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Original Article

DCAF4L2 promotes colorectal cancer invasion and metastasis via mediating degradation of NFκb negative regulator PPM1B

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Abstract: DCAF4L2 is a member of WD-repeat proteins, which commonly serve as mediators of protein-protein interplay. In this study, we reported that elevated DCAF4L2 expression in human colorectal cancer (CRC) significantly correlated with a more advanced clinical stage as in lymphatic and distant metastasis. More importantly, elevated DCAF4L2 expression is an independent prognosis factor for survival. Genetic perturbations demonstrated that DCAF4L2 overexpression in CRC cells promoted cell migration and invasion, whereas knockdown of which had opposing effects. Moreover we discovered that DCAF4L2 overexpression could promote epithelial-mesenchymal-transition (EMT) through activating NFκB signal pathway. Mass spectrometry analysis showed that DCAF4L2 could form an E3 ligase complex with Cul4A and DDB1 thus mediated degradation of PPM1B, which has been reported to negatively regulate NFκB signaling. We identified PPM1B as a substrate of Cul4A-DDB1-DCAF4L2 E3 ligase complex, as knockdown of PPM1B abrogated shDCAF4L2 mediated inhibition of cell invasion in CRC cells. For further verification, DCAF4L2 expression inversely correlated with PPM1B expression in a cohort of 87 CRC patients. These findings may provide insight into the understanding of DCAF4L2 as a novel critical factor and a candidate target for CRC treatment.

Keywords: DCAF4L2, colorectal carcinoma, Cul4A-DDB1 E3 ligase complex, PPM1B, NFκB

Introduction

Colorectal carcinoma (CRC) is the third most common malignant disease in men and the second in women worldwide and it is seen with substantially increasing mortality in the Asia-Pacific region. Surgery is considered the most effective treatment for CRC patients [1]. Neo-adjuvant treatments only benefit high-risk colon cancer patients slightly, which are not nearly as satisfactory considering its high financial cost and toxic effects [2]. Hence, it is of great importance to identify features of advanced CRC and establish new therapeutic strategy for clinical treatments.

Metastasis is the hallmark among aggressive characterizations of CRC. Although the precise mechanisms underlying metastasis remain mostly elusive, several studies have postulated a link between epithelial-mesenchymal transition (EMT) and invasive characteristics. The epithelial-mesenchymal transition (EMT) is a reversible process in which epithelial cells adopt mesenchymal properties by altering their morphology, and most importantly migratory capacity, which allows tumor cells to infiltrate surrounding tissue and metastasize to distant sites [3]. Moreover, EMT also takes an active part in anticancer drug resistance, reinforcing the idea that EMT is closely linked to tumor progression. Induction of EMT involves a complicated molecular network, which comprises of well-established transcription factors and cytokines, such as Snail/Twist, MMP2/ MMP9. However, mechanistic understanding of how EMT is specifically initiated in certain solid tumors is still lacking.

DDB1 and Cul4 associated factor 4 like 2 (DCAF4L2) belongs to WD domain repeat containing protein family which mainly act as a platform for protein complex assembly or mediator for protein-protein interplay and is frequently

seen in E3 ligase CRL4 [4]. Many cognate molecules of DCAF4L2 referred to as DDB1-CUL4-associated factors (DCAF) interact with DDB1 and CUL4 thus forming a E3 ligase complex, among which DCAFs are mainly responsible for substrate recognition [5]. Cul4-DDB1-DCAF is implicated in multiple physiological processes and recently in breast, ovarian, gastric and colorectal cancers. However, its adaptor protein and specific substrate carrying out its potential oncogenic roles, exact molecular mechanisms during CRC tumorigenesis remain unknown. DCAF4L2 has been previously reported amplified in lung cancer and liver cancer indicating its oncogenic role. However, DCAF4-L2 expression in other tumor types remained to be investigated.

In this study, we identified elevated expression of adaptor protein DCAF4L2 in colorectal carcinoma and its associations with malignant phenotype in terms of advanced clinical stage, poor prognosis and survival. We systematically assessed DCAF4L2 function by overexpression and shRNA interference, which suggested that DCAF4L2 played crucial role in CRC cell migration and invasion mostly through activating epithelial-mesenchymal-transition which was achieved by up-regulating NFκB signal pathway. Further study of the pathway leading from DCAF4L2 to migration revealed a role for Cul4A-DDB1 E3 ligase and its substrate PPM1B, an announced negative regulator of NFκB signal pathway. These data collectively established DCAF4L2 as a driver of EMT in CRC cell culture and further clinical analysis highlight DCAF4L2 as a potential oncogenic biomarker and therapeutic target for late-stage and metastatic CRC patients.

Materials and methods

cell culture

Colorectal cancer cell line HT-29, SW480, SW620, SW1116 and embryonic kidney cell line HEK293T were purchased from Cell Bank of Chinese Academy of Sciences. HT-29 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) and were incubated at 37°C in a humidified incubator under 5% CO_2 condition.

SW480, SW620, SW1116 cells were cultured in L-15 medium (DMEM) supplemented with 10% fetal bovine serum (FBS) , penicillin (100 U/ml) and streptomycin (100 μg/ml) and were incubated at 37°C in a humidified incubator under 0.038% CO_2 condition.

Immunohistochemical staining (IHC)

Paraffin-embedded CRC and adjacent normal tissue specimens were obtained from the Division of General Surgery at Zhongshan hospital. Formalin-fixed paraffin-embedded (FFPE) 6 mm sections were used for IHC. DCAF4L2 primary antibody (proteintech, cat# 21571-1-AP, 1:50 dilution) was incubated overnight at 4°C. Immunostaining was performed using diaminobenzidine reaction and controlled under microscope. Slides were counterstained with hematoxylin.

All IHC staining was assessed independently by two pathologists and scored according to the ratio and intensity of positive staining. Briefly, the ratio was graded from 1 to 4 based on the percentage of positive staining cells (1, 0%-5%; 2, 6%-50%; 3, 51%-75%; 4, 76-100%). The intensity was graded from 1 to 4 (1, no staining; 2, weak staining; 3, moderate staining; 4, strong staining). A final score from 1 to 16 was calculated by multiplying ratio and intensity score. For each sample, it was indicated as negative (1-4), weakly positive (5-8), moderately positive (9-12) or strongly positive (>12).

Quantitative real-time PCR

Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen, Carlsbad, California, USA) and dissolved in diethylpyrocarbonate treated (DEPC) water. cDNA was synthesized using the Takara Reverse Transcription System Kit (Takara Biotechnology Co. Ltd., Japan) according to the manufacturer's instruction. Real time quantitative RT-PCR was performed using the Sybr green premix kit (BioRad). All reactions were done in triplicates. GAPDH was used as a housekeeping gene. The following primers are used in this assay: DCAF4L2: sense CGCCAACTATTGCCGTATAGC, and antisense TCGCCAGTATGCGGTTAAATC; PPM1B: sense TGGGAATGGTTTACGTTATGGC, and antisense GCCGTGAGGAATACCTACAACAG; E-cadherin: sense CGAGAGCTACACGTTCACGG, and

anti-sense GGGTGTCGAGGGAAAAATAGG; ZO1: sense ACCAGTAAGTCGTCCTGATCC, and anti-sense TCGGCCAAATCTTCTCACTCC; N-cadherin: sense TGCGGTACAGTGTAACTGGG, and anti-sense GAAACCGGGCTATCTGCTCG; FN1: sense AGGAAGCCGAGGTTTTAACTG, and anti-sense AGGACGCTCATAAGTGTCACC; GAPDH: sense ACAACTTTGGTATCGTGGAAGG, and anti-sense GCCATCACGCCACAGTTTC.

