

# TIMP-2 Regulates 5-Fu Resistance via the ERK/MAPK Signaling Pathway in Colorectal Cancer

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## Research

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# Abstract

**Background:** 5-Fluorouracil (5-Fu) is the first-line chemotherapeutic drug in the treatment of colorectal cancer. The efficiency of 5-Fu is limited by drug resistance in colorectal cancer patients. This study was aimed to define the functions of tissue inhibitor metalloproteinases 2 (TIMP-2) in the 5-Fu resistance to colorectal cancer and investigate its potential mechanism.

**Methods:** Cytokine array, ELISA and RT-qPCR were performed to detect cytokine expression levels. Western blot and immunohistochemistry were used to show the differential expression of proteins. In addition, cell viability was detected by CCK-8.

**Results:** We established that there is an up-regulation in the expression of the TIMP-2 in colorectal cancer patients. This up-regulation in TIMP-2 expression was evident in 5-Fu resistant colorectal cancer patients and resulted in a poor prognosis. Besides, *in vivo*, clinical studies and patient-derived xenograft (PDX) models confirmed that TIMP-2 was highly expressed in the 5-Fu-resistant colorectal cancer. We deduced an autocrine mechanism through which elevated TIMP-2 protein levels sustained colorectal cancer cell resistance to 5-Fu by constitutively activating the ERK/MAPK signaling pathway via an autocrine mechanism. The 5-Fu resistance could overcome by the inhibition of TIMP-2 by anti-TIMP-2 antibody or ERK/MAPK by U0126.

**Conclusion:** Our findings identify a TIMP-2-ERK/MAPK mediated 5-Fu resistance mechanism in colorectal cancer. Moreover, we recommend the use of an ERK/MAPK signal pathway inhibitor or TIMP-2-mediated immunotherapy for 5-Fu resistant colorectal cancer.

## Background

Colorectal cancer (CRC), particularly advanced colorectal cancer, is a significant challenge for clinicians because of its high mortality rates(1). Continuous medical research and clinical practice have not improved the prognosis of patients with advanced CRC(2). 5-fluorouracil (5-Fu) is an essential first-line chemotherapy drug for the treatment of CRC. 5-Fu acts by interfering with the cells' DNA synthesis and histone deacetylation(3, 4). Clinically, 5-Fu administered in combination with irinotecan or oxaliplatin is generally considered a relatively standard chemotherapy regimen(5). The vast majority of patients initially responded effectively to 5-Fu but developed tumor progression afterward which is indicative of resistance to 5-Fu(6–8). Many research and experimental data show the potential mechanism of 5-Fu drug resistance. However, the specific molecular mechanism of 5-Fu drug resistance remains is yet to be understood.

Increasing evidence indicates that tumor resistance is closely linked to miRNAs dysregulation(9), promoter hypermethylation(10), and abnormal expression of cell cycle-related proteins(11). Due to the prominent role of cytokines in the physiological and pathological activities of cells, researchers have turned their attention to cytokines to understand the mechanism of tumor drug resistance(12–16).

Indeed, cytokines contribute drug metabolism, drug delivery, drug targeting and drug resistance(13, 17, 18).

Acting as endoproteases, matrix metalloproteinases (MMPs) affect the integrity of extracellular matrix components(19). Particularly, matrix metalloproteinase 2 (MMP-2) is associated with the movement, migration, and metastasis of malignant cells(20–22). Tissue inhibitor of matrix metalloproteinase 2 (TIMP-2) is a natural inhibitor of MMP-2 and finely regulates it(23, 24). TIMP-2 plays a dual role in regulating cell physiology. It promotes tumor growth through angiogenesis and is also involved in the inhibition of malignant cells through apoptosis(25–28). Recent studies have shown that TIMP-2 is associated with tumor malignancy and resistance to chemotherapy in hepatoma, melanoma, and breast cancer(29–31). As for colorectal cancer, Zhang M. et al. found that TIMP-2 siRNA effectively inhibits the invasion of colorectal tumor cells HCT116 *in vitro*(32). In addition, many clinical reports suggested that MMP-2 and TIMP-2 were more frequent in CRC tissues than normal tissues, were higher expression in metastatic CRC than in those without metastasis(33–35). In particular, higher TIMP-2 levels were found in the unfavourable response to chemotherapy in CRC patients(33). However, whether it has the same effect in all these tumors and the specific mechanism of action remains unknown. Therefore, the role of TIMP-2 has yet to be studied thoroughly in the context of tumor resistance to 5-Fu.

ERK/MAPK signaling pathway is present in various types of cells. Its activation requires dual phosphorylation of MAPKK kinase, catalyzed by the Thr-X-Tyr motif(36, 37). Upon activation, MAPKs phosphorylate several protein kinases and transcription factors, including ERK1/2, JNK, p38MAPK, and ERK5<sup>32–35</sup>. This signaling pathway regulates many critical physiological effects, such as cell growth, signal transduction, stress, and inflammatory response(32, 38).

Previous studies have shown that various cytokines simultaneously activate ERK1/2 and ERK5, which subsequently affects cell proliferation and differentiation(39–42). More specifically, Peng L et al. found that the role of ERK1/2 signaling pathway plays an important regulatory role in CRC invasion and metastasis(43). In the field of drug resistance to tumors, ERK/MAPK signaling pathway has been shown to play an essential role in melanoma prognosis(44–46). And the combined use of BET and MEK inhibitors can significantly inhibit the growth of NRAS mutant melanoma and improve the survival rate of cancer patients(45). Another important evidence is that signaling pathways reactivate and play an important role in metastatic melanoma which is resistant to BRAF inhibition(46). However, its role in CRC 5-Fu resistance is not yet well established.

In our study, we investigated the difference in cytokine expression profiles of 5-Fu drug-resistant in CRC patients' serum by cytokine array. From this analysis, we found that 5-Fu resistant CRC patients expressed high levels of TIMP-2 and presented with poor clinical prognosis. TIMP-2 was also highly expressed in the 5-Fu resistant CRC PDX model. Furthermore, we indicated that TIMP-2 promotes the CRC cells resistance to 5-Fu *in vitro*. Detailed mechanistic analyses validated that ERK/MAPK signaling pathway is actively involved in the process of 5-Fu resistance caused by TIMP-2, and its inhibitor U0126

could largely overcome this drug resistance. Based on our findings, we hypothesized that TIMP-2 and ERK/MAPK signaling pathway would be excellent therapeutic targets to overcome 5-Fu resistance in CRC.

## **Materials And Methods**

### **Antibodies and reagents**

5-Fluorouracil (5-Fu) was obtained from MedChemExpress. The recombinant TIMP-2 was obtained from PeproTech. The antibody for TIMP-2 neutralization was obtained from R&D systems. Antibodies to MAPK (Erk1/2) (Cat No.4695), phospho-MAPK (Erk1/2) (Thr202/Tyr204) (Cat No.4370), Erk5 (Cat No.3552), phospho-Erk5 (Thr218/Tyr220) (Cat No.3371) and GAPDH (Cat No. 97166) were purchased from Cell Signaling Technology (CST). HRP-conjugated antibodies were obtained from Hangzhou Fude Biological Technology.

### **Enzyme-linked immunosorbent assay (ELISA)**

Each sample was duplicated. The cell culture supernatants or patient's serum levels of TIMP-2 were measured by sandwich ELISA. The ELISA assay was carried out using an ELISA kit (Elabscience) according to instructions. Each sample was assayed at least in triplicate.

### **Ethics statement**

The study of animal and human tissue specimens used in this experiment received approval of the local ethics committee at the Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University (study number: 20140213-19). All animal experiments were complied with the approval of the local ethics committee and in accordance with standard animal care guidelines.

### **Patients**

Briefly, serum samples were collected from CRC patients at the Key Laboratory of Biotherapy of Zhejiang province, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University from 2008 to 2018. Two experienced pathologists analyzed the cancer cells' content, histological tissue types, as well as tumor staging. 5-Fu based chemotherapy was administered to CRC patients who were then operated by senior surgeons. The World Health Organization (WHO) approved indices of Overall survival (OS) and Disease-free survival (DFS) were used to evaluate the efficacy of the treatments. Further, the patients' response to 5-Fu was divide into two categories; 5-Fu sensitive and 5-Fu resistant CRC. This classification was based on the tumor regression within six months following the administration of 5-Fu. As for the PDX model, tumor cells were extracted from a 66-year-old male rectal cancer patient, diagnosed with pathologic stage III adenocarcinoma. This patient was untreated and had received neither chemotherapy nor radiotherapy before surgery. Subsequent chemotherapy showed that the tumor was sensitive to 5-Fu.

### **Cytokine array**

Cytokine levels in the serum of patients were detected by a protein cytokine array: Human Cytokine Antibody Array-Membrane (ab193656), purchased from Abcam, Cambridge, UK. This technique is based on the principle of sandwich immunoassay. It comprises 120 coupled targets anti-cytokines along with appropriate controls in duplicate. DLD-1 and DLD-1 5-FuR cells were incubated in RPMI-1640 medium without fetal bovine serum cultured in a cell incubator at 37 ° C and 5% CO<sub>2</sub> for 24 hr. Membranes were exposed to the chemiluminescence imaging system (LUMIPULSE G1200). Conditioned medium containing cytokines were detected according to the manufacturer's protocol. The results were then normalized using internal controls, and the relative protein levels were determined across four biological replicates.

## **Cell culture**

The DLD-1 cells and HCT116 cells, two of the CRC cell lines, were obtained from the American Type Culture Collection (ATCC, Manassas). They were cultured respectively in RPMI-1640 (Genom) or Dulbecco's Modified Eagle Medium (DMEM) with higher glucose levels (Genom) containing 10% fetal bovine serum (GIBCO) medium at 37 ° C in 5% CO<sub>2</sub>/95% air.

## **Cell viability assay**

Cell viability was monitored with Cell-Counting Kit-8 (CCK8) assays (Dojindo Molecular Technologies) following the instructions of the kit. The absorbance was measured at a wavelength of 450 nm on the microplate reader. All experiments contain three independent trials in triplicate to guarantee the stability of the results.

## **RNA isolation and RT-qPCR**

Total RNA was extracted from the cells using Trizol (Invitrogen), and cDNA was prepared using the cDNA reverse transcriptase kit (Takara). The LightCycler 480 real-time PCR system (Roche, Mannheim) was used to perform SYBR Green-based (Takara) quantitative real-time PCR (RT-qPCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The  $2^{-\Delta\Delta Cq}$  relative quantification method was used to determine the mRNA levels of the target genes.

## **siRNA interference**

Small interfering RNA (siRNA) against TIMP-2 was obtained from Thermo Fisher Scientific. Transient transfection assays were performed using Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturers' protocols. Cellular drug resistance and cytokine secretion were analyzed by treating cells with 30 pg/ml TIMP-2 siRNA for two days.

## **Western blot analysis**

Cells were lysed in RIPA lysis buffer (Solarbio Life Sciences). Protein concentration was determined using the Bicinchoninic acid method (BCA) (Beyotime Institute of Biotechnology) method. Proteins from each sample (25 µg) were then separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred to polyvinylidene fluoride membranes (Immobilon-P). The membranes were blocked with 5%

dried skimmed milk for 1 h at room temperature and incubated with primary antibodies at 4 °C overnight. Subsequently, IgG conjugated goat anti-rabbit or IgG conjugated goat anti-mouse secondary antibodies were added for 1 h at room temperature. The blots were developed using enhanced chemiluminescence detection reagent (Hangzhou Fude Biological Technology).

## Animal experiments

Four weeks old female BALB/c- nude mice (SiBeiFu (Beijing) Biotechnology Co., Ltd.) were used for this study. Briefly, tumor cells from CRC patients were subcutaneously implanted in the groin of the nude mice. The mice were then divided into two groups; control and experimental groups. The test group was given an intraperitoneal injection of 5-Fu (30 mg/kg) until the average tumor diameter reached 1.0 cm. An initial reduction in the tumor size of the experimental group followed by a re-growth of more than 2.0 cm diameter represented a successful PDX model of colorectal tumors resistant to 5-Fu. Once the PDX model was obtained, blood samples were collected from the eyelids of nude mice and the mice were sacrificed to obtain tumor tissues.

## Immunohistochemistry

Tumor tissue samples were fixed in 4% buffered paraformaldehyde solution, dehydrated and immersed in paraffin, then made into 4 µm thick sections. Epitope retrieval was carried out by cooking the de-paraffinized sections under pressure in Tris-EDTA buffer (pH 9.0) for 20 minutes. Hydrogen peroxide (3%) in methanol solution was applied for 10 minutes to block endogenous peroxidase activity. Normal goat serum (10%) was then used to prevent the nonspecific binding for 30 minutes. Slides were incubated for 1 hour at 4 °C in TIMP-2 antibody solution diluted 1:20 at followed by incubating in secondary antibody for 30 minutes at room temperature. The sections were then developed using a DAB kit (Shanghai Gene Co., Ltd) and counterstained with hematoxylin (Sigma).

## Statistical analysis

Results from three independent experiments tested in triplicates were represented as means ± SD. Data were processed using SPSS (version 22.0). Image J, (version 2.0) the Graph Pad Prism, (version 7.0) analysis software were used for statistical evaluation. A Combination index (CI) of 1.0 indicated an additive effect, while CI < 1 suggested synergy. Alternative CI values indicated antagonism. Experimental data were examined for consistency to a normal distribution using the one-sample Kolmogorov-Smirnov test. An independent sample t-test or nonparametric test was then used to analyze the experimental results. Comparisons between survival curves were tested for statistical significance using either a Log-rank test or COX regression analysis. In all cases, p values were two-sided. A p-value of less than 5% was considered significant.

## Results

**TIMP-2 was established in 5-Fu resistant patients and was correlated with the outcome of CRC patients**

Previous research has shown that cytokines close to the tumor and circulating throughout the body is crucial for drug resistance. Firstly, we selected three typical 5-Fu-resistant and three typical 5-Fu-sensitive CRC patients from the clinic. Cytokine levels in these patients serum were then screened using cytokine array studies. Cytokines such as TIMP-2, GRO, ANGPT2, and EGF, among others, are significantly elevated in the serum of 5-Fu-resistant patients (Fig. 1A). Since TIMP-2 had the greatest change in expression levels, we hypothesized that TIMP-2 is the key cause of CRC resistance to 5-Fu. To validate our hypothesis, we looked at nine 5-Fu-resistant and nine 5-Fu-sensitive CRC patients, respectively, and tested the level of TIMP-2 protein in serum using enzyme-linked immunosorbent assay (ELISA). TIMP-2 protein in 5-Fu resistant CRC patients' serum was 73.61 pg/ml, 5.3 times higher than that of 5-Fu sensitive CRC patients (13.57 pg/ml) (Fig. 1B). Patient details are recorded in Table 1. The prognosis of clinical patients is of great concern to oncologists. Therefore, we examined the expression of TIMP-2 protein in the serum of 84 CRC patients undergoing 5-Fu-based chemotherapy and attempted to link it to the patient's prognosis. The characteristics of the above patients are as detailed in Table 2. The patient's median follow-up time was up to 54.4 months. Using the median value (36.60 pg/ml) of TIMP-2 protein expression in serum as a cut-off value, we divided the patients into two groups: TIMP-2 high expression group (n = 42) and TIMP-2 low expression group (n = 42). According to the major clinical outcomes of Overall survival (OS) and Disease-free survival (DFS), the TIMP-2 high expression group showed a worse prognosis than the TIMP-2 low expression group (Fig. 1C and D).

Table 1  
Patient characteristics of 5-Fu sensitive or resistance set

Patient	Age (years)	Sex	Stage	Histology	Chemotherapy
P1631	66	M	IVA	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Bevacizumab
P1692	61	F	IVA	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Bevacizumab
P1699	78	F	IVA	Mucus adenocarcinoma	5-Fu + Oxaliplatin + Bevacizumab
P1804	21	F	IIIC	Mucus adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin
P1822	76	M	IVB	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Bevacizumab
P1863	47	F	IVB	Mucus adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Bevacizumab
P1893	55	M	IVA	Adenocarcinoma	5-Fu + Oxaliplatin
P1957	55	M	IVB	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Cetuximab
P1977	51	F	IVB	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Bevacizumab
P2132	55	M	IVB	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Bevacizumab
P2167	66	M	IVA	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Bevacizumab
P2180	53	F	IVB	Adenocarcinoma	5-Fu + Oxaliplatin
P2186	65	M	IIIC	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin
P2286	59	M	IVA	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Cetuximab + Bevacizumab
P2295	48	M	IIIC	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Bevacizumab
P2338	66	M	IVB	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Cetuximab
P2357	70	M	IIIB	Adenocarcinoma	5-Fu + Irinotecan + Bevacizumab
P2653	65	M	IIIC	Adenocarcinoma	5-Fu + Irinotecan + Oxaliplatin + Cetuximab

Table 2  
Correlation between patient serum TIMP-2 level and clinical characteristics of ELISA set

Characteristics	Total	serum TIMP-2 level		OR	95% CI	P-value
		< 36.6 pg/ml	≥ 36.6 pg/ml			
<b>All Cases</b>	84	42(39.0%)	42(61.0%)			
<b>Age (years)</b>						
≥ 65	45	19(42.2%)	26(57.8%)			
< 65	39	23(59.0%)	16(41.0%)	0.508	0.213–1.214	0.189
<b>Gender</b>						
Male	48	19(39.6%)	29(60.4%)			
Female	36	23(63.9%)	13(36.1%)	0.37	0.151–1.043	0.179
<b>Stage</b>						
IIIA	7	3(42.9%)	4(57.1%)			
IIIB	62	33(53.2%)	29(46.8%)			
IIIC	10	6(60.0%)	4(40.4%)			
IVA	3	0	3(100%)			
IVB	2	0	2(100%)			0.215
<b>Histological type</b>						
Adenocarcinoma	67	33(49.3%)	34(50.7%)			
Mucus adenocarcinoma	14	8(57.1%)	6(42.9%)			
Others	3	1(33.3%)	2(66.7%)			0.728
P-value calculated by Chi-square test.						

