

# UHMK1 Dependent Phosphorylation of Cajal Body Protein Coilin Altered 5-FU Sensitivity in Colon Cancer Cells

**Huan Niu**

Capital Medical University

**Meng Zhao**

Capital Medical University

**Jing Huang**

Capital Medical University

**Jing Wang**

Capital Medical University

**Yang Si**

Capital Medical University

**Shan Cheng** (✉ [chengs@ccmu.edu.cn](mailto:chengs@ccmu.edu.cn))

Capital Medical University <https://orcid.org/0000-0002-8658-6916>

**Wei Ding**

Capital Medical University

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## Research

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# Abstract

**Background:** Resistance to 5-fluorouracil (5-FU) in chemotherapy and recurrence of colorectal tumors is a serious problem to be resolved for the improvement of clinical outcomes.

**Methods:** In the present study, the effects of conditioned medium (CM) derived from 5-FU-resistant HCT-8/FU on cell functions were evaluated. The methods of immunofluorescence and RNA-seq analyses were used to investigate the molecular mechanism underlining the roles of CM from resistant cells.

**Results:** we found that CM derived from 5-FU-resistant HCT-8/FU was able to reduce 5-FU chemosensitivity of HCT-8 colon cancer cells, with correlating changes in the number and morphology of the Cajal bodies (CBs) as observable nuclear structures. We identified UHMK1 was able to change the disassembly and reassembly of CBs regulated by the phosphorylation of coilin, a major component of CBs, and subsequently resulted in a large number of variations of RNA alternative splicing, affecting the cell survival following 5-FU treatment through changes in intracellular phenotype and transmitted preadaptive signals to adjacent cells in tumor microenvironment (TME).

**Conclusion:** Our finding provided evidence to demonstrate CBs of their disassembling/reassembling dynamics to indicate drug sensitivity or resistance in tumor cells in response to stress signal. The results also suggested that UHMK1 could be an important factor to maintain CB structure and morphology with its possible roles in the regulation of splicing events, especially when cells exposed to cytotoxic drugs.

## Background

Chemotherapy remained to be a major procedure for the treatment of colorectal cancers (CRC), as proper targeting drugs were merely able to match the majority of patient cases. Increasing evidence from both animal studies and clinical trials implied that the development of drug resistance could occur even at the early phases of treatments and resulted in tumor expansion and metastasis [1, 2]. The compound of 5-fluorouracil (5-FU) or its derivatives is a most frequent constituent to formulate chemotherapy protocols for treating a variety of tumors [3], especially in CRC. Despite 5-FU induced massive cell deaths and was able to quickly reduce the tumor burden, the increased levels of apoptosis did not indicate better prognosis as frequently observed in CRC patients. It was disappointing that some patients received adjuvant 5-FU exhibited even shorter overall survival comparing to patients who subjected to only surgery operations, especially in cases of CRC reoccurrence [4]. The molecular mechanism for the development of drug resistance can be extremely complex, and to date, the nature of 5-FU resistance was not well understood, left alone the strategy to overcome this issue.

Recent research has started to emphasize tumor microenvironment (TME) for its vital role in the development of drug resistance, which extensively involved the intercellular communication through direct cell-to-cell contact or canonical paracrine pathways [5]. The factors secreted by tumor cells in conditioned medium (CM) include metabolites, cytokines and growth factors, all of which contributed to the changes in cancer cell phenotypes as if they are functioned similarly to in vivo situations [6].

Therefore. In laboratory researches, the culture of cancer cells using CM from defined sources is a commonly accepted approach to discover and dissect the association between the tumor microenvironmental conditions and inheritable resistance factors.

The cell responses to CM exposure, especially related to changes in resistance, are often dynamic and chronic processes, hence, morphology indicators can be advantageous and desired for technical convenience. Cajal body (CB) is a membrane-less organelle and largely consists of proteins and RNA observed in different cell types, which is recently rediscovered for its important biological functions [7]. Such highly organized and nucleus structures in eukaryotes were previously monitored in cell cycle or DNA repair studies, which were also important and overlapped the biological processes defining drug sensitivities. In tumor cells, the morphology of CBs was reported as a rapidly-response index that was extremely sensitive to DNA damages [8]. Coilin is a hallmark protein of the CB and the morphology of CBs, including number per nucleus, shapes or sizes, were reported to characterize changes in cell phenotype following DNA damages, such as treatments of cisplatin, daunorubicin, etoposide, as well as UV-C or gamma irradiation [9].

As a major scaffold component of CBs, coilin is known to functionally involve many associated nuclear events of RNA processing, including small nuclear ribonucleoproteins (snRNPs) or Cajal body-specific RNPs (scaRNPs) of their biogenesis, maturation and recycling, as well as the modification of histone pre-mRNAs at the 3'-ends [10]. Reports showed that suppressing coilin decreased cell proliferation and the splicing of certain pre-mRNAs [11]. It was suggested that rapid recruitment of coilin to DNA lesions could mediate chromatin conformational changes in response to genotoxic stress. Defected snRNP biogenesis and splicing in Coilin<sup>-/-</sup> mice exerted a survival problem with reduced viability [12]. In stable coilin knockdown cells, promoted apoptosis and increased chemosensitivity to daunorubicin was observed [13]. The molecular mechanism explaining the changes in CB formation was yet unclear. It was hypothesized that either CBs directly participate the cellular stress response pathways, interacting certain nuclear events under conditions of drug treatment. Alterations in other essential CB components, such as WRAP53 $\beta$  and TCAB1 have also been linked to carcinogenesis and associated with the poor prognosis of cancer patients [14, 15]. Recent studies indicated that CB was able to alter the consequence of transcription-related events following DNA damages by changing the dynamics of RNA processing. Aberrant splicing events conferring drug/therapy resistance in cancer can be more frequently occur than previously expected. How aberrant splicing is influenced in response to environmental stimuli and subsequently sculpt cellular phenotypic properties, particularly in highly heterogeneous and progressing malignant tumors, is poorly understood.

In the present study, we found that CM derived from the drug-resistant HCT-8/FU cells inhibited the cell sensitivity of the native HCT-8 to 5-FU, accompanied with the significant changes CB morphologies. Following a screening from high-throughput transcriptome analysis, we identified UHMK1 could serve as a modulator related to the adaptive reduction of 5-FU chemosensitivity by microenvironment factors in CM. The overexpression of UHMK1 promoted the phosphorylation of coilin and altered the formation and reassembly of CBs, resulted in a massive profile change of RNA alternative splicing in addition to a

variety of differentially expressed genes responsible for cell growth and survival. Our finding provided not only evidence to understand the cellular response to environmental stimulation of cancer cells, but also reemphasized the importance of Cajal bodies of their disassembling/reassembling dynamics to indicate the development of adaptive phenotype of cancer cells in conditioned stress microenvironment.

## Materials And Methods

### Tissue culture and cell treatments

The experiments used human colon cancer cells that were 5-FU-sensitive HCT-8 cells and 5-FU-resistant HCT-8/FU cells, which were obtained from MEIXUAN Bioscience & Technology Co. Ltd. (Shanghai, China). The human colon cancer cells SW480 were obtained from the Cell Resource Centre of Chinese Academy of Medical Science (Beijing, China). HCT-8 and SW480 were maintained at 37°C in a 5% CO<sub>2</sub> incubator in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) (Biological Industries, Kibbutz BeitHaemek, Israel) and 1% penicillin-streptomycin (Keygen Biotech, Nanjing, China). Research grade 5-FU (MCE, Monmouth Junction, USA) was used in the assays to evaluate the cell viability. Apatinib (Hengrui medicine Co, Ltd., Jiangsu, China), raltitrexed (Zhengda Tianqing Pharmaceutical Co., Ltd., Jiangsu, China), artemisinin (National Institutes for Food and Drug Control, Beijing, China), cisplatin (QILU Pharmaceutical Co. Ltd., Shandong, China) were used in the immunofluorescence assays to evaluate the morphology changes of CBs.

