RESEARCH ARTICLE

Functional Significance of Hippo/YAP Signaling for Drug Resistance in Colorectal Cancer†

Running title: Mechanism of colorectal cancer drug resistance

Ruolan Song[1, 2#], Dongsheng Gu[2#], Lining Zhang[1], Xiaoli Zhang[2], Beiqin Yu[1], Bingya Liu[1,*], Jingwu Xie[2, *]

[1]Shanghai Key Laboratory of Gastric Neoplasms, Shanghai Institute of Digestive Surgery, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

[2]Indiana University Departments of Pediatrics, Biochemistry and Molecular Biology, Pharmacology and Toxicology, The Wells Center for Pediatrics Research and IU Simon Cancer Center, Indianapolis, IN 46202, USA.

***Correspondence to:**

Jingwu Xie, E-mail: jinxie@iu.edu; Bingya Liu, Email: liubingya@sjtu.edu.cn

#the first two authors contributed equally to this work

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Abstract

Colorectal cancer is a leading cause of cancer-related death worldwide. While early stage colorectal cancer can be removed by surgery, patients with advanced disease are treated by chemotherapy, with 5-Fluorouracil (5-FU) as a main ingredient. However, most patients with advanced colorectal cancer eventually succumb to the disease despite some responded initially. Thus, identifying molecular mechanisms responsible for drug resistance will help design novel strategies to treat colorectal cancer. In this study, we analyzed an acquired 5-FU resistant cell line, LoVo-R, and determined that elevated expression of YAP target genes is a major alteration in the 5-FU resistant cells. Hippo/YAP signaling, a pathway essential for cell polarity, is an important regulator for tissue homeostasis, organ size and stem cells. We demonstrated that knockdown of YAP1 sensitized LoVo-R cells to 5-FU treatment in cultured cells and in mice. The relevance of our studies to colorectal cancer patients is reflected by our discovery that high expression of YAP target genes in the tumor was associated with an increased risk of cancer relapse and poor survival in a larger cohort of colorectal cancer patients who underwent 5-FU-related chemotherapy. Taken together, we demonstrate a critical role of YAP signaling for drug resistance in colorectal cancer.

Keywords: YAP, drug resistance, colorectal cancer, 5-Fluorouracil (5-FU), cancer therapy

Introduction

Colorectal cancer (CRC) patients have benefited from research advancements in early screening and preventative measures in life styles. The overall incidence of CRC has significantly declined in the last two decades although CRC is still the leading cause of cancer-related deaths, with nearly 1.4 million cases a year and ~774,000 deaths worldwide [1].

Chemotherapy, particularly 5-Fluorouracil (5-FU) -based adjuvant chemotherapy, has been widely used to treat CRC since early 1990s. Addition of oxaliplatin to 5-FU-based regiment has resulted in risk reduction (~20%) for disease-free survival. In the clinic, the first line treatment of CRC includes mFOLFOX6 with or without targeted drugs bevacizumab or cetuximab [2,3]. The mFOLFOX6 regimen contains leucovorin calcium (folinic acid), 5-fluorouracil, and oxaliplatin. Many advanced CRC patients eventually develop relapsed disease despite of initial response to chemotherapy. Thus, drug resistance is a major barrier to achieve effective CRC treatment.

5-FU is in the antimetabolite and pyrimidine analog families of medications, commonly used to treat a variety of cancer including breast, gastric, colorectal, pancreatic cancers, and squamous cell carcinomas arising in the head and neck [4]. The mechanisms of action include 1) incorporation of fluorouridine triphosphate into RNA to interfere with RNA synthesis and function; 2) inhibition of thymidylate synthase; 3) incorporation of fluorodeoxyuridine triphosphate and deoxyuridine triphosphate into DNA, and 4) genotoxic stress to trigger programmed cell death. Resistance to 5-FU in CRC is a major clinical problem. While there are a number of mechanisms reported to be responsible for drug resistance[5-9], activation of several tissue polarity regulation pathways is quite

appealing[10-25]. Like wnt, notch and hedgehog signaling pathways, YAP1 is an important regulator for embryonic development, organ size, cell differentiation and cancer development[5,9,26-28]. Thus, specific inhibitors for these signaling pathways may be used to sensitize cancer cells to 5-FU treatment.

To elucidate the underlying mechanism for 5-FU resistance in CRC, we used an acquired resistant cell line, LoVo-R, to identify gene expression profiles in comparison with that of the parental LoVo cells. We discovered elevated expression of YAP1 target genes in LoVo-R cells. We demonstrated both in cultured cells and in mice that YAP1 is significant for 5-FU resistance. We also investigate the relevance of our results to colorectal cancer patients by examining a large cohort of patients with colorectal cancer who underwent 5-FU-based chemotherapy (n=663) to compare the rate of cancer relapse and patient survival in patients with high and low expression of YAP1 target genes.