DCAF4L2 overexpression and shRNA transfection

DCAF4L2 cDNA was cloned in plenti vector and additional VSVG and Δ8.9 were used for recombinant lentivirus packaging. DCAF4L2 shRNAs were obtained from Institute of Biochemistry and Cell Biology, SIBS, CAS. A scrambled sequence was used as a control. Cell transfection was performed with PEI reagent (Sigma-Aldrich, MO).

Matrigel invasion assay

For transwell invasion assay, 50μl matrigel (BD Bioscience, Franklin Lakes, NJ) was added into top chamber for 30 minutes at 37°C. Cells (1×10^4 cells/well) was starved in serum free medium for 24 hours and plated to the top chambers. The bottom chambers were filled with completed medium. Any non-invading cells remaining in the top chamber were removed carefully in 48 hours culturing. After fixed in methanol and stained with crystal violet, cells adhering to the lower membrane of the well were counted and imaged under ×200 magnification. Crystal violet staining was dissolved in 33% acetic acid and optical density was detected at 570 nm. The assay was carried out in three times.

Wound healing assay

Cells were cultured to approximately 80% confluence in 6-well dishes. Wounds were scratched using a sterile 200 μl tip, after rinsed with PBS, cells were starved in media with no serum. Cells were monitored every 6 hours. Pictures of representative fields were taken using phase contrast microphotography (×200 magnification).

Cell viability assay

Cells were seeded at a density of 10^4 cells per well in 96-well plates. 10 μl MTT (Sigma, MO) was added per well every 24 hours. Continuously incubated at 37°C for 2 hours, 190 μl DMSO was additionally added per well. Gently vibrated for 10 minutes and absorbance was detected at 450 nm using microplate reader. Experiments were repeated three times.

Western blotting

Equal amounts of protein were separated by 10% SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. After blocking with 5% non-fat milk in TBST for 1 h, membranes were incubated overnight at 4°C with DCAF4L2, GAPDH primary antibodies followed by incubation with HRP-conjugated secondary antibodies. Immunoreactive bands were detected with enhanced chemiluminescent HRP substrate (Millipore, Bollerica, MA).

Flag-tagged pulldown and mass spectrometry analysis

SW480 was infected with DCAF4L2 lentivirus, after 7 days hygromycin (Sigma-Aldrich, MO) selection, over expression stable cell line was constructed. 2.5×10^8 cells were harvested and lysed at 4°C. Incubate the samples at 4°C with IgG beads for 2 hours and then centrifuge at 5,000 RPM for 10 minutes. Save 20 μl of the supernatant as (Flag-Input). Add anti-Flag M2 affinity beads (Sigma, MO) to the remaining cell lysis which have been pre-washed with TBS buffer for 3 times. Gently rotate the cell lysate with Flag-M2 beads overnight at 4°C. Centrifuge 2 minutes at 5,000 RPM and harvest Flag-M2 beads. Wash the beads 3 times in TBS and elute the beads with Flag-peptide by incubating 5 minutes at room temperature with gentle tapping. Centrifuge for 30 seconds, transfer the supernatants to fresh tubes, and send the samples to Institute of Biochemistry and Cell Biology, SIBS, CAS for mass spectrometry and further analysis.

Statistical analysis

Statistical analyses were performed using SPSS 21.0 (IBM, Chicago, IL). Relations between clinical parameters and DCAF4L2 expression levels were analyzed using chi-squared test. In vitro studies were evaluated with student's t test. Overall survival rate was estimated by Kaplan-Meier method. *P* value less than 0.05 was considered statistically significant.

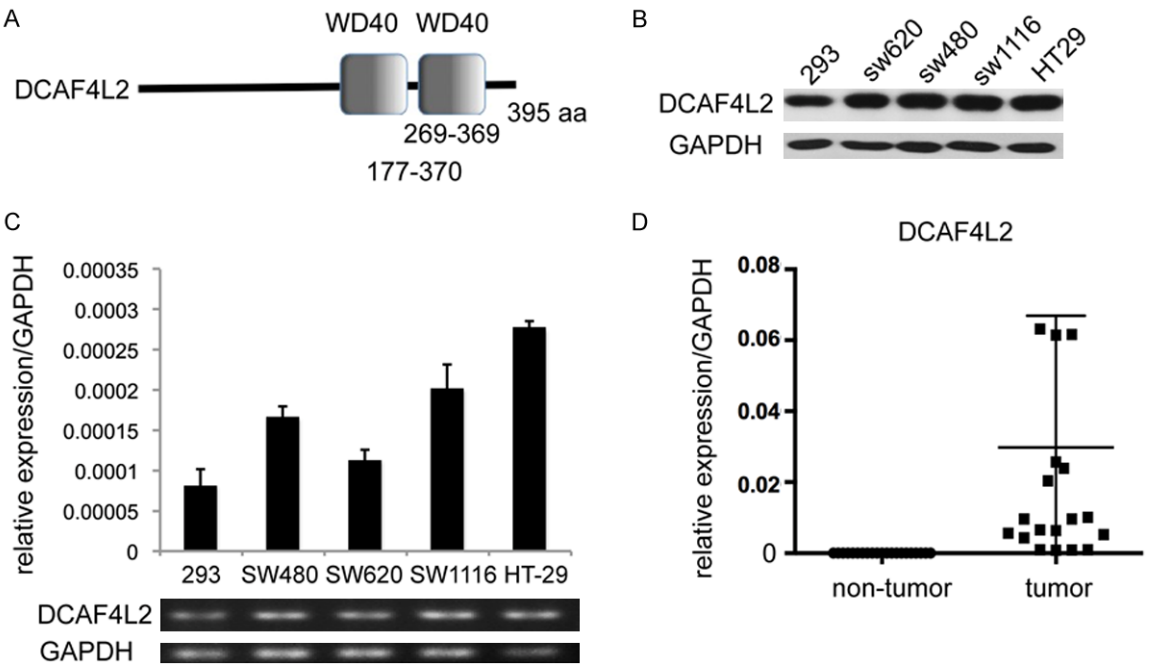


Figure 1. DCAF4L2 is over expressed in CRC cell lines and CRC tissues. A. A schematic protein structure of DCAF4L2 mainly contains WD-repeats domain. B. Western blotting analysis of DCAF4L2 protein expression in four CRC cell lines. C. Quantitative RT-PCR and semi-quantitative PCR analysis of DCAF4L2 expression in four CRC cell lines (Student's t test). D. Quantitative RT-PCR analysis of DCAF4L2 expression in 18 pairs of colorectal tumor and its corresponding normal tissues ($P<0.05$, Student's t test).