### Vector preparation and transfection

The expression plasmid of FLAG tagged UHMK1 were cloned individually into pENTER vectors by Vigene Bioscience Co., Ltd. (Shandong, China). The construct of the UHMK1-K54A mutant was generated from the wild type by Vigene Bioscience Co., Ltd. (Shandong, China). The siRNA targeting UHMK1 duplexes of 5'-AAGCAGUUCUUGCCGCCAGGA-3' and 5'-CGAGUAUGGUUCCGCAAATT-3' were purchased from General Biosystems, Inc. (General Biosystems, Inc, Chuzhou, China). Coilin-targeting siRNA of 5'-GAGAGAACCUGGGAAAUUUTT-3' was obtained from General Biosystems, Inc. (Chuzhou, China). A scrambled siRNA 5'-UUCUCCGAACGUGUCACGUTT-3' was used as the control. The cells were transfected with either the UHMK1 plasmids or paired siRNA oligos using a Lipofectamine™ RNAiMAX Kit (Invitrogen, Waltham, Massachusetts, USA) following the vendor's recommended protocols. Western blotting was performed to detect the protein levels of the corresponded genes at 48 h post transfection.

### Cell viability assay

A number of 5,000 cells were seeded in each well of 96-well plates and cultured with 5-FU at 0, 1, 5, 10, 20, 40, 60, 80 µg/ml for 48 h. The cell viability was determined using cell counting kit (CCK8) (KeyGENBioTECH, Jiangsu, China) according to the vendor's standard protocols. The plates were scanned at 450 nm for absorbance using a spectrophotometer (BioTek, Winooski, VT, USA). Each data point was measured for the average from six duplicates. The experiments were repeated independently for 3 times.

## Immunofluorescence

The method has been widely implemented by our laboratories. Cells of  $5.0 \times 10^4$  were plated onto a glass coverslip placed into the well of a 12-well plate. The cells on coverslips were fixed, permeabilized, blocked and washed with phosphate-buffered saline (PBS). Anti-coilin (Proteintech Group, Rosemont, USA) was used as the primary antibody and an Alexa Fluor® 594 secondary antibody (Life Technologies, MA, USA) was used for incubation in the dark. The nuclei were stained with Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA) prior to the examination and image acquisition under a confocal system (Leica Microsystems TCS SP8, Wetzlar, Germany). Control samples without adding the primary antibody were prepared for determining the level of non-specific noise.

## mRNA-sequencing and data processing

RNA-seq analyses were performed as earlier described. Cells were scraped off from the surface in trypsin-versene solution and collected by 500 g centrifugation. The pellet was washed with PBS to remove residual media. Total RNA extractions were performed with the RNA-Quick Purification Kit (Yishan Biotechnology Co., Ltd., Shanghai, China) following the manufacturer's protocol. The RNA concentrations were determined using a Nanodrop ND1000 spectrophotometer (Thermo Scientific). The quality assessments and mRNA sequencing libraries were performed in the laboratory of VAHTS Universal V6 RNA-seq Library Prep Kit for Illumina (Vazyme Biotech, Nanjing, China), VAHTS RNA Multiplex Oligos Set1- Set2 for Illumina (Vazyme Biotech, Nanjing, China), VAHTS DNA Clean Beads (Vazyme Biotech, Nanjing, China), VAHTS mRNA Capture Beads (N401-01, Vazyme Biotech, Nanjing, China). All prepared samples subjected to paired-end multiplex sequenced ( $2 \times 150$  bp) on the Illumina HiSeq X10 platform. Approximately 8 Gb sequencing data was generated for each sample.

The clean reads in compressed FASTQ format were aligned using HISAT2 (version 2.1.0) to the reference of human genome (Homo\_sapiens.GRCh38.dna.primary\_assembly.fa) with matched rates over 90%. The resulted SAM files output in the BAM format used with SAMtools (version 1.18, <http://samtools.sourceforge.net>). The resulted BAM files were sorted with SAMtools. The depth counts were called with HTSeq (version 0.11.2, Linux\_x86\_64, Simon Anders (sanders@fs.tum.de)) with the reference of human genome (Homo\_sapiens.GRCh38.94.gtf), European Molecular Biology Laboratory (EMBL)) used to calculate the Fold change (FC) of FPKM and p value among sample groups according to an over-dispersed Poisson model. Differentially expressed (DE) genes were identified with the thresholds of both 1.5 fold change ( $|\log_2 FC| \geq 0.58$ ) in mean expression and  $FDR \leq 5\%$  using Benjamin-Hochberg procedure. The p-value was identified using DESeq2 (version 1.30.0). The enrichment of DE genes was performed using Gene Ontology (<https://go.princeton.edu/>) and Cluster 3.0 (Michael Eisen, Stanford University), then displayed with TreeView (version 1.1.6r4, Alok Saldanha). The analyses for alternative splicing events of expressed genes were performed using the rMATS software package (version 4.1.0, <http://rnaseq-mats.sourceforge.net/>, Xing Lab, Children's Hospital of Philadelphia). The differences in splicing isoforms (SIs) were identified with  $FDR \leq 5\%$  and a threshold of  $p \leq 0.01$  in the mean expression between the samples.

## **RNA extraction and qRT-PCR**

Total RNA was isolated using Trizol (Life Technologies, Carlsbad, CA, USA). HiScript II Q RT Kit (Vazyme, Nanjing, China) was used for reverse transcription. NovoStart® SYBR qPCRSuperMix Plus (Novoprotein, Shanghai, China) was used to quantify gene expression level from the obtained cDNA. The primers for detecting are listed in Table S1. GAPDH was used as the loading reference. The cDNA were determined using a Quantitative Real-time PCR (Archimed X6, Rocgene, Beijing, China)

## **Western blotting**

Western blot analyses were performed as earlier described. Briefly, samples of cell lysates were prepared and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), then transferred onto polyvinylidene fluoride (PVDF) filters. The probing antibodies were against the following antigens: UHMK1 (SC-393605, Santa Cruz Biotechnology, Santa Cruz, CA, USA), coilin (10967-1-AP, Proteintech Group, Rosemont, USA) and GAPDH (TA-08, ZSGB-BIO, Beijing, China).

## **Co-Immunoprecipitation (Co-IP) assay**

Cells were harvested and lysed in 1000 µl of ice-cold lysis buffer (10 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1 mM Benzamidine, 0.5% Triton X-100). The lysate was solubilized via end-over-end rotation at 4°C and clarified via centrifugation at 12,000 rpm for 30 min. A small fraction of the supernatant was taken at this point and incubated with SDS-PAGE sample buffer in order to examine expression of proteins in the whole cell extract. The remaining supernatant was divided equally into two tubes, and then incubated with 2 µg phospho-Ser antibody (SPC-149F, StressMarq Biosciences Inc, Victoria, British Columbia) antibody or 2 µg IgG (C2170, Applygen Technologies Inc, Beijing, China) respectively with end-over-end rotation at 4°C overnight. After incubated with 30 µl of Protein A/G PLUS-Agarose (sc-2003, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h with end-over-end rotation at 4°C, the immunoprecipitated proteins were eluted from the beads with sodium dodecyl sulfate (SDS) sample loading buffer, resolved by SDS-PAGE and subjected to Western blot analyses using an antibody against coilin (10967-1-AP, Proteintech Group, Rosemont, USA)

## **Statistical analysis**

The analysis of variance (ANOVA) was used to determine the statistical significance of data in multiple groups. The Student's t-test was used to compare cell functions between paired groups. Cases of p-value <0.05 was defined as statistically significant. The program of Prism 8 (GraphPad Software, Inc., La Jolla, CA, USA) was used for data plotting.

# **Results**

**Morphology changes of Cajal bodies indicated sensitivity to 5-FU in colon cancer cell, and CM induced CB morphology associated with the phosphorylation of coilin**

It has been reported that morphology of Cajal bodies, such as the numbers, sizes or shapes, subjected to significant changes when cells were exposed to DNA damage agents [16]. We observed that CB counts significantly decreased in HCT-8 colon cancer cells following 5-FU treatments, as shown from the immunofluorescence of coilin (Fig. 1a). Such changes in CB staining could also be observed when HCT-8 cells exposed to other chemotherapy reagents known with DNA damage activities, including 5-FU, apatinib, raltitrexed, penicillin, artemisinin and cisplatin (Fig. 1b). In order to better characterize changes in CB morphology, we categorized several major types of CB patterns as normal, deformed, diffused and multi-scattered (Fig. 1). Comparing a pair of 5-FU-resistant and -sensitive colon cancer cell lines, HCT-8/FU and HCT-8 (Fig. S1c), we found that there were significant differences in CB morphologies in various categories ( $p < 0.01$ ) (Fig. 1d). Coilin acts as a scaffold for the correct assembly of CBs, both the aberrant expression and modification could influence the formation of CBs [17, 18]. We determined that no significant difference in coilin expression at protein levels between HCT-8 and HCT-8/FU cells (Fig. 1e), however, the phosphorylation of coilin in HCT-8/FU, detected from immunoprecipitation assays using a phospho-Ser antibody, was significantly increased than in HCT-8 cells (Fig. 1f). These results supported previous findings that coilin phosphorylation could be a key factor determining the assembly of CBs, or associated with cell phenotype of drug sensitivity or resistance.

