

Original Article

FLNA promotes chemoresistance of colorectal cancer through inducing epithelial-mesenchymal transition and smad2 signaling pathway

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Abstract: Chemoresistance is a leading cause of tumor relapse and treatment failure in colorectal cancer (CRC) patients and is correlated with epithelial-mesenchymal transition (EMT). This study was aimed to explore the mechanism of EMT in chemoresistant CRC. Bioinformatic method was used to screen differentially expressed genes between 5-FU sensitive and resistant CRC cells. Immunohistochemistry staining was utilized to analyze the expression of FLNA in CRC tissues. The roles of FLNA in chemoresistance were validated via loss-of-function and gain-of-function experiments in vitro and in an orthotopic CRC animal model. The regulation of c-Met signaling by FLNA was explored via Co-Immunoprecipitation and luciferase reporter assays. Our results suggested FLNA directly regulated the metastasis and EMT of chemoresistant CRC cells. Moreover, c-Met-AKT mediated ser2152 phosphorylation of FLNA was demonstrated to be correlated with EMT. In turn, FLNA enhanced c-Met promoter activity by its interaction with smad2. Clinically, the expression of FLNA was significantly associated with c-Met protein levels in CRC tissues. These data established that FLNA could be a novel and reliable CRC marker and a potential therapeutic target against CRC.

Keywords: Colorectal cancer, chemoresistance, FLNA, smad2, EMT

Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of cancer-related mortality worldwide [1]. Although chemotherapy is well demonstrated to reduce tumor burden and prolong survival, it remains a palliative treatment since most CRC patients eventually exhibit drug-resistance [2]. Currently, 5-fluorouracil (5-FU) is widely used as one of the classic and basic drugs in adjuvant chemotherapy and palliative chemotherapy of CRC patients. 5-FU resistance is often coupled with local recurrence and distant metastasis, which always contribute to failure of anticancer therapy [3]. Thus, it is necessary to reveal the potential mechanisms of 5-Fu resistance in CRC

patients. Epithelial-mesenchymal transition (EMT), characterized by cytoskeletal transformation and decreased expression of cell adhesion molecules (such as E-cadherin), exhibited important roles in embryonic development, chronic inflammation, tissue remodeling, cancer metastasis as well as fibrosis [4]. Tumor cells could obtain aggressive phenotypes including migration, invasion, anti-apoptosis, and degradation of extracellular matrix through EMT [4]. Recent evidences indicated that EMT might be responsible for the aggressive phenotype in chemotherapy-resistant cancer cells. EMT can be observed in adriamycin-resistant gastric cancer, cisplatin-resistant neuroblastoma and folate-resistant lung cancer [5-7]. Several studies revealed that EMT is cor-

related with 5-FU resistance in CRC [8, 9]. However, the molecular mechanism that regulates EMT in 5-FU resistant colorectal cancer is still elusive and needs further investigation.

Filamin A (FLNA), the most abundant and widely distributed member of the filamin family (Filamin A, Filamin B and Filamin C), was first identified as a non-muscle actin filament cross-linking protein in 1975 [10]. FLNA crosslinks actin filaments into an orthogonal network, which contributes to stabilize the cytoskeleton network and supports cell integrity [11, 12]. Interestingly, several studies showed that FLNA associates with multiple functional non-cytoskeletal proteins and is involved in several unrelated pathways that regulate cell migration and adhesion [13]. FLNA was generally revealed as a cancer promoting protein in multiple human malignancies including metastatic melanoma, lung cancer and hepatocellular carcinoma [14-16]. However, Xu et al. reported that FLNA expression was decreased in breast cancer tissues and was negatively correlated with lymph node metastasis. Their biological studies indicated that knockdown of FLNA could promote cell migration and invasion [17]. These studies revealed controversial roles of FLNA in human malignancies. Accumulating evidences showed that FLNA ser2152 phosphorylation was critical for its biological functions [18]. Woo et al. first reported that N-terminal kinase domain of RSK could phosphorylate FLNA on Ser2152 in response to mitogens [19]. Later studies revealed that epidermal growth factor (EGF) stimulation promoted $\alpha 5\beta 1$ integrin activation of human cancer cell lines through p90RSK-dependent FLNA phosphorylation [20]. The carcinogenic effect of FLNA ser2152 phosphorylation was also well demonstrated in breast cancer. Ravid et al. reported that caveolin-1 up-regulated FLNA ser2152 phosphorylation via Akt pathway, thereby mediating breast cancer cell migration [21]. Cyclin D1 interacted with FLNA and induced its ser2152 phosphorylation in breast cancer cell adhesion and motility [22]. Therefore, it seemed that the role of FLNA expression level and phosphorylation status in tumor progression should be discussed separately according to the types of tumors.

The clinical expression and biological function of FLNA in colorectal cancer seemed to be controversial. Tian et al. reported that FLNA was

lower expressed in 46 colorectal adenocarcinoma tissues compared with normal mucosa [23]. However, in another study, FLNA overexpression and ser2152 phosphorylation were demonstrated to be correlated with snail-induced EMT and cell adhesion [24]. In current study, we further analyzed the expression and clinical significance of FLNA in CRC clinical samples, and initially elucidated the potential role of FLNA in 5-FU resistant CRC cells. Developing drugs that target FLNA or inhibit FLNA ser2152 phosphorylation might contribute to overcome 5-FU resistance of CRC.

Materials and methods

Biological information analysis

Colorectal cancer cell lines and 5-FU treatment information were obtained from the CCLE database and the CTRP database. The differentially expressed genes were compared by 'limma' package of R. Functional association network of the differentially expressed genes were constructed by Cytoscape software. RNA-seq data of CRC tissues in TCGA database and the matched clinical data were downloaded. The functional enrichment analysis of FLNA was performed using the 'clusterProfiler' package of R, and the bubble map was drawn using the "ggplot2" package of R.

Clinical specimens

The clinical research protocol was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (Zhengzhou, China). All patients provided written informed consent. A total of 152 patients were enrolled in this study. Paired tumor and adjacent non-tumor CRC tissues were collected from patients who underwent surgical resection in The First Affiliated Hospital of Zhengzhou University between January 2011 and December 2018. The specimens were immediately frozen in liquid nitrogen or fixed in 10% formalin and paraffin embedded. No patient had received any anti-tumor treatment preoperatively. Immunohistochemistry staining and scoring of each slide were performed as previously described [25]. Slides were examined by two researchers (Yang and Zhao) independently. FLNA expression status was determined according to the staining intensity and percentage of positive cells. Percentage of positive cells was deter-

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mined as 0 (<10%), 1 (10%-25%), 2 (26-50%) and 3 (>50%). Staining intensity was determined as no staining (0), weak staining (1), moderate staining (2) and strong staining (3). The final score of each case was determined by multiplying the score of intensity and score of positive cells. Therefore, cases were considered negative if the final score was 0-3 and positive if the final score was 4-9.

Cell culture conditions and reagents

Human CRC cell lines, HCT116 and HT29, were purchased from the American Type Culture Collection (Manassas, VA, USA). 5-FU resistant HCT116 and HT29 (HCT116R and HT29R) cell lines were established by continuous culture in media containing increasingly higher concentrations of 5-FU (Selleckchem, Houston, TX, USA). HCT116 and HT29 cell lines were cultured in RPMI 1640 (Corning Cellgro®, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). HCT116R and HT29R cell lines were cultured in the same medium containing 1 µg/mL of 5-FU. All cells were maintained in a humidified incubator at 37°C with 5% CO₂. Crizotinib (c-Met inhibitor), LY294002 (PI3K/AKT pathway inhibitor) and LY2109761 (smad2 signaling inhibitor) were purchased from Selleckchem (Houston, TX, USA) and used according to manufacturer's instructions.

Short hairpin RNA (shRNA) and constructs

The short hairpin RNA (shRNA) targeting FLNA were established using the following targets: sequence 1, 5'-GGGCTGACAACAGTGTGGTGC-3' (targeting nt 3323-3343); sequence 2, 5'-GGTGCTGCCTACTCATGATGC-3' (targeting nt 4663-4683). Stably transfected cells were selected with 5 µg/ml puromycin and tested regularly by immunoblotting to ensure downregulation of FLNA. pGL3-c-Met and pcDNA3-smad2 plasmids were obtained from Sangon Biotech (Shanghai, China) and amplified by PureLink HiPure Plasmid Filter Midiprep Kit (Invitrogen). pcDNA3-myc expression vectors coding for wild-type and S2152A FLNA were obtained from Addgene (Cambridge MA, USA). Three µg of empty vector pcDNA3 (control) or the vector expressing pcDNA3-myc-FLNA (WT) or mutant pcDNA3-myc-FLNA (S2152A) were transfected into HCT116 or HT29 cells using Lipofectamine 2000 (Invitrogen). Stable clones were obtained

by G418 (500 µg/ml) selection. All plasmids transfection was performed according to the manufacturer's instruction.

Cell proliferation and colony formation assay

Cells were seeded in a 96-well plate overnight at a density of 5×10^3 cells per well and treated with indicated doses of 5-FU (2, 4, 6, 8, 16, 32, 64, 128, 256, 512 µM) for 48 h. Then, cells were incubated with 10 µL CCK-8 for 60 min at 37°C, 5% CO₂. The absorption value was detected at 450 nm with a spectrophotometer. Each assay was conducted in triplicate and repeated three times. For colony formation assay, 1×10^3 treated cells were seeded into 6-well plates. The drug-containing medium was changed every 3 days. At 15th day, these plates were washed with phosphate buffered saline (PBS) twice, fixed by methanol for 10 min and stained with 0.1% crystal violet solution for further analysis.

Flow cytometry analysis

Apoptosis was examined by flow cytometric analysis. Cells were collected, washed with PBS and incubated with Annexin V and propidium iodide (BD Biosciences, San Jose, CA, USA) for 15 min. Then cell apoptosis was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Sparks, MD) according to the manufacturer's instruction. All the assays were performed in triplicate.

Cell migration, invasion and adhesion assay

Cell migration/invasion assays were performed to analyze the migration and invasiveness of CRC cells according to a previously described protocol [25]. The adhesion of CRC cells to ECM components was evaluated using a CytoSelect 48-Well Cell Adhesion Assay Kit (Cell Biolabs, San Diego, CA, USA) following the manufacturer's instructions. Firstly, cells were seeded onto the coated substrate and the adherent cells were captured. After unbound cells were washed away, the adherent cells were fixed and stained. Finally, the stain is extracted and quantified colorimetrically.

Quantitative reverse transcription-PCR

Total RNA was extracted from CRC cells and subjected to qRT-PCR as previously described

[25]. Primers for qRT-PCR were listed in [Supplementary Table 1](#).

Immunoblotting and Co-Immunoprecipitation assay

Immunoblotting and Co-Immunoprecipitation (Co-IP) assays were performed as previously described [25]. The primary antibodies were: anti-GAPDH and anti-HGF from Abcam (Cambridge, MA, USA); anti-FLNA from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-FLNA, anti-phospho-AKT, anti-smad2, anti-phospho-smad2, anti-c-Met, anti-vimentin, anti-N-cadherin and anti-E-cadherin from Cell Signaling Technology (Beverly, MA, USA).

Immunofluorescence assay

Cells were cultured on confocal dishes, fixed in methyl alcohol, permeabilized with 0.5% Triton X-100, blocked with 5% BSA (in TBST), and incubated with anti-smad2 (1:100) at 4°C overnight. Cells were then incubated with secondary antibody (Cell Signaling Technology) at room temperature for 30 min. Immunofluorescence (IF) images were obtained with confocal microscopy (Olympus, Japan).

Nude mouse tumor transplantation model

All animal procedures were performed with the approval of the Local Medical Experimental Animal Care Commission of the First Affiliated Hospital of Zhengzhou University. Mice were randomly grouped (5 mice per group). Four-week-old male nude BALB/C mice were subcutaneously injected with HCT116/shFLNA or control cells (1×10^6). When tumor volumes reached approximately 0.2 cm³, mice were subcutaneously injected with control vehicle (DMSO) or 5-FU (20 mg/kg every week). Body weights, tumor length (L) and width (W) were measured every three days. Tumor volumes were calculated by the following formula: $V=0.5 \times LW^2$. Thirty days later, mice were sacrificed. Subcutaneous tumor grafts were excised and weighed. For lung metastasis assay, 2×10^6 cells re-suspended in 100 µl PBS were injected into caudal vena. On the 60th day, mice were killed by cervical decapitation. The pulmonary metastatic masses were removed and fixed with 10% buffered formalin for hematoxylin-eosin (HE) staining.