Results

Analysis of DCAF4L2 expression level in CRC cell lines and samples of CRC patients

DCAF4L2 is a small protein, which only contains WD-repeat domain (**Figure 1A**). We examined DCAF4L2 protein and mRNA level in four CRC cell lines and non-CRC 293FT cells. As shown in **Figure 1B** and **1C** both mRNA and protein level is relatively high in all four CRC cell lines (SW480, SW620, SW1116 and HT-29), among which SW1116 and HT-29 cells displayed significant higher levels of DCAF4L2. For further confirmation, we also determined DCAF4L2 mRNA expression level in 18 pairs of CRC and corresponding noncancerous tissues by quantitative reverse transcription (RT)-PCR. As shown in **Figure 1D**, CRC tissues exhibited remarkably elevated DCAF4L2 mRNA expression as compared to almost no expression in adjacent normal tissues.

DCAF4L2 overexpression promotes migration and invasion capacity in CRC cells

Since DCAF4L2 is highly expressed in CRC patients and across various CRC cell lines, we undertook experiments to explore its function during CRC tumorigenesis. Based on previous finding that SW480 and SW620 had relative lower expression of DCAF4L2, we constructed DCAF4L2 overexpression stable cell line through lentiviral infection in these two cell lines which confirmed efficiently that DCAF4L2 expression level at least doubled in both two stable cell lines (**Figure 2A**). We proceeded gain of function analysis involving proliferation, migration, invasion, apoptosis and necrosis. Wound healing and matrigel invasion assay results demonstrated that exogenous expression of DCAF4L2 in SW480 and SW620 cells reinforced both migration and invasion abilities (**Figure 2C** and **2D**) while MTT assay and Annexin V/PI assay revealed no visible effects of DCAF4L2 regarding proliferation, apoptosis and necrosis (**Figure 2B** and **2E**). These results suggested that DCAF4L2 overexpression enhanced tumor migration and invasion in vitro.

Knockdown of DCAF4L2 attenuates migration and invasion of CRC cells

We performed RNA interference in SW1116 and HT-29 cell lines, which harbored relatively

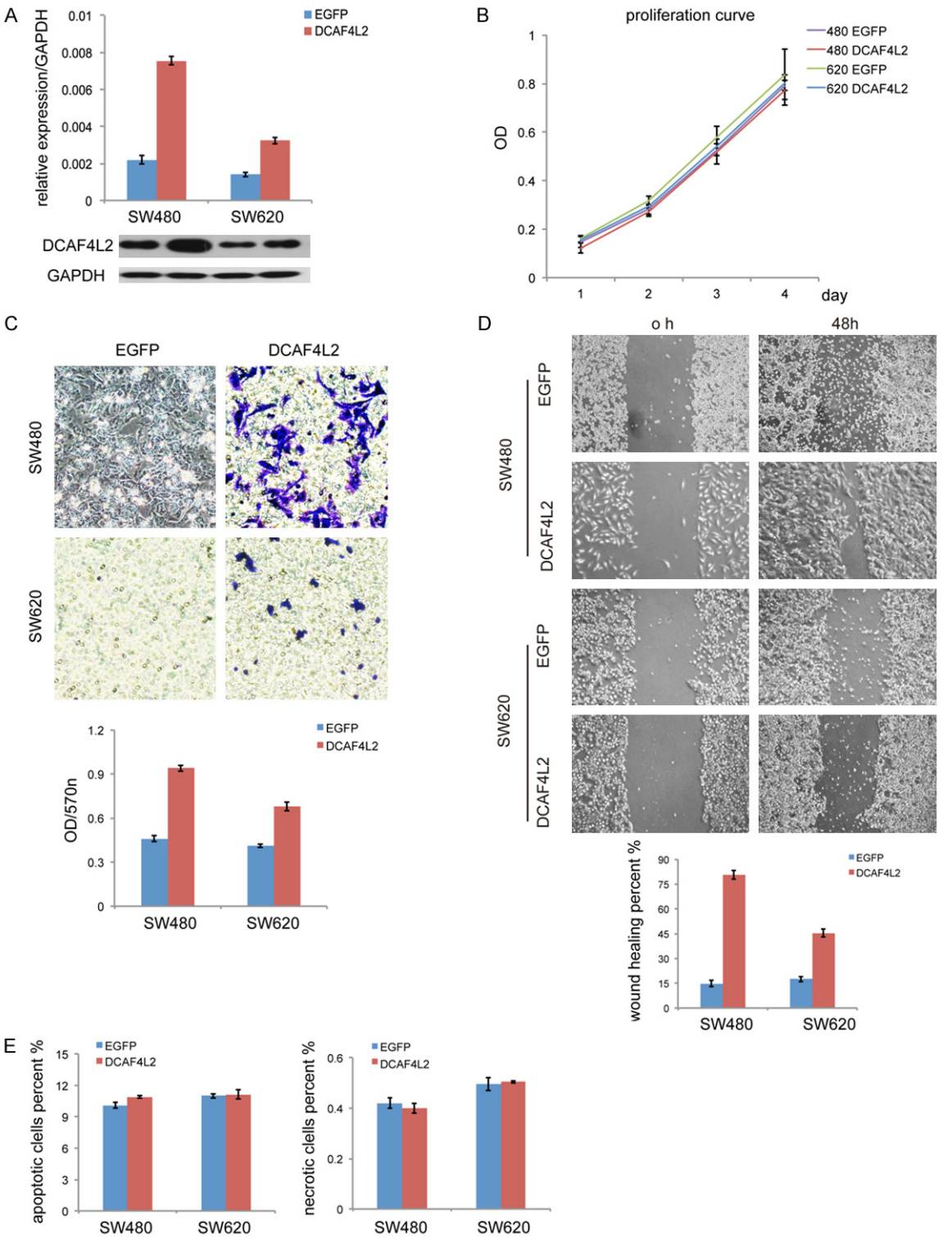


Figure 2. DCAF4L2 overexpression induced migration and invasion in CRC cells. A. Western blotting and quantitative RT-PCR analysis of over expression efficiency in DCAF4L2 stable cell lines (Student's t test). B. MTT assay showed no obvious differences in proliferation when DCAF4L2 is over expressed (Student's t test). C. Matrigel invasion assay showed that DCAF4L2 overexpression increased invasive capacity. The graph below showed absorbance at 570 nm after 24 hours ($P<0.05$, Student's t test). D. Wound-healing assay showed that DCAF4L2 overexpression increased motility of CRC cells. The graph below showed mean opening distances after 48 hours ($P<0.05$, Student's t test). E. Annexin V/PI assay showed no obvious differences in apoptosis and necrosis when DCAF4L2 is over expressed (Student's t test).