### **HCT-8 cells cultured in HCT-8/FU condition medium exerted reduced 5-FU sensitivity accompanied with the morphology changes of CBs**

Recent studies reported that TME induced drug-resistant phenotype by transmitting stress signal to adjacent cells and promote cell survival [15]. Our CCK-8 results showed that HCT-8/FU CM treatments reduced HCT-8 viability in 5-FU dose responses as compared to the control (Fig. 2a). Aberrant CBs in both numbers and shape categories were also changed (Fig. 2b). The protein expression of coilin were stable between groups of HCT-8 and HCT-8/FU in CM cultures (Fig. 2c), except the phosphorylation of coilin in HCT-8/FU CM treated cells was significantly increased (Fig. 2d). These data indicated that exposure in CM derived from the drug-resistant HCT-8/FU was able to reduced 5-FU sensitivity in HCT-8 cells, supporting the importance of environmental factors and stimulation for the development of chemoresistance. In CM cultured cells, not only the 5-FU sensitivity was once again accompanied with significant morphological changes of CBs, interestingly, the changes in counts and percentages in CB types seemed to be more significant than cells in normal cultures under the similar levels of coilin phosphorylation. This suggested that using CB as an indicator for the cell response to environmental stimulation could be sensitive, rapid and dynamic.

## **Transcriptome analyses and validation revealed UHMK1 involved in coilin phosphorylation**

The importance of coilin phosphorylation in the formation and maintenance of CB structure was previously demonstrated [19], however, the kinases responsible for the phosphorylation of coilin were not characterized, especially in context of drug pressure. We therefore used an RNA-seq approach attempt to search for possible candidate serine kinases. The 5-FU sensitive and resistant HCT-8 cells in pairs were

compared in both conventional and CM culture conditions. The MA plot was used to show the differentially expressed genes of fold changes (FC) attributable to a given variable over the mean of normalized counts (Fig, S2a, b). After correction for multiple testing ( $FDR \leq 5\%$ ), we found 6,013 DE genes between HCT-8/FU and HCT-8, and 4,001 DE genes between HCT-8 with or without HCT-8/FU CM treatments, using the  $FC > 1.5$  ( $|\log_2 FC| \geq 0.58$ ) as cutoff thresholds (Fig. 3a). A total of 2,962 genes were shared in common of both comparisons, mainly enriched in cell differentiation, response to stress, cell cycle, cell death and cell proliferation by gene ontology (Fig. 3c). Twelve genes of different abundance were selected and subjected to RT-qPCR analyses, the results showed a confirmation rate of 68.8% (Fig. S2c). It was not a total surprise that not a single known serine/threonine kinase was outstood as the unique prominent candidate of over-representation.

Besides DE analysis, we also performed a comprehensive protocol supplied with rMATS software package to identify alternatively spliced transcripts between paired experimental groups [20]. rMATS provides functions to detect, quantify, and visualize complex SIs, including de-novo variations. Skipped exon was the most common event (nearly  $\sim 70\%$  across the different AS types) followed by intron retention, mutually exclusive exons, alternative 5' splice site and alternative 3' splice site (each  $\sim 10\%$  across the different AS types) (Fig. S3a). A total of 3,632 differential SIs was reported in between HCT-8/FU and HCT-8 cells; a similar number of 3,794 was found between HCT-8/FU CM and the control group, including 1668 shared SIs (Fig. 3b). About 30% of differential SIs was differential expressed which was an astonishing high percentage (Fig. 3c). The differential SIs showed significant enrichment in response to stress, cell differentiation, cell cycle, and cell death, agreeing with previous RNA-seq transcriptome analysis, suggesting that the differential SIs could contribute to cell survival (Fig. 3c). We also examined the 12 transcripts for the presence of alternative exons in the coding sequences (Table S2) and determined the existence of 8, making up to 76% of the verifiable AS events (Fig. S3b, c). Coilin has been suggested to have a crucial function in CB-related RNA processing, which known to affect drug resistance in acute lymphoblastic leukemia (ALL) [21]. Therefore, we reasoned that reported kinases with activities to induce massive splicing changes could also be a source for the identification of potent or novel kinases of coilin phosphorylation, at least in responses to 5-FU treatments.

We predicted the properties of candidate kinase would like to include nuclear localization, with known substrate of RNA binding or splicing regulator proteins, reported or verified upregulation following drug treatments (especially 5-FU and analogues), and/or associated with multiple categories as indicated from the GO enrichment in the DE analyses of the present study (Fig. 3c), particularly in those that also overlapped with the functional annotation of coilin or CBs. The narrowed down candidate list from the cross-reference of kinase activity (GO: 0016301), RNA binding (GO:0003723) and nucleus (GO:0005634) (Fig. 3d) contained only a limited number of choices (Table S3). By sifting through our data reports, the related database queries, and the literatures, a serine/threonine protein kinase U2AF Homology Motif Kinase 1 (UHMK1) appeared to be a top selection with a best p value confidence (Fig. 3e, f) [22]. The UHMK1 mRNA levels were determined by RT-qPCR and confirmed the upregulation in the paired compared comparison groups as revealed from the RNA-seq results (Fig. 3g, h). We also performed

western blotting for the detection of UHMK1 expression at the protein levels, the upregulation of UHMK1 were verified in consistency with the findings from the mRNA quantifications (Fig. 3g, h).

## **Knockdown of UHMK1 influenced 5-FU sensitivity and coilin phosphorylation in HCT-8 cells**

We next started to address whether UHMK1 is functionally involved in the regulation of 5-FU sensitivity in colon cancer cells. Transfection of interfering siRNAs against UHMK1 were used in HCT-8 cells. The expression level of UHMK1 was decreased as compared with the scramble controls from the western blots (Fig. 4a). From immunoprecipitation assays, the knockdown of UHMK1 significantly reduced the phosphorylation of coilin (Fig. 4a), with significant changes in CB morphologies, especially in the counted percentage of the aberrant CBs of the deformed, diffused and multi-scattered types (Fig. 4b). In addition, we observed about 50% decrease in cell growth in UHMK1 siRNAs transfected cells (Fig. 4c). The cell survival in the presence of 5-FU was also reduced in the UHMK1 knockdown samples as compared to the scramble controls (Fig. 4d). These data indicated that suppression of UHMK1 expression was able to reduce the phosphorylation of coilin, alter the CB morphology, and influence the cell growth and the sensitivity to 5-FU in HCT-8 cells.

### **The kinase activity of UHMK1 was required for coilin phosphorylation and associated impact on the sensitivity to 5-FU**

As UHMK1 was a serine/threonine protein kinase [23], to determine whether the kinase activity is necessary to mediate the effects on cell responses to 5-FU, we deployed a UHMK1 mutant lacking its kinase activity as a dominant-negative agent for transfection into HCT-8 cells (Fig. 5a). Increase of coilin phosphorylation was observed in wild type UHMK1 overexpression cells over the vector controls, whereas transfection of the kinase-dead mutant, UHMK1-K54A [24], reduced the relative levels of coilin phosphorylation (Fig. 5a). To further examine whether UHMK1 transfection was able to alter the growth and sensitivity to 5-FU in HCT-8 cells, CCK-8 assays were performed. The cell growth was promoted to 160% in UHMK1 transfected cells as compared to the control (Fig. 5b), and the survival of cells was 5-FU treatment was also increased (Fig. 5c). However, no significant changes were shown in UHMK1-K54A overexpressed HCT-8 cells (Fig. 5b, c). To verify whether UHMK1 regulation on cell survival is dependent to the level of coilin expression, we carried out experiments on UHMK1 overexpression in coilin knockdown HCT-8 cells (Fig. S4). The results showed that the previously observed effects of UHMK1 were abolished in coilin siRNA cotransfection samples (Fig. 5d, e).

