.

**Materials and Method**

**Mice**

REGγ knockout mice were kindly provided by Dr. John J. Monaco at the University of Cincinnati and backcrossed for more than 10 generations in our specific pathogen free animal center (21). 8-10 weeks old C57BL/6 male mice were used. Animals were maintained according to the ethical and scientific standards by the Animal Center at East China Normal University.

**Cell Culture**

HCT116, HT29, HEK293T, MEF SV40 and HeLa and were purchased from American Type Culture Collection (ATCC, USA) or acquired from the Cell Culture Core at Baylor College of Medicine. The Core has regular examination of cell contamination or authentication. All the cells were maintained for no more than 50 passages before we thaw up a new stock or acquire a low passage batch. All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 100 ug/ml penicillin/streptomycin. HCT116 sh-N, HCT116 sh-R, HT29 sh-N, HT29 sh-R cells were generated by retroviral shRNA vectors specific for REGγ or a control vector from OriGene (Rockville, MD). The 293- REGγ inducible cell line and MEF SV40 WT, MEF SV40 REGγ KO, HeLa sh-N, HeLa sh-R cells were previously generated.
Plasmids, constructs and expression
pCDNA3.1-Lats1, pCDNA3.1-YAP, pCDNA3.1-YAP(S127A), pCDNA3.1-HA/Flag-
REGγ and PCDH-YAPS127A were constructed in our lab. Lats1 siRNA
(GGTTCCTGAGAGTAAATTATT) and YAP siRNA (GACAUCUUCUGGUCCAGAG
ATT) were synthesized by GenePharma. Plasmids or siRNA were transfected
to different cells and cultured for 36h or 72h by using transfection reagents (Cenji,
Shanghai, China).

Antibodies and Drugs
β-actin (Sigma), anti-REGγ (Invitrogen), anti-Lats1, anti-p-Lats1(S909), anti-Lats2,
anti-YAP, anti-p-YAP (S127), anti-p-YAP (S397), anti-Mst1 (Cell Signaling
Technology), anti-p53 (Santa Cruz), anti-p21 (BD Pharmingen), anti-Flag-mouse
(MBL), anti-HA-mouse (Abmart), anti-p65 (Santa Cruz), anti-p-p65(S536) (Abcam).
For drugs: Verteporfin (Sigma), Human TNFα and Human IL-6 (Novoprotein).

Western blotting
Cell were lysed in cold RIPA buffer (50 mM Tris-HCl pH 7.4, 1mM EDTA, 150mM
NaCl, 1% NonidetP-40, 0.25% sodium deoxycholate, 0.1% SDS and protease inhibitors)
and run on SDS-PAGE gels. After transferring to nitrocellulose membrane,
immunoblots were analysed using the primary antibodies at 4°C overnight. They were
then incubated 1~2 hours with Fluorescent secondary antibodies (Jackson
ImmunoResearch) and detected by using Odyssey CLx (LI-COR).

Immunoprecipitation
Cells were harvested in lysis buffer (50 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM
NaCl, 1% TritonX-100, 1 mM Na3VO4, 5 mM NaF) and centrifuged for 15 mins at
10,000g to discard the insoluble debris. Cell lysate was immunoprecipitated with anti-
Flag-M2 agarose or anti-HA agarose beads for 4-8 hrs at 4°C. Immunoprecipitates were
washed three times with wash buffer (50 mM Tris-HCl pH7.5, 5 mM EDTA, 150 mM
NaCl, 1 mM Na3VO4, 5 mM NaF) followed by Western Blot analysis.

ChIP Assay
Cells were treated with TNF α or/and VP (verteporfin) beforehand. The detailed steps
can refer to this paper (22). The primer sequences are described in Supplementary Table
In vitro protein degradation assay

Recombinant REGγ protein was purified in our laboratory as described previously (13). The substrate proteins were generated by in vitro translation. The protein decay experiment was conducted by incubating substrate, 20S proteasome (Boston Biochem) and purified REGγ for 1–3 h in 25 μl reaction volume at 30°C with appropriate controls. The results were analyzed by Western blotting.

Immunofluorescence staining

Cells were seeded on coverslips in 24-well plates were washed in cold PBS three times, fixed in 4% formaldehyde, and immunostained by monoclonal anti-YAP antibodies, as well as DNA staining with 4, 6-diamidino-2-phenylindole (DAPI). The Alexa Fluor 546 (red) goat anti-rabbit antibody (Molecular Probes, OR) was used for YAP. Immunofluorescence was visualized by confocal microscopy (Leica TCS SP5).

Immunohistochemistry and tissue array analysis

Human samples were provided by Xinhua Hospital Affiliated to Shanghai Jiaotong University. Patient organization and case access are in line with the ethical requirements of University Committee on Human Research Protection in East China Normal University.