Luciferase assay

The c-Met promoter fragments were amplified from human genomic DNA, and were inserted into pGL3-basic vector. The smad2/FLNA (WT)/FLNA (S2152A) overexpressing or control cells were co-transfected with pGL3-c-Met-promotor constructs. Luciferase assays were performed 24 h after transfection using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. The ratios of luminescence intensities were measured relative to that of the pGL3 empty vector.

Statistical analysis

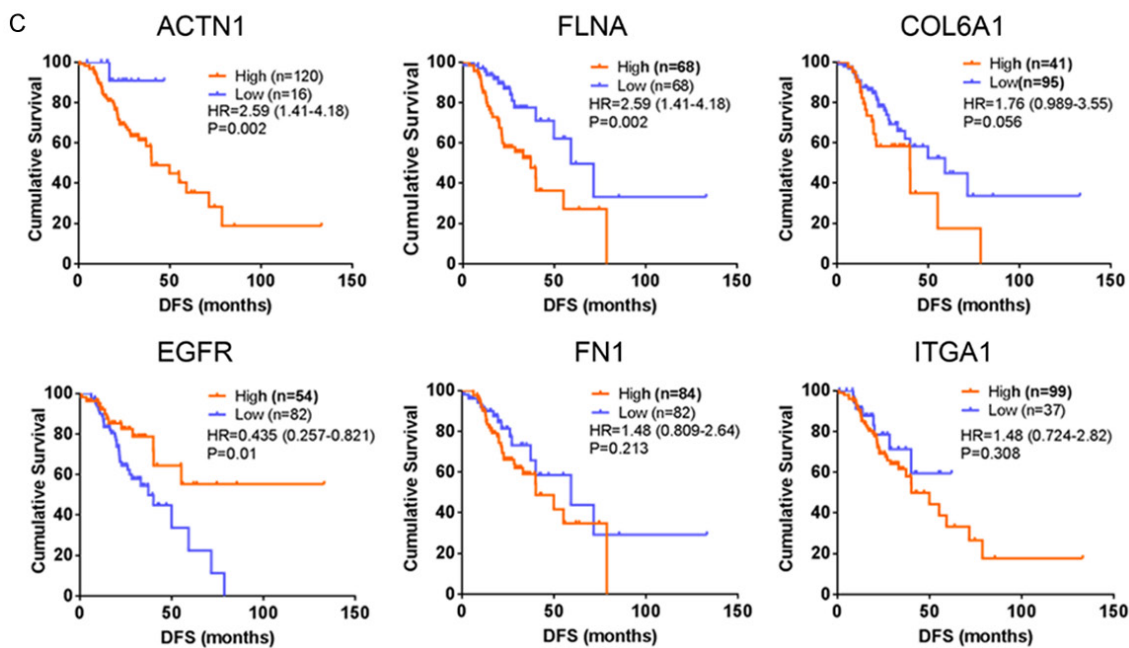
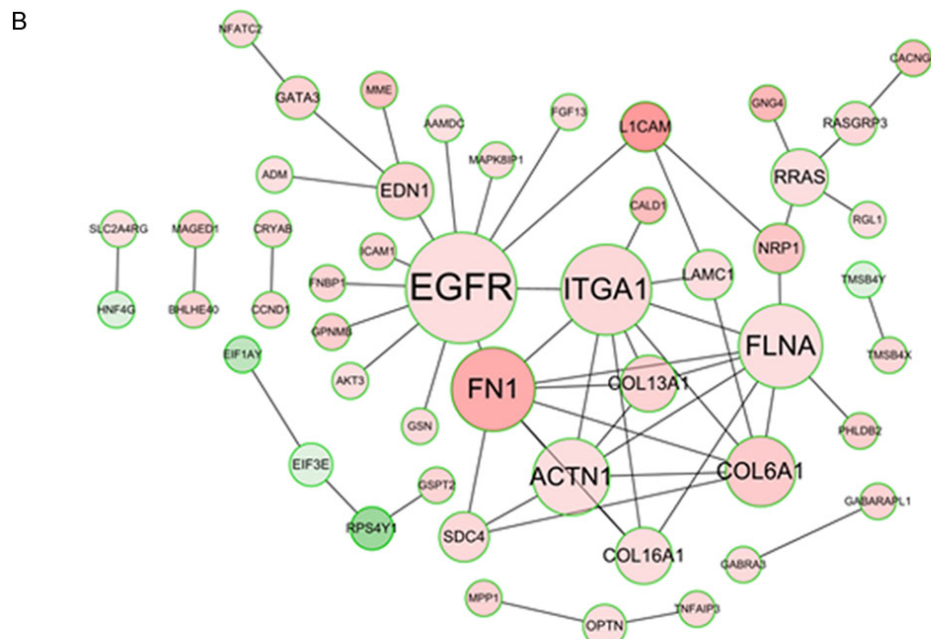
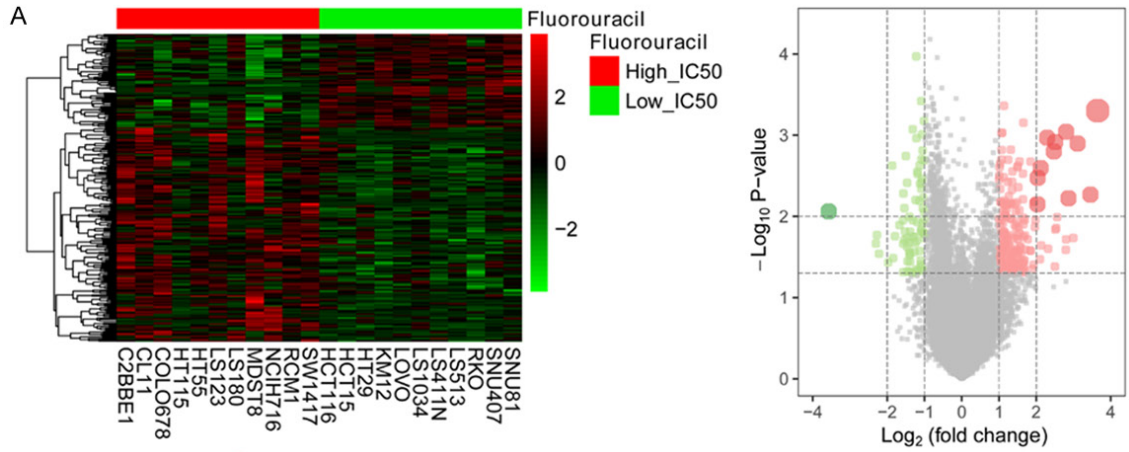
The statistical analysis was performed using SPSS version 25.0 for Windows (SPSS, Chicago, USA). All data were presented as the mean \pm SD and repeated from at least three independent experiments. Statistical difference between two groups was examined by Student's t-test. Multiple comparisons were conducted by one-way analysis of variance. *P* value less than 0.05 was considered statistically significant.

Results

Identification of FLNA as a potential target of 5-FU resistance in CRC

To explore potential target of 5-FU resistance in CRC, we first extracted the 5-FU treatment information of CRC cell lines by using the CCLE database and the CTRP database. As shown in [Supplementary Table 2](#), CRC cell lines were divided into 3 groups (Low IC50, Moderate IC50, High IC50) according to different sensitivities to 5-FU. The differentially expressed genes between low IC50 group and high IC50 group were compared by "limma" package in R, and 254 genes (fold change >2, *P*<0.05) were obtained (176 of which were up-regulated in High IC50 group, 77 were down-regulated, **Figure 1A** and [Supplementary Table 3](#)). We next used the above genes to build a functional association network, and the most relevant 6 genes (ACTN1, COL6A1, EGFR, FLNA, FN1 and ITGA1) were used for further analysis (**Figure 1B**). We further analyzed the prognostic significance of these genes in patients receiving 5-FU based chemotherapy. As the result, only FLNA was up-regulated in High IC50 group and correlated with poorer survival status of CRC

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Figure 1. Comprehensive analysis of FLNA mediated 5-FU resistance in CRC. A. The heatmap and volcano plot of differentially expressed genes in 5-FU sensitive and resistant CRC cells. B. Functional association network of the differentially expressed genes. C. Prognostic significance of ACTN1, COL6A1, EGFR, FLNA, FN1 and ITGA1 in patients receiving 5-FU based chemotherapy.

patients (**Figure 1C**). Thus, we selected FLNA for further characterization.

High FLNA expression correlates with poor survival of CRC patients

To clarify whether FLNA expression is aberrantly elevated in CRC tissues, we first detected the mRNA levels of FLNA in 40 paired CRC tissues. As shown in [Supplementary Figure 1A](#), FLNA mRNA levels were highly expressed in CRC tissues compared with normal tissues. The clinical significance of FLNA was further analyzed by IHC assay of FLNA protein expression in 152 paired CRC tissues (**Figure 2A**). High levels of FLNA expression were found in 86 of 152 (56.6%) CRC samples. To investigate the clinical significance of FLNA in CRC, we analyzed the association between FLNA expression and the clinicopathologic characteristics of CRC patients. As shown in [Supplementary Table 4](#), FLNA expression was significantly associated with tumor size ($P=0.048$), extent of invasion ($P=0.022$) and lymphatic metastasis ($P=0.009$), while there was no significant association between FLNA expression and age, gender, location, tumor histology and CEA level. Additionally, FLNA high expression was associated with short Overall Survival (OS, $P<0.01$) and Disease Free Survival (DFS, $P<0.01$, **Figure 2B**) time. We next assessed the prognostic significance of FLNA in a TCGA pan-cancer data set obtained from Gene Expression Profiling Interactive Analysis (GEPIA) online database (<http://gepia.cancer-pku.cn>). The result indicated that higher FLNA expression was correlated with shorter Disease Free Survival (HR=1.9, $P=0.012$) but not Overall Survival (HR=1.6, $P=0.058$) of CRC patients (**Figure 2C**).

FLNA regulates the sensitivity of CRC cells to 5-FU treatment

Two 5-FU resistant cell models (HT29R and HCT116R) were established from the human CRC cell line HT29 and HCT116 through serial 5-FU induction. We evaluated the IC₅₀ values of HT29, HT29R, HCT116 and HCT116R cells under 5-FU treatment. HT29R and HCT116R cells exhibited significantly resistance to 5-FU treatment in vitro compared with parental HT29

and HCT116 cells (**Figure 3A**). As to immunoblotting assay, protein levels of FLNA and its phosphorylated form p-FLNA (Ser2152) were up-regulated in HT29R/HCT116R cells compared with the parental cells (**Figure 3B**). We investigated the role of FLNA in chemoresistance by knocking down its expression in the two acquired 5-FU resistant cells. As indicated in **Figure 3C**, both shRNAs targeting FLNA suppressed FLNA expression in HCT116R or HT29R cells compared with the mock shRNA. Meanwhile, FLNA knockdown in HCT116R or HT29R cells resulted in a fifty percent decrease ($P<0.01$) in its sensitivity to 5-FU. Engineered cell lines were then treated with 5-FU for colony formation. shRNA HCT116R and shRNA HT29R cells had relatively lower colony survival rates compared to mock cells under 5-FU treatment (**Figure 3D**). To examine whether FLNA mediated 5-FU resistance through the apoptosis, we performed flow cytometry analysis by staining cells with annexin V-FITC and PI. As the result, knockdown of FLNA only slightly promoted the apoptotic rate of HCT116R or HT29R cells without 5-FU treatment, while knockdown of FLNA in HCT116R or HT29R cells significantly promoted apoptosis with 5-FU treatment (**Figure 3E**). Since AKT could phosphorylate FLNA at Ser2152. We then stimulated the parental HCT116 cells with different concentrations of HGF to activate c-Met-AKT signaling pathway. Immunoblotting analysis of HCT116 cell lysates showed that Ser2152 phosphorylation of FLNA began 5-10 min following HGF stimulation and was increased by 3-fold within 30 min (**Figure 3F**). To address the role of FLNA overexpression and phosphorylation in the regulation of 5-FU resistance, HCT116 or HT29 cell lines that expressed either FLNA (WT) or the non-phosphorylatable mutant FLNA (S2152A) were constructed (**Figure 3G**). In the presence of HGF, stable cells expressing FLNA (WT) exhibited higher colony survival rates and lower apoptosis rates, while cells expressing FLNA (S2152A) showed no difference compared with the control cells. Following the addition of HGF, cells expressing FLNA (WT) exhibited significantly higher colony survival rates (**Figure 4A**) and lower apoptosis rates (**Figure 4B**) than other groups.

