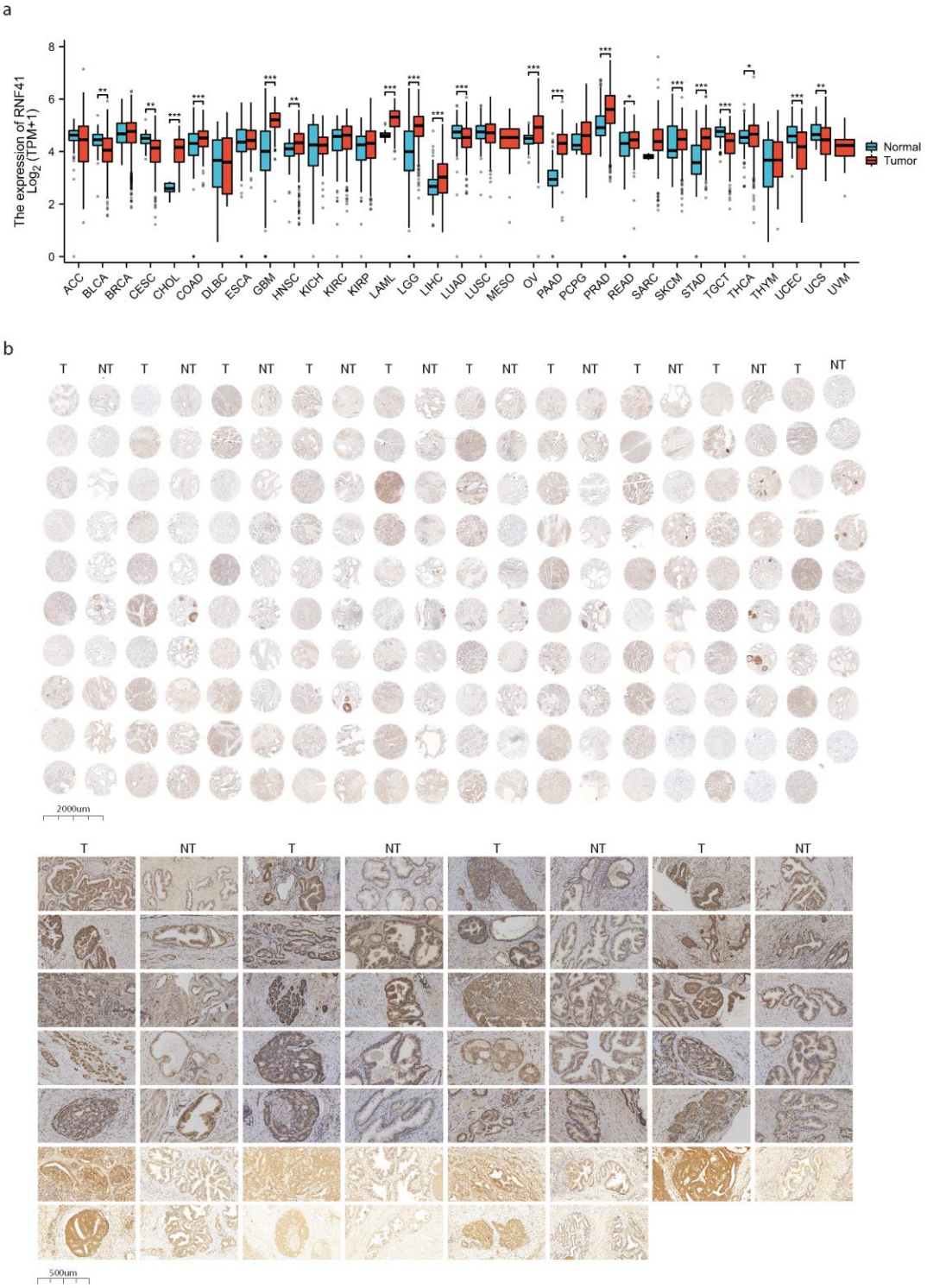
Supplementary information

Supplemental Figures, Tables, Methods and References

Supplementary Figures

Supplementary Fig. 1

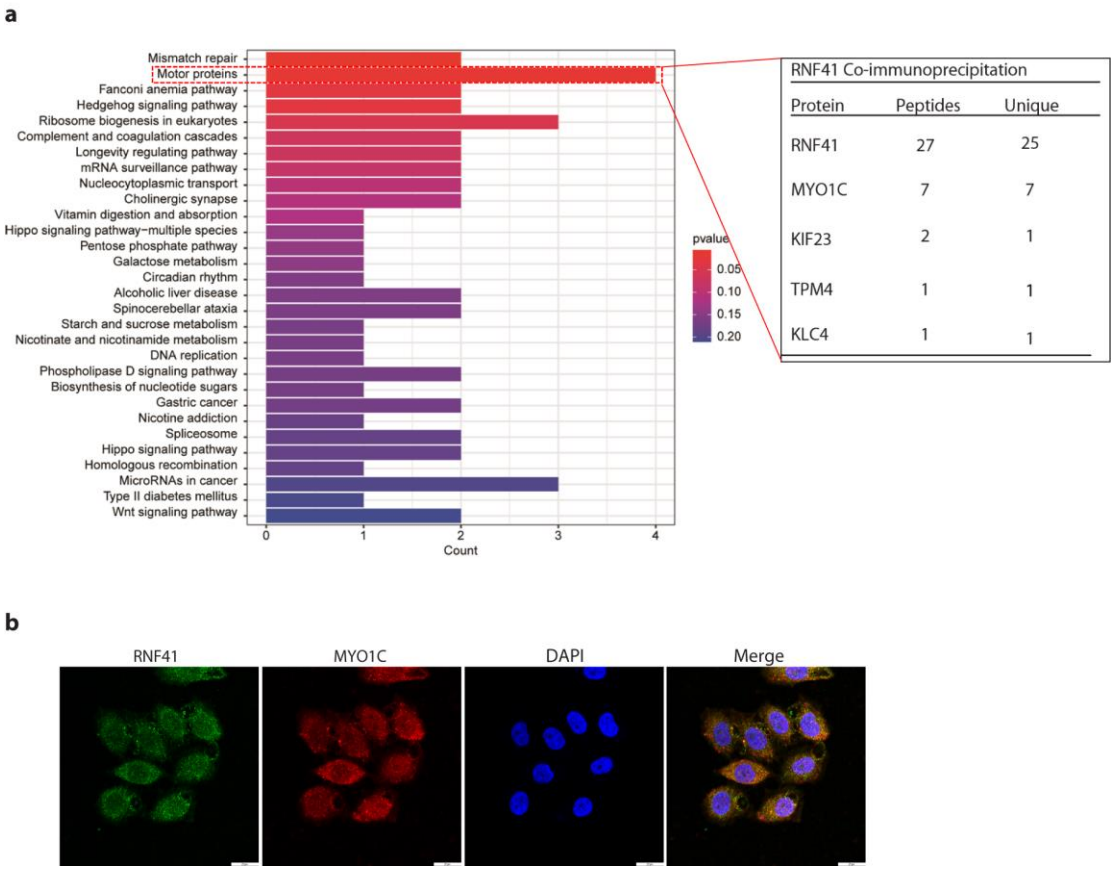


Supplementary Fig. 1

(**a**) The expression of the RNF41 gene in 33 different types of tumors and normal tissues in the TCGA database (https://portal.gdc.cancer.gov/). (**b**) IHC staining of

RNF41 protein in metastatic PCa tissues containing 27 samples of metastatic PCa tissues and 27 samples of adjacent metastatic non-tumor tissues, and a tissue microarray containing 100 samples of PCa tissues and 99 samples of adjacent non-tumor tissues.