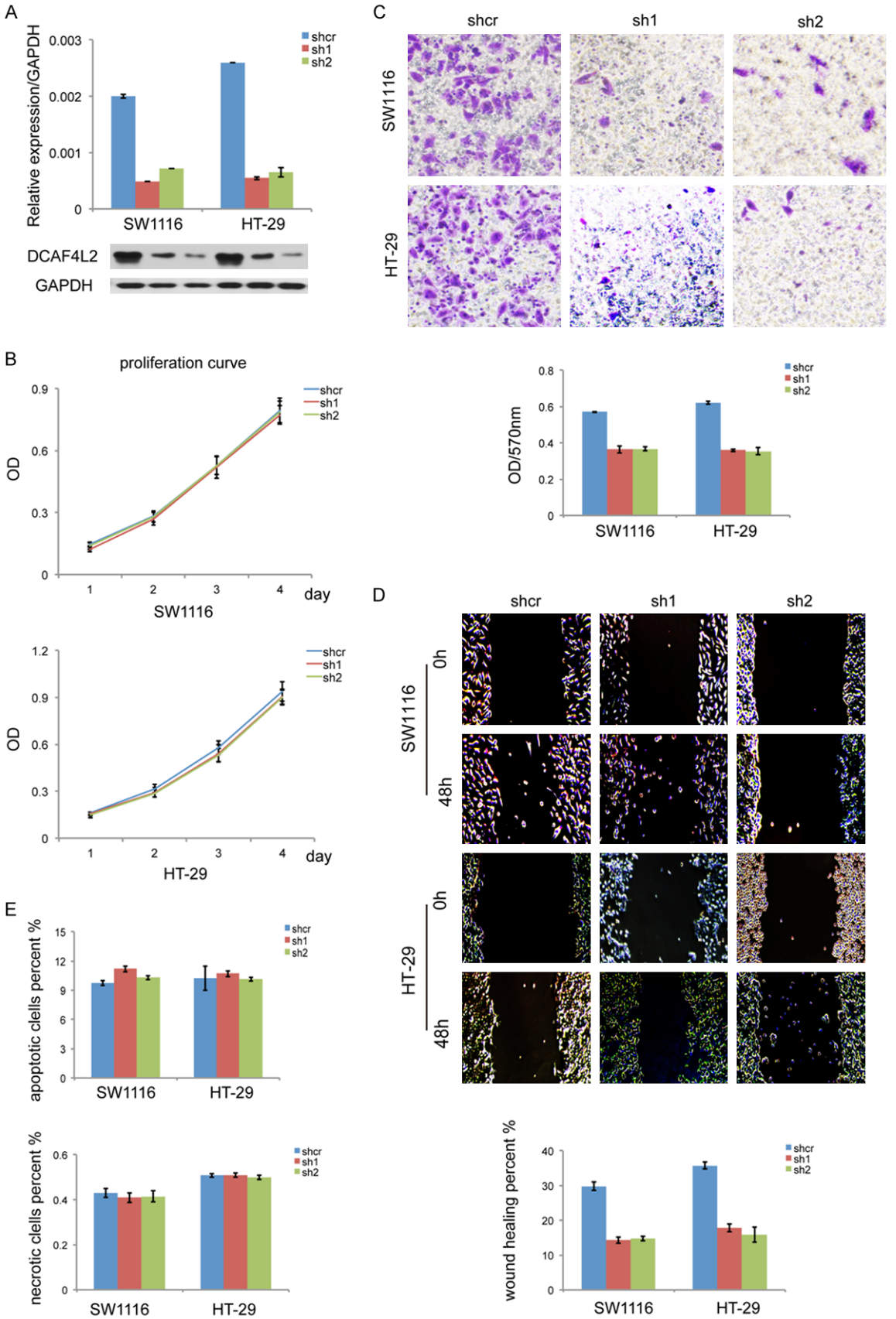

A
Relative expression/GAPDH
0.003
0.002
0.001
0
shcr
sh1
sh2
SW1116
HT-29
DCAF4L2
GAPDH
C
shcr
sh1
sh2
SW1116
HT-29
B
proliferation curve
OD
0.9
0.6
0.3
0
1
2
3
4
day
SW1116
HT-29
OD/570nm
0.8
0.6
0.4
0.2
D
0h
48h
wound healing percent %
40
30
20
10
E
apoptotic clells percent %
15
12
9
6
3
necrotic clells percent %
0.6
0.4
0.2

Figure 3. DCAF4L2 knockdown inhibited migration and invasion in CRC cells. A. Western blotting and quantitative RT-PCR analysis of knockdown efficiency in DCAF4L2 stable cell lines. B. MTT assay showed no obvious differences in proliferation when DCAF4L2 is knocked down (Student's t test). C. Matrigel invasion assay showed that DCAF4L2 knockdown decreased invasive capacity. The graph below showed absorbance at 570 nm after 24 hours ($P<0.05$, Student's t test). D. Wound-healing assay showed that DCAF4L2 knockdown decreased motility of CRC cells. The graph below showed mean opening distances after 48 hours ($P<0.05$, Student's t test). E. Annexin V/PI assay showed no obvious differences in apoptosis and necrosis when DCAF4L2 is knocked down (Student's t test).

high expression of DCAF4L2 via lentivirus infection, shDCAF4L2 stable cell lines were successfully constructed and DCAF4L2 expression was significantly reduced in both cell lines (**Figure 3A**). Results of other analysis concerning pivotal cellular biological functions came in consonance with prior ones as knockdown of DCAF4L2 attenuated cell migration and invasion (**Figure 3C** and **3D**) while imposing minimal influence on proliferation and cell death (**Figure 3B** and **3E**).

DCAF4L2 promotes epithelial-mesenchymal-transition via NFκB signaling

Since epithelial-mesenchymal-transition (EMT) has a fundamental role in enabling tumor cells to be invasive and metastatic, we sought to determine whether DCAF4L2 induces EMT in CRC cells. As expected, overexpression of DCAF4L2 resulted in EMT, as evidenced by induction of the mesenchymal markers N-cadherin (CDH2), and FN1 and repression of the epithelial marker E-cadherin (CDH1) and ZO1 (**Figure 4A**), which was reversed by down-regulation of DCAF4L2 (**Figure 4B**). Recent studies have provided stacking evidences suggesting that abnormally activated pathways like TGFβ, Hedgehog/Gli, Wnt/β-catenin and NFκB could facilitate EMT [6, 7]. To elucidate the molecular mechanism through which excess DCAF4L2 leads to the EMT activating phenotype, we examined multiple signaling pathways participated [8]. Indeed, We saw a prominent increase in NFκB signal pathway while observed no difference in Hedgehog/Gli, Wnt/β-catenin (**Figure 4C**) [9, 10]. There was a modest increase in TGFβ signal pathway, consistent with previous findings that NFκB could mediate TGFβ inducing extracellular matrix degradation, cell invasiveness and epithelial-mesenchymal transition during metastasis. In summary, these results show that DCAF4L2 could emerge as important regulators of EMT, enabled by NFκB signal pathway activation, which ultimately endows tumor cell high potential of dissemination.

DCAF4L2 forms an E3 ligase complex with Cul4A/DDB1 and mediates degradation of NFκB negative regulator PPM1B

DCAF4L2 is a WD-repeats protein as commonly implicated in E3 ligase complex, which suggests that it may carry out its function through protein-protein interplay [11]. To understand the underlying molecular connection between DCAF4L2 and NFκB activation, we performed Flag-tagged pulldown assay and final product was sent for mass spectrometry analysis. Silver staining displayed a clear interaction with numerous proteins, further confirmed by mass spectrometry (**Figure 5A** and **5B**). Among all proteins detected, DDB1, Cul4A, PPM1B had the most peptide binding. Cul4A-DDB1 E3 ligase is a multi-subunit protein complex, with the scaffold protein Cul4A serving as a hub bringing together catalytic and substrate-recognition components, which comprises an adaptor protein and a substrate receptor protein. Damage-specific DNA binding protein 1 (DDB1) is the canonical adaptor protein of CUL4A, linking the Cul4A with the substrate receptor, which in turn determines substrate specificity. To investigate the possible interaction between potential substrate receptor DCAF4L2 and DDB1, we co-expressed DCAF4L2 and DDB1 in HEK293T cells. As depicted in **Figure 5C**, DCAF4L2 was indeed co-immunoprecipitated with DDB1, which was consistent with the idea that DCAF4L2 formed an E3 ligase complex with Cul4A/ DDB1. Now we need to address whether PPM1B is the specific substrate considering this DCAF4L2-induced aggressive phenotype. We first examined its interaction and results came in expected as **Figure 5C** clearly demonstrated that DCAF4L2 was co-immunoprecipitated with PPM1B. In the next line of experiments, we asked whether DCAF4L2 regulated protein ubiquitination and protein stability of PPM1B. The foregoing observations implied that overexpressing DCAF4L2 enhanced the polyubiquitination of PPM1B hence notably reduced the total protein of PPM1B (**Figure 5D**), which may