Results

Elevated YAP signaling in 5-FU resistant colon cancer cells

To understand the molecular basis underlying drug resistance of 5-FU in colorectal cancer, we first generated 5-FU resistant LoVo-R cell line from the parental LoVo cells through gradual addition of 5-FU for nearly a year [29]. We compared gene expression between LoVo-R and LoVo cells via RNA-seq. Several YAP target genes were among the top differentially expressed genes, including CTGF, CYR61, ANKRD1, NRG1 and TEAD2 (Fig.1), indicating elevated YAP signaling in 5-FU resistant cancer cells. We have confirmed elevated YAP and CYR61 protein levels by Western blotting with available specific antibodies (Fig.1C).

The Hippo/YAP signaling pathway involves a kinase cascade including Mst1/2 and Lats1/2. Lats1/2 phosphorylate YAP1, leading to degradation and inactivation of YAP1 [30,31]. In the active form, YAP1 is a co-activator to promote DNA binding of TEAD1-4, causing elevated expression of YAP1 target genes. To confirm the data from target gene expression, we detect protein levels of YAP1 in 5-FU resistant LoVo-R and the parental LoVo cells. Nuclear YAP1 is an indicator for activated YAP1 signaling, and we detected YAP1 protein level by two approaches: immunofluorescent staining, cell fractionation followed by Western blotting. By immunofluorescent staining, we detected more nuclear staining of YAP1 in the 5-FU resistant LoVo-R cells, but not in the parental LoVo cells (Fig.2A). By cell fractionation analysis, we detected a high ratio of nuclear YAP1/Lamin C in 5-FU resistant LoVo-R cells (Fig.2B). These results indicate that more YAP1 protein is accumulated in the nucleus in the 5-FU resistant cells, suggesting elevated YAP signaling in the 5-FU resistant cells.

Molecular mechanisms by which YAP signaling is elevated in 5-FU resistant LoVo-R cells

To determine the molecular mechanisms responsible for elevated YAP1 target gene expression, we detected phosphor- and total- protein levels of Mst1/2 and Lats1/2. We observed reduced levels of phosphor-Mst1/2 and Lats1/2 in the 5-FU resistant LoVo-R cells (Fig.3A), indicating that elevated YAP signaling is at least partially due to up-stream signaling activation.

There are several upstream signaling pathways that regulate the Hippo pathway. Elevated receptor tyrosine kinases (RTKs) can suppress Mst1/2 phosphorylation, leading to more nuclear YAP1 protein and elevated YAP1 target gene expression. Tight junction and adherent junction can promote Mst1/2 phosphorylation. Mst1/2 can also be activated by G-protein coupled receptors, including receptors for Wnts, the frizzled molecules. Gene expression comparison between the 5-FU resistant LoVo-R cells and the parental LoVo cells revealed elevated expression of several growth factors, such as PDGFD, EGF, FGF5 and VEGFC in the 5-FU resistant LoVo-R cells (Fig.1S), suggesting that several RTKs may be activated in the resistant cells. If activation of RTKs is the major reason for reduced Lats1/2 phosphorylation, inhibiting RTKs using downstream inhibitors should increase the relative level of phosphorylated Lats1/2, which is indicated by the ratio of phosphorylated Lats1/2 vs. total Lats1/2. We treated LoVo-R cells with MEK inhibitor AZD4266 and

PI3K inhibitor BEZ235. We observed an increase in the ratio of phosphor-Lats1/2 vs. total Lats1/2 (Fig.3B). This result indicates that RTKs are involved in regulation of Hippo/YAP1 signaling.

In addition, we also noticed morphological changes in 5-FU resistant LoVo-R cells (Fig.2A). 5-FU resistant LoVo-R cells are mesenchymal-like whereas the parental cell is round with a large nucleus. Furthermore, the 5-FU resistant LoVo-R cells have more expression of vimentin, N-Cadherin and integrin $\alpha 5\beta 1$, suggestive of an EMT phenotype.

In addition, we found elevated expression of YAP1 and TEAD2 in 5-FU resistant LoVo-R cells (Fig.1). Thus, it appears that there are several mechanisms by which expression of YAP1 target genes are up-regulated in 5-FU resistant LoVo-R cells.

Significance of YAP1 for 5-FU resistance in LoVo-R cells

Although RTKs and EMT regulate YAP1 signaling, knocking down YAP1 seems to be more effective in suppression of YAP1 signaling because up-regulation of YAP1 and TEAD2 is one important mechanism by which YAP1 target genes are induced. We knocked down YAP1 by specific shRNAs in LoVo-R cells. We examined YAP1 target genes expression. As shown in Fig.4A, expression of ANKRD1 and CTGF were significantly suppressed whereas NRG1 and Tead2 were not affected.

We further tested the IC50 of LoVo-R shYAP cells (Fig. 4A). The IC50 value of LoVo-R shYAP is around 0.4mM which is lower than 13.803mM, the value observed for LoVo-R control cells (Fig. 4B).

Next, we determined whether 5-FU sensitivity is altered by YAP1 knocking down in LoVo formed tumors in mice. After injection of LoVo-R-shYAP or the LoVo-R control cells into immune deficient NSG mice and tumor formation, we treated mice with 5 - FU (50 mg/kg) once a week. We measured the tumor size at different time points. As shown in Fig. 5, we found that the tumors derived from the LoVo-R control cells were not significantly different from the tumors from 5-FU

treated mice (Fig.5A). In contrast, tumors from LoVo-R-shYAP1 cells without 5-FU treatment continues to grow whereas 5-FU treatment significantly reduced tumor growth (Fig.5B), indicating that knocking down YAP1 sensitizes tumors to 5-FU treatment.