Tissues were embedded in paraffin and then cut into different sections (4–5 μm thick). Immunohistochemical staining was scored according to the following standards: staining intensity (I) was classified as 0 (lack of staining), 1 (mild staining), 2 (moderate staining) or 3 (strong staining); staining percentage (P) was designated as 1 (<25%), 2 (25-50%), 3 (51-75%) or 4 (>75%). For each section, the semi-quantitative score was calculated by multiplying I and P (which ranged from 0 to 12). Score 0-3 was as not significant (negative), 4-8 as weak positive and 9-12 as strong positive. In the analysis, low expression meant negative or weak positive, high expression meant strong positive.

The log-rank test was performed to assess statistical significance.

Reverse Transcriptase–PCR

Total RNA was extracted from cells or pulverized colons (liquid nitrogen treatment) using TRIZOL (TakaRa). 2μg of total RNA was reverse-transcribed in a total volume
of 20μL. Aliquots of the RT products were used for quantitative RT–PCR analysis with Mx3005P (Stratagene). Each experiment was repeated three times. Primer sequences are described in Supplementary Table 1.

**Luciferase assays**

The cells were washed with cold PBS three times 24h after transfection, harvested in the lysis buffer provided with the Luiferase Assay Kit (Promega). After one cycle of freezing and thawing, the cell lysates were centrifuged in 4°C at 12,000g for 10min. Then 20μl of supernatant was added to equal amount of luciferase assay substrate, with two repeats. Luminescence was measured as relative light units using the LUMIstar OPTIMA (BMG Labtech) illuminometer. Each individual experiment was repeated for three times.

**MTT assay**

Logarithmic phase cells were seeded in 96-well plates at 2.5×10³ cells per well and incubated for 24h. Cells were incubated with 0.5mg/ml MTT for 4h and add DMSO for 15min. Absorbance (490nm) was measured and analyzed as described (13).

**Bioinformatics analysis**

21 samples (Healthy control (n=11) and Ulcerative colitis (n=10)) were chosen from the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) to detect the correlation of mRNA expression between REGγ and Hippo pathway, the accession number is GSE10616. Another 53 UC data and 68 CRC data were used to investigate the correlation of REGγ, p-65 and YAP. Raw data were downloaded from GSE3629.

**Xenograft Tumorigenicity Analysis**

HCT116 sh-N&sh-R stable cells overexpressing YAP (S127A) cell lines were constructed with PCDH-yapS127A. Eight-week-old BALB/c male nude mice were used. Cells were implanted into the dorsal flanking sites of nude mice at 2×10⁶ cells in 200 μl per spot. 30 Days after injection, mice were examined and tumor volumes were measured three times. Tumor volume = 1/2(length × width²).

**Induction of colitis and CRC (colorectal cancer) Model**

Colon inflammation was induced by treatment with 2% (w/v) DSS (MP Biomedicals) in drinking water for 3~5 days. For CRC Model, mice were given intraperitoneal
injection of azomethane (AOM) (Sigma) at 10 mg/kg. After seven days, 2% DSS was given to mice in drinking water for seven days, followed by normal water for seven days; this process was repeated three times. Then given normal water until mice were sacrificed. Four weeks later after completing the DSS treatment, tissues were collected for the followed experiments.

Statistics
Quantitative data were displayed as mean ± SEM of independent samples by using Prism software (GraphPad Software). Statistical analysis of values was performed using One Way Analysis of Variance (ANOVA). P-values < 0.05 were considered significantly different.

Results
REGγ regulates the Hippo-YAP activities in human colon cancer cells
Given that patients with ulcerative colitis have an increased risk of developing colorectal cancer, our discovery of REGγ as an activator of bowel inflammation prompted us to investigate its link with human colon cancer. Bioinformatics analyses revealed a strong correlation between REGγ expression and key components in the Hippo-YAP pathway (Figure 1A). With the hypothesis that REGγ might be a positive regulator of YAP signaling, we generated stable REGγ knockdown (sh-REGγ), using a previously well-defined shRNA (23), in HCT116 and HT29 human colon cancer cell lines to determine whether REGγ can modulate the Hippo-YAP activities. Silencing REGγ in HCT116 and HT29 cells resulted in a significant increase in Lats1 and p-YAP levels (Figure 1B). Immunofluorescence with antibody against total YAP revealed an increased nuclear localization of YAP in REGγ-high HCT116 cells (sh-N), whereas a more dispersed YAP staining with less nuclear intensity was found in REGγ-deficient (sh-R) HCT116 cells (Figure 1C). Quantitative analysis indicated a 30% decrease in the ratio of nuclear localization in REGγ silenced sh-R cells compared to the REGγ sh-N cells (Figure 1D), suggesting a positive link between REGγ and YAP. We then measured transcriptional activities of YAP in cells with varying REGγ levels. There was a
significant reduction in the expression of connective tissue growth factor (CTGF), cysteine-rich angiogenic inducer 61 (Cyr61) and amphiregulin (AREG) genes, positively regulated by YAP (24,25), in REGγ-deficient sh-R HCT116 cells; however, genes DDIT4 and Trail, negatively regulated by YAP (26), had higher expression in the sh-R cells (Figure 1E).