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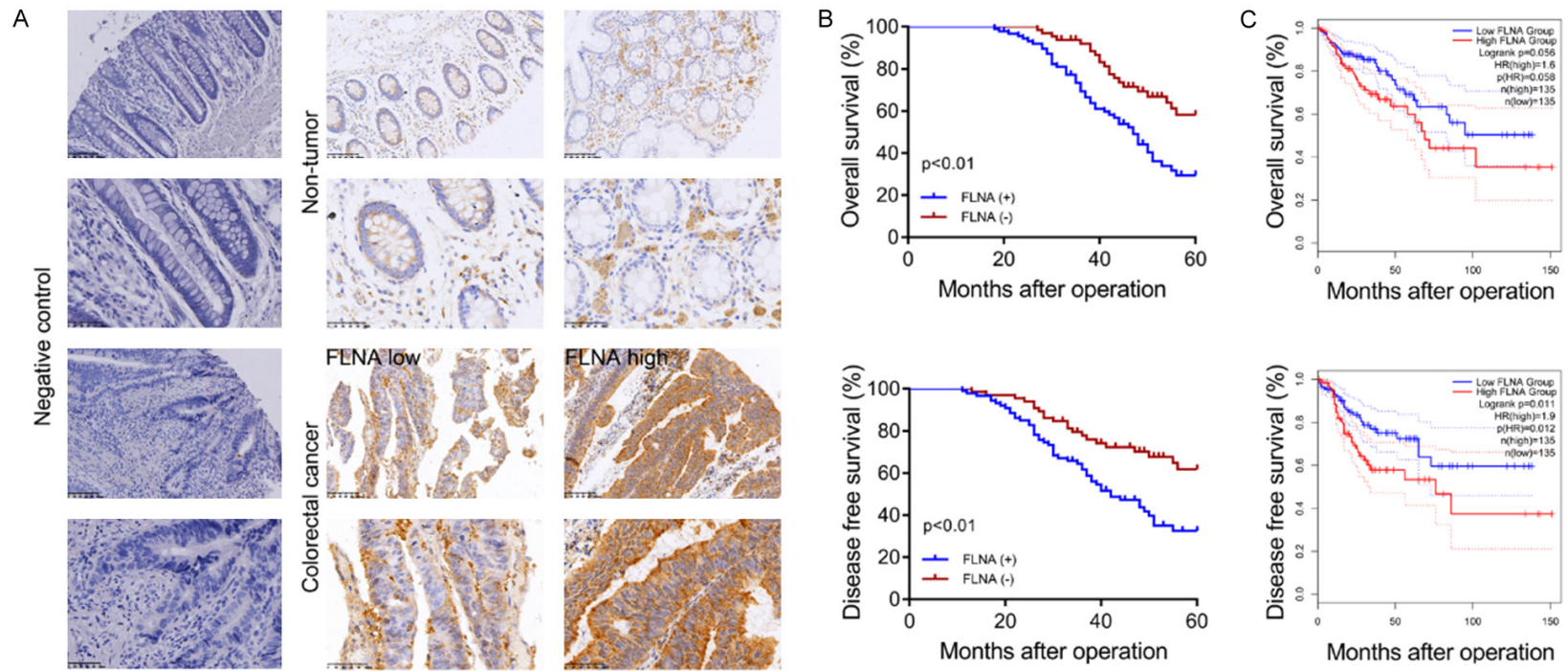
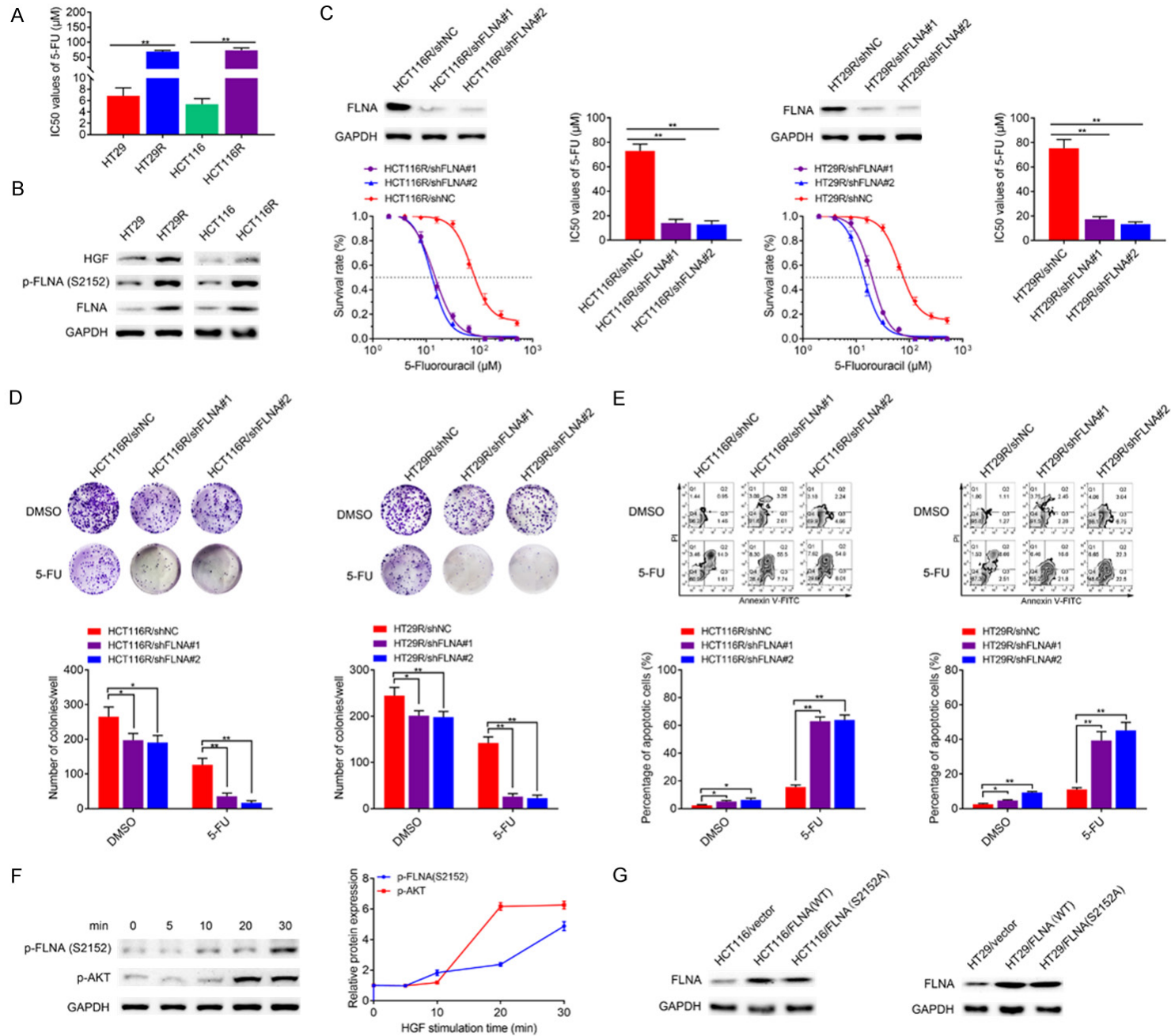


Figure 2. FLNA is highly expressed in CRC and associated with poor prognosis. A. The typical IHC images of FLNA expression in CRC tissues. B. Kaplan-Meier analysis of OS and DFS of 152 CRC patients. C. OS and DFS curves of CRC patients from TCGA database.

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Figure 3. FLNA is critical for 5-FU resistance of CRC cells. A. IC50 dose of acquired 5-FU resistant cells and the parental cells. B. Immunoblotting analysis shows 5-FU resistant cells exhibit higher FLNA, p-FLNA (Ser2152) and HGF expression than parental cells. C. Protein of HCT116R/shNC, HCT116R/shFLNA#1, HCT116R/shFLNA#2, HT29R/shNC, HT29R/shFLNA#1 and HT29R/shFLNA#2 were extracted and the knockdown efficacy of FLNA were verified by immunoblotting. Statistical chart showed IC50 dose of 5-FU calculated from measurement of cell viability in different concentrations of 5-FU. D. Representative images of colony formation of transfected CRC cells after 5-FU treatment. E. Representative images of apoptosis of transfected CRC cells after 5-FU treatment. F. HCT116 cells were stimulated with HGF (50 ng/ml) at the indicated times. Cell lysates were immunoblotted using anti-p-FLNA and anti-p-AKT. Bands for p-FLNA and p-AKT were quantified and normalized to GAPDH. G. Lysates from control cells (HCT116/vector, HT29/vector), clonal cell lines expressing either wild-type FLNA (HCT116/FLNA (WT), HT29/FLNA (WT)) or the mutant FLNA (HCT116/FLNA (S2152A), HT29/FLNA (S2152A)) were immunoblotted to verify FLNA expression. All data are presented as the mean \pm SD of three experiments. * $P < 0.05$, ** $P < 0.01$.

FLNA maintains 5-FU resistance of CRC cells through inducing EMT

To clarify the mechanism of FLNA in 5-FU resistant CRC cells, we analyzed FLNA related signaling pathways by GSEA analysis. Among the top 20 enriched pathways, FLNA was significantly correlated with regulation of actin cytoskeleton, cell adhesion (focal adhesion and cell adhesion molecules) and PI3K-AKT signaling pathway (**Figure 5A**). Since EMT of cancer cells is tightly correlated with drug resistance and is always characterized as enhanced migration and invasion ability, anti-apoptosis, extracellular matrix remodeling as well as cytoskeletal reconstruction, we suspected that FLNA might maintain 5-FU resistance of CRC cells through inducing EMT. We first compared cell motility between the chemoresistant and parental CRC cells. As shown in **Figure 5B**, the parental HCT116 and HT29 cells exhibited a flat epithelial phenotype with extensive cell-cell contacts, whereas HCT116R and HT29R cells exhibited spindle-shaped morphology with thin pseudopods and reduced cell-cell contacts. Since distinct morphology is associated with cell invasiveness and typical EMT characteristics, we compared the adhesion, migration and invasion ability of 5-FU resistant cells and parental cells. As expected, HCT116R and HT29R cells exhibited higher adhesion rates to fibronectin, collagen I, collagen IV, Laminin I and fibrinogen compared with their parental cells, respectively (**Figure 5C**). Additionally, transwell assays showed that migration and invasion capacities of resistant cells were higher than parental cells (**Figure 5D**). Epithelial marker protein E-cadherin levels were reduced, while the levels of mesenchymal marker proteins vimentin, N-cadherin, fibronectin and β -catenin were increased in acquired 5-FU resistant cells (**Figure 5E**). These results suggested that

migration and invasion ability of CRC cells were elevated upon acquired 5-FU resistance. To confirm the necessity of FLNA in EMT process of 5-FU resistance, cell adhesion, migration and invasion assays were performed to detect alterations after shFLNA or shNC transfection. The cells exhibited lower adhesion rates to fibronectin, collagen I, collagen IV, Laminin I and fibrinogen when FLNA was down-regulated (**Figure 6A**). Transwell assays showed that invasion and migration capacities of the resistant-shFLNA cells were lower than that of resistant-shNC cells (**Figure 6B**). We also found that the levels of E-cadherin were increased, whereas the levels of vimentin, N-cadherin, fibronectin and β -catenin were reduced in cells transfected with shFLNA (**Figure 6C**). Using the nude mice model, we noticed that knockdown of FLNA significantly inhibited lung metastases of HCT116R cells (**Figure 6D**).

c-Met-AKT mediated ser2152 phosphorylation of FLNA is essential for its function

We then conducted FLNA gain-of-function assay in parental HCT116 and HT29 cells. In cell adhesion assays, overexpression of FLNA (WT) but not FLNA (S2152A) promoted the adhesion ability compared with control cells (**Figure 7A**). FLNA (WT) expressing cells but not FLNA (S2152A) expressing cells exhibited enhanced cell migration and invasion ability compared with control cells (**Figure 7B**). In addition, the level of E-cadherin was decreased while the levels of vimentin, N-cadherin, fibronectin and β -catenin were increased in FLNA (WT) group but not FLNA (S2152A) group compared with control group (**Figure 7C**). Since mutation of FLNA ser2152 attenuated its oncogenic roles, we attempted to determine whether inhibition of c-Met-AKT pathway, a well-established regulator of FLNA Ser2152 phosphorylation, influ-