These experiments demonstrate that elevated YAP1 signaling is important for 5-FU resistance both in cultured LoVo cells and in LoVo-derived tumors.
[32]

Relevance of YAP1 signaling to cancer relapse and survival of colorectal cancer patients

To determine the relevance of our studies in 5-FU resistant LoVo-R cells and the matched parental LoVo cells, we used a public database to find the association between expression of YAP1 and its target genes in human colorectal cancer with cancer relapse and survival of colorectal cancer patients (through CBioportal database). All patients with colon cancer received 5-FU-containing chemotherapy as a standard treatment, and we determined the rate of cancer relapse in patients with high or low expression of YAP1 target genes in the tumor. We found that cancer relapse occurred in >33% of the patients with high expression of YAP1 target genes in the tumor. In contrast, the rate of cancer relapse in patients with low YAP1 target gene expression was only about 10%. The relevance of YAP1 target gene expression with drug resistance can be reflected from survival of colorectal cancer patients with high or low YAP1 target gene expression in the tumor. All patients underwent 5-FU containing chemotherapy as a standard treatment option. According to expression levels of YAP and its target genes, we analyzed the relationship between YAP1 target gene expression in the tumor with the survival time of the patients. We found that patients with low YAP1 target gene expression survived better than those with high YAP1 target gene expression (Fig.6).

Taken all the data together, we found that YAP1 and its target genes are highly expressed in 5-FU resistant LoVo-R cells. Although the exact mechanisms underlying YAP1 signaling activation remain incompletely understood, we believe that activated RTKs and YAP1 gene expression are responsible for elevated YAP1 signaling in drug resistant cells. We demonstrated that down-regulation of YAP1 by shRNAs sensitized LoVo-R cells to 5-FU treatment both in cultured cells and in mice. Our results are relevant to human colorectal cancer patients as high expression of

YAP1 and its target genes is associated with a high cancer relapse rate and poor patient survival. Our data indicate that reducing YAP1 signaling may be one way to improve the efficacy of 5-FU – containing chemotherapy of colorectal cancer.

Discussion

Drug resistance has been a clinical issue for treatment of cancer, including traditional chemotherapy, targeted therapy and immune therapy [33-35]. Drug resistance to chemotherapy in colorectal cancer is a significant issue as most colorectal treatment includes 5-FU. While numerous studies have been conducted on drug resistance in colorectal cancer, thus far, no effective counter measures have been developed to reverse drug resistance. In this study, we tried to identify drug resistance mechanisms of colorectal cancer cells using cells with acquired 5-FU resistance in comparison with the parental cells. Through RNA-seq analyses, we discovered elevated YAP1 target gene expression as the major alteration in the 5-FU resistant cells. By knocking down YAP1, we demonstrated that YAP1 is required for 5-FU resistance. We also showed the relevance of our results to human colorectal cancer patients through gene expression association studies. We showed that patients with high YAP1 target gene expression had a high risk of cancer relapse and poor survival. Thus, we identified YAP1 signaling as the major factor for 5-FU resistance, and thus, down-regulation of YAP1 signaling may be effective in promoting the efficacy of 5-FU-based chemotherapy.

Previous studies have linked YAP1 signaling to colorectal cancer development. In one study, a tyrosine kinase YES1 was shown to be up-regulated in the 5-FU resistant cells, and YES1 regulates drug resistance through regulation of YAP1[36]. In other studies, YAP1 signaling is shown to be associated with cell proliferation in colorectal cancer cells as well as metastasis of colon cancer[31]. In our studies, we revealed several possible mechanisms by which YAP1 signaling is activated in 5-FU resistant colorectal cancer cells. These include elevated activation of RTKs, EMT and elevated expression of YAP1 itself. It thus appears that there are different mechanisms by which YAP1 signaling is activated in drug resistance in colorectal cancer. Using a large cohort of colorectal cancer patients (n=633), we were able to show that high expression of YAP1, TEAD2 and YAP1 target genes CYR61 and ANKRD1 is associated with a high risk of cancer relapse (>33% vs. ~10%) and

poor survival (Fig.6), suggesting that our findings are relevant to colorectal cancer patients. As complicated as drug resistance, it is likely that multiple mechanisms are involved in regulation of 5-FU resistance in colorectal cancer.

It remains unclear how YAP1 signaling regulates 5-FU drug resistance. It is known that YAP1 signaling is an important pathway for regulation of tissue polarity. Like other cell polarity regulators, including hedgehog and Wnt signaling, YAP1 is known to regulate residual cancer cells or cancer stem cells. Efficiency of tumor sphere formation is one feasible biology assay for cancer stem cells. However, LoVo-R or its parental LoVo cells do not form tumor spheres, which preventing further analyses. Additional 5-FU resistant cell lines with activated YAP1 signaling will be sought to further the mechanism study.