### TIMP-2 was upregulated in 5-Fu resistant CRC cells and PDX model.

By gradually increasing the concentration of 5-Fu in the culture medium, we cultivated resistant cell lines from two CRC cell lines, DLD-1 and HCT116(47). These were named DLD-1 5-FuR and HCT116 5-FuR, respectively. Cell activity data obtained at different concentrations were used to achieve 50% inhibitory

concentration ( $IC_{50}$ ) calculations. The  $IC_{50}$  value for 5-Fu is 11.8-fold in DLD-1 5-FuR compared to DLD-1. In HCT116 5-FuR and HCT116, the  $IC_{50}$  is 3.81 (Fig. 2A and B). Given the relationship between cytokines and tumor resistance(12, 48), we designed a cell culture medium (CM) exchange experiment to verify the effect of cytokines on tumor cell resistance. We used DLD-1 5-FuR cells culture medium to culture DLD-1 cells. These experiments showed that DLD-1 cells co-cultured in the DLD-1 5-FuR medium were less tolerant of different concentrations of 5-Fu than those cultured in conditioned medium (Fig. 2C). The same experiment was repeated in HCT116 cells, and similar results were obtained (Fig. 2D). Both studies revealed that 5-Fu-resistant cell lines secrete cytokines that cause drug resistance.

To further clarify whether the TIMP-2 protein in the supernatant play role in drug-resistant cells, we tested the expression of TIMP-2 using real-time quantitative PCR and enzyme-linked immunosorbent assay (ELISA). Semi-quantitative mRNA analysis showed the level of TIMP-2 transcription in drug-resistant cell lines was dramatically higher than that of insensitive cell lines (Fig. 2E). The detection of secreted TIMP-2 proteins by ELISA showed that TIMP-2 was highly secreted in drug-resistant cell lines, including DLD-1 5-FuR and HCT116 5-FuR (Fig. 2F).

To further demonstrate TIMP-2 was also up-regulated during 5-fu treatment *in vivo*, since it is difficult to obtain the human specimen *in vivo*, we used the patient-derived xenograft (PDX) models for this research. The PDX model has received much attention because it maintains the donor's original biological behavior and molecular characteristics(49–52). We successfully constructed a PDX model of colorectal tumors resistant to 5-Fu, while the necessary construction process were showed in Fig. 3A. We found that the level of TIMP-2 protein in the serum of nude mice with 5-Fu-resistant PDX model was significantly higher than that of sensitive strains (Fig. 3B). Immunohistochemical (IHC) analysis revealed that tumor tissues which showed high TIMP-2 expression levels exhibited resistance to 5-Fu (Fig. 3C). The semi-quantitative immunohistochemical analysis further affirmed these results (Fig. 3D). Thus, we obtained evidence of elevated TIMP-2 expression in 5-Fu-resistant tumor tissues under *in vivo*. Similar results were obtained from tested patient serum.

### **TIMP-2 promotes CRC cells resistance to 5-Fu through an autocrine mechanism**

Our above clinical and animal experiments suggested that TIMP-2 holds a close relationship with 5-Fu resistance in CRC cells. We studied the role of this cytokine in tumor resistance behavior by artificially increasing or decreasing TIMP-2 protein levels. We studied the action cytokines by using a corresponding antibody to neutralize specific cytokines in the culture medium. Following the treatment of CRC cell lines DLD-1 and HCT116 culture medium using recombinant TIMP-2, less sensitivity to 5-Fu and increased  $IC_{50}$  was observed (Fig. 4A and B). When TIMP-2 neutralization antibody was added to the culture medium of DLD-1 5-FuR and HCT116 5-FuR cells, the  $IC_{50}$  of both cell lines were significantly decreased, indicative of the increased sensitivity of the cells to drugs (Fig. 4C and D).

To further validate the relationship between 5-Fu resistance and TIMP-2 protein expression in colorectal tumors, we used small interfering RNA (siRNA) to knock down TIMP-2 expression in the cell lines. siRNA against TIMP-2 showed excellent knock-down efficiency in DLD-1 5-FuR and HCT116 5-FuR cells (Fig. 5A). Besides, DLD-1 5-FuR and HCT116 5-FuR cells regained sensitivity to 5-Fu after knock-down of TIMP-2 expression by siRNA (Fig. 5B and C). Remarkably, the higher the concentration of 5-Fu in the culture solution, the more apparent the above effect. The addition of recombinant TIMP-2 protein to the siRNA-treated DLD-1 5-FuR and HCT116 5-FuR cells restored the resistance of the cell lines to 5-Fu. (Fig. 5B and C). The  $IC_{50}$  of each group of cells in the above experiment are shown in Fig. 5D. Thus, these experimental results demonstrated that TIMP-2 induces resistance of CRC cells to 5-Fu.

### **TIMP-2 induces 5-Fu resistance via ERK/MAPK activation in CRC cells**

To understand which signaling pathway regulates the TIMP-2 protein causing 5-Fu resistance in CRC. Previous studies showed that TIMP-2 mediates endothelial proliferation, the formation of a capillary tube in obesity, and promotes tumor invasion in advanced squamous cell carcinomas(53, 54) by activating ERK/MAPK signaling pathway. The role of the ERK/MAPK signaling pathway in tumor resistance has been widely reported(55–57). Therefore, we explored the underlying mechanisms driving TIMP-2 mediated drug resistance by analyzing the expression of key proteins in the ERK/MAPK signaling pathway.

The ERK1/2 protein levels were not altered in the DLD-1 5-FuR and HCT116 5-FuR cells in comparison to DLD-1 and HCT116 cells (Fig. 6A). However, phosphorylation of ERK1/2 prominently increased in the DLD-1 5-FuR and HCT116 5-FuR cells compared to the sensitive cells. The activation of ERK1/2 was accompanied by phosphorylation of ERK5. Both showed high expression in the resistant cells (Fig. 6A).

To confirm the ERK/MAPK signaling pathway on drug resistance to CRC, We conducted a series of experiments to verify the role of the ERK/MAPK signaling pathway in CRC drug resistance. U0126 is a well-studied and extensive ERK/MAPK signaling pathway inhibitor. Indeed, the phosphorylation of ERK1/2 and ERK5 in both DLD-1 5-FuR and HCT116 5-FuR cells were markedly reduced when added the U0126 (Fig. 6B). We also examined the role of TIMP-2 in the activation of the ERK/MAPK signaling pathway in CRC cells. Recombinant TIMP-2 treatment dramatically increased the phosphorylation of ERK1/2 and ERK5 in both DLD-1 and HCT116 cells (Fig. 6C). On the other hand, the addition of the TIMP-2 neutralization antibody resulted in a significant decrease in the expression of the phosphorylation of ERK1/2 and ERK5 in both resistant cell lines (Fig. 6D).

### **U0126 overcomes 5-Fu resistance in CRC via ERK/MAPK signaling pathway**

Since our data has proven that ERK/MAPK signaling pathway is vital for 5-Fu resistance in CRC, we next explored whether U0126 could overcome the process of drug resistance. We used synergistic effects to analyze the impact of U0126 on the ERK/MAPK signaling pathway in CRC cell resistance. The treatment of CRC resistant cell lines with different concentrations of 5-Fu and U0126, alone or in combination, exhibited different effects. We calculated the Combination index (CI) values, which used to quantitatively calculating the interaction between two drugs, for evaluating the combined use of U0126 and 5-Fu. With this, we established a strong synergy of U0126 on both DLD-1 5-FuR and HCT116 5-FuR cells (Fig. 7A and B).

To further determination that U0126 could reverse TIMP-2-induced 5-Fu resistance in CRC via ERK/MAPK signaling pathway, we downregulated ERK/MAPK by U0126 in DLD-1 and HCT116 cells with recombinant TIMP-2 treatment and then followed by increasing concentrations of 5-Fu culture (Fig. 7C and D). Although the addition of recombinant TIMP-2 could induce resistance to 5-Fu in DLD-1 and HCT116 cells containing ERK/MAPK, TIMP-2 no longer induced 5-Fu resistance in the CRC cells with downregulated ERK/MAPK signaling pathway via U0126 (Fig. 7C and D). Taken together, these results suggest that ERK/MAPK signaling pathway is a major mechanism involved in TIMP-2-induced 5-Fu resistance in CRC cells. What's more, this 5-Fu resistance could overcome by U0126, the inhibitor of ERK/MAPK.

## Discussion

In recent years, CRC has gradually increased worldwide, especially among the elderly. Advanced CRC has been attributed to resistance to 5-Fu. Consequently, this research seeks to understand the role of 5-Fu in CRC disease prognosis. Previous studies show that cytokines in the Para cancerous and circulating system affect the immune response, occurrence, metastasis of tumors as well as tumor resistance to drugs(12–15). Using cytokine array studies, we compared cytokine changes in serum from 5-Fu-resistant and 5-Fu-sensitive CRC patients. This assay identified elevated TIMP-2 in the serum from 5-Fu resistant CRC patients. This result was further validated using ELISA assays for TIMP-2 detection in the serum of both groups.

TIMP-2 belongs to the tissue inhibitor of metalloproteinase (TIMP) family. This gene family encodes for natural inhibitors of the matrix metalloproteinases (MMPs), a group of peptidases involved in the degradation of the extracellular matrix (ECM). Also, the encoded proteins have a unique role in suppressing the proliferation of endothelial cells, inhibiting protease activity in tissues undergoing remodeling of the extracellular matrix, and possessing erythroid-potentiating activity(58–61). Previous studies have shown the role of TIMP-2 in clinical practice. High expression levels of TIMP-2 in breast cancer is associated with poor prognosis(62), whereas low expression of TIMP-2 in lung cancer is related to poor prognosis(63). Further, metastasis studies, have shown that high expression of TIMP-2 in tumor tissue and serum of liver cancer patients was associated with decreased metastases(64). However, the role of TIMP-2 in CRC prognosis and CRC drug resistance has not been studied.

Our clinical research findings show that CRC patients with elevated TIMP-2 production are exhibited a poor disease prognosis as well as Overall survival (OS) and Disease-free survival (DFS). The findings were a confirmation of preliminary clinical and 5-Fu-resistant PDX model results showed a high expression of TIMP-2 in drug-resistant CRC. TIMP-2 may, therefore, act as a remarkable indicator for 5-Fu drug resistance in CRC patients. Since elevated TIMP-2 levels are instructive on the prognosis of 5-Fu-resistant CRC patients, it is crucial to detect TIMP-2 levels in the blood during the chemotherapy cycle of patients with CRC to catch sight of 5-Fu resistance as early as possible. Elevated levels of TIMP-2 expression, is accompanied by changes in the patient's 5-Fu resistance status. Consequently, doctors can act appropriately to prevent tumor progression in advance. These notwithstanding, more detailed clinical data on the relationship between TIMP-2 expression levels and clinical patient characteristics need to ascertain these findings.

Our cellular experiments confirmed the ability of TIMP-2 to cause resistance in CRC cells. Since Li S *et al.* reported a new autocrine expression of cytokine following drug resistance in leukemia(65), it led us to think about whether TIMP-2 also induces 5-Fu resistance through this way. 5-Fu sensitive cells co-culturing with the medium of 5-Fu resistant cells imparted cells with a survival advantage, which represents potential cytokines endowing CRC with 5-Fu resistance. Specifically, the effect of recombinant TIMP-2 treatment on CRC cells revealed that secreted TIMP-2 acts as an autocrine factor to induce 5-Fu resistance. Following the withdrawal of TIMP-2 by neutralization antibody or siRNA appeared to reverse the drug resistance in 5-Fu resistant cells. Thus, the up-regulation, down-regulation and rescue experiments proved an autocrine mechanism through which elevated TIMP-2 protein levels sustained colorectal cancer cell resistance to 5-Fu. Now that 5-Fu treatment stimulates the expression of TIMP-2 in CRC cells, it provide an insight into the role of anti-TIMP-2 antibody to prevent CRC patients from acquiring resistance to the 5-Fu drugs during treatment. What's more, the serum TIMP-2 level in CRC patients may be an available biomarker to evaluate the potential resistance of patients following the 5-Fu treatment.

Furthermore, we explored TIMP-2 activates specific signaling pathways leading to drug resistance in tumor cells. Then, we demonstrated the differential expression of major proteins in the ERK/MAPK signaling pathway, including the phosphorylation of ERK1/2 and ERK5. A small-molecule inhibitor known as U0126 has been shown to target key proteins in ERK/MAPK signaling pathway(39, 40). From our data, for the first time we established that TIMP-2 leads to 5-Fu resistance through ERK/MAPK signaling pathway in CRC cells. Targeting of the ERK/MAPK signaling pathway could re-sensitize 5-Fu resistant CRC cells to the 5-Fu. Based on our results, we infer that U0126 could efficiently switch 5-Fu-resistant CRC cells to 5-Fu sensitive CRC cells due to its ability to inhibit the ERK/MAPK signaling pathway and blocking the TIMP-2 autocrine mechanism for 5-Fu resistance.

Consequently, the combined use of an agent targeting TIMP-2 and 5-Fu will be an excellent choice to prevent or treat CRC resistance to 5-Fu in CRC patients treated with 5-Fu as the base drug. Alternatively, small molecule inhibitors that target the ERK/MAPK signaling pathway, such as U0126, could also

effectively cut off the pathway, thereby increasing the sensitivity of colorectal tumors to 5-Fu. Nevertheless, it is essential to conduct animal experiments and clinical trials to ascertain these findings.

## **Conclusions**

The results of this study show that TIMP-2 is overexpressed in CRC patients. The Overexpression of TIMP-2 in CRC patients' serum promotes drug resistance to 5-Fu via the EPK/MAPK signaling pathway and is indicative of poor disease prognosis. Consequently, CRC resistance to 5-Fu can be regulated by the inhibition of the TIMP-2 or ERK/MAPK signaling pathway. Finally, the combined use of TIMP-2 or ERK/MAPK signaling pathway inhibitors and 5-Fu is a promising chemotherapeutic regimen for the treatment of first time CRC patients as well as relapsed CRC patients previously treated using 5-Fu-based chemotherapy.

## **Abbreviations**

TIMP-2	tissue inhibitor metalloproteinases 2
5-Fu	5-Fluorouracil
ERK	extracellular regulated protein kinases
MAPK	mitogen-activated protein kinase
ELISA	enzyme linked immunosorbent assay
RT-qPCR	quantitative reverse transcription polymerase chain reaction
CCK-8	Cell Counting Kit-8
PDX	patient-derived xenograft
CRC	colorectal cancer
DNA	DeoxyriboNucleic Acid
RNA	Ribonucleic Acid
MMPs	matrix metalloproteinases
JNK	c-Jun N-terminal kinase
DFS	Disease-Free Survival
OS	Overall Survival
siRNA	Small interfering Ribonucleic Acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
PBS	Phosphate Buffered Saline
DAB	Diaminobenzidine
IC <sub>50</sub>	the 50% Inhibitory Concentration

## Declarations

### *Ethics approval and consent to participate:*

The study of animal and human tissue specimens used in this experiment received approval of the local ethics committee at the Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University (study number: 20140213-19). All animal experiments were complied with the approval of the local ethics committee and in accordance with standard animal care guidelines.

### ***Consent for publication:***

Not applicable.

### ***Availability of data and materials***

The data that support the findings of this study are available from Department of colorectal surgery, Sir Run Run Shaw Hospital of Zhejiang University but restrictions apply to the availability of these data, which were used under license for the current study, and so are not publicly available. Data are however available from the authors upon reasonable request and with permission of Department of colorectal surgery, Sir Run Run Shaw Hospital of Zhejiang University.

### ***Competing interests:***

The authors have no conflicts of interest to declare.

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### ***Author Contributions:***

Study design: Zhou W, Song Z, Huang X; Experimental operation: Zhang G, Luo X, Zhang W; Data analysis: Xu J, Chen E; Data collection: Meng Q, Wang D.

### ***Acknowledgements:***

Not applicable.