### **UHMK1 played an important role in the reassembly dynamics of CBs during 5-FU drug release experiment**

To explore on whether UHMK1 has a physiological significant in cellular responses to 5-FU treatments, as well as to drug-related microenvironment changes, we performed drug release experiments for dynamic observation. The CBs disassembled and decreased in numbers following 5-FU treatments in HCT-8 cells, usually progressed during the time course. When 5-FU was removed, CBs could be reassembled in the survived cells and recovering the normal counts and morphology as time prolonged (Fig. 6a). During the

entire process, the levels of phosphorylated coilin varied accordingly, where the coilin total protein levels remained stable (Fig. 6b). We analyzed the UHMK1 expression at protein and mRNA levels in both of the 5-FU drug maintain and release phases during the entire time course, and found the expression levels of UHMK1 also correlated well with both coilin phosphorylation levels and counts of CB numbers per cell (Fig. 6c, d). These findings strongly suggested that UHMK1 and its serine phosphorylation activity were important required factors for CB functions in response to 5-FU treatment and associated drug-induced cellular stress.

### **Phosphorylation modification of coilin activated by UHMK1 contributed to the cell resistance of SW480 to 5-FU**

To verify the findings in HCT-8 cells, we used 5-FU to treat SW480 cells, another human colon cancer cell line. Exposed in 5-FU (1 µg/ml) for 3 d, the CBs disassembled and decreased in numbers during the time course comparing to the 0 d untreated controls ( $p < 0.01$ ). When 5-FU was removed, CBs could be reassembled in the survived cells and recovering the normal counts and morphology as time prolonged (Fig. 7a). The expression levels of UHMK1 varied accordingly and correlated well with both coilin phosphorylation levels and counts of CB numbers per cell during the entire time course (Fig. 7b). The knockdown of UHMK1 significantly reduced the phosphorylation of coilin (Fig. 7c), with significant decrease in cell growth and increase of cell sensitivity to 5-FU in UHMK1 siRNAs transfected cells (Fig. 7e and 7f). Increase of coilin phosphorylation was observed in wild type UHMK1 overexpressed SW480 cells, whereas transfection of UHMK1-K54A reduced the relative levels of coilin phosphorylation (Fig. 7d). Overexpression of UHMK1 significantly promoted the cell growth and increased the survival of cells upon 5-FU treatment (Fig. 7g and 7h). However, no significant changes were shown in UHMK1-K54A overexpressed SW480 cells (Fig. 7g and 7h). Moreover, effects of UHMK1 were abolished in coilin siRNA cotransfection samples (Fig. S5) which confirmed that UHMK1 regulation on cell survival is dependent to the level of coilin expression. These data indicated that UHMK1 and its serine phosphorylation activity were essential for CB functions and able to influence SW480 cell growth and the sensitivity to 5-FU.

## **Discussion**

The number and sizes of CBs altered in cell cycle, development, transformation, temperature, DNA damage, and coilin mutations. Earlier studies suggest that the morphology of CBs appeared to be more dynamic as a nuclear organelle in cells with higher metabolic rates, such as in neurons or cancer cells. Several recent studies have demonstrated the reassembly of CBs responded to DNA damages and indicated the repair process in solid tumor cells. In the present study, we found that exposure in CM derived from the drug-resistant HCT-8/FU was able to reduced 5-FU sensitivity in HCT-8 cells, accompanied with morphological changes of CBs (Fig. 2). It suggested that secretory factors from the resistant cells, including metabolites, cytokines and growth factors in CM, could confer chemoresistance to sensitive cells through direct cell-to-cell contact or by classical paracrine. CBs could be not only an sensitive indicator for the cell response to cytotoxic pressure, but also involved in cell adaptation against environmental stress. Coilin is a scaffold protein that is necessary for the organization of CB, whose

amino acid sequence contains at least 17 amino acid residues that can be modified by phosphorylation. The phosphorylation of coilin at different amino acid residues triggered the formation or destabilization of CBs. Coilin is known to be phosphorylated in several residues by VRK1 (vaccinia-related kinase 1) [25]. The VRK1 pathogenic variants with reduced protein stability or kinase activity is a functional insufficiency of VRK1 cause a defective formation of 53BP1 (repair) foci in response to DNA damage, and loss CBs assembled on coilin in patients with neuromotor developmental syndromes [26, 27]. In the present study, the phosphorylation at serine residues of coilin (Fig. 3) were suggested to be activated by UHMK1, a serine/threonine protein kinase.

UHMK1 was initially identified to regulate the function of stathmin [28]. Dysregulation or mutation of UHMK1 has been indicated as a high-penetrant factor in different types of tumors of pancreatic, ovarian or gastric cancer in humans. From public database queries for UHMK1 genetic and epigenetic modifications in colon cancers, we found that gene amplification, nucleotide substitution and other genomic changes in UHMK1 associated with poor overall survival in patients (Fig. S6). UHMK1 was known to bind a range of proteins, such as eEF1A, FAM64, CDKI, p27KIP1, SF3b155, and CPEB1, suggesting the sophisticated roles of UHMK1 in different cellular processes [29]. We found that UHMK1 enhanced the phosphorylation of coilin and subsequently regulated the formation of CBs and contribute to the resistance of cells to 5-FU (Figs. 4, 5 and 7). This expanded our understanding about functional natural substrates of UHMK1, and meanwhile demonstrated coilin phosphorylation for its importance in colon cancers, especially in context of drug responses.

CBs as a structure of multiple components contain splicing snRNPs in large abundance, suggesting their functions in RNA modification post-transcription. The RNA association profile of coilin changes in mitosis with respect to that during interphase, which require coilin function besides of just being a scaffold protein. Ectopic expression of mutant coilin could induce transcriptional and/or processing dysregulation of a number of CB-related RNA transcripts. Knockdown of coilin decreased cell proliferation and could alter mRNA splicing as shown from minigene reporter assays. These results implied that the involvement of coilin in splicing regulation possibly related to its phosphorylation status [30], which was also supported from our RNA-seq results showing a large number of differential SIs in 5-FU resistant cells and CM-induced cells (Fig. 3). Aberrant splicing events conferring drug/therapy resistance in cancer is gradually becoming a focus in recent researches, since the differential expression of the primary transcript of the genes often do not explain the phenotype of drug responses [31]. However, the challenge of profiling SIs and used for drug sensitivity assessment not only due to the dynamic and complex nature of splicing events alone, also can be cumbersome when lacks of cellular indicators for monitoring the immediate response of cells to drug or environmental stimuli. Fortunately, CB appeared to be a suitable visual maker to evaluate cell responses to cytotoxic pressure and resistance development to cytotoxic drugs, in aspects of both environmental stimulation and potential alternative splicing profile changes.

Another interesting finding from this study was that the environmental factors could be very potent to change the resistance to anticancer drugs in cancer cells. Various genetic and epigenetic modifications are the main ways for cells to cope with environmental stress. Recent studies demonstrated that cancer

cell-secreted molecules were functionally involved in conferring chemoresistance by altering TME. In our study, the paired 5-FU sensitive and resistant HCT-8 cells were used to find the factors related to resistance of cells in different genetic backgrounds. Using CM prepared in vitro from tumor cell of resistant phenotypes could be a convenient approach to reconstruct TMEs for laboratory experiments. In such systems, using CB morphology as index for the evaluation of fast and reversible drug responses appeared to be most advantageous in cultured cancer cells, it allows the identification of other important kinases, like UHMK1, to be responsible for coilin phosphorylation, altering the signals of cellular stress response, and influence the resistance to chemotherapy drugs.

## Conclusion

In conclusion, our study discovered that the phosphorylation modification of coilin at serine residues could be activated by UHMK1 in colon cancer cells, which subsequently regulated the assembly of CBs and contributed to the cell resistance of colon cancer cells to 5-FU. The findings improved the understanding about how coilin appeared to be considered as a visual maker to indicate the cell response under cytotoxic pressure and the development of cell resistance to toxins. The reassembly of CBs induced by CM of resistant cells or in cells upon cytotoxic pressure involved complex interactions of snRNAs and snRNP proteins, which led to the spectrum changes in splicing isoforms, as well as the selective production of specific SIs which could be secreted in TME and remodel the intracellular phenotype of adjacent cells. The mechanism was possibly involved in the role of TME in the development of drug resistance through CB-edited RNA processing, during which UHMK1 was an important participator with crucial functions.