5-FU is widely used in cancer treatment for chemotherapy or neoadjuvant chemotherapy. In addition to colorectal cancer, 5-FU has been used for treatment with pancreatic cancer, head and neck cancers, skin and pediatric cancers. We predict that similar mechanisms may also exist for 5-FU resistance in other cancer types. In addition to be a drug target, elevated expression of YAP1 and its target genes may be also used to predict cancer relapse in chemotherapy.

Materials and methods

Cell culture

Human colon cancer cell lines LoVo, and HEK293T were purchased from ATCC. LoVo cells were cultured in DMEM: F12=1:1 medium and HEK293T cells were cultured in DMEM, supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin at 37 ℃ under a humidified environment containing 5% CO2. The cells in logarithmic growth were selected for subsequent experiments.

Reagents and antibody

5-fluorouracil reagent was purchased from Sigma, F6627-5G. Powder was dissolved in growth medium (DMEM: F12), then stocked at -80 ℃ until use. Western Blotting was performed using

antibodies against YAP (Cell Signaling Technology Inc, Cat#14074S), p-YAP (Cell Signaling Technology Inc, #46931), p-LATS1 (Cell Signaling Technology Inc, #9157), LATS1 (Cell Signaling Technology Inc, #9153), p-MST1 (Cell Signaling Technology Inc, #3681S) and β-actin antibody (Santa Cruz Biotechnology).

Cell viability and test cell growth inhibition rate

Day 1, cells (4000/well for LoVo cells, 3500/well for LoVo-R cells) were seeded into 96-well plates, with 100 μl growth medium for a well. After cultured for 24 h, chemosensitivity was assssed, with three treatment groups: group A- only growth medium for the background; group B- cells with fresh medium without 5-FU for basic metabolism; group C - the growth medium was replaced with various concentrations of 5-FU (LoVo cells, 0mM, 0.0154mM, 0.0308mM, 0.0616mM, 0.1232mM, 0.2464mM, 0.4928mM; LoVo-R cells, 0 mM, 0.3125mM, 0.625mM, 1.25mM, 2.5mM, 5mM, 10mM, 12mM, 15mM). After treatment for 48 h, cell viability was performed by AlamarBlue (Thermo scientific, FL, USA) with 10 μl for each well. After 1.5h, the absorbance was measured by Microplate Reader (Thermo Scientific) at the 530/590 nm. The value was used as an indicator of cell viability. Eight wells were used for each drug concentration, and the experiment was repeated at least 3 times.

Lentiviral infection

The plasmid siRNAs were purchased from Sigma (Broad Institute). To generate YAP knockdown cells, HEK293T cells were used to generate lentiviruses after transfection with plasmids containing a specific shYAP. PRRE, RSV/REV and CMVG plasmids using Lipofectamine 3000 (Invitrogen, USA). Viruses-containing cell culture supernatant was used to infect LoVo-R cells. After infection for three times, cells were selected with 1 μg/ml puromycin (Sigma, USA) to establish stable shRNA expression.

RNA extraction and real-time PCR

Total RNAs were extracted from cells using TRIzol reagent (Sigma) and performed real-time PCR according to the manufacturer's instructions. In brief, 1 μg of total RNA was reversely transcribed into cDNA using the First-Strand Synthesis Kit (Roche, USA). Real-time PCR was carried out with

specific probes according to manufacturer's instructions. All probes for real-time PCR were purchased from the Applied Biosystems. (YAP-HS00902712-g1; CTGF-HS01026927; CYR61-HS00998500; ANKRD1-HS00173317; TEAD2-HS01055894; NRG1-HS01101538)

Western blot analysis

Cells were lysed using cell lysis buffer (50mM Hepes, 2mM EDTA, 100mM NaCl, 1% Glycerol, 1% triton X-100, protease and phosphatase inhibitors). Protein were denatured at 100 ℃ for 5min.

Proteins were separated by SDS-PAGE, and then transferred onto PVDF membrane. Specific proteins were detected after incubation with the first antibodies (anti-MST1-P antibody, anti-LATS1-P antibody, anti-LATS1 antibody, anti-YAP-P antibody, anti-YAP antibody, which were diluted at 1:1000, Cell Signaling Technology, USA; mouse anti-β actin antibody, diluted at 1:10000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by the secondary antibody (goat anti-rabbit-HRP/goat anti-mouse-HRP (diluted at 1:20000, Fisher Scientific). Proteins bands were detected by using an ECL Western blotting detection kit (Fisher scientific).

Immunofluorescence Staining

To test the expression of YAP between LoVo cells and LoVo-R cells. Cells were seeded in 6-well plates with coverslips at the bottom when cell confluence reaches 50-80%, cells were fixed with 4% of PFA /PBS for 15 min at room temperature. There were two groups: one group with the first antibody (anti-YAP antibody 1:400 dilution）and the second antibody (1:300); another group with the second antibody (1:300) only. DAPI was used for nuclear staining.