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## Figures

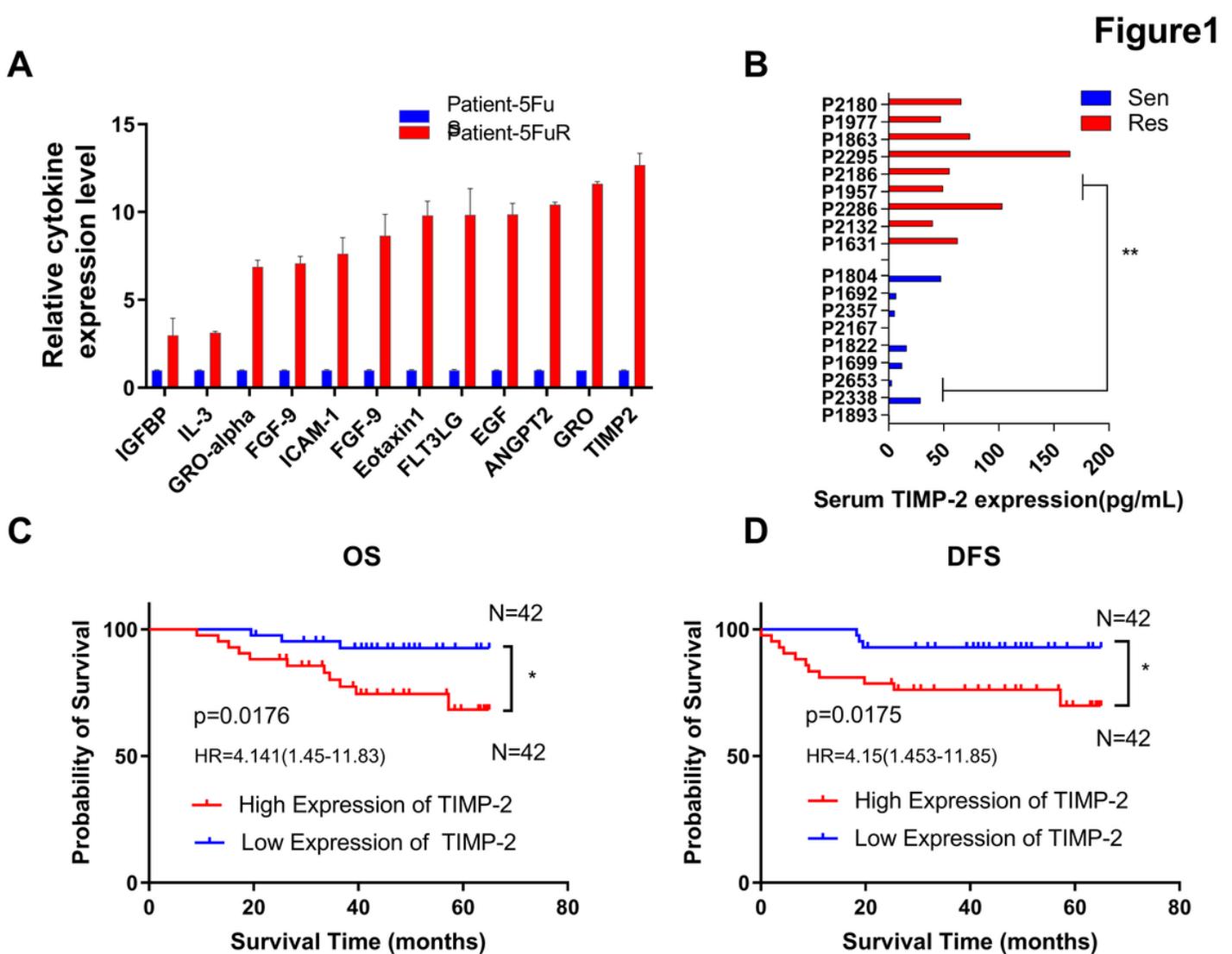


Figure 1

TIMP-2 is elevated in 5-Fu resistant CRC patient and predicts clinical outcomes (A) Semi-quantitative of relative cytokine expression levels of different using a cytokine array and measured in the serum of 5-Fu non-resistant and resistant patients. (B) Difference in TIMP-2 protein expression levels in non-resistant (n=9) and resistant patients (n=9) with colorectal cancer, which measured by ELISA. Patient details are shown in Table1. Sen, sensitive patients. Res, resistant patients. (C) 6-year OS Kaplan–Meier survival curves of 84 colorectal cancer patients, differential grouping of TIMP-2 expression (36.6 pg/ml) in serum. Table 2 counts patient information. (D) 6-year DFS Kaplan–Meier survival curves of 84 colorectal cancer patients, differential grouping of TIMP-2 expression (36.6 pg/ml) in serum. Table 2 counts patient information. (B)\*p<0.05, \*\*p<0.01 by unpaired Student’s t-test. (C) (D)\*p<0.05 by logrank (Mantel-Cox), HRs are shown in the figures.

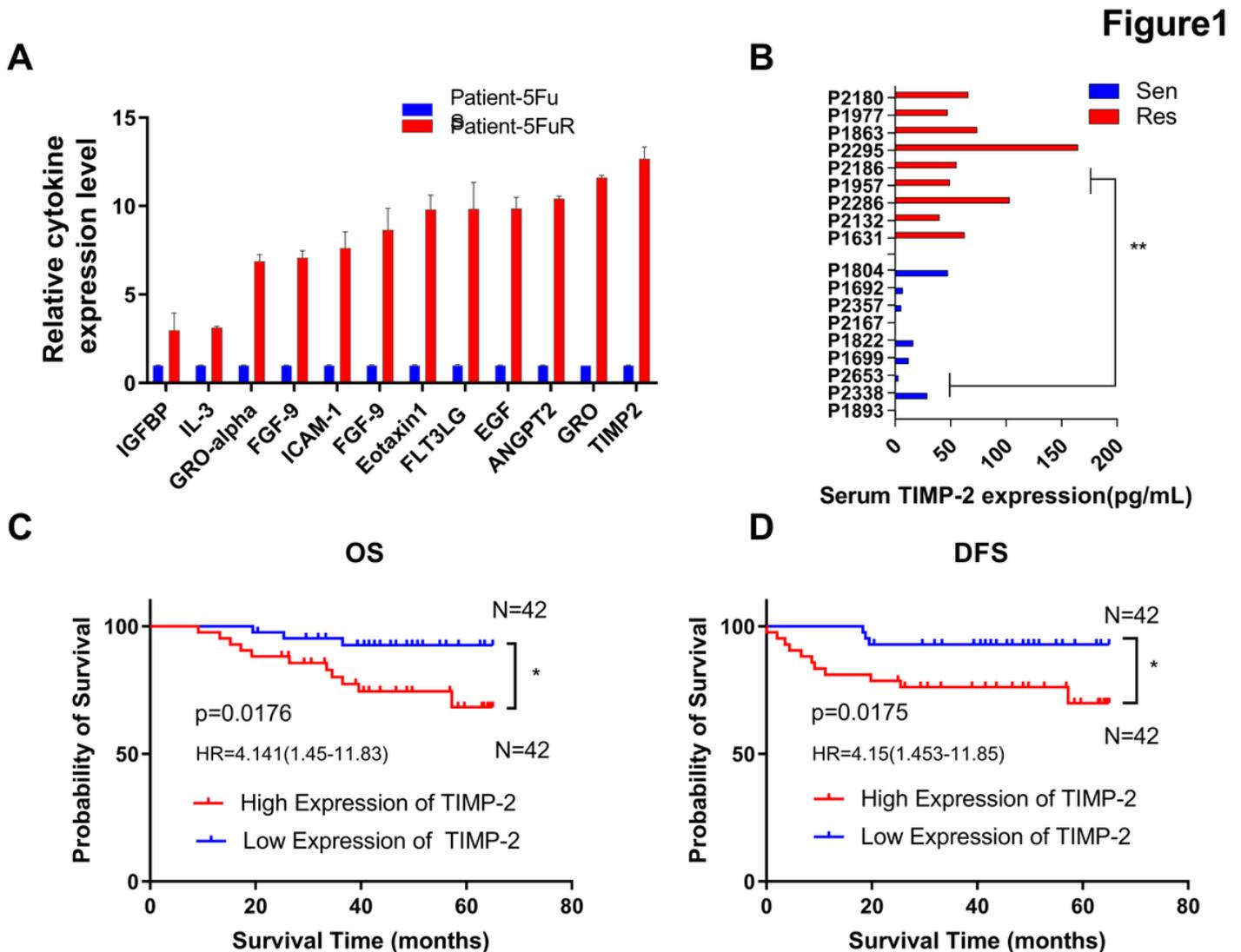
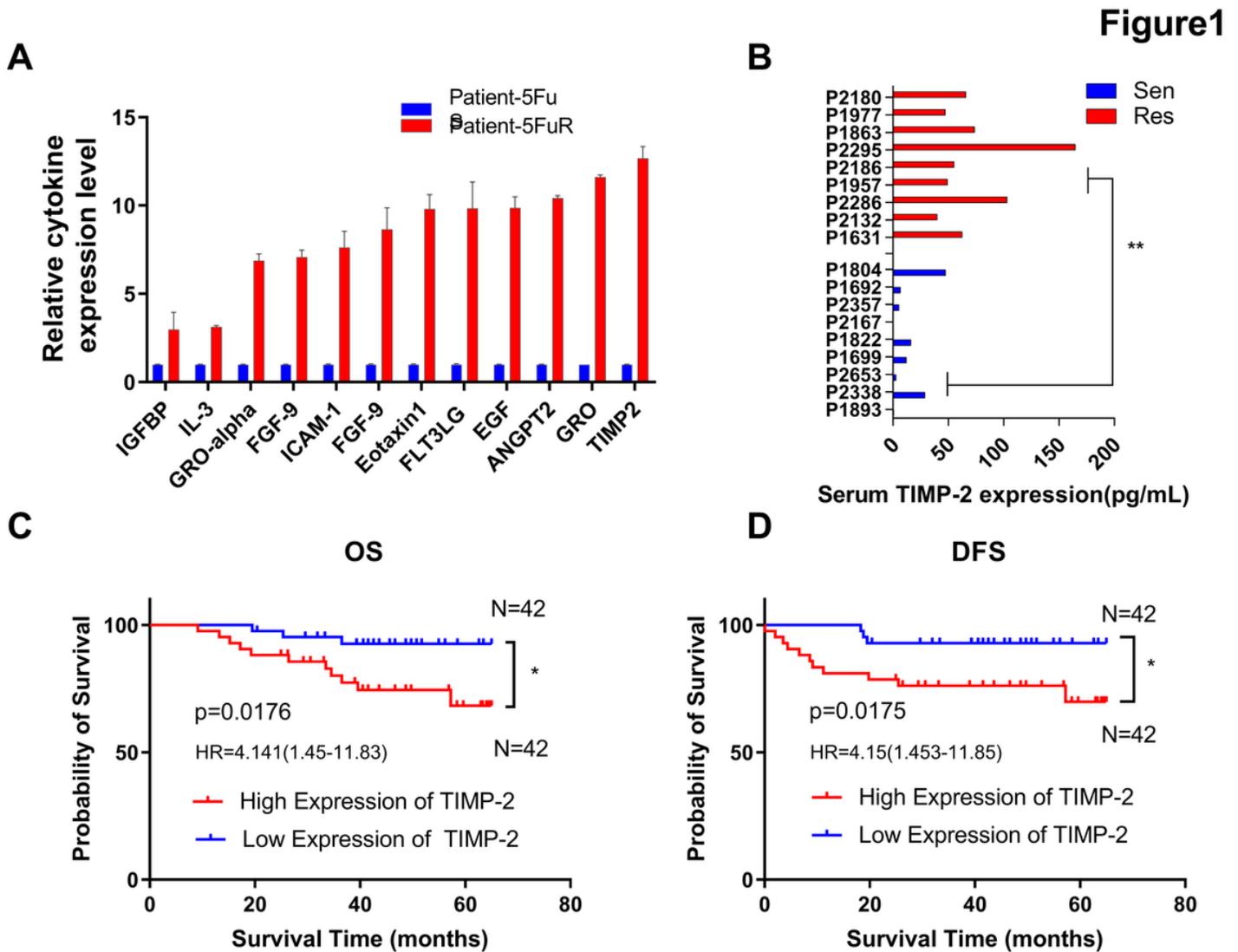


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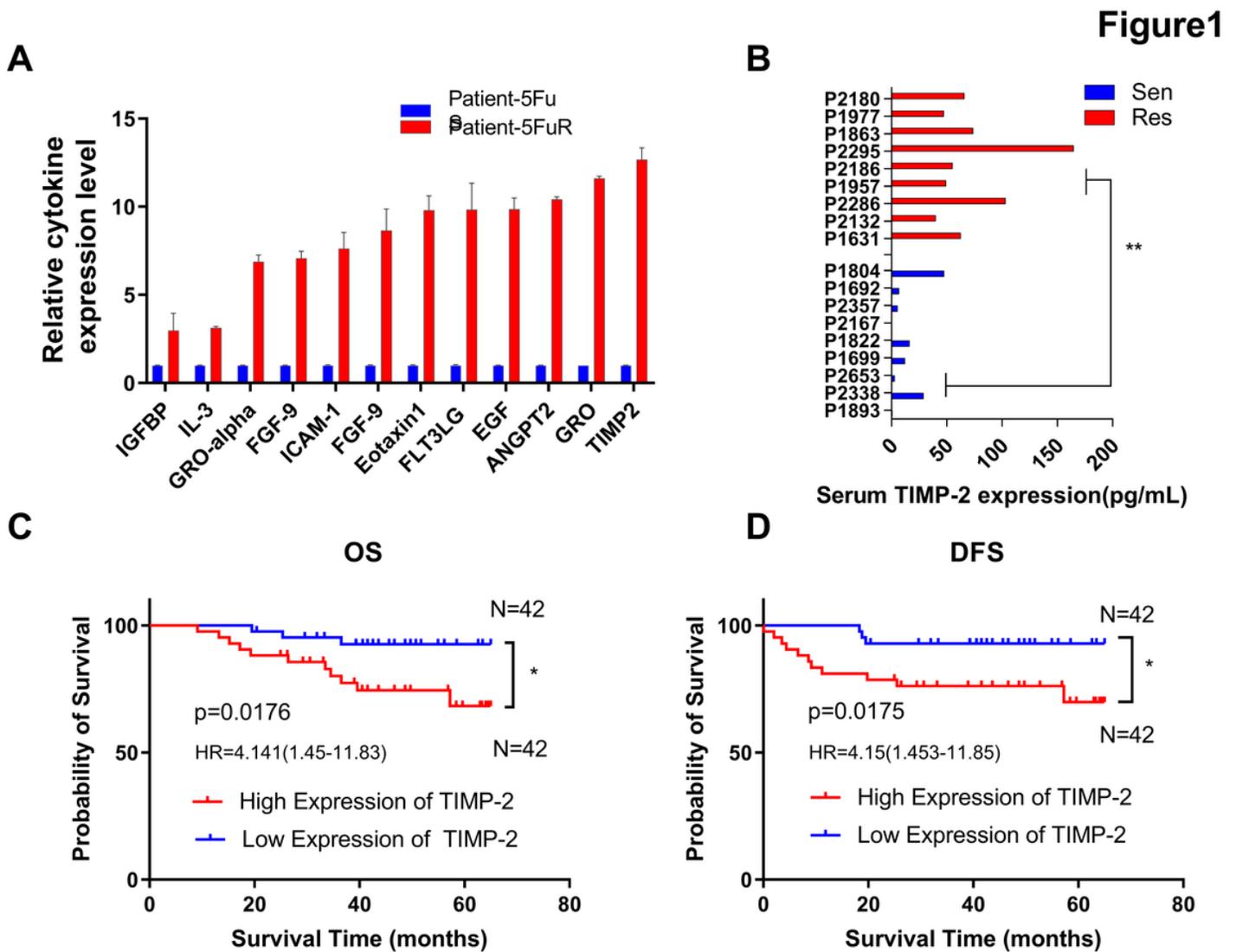
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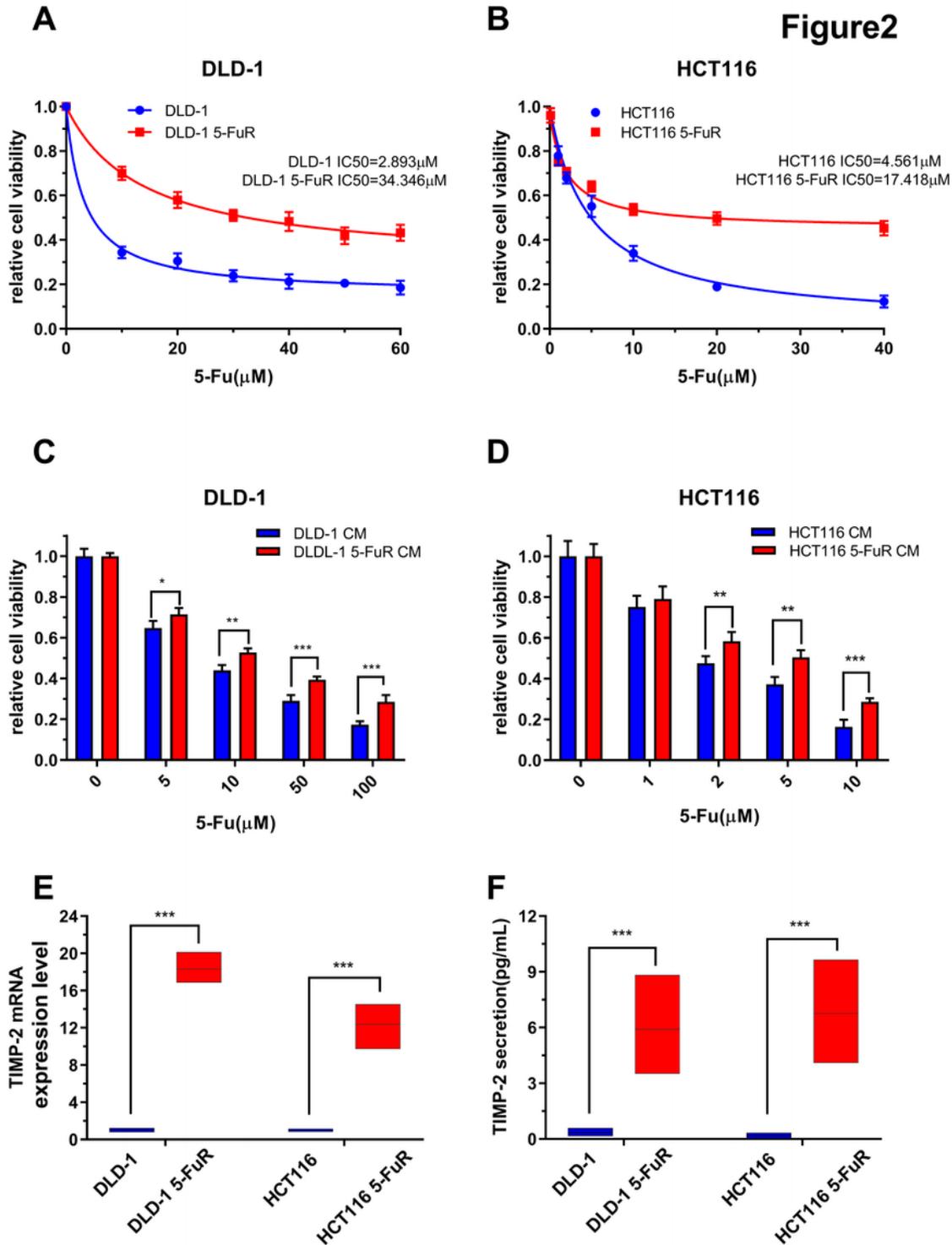


Figure 2

Upregulation of TIMP-2 in 5-Fu resistant CRC cells in vitro (A)(B) Relatively cell viability of DLD-1 cells and DLD-1 5-FuR cells, HCT116 cells and HCT116 5-FuR cells in the condition of increasing concentration of 5-Fu for 3 days. (C)(D) Relatively cell viability of DLD-1 cells and HCT116 cells in the condition of

increasing concentration of 5-Fu for 3 days after culturing in conditioned medium of DLD-1 5-FuR cells or HCT116 5-FuR cells for 2 days. (E) Semi-quantitative expression level of TIMP-2 mRNA in the paired DLD-1 cells and DLD-1 5-FuR cells, HCT116 cells and HCT116 5-FuR cells. (F) Difference in TIMP-2 protein expression level in the paired DLD-1 cells and DLD-1 5-FuR cells, HCT116 cells and HCT116 5-FuR cells. Data from triplicate wells in 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by Student's t-test.