Considering the stability of YAP is also regulated by Lats1-independent phosphorylation at S397 (27), we examined if REGγ may affect its level in colon cancer cells. As expected, expression of p-YAP (S397) in colon cancer cells and tissues was not altered when REGγ was deleted (Supplemental Figure 1B and 1C). Unlike Lats1, Lats2 had no change in REGγ knockdown cells (Supplemental Figure 1A). The upstream kinase of Lats1 and Lats2, Mst1, was unchanged in either sh-N or sh-R colon cancer cell lines (Supplemental Figure 1D). These data indicate that REGγ may negatively regulate Lats1 to activate YAP in human colon cancer cells and modulate YAP downstream gene expression. Also, we observed that REGγ deletion resulted in a significant increase of Lats1 and p-YAP in HeLa, but not in REGγ−/− MEFs (Supplemental Figure 1E) probably due to cell specificity.

**REGγ binds Lats1 and promotes its degradation**

To determine a possible role for REGγ in degradation of Lats1, the degradation dynamics of Lats1 in the presence of cycloheximide, a protein synthesis inhibitor, in REGγ-deficient sh-R cells and the control cells (sh-N) was measured. Lats1 decayed much slower in the REGγ-depleted HT29 or HCT116 sh-R cells than in the sh-N cells (Figure 2A, 2B, and Supplemental Figure 2A), indicating that REGγ affects the stability of Lats1 in these cells. Lats1-dependent p-YAP (S127) displayed an increase of stability in REGγ-knockdown cells (Figure 2A, 2B, and Supplemental Figure 2A) while Lats1-independent p-YAP (S397) stability was not regulated by REGγ (Supplemental Figure 2C and 2D). The mRNA levels of Lats1, evaluated by semi-quantitative RT-PCR, had no changes in these cells regardless of REGγ abundance (Figure 2C), suggesting a post-transcriptional regulatory mechanism.

We carried out gain-of-function experiments by using doxycycline-inducible 293
cells, a previously well-defined cellular system for conditional study of REGγ functions(6), that can overexpress either wild-type REGγ or a dominant-negative mutant REGγ unable to activate the 20S proteasome. As expected, induction of REGγ triggered degradation of Lats1 (Supplemental Figure 2B), whereas induced expression of the REGγ-N151Y dominant-negative mutant failed to promote degradation of endogenous Lats1 as compared with the wild-type REGγ control.

Next, we analyzed if REGγ and Lats1 bind to each other intracellularly by reciprocal co-immunoprecipitation experiments. we found physical interactions between endogenous REGγ and Lats1 respectively (Figure 2D, 2E). To elucidate if the effect of REGγ on Lats1 degradation is direct, we used cell-free proteolysis. Incubation of in vitro translated Lats1 with 20S proteasome or purified REGγ alone exhibited no significant degradation of Lats1, but a combination of REGγ and 20S proteasome promoted much faster turnover of Lats1 than did the 20S proteasome alone (Figure 2F). Taken together, our results demonstrate a direct role of REGγ in degradation of Lats1 in vitro and in cells.

REGγ promotes YAP signaling via degradation of Lats1

The finding that Lats1 is a target of REGγ-proteasome led us to verify the causal relation and specificity in the Hippo-YAP signaling. We transiently silenced Lats1 in sh-N and sh-R HCT116 cells and assessed expression of YAP downstream genes by real-time RT-PCR. While REGγ-depleted sh-R cells had reduced expression of AREG, CTGF and Cyr61, silencing Lats1 markedly increased their expressions (Supplemental Figure 3A). The expression of Trail, a gene negatively regulated by YAP, was down-regulated with RNA interference of Lats1 in REGγ-depleted sh-R cells (Supplemental Figure 3A, 3B). In gain-of-function experiments, exogenous Lats1 was introduced to sh-N and sh-R HCT116 cells with viral infection followed by evaluation of AREG, CTGF, Cyr61 and Trail expression. Lats1 overexpression drastically repressed transcription of AREG, CTGF and Cyr61 in sh-N HCT116 cells (Supplemental Figure 3C, 3D). In contrast, overexpressed Lats1 greatly enhanced Trail levels in sh-N HCT116 cells (Supplemental Figure 3C, 3D). We also checked AREG, CTGF and
Cyr61 expressions in sh-N and sh-R HT29 cells with siLats1 or overexpression of Lats1. Similar result of REGγ dependent YAP regulation was observed as in HCT116 cells (Supplemental Figure 3E-3H). Therefore, we conclude that REGγ-mediated regulation of YAP activity is Lats1-dependent.