Tumor xenografts and anticancer chemotherapy

All procedures about animal experiments were approved by the Institutional Animal Care and Use Committee in Indiana University of Medicine. To generate tumors in mice, we injected 1×10^7 cells re-suspended in 100 μl PBS into NSG mice. Tumor volume was measured with a caliper twice a week ($0.5 \times \text{length} \times \text{width}^2$). Mice with tumor were treated twice a week with 5-FU (50mg/kg/body weight). Tumor was removed, weighted and fixed in formalin for further study and analysis.

Statistical analysis

All the data are presented as mean ± SD and from three independent experiments. IC50 values of 5-FU were calculated with the GraphPad Prism 7.0 software. Student's test was done for statistical analysis with two groups and P value was calculated by two-tail unpaired t-test. Significance was distinguished as *p < 0.05, **p < 0.01, ***p < 0.001. Bands of Western blotting and pictures of immunofluorescence staining were quantified by ImageJ software. Analyses of a large cohort of colorectal cancer patients was through a public CTGA database (Cbioportal) with Kaplan-Meier Survival generated automatically.

Declarations

Authors' contributions

Ruolan Song and Dongsheng Gu performed the initial experiments. Lining Zhang and Xiaoli Zhang help with real-time PCR and Western blot, Beiqin Yu help with LoVo-R cell line establishment. Bingya Liu and Jingwu Xie designed the experiments. Ruola Song and Jingwu Xie wrote the manuscript.

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Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics app

Ethics, consent and permissions
Our studies have been approved by IACUC (protocol number 11370) and IRB (Ex0909-22) from our institutions.

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FIGURE LEGENDS

Figure 1- Gene expression analysis in 5-FU resistant LoVo-R cells vs parental LoVo cells. Total RNAs were extracted from LoVo-R and LoVo cells, and real-time PCR was performed using taqman primers and probes. **A** shows a diagram of the Hippo/YAP1 pathway. **B** shows relative levels of YAP1, TEAD2 and several YAP target genes. **C** shows protein levels of YAP1 and CYR61. All the data were shown as mean ± SD and from three independent experiments.

Figure 2- Nuclear localization of YAP1 protein. We detected YAP1 protein in LoVo and LoVo-R cells by immunofluorescent staining and cell fractionation analysis. **A** shows typical YAP1 staining. Please note the different cell morphology between LoVo-R cells and LoVo cells. YAP1 was indicated by red staining, counter stained with DAPI. **B** shows detection of YAP1 in nuclear fractionated proteins by Western blotting, with laminA/C as the internal control. Left is the Western blotting results, and the right is the quantitative analysis of the nuclear YAP1/LaminA/C ratios. Cells treated with trypsin or without trypsin treatment had no significant difference. Significant difference was indicated by *** ($p < 0.0005$).

Figure 3- Analysis of protein phosphorylation in the Hippo/YAP1 signaling in LoVo-R and LoVo cells. Total proteins from cultured cells with or without signaling inhibitors were subjected to Western blotting analyses. **A** shows data from cultured untreated cells. **B** shows data from cells treated with signaling inhibitors or a control solvent. The top shows the diagram of the targets of signaling inhibitors whereas the bottom shows the actual Western blotting. **C** shows analysis of Lats-p/Lats ratios. The ratios were generated from band intensity from triplicated samples, and the p values were calculated from the mean and STDEV values.

Fig.4 The effect of *YAP1* knockdown on YAP1 target gene expression and on 5-FU response in culture. A. Real-time PCR detection of gene expression. **B.** The effect of *YAP1*-ShRNAs (shown as shYAP) on the IC50 of 5-FU. Significant difference was indicated by * ($p < 0.05$); ** ($p < 0.005$), or *** ($p < 0.0005$).

Fig.5- The effects of *YAP1 knockdown* on 5-FU response in tumors formed in NSG mice. Tumor growth with and without 5-FU treatment (50mg/kg/body weight) was measured for caliper twice a week. **A** shows tumor growth curves from LoVo-R control cells. **B** shows tumor growth curves from LoVo-R shYAP cells. Significant difference was indicated by ** ($p<0.005$).

Fig. 6- Kaplan-Meier Survival analysis comparing patients with high YAP1 signaling axis in the tumor with those patients with low YAP1 signaling. The results here were derived from a public database generated by the TCGA Research Network: http://cancergenome.nih.gov/ and http://www.cbioportal.org/