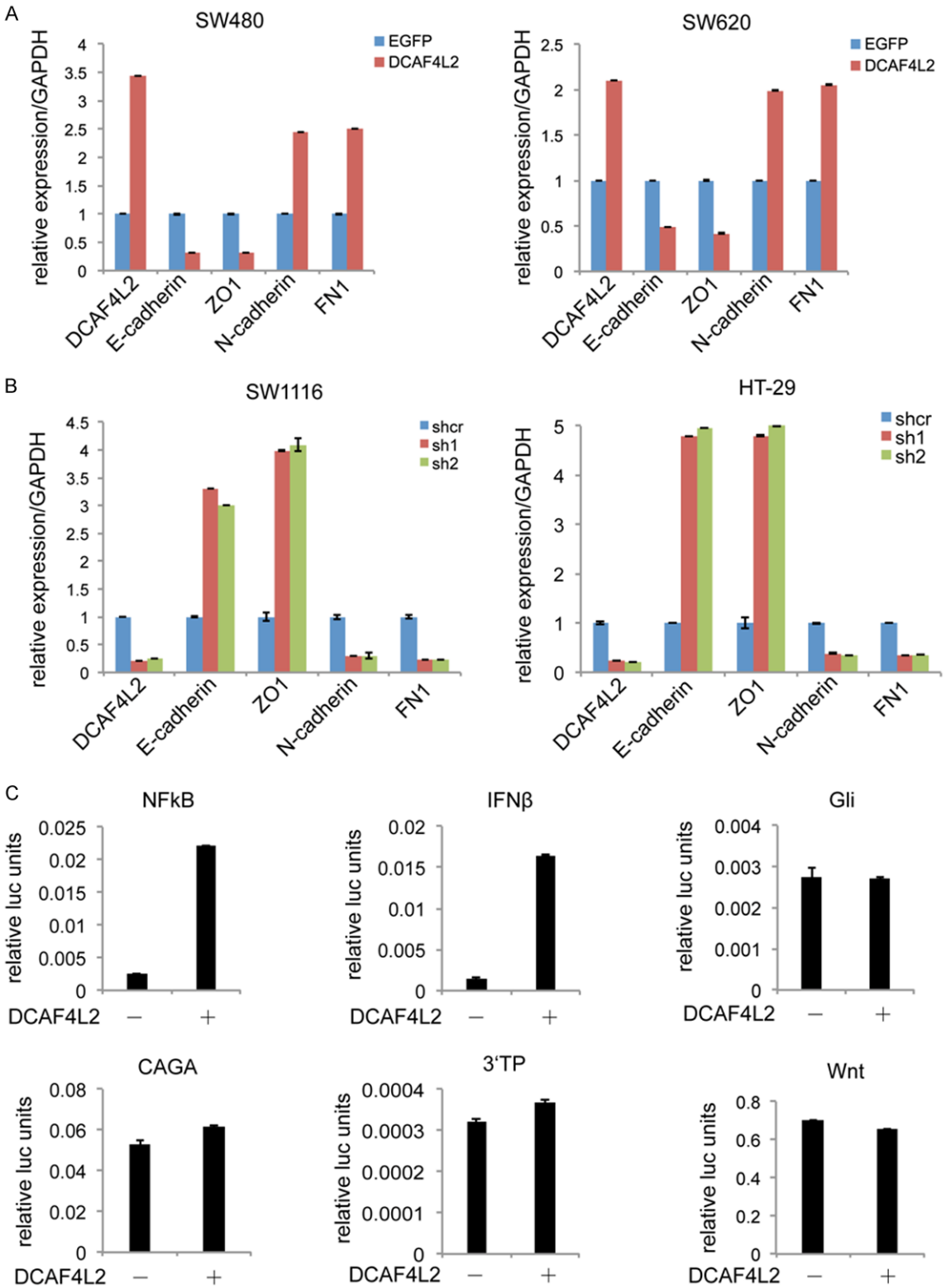
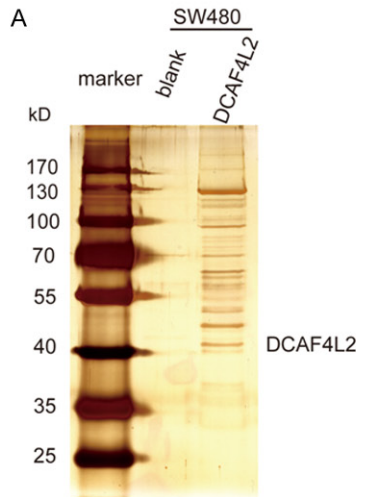


Figure 4. DCAF4L2 overexpression induced EMT through activating NFkB signal pathway in CRC cells. (A) Quantitative RT-PCR assay showed that DCAF4L2 overexpression induced EMT in CRC cells, characterized by an increase in epithelial marker such as E-cadherin, ZO1 and a decrease in mesenchymal marker such as N-cadherin and FN1 ($P<0.05$, Student's t test). (B) Quantitative RT-PCR assay showed that DCAF4L2 knockdown induced a reversed EMT in CRC cells ($P<0.05$, Student's t test). (C) Dual luciferase assay showed that DCAF4L2 overexpression activated NFkB signaling in HT-29 cells (Student's t test). NFkB and IFNβ is the promoter plasmid commonly used for NFkB

signaling; Gli is the promoter plasmid commonly used for Gli signaling; CAGA and 3'TP is the promoter plasmid commonly used for TGFβ signaling; TOP/FOP is the promoter plasmid commonly used for Wnt signaling.