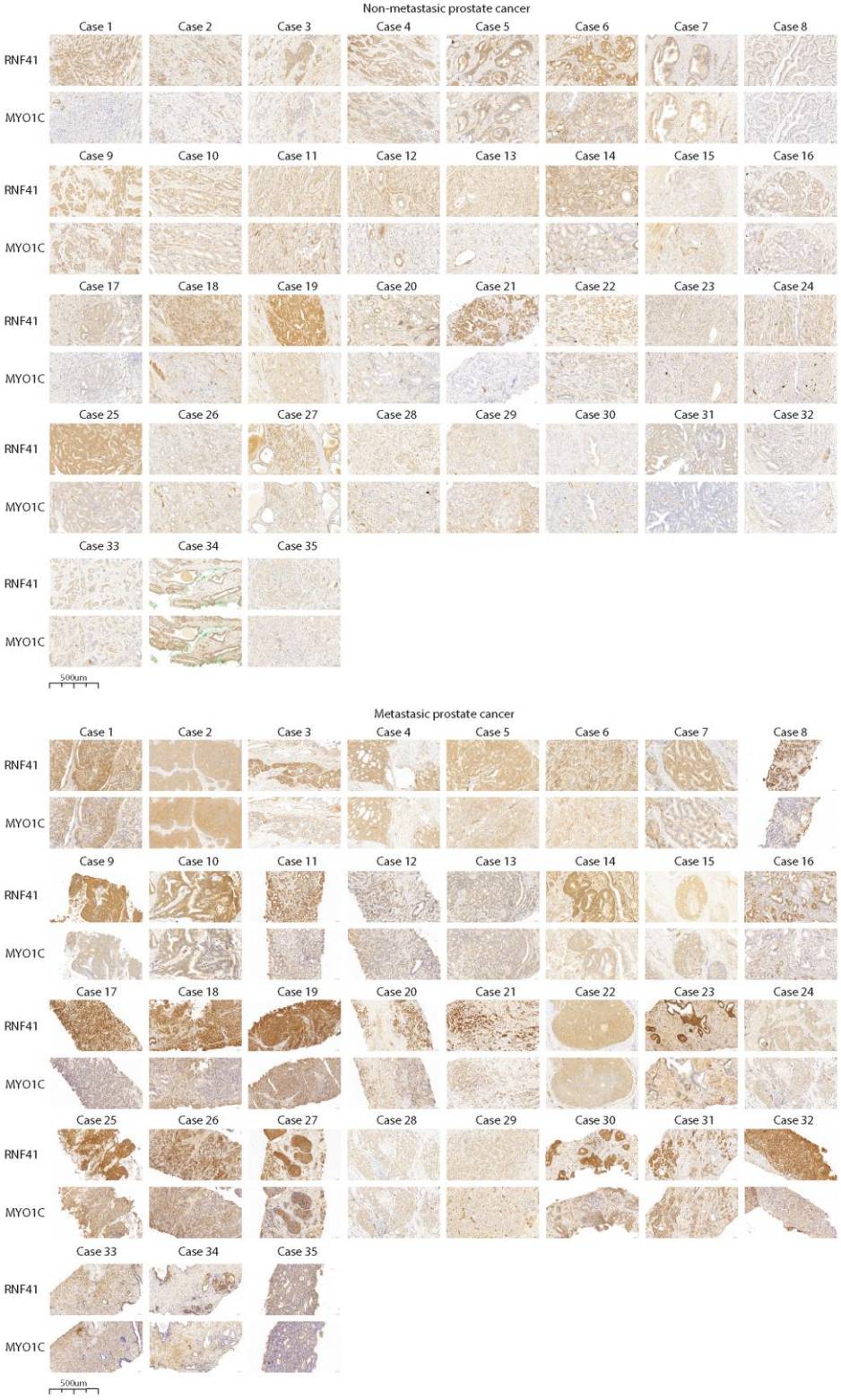
Supplementary Fig. 2



Supplementary Fig. 2

(**a**) Concept-protein networks of enriched pathway of RNF41-interacted proteins were analyzed by protein ontology annotation (KEGG). (**b**) Immunofluorescent staining of RNF41 (green) and MYO1C (red) in DU145 cells. Nuclear 4', 6-diamidino-2-phenylindole (DAPI; blue).