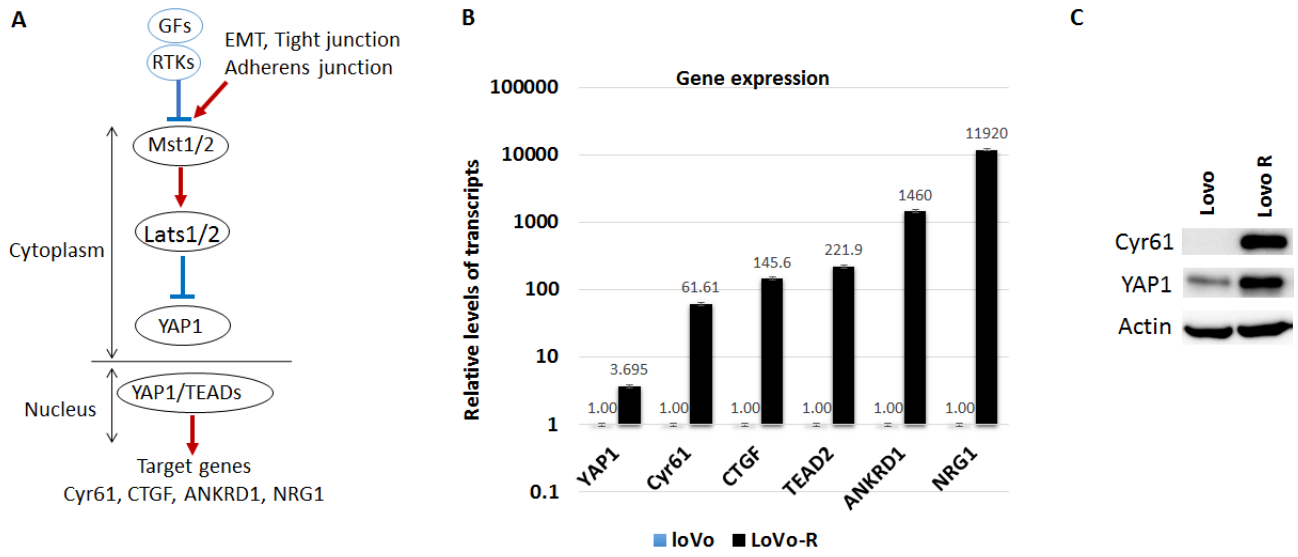


Figure 1

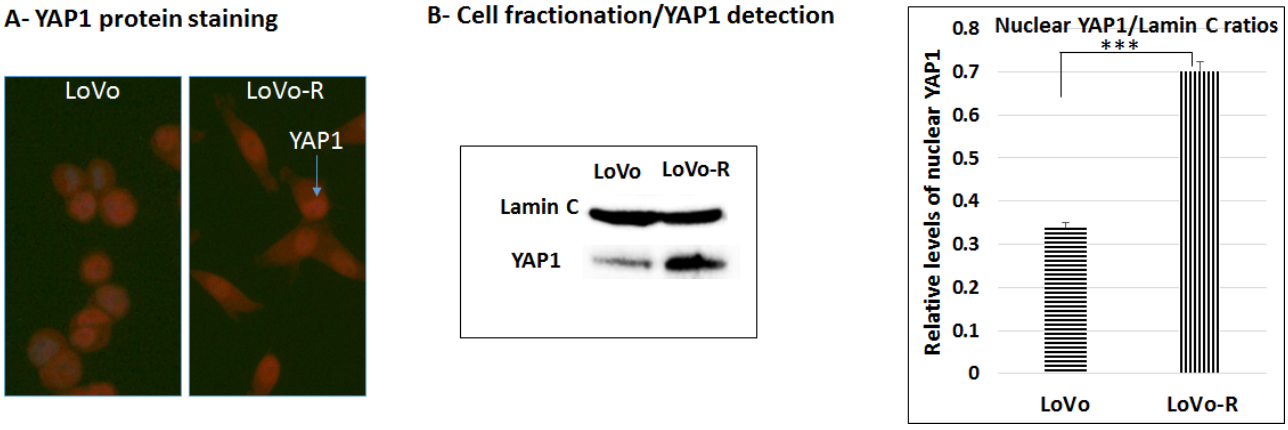