The REGγ-YAP pathway promotes colon cancer cell proliferation and tumor growth

With the findings that REGγ drives the tumor promoter YAP (15) and hyperexpression of REGγ exists in human CRC, we investigated the role of the REGγ-YAP pathway in colon cancer cell growth and experimental tumor formation. In both HCT116 and HT29 cells, cell viability assays were performed to demonstrate that depletion of REGγ markedly attenuated the growth of these human colon cancer cells 4 days post-seeding (Supplemental Figure 4A, 4B). Silencing YAP had a similar effect to REGγ depletion on cell growth (Supplemental Figure 4A, 4B). However, Lats1 knockdown increased cell viability in HCT116 and HT29 cells (Supplemental Figure 4C), indicating Lats1-dependent effects on cell growth. We established a murine colitis-associated cancer model by injecting a single dose of DNA-methylating agent azoxymethane (AOM) followed by three rounds of 2% Dextran sodium sulfate (DSS) treatment (one week each round) at intervals of two weeks. Colon tumors developed in the REGγ wildtype mice were significantly more severe than those in the REGγ−/− mice (Figure 3A). We found that REGγ+/+ mice developed 13.5 tumors per mouse while REGγ−/− mice had only 6.8 (Figure 3B). The average tumor size in WT mice was 6.5 mm in diameter while it was 2.1 mm in REGγ−/− mice (Figure 3C). Expression of proliferating cell nuclear antigen (PCNA) at the protein and mRNA levels was much higher in tumor tissues from REGγ+/+ mice than from REGγ−/− mice (Supplemental Figure 4D, 4E). In line with molecular analysis, immunohistochemical staining with anti-PCNA demonstrated significantly higher numbers of PCNA-positive cells in colon tumors from WT mice than in REGγ−/− mice (Supplemental Figure 4F). These findings indicate that lack of REGγ inhibits the proliferation of human colon
cancer cells and the progression of colitis-associated tumor formation.

We compared the variation of YAP signal-related molecules in colon tumors from $REG\gamma^{+/+}$ and $REG\gamma^{-/-}$ mice. While Lats1 and p-YAP protein levels in $REG\gamma^{-/-}$ tumors were considerably higher than those in $REG\gamma^{+/+}$ tumors, YAP levels were significantly lower in $REG\gamma^{-/-}$ tumors by Western blot analysis (Figure 3D), likely due to sustained increase of REG$\gamma$ in tumor areas. Immunohistochemical studies supported the observation of elevated Lats1 and p-YAP in REG$\gamma$-high tumors vs. reduced total YAP in REG$\gamma$-defective tumors (Figure 3E). By RT-PCR, expression of genes ($AREG$, $CTGF$ and $Cyr61$) positively regulated by YAP was significantly reduced in $REG\gamma^{-/-}$ tumors; however, transcription of genes ($DDTI4$ and $Trail$) regulated by YAP as a suppressor was greatly reinforced in REG$\gamma$ deficient tumors (Figure 3F), exhibiting a positive correlation between REG$\gamma$ and YAP activity in the development of experimental colon tumors.

REG$\gamma$ is known to regulate multiple tumor suppressors and lack of REG$\gamma$ is associated with tumor suppression (6,7,13,14). To determine if activation of YAP signaling in sh-R HCT116 cells may override tumor suppressive functions with REG$\gamma$ deficiency, we generated a constitutively active YAP (S127A) construct (28) and stable cell lines expressing activated YAP were created in sh-N and sh-R HCT116 cells. Cells expressing active YAP or control vectors were injected into dorsal sides of nude mice as previously described to generate xenograft tumors (23). Mice with REG$\gamma$-defective sh-R HCT116 cells had smaller tumor loads than mice with normal REG$\gamma$ (Supplemental Figure 5A), substantiating the proliferative action of REG$\gamma$ on colon cancer cells. In contrast, expressing constitutively active YAP in sh-R HCT116 cells restored tumor size like that derived from sh-N HCT116 cells (Supplemental Figure 5A-C). These results suggest that active YAP, acting downstream of REG$\gamma$, can fully overcome the tumor suppressive effect by REG$\gamma$-depletion in colon cancer cells. Therefore, we conclude that the REG$\gamma$-YAP pathway promotes colon cancer cell proliferation and tumor growth.

**The crosstalk between the NF-κB and YAP pathways is enhanced by REG$\gamma$**
An interesting observation was that the differences of Lats1 and YAP levels in non-tumor colon tissues between REGγ+/+ and REGγ−/− mice were boosted in tumor samples (Figure 3D). These data raise a possibility that Hippo-YAP activity was further enhanced by crosstalk with other signals regulated by augmented REGγ during tumorigenesis. The fact that REGγ promotes both NF-κB (14) and YAP signaling led us to investigate whether REGγ promotes cross-talks between the NF-κB and YAP pathways.