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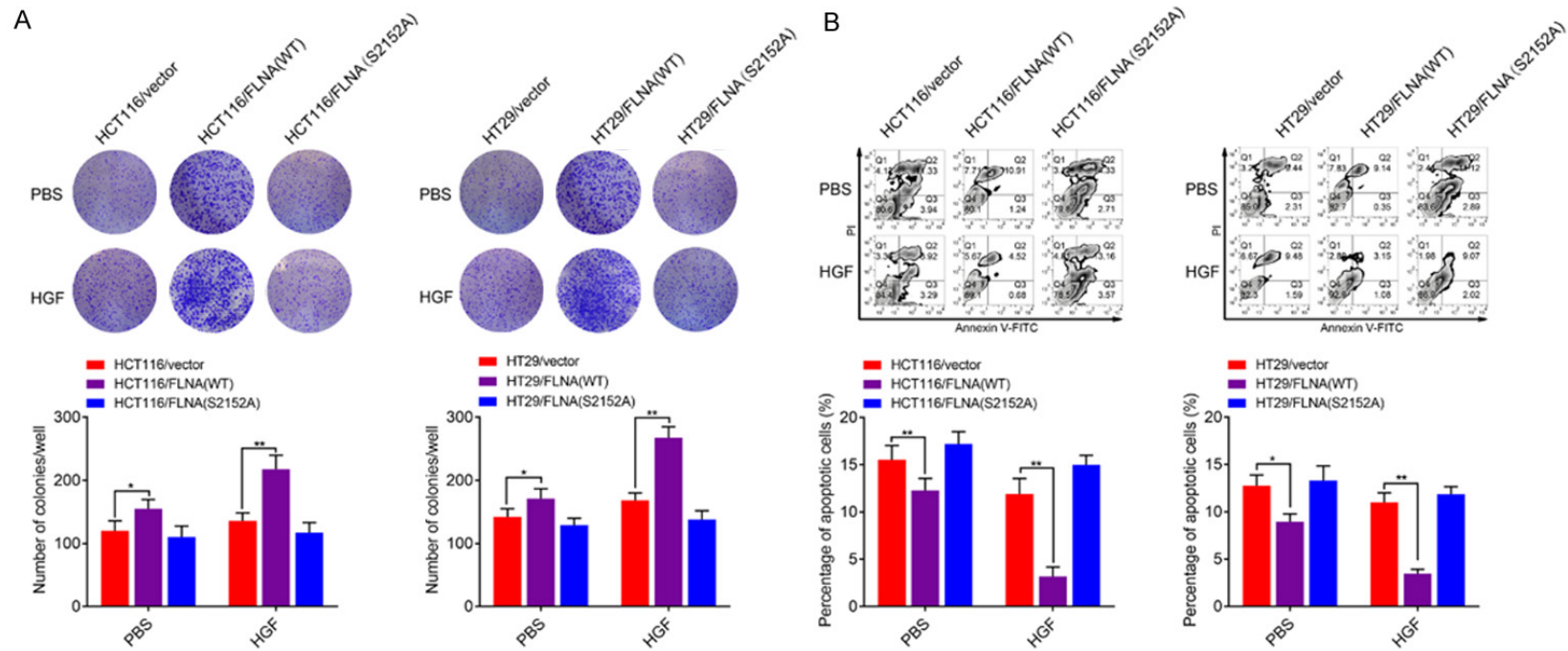
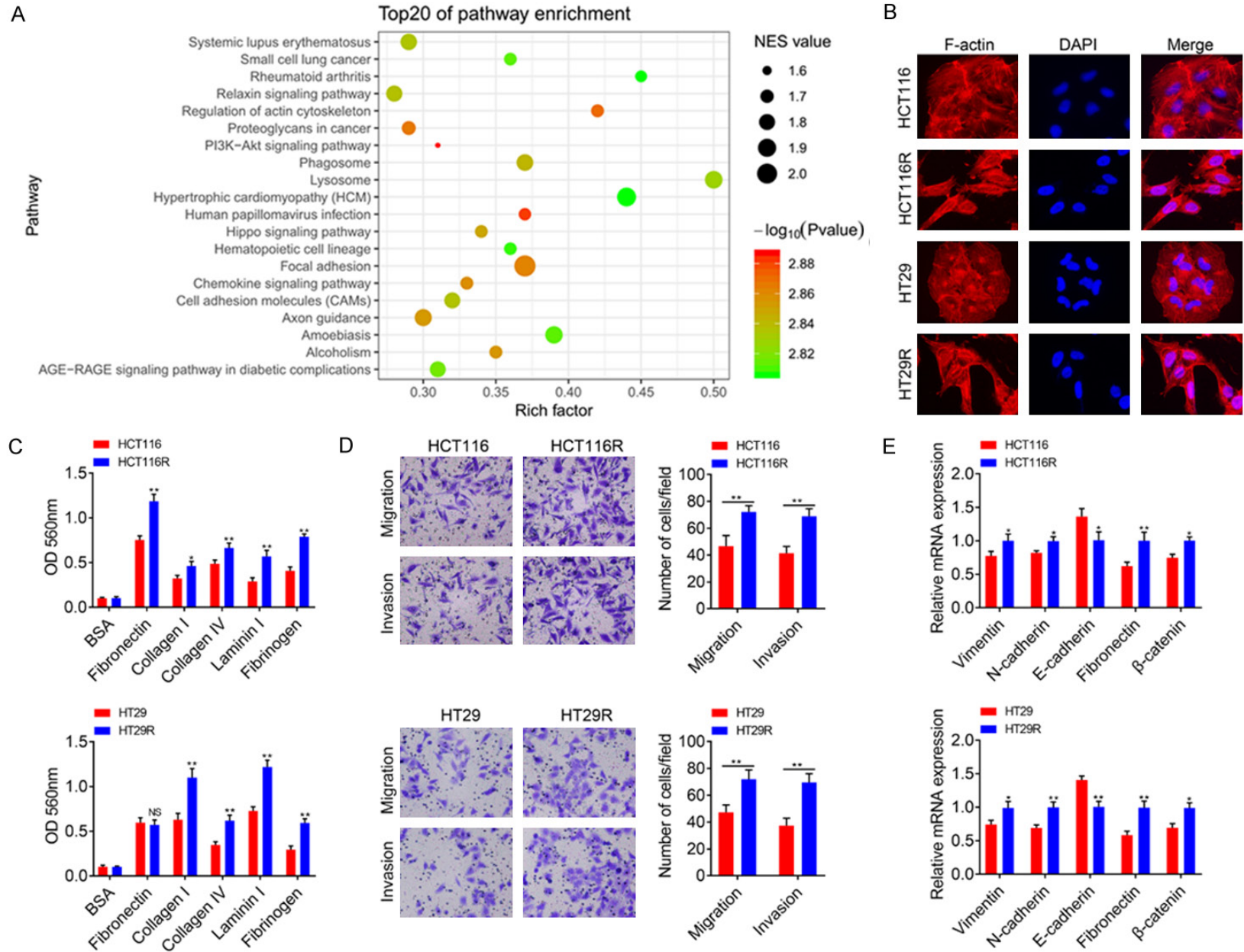


Figure 4. Clonal cell lines expressing either wild-type FLNA or the mutant FLNA were pre-treated with HGF (50 ng/ml) or PBS for 30 min. The colony formation (A) and apoptosis (B) were compared after cultured with 5-FU (10 μ M) for 48 h. All data are presented as the mean \pm SD of three experiments. *P<0.05, **P<0.01.

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Figure 5. FLNA is associated with metastasis and EMT of chemoresistant CRC. A. Bubble chart of top 20 pathways enriched from TCGA data according to FLNA expression. B. Phalloidin staining (red) in 5-FU resistant CRC cells and parental cells. C. HCT116, HCT116R, HT29 and HT29R cells that adhered to plates coated with different ECM components after 30 min of incubation were quantified at OD 560 nm after 30 min incubation. D. Representative figures and data from the transwell assay of HCT116, HCT116R, HT29 and HT29R cells. E. qRT-PCR analysis for the expression of EMT-associated markers in HCT116, HCT116R, HT29 and HT29R cells. All data are presented as the mean \pm SD of three experiments. * $P < 0.05$, ** $P < 0.01$.

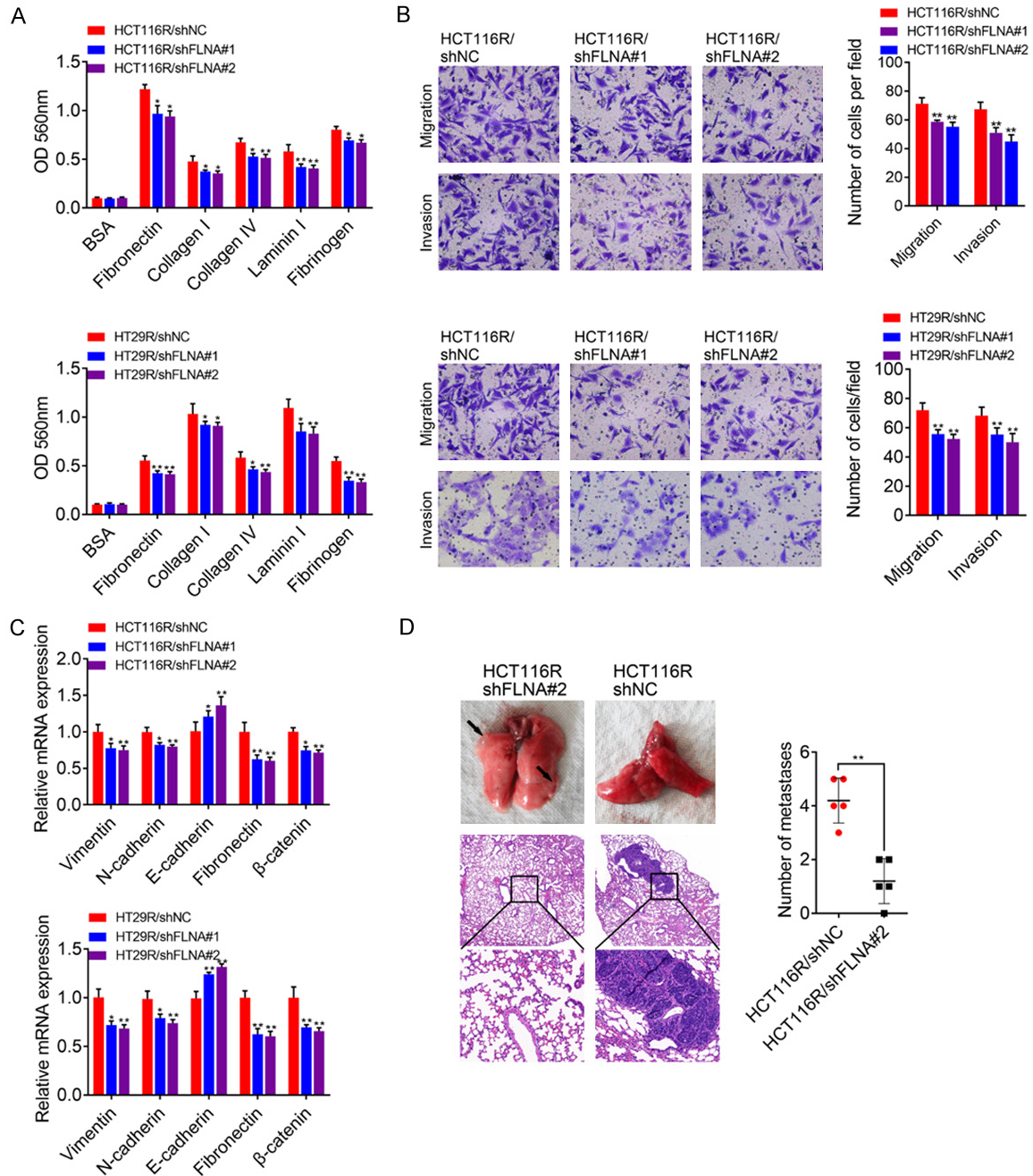


Figure 6. A. Adhesion assay of CRC cells in which FLNA was knocked down. B. Transwell analysis of HCT116R and HT29R cells transfected with shNC, shFLNA#1 or shFLNA#2. Five randomly selected fields were assessed under a microscope. C. qRT-PCR analysis of EMT-associated markers in HCT116R and HT29R cells transfected with shNC, shFLNA#1 or shFLNA#2. D. 2×10^6 HCT116R/shFLNA#2 or HCT116R/shNC cells were injected into nude mice for 60 days to evaluate the lung metastasis ability. Representative figures for lung metastatic tumor were presented by HE staining.