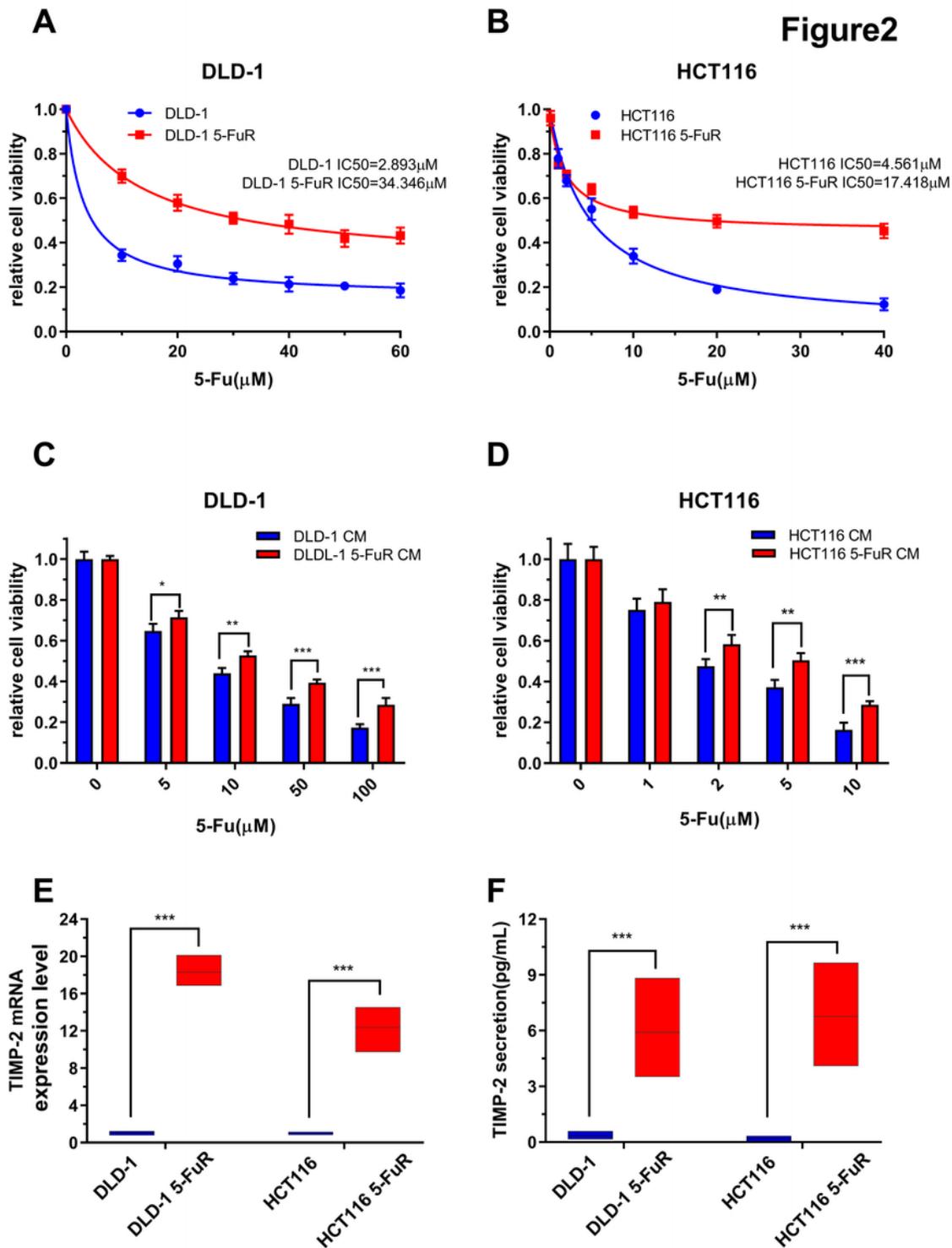
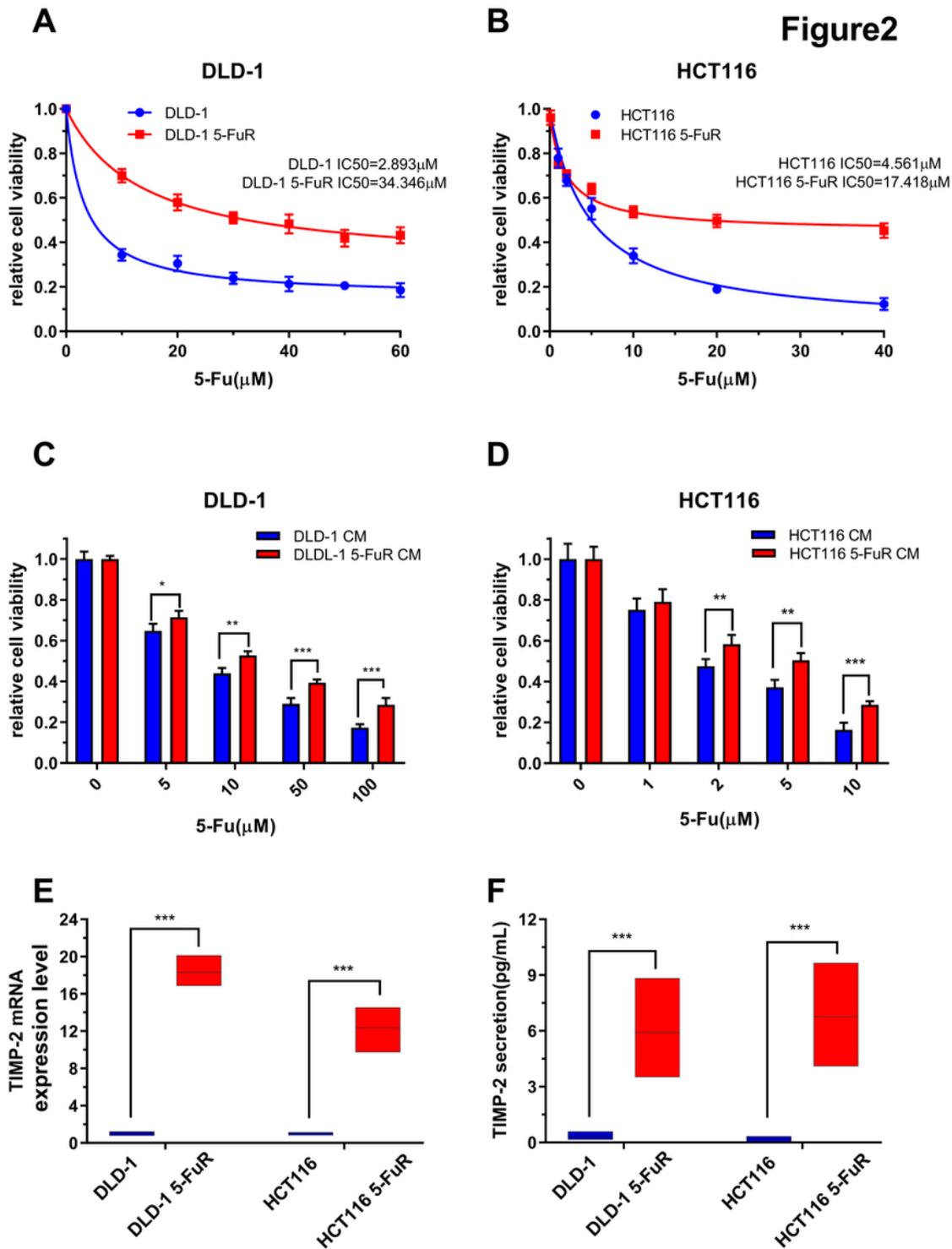


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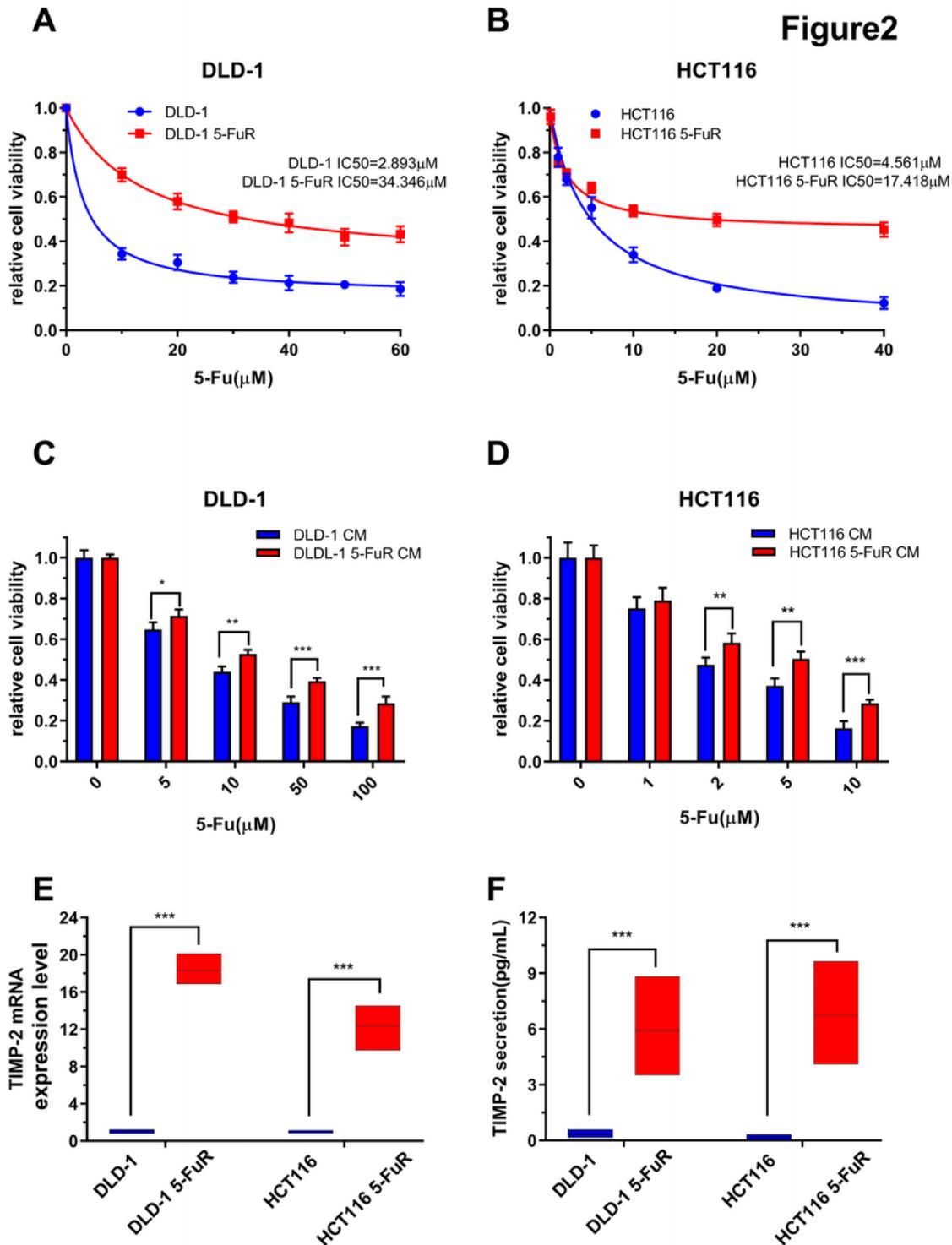
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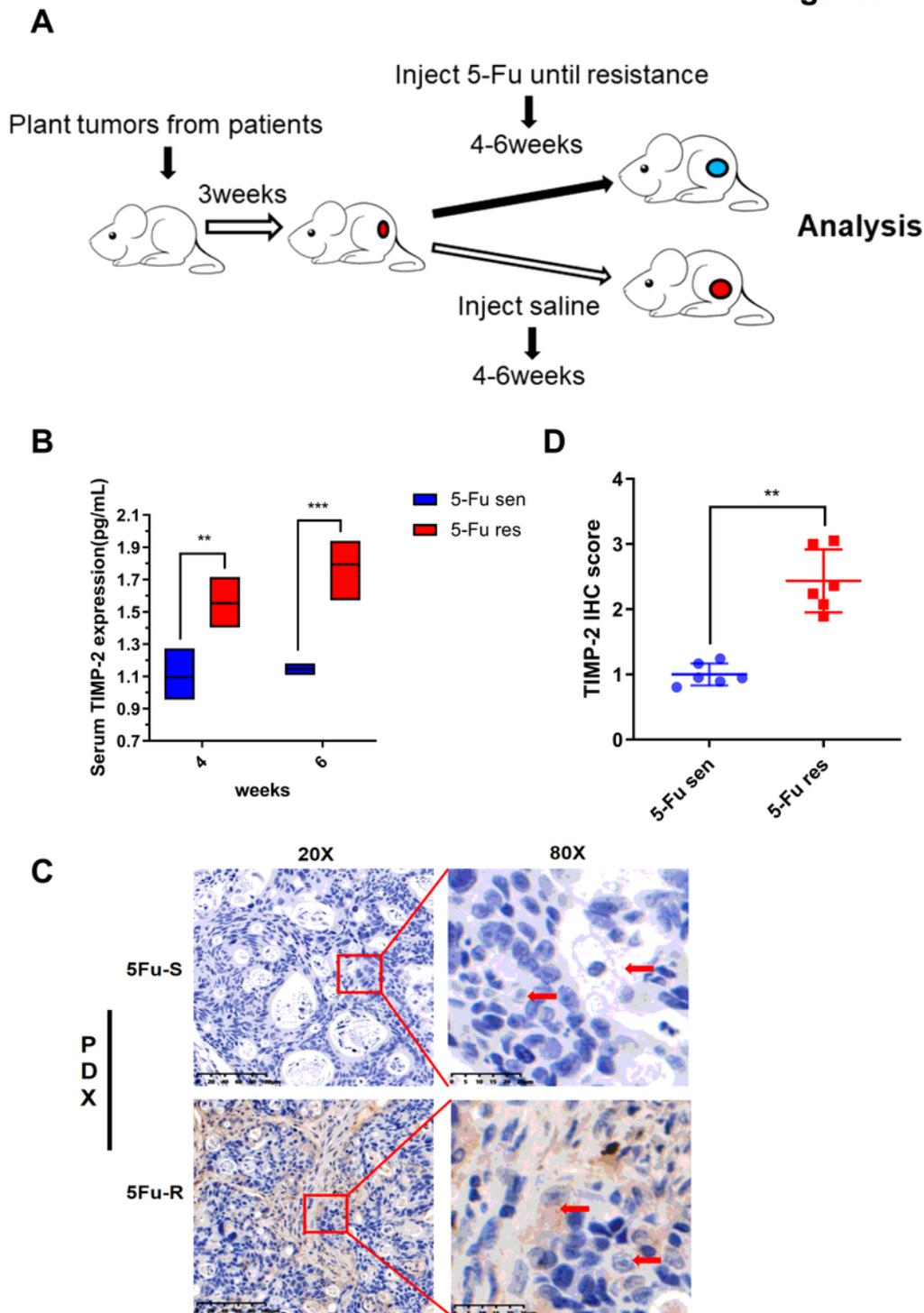


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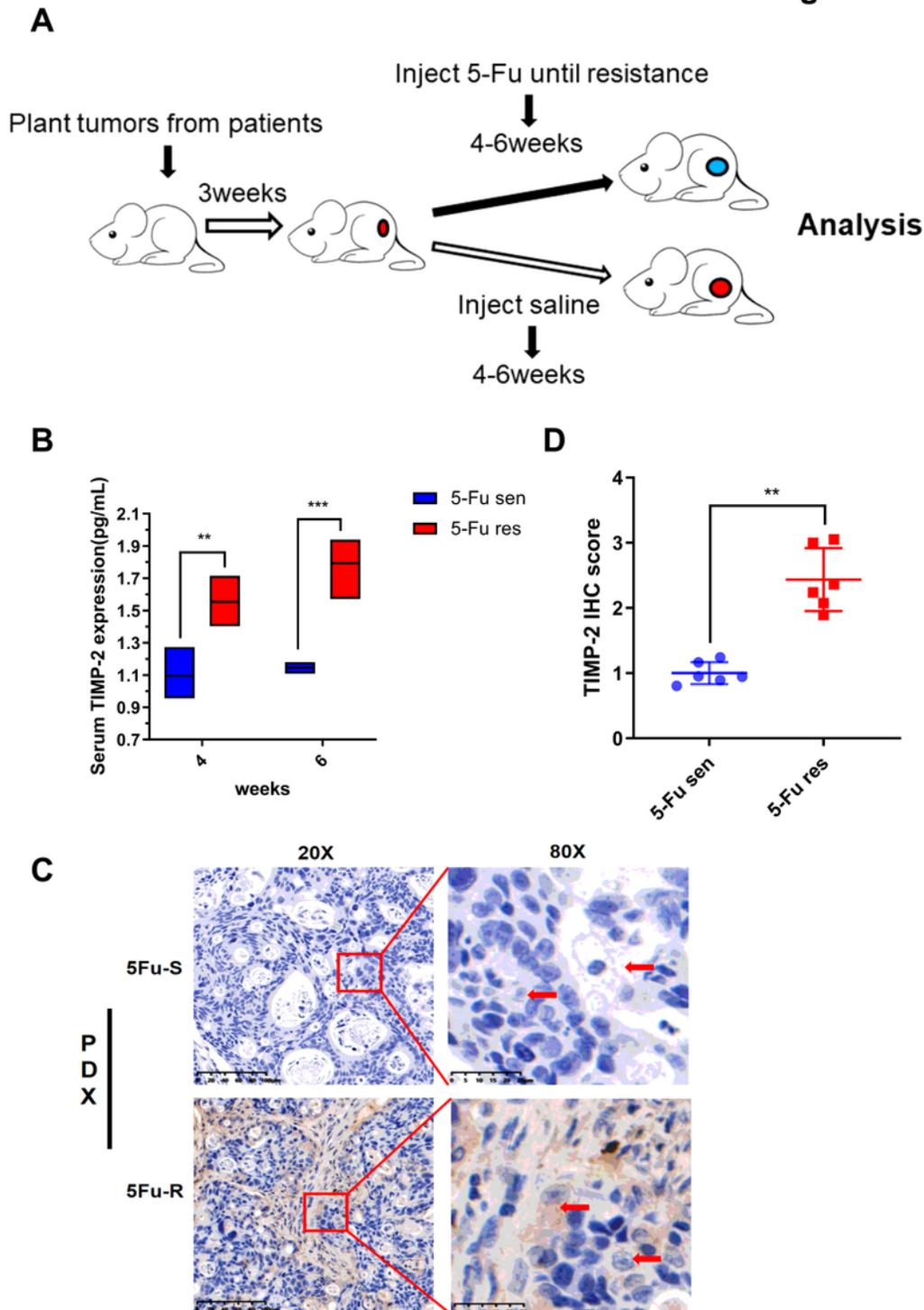
**Figure3**



**Figure 3**

Activation of TIMP-2 in 5-Fu resistant PDX model of CRC in vivo (A) A brief experimental diagram of constructing a PDX-drug resistance model. (B) Difference in TIMP-2 protein expression levels in 5-Fu resistant and sensitive PDX mice model in the following 5-Fu treatment. (C) IHC for typical TIMP-2 staining images of subcutaneous tumors formed in mice bearing patient-derived tumor xenograft tumors and treated with 5-Fu until resistance or not. (D) Semi-quantitative of IHC staining scores for TIMP-2 as shown in Figure 3(C). Mean  $\pm$  SD is the way of the data presentation. 3 mice and 6 tumors per experimental group. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by Student's t-test or two-way ANOVA.