## Abbreviations

5-FU: 5-fluorouracil; CM: conditioned medium; CBs: Cajal bodys; TME: tumor microenvironment; CRC: colorectal cancers; snRNPs: small nuclear ribonucleoproteins; scaRNPs: Cajal body-specific RNPs; FBS: fetal bovine serum; CCK8: cell counting kit; PBS: phosphate-buffered saline; Sis: splicing isoforms; SDS-PAGE: SDS-polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride; CO-IP: Co-Immunoprecipitation; SDS: sodium dodecyl sulfate; ANOVA: analysis of varianc; FC: fold changes; ALL: acute lymphoblastic leukemia; UHMK1: U2AF Homology Motif Kinase 1; VRK1: vaccinia-related kinase 1;

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable; manuscript contains no individual person's data in any form.

## Availability of data

Raw data of RNAseq were available at GEO database repository; accession ID: GSE168888 (all data), GSM5171962 (HCT-8 rep1), GSM5171963 (HCT-8 rep2), GSM5171964 (HCT-8/FU rep1), GSM5171965 (HCT-8/FU rep2), GSM5171966 (CM of HCT-8 rep1), GSM5171967 (CM of HCT-8 rep2), GSM5171968 (CM of HCT-8/FU rep1) and GSM5171969 (CM of HCT-8/FU rep2).

## Competing interests

The authors declare no conflict of interest.

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## Authors' contributions

H.N., M.Z., J.H., J.W. and S.Y. contributed significantly to the designing and implementation of the research project. H.N. conducted most of the experiments, acquisition of data and analysis. S.C. was responsible for the data analyses and interpretation of the results, also contributed most for editing and revision of the written manuscript. W.D. contributed to conception and revision of the written manuscript. All authors have read and agreed to the published version of the manuscript.

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## Figures

Fig. 1

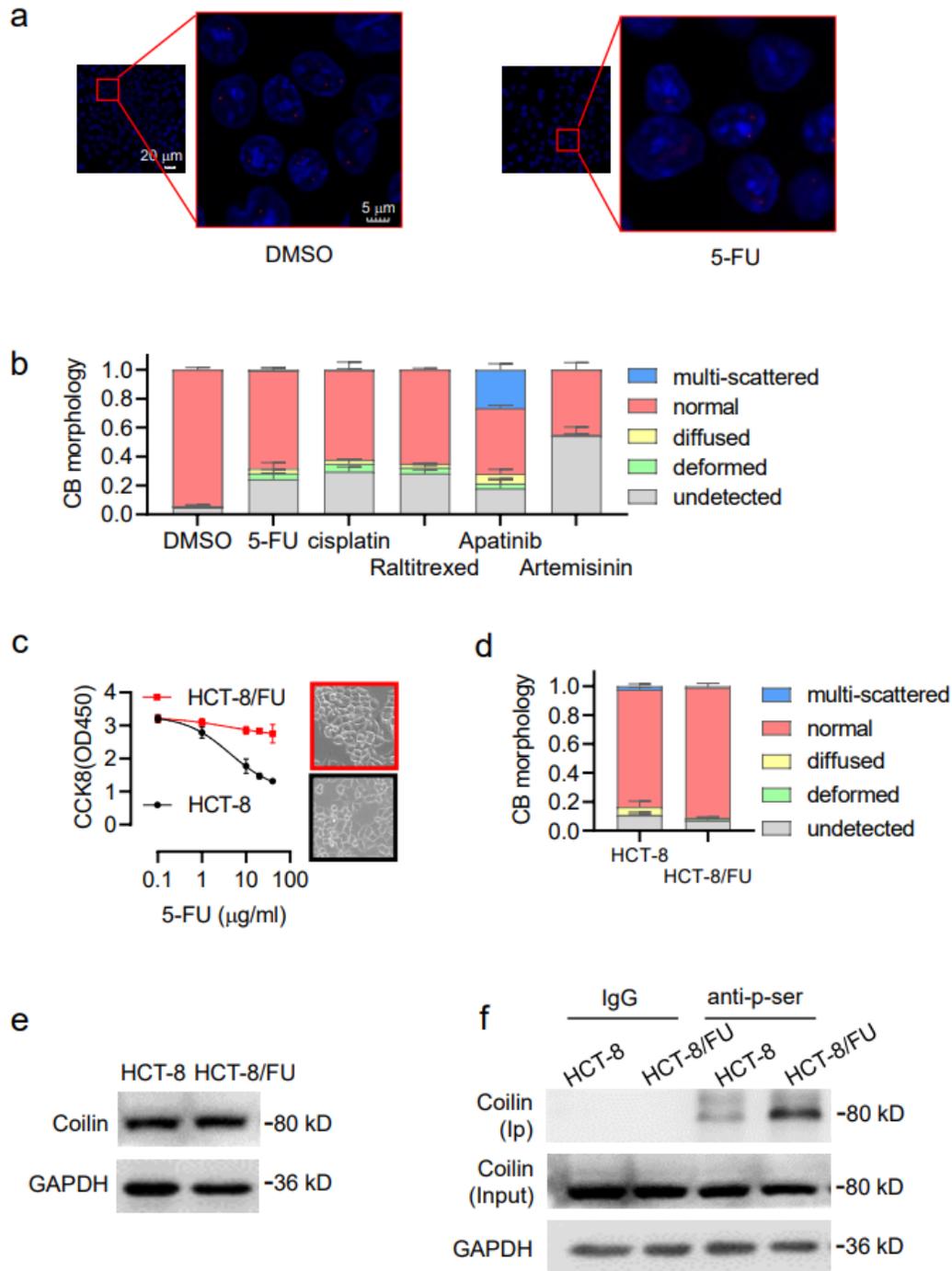
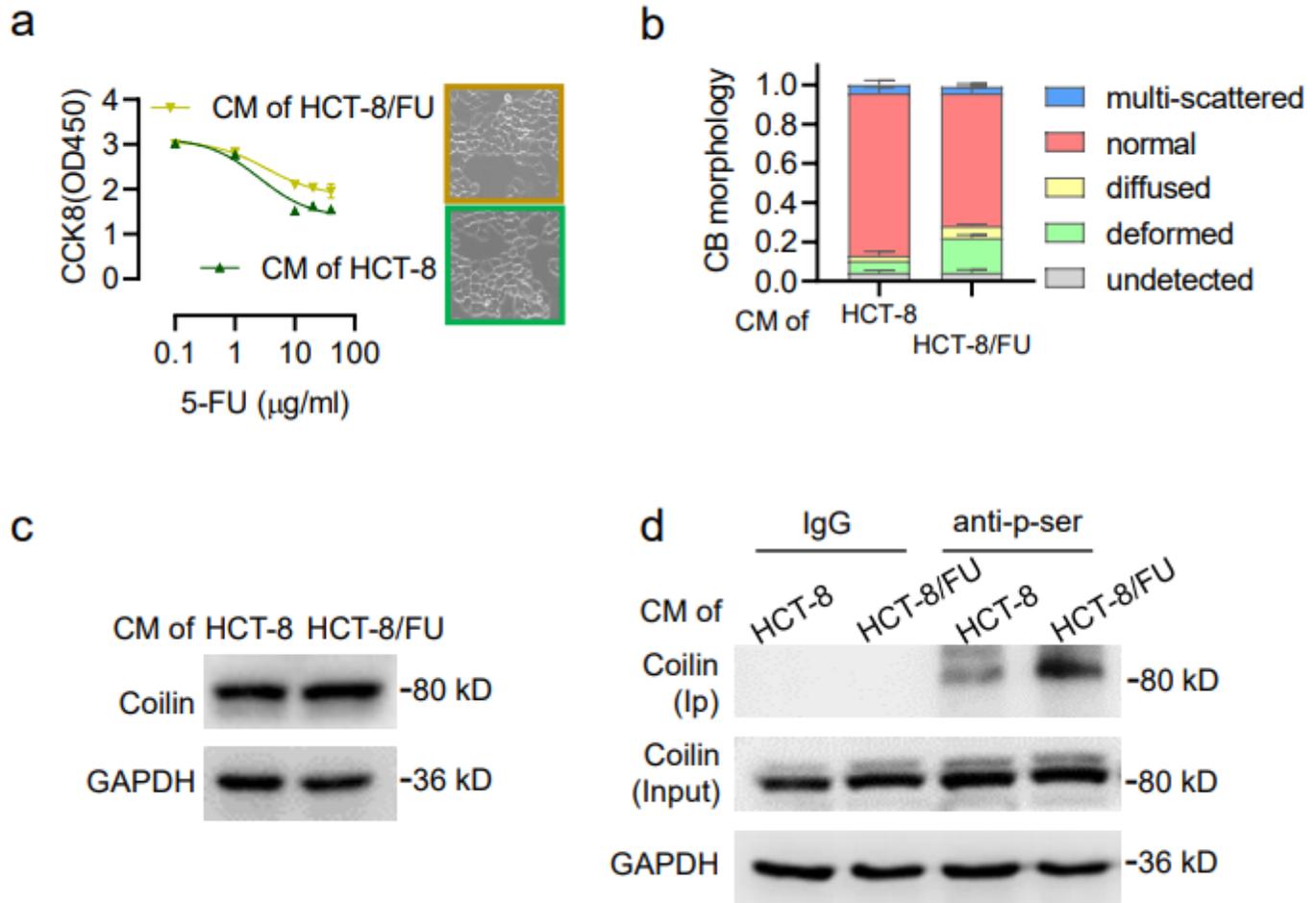