First, we inspected whether inflammatory microenvironment, such as activation of NF-κB, may promote YAP activity in human colon cancer cells. HCT116 and HT29 human colon cancer cells treated with TNF-α or IL-6 induced marked increase of YAP transcription (Figure 4A, Supplemental Figure 6A). Chromatin immunoprecipitation (ChIP) assays showed recruitment of p65 to the YAP1 promoter following TNF-α incubation (Figure 4B), suggesting a direct role of p65 in YAP transcription. In addition, TNF-α stimulation enhanced YAP1 binding to the promoter of its downstream gene, Cyr61 (Supplemental Figure 6B). Activation of NF-κB by TNFα or IL-6 significantly induced expression of AREG, CTGF and Cyr61, but attenuated transcription of Trail and DDIT4 in HCT116 or HT29 cell lines (Figure 4C, 4D, Supplemental Figure 6C, 6D), establishing a positive regulation by NF-κB signal on the YAP pathway. To determine whether inflammation influenced YAP activation, mice were treated with DSS for five days and analyzed for the expression of YAP target genes in colon epithelium. RT-PCR results demonstrated that an inflammatory microenvironment, as evidenced by elevation of NF-κB target genes (Supplemental Figure 6F), strikingly correlated with an increase in the expression of YAP (Figure 4E) and its target genes AREG, CTGF and Cyr61 (Figure 4F, Supplemental Figure 6E), an indication of YAP activation.

Next, we analyzed if YAP activation may regulate NF-κB signaling. To our surprise, transcription of p65 was significantly enhanced by expression of a constitutively active YAP protein (YAP-S127A) but attenuated by a YAP inhibitor (Figure 5A, Supplemental Figure 7A). ChIP analysis with an anti-YAP1 antibody revealed YAP1 enrichment at the promoter region of p65 (Figure 5B), suggesting YAP1 can directly promote the
transcription of p65 in human colon cancer cells. To validate our observation, an NF-κB luciferase reporter assay was performed in the presence or absence of active YAP. Constitutively active YAP (S127A) strongly enhanced NF-κB activity in HCT116 or HT29 cells (Figure 5C, Supplemental Figure 7B). Moreover, we applied a YAP inhibitor to TNFα or IL-6 treated cells, followed by assessing transcription of NF-κB target genes. Consistently, TNFα or IL-6 treatments led to a robust increase in transcription of IL-8 and IL-6, downstream genes of NF-κB, whereas IL-8 and IL-6 expression were blocked by YAP inhibitor Verteporfin (VP) in HCT116 or HT29 cells (Figure 5D, 5E; Supplemental Figure 7C, 7D). To rule out the potential off-target effect by YAP inhibitor, we employed siRNA against YAP to elucidate its function in crosstalk with NF-κB. Antagonizing YAP function markedly attenuated TNFα- or IL-6-induced expression of IL-8 and IL-6 (Figure 5F, 5G, Supplemental Figure 7E, 7F) in two different colon cancer cell lines. Consistently, ChIP assays indicated that a YAP inhibitor effectively blocked TNF-α-induced p65 binding to IL-8 promoter (Figure 5H). Thus, the YAP signal pathway could intensify the activity of NF-κB in human colon cancer cells.

Clearly, REGγ-deficient cells had impaired responses to TNFα or IL-6 treatments (Figure 4C-D, 5D-G). Moreover, the reciprocal effects of NF-κB and YAP signals were simultaneously suppressed in cells lacking REGγ (Figure 4C-D, 5D-G), indicating that REGγ modulates the interaction between the NF-κB and YAP pathways. Collectively, our study identifies a previously unknown mechanism by which the REGγ-proteasome, a new regulator of the Hippo-YAP pathway, promotes a positive feedback regulation between YAP and NF-κB to coordinate the growth of inflammation-associated colon tumor.

**Positive correlation among REGγ, YAP, and NF-κB are associated with poor prognosis of human colorectal cancer**

To delineate the clinical implication of REGγ-YAP-NF-κB signaling in human colon cancer, a cohort of 68 human Colorectal Cancer samples and 53 UC samples obtained from dataset GSE3629 was analyzed for correlation of gene expression. Strong positive
correlations for REGγ-YAP1, REGγ-RELA (p65), and YAP1-RELA were observed in colorectal cancer tissues (Figure 6A) as well as in UC tissues (Supplemental Figure 8A).

We analyzed 172 cases of Chinese human colon tumors with matched non-tumor controls by immunohistochemistry. REGγ was highly expressed in over 60% human colon cancer samples in all stages compared with paired non-tumor tissues (Figure 6B, Supplemental Figure 8D). Approximately 53% of samples had high expression of YAP and 76% cases had positive p-p65 staining (Figure 6C, Supplemental Figure 8B). While Lats1 and p-YAP levels were lower in tumor samples compared with non-tumor controls, total YAP exhibited a high correlation to REGγ levels (Supplemental Figure 8C). REGγ in tumor specimens displayed a strong positive correlation with YAP and a negative association with Lats1 and p-YAP (Figure 6B, Supplemental Figure 8C), consistent with our observation in vitro and in animal studies.