Figure 2

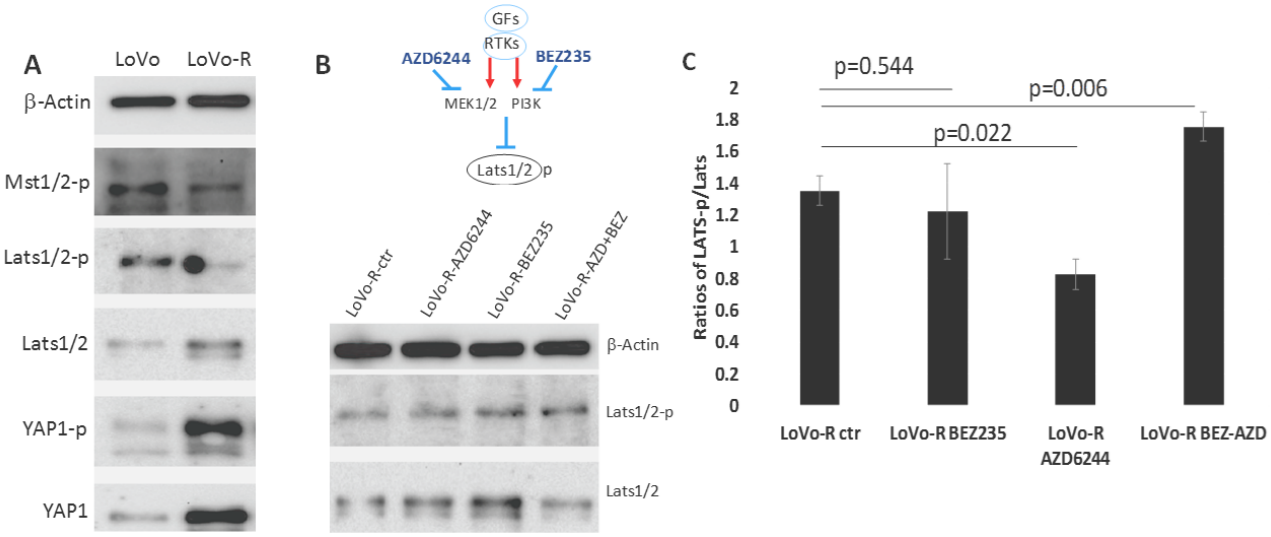
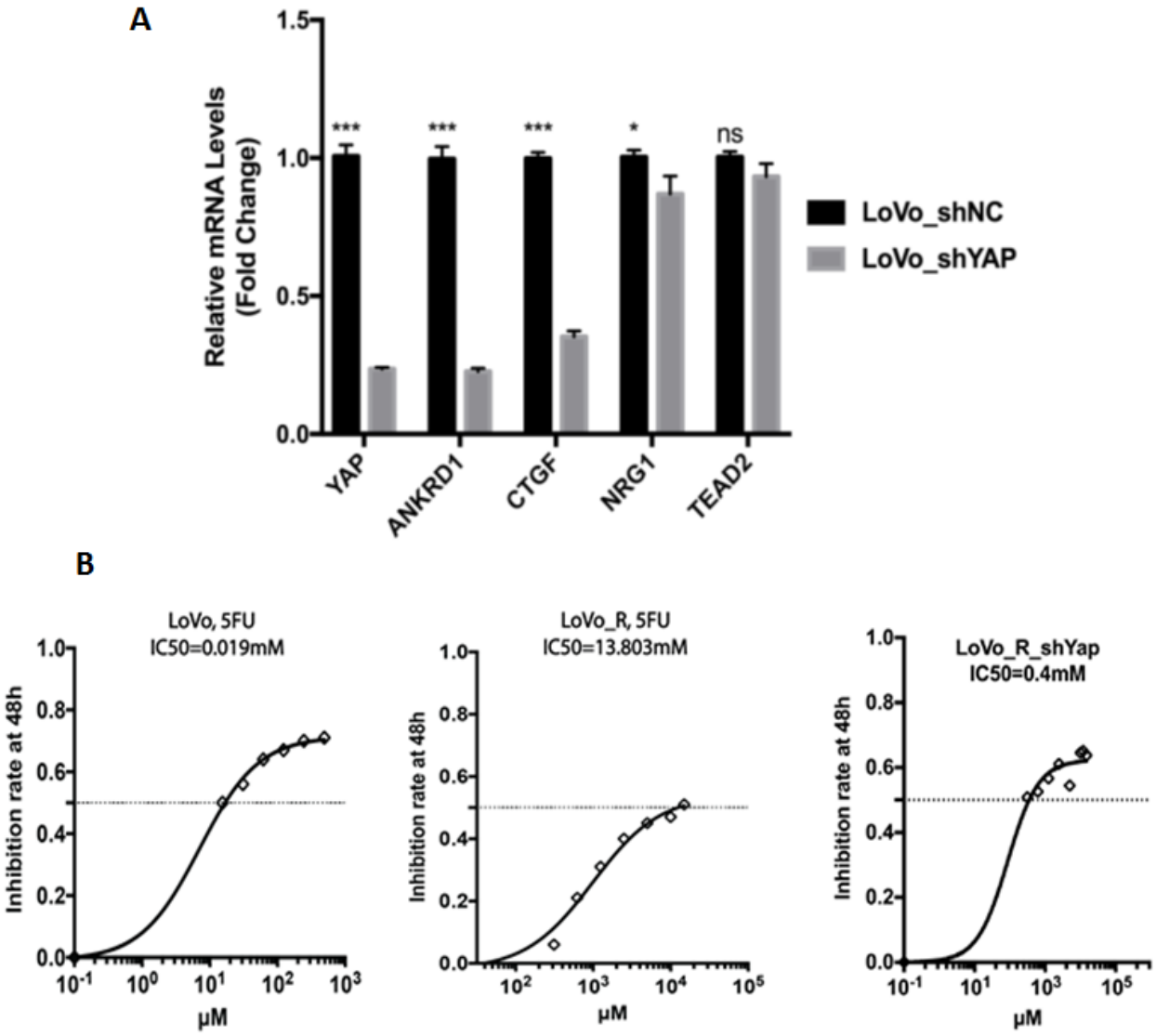