B

Reference FileScan	PepCount Sequen	UniquePep MH+	CoverPerc Diff(M	MW DiffTPP	PI Charge	
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sp|Q13619|CUL4A_HUMAN Cullin-4A OS=Homo sapiens GN=CUL4A PE=1 SV=3	21	14	20.55%	87679.25	8.29	
sp|Q9BT78|CSN4_HUMAN COP9 signalosome complex subunit 4 OS=Homo sapiens GN=COPS4 PE=1 SV=1	28	13	42.12%	46268.28	5.57	
sp|O75688|PPM1B_HUMAN Protein phosphatase 1B OS=Homo sapiens GN=PPM1B PE=1 SV=1	40	12	42.38%	52642.04	4.95	
sp|P07355|ANXA2_HUMAN Annexin A2 OS=Homo sapiens GN=ANXA2 PE=1 SV=2	22	12	35.99%	38603.6	7.57	
sp|P19338|NUCL_HUMAN Nucleolin OS=Homo sapiens GN=NCL PE=1 SV=3	17	9	15.77%	76613.47	4.6	
sp|P11021|GRP78_HUMAN 78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2	13	9	20.18%	72332.07	5.07	
sp|P19474|RO52_HUMAN 52 kDa Ro protein OS=Homo sapiens GN=TRIM21 PE=1 SV=1	16	8	22.32%	54169.15	5.98	
sp|Q92905|CSN5_HUMAN COP9 signalosome complex subunit 5 OS=Homo sapiens GN=COPS5 PE=1 SV=4	16	8	29.64%	37578.39	6.1	
sp|Q13620|CUL4B_HUMAN Cullin-4B OS=Homo sapiens GN=CUL4B PE=1 SV=3	14	8	9.39%	102297.9	8.06	
sp|O14744|ANM5_HUMAN Protein arginine N-methyltransferase 5 OS=Homo sapiens GN=PRMT5 PE=1 SV=4	13	8	15.86%	72683.02	5.88	
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sp|P11142|HSP7C_HUMAN Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1	9	7	14.24%	70897.24	5.37	
sp|P67809|YBOX1_HUMAN Nuclease-sensitive element-binding protein 1 OS=Homo sapiens GN=YBX1 PE=1 SV=3	9	7	41.67%	35923.75	9.87	
sp|O75153|K0664_HUMAN Protein KIAA0664 OS=Homo sapiens GN=KIAA0664 PE=1 SV=2	8	7	8.63%	146668.1	5.75	
sp|Q8NA75|DC4L2_HUMAN DDB1- and CUL4-associated factor 4-like protein 2 OS=Homo sapiens GN=DCAF4L2 PE=1 SV=1	15	6	18.73%	43746.73	8.85	
sp|Q06830|PRDX1_HUMAN Peroxiredoxin-1 OS=Homo sapiens GN=PRDX1 PE=1 SV=1	12	6	31.66%	22110.09	8.27	
sp|P10809|CH60_HUMAN 60 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPD1 PE=1 SV=2	11	6	15.71%	61053.94	5.7	
sp|Q13098|CSN1_HUMAN COP9 signalosome complex subunit 1 OS=Homo sapiens GN=GPS1 PE=1 SV=4	10	6	15.07%	55535.98	6.3	
sp|P61978|HNRPK_HUMAN Heterogeneous nuclear ribonucleoprotein K OS=Homo sapiens GN=HNRNPK PE=1 SV=1	9	6	13.61%	50975.66	5.39	
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sp|P60660|MYL6_HUMAN Myosin light polypeptide 6 OS=Homo sapiens GN=MYL6 PE=1 SV=2	9	5	37.75%	16929.88	4.56	
sp|P11940|PABP1_HUMAN Polyadenylate-binding protein 1 OS=Homo sapiens GN=PABPC1 PE=1 SV=2	8	5	9.43%	70670.07	9.52	
sp|P22626|ROA2_HUMAN Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1 PE=1 SV=2	8	5	15.58%	37429.25	8.97	
sp|P50990|TCPQ_HUMAN T-complex protein 1 subunit theta OS=Homo sapiens GN=CCT8 PE=1 SV=4	5	5	11.13%	59619.93	5.42	
sp|Q7L5N1|CSN6_HUMAN COP9 signalosome complex subunit 6 OS=Homo sapiens GN=COPS6 PE=1 SV=1	12	4	14.07%	36163.09	5.47	
sp|Q99627|CSN8_HUMAN COP9 signalosome complex subunit 8 OS=Homo sapiens GN=COPS8 PE=1 SV=1	9	4	30.14%	23225.35	5.25	
sp|Q9H9Q2|CSN7B_HUMAN COP9 signalosome complex subunit 7b OS=Homo sapiens GN=COPS7B PE=1 SV=1	8	4	20.08%	29621.62	5.83	
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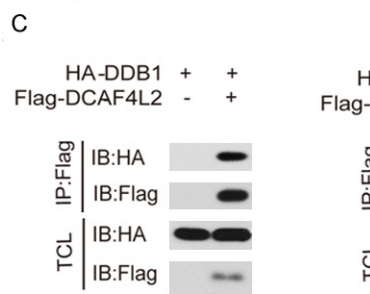
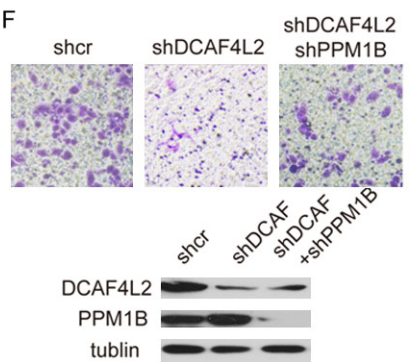
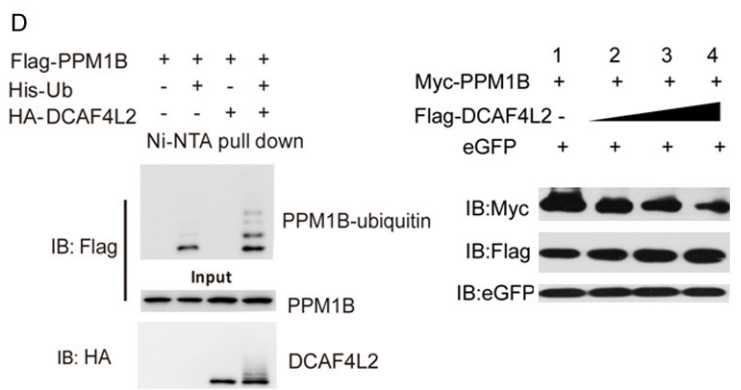
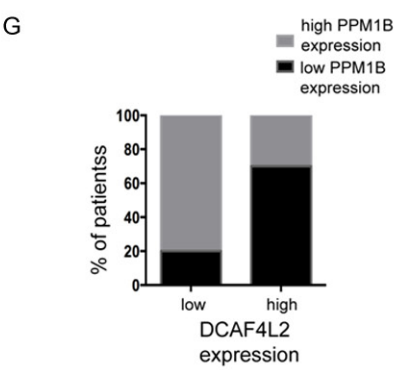
sp|P23528|COF1_HUMAN Cofilin-1 OS=Homo sapiens GN=CFL1 PE=1 SV=3	7	4	33.13%	18502.29	8.22	