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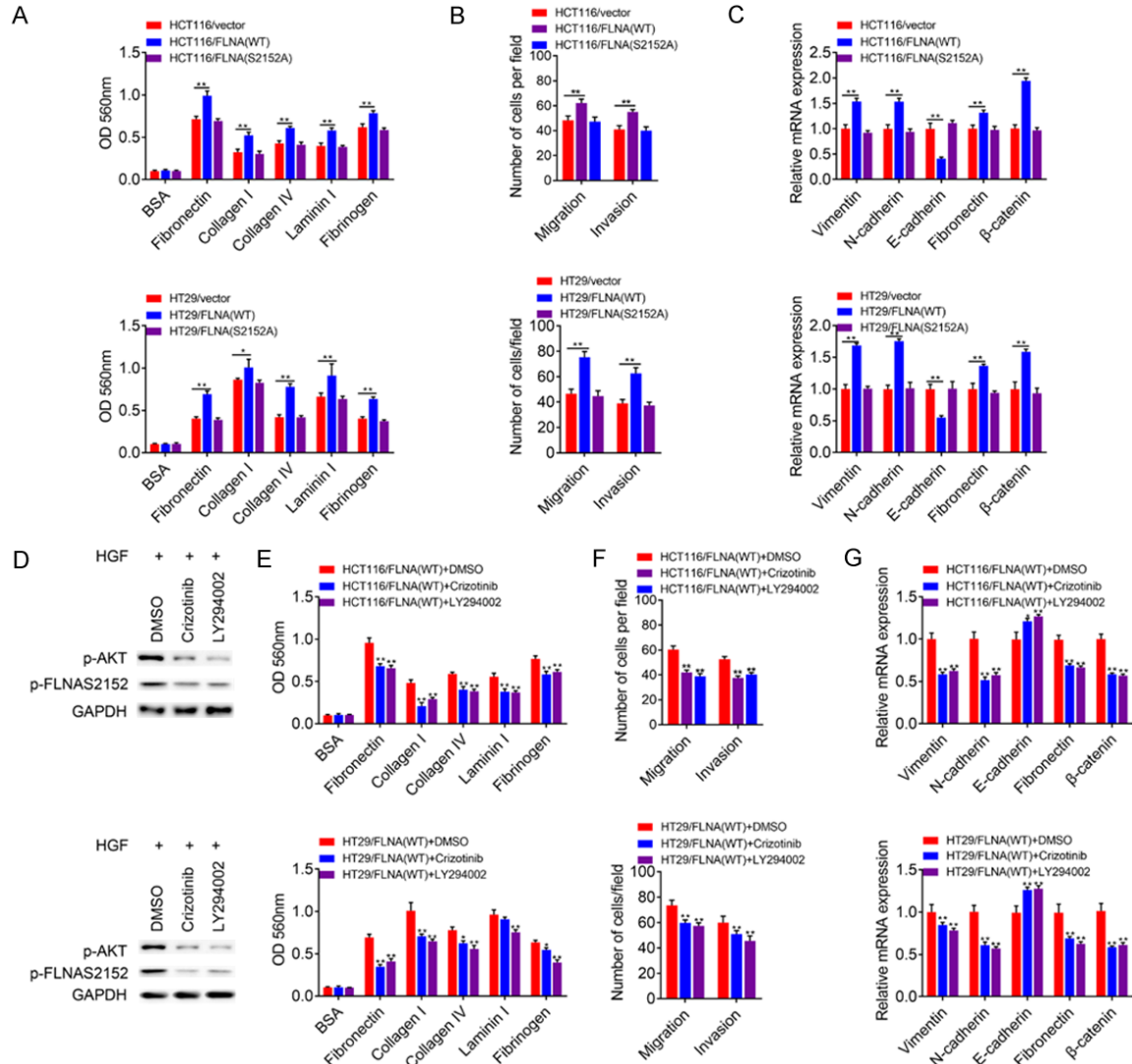


Figure 7. c-Met-AKT mediated ser2152 phosphorylation of FLNA was essential for EMT. (A) HCT116 or HT29 cells were transfected with empty vector, wild-type FLNA, or the mutant S2152A. Then, clonal cell lines expressing either WT-FLNA or the mutant S2152A were compared in ECM-based adhesion assay (A) and transwell assay (B). (C) RNA were extracted from clonal cells expressing empty vector, or clonal cell lines expressing either WT-FLNA or the S2152A mutant to determine the mRNA levels of EMT-associated markers. (D-G) HCT116 or HT29 cells expressing WT-FLNA were pre-treated with HGF (50 ng/ml) for 30 min. Then, cells were cultured with DMSO, Crizotinib (2 μ M) or LY294002 (5 μ M) for 48 h to evaluate the adhesion, migration, invasion ability and the mRNA levels of EMT-associated markers. All data are presented as the mean \pm SD of three experiments. * P <0.05, ** P <0.01.

ences the downstream biological processes. In HGF stimulated HCT116/FLNA (WT) or HT29/FLNA (WT) cells, Crizotinib (c-Met inhibitor) or LY294002 (PI3K-AKT inhibitor) treatment significantly decreased FLNA ser2152 phosphorylation (Figure 7D). Crizotinib or LY294002 treatment also attenuated the adhesion/migration/invasion ability of HCT116/FLNA (WT) or HT29/FLNA (WT) cells (Figure 7E, 7F). In addition, Crizotinib or LY294002 treatment also decreased the expression of vimen-

tin, N-cadherin, fibronectin, β -catenin and increased the expression of E-cadherin of HCT116/FLNA (WT) or HT29/FLNA (WT) cells (Figure 7G).

FLNA interacts with smad2 to promote EMT

To further clarify the molecular mechanisms of FLNA induced EMT, we performed a signal transduction RT² Profiler PCR Array to profile differentially expressed genes between each

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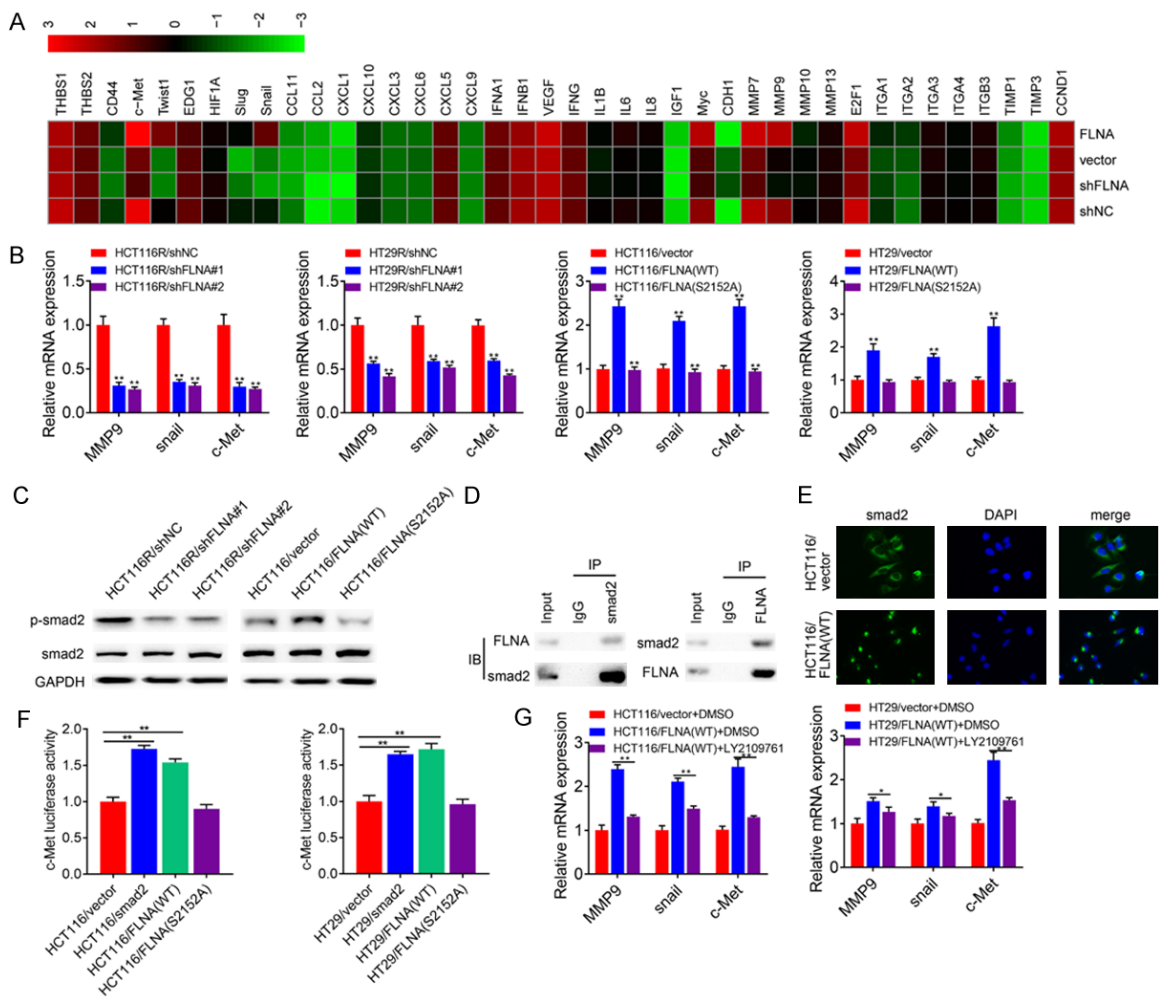


Figure 8. FLNA regulates EMT through smad2 signaling. A. A total of 40 genes are quantified using a human signal transduction PCR array. The color scheme represents gene expression changes on a log₂ scale. B. Three smad2 target genes (MMP9, snail and c-Met) reached the cutoff value (fold change >2 or <0.5) and their expression levels were confirmed in both two groups of cells. C. Expression of smad2 and p-smad2 in the indicated cell lines were determined by immunoblotting. D. Co-IP of smad2 and FLNA. HCT116 cells were immunoprecipitated using smad2 or FLNA Abs and immunoblotted with FLNA or smad2 Abs, respectively. E. Smad2 expression and its cellular location were detected by immunostaining with anti-smad2 antibody. DAPI stains nuclei. F. Promoter activity of c-Met in clonal cell lines expressing empty vector, smad2, WT-FLNA or the mutant S2152A were analyzed by luciferase reporter assay. G. The mRNA expression levels of MMP9, snail and c-Met in HCT116/vector cells and HCT116/FLNA (WT) cells treated with LY2109761. All data are presented as the mean \pm SD of three experiments. *P<0.05, **P<0.01.

group (HCT116R/shNC, HCT116R/shFLNA#2; HCT116/vector, HCT116/FLNA (WT)). The heat-map produced by this array shows gene expression levels relative to GAPDH on a log₂ scale (Figure 8A). As the result, seven genes were significantly (fold change >2) down-regulated and one gene was up-regulated in HCT116R/shFLNA#2 cells compared with HCT116R/shNC cells. Eight genes were up-regulated and one gene was down-regulated in HCT116/FLNA (WT) cells compared with HCT116/vector cells. Among these genes, MMP9, snail and c-Met

were well-reported targets of smad2. Thus, we suspected that FLNA might be involved in smad2 signaling. We first confirmed the results of PCR array by qRT-PCR assay. As shown in Figure 8B, knockdown of FLNA in HCT116R or HT29R cells significantly suppressed the expression of all the three genes, while overexpression of FLNA (WT) in HCT116 or HT29 cells promoted the expression of these genes. Correlation between FLNA and smad2 downstream genes prompted us to examine whether FLNA could regulate smad2 signaling. As indi-