Figure 1

Morphology of Cajal bodies (CB) and coilin phosphorylation in HCT-8 and 5-FU resistant HCT-8/FU cells. a Immunofluorescent of coilin in cells treated with 1  $\mu\text{g/ml}$  5-FU for 24 h. b CB morphology in HCT-8 cells following treatments of chemotherapeutic reagents of 5-FU (1  $\mu\text{g/ml}$ ), apatinib (20  $\mu\text{M}$ ), raltitrexed (2.5  $\mu\text{M}$ ), artemisinin (100  $\mu\text{M}$ ) and cisplatin (10  $\mu\text{g/ml}$ ) for 24 h. c Survival of HCT-8 and HCT-8/FU cells 5-FU of increasing doses (0, 1, 10, 20, 40  $\mu\text{g/ml}$ ) for 48 h by CCK8 assays. d Percentage in counts of various

CB morphological categories in HCT-8 and HCT-8/FU cells. e Western blots for coilin expression in HCT-8 and HCT-8/FU cells. f Determination of coilin phosphorylation in HCT-8 and HCT-8/FU cells.

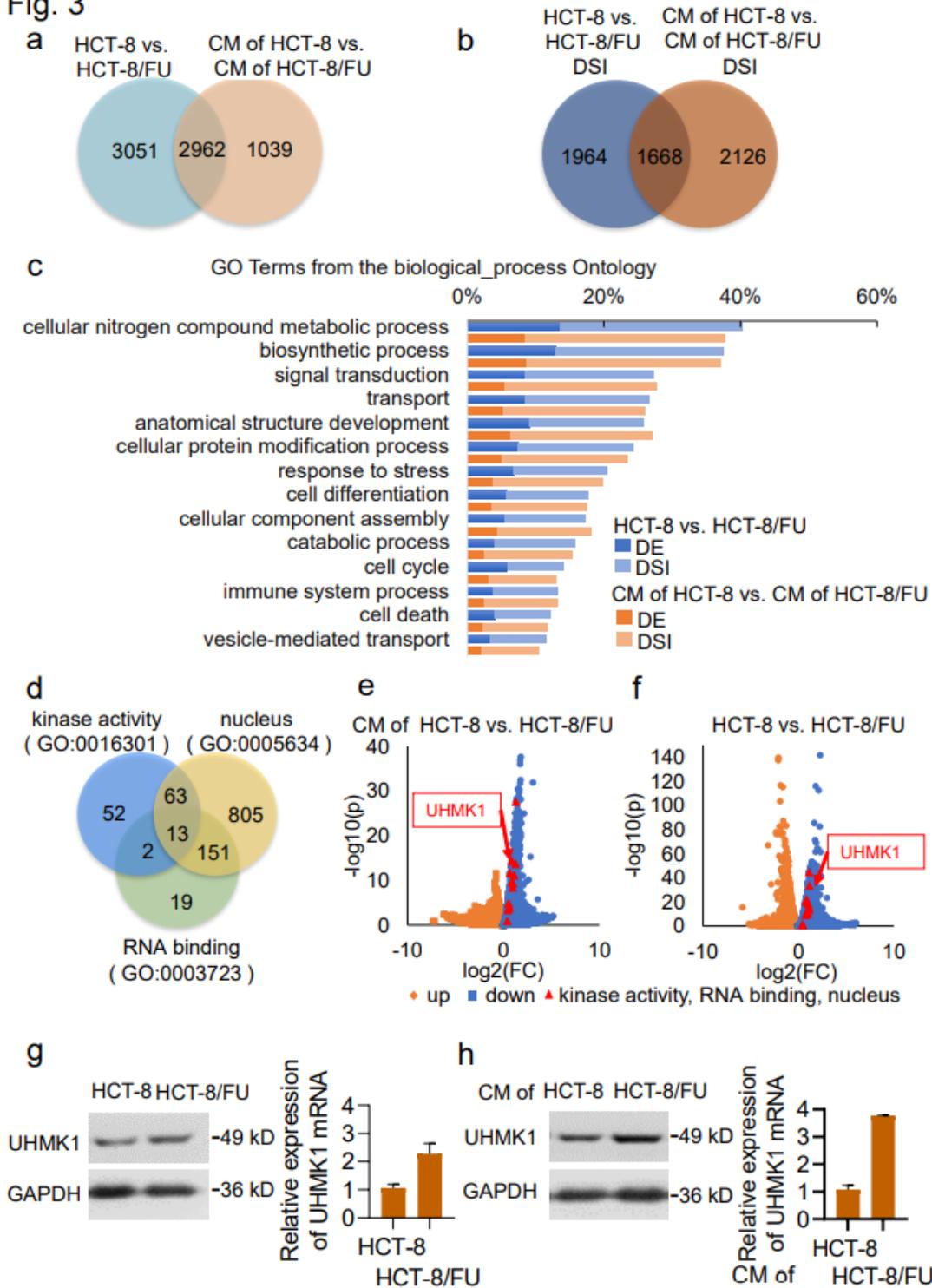
**Fig. 2**



**Figure 2**

Morphology of Cajal bodies and coilin expression in HCT-8 cells treated with CM from the 5-FU resistant HCT-8/FU cells. a Dose responsive results of CCK8 assays in HCT-8 cells treated with CM from HCT-8/FU. b Quantitative analyses of CB morphology in different categories. c Protein expression of coilin in HCT-8 cells cultured in CM from HCT-8 or HCT-8/FU. d Coilin phosphorylation in HCT-8 cells treated with CMs.

**Fig. 3**



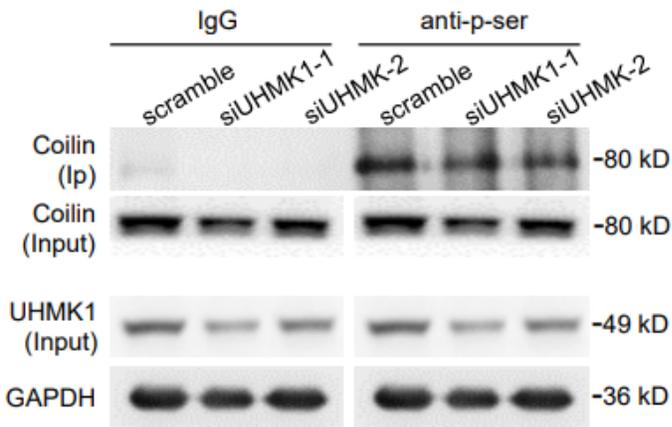
**Figure 3**

RNA-seq analyses for differentially expressed (DE) genes and alternative splicing events comparing HCT-8/FU cells and HCT-8 cells in both normal culture and CM treatment from HCT-8/FU cultures. a Venn diagram illustrating the total and shared numbers of DE genes. b Venn diagram summarizing the number of differences in identified splicing isoforms (SIs). c Enrichment from Gene Ontology for biological processes on DE genes and differential SIs. d Venn diagram on gene sets of kinase activity (GO:

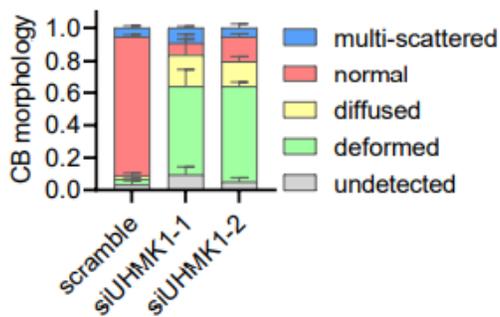
0016301), RNA binding (GO: 0003723) and nucleus (GO: 0005634). e Volcano plots and identification of DE genes between HCT-8/FU and HCT-8 cells. f Volcano plots of DE genes between HCT-8 treated with CM of HCT-8/FU and CM of HCT-8. g Protein (left) and mRNA (right) expression of UHMK1 in HCT-8 and HCT-8/FU cells. h UHMK1 expression in HCT-8 cells treated with CM from HCT-8 and HCT-8/FU.