Figure 3



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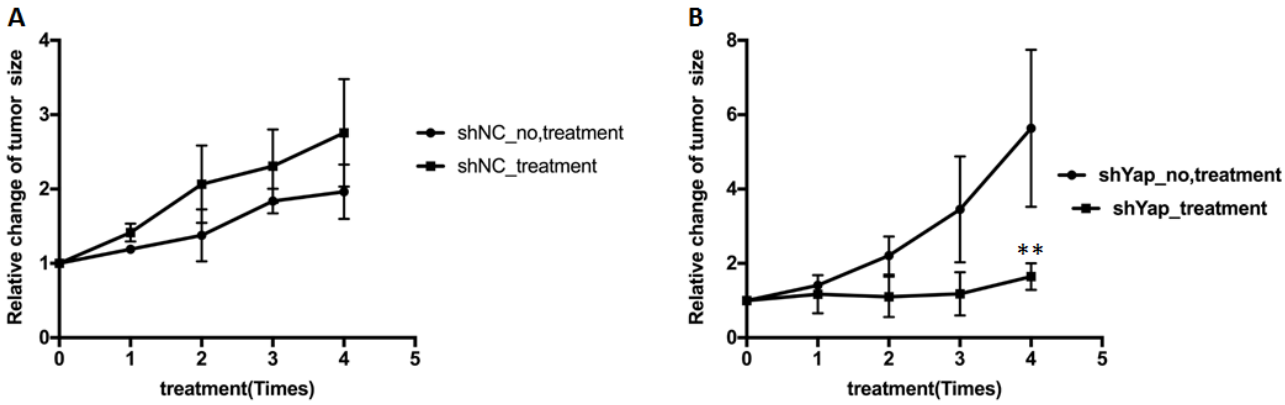

Figure 5

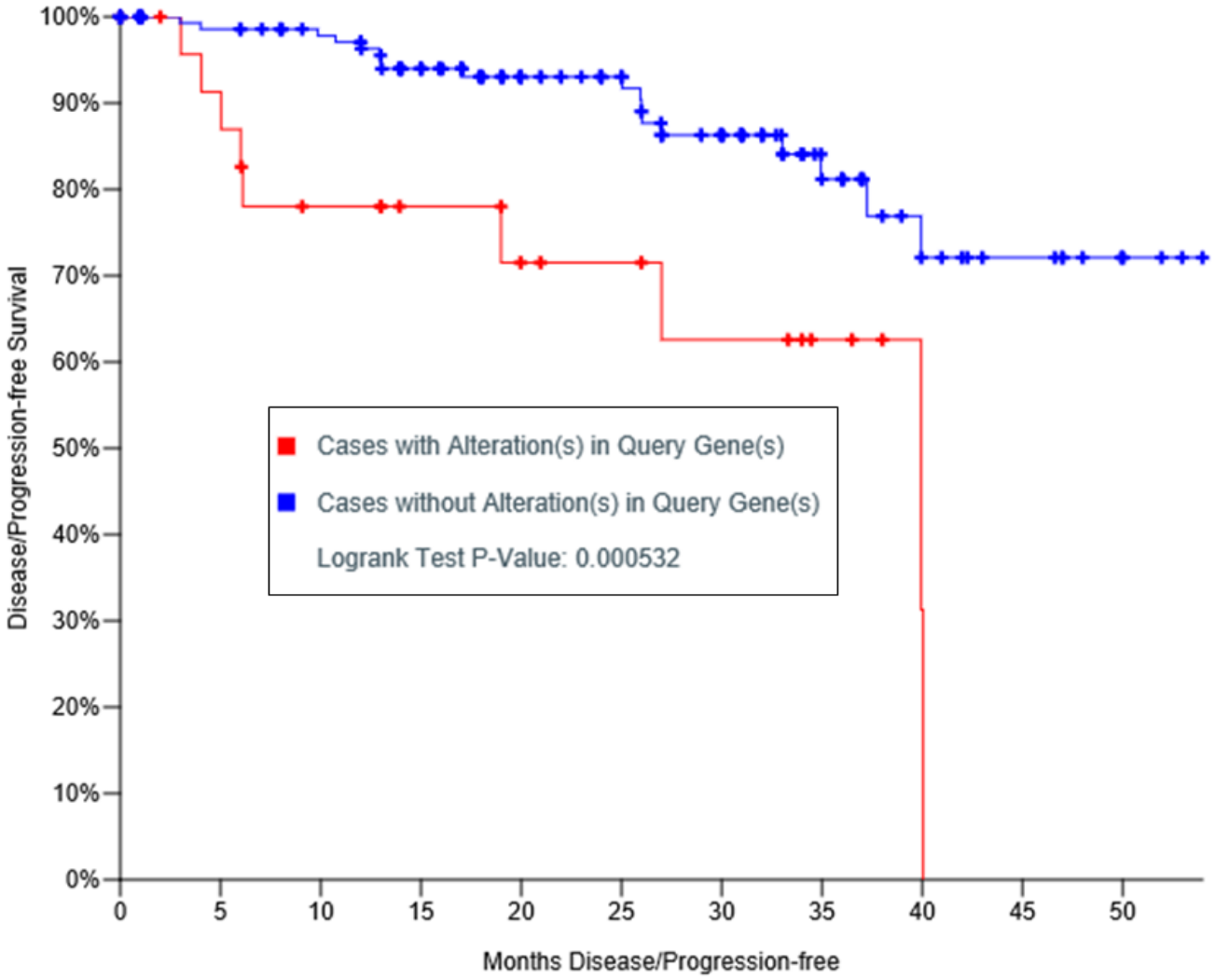

Figure 6