Among the 172 colon cancer cases with complete clinical records and post-operative follow-up for more than 2 years, Kaplan-Meier analysis indicated a significantly lower survival rate in REGγ-high patient groups compared to REGγ-low patients (Figure 6C, upper panel). Importantly, overexpression of REGγ was not correlated with age, sex, or tumor size (Supplemental Figure 8D). Meanwhile, high expression of YAP was also associated with marked reduction of survival rate (Figure 6C, middle panel). Correlation of lower survival rate with p-p65 overexpression appeared to have a trend although it was not statistically significant (Supplemental Figure 8B). In patients with high expression of REGγ/YAP and positive p-p65, the survival rate was further reduced compared with the other cases (Figure 6C, lower panel). Taken together, we conclude that REGγ overexpression is likely a marker for poor prognosis of human colon cancers.

**Discussion**

It is thought that YAP is constitutively inhibited by the Hippo kinase cascade, which, in epithelia, is activated by physiological cell-cell contact during normal homeostasis, but intestinal damage increases YAP abundance and nuclear residence dependent on Hippo (19). However, it is still unclear how Hippo signal molecules may be regulated
in response to inflammation. In this study, we have shown that Hippo kinase signal transduction is inhibited by REGγ proteasome-dependent degradation of Lats1 to enhance oncogenic YAP in colon cancer cells (Supplemental Figure 9). As a result, REGγ promotes colon cell proliferation \textit{in vitro} and \textit{in vivo} in a YAP-dependent manner. Furthermore, we have discovered a REGγ-dependent positive feedback loop for reciprocal regulation between the YAP and NF-κB signaling, suggesting that cancer cells hijack proteasome degradation, inflammation and YAP pathways (Supplemental Figure 9). Corresponding with these findings, overexpression of REGγ is found in over 60% of human colon cancer samples, correlated with elevated levels of YAP/NF-κB in those samples, and associated with a poor prognosis, suggesting that REGγ is a master regulator for the development of human colon cancer.

REGγ-mediated degradation of Lats1 but not Lats2 or Mst1/2, in epithelial cells but not in MEFs, reflects a specific mechanism, suggesting additional layers of inhibitory regulation by REGγ in the Hippo kinase cascade, activated by physiological cell-cell contact during normal homeostasis in epithelia, but disturbed upon intestinal damage (19). In fact, REGγ is transcriptionally activated by NF-κB upon colon epithelial damage during experimental colitis (14). Elevated REGγ in colon epithelial cells not only exacerbates inflammation, but also bridges activation of the YAP pathway by proteasome dependent degradation of Lats1, explaining in part how disrupted cell-cell contact by inflammatory microenvironment may lead to loss of Lats1 and increased residence of nuclear YAP.

REGγ-triggered negative regulation of Lats1 and positive regulation of YAP are shown here via loss-of-function and gain-of-function experiments. We verified Lats1 is indeed a direct target of the REGγ-proteasome in a cell-free \textit{in vitro} degradation system, where no ATP or ubiquitin is required. REGγ depletion results in differential regulation of total YAP and phosphorylated YAP, likely due to quick degradation of phosphorylated YAP by ubiquitin proteasome pathway in the cytosol. The physiological functions of the non-canonical REGγ-proteasome in Lats1 degradation appear to maintain the homeostasis of Lats1 for rapid proliferative needs of colon epithelial cells, whereas the traditional ubiquitin-proteasome system may execute transient and robust
clearance of Lats1 upon oncogenic signals. Changes in oncogenic p53 mutation, loss of TGF-β components, or increased NF-κB activities can dramatically promote REGγ levels (14,22), leading to pathological activation of YAP. Expressing constitutively active YAP completely abrogates inhibition of tumor growth in REGγ-depleted colon cancer cells in mouse models. However, no striking increase in p53 expression was found after REGγ deletion in vitro or in vivo (Supplemental Figure 10A, 10B), indicating that p53 might play a minor role in this context.

Accumulating evidence indicate that YAP is overexpressed in human colon cancers and most colon cancer-derived cell lines (2,18,19). Hyperactivation of YAP results in widespread early-onset polyp formation following DSS treatment (3). Co-overexpression of YAP and transcriptional co-activator with PDZ-binding motif (TAZ) is proposed as an independent predictor of prognosis for patients with CRC (2). In this study, we have also found significant elevation of YAP in both mouse colitis-associated colon tumor and human colorectal cancer tissues, which is significantly associated with a poor prognosis in human CRC patients. There is a strong positive-correlation between overexpression of REGγ and total YAP but not phosphorylated YAP, with concomitant reduced Last1 in tumor tissues. Perhaps phosphorylated YAP is quickly degraded by the ubiquitin proteasome pathway in the cytosol.