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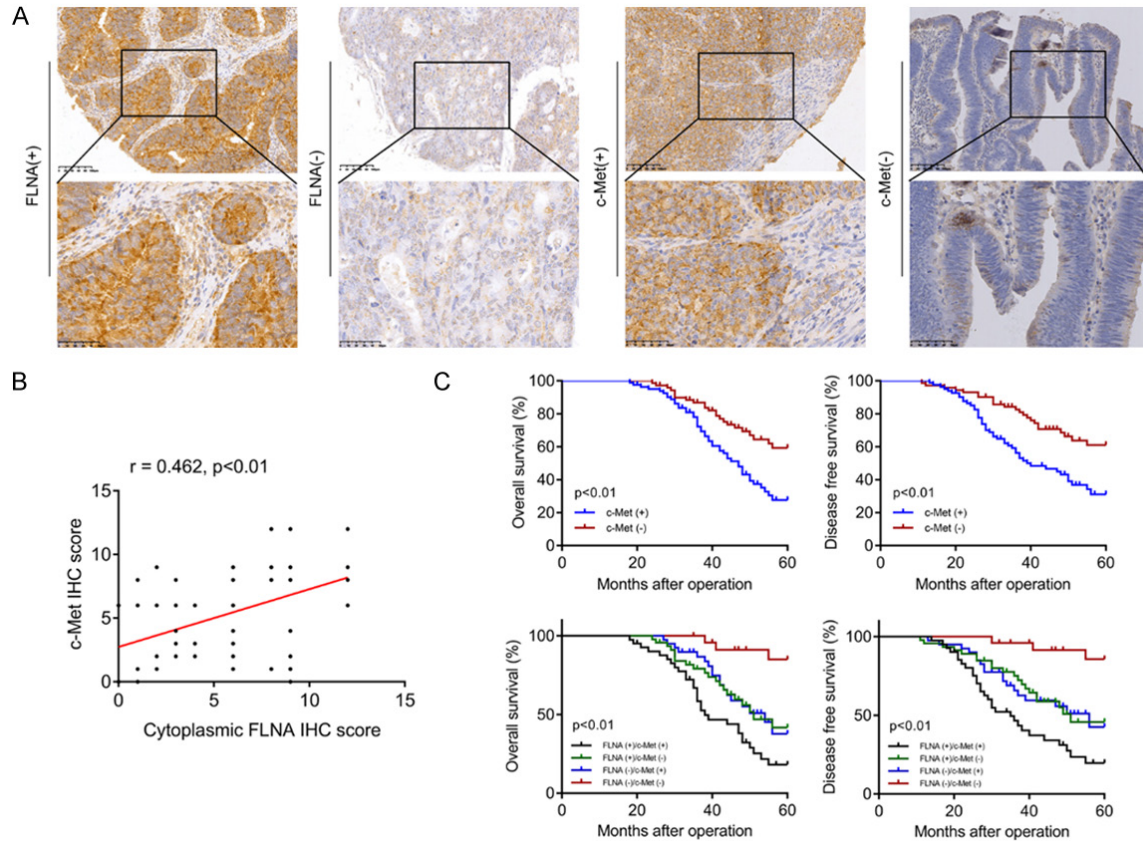


Figure 9. Clinical significance of FLNA and c-Met expression in CRC patients. A. Microphotographs of FLNA and c-Met protein expression in CRC specimens. B. The correlation between FLNA and c-Met expression was analyzed in a cohort of 152 CRC tissues (Pearson's correlation, $P < 0.01$, $r = 0.462$). C. Kaplan-Meier curves showing OS and DFS based on c-Met protein expression or FLNA combined with c-Met.

cated in **Figure 8C**, knockdown of FLNA decreased the levels of p-smad2, whereas overexpression of FLNA promoted the levels of p-smad2. To elucidate how FLNA regulated smad2 signaling, we analyzed the relationship between FLNA and smad2. Co-IP assay indicated that FLNA could interact with smad2 in HCT116 cells (**Figure 8D**). Immunofluorescence assay demonstrated that HCT116/vector cells mainly exhibited cytoplasmic smad2 staining, while smad2 mainly accumulated in the nucleus in HCT116/FLNA (WT) cells (**Figure 8E**). The promoter activity of c-MET in HCT116 cells was significantly increased after transfection with plasmid vectors driving either smad2 or FLNA (**Figure 8F**). To clarify FLNA-mediated function/chemoresistance was dependent on FLNA, we blocked smad2 signaling by a specific inhibitor LY2109761 in CRC cells expressing FLNA (WT). As shown in **Figure 8G**, LY2109761 treatment suppressed MMP8, snail and c-Met expression in HCT116/FLNA (WT) and HT29/FLNA (WT)

cells. Blockage of smad2 signaling decreased the IC50 value of HCT116/FLNA (WT) and HT29/FLNA (WT) cells (**Supplementary Figure 1A**). Moreover, Blockage of smad2 signaling also significantly inhibited the migration and invasion ability of HCT116/FLNA (WT) and HT29/FLNA (WT) cells (**Supplementary Figure 1B**).

Clinical significance of FLNA and c-Met expression in CRC specimens

Given the regulatory relationship between FLNA and c-Met in CRC cells, we investigated the clinical significance of FLNA and c-Met in CRC specimens. Representative images of FLNA and c-Met staining were shown in **Figure 9A**. Pearson's correlation analysis indicated that FLNA expression was positively correlated with c-Met expression (**Figure 9B**, $P < 0.01$, $r = 0.462$) in this cohort. We then performed Kaplan-Meier analyses to evaluate whether combina-

tion of FLNA and c-Met could better predict the prognosis of CRC patients. As illustrated in **Figure 9C**, the FLNA(+)/c-Met(+) group had the shortest 5-year overall survival and disease-free survival, and the FLNA(-)/c-Met(-) group had the longest survival ($P < 0.01$); there was no significant difference of survival between the FLNA(+)/c-Met(-) group and FLNA(-)/c-Met(+) group ($P > 0.05$).

Knockdown of FLNA promotes chemosensitivity in a xenograft model

A xenograft mouse model was employed to further validate whether silencing FLNA could promote chemosensitivity in vivo. The tumor weight and growth rates were lower in HCT116/shFLNA group compared to HCT116/shNC group. Moreover, xenografts derived from HCT116/shFLNA cells exhibited decreased volume and weight than that from controls in response to 5-FU (**Figure 10A, 10B**). Decreased ki67 signals were observed in the shFLNA group compared to the shNC group. Correspondingly, TUNEL assay results indicated that knockdown of FLNA could promote apoptosis in vivo (**Figure 10C**). Furthermore, three specimens were randomly selected from each group for IHC and immunoblotting assay. Knockdown of FLNA could inhibit EMT in vivo, as demonstrated by the up-regulation of E-cadherin and the down-regulation of vimentin and N-cadherin. Moreover, knockdown of FLNA could suppress the c-Met-AKT signaling pathway in vivo (**Figure 10D, 10E**). Altogether, the schematic diagram of our study is shown in **Figure 10F**.

Discussion

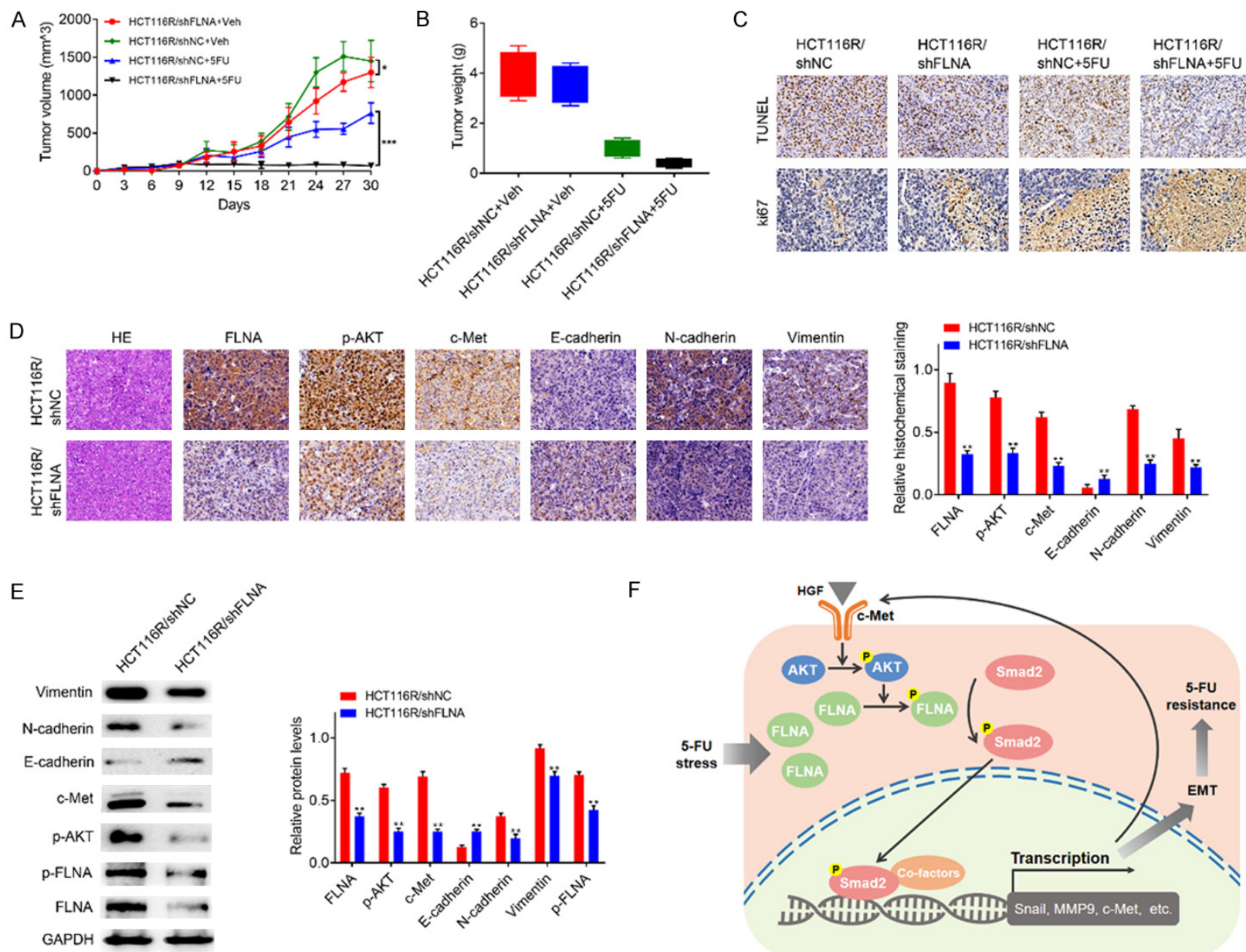
Acquisition of 5-FU resistance is a major clinical obstacle for successful treatment of CRC. Although multiple mechanisms of 5-FU resistance had been reported, we focused on FLNA since bioinformatics analysis revealed that FLNA was up-regulated in a series of primary 5-FU resistant CRC cell lines. Moreover, Kaplan-Meier survival analysis of public database and our cohort showed a negative correlation between FLNA expression and disease free survival of CRC patients. Thus, FLNA may be a potential chemotherapeutic drug sensitivity marker for CRC patients. We then used common CRC cell lines that were selected over time for acquired resistance to 5-FU. After weeks of exposure to increasing concentrations of 5-FU,

we observed remarkably elevated FLNA levels in two acquired 5-FU-resistant cells HCT116R and HT29R compared to parental cells, which prompted us to further analyze the exact roles of FLNA in chemoresistance.

Our study highlights the function and mechanisms of FLNA in regulating chemosensitivity of CRC. Though FLNA has been demonstrated to participate in several cancer-related processes, little is known about its role in regulation of drug resistance. In the present study, FLNA knockdown sensitized the CRC cells to 5-FU while FLNA overexpression promoted 5-FU resistance. Further functional studies confirmed that FLNA down or up-regulation altered the colony formation ability or apoptotic rate under 5-FU treatment. To be noticed, we also found that p-FLNA (ser2152) was up-regulated in acquired 5-FU resistant HCT116R and HT29R cells compared with the parental cells. Transfection of wild-type FLNA into HCT116 or HT29 cells promoted 5-FU resistance while transfection of mutant FLNA (S2152A) didn't alter 5-FU sensitivity. Thus, FLNA mediated 5-FU resistance might be dependent on ser2152 phosphorylation, at least partly.

Chemoresistance is closely related to EMT during malignant tumor progression. In lung adenocarcinoma specimens, Uramoto et al. found that sensitive tumors exhibited positive expression of epithelial markers [26]. Biological studies have linked EMT to the emergence of drug resistance such as gefitinib, adriamycin, and cisplatin [5, 27, 28]. In CRC, there are multiple known factors responsible for EMT induced 5-FU resistance. Sun et al. reported that HES1 promotes 5-FU resistance of colorectal cancer cells (RKO and HCT8, LOVO) by prompting EMT and several ABC transporter genes such as ABCC1, ABCC2 and P-gp1 [9]. In comparison with parental HT29 cells, 5-FU-resistant HT29R cells exhibited enhanced migration, mesenchymal-like morphology, increased expression of mesenchymal marker fibronectin and EMT-inducing transcription factors Twist, Zeb1, and Zeb2 [8]. In accordance with the findings of the previous studies, our established 5-FU resistant HT29R cells were observed obvious acquisition of the mesenchymal properties. Moreover, another 5-FU-resistant cell line HCT116R also acquired mesenchymal properties compared with the parental HCT116 cells.