**Fig. 4**

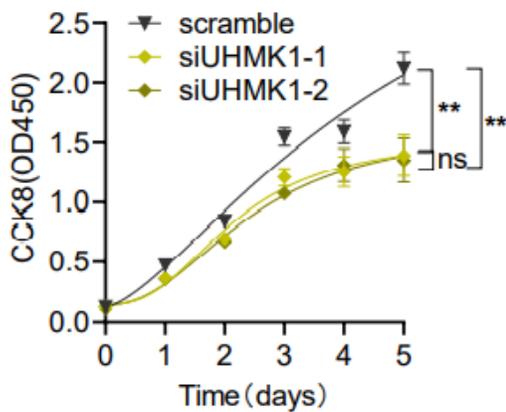
**a**



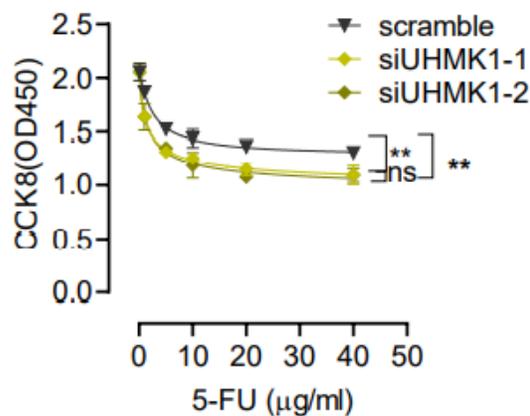
**b**



**c**



**d**



**Figure 4**

Effects of UHMK1 knockdown on coilin phosphorylation and 5-FU sensitivity in HCT-8 cells. a Co-immunoprecipitation assay for coilin serine phosphorylation in UHMK1 knockdown HCT-8 cells. b CB morphology and statistics in UHMK1 knockdown HCT-8 cells. c CCK8 assays for cell growth of UHMK1 knockdown cells. d Survival of UHMK1 knockdown HCT-8 cells following 5-FU treatments (0, 1, 5, 10, 20 and 40  $\mu\text{g/ml}$ ) for 48 h. Data were presented as the mean  $\pm$  SD,  $n=6$ . \*\*  $p<0.01$

Fig. 5

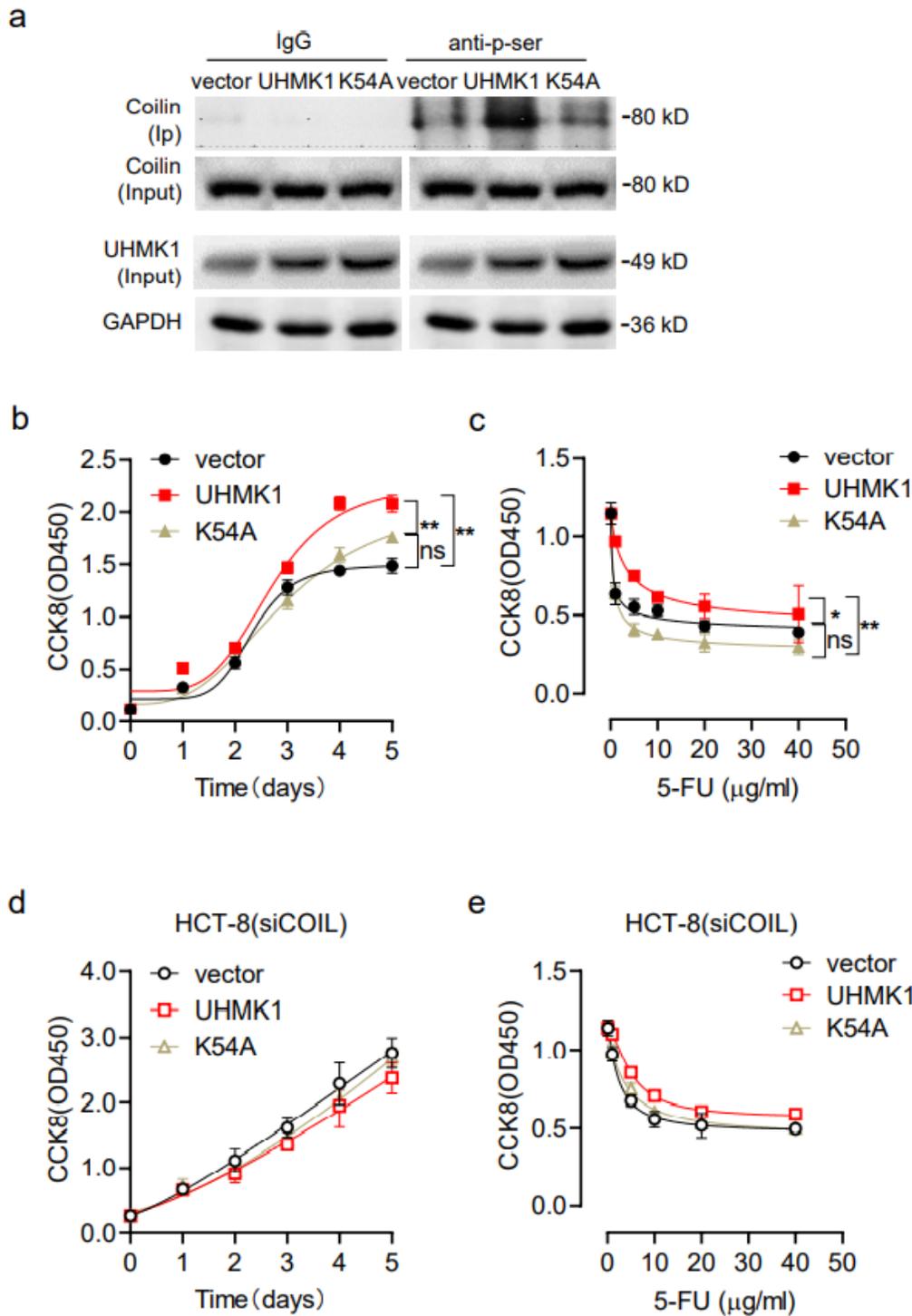
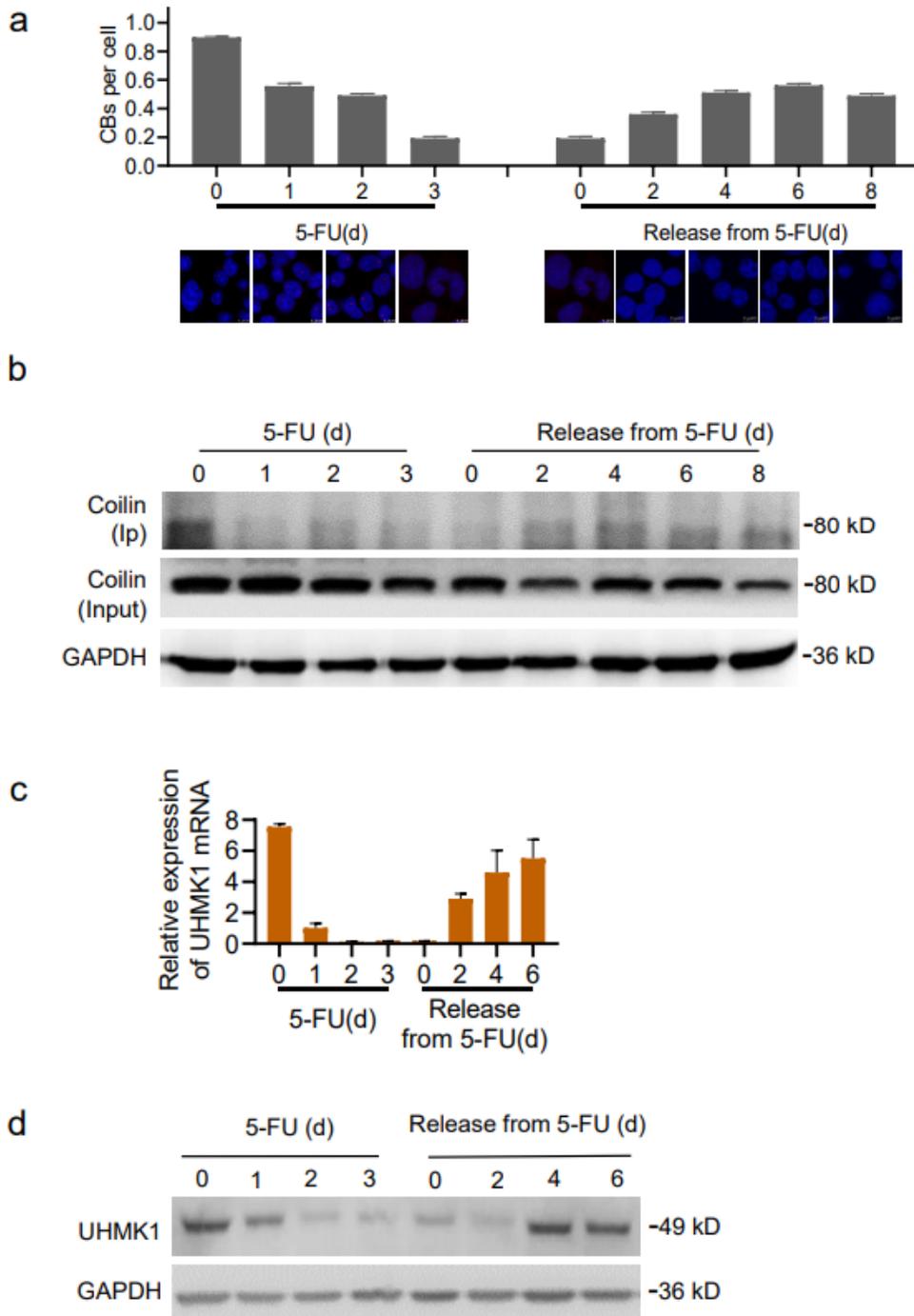