Although YAP functions as an oncogenic factor, a second signal pathway provided by tissue damage or inflammation is required for growth-promoting function of YAP in hepatocytes (29). Many studies have revealed the connection between colon carcinogenesis and inflammation, in which NF-κB is a central player (30,31). In this study, we have found that NF-κB is able to enhance YAP transcription and its activity in human colon cancer cells and mouse-colitis model, providing a direct link between inflammatory signals and activation of YAP. Meanwhile, YAP can bind the p65 promoter to enhance NF-κB signaling, reinforcing a positive feedback between NF-κB and YAP signals during colon tumor formation. Our findings are supported by a previous high-throughput ChIP-seq study suggesting recruitment of NF-κB to YAP1 promoter in head and neck squamous carcinoma cells (32).

As a critical transcriptional mediator of inflammatory responses, p65 is likely to
play a key role in the initiation of cancer formation. With the notion that NF-κB enhances REGγ transcription during colonic inflammation (14), there is a crosstalk network among REGγ, NF-κB, and YAP signals, in which REGγ appears to be the hub. We believe the REGγ regulated network is much more complicated than we have mentioned here and illustrated in Supplemental Figure 9. For instance, we have previously found REGγ-dependent regulation of Wnt signaling (10), which is important for the development of colon cancer (33). Wnt/β-catenin signaling has been reported to regulate transcription of YAP in colorectal carcinoma cells (34) and nuclear accumulation of YAP correlates with β-catenin activation (18). Future studies will integrate all these signal pathways to elucidate REGγ functions in CRC carcinogenesis.

References


Figure Legends

Figure 1. REGγ regulates Hippo-YAP activities in human colon cancer cells.  
A. REGγ mRNA level is strongly correlated with the Hippo-YAP signal pathway in a bioinformatics analysis of 21 normal and UC data, p<0.01. B. Stably silencing REGγ (sh-R) increased expression of Lats1 and p-YAP. Western Blot analyses were performed in human colon cancer cells with stable integration of RNAi against REGγ or a control (sh-N). C. REGγ depletion inhibited YAP nuclear localization by immunofluorescence. D. Quantitative results of C. Cells were stained for YAP (red) and analyzed by confocal microscopy. A minimum of 200 cells per sample were measured in triplicate. Data are represented as the mean ± SEM. E. REGγ deficiency suppressed the transcriptional activity of YAP. Gene expression was assessed by Real-time RT-PCR. Data are presented as the mean ± SEM (One Way Analysis of Variance (ANOVA), n=3, *p<0.05, **p<0.01). All data in this figure are representatives of three independent repeats.

Figure 2. REGγ promotes YAP signal pathway by degrading Lats1.  
A. REGγ regulates Lats1 stability. HT29 REGγ sh-N or sh-R cells were treated with cycloheximide (100μg/ml) for indicated time followed by Western Blotting. B. Quantitative results in (A) were obtained by Image J software and plotted to indicate dynamic changes (One Way Analysis of Variance (ANOVA), n=3. *p<0.05, **p<0.01, ***p<0.001). C. Alteration of REGγ had no effect on Lats1 mRNA levels as indicated by RT-PCR analysis. Data represent mean ± SEM (Analysis of Variance, n=3). D & E. REGγ interacted with Lats1. Reciprocal interactions between REGγ and Lats1 in 293T cells were determined by co-immunoprecipitation and Western blot analysis. F. REGγ directly promoted the degradation of Lats1. In vitro proteolytic analyses were performed using purified REGγ, 20S proteasome and Lats1 protein at 30℃ for 3h. A known substrate of REGγ, p21, was shown as a positive control. Data in this figure are representatives of three independent repeats.

Figure 3. REGγ promotes proliferation of colon cancer cells and colon tumor Research.
growth dependent on the YAP signal pathway

A. REGγ deletion inhibited mouse colitis-associated colon tumor formation. Micrographs of mouse colitis-associated colon tumor in REGγ+/+ and REGγ−/− mice. B. The average number of tumors in each mouse in the colons were quantitated. One representative experiment of three repeats is depicted. Data refer to mean ± SEM (Analysis of Variance, n=9 each group, **P<0.01). C. The average tumor size was measured with calipers in REGγ+/+ and REGγ−/− mice. Data represent the mean ± SEM (Analysis of Variance, n=9 each group, ***P<0.001). D. REGγ deletion had more dramatic impact on Lats1 and YAP levels in colon tumor than in normal colon tissues. Western blot analysis was performed with normal colon tissues and colon cancer tissues from REGγ+/+ and REGγ−/− mice. E. Immunohistochemical staining of Last1, p-YAP (S127) and YAP in normal colon tissues and colon cancer tissues of REGγ+/+ and REGγ−/− mice. Scale bars: 50 µm (Magnification ×20). F. Attenuated YAP transcriptional activity in the REGγ−/− colon cancer tissues compared to WT by quantitative real-time PCR analysis. Data represented mean ± SEM (Analysis of Variance, n=3, *P<0.05, **P<0.01). All results in this figure are representatives of three independent repeats.