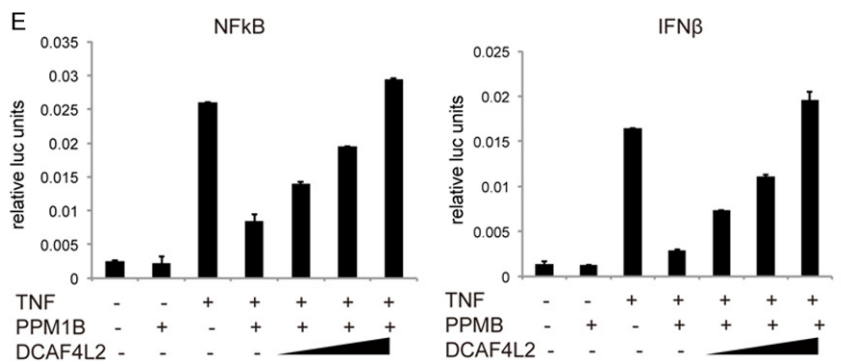


Figure 5. DCAF4L2 formed an E3 ligase complex with Cul4A and DDB1 thus mediated PPM1B degradation which contributing to DCAF4L2-induced NFkB activation and EMT. (A) Silver staining confirmed that DCAF4L2 interacted with various proteins via Flag-tagged pulldown. (B) Mass spectrometry analysis of pulldown product in (A). (C) Co-immunoprecipitation demonstrated a direct binding between DCAF4L2 and DDB1/PPM1B. (D) Immunoblot analysis displayed that DCAF4L2 overexpression could enhance PPM1B ubiquitination and mediated PPM1B degradation. (E) Dual luciferase assay revealed that PPM1B could efficiently inhibited NFkB signaling which, could be revoked and even reversed with DCAF4L2 overexpression in HT-29 cells. (F) Matrigel invasion assay confirmed that shPPM1B transfection partly relieved the inhibitory effect of shDCAF4L2 transfection considering the invasive ability of HT-29 cells. Immunoblot assay verified transfection efficiency. (G) Quantitative RT-PCR analysis showed that the expression of DCAF4L2 and PPM1B is inversely correlated in 40 pair of CRC tissues ($P<0.05$, chi-square test).

imply that HEK293T cells are endowed with a high expression level of endogenous DDB1. To investigate whether PPM1B is the designated substrate for DCAF4L2-mediated changes in cellular function, we examined effects of PPM1B on NFκB signaling and cell invasion. PPM1B has been established as an IKKβ phosphatase to terminate TNFα-induced IKKβ/NFκB activation [12]. Our dual luciferase assay substantiated previous studies and further confirmed our story by showing that PPM1B could remarkably inhibit TNFα-induced NFκB activation, which accordingly could be revoked and even reversed as DCAF4L2 level incremented (**Figure 5E**). Matrigel invasion assay showed that PPM1B knockdown relieved the shDCAF4L2-induced abrogation of cell invasion in SW1116 cells (**Figure 5F**). Moreover, shRNA knockdown of endogenous DCAF4L2 resulted in increased PPM1B protein expression (**Figure 5F**). In a cohort of CRC patient tissues, DCAF4L2 expression was inversely correlated with PPM1B expression (**Figure 5G**). Together these observations support the notion that DCAF4L2 assembled an E3 ligase complex with Cul4A and DDB1 to mediate polyubiquitination and control the protein turnover of PPM1B, which led to NFκB activation and thus contributed to increased invasiveness in CRC.

DCAF4L2 expression is negatively correlated with prognosis and survival in CRC

Our studies have verified higher level of DCAF4L2 in CRC samples compared to adjacent non-tumorous tissues, yet its relevance with clinical outcome remained unclear. We examined DCAF4L2 mRNA level in 40 pairs of CRC samples consisting of all four pathological stages and discovered a positive correlation. DCAF4L2 expression drastically increased as CRC evolved and progressed through each stage (**Figure 6A**). In addition, we conducted tissue microarray on 87 pairs of CRC specimens and corresponding normal colorectal mucosal tissues, high expression level of DCAF4L2 was found in 50.57% (43/87) of primary CRCs. Correlation analysis demonstrated that high expression level of DCAF4L2 was positively correlated with CRC lymphatic and distant metastasis, as a more advanced pathological TNM stage while tumor volume is independent (**Table 1**). Kaplan-Meier analysis showed that CRC patients with higher level of DCAF4L2 had a rather shorter length of mean survival time (**Figure 6B**), which indicating that DCAF4L2 could serve as an independent prognosis factor for poor survival in CRC patients. Representative images of DCAF4L2 IHC staining of were shown in **Figure 6C**.

Discussion

Colorectal carcinoma (CRC) is one of the most common cancer worldwide with high incidence of mortality and poor prognosis [13]. Although apprehension of CRC pathology and characteristics continued evolving and new therapeutic strategy and targeted drugs kept surfacing, patients still suffered from relapse and metastatic complications, indicating that precise mechanisms underlying remained unknown, especially when it comes to that of metastasis [14]. Therefore it is high-priority that we investigate molecules contributing to metastasis and identify new biomarkers to better predict prognosis, which could have broad biological significance and potentially pilot new therapies targeting cancer metastasis.

DCAF4L2 has only been reported to have a potential pathogenicity in cleft palate and a high expression in liver cancer [15]. We provide the first evidence of DCAF4L2 expression in CRC, showing that DCAF4L2 is markedly overexpressed in CRC cell lines and tissues compared to almost no expression in non-cancerous tissues. To understand the role of DCAF4L2

Table 1. Clinicopathologic correlation of DCAF4L2 expression in human sporatic colorectal carcinoma

	DCAF4L2			
Feature	low	high*	χ^2	*P* value#
All cases	43	44		
Gender			0.3118	0.577
Male	26	24		
Female	17	20		
Age			0.0975	0.755
>65	23	25		
≤65	20	19		
Intravascular cancer embolus			2.351	0.125
Present	6	12		
Absent	37	32		
Perineuronal invasion			3.433	0.064
Present	8	16		
Absent	35	28		
Tumor size (cm)			3.745	0.053
>4	11	20		
≤4	32	24		
T stage			3.832	0.280
T1	4	1		
T2	12	8		
T3	10	11		
T4	17	24		
N stage			10.418	0.005
N0	18	9		
N1	15	10		
N2	10	25		
M stage			4.679	0.031
M0	39	32		
M1	4	12		
Tumor stage				
I	6	2	9.509	0.023
II	15	7		
III	18	23		
IV	4	12		

*The median expression level was used as the cutoff. Low expression of DCAF4L2 in 43 patients was classified as values below the 50th percentile. High DCAF4L2 expression in 44 patients was classified as values at or above the 50th percentile. #Pearson's chi-square tests were used for analysis of correlation between DCAF4L2 and clinical features. Results were considered statistically significant at P<0.05.

in CRC tumorigenesis and development, we employed a series of in vitro assays to investigate its function in cell proliferation, death, motility, invasion and metastasis. Results showed that the ectopic overexpression of DCAF4L2 in CRC cells substantially promoted cell motility and invasiveness while shRNA-mediated knockdown inhibited the ability of either cell migration or invasion. As for cell proliferation and cell death, neither overexpression nor knockdown of DCAF4L2 had apparent effects. EMT is a transcriptional program in which epithelial cells are converted to migratory and invasive cells [16]. This process has been considered a fundamental event in the generation of tissues and organs during embryogenesis, wound healing and to be intimately involved in cancer progression in this near decade. We reinforced this conviction by demonstrating that in the presence of excess DCAF4L2, EMT occurs and when DCAF4L2 is lacking, EMT is reduced. So far, it has become clear that EMT reprogramming occurs systematically at transcriptional, post-transcriptional, translational and post-translational levels and deregulated signal pathway at alternative levels affect EMT regulation. TGFβ, Wnt, Gli, NFκB and many other signal pathways have been featured during this process [17]. Utilizing dual luciferase report assay, we were able to elaborate that DCAF4L2 could exert great stimulation upon NFκB signaling other than Wnt and Gli. In the process, we also found that DCAF4L2 could slightly activated TGFβ, indicating an interwinding interplay existing for EMT control [18].