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Figure 10. Knockdown of FLNA promotes tumor 5-FU sensitivity in a xenograft model. Nude mice were injected with HCT116R/shNC or HCT116R/shFLNA cells, followed by 5-FU or vehicle treatment. A. Tumor growth rate in each group. B. Average weight of subcutaneous xenografts. C. Representative immunohistochemical staining of TUNEL and ki67 from tumor samples in each group. D. Representative immunohistochemical staining of FLNA, p-AKT, c-Met, E-cadherin, N-cadherin and vimentin from tumor samples in each non-treated group. E. Expression of FLNA, p-AKT, c-Met, E-cadherin, N-cadherin and vimentin were determined using Immunoblotting. F. Schematic illustration of the mechanism by which FLNA promotes 5-FU resistance of CRC cells.

FLNA is well recognized to regulate EMT-like phenotypes such as cell adhesion, migration and cytoskeletal remodeling [16, 18]. In our research, knockdown of FLNA in HT29R and HCT116R cells suppressed EMT while overexpression of FLNA (WT) in HT29 and HCT116 cells promoted EMT, which demonstrated FLNA was essential for EMT in CRC cells. However, overexpression of FLNA (S2152A) in HT29 and HCT116 cells had no effect on EMT, suggesting FLNA ser2152 phosphorylation is essential to regulate EMT. There are several known signaling pathways responsible for chemoresistance as well as EMT, including PI3K/AKT, MAPK/ERK and Hippo/YAP [29-31]. Among these pathways, AKT activation was well reported to promote FLNA ser2152 phosphorylation [19]. Our results indicated HGF stimulation promoted phosphorylation of AKT and FLNA (ser2152), while blockage of AKT signaling by c-Met inhibitor Crizotinib or PI3K inhibitor LY294002 significantly decreased FLNA ser2152 phosphorylation. Furthermore, Crizotinib or LY294002 treatment also suppressed the adhesion/migration/invasion ability of HCT116/FLNA (WT) and HT29/FLNA (WT) cells. Based on these findings, we concluded that FLNA dephosphorylation with the c-Met inhibitor or PI3K inhibitor resulted in decreased invasiveness of CRC cells.

Dissection of the downstream signaling responsible for FLNA-mediated 5-FU resistance and EMT is another challenge. Firstly, we used a PCR array to profile differentially expressed signal transduction-related genes in two groups (HCT116R/shNC vs HCT116R/shFLNA#2 cells, HCT116/vector vs HCT116/FLNA). Among these differential genes, snail and MMP9 are well recognized targets of smad2. Snail is a well-known EMT-promoting transcription factor; MMP9 is mainly involved in the degradation of extracellular matrix to promote tumor invasion and metastasis. Thus, our results suggested smad2 signaling might play a crucial role in FLNA mediated EMT. To date, the most well recognized mechanism for smad2 mediated func-

tion is the phosphorylation and nuclear translocation. In our results, knockdown of FLNA in HCT116R cells led to a significant decrease of smad2 phosphorylation. On the contrary, overexpression of FLNA (WT) but not FLNA (S2152A) in HCT116 cells drastically promoted the protein level of p-smad2. Immunofluorescence assay indicated smad2 mainly exhibited cytoplasmic staining in HCT116/vector cells and nuclear staining in HCT116/FLNA (WT) cells, suggesting FLNA led to an accumulation of smad2 in the nucleus. Intriguingly, c-Met was also identified as a target of FLNA-smad2 signaling. On one hand, c-Met is a tyrosine kinase receptor that can participate in the activation of PI3K/AKT, MAPK and other signaling pathways when stimulated by the ligand HGF. On the other hand, c-Met-AKT pathway could induce FLNA ser2152 phosphorylation. Thus, our results highlighted a novel mechanism that c-Met-AKT-FLNA formed a positive feedback loop to regulate EMT. We then investigated whether smad2 activation induced by FLNA was essential for the expression of downstream effectors. LY2109761 was used to block smad2 signaling in HCT116/FLNA (WT) cells and HT29/FLNA (WT) cells. qRT-PCR assay suggested LY2109761 treatment suppressed the expression of c-Met, snail and MMP9, which meant an attenuation of FLNA mediated EMT. Clinically, IHC analysis revealed a positive correlation between FLNA and c-Met expression in a cohort of CRC tissues. Furthermore, combination of FLNA and c-Met was a better prognostic marker for CRC patients.

A limitation of our current work is that cell motility could be influenced by various pathways, such as NF- κ B, Wnt and Notch signaling, which also play important roles in EMT induced chemoresistance. Despite the chemoresistant CRC model used in our study exhibited enhanced adhesion/migration/invasion ability, we could not definitively conclude that increased cell motility was driven by EMT. Actually, several recent studies show that EMT might be

necessary for chemoresistance but may not be essential for distant metastasis [32, 33]. Therefore, whether changes in cell motility and EMT in chemoresistant CRC cells occur in parallel or have a cause-and-effect relationship still need further study. Secondly, despite FLNA could interact with smad2 to promote smad2 phosphorylation, the mechanism seemed to be complicated. Zhou et al. reported that FLNA interacted with smad2 but didn't affect its stability [34]. Several researchers speculated that FLNA might serve as an anchor protein to control localization of smad proteins near the cell surface receptors, but still lack of powerful evidences [35, 36]. In CRC cells, we confirmed the result that FLNA showed no effect on ubiquitin-dependent smad2 degradation (data not shown). However, we cannot elucidate deeper molecular mechanisms except that FLNA has an effect on smad2 phosphorylation. Thus, how does FLNA work in the phosphorylation of smad2 also need further investigation.

In conclusion, we elucidated the potential roles of FLNA overexpression and ser2152 phosphorylation in acquired 5-FU resistance of CRC for the first time. We provided evidences that c-Met-AKT-FLNA formed a positive feedback loop to enhance EMT, which contributes to 5-FU resistance of CRC cells. In the future, disruption of the functionally relevant FLNA-smad2 interaction and c-Met-AKT mediated FLNA ser2152 phosphorylation might contribute to development of biomarkers and treatment strategies for CRC patients.

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Disclosure of conflict of interest

None.

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Supplementary Table 1. Primer sequences used in the study

Gene name	Primer sequence (5'-3')
GAPDH (F)	GGACCTGACCTGCCGTCTAG
GAPDH (R)	GTAGCCCAGGATGCCCTTGA
MMP9 (F)	AGACCTGGGCAGATTCCAAAC
MMP9 (R)	CGGCAAGTCTTCCGAGTAGT
snail (F)	TTACCTTCCAGCAGCCCTAC
snail (R)	AGCCTTTCCCACTGTCCTC
c-Met (F)	CATGCCGACAAGTGCAAGTA
c-Met (R)	TCTTGCCATCATTGTCCAAC

Supplementary Table 2. 5-FU treatment information of colorectal cancer cell lines using CCLE database and CTRP database

Cell line	5-FU IC50 (μ M)	group
LS513	2.06	Low IC50
LS411N	2.57	Low IC50
HCT116	2.7	Low IC50
RKO	3.5	Low IC50
SNU407	3.65	Low IC50
LOVO	3.79	Low IC50
LS1034	3.87	Low IC50
HT29	4.32	Low IC50
HCT15	4.41	Low IC50
SNU81	4.71	Low IC50
KM12	5.83	Low IC50
SNU61	6.61	Moderate IC50
CCK81	7.28	Moderate IC50
CW2	7.91	Moderate IC50
SW948	8.36	Moderate IC50
HCC56	12.1	Moderate IC50
SW48	12.2	Moderate IC50
T84	17.7	Moderate IC50
NCIH747	23	Moderate IC50
SW620	25.7	Moderate IC50
COLO320	26.8	Moderate IC50
SNUC1	27.7	Moderate IC50
SNUC5	34.7	Moderate IC50
LS180	43.2	High IC50
C2BBE1	57.1	High IC50
NCIH716	88.1	High IC50
RCM1	129	High IC50
CL11	132	High IC50
MDST8	147	High IC50
HT115	185	High IC50
SW1417	195	High IC50
LS123	323	High IC50
COLO678	340	High IC50
HT55	340	High IC50

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Supplementary Table 3. The differentially expressed genes were compared by 'limma' package of R

gene_name	logFC	P.Value
RPS4Y1	-3.572743	0.0086898
EIF1AY	-2.305443	0.021553
DDX3Y	-2.275582	0.0169778
ERN2	-2.219026	0.0288828
TESC	-1.975507	0.0371564
RPL22L1	-1.869026	0.0023625
TRABD2A	-1.841831	0.0326016
KDM5D	-1.790969	0.0175696
HMG5	-1.669876	0.0127951
PLEKHH1	-1.573925	0.0111557
GJB2	-1.571249	0.0485949
BTNL9	-1.571185	0.0217055
WNK2	-1.565702	0.0052219
MGST1	-1.540462	0.0079041
MCOLN2	-1.540096	0.0195712
GSTO2	-1.525054	0.0153918
WDR52	-1.501511	0.0018068
BTN3A2	-1.465021	0.0186455
TUBE1	-1.463964	0.0038643
TRIM7	-1.461913	0.0237368
PAPSS2	-1.457356	0.0474631
CPNE7	-1.451979	0.0357487
ATF7IP2	-1.41746	0.0211244
CA2	-1.406851	0.0477544
EFCAB4B	-1.40409	0.0100879
ELL3	-1.401725	0.0274423
MARC1	-1.394908	0.0298081
GPC4	-1.390775	0.0289078
C6orf141	-1.387172	0.036294
MAPK15	-1.385002	0.0154413
SGPP2	-1.346984	0.0492607
SLC27A5	-1.32007	0.0494203
ALDH3A2	-1.319167	0.0111243
SLC18B1	-1.316954	0.0008461
MTAP	-1.314032	0.0177499
C14orf159	-1.277791	0.0276194
GALNT12	-1.277711	0.0178543
FAM149A	-1.252761	0.021387
ZDHHC11	-1.237293	0.0062797
PALD1	-1.221994	0.049685
CYB5D1	-1.221981	0.0001071
FAR2	-1.218145	0.0450456
TMCC3	-1.215354	0.001091
GCAT	-1.20454	0.0199988
KIAA1257	-1.194838	0.0117722
YJEFN3	-1.190704	0.0028194

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ZNF239	-1.184326	0.0030038
NPIPA8	-1.179058	0.0465758
SEC31B	-1.134947	0.0064415
DOK3	-1.131935	0.0023691
HNF4G	-1.126805	0.0266304
MLKL	-1.118855	0.0039544
GRTP1	-1.104439	0.0349225
F12	-1.102336	0.0422956
RGS16	-1.09989	0.0153885
CDC42EP1	-1.097299	0.0393634
MTHFD2	-1.086987	0.0145696
AHI1	-1.086865	0.0003798
CYP27B1	-1.084921	0.0045728
ZFAND1	-1.080715	0.0019824
ELOVL7	-1.078182	0.0451788
TTC12	-1.06987	0.0037891
PRR22	-1.068687	0.0034164
EIF3E	-1.065351	0.0009801
TMSB4Y	-1.056627	0.0121693
MRPL1	-1.050661	0.0015471
NPIPB4	-1.04737	0.0078965
ZFY	-1.043913	0.0159866
CDCA7	-1.033136	0.0059048
IL17RB	-1.031564	0.0466385
CTAGE8	-1.022692	0.016042
ANKRD18A	-1.016917	0.0161195
NFXL1	-1.013532	0.0006676
TLR3	-1.007275	0.0111812
ZNF121	-1.007224	0.0088681
GPR114	-1.0072	0.0020524
SMIM20	-1.00239	0.0185853
SLC2A4RG	1.001076	0.0107819
GPR1	1.0044609	0.0035374
PPDPF	1.0084342	0.0031557
PEA15	1.0154623	0.0253985
YPEL5	1.0165028	0.0024859
SGK223	1.0191967	0.0319514
RIPK4	1.0296183	0.002249
MID1	1.0298463	0.0496859
DLX3	1.0333336	0.0045934
CLCN5	1.0339093	0.0333668
IER5	1.0376947	0.0132575
C6orf1	1.0396874	0.0066102
AKT3	1.051459	0.0387334
ACSS2	1.0525482	0.006725
ADM	1.0551786	0.0313646
CYP27A1	1.0608716	0.0301833
SYNE3	1.0632054	0.011207
BCL9L	1.0645164	0.0261031