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Figure 3

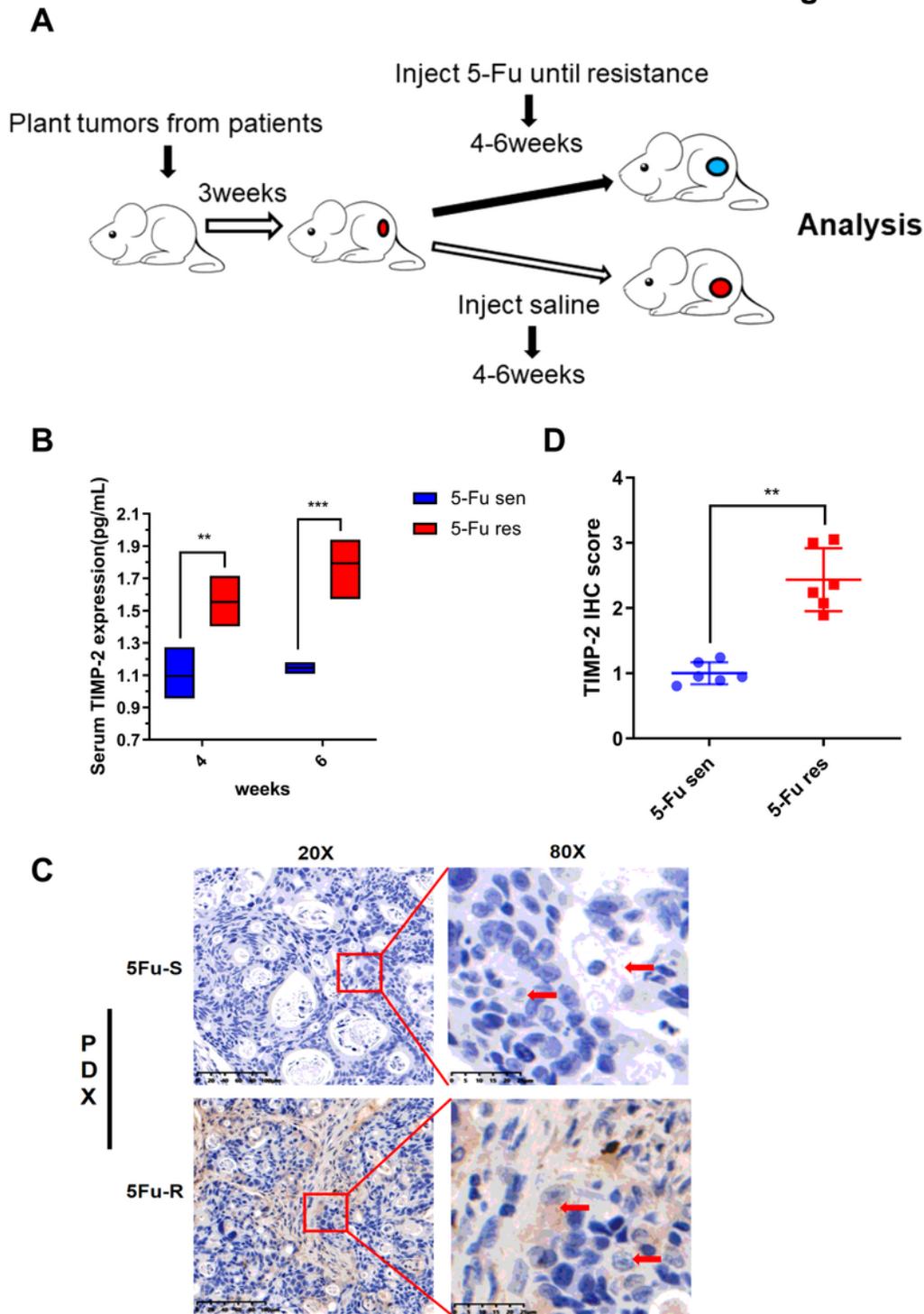
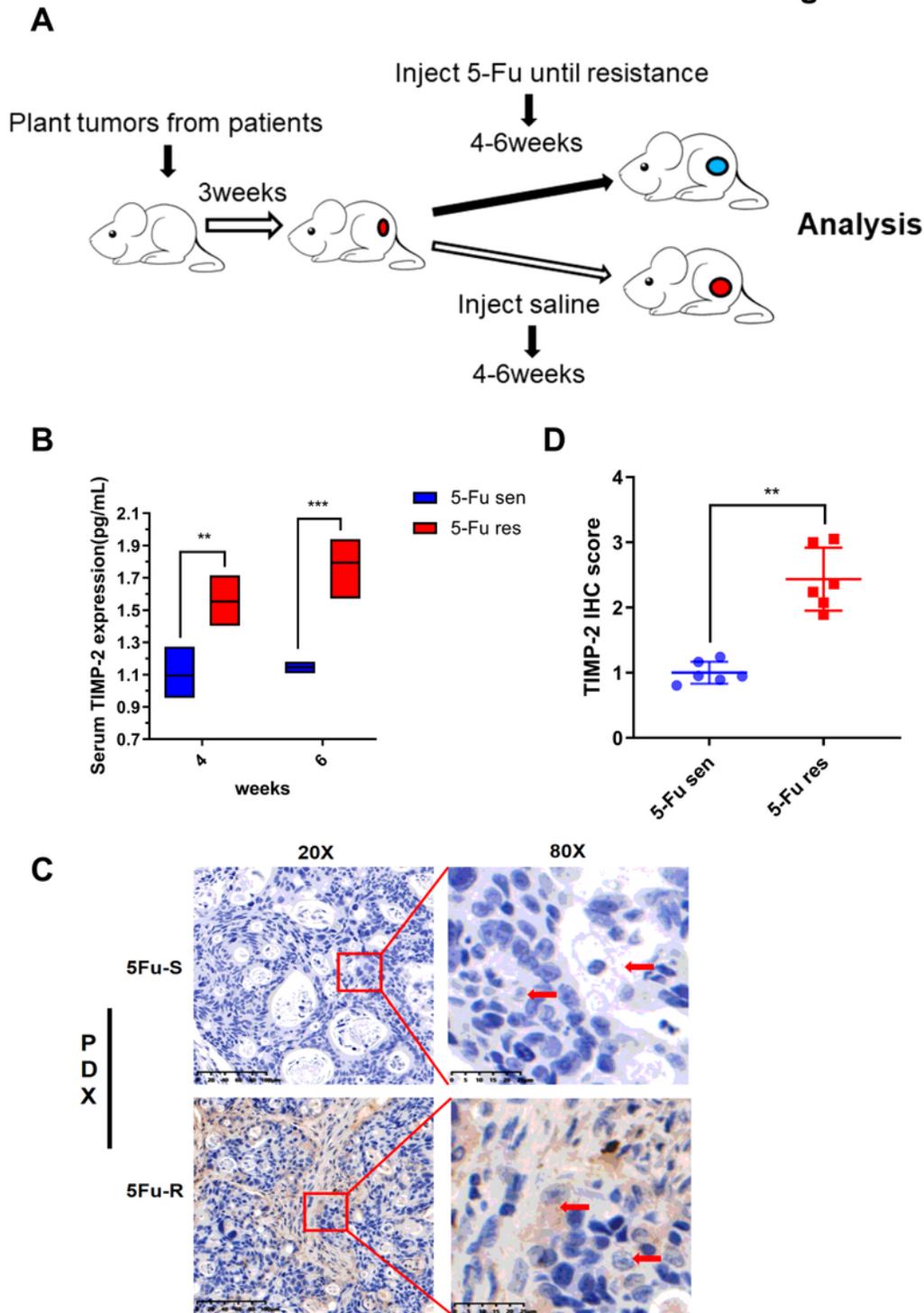


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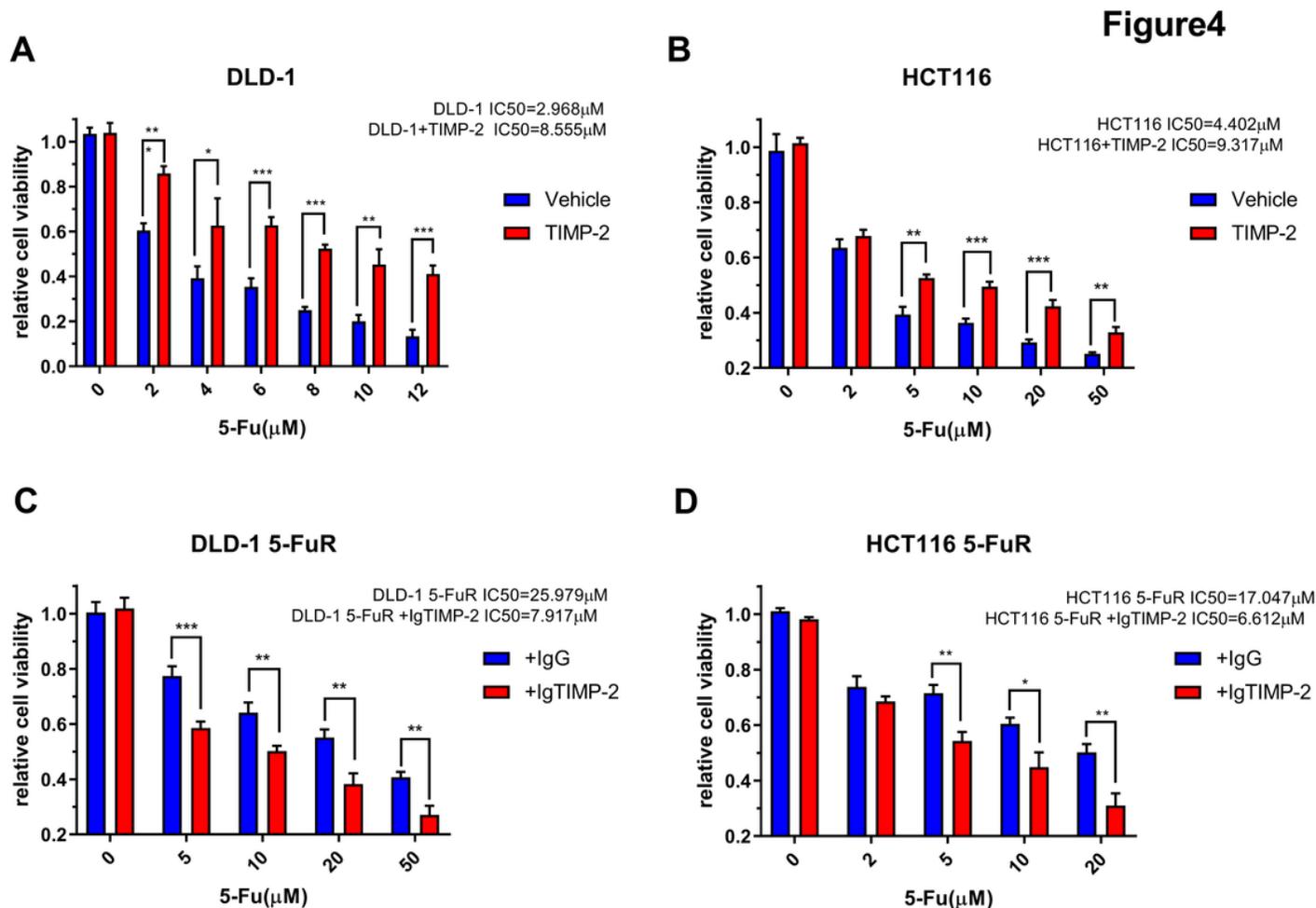
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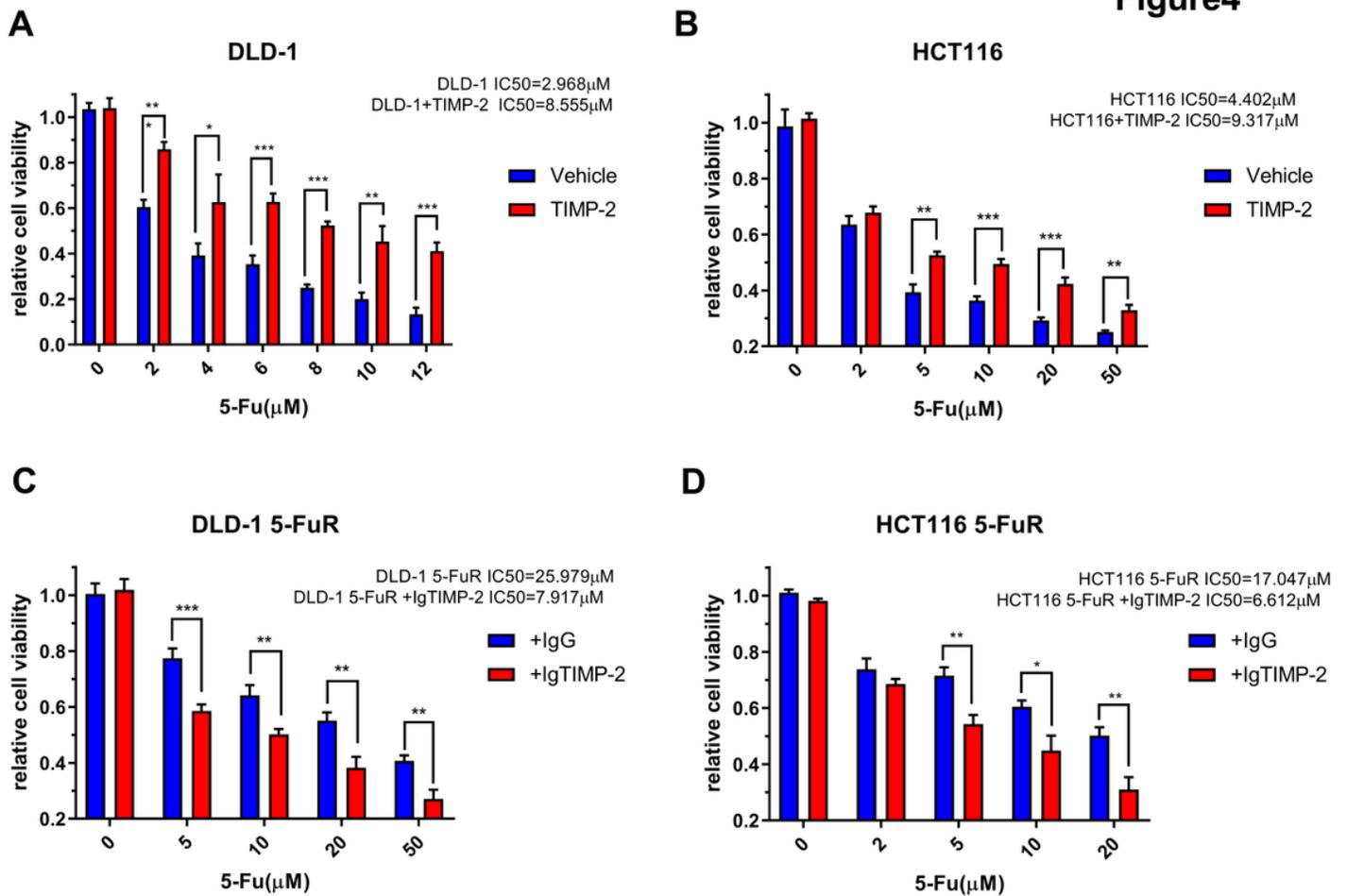
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**Figure 4**

TIMP-2 promotes CRC cells resistance to 5-Fu through autocrine mechanism (A)(B) Relatively cell viability of DLD-1 cells and HCT116 cells in the condition of increasing concentration of 5-Fu for 3 days after culturing with 10 ng/mL of recombinant TIMP-2 for 6 hr. (C)(D) Relatively cell viability of DLD-1 5-FuR cells and HCT116 5-FuR cells in the condition of increasing concentration of 5-Fu for 3 days after culturing with control IgG or 5  $\mu$ g/mL of TIMP-2 neutralizing antibody for 6 hr. Data from triplicate wells in 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by Student's t-test or one-way ANOVA.