Figure 4. P65 strengthens YAP transcription and its signal pathway

A. Quantitative PCR analysis of YAP expression in HCT116 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<0.01, ***p<0.001). B. HCT116 cells were treated with or without TNFα (20 ng/ml) for 3 hours and proceeded to ChIP assay using anti-p65 antibody. Immunoprecipitated chromatin was analyzed by RT-PCR using the specific primers for YAP1 promoter. C&D. Activation of NF-κB by TNFα or IL-6 reinforces YAP signaling. HCT116 and HT29 cells were treated with TNFα or IL-6 in the presence or absence of VP for 3 h followed by real-time RT-PCR analyses. (Analysis of Variance, n=3, *p<0.05, **p<0.01, ***p<0.001). E. In vivo stimulation of NF-κB by DSS treatment promotes YAP activity. REGγ+/+ and REGγ−/− mice were treated with 2% DSS for five days and the mRNA levels of YAP1 in the mouse colon tissues were quantified by real-time RT-PCR analysis (Analysis of Variance, n=3, ***p<0.001). F. In vivo stimulation of NF-
κB by DSS treatment promotes YAP activity. REGγ+/+ and REGγ−/− mice were treated with 2% DSS for five days and the mRNA levels of AREG and CTGF in the mouse colon tissues were quantified by real-time RT-PCR analysis (Analysis of Variance, n=3, **p<0.01, ***p<0.001). All the experiments were repeated three times.

**Figure 5. YAP enhances p65 transcription and its signal pathway**

A. Quantitative PCR analysis of p65 expression in HCT116 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<0.001). B. ChIP assays in HCT116 cells using a YAP antibody were performed and RT-PCR was then carried out on immunoprecipitated DNA using primers amplifying the promoter of p65 gene. C. YAP activation increased the transcriptional activity of NF-κB in HCT116 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 mean ± SEM (Analysis of Variance, n=3, ***P<0.001). D-G. Interference of YAP activity attenuates cytokine-induced increase in NF-κB responsive IL-8. HCT116 and HT29 cells were treated with IL-6 (20 ng/ml) (D, F) or TNFα (20 ng/ml) (E, G) in the presence or absence of Verteporfin (VP) (10 μM) for 3 h or RNAi against YAP followed by real-time RT-PCR analyses. Data are presented as the mean ± SEM (Analysis of Variance, n=3, *p<0.05, ***p<0.001). H. HCT116 cells were treated with TNFα (20 ng/ml) in the presence of absence of Verteporfin (VP) (10 μM) for 3 h followed by ChIP assay using an anti-p65 antibody. Immunoprecipitated chromatin was analyzed by gel-based PCR using the specific primers for IL8 promoter. All the experiments were repeated at least three times.

**Figure 6. REGγ-YAP-NF-κB signaling cascades are associated with poor prognosis for human colorectal cancer**

A. Correlation between REGγ, YAP and RELA mRNA expression in patients with colorectal cancer, displayed by scatter plot. n=68, p<0.0001. B. REGγ negatively correlated with Lats1 and p-YAP, but positively with YAP and p-p65, in human colon cancer tissues. IHC analyses of Lats1, YAP, p-YAP and p-p65 were
performed with adjacent sections from normal colon and cancer tissues. Scale bar, 50μm (magnification, ×20). C. Survival curve in 172 Chinese CRC patients based on the protein levels of REGγ, YAP and p-p65. The overall survival rate was lower in the high expression of REGγ, YAP and p-p65 compared to their respective low expression group. The 28-month survival rates of the patients with high co-expression of REGγ, YAP and p-p65 and low co-expression were 62.9% and 91.3 %, respectively. P<0.001.
Figure 1

A

B

C

D

E

HCT116 sh-N  HCT116 sh-R  HT29 sh-N  HT29 sh-R

Total-lats1

1  4.5  1  2.6

P-YAP(S127)

1  2.8  1  2.9

Total-YAP

1.8  1  1.7  1

REGy

1  1  1  1

Actin

6.5  1  7.8  1

Total-YAP

Merge(YAP/DAPI)

The ratio of YAP nuclear localization

HCT116 sh-N  HCT116 sh-R

AREG  CTGF  Cyr61  DDIT4  Trail

Fold

Fold

Fold

Fold

Fold
REGγ controls Hippo signaling and reciprocal NF-κB-YAP regulation to promote colon cancer

Xiaotao Li, Qingwei Wang, Xiao Gao, et al.

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