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PSG5	1.0653794	0.0165726
FOXL1	1.067667	0.0378144
CTSA	1.0689501	0.0019938
PPAP2B	1.0700926	0.0015198
AAMDC	1.0708091	0.000939
SLC37A2	1.0771493	0.0291757
PRRG1	1.0844803	0.0009331
CDK5R2	1.0893196	0.0258112
RGL1	1.0926763	0.0385479
GYG2	1.1025715	0.0490457
FOXJ1	1.1059351	0.0258056
OPTN	1.1062066	0.0121727
C10orf11	1.1098336	0.0087706
SLX1B	1.1119867	0.0212882
CNPY4	1.1177176	0.0195052
TMSB15B	1.1181304	0.0270107
CLDN9	1.1198302	0.0163566
TCEAL4	1.1265695	0.04461
ACTN1	1.1280306	0.0032954
BMF	1.1280836	0.0068954
DDAH2	1.1324977	0.0154418
DYNLRB1	1.1340422	0.0004342
FLNA	1.1350651	0.0063774
MARCH4	1.1367011	0.0445225
CCDC71L	1.1375298	0.0211936
RRAS	1.1383358	0.0072237
MAP7D3	1.1423229	0.022041
CTSV	1.1433873	0.024386
CPPED1	1.1456454	0.0168203
LAMC1	1.1459608	0.0155997
COL16A1	1.1541839	0.008941
CROT	1.167786	0.0243835
HIST2H4A	1.1711899	0.0444297
TBC1D2	1.1719227	0.0097014
EGFR	1.1731725	0.0097676
MMP24	1.1778021	0.0407198
ARID3A	1.1850501	0.0200305
PLCXD2	1.188407	0.0101642
SOGA1	1.1902871	0.0357805
RASL10B	1.1903838	0.049327
IDS	1.1953171	0.0071957
MAPK8IP1	1.198123	0.0368459
PXMP4	1.203958	0.0081473
ADAMTS9	1.2194872	0.0175291
C1QTNF1	1.2210263	0.0429151
HIST1H2BG	1.225864	0.0189555
GULP1	1.2288286	0.0236048
SNTA1	1.2362507	0.0037597
GABRA3	1.2462989	0.0020082

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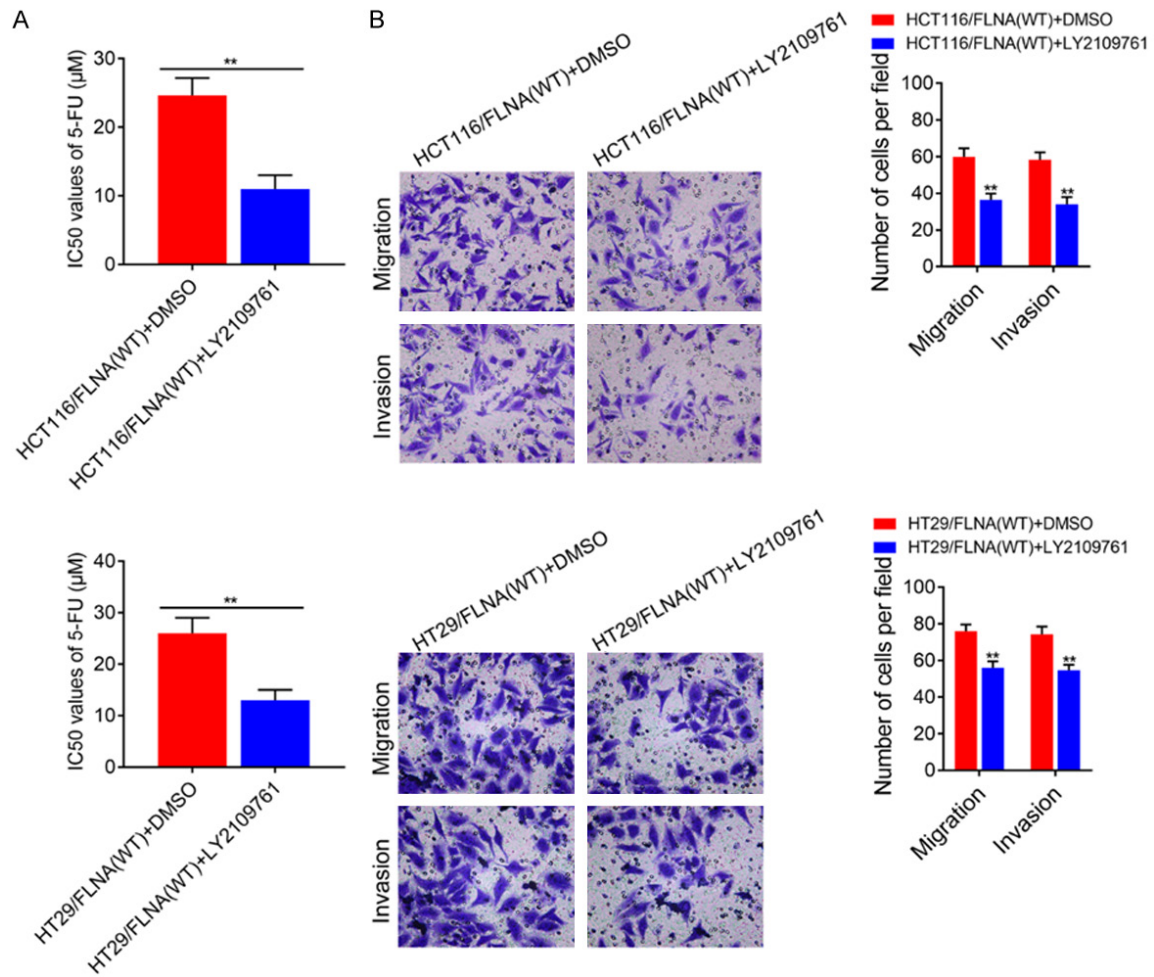
FAM43A	1.2530825	0.04804
C7orf13	1.256296	0.0070784
FGF13	1.2583102	0.021851
NFATC2	1.2650941	0.0056464
GSN	1.269893	0.0462424
PTPRN2	1.2736077	0.0375718
SEZ6L2	1.290705	0.0317641
NFATC4	1.2934089	0.0296552
MAP7D2	1.2953942	0.0284432
RNLS	1.2968852	0.0233487
EPHA4	1.3040038	0.0170442
SDC4	1.3123346	0.0068693
DCLK1	1.3147031	0.0106231
WWC3	1.3169111	0.0015104
FLRT3	1.3178727	0.0312042
TMEM198	1.321749	0.0107683
CST3	1.330145	0.0071187
TNFAIP3	1.336429	0.034879
ASB4	1.340769	0.0495004
TMSB4X	1.3460361	0.0047125
RASGRP3	1.3515531	0.0037438
CDIP1	1.3546707	0.0382346
DUSP5	1.3547894	0.0494233
CRYAB	1.360354	0.0027928
ITGA1	1.3617858	0.0283648
GGT7	1.3769537	0.0356199
CCDC80	1.3814326	0.042102
MGLL	1.3818126	0.0466398
FSTL3	1.3977212	0.0185551
HEPHL1	1.4042052	0.0449309
FAM213A	1.4111112	0.0275051
FAM127C	1.412455	0.0407849
OLFML3	1.4175337	0.0378975
LTBP3	1.4224445	0.0126886
CCND1	1.4243439	0.0070266
LYPD3	1.4256059	0.0373106
FNBP1	1.4559988	0.0087361
GABARAPL1	1.4581167	0.0256492
GADD45B	1.4594439	0.0137379
GSPT2	1.4629921	0.0264832
ZNF512B	1.4637379	0.0317648
BHLHE40	1.4729298	0.011552
WFDC2	1.4742625	0.0493103
DHRS2	1.4781781	0.0067021
AMOT	1.4793771	0.0174441
MPP1	1.49227	0.0438778
ICAM1	1.4945233	0.0392047
ATP9A	1.4967417	0.0024842
ARL4D	1.4990035	0.0191794

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GRAMD1A	1.5040049	0.0023878
IGFBP7	1.5082676	0.0306472
SLC2A14	1.512471	0.0265398
EDN1	1.5158116	0.028233
CREG2	1.518008	0.0043775
TFAP2C	1.5249876	0.0138253
JPH2	1.5475308	0.0084172
CRIM1	1.5518521	0.0162932
GATA3	1.5519704	0.0021061
COL13A1	1.5572255	0.0231164
NRP2	1.5673004	0.0470809
HEG1	1.5853585	0.0241379
TCEA3	1.5966381	0.037789
SLC16A6	1.602773	0.0293255
NRSN2	1.6265248	0.0376962
SMPD1	1.6438362	0.0029717
CDC42EP3	1.6460499	0.0061747
CSAG1	1.6488485	0.0494676
IGF2BP1	1.6541411	0.0179208
RBMS1	1.6550997	0.012552
SMIM10	1.6572238	0.0007011
MAGEA12	1.6599372	0.0474948
MAGED1	1.6639403	0.0359428
NAV2	1.6652217	0.0104491
PHLDB2	1.6692058	0.0410342
TMEM47	1.6753053	0.0455661
SLC2A1	1.6823568	0.0345998
DTX3	1.6888683	0.0111396
TUBB3	1.6938611	0.017124
GPNMB	1.7066188	0.025566
EVA1A	1.7730414	0.019159
COL6A1	1.8429029	0.0263776
ARHGDI3	1.8436822	0.0265348
FAM126A	1.8463549	0.0021101
C3orf14	1.9010152	0.0445696
OBSL1	1.9280197	0.0394599
GREM1	1.9442329	0.0066275
AMOTL1	1.9645632	0.0135409
KRTAP3-1	1.9798673	0.0110692
RAB32	2.0325781	0.0070952
NRP1	2.0398375	0.0033524
MME	2.1011279	0.0337081
CACNG4	2.1200536	0.002544
TIMP2	2.1306246	0.0227989
ARL4C	2.2942569	0.001076
CALD1	2.3198665	0.0250888
PLTP	2.4749128	0.0015851
DKK1	2.5007263	0.0415348
GNG4	2.5084262	0.0012176

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STC2	2.5132967	0.0145186
HPGD	2.5369741	0.0139095
UPK2	2.5611154	0.010227
TGFBI	2.7942066	0.0258365
LBH	2.8033036	0.0009089
ANKRD1	2.8049553	0.0192149
FAM127A	2.8673196	0.0059829
FN1	2.994706	0.0184628
FAM127B	3.1107127	0.001268
TGM2	3.4555036	0.0054046
L1CAM	3.6496545	0.0004995



Supplementary Figure 1. Blockage of smad2 signaling abrogated FLNA-mediated function/chemoresistance. HCT116/FLNA (WT) or HT29/FLNA (WT) cells were pre-treated with DMSO or LY2109761 for 30 min. A. Statistical chart showed IC50 dose of 5-FU calculated from measurement of cell viability. B. Cell migration and invasion were analyzed by transwell analysis. Five randomly selected fields were assessed under a microscope. All data are presented as the mean \pm SD of three experiments. * $P < 0.05$, ** $P < 0.01$.

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Supplementary Table 4. Association between FLNA expression and clinicopathological factors of CRC patients

Clinicopathological Variables	N=152	FLNA Expression		
		Low (66)	High (86)	P value
Age (years)				
≤60	64	30	34	0.509
>60	88	36	52	
Gender				
Female	67	32	35	0.410
Male	85	34	51	
Location				
Left hemicolon	23	11	12	0.925
Right hemicolon	43	17	26	
Sigmoid colon	32	14	18	
Rectum	54	24	30	
Tumor size (cm)				
≤4 × 3	68	36	32	0.048*
>4 × 3	84	30	54	
Tumor histology				
Tubular	125	57	68	0.709
Mucinous	24	9	15	
Papillary	3	1	2	
Extent of invasion				
T1 + T2	47	27	20	0.022*
T3 + T4	105	39	66	
Lymphatic metastasis				
N0	71	39	32	0.009*
N1 + N2	81	27	54	
CEA level				
≤5.0	110	46	64	0.584
>5.0	42	20	22	

*P<0.05.