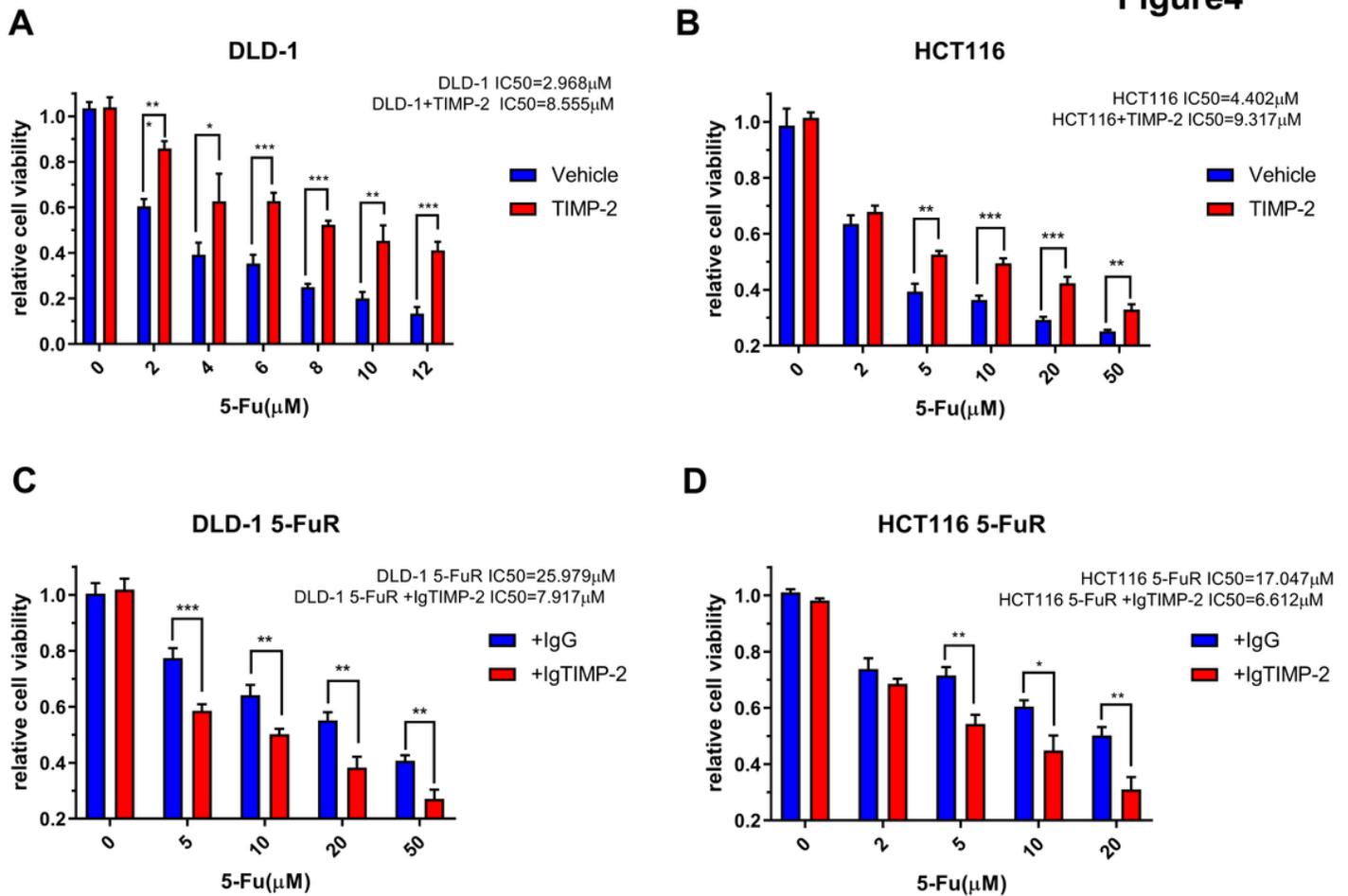
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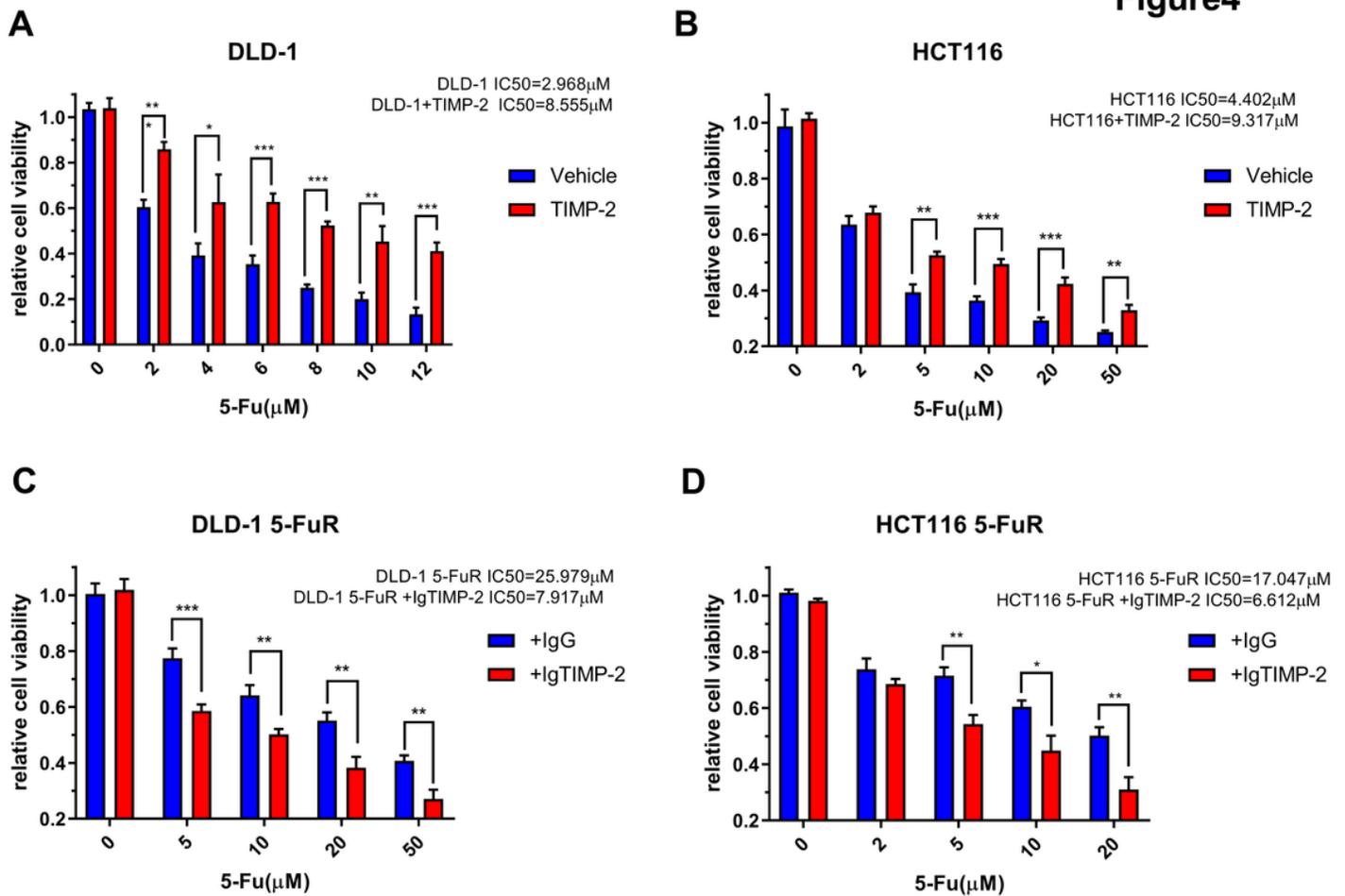
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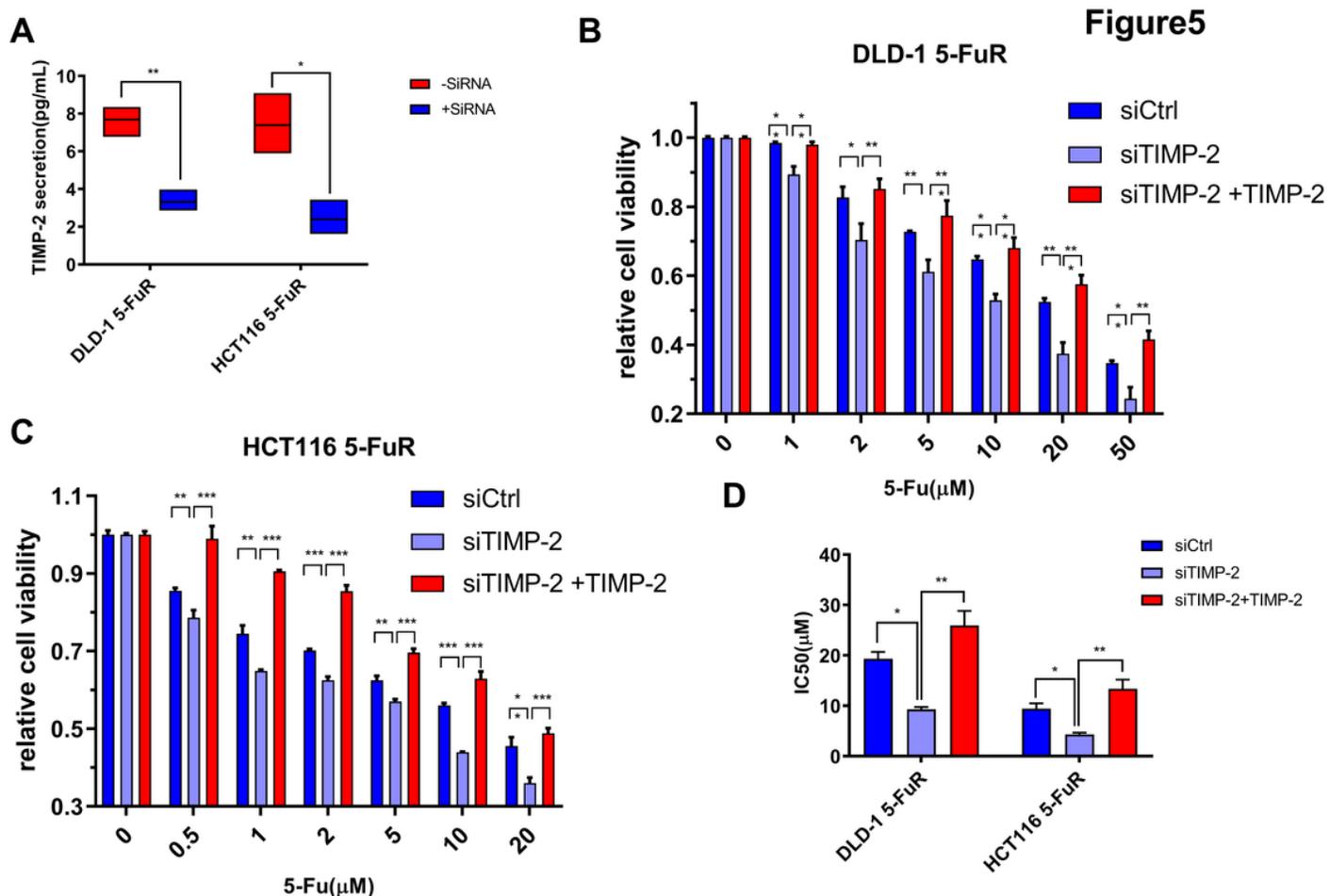
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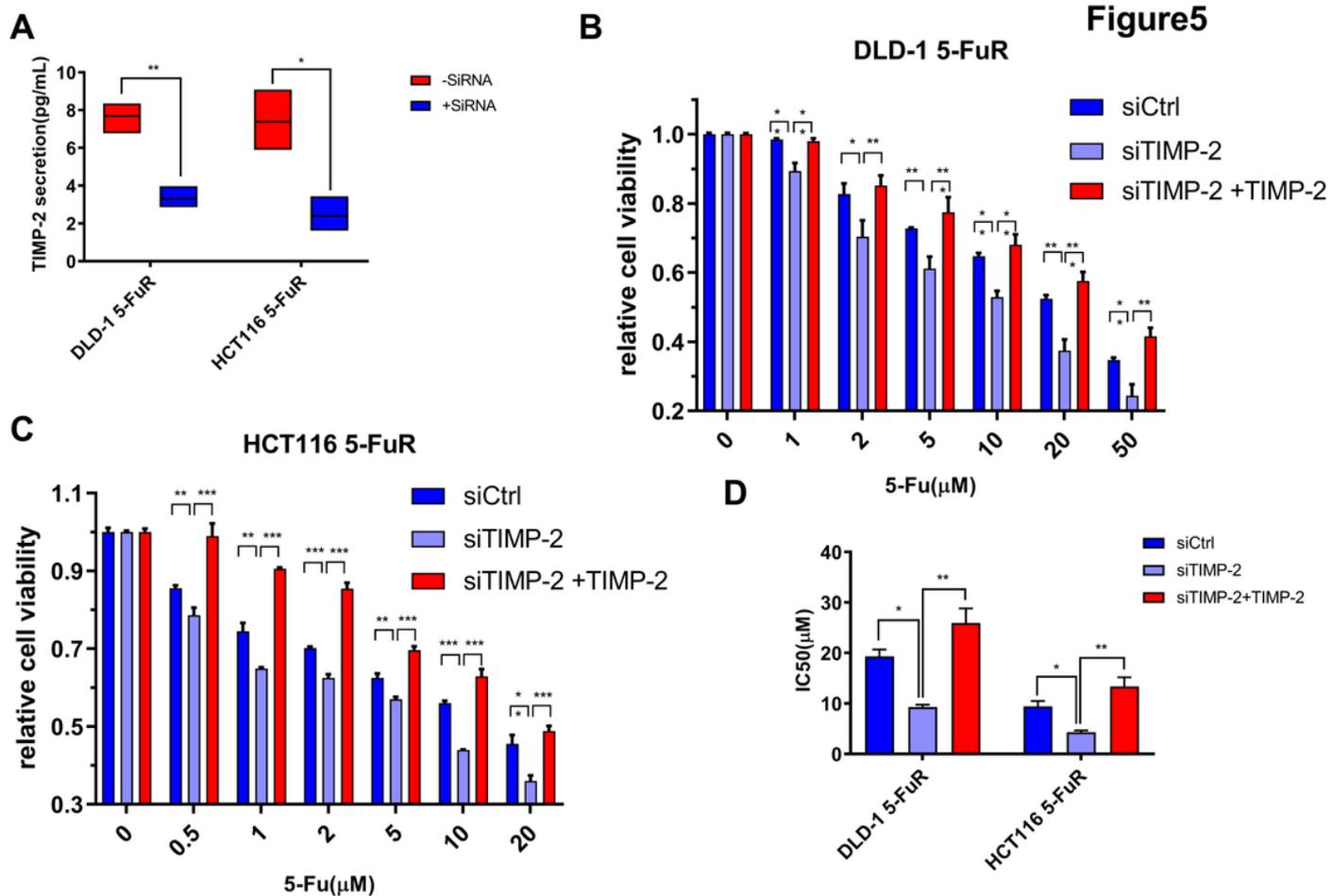
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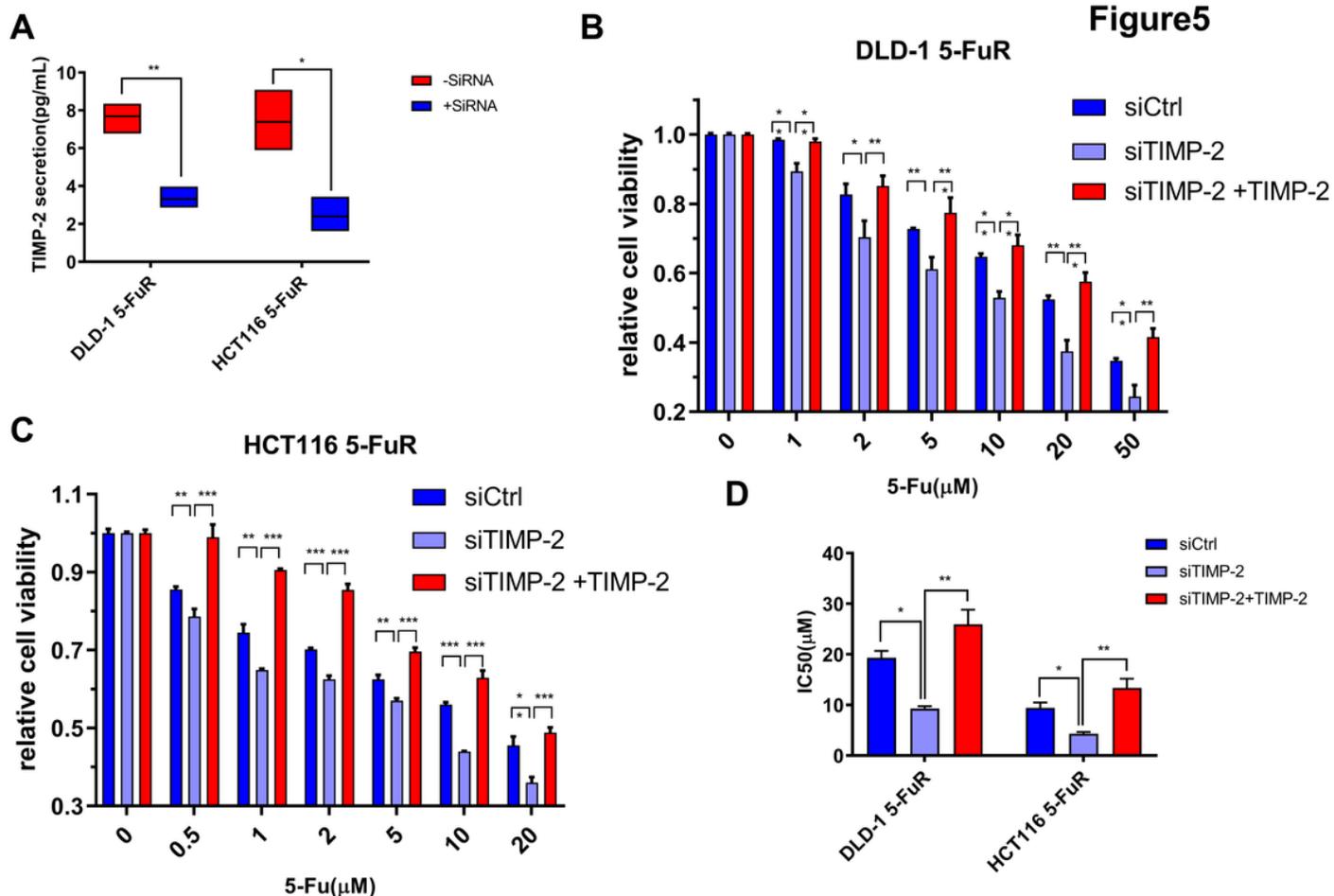
**Figure 5**

Knockdown of TIMP-2 overcomes 5-Fu resistance in CRC cells (A) Changes in expression level of TIMP-2 in DLD-1 5-FuR cells and HCT116 5-FuR cells by TIMP-2 siRNA (30 pg/ml). (B) (C) Relatively cell viability of DLD-1 5-FuR cells and HCT116 5-FuR cells in the condition of increasing concentration of 5-Fu for 3 days after culturing with TIMP-2 siRNA (30 pg/ml) or TIMP-2 siRNA (30 pg/ml) and recombinant TIMP-2 (10 ng/ml) together. (D) The differences in 5-Fu concentration of 50% inhibition of cell growth (IC50) between the six groups of cells in Figure 5 (B) (C) above. Data from triplicate wells in 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by Student's t-test or one-way ANOVA.