DCAF4L2 is a WD-repeats domain containing protein mostly acting as a platform for protein-protein interaction, which is commonly seen in E3 ligase complex [19]. The largest subfamily of human E3 ubiquitin ligases is the cullin RING ligase, which is an evolutionarily conserved protein family comprised of up to 400 distinct E3 ubiquitin ligase complexes regulating diverse cellular pathways [20]. CUL4, among three founding cullins, uses DDB1, as a linker to interact with a subset of WD40 proteins that serve as substrate receptors, forming as many as 90 E3 complexes in mammals, regulating a broad spectrum of critical cellular processes [21]. With Flag-tagged pulldown and mass spectrometry analysis, we took a proteomic approach and discovered that DCAF4L2 could bind with DDB1 and Cul4A to a great extent and the binding with Cul4A is most likely an indirect binding through DDB1. A thorough screening into mass spectrometry came across

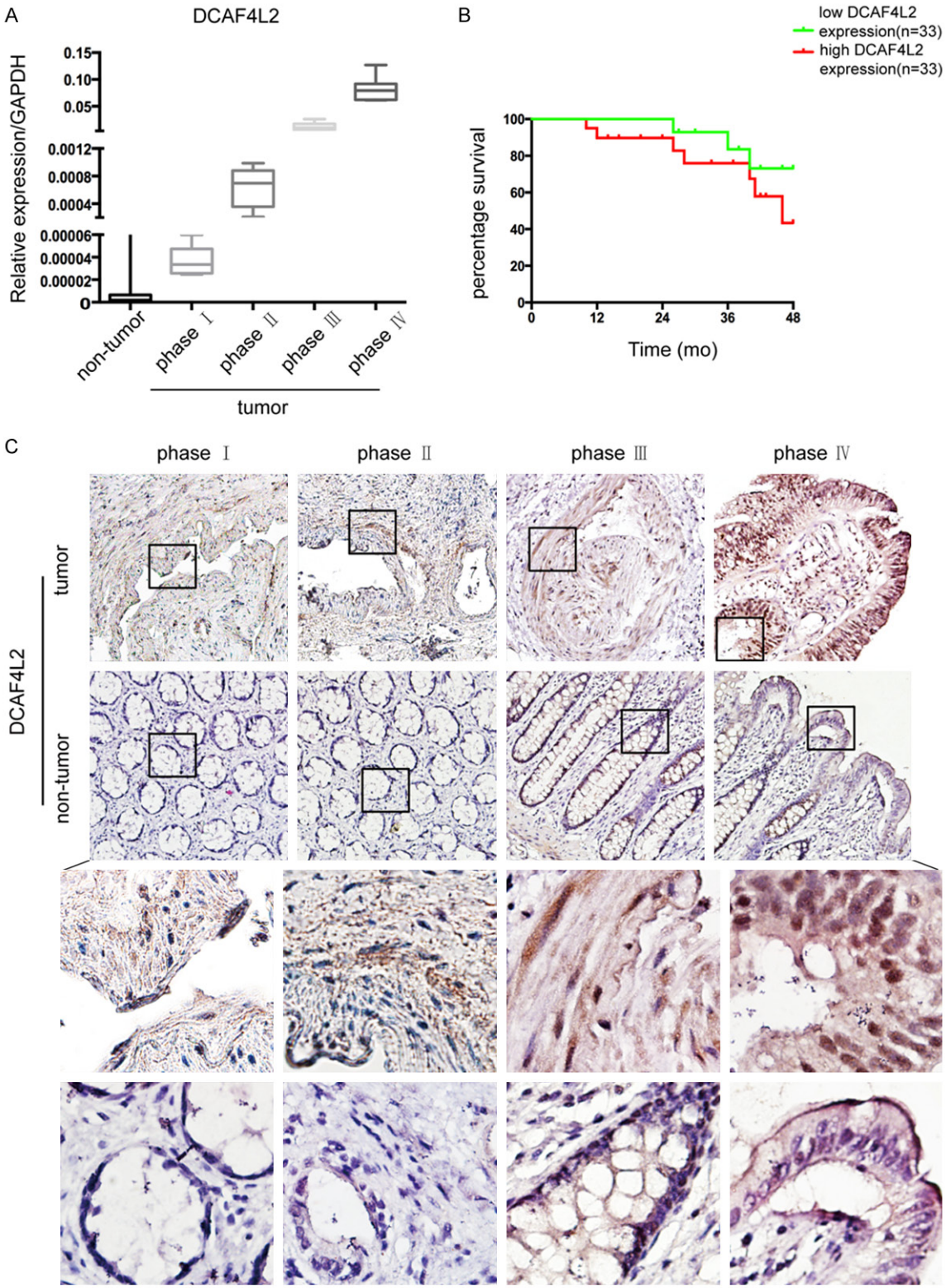


Figure 6. DCAF4L2 expression conversely correlated with clinical stage in CRC patients and is an independent indicator for poor prognosis and poor survival. A. Quantitative RT-PCR analysis showed that DCAF4L2 expression drastically increased as CRC progressed ($P<0.05$, Student's t test). B. Kaplan-Meier survival analysis according to DCAF4L2 expression in 66 CRC patients (log-rank test). C. Representative immunohistochemistry images showed the expression of DCAF4L2 peeked as CRC progressed.

a candidate substrate PPM1B, which is the fourth most binding protein on the list and could terminate NFκB activation by phosphorylating IKKβ [22]. Indeed, we performed co-immunoprecipitation in vitro and it turned out that DCAF4L2 could directly interact with DDB1 and PPM1B [23]. We then investigated the function of DCAF4L2 considering PPM1 protein ubiquitination and stability. DCAF4L2 over expression enhanced the polyubiquitination of PPM1B and reduced its total protein level whereas DCAF4L2 knockdown up regulated PPM1B protein level [24]. However, PPM1B was the particular substrate responsible for DCAF4L2-induced metastasis remained further elucidated. Reverse experiments showed that DCAF4L2 lifted inhibition PPM1B wielded upon TNFα-induced pronounced activation in NFκB signaling. Moreover, additional PPM1B knockdown notably antagonized DCAF4L2 insufficiency retarded cell migration and invasion. Clinical data in a cohort of CRC patient tissues illustrated a negative correlation between the expressions of DCAF4L2 and PPM1B, which is also in consistent with our hypothesis.

Subsequently, we examined expression dynamics of DCAF4L2 in all stages of CRC, utilizing an immunochemistry tissue microarray combined with complete clinical follow-up, suggesting that the up-regulated expression of DCAF4L2 in CRC may facilitate the invasive and metastatic phenotype. More importantly, overexpression of DCAF4L2 in CRC was an independent prognosis factor and an indicator for poor survival, which could be applied clinically in the future as a tool for metastatic risk screening, underscoring its significance.

Collectively our work provided better insights into sophisticated and interconnected control mechanisms during EMT by shedding lights on a post-translational regulatory mechanism of E3 ligase complex Cul4A-DDB1-DCAF4L2 mediated EMT in CRC through PPM1B degradation, accompanied with NFκB aberrant activation. DCAF4L2 clearly exemplifies a regulatory network connecting multiple levels of EMT control, which allows versatile therapeutic interventions aimed at blocking its pro-metastatic activity. This discovery holds the promise of resolving unsettled problems and yield innovative therapeutics when treating colon cancer and other EMT-related phenomena in the near future when EMT regulators are not available as druggable targets at present.

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Disclosure of conflict of interest

None.

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