Figure 5

Effects of UHMK1 overexpression in comparison to UHMK1 mutant lacks of kinase activity in HCT-8 cells. a Coilin phosphorylation in transfected HCT-8 cells. b Cell growth by CCK8 assays. c Dose-dependent survival from 5-FU treatments (0, 1, 5, 10, 20 and 40  $\mu\text{g}/\text{ml}$ ) for 48 h. d Cell growth in UHMK1 or mutant transfected HCT-8 cells with coilin knockdown. e Cell survival in coilin knockdown HCT-8 cells. Data were presented as the mean  $\pm$  SD, n= 6. \*  $p < 0.05$  and \*\*  $p < 0.01$

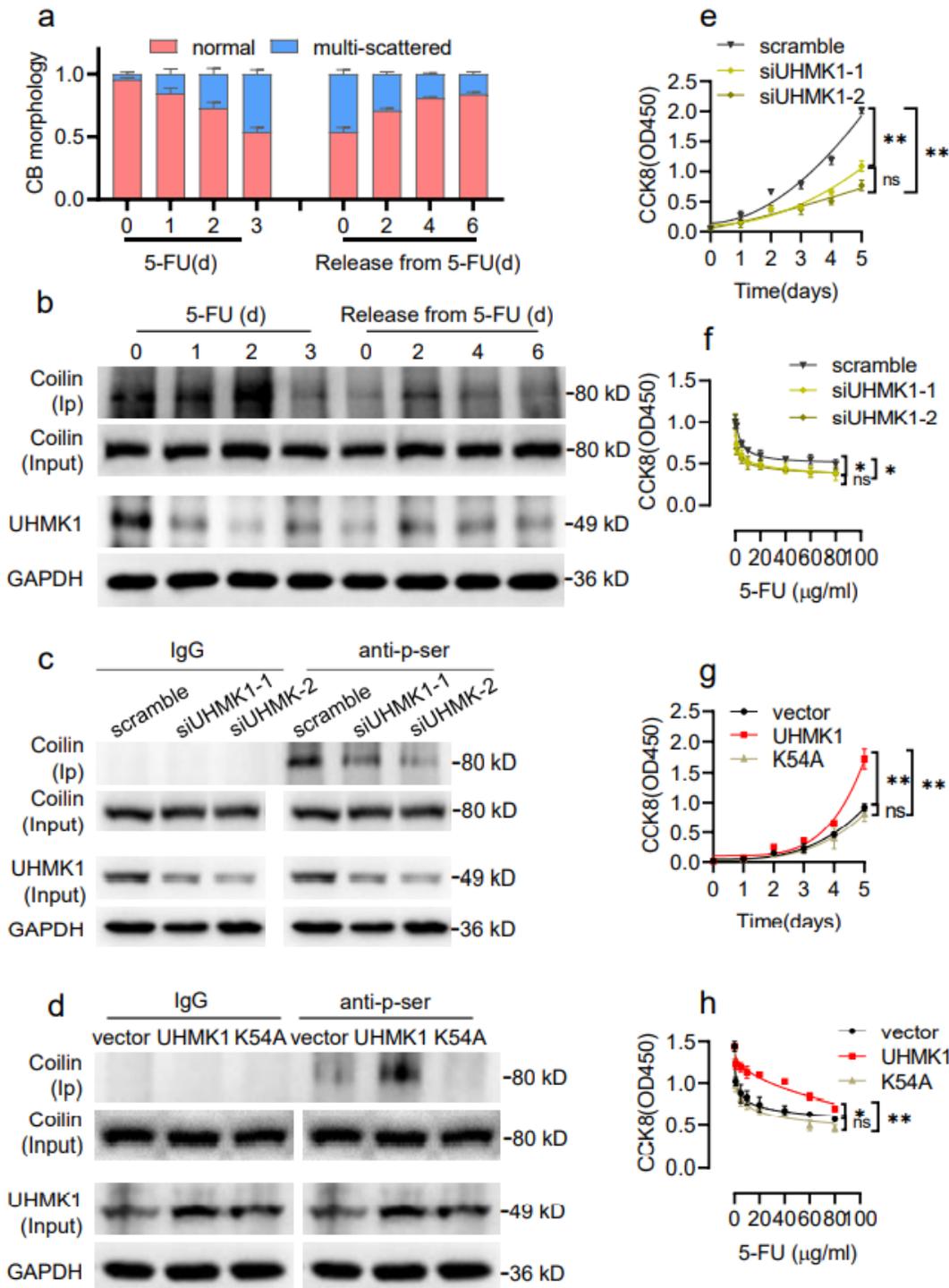
**Fig. 6**



**Figure 6**

Role of UHMK1 in 5-FU exposure and release in HCT-8 cells. a Morphology changes of CBs in 1  $\mu\text{g/ml}$  5-FU and removal. Significant differences in all the 5-FU drug maintain groups of 1, 2 and 3 days compared to 0 d controls ( $p < 0.01$ ); significant differences in all the release groups of 2, 4, 6 days compared with 0 d group ( $p < 0.01$ ). b Western blots for coilin phosphorylation. c UHMK1 expression in the HCT-8 cells determined by RT-PCR.  $p < 0.01$  for all the 5-FU drug maintain groups of 1, 2 and 3 days compared to 0 d controls;  $p < 0.01$  for all the release groups of 2, 4, 6 days compared with 0 d group. d Western blots for UHMK1 expression in the HCT-8 cells.

Fig. 7



## Figure 7

Effects of UHMK1 on coilin phosphorylation and 5-FU sensitivity in SW480 cells. a Morphology changes of CBs in 1  $\mu\text{g/ml}$  5-FU and removal. b Western blots for coilin phosphorylation and UHMK1 expression. c Co-immunoprecipitation assay for coilin serine phosphorylation in UHMK1 knockdown cells. d Coilin phosphorylation in UHMK1 and its mutant transfected SW480 cells. e CCK8 assays for cell growth of UHMK1 knockdown cells. f Survival of UHMK1 knockdown SW480 cells following 5-FU treatments (0, 1, 5, 10, 20, 40, 60 and 80  $\mu\text{g/ml}$ ) for 48 h. g Cell growth of transfected SW480 by CCK8 assays. h Dose-dependent survival from 5-FU treatments (0, 1, 5, 10, 20, 40, 60 and 80  $\mu\text{g/ml}$ ) for 48 h of transfected SW480 cells. Data were presented as the mean  $\pm$  SD, n= 6. \*  $p < 0.05$ , \*\*  $p < 0.01$

## Supplementary Files

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