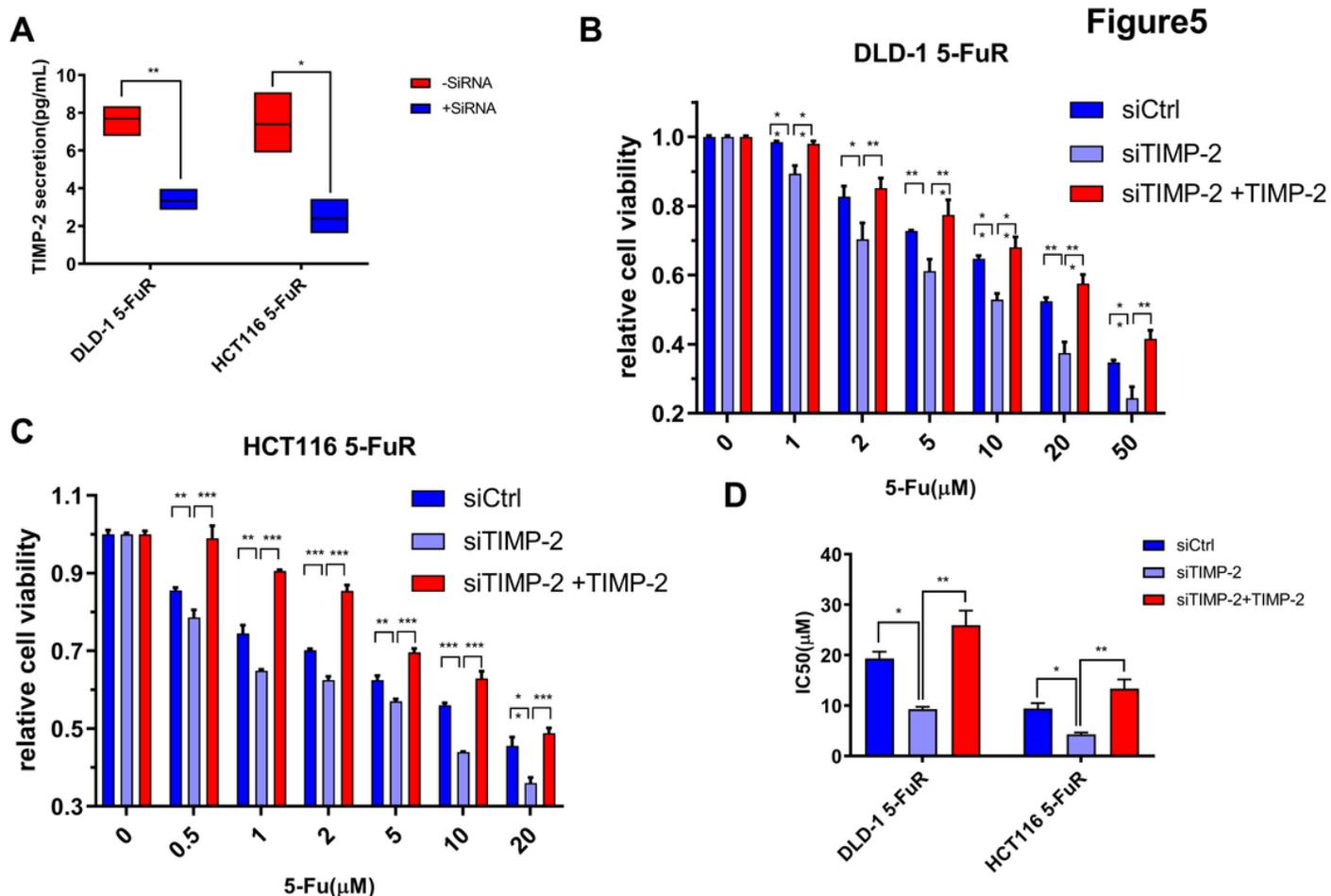
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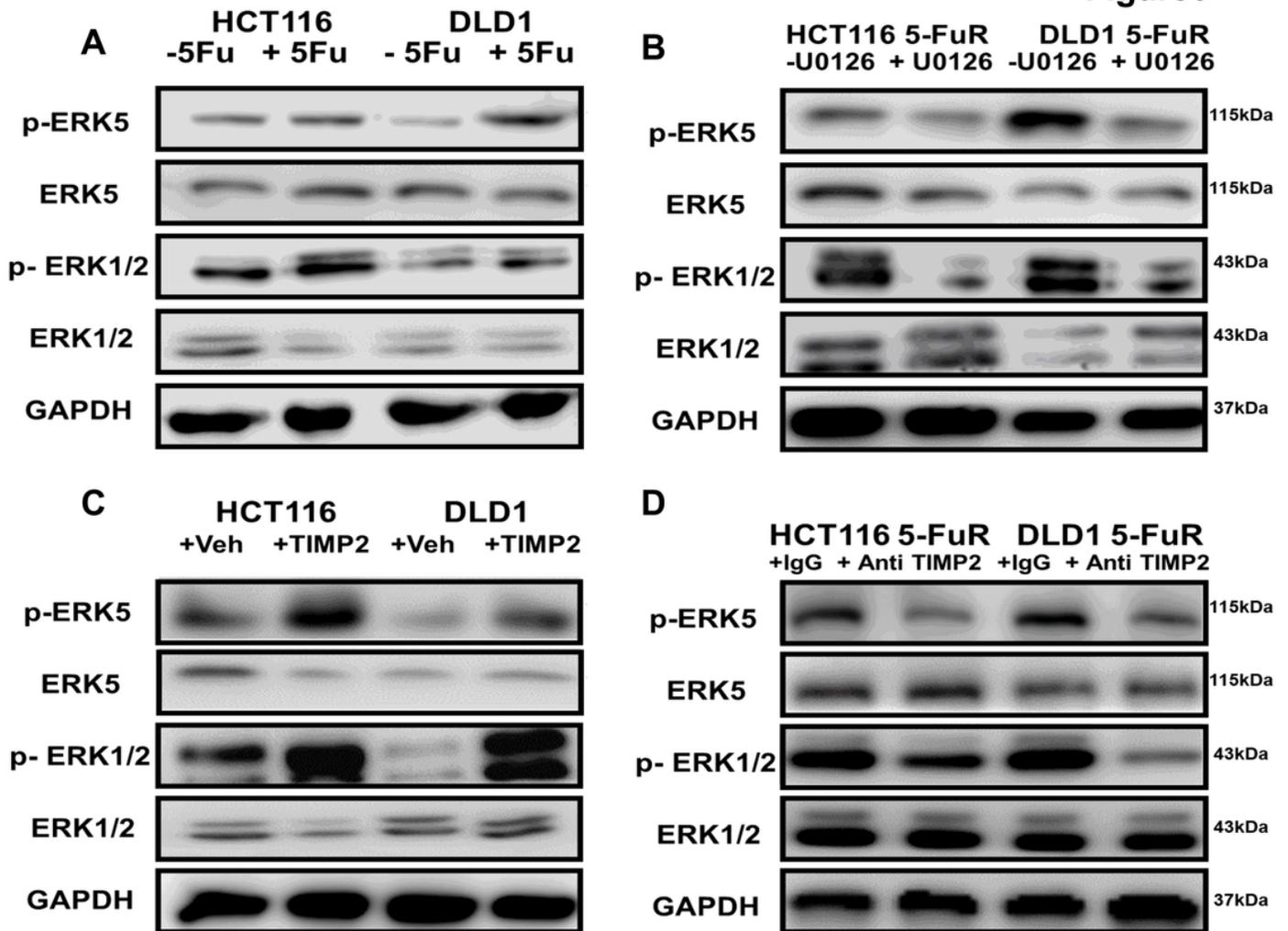
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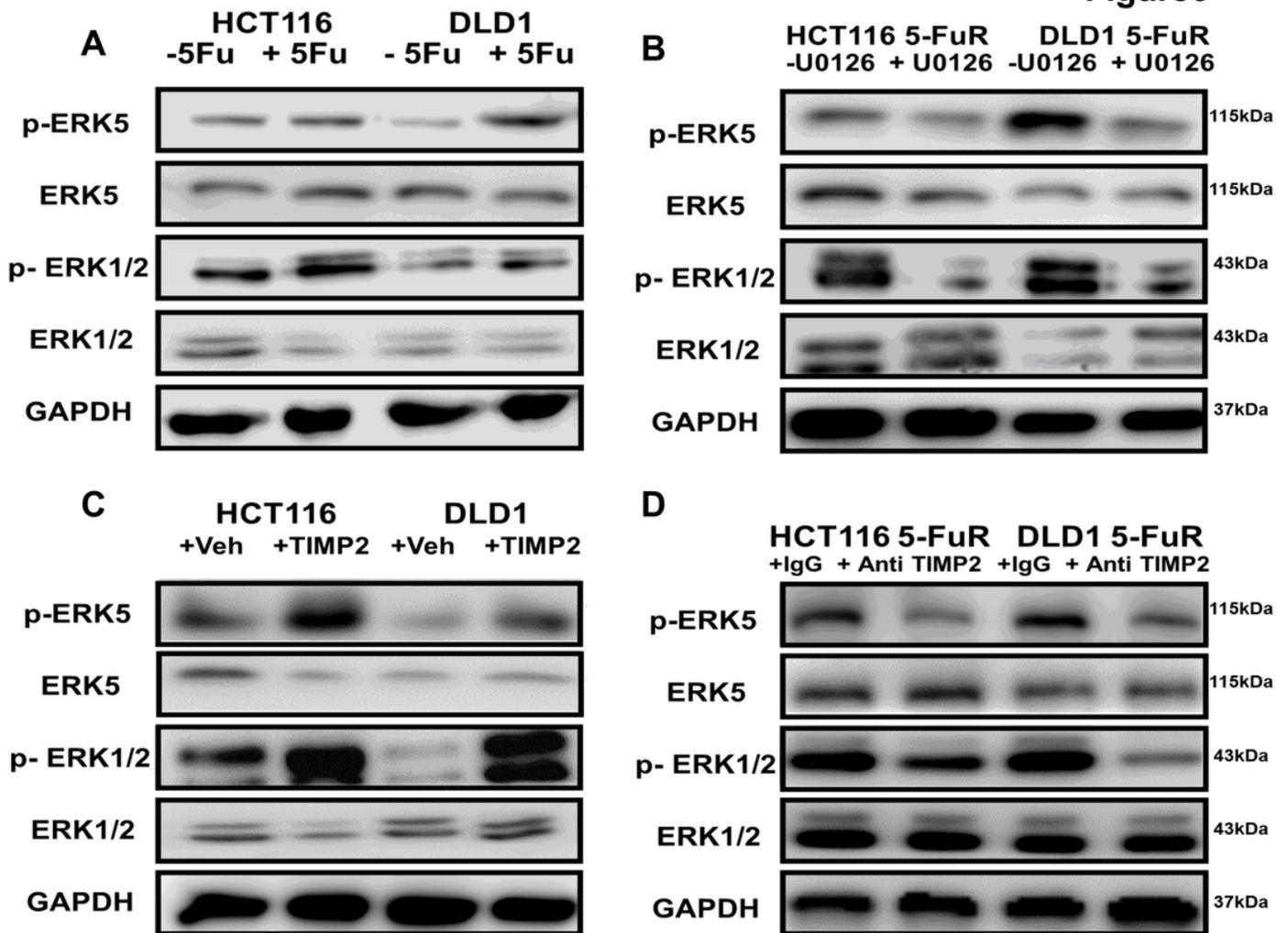


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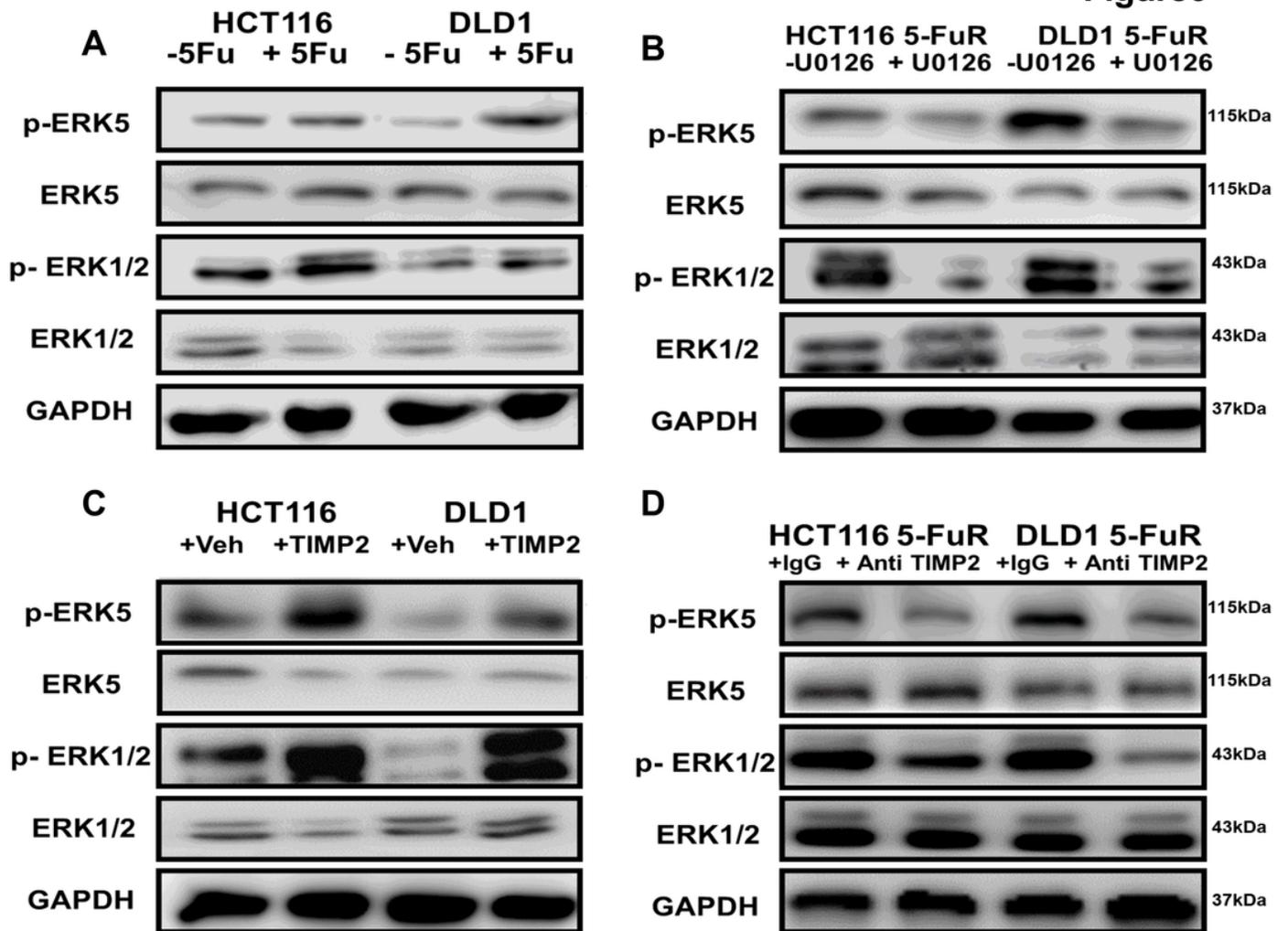
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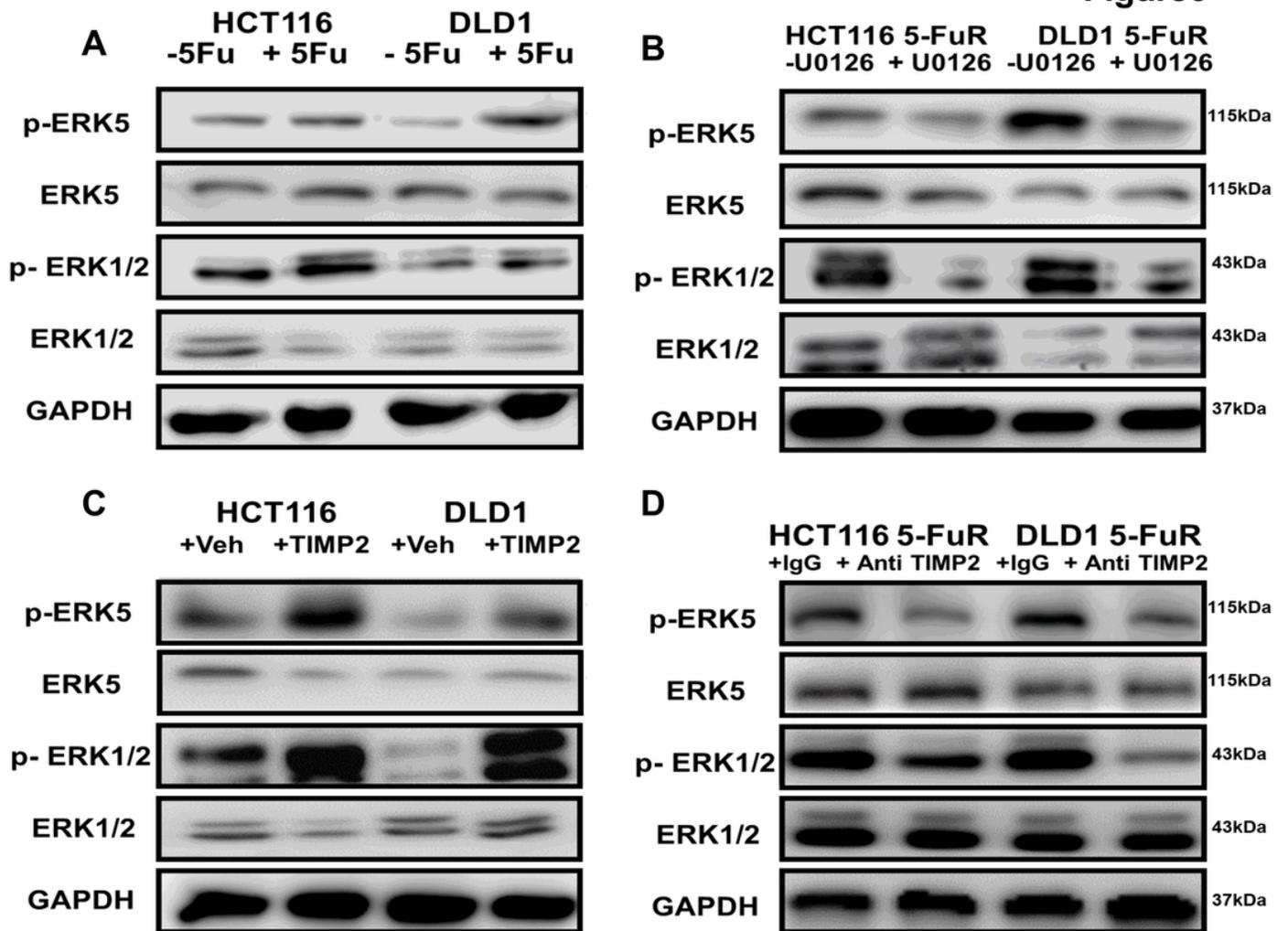
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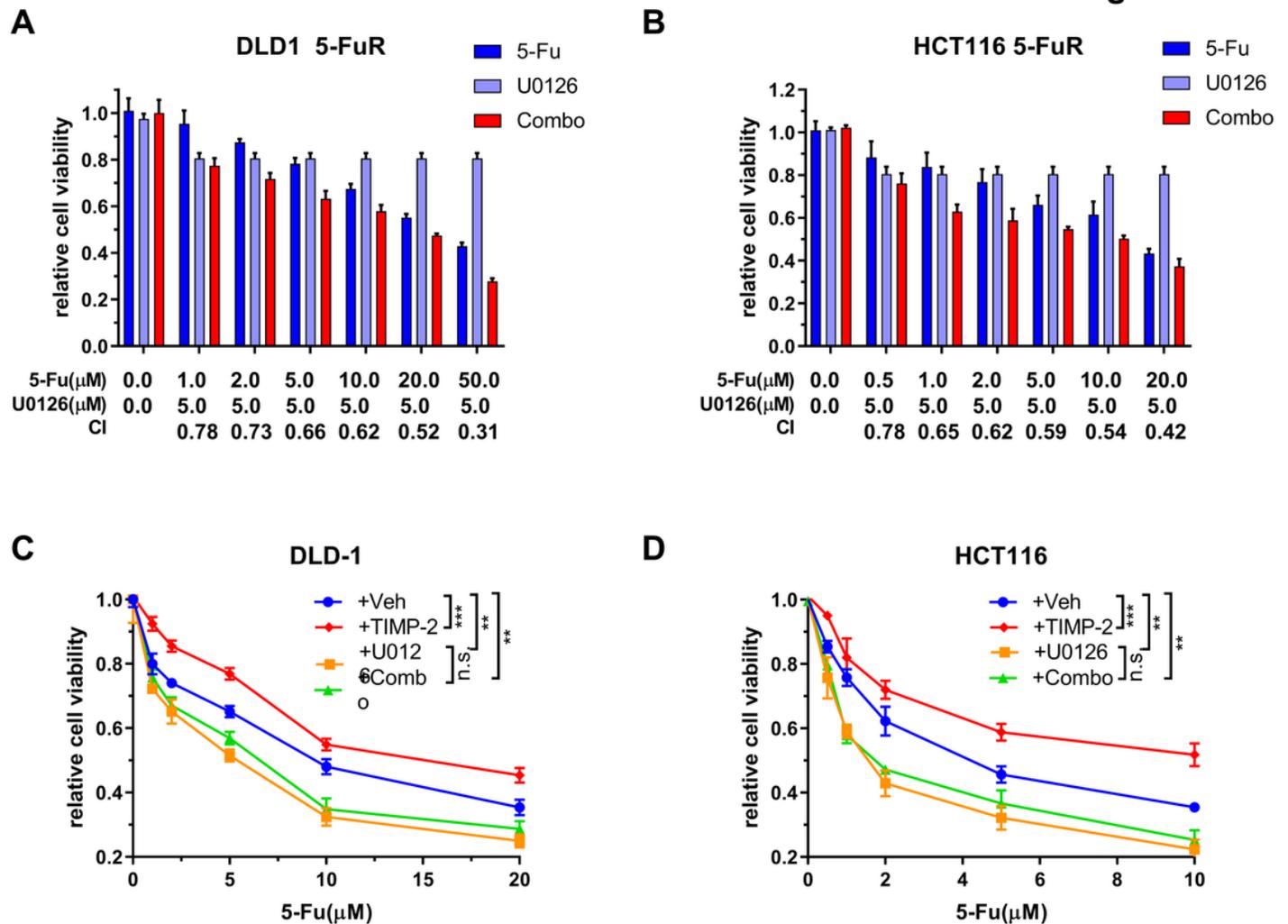
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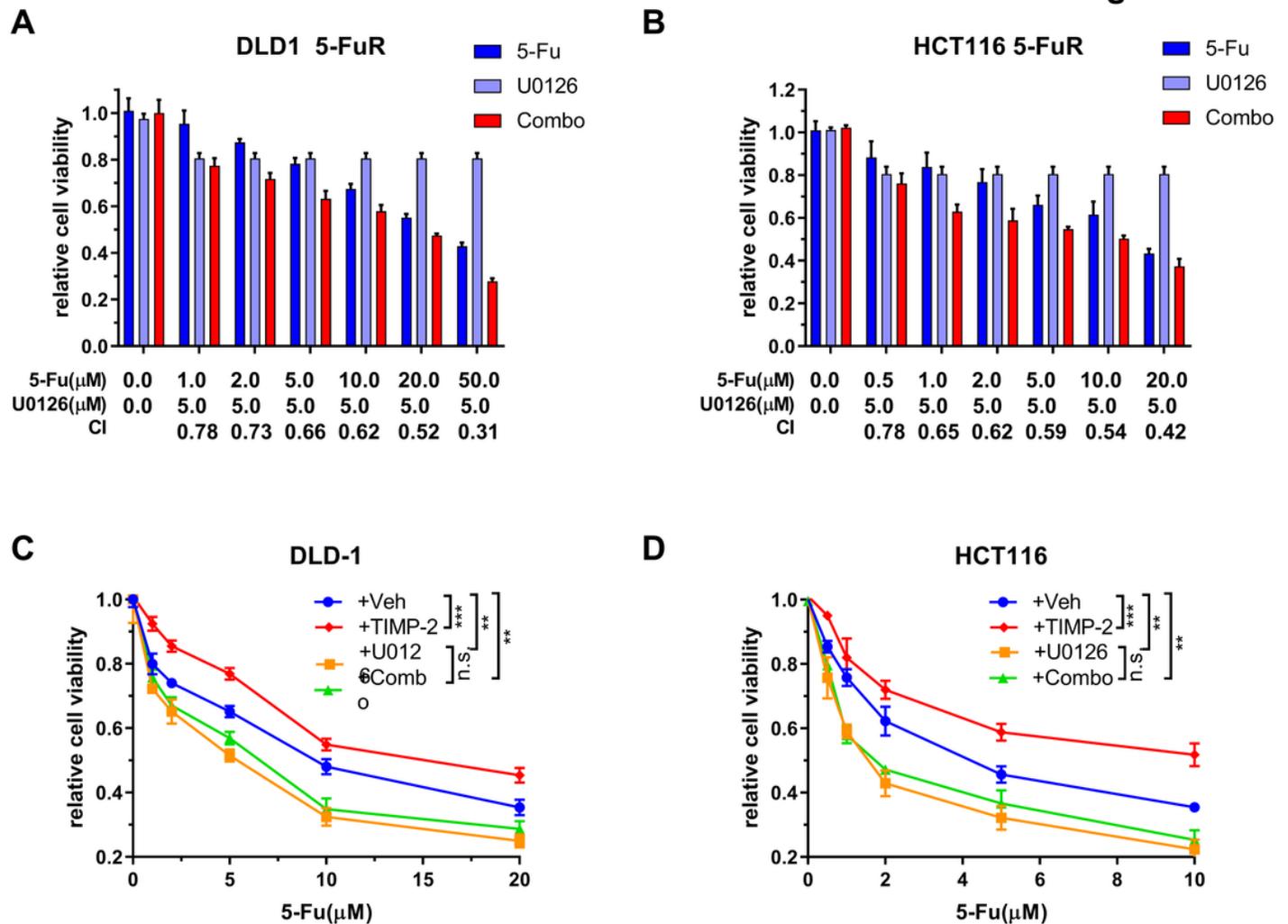
**Figure 7**



**Figure 7**

U0126 overcomes 5-Fu resistance in CRC via ERK/MAPK signaling pathway (A) (B) The synergistic effects of U0126 and 5-Fu on DLD-1 5-FuR and HCT116 5-FuR cells. Combo = 5-Fu + U0126. (C) (D) Knockdown ERK/MAPK by U0126 block TIMP-2 mediated resistance of CRC cells to 5-Fu. DLD-1 and HCT116 cells were cultured with 5 μM of U0126 for 24hr and then cultured with recombinant TIMP-2 (10 ng/ml) for 6 hr, following by increasing concentrations of 5-Fu treatment for 3 days. Combo = TIMP-2 + U0126. The combination index (CI) is presented below the bars. Data from triplicate wells in 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by one-way ANOVA or two-way ANOVA.

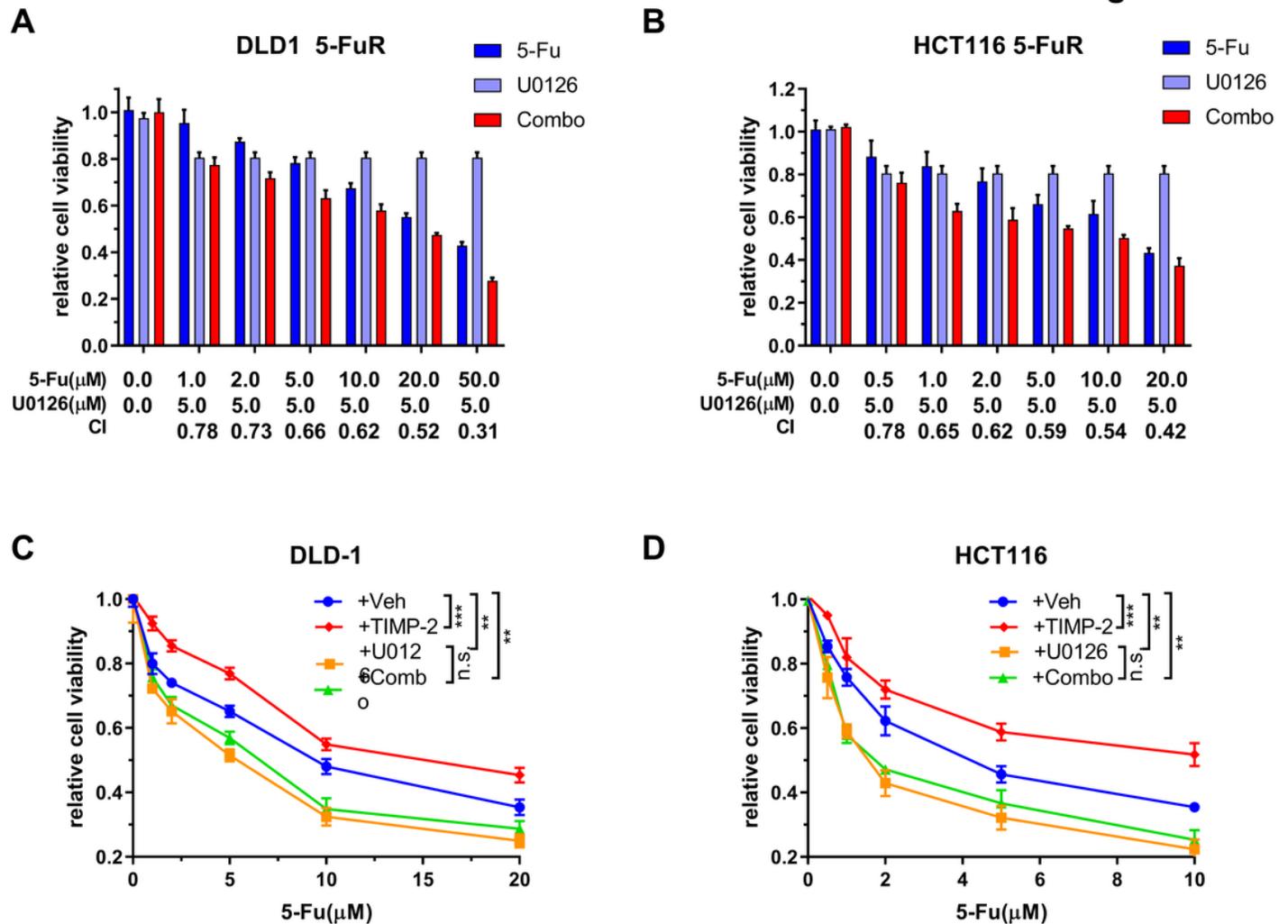
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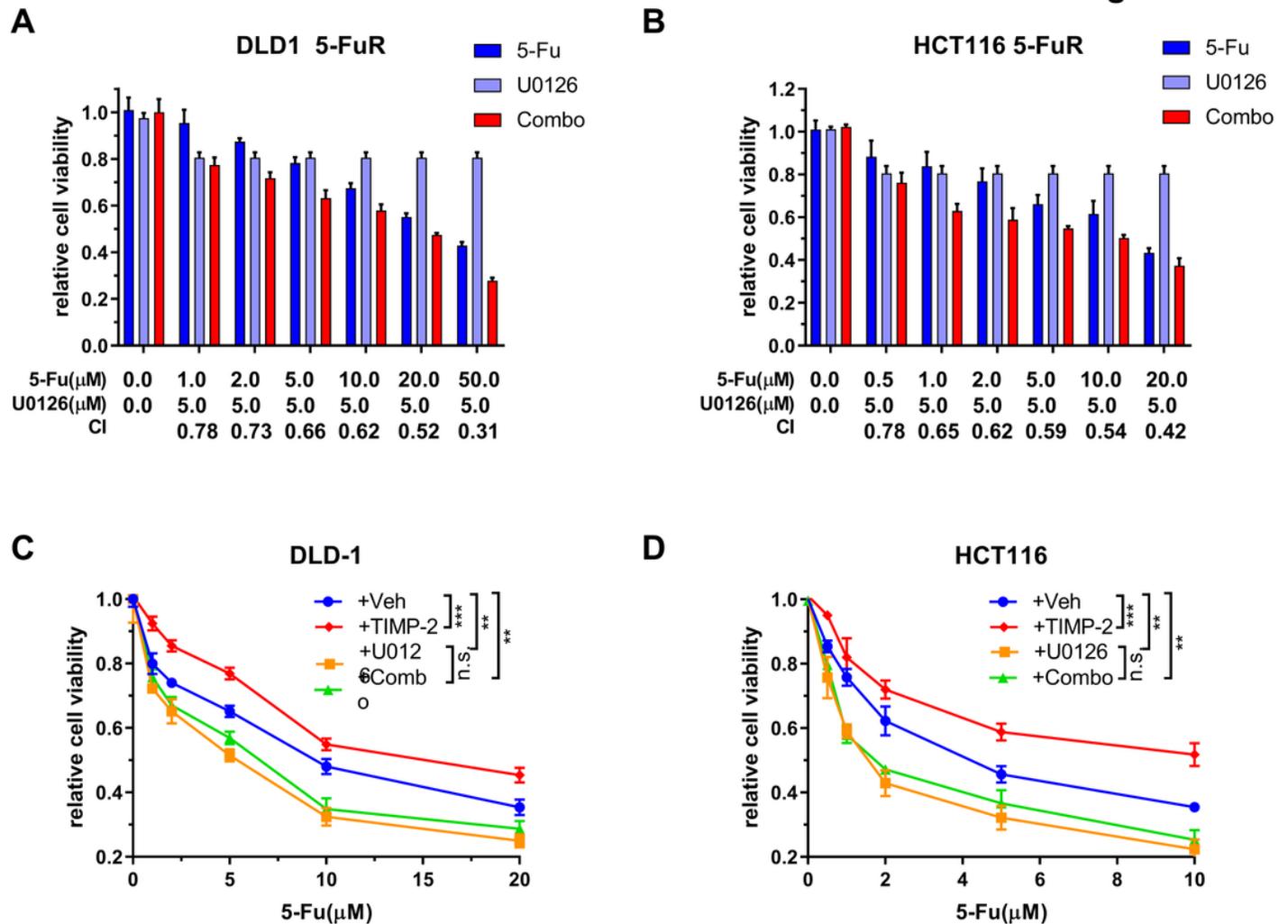
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