Supplementary Fig. 3

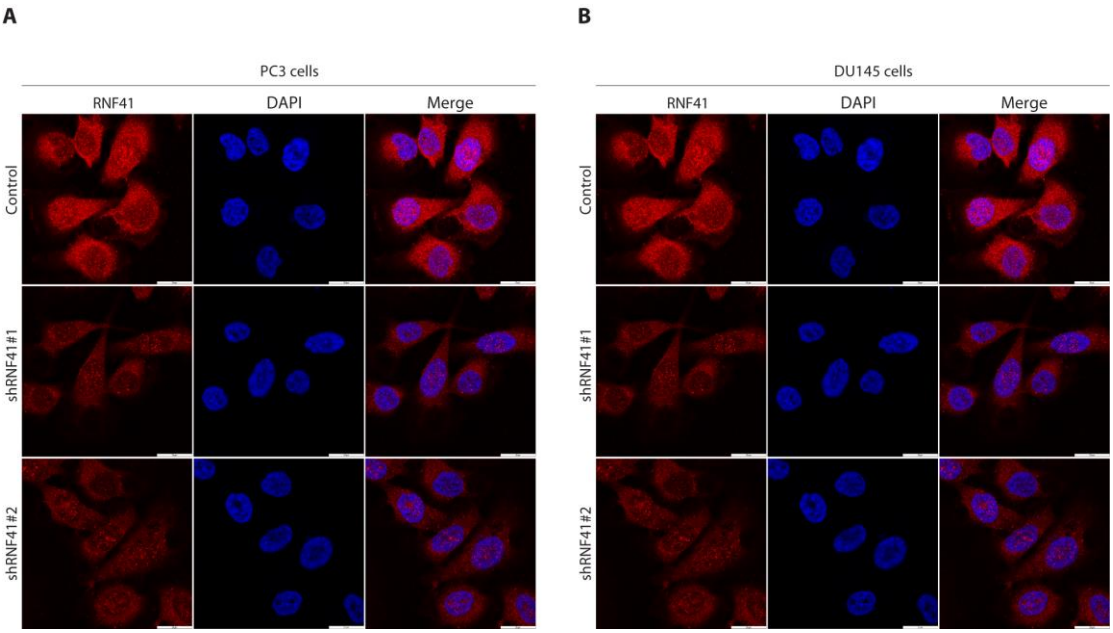


Supplementary Fig. 3

Immunohistochemical staining of RNF41 and MYO1C in PCa tissues containing 35

samples of metastatic PCa tissues and matched 35 samples of none-metastatic PCa tissues

Supplementary Fig. 4



Supplementary Fig. 4

(**a**) The subcellular localization of RNF41 in PC3 cells stably expressing Control or RNF41 shRNA were visualized by immunofluorescence assay. (**b**) The subcellular localization of RNF41 in DU145 cells stably expressing Control or RNF41 shRNA were visualized by immunofluorescence assay.

Supplementary Tables

Supplementary Table 1 Clinic-pathological variables and RNF41 expression in 126 prostate cancer patients assessed by IHC from TMA.

Variables	All	RNF41		*P*
	n=126	n=24 (Low)	n=102 (High)	Value#
Age (%)				0.100

＜70	65 (51.6)	16 (66.7)	49 (48.0)	
⩾70	61 (48.4)	8 (33.3)	53 (52.0)	
pT stage (%)				**0.019***
T2	52 (41.3)	15 (62.5)	37 (36.3)	
T3/T4	74 (58.7)	9 (37.5)	65 (63.7)	
Gleason score (%)				**0.028***
⩽7	44 (34.9)	13 (54.2)	31 (30.4)	
＞7	82 (65.1)	11 (45.8)	71 (69.6)	
Tissue grade (%)				**0.011***
＜3	28 (22.2)	10 (41.7)	18 (17.6)	
⩾3	98 (77.8)	14 (58.3)	84 (82.4)	
Metastasis (%)				**0.040***
No	106 (84.1)	24 (100)	82 (80.4)	
Yes	20 (15.9)	0 (0)	20 (19.6)	
Pathological stage (%)				0.072
＜IIIA	31 (24.6)	10 (41.7)	24 (23.5)	
⩾IIIA	95 (75.4)	14 (58.3)	78 (76.5)	

#*P* value was analyzed by chi-sequared test; * indicates $P < 0.05$.

Supplementary Table 2

Supplementary Table 2. The candidate ubiquitin-proteins interacted with RNF41 were identified by Mass Spectrometry.

Protein Names	Gene Names	#Peptides	Unique Peptides	Mol. Weight [kDa]
E3 ubiquitin-protein ligase NRDP1	RNF41	27	27	35.9
Unconventional myosin-Ic	MYO1C	7	7	121.7
Kinesin-like protein KIF23	KIF23	2	1	110
Tropomyosin alpha-4 chain	TPM4	1	1	28.5
Kinesin light chain 4	KLC4	1	1	68.6

Supplementary Table 3 Clinic-pathological variables and MYO1C expression in prostate cancer patients assessed by IHC from TMA.

Variables	All	MYO1C		*P*
	n=70	n=35 (Low)	n=35 (High)	Value#
Age (%)				0.089

＜70	29 (41.4)	18 (51.4)	11 (31.4)	
⩾70	41 (58.6)	17 (48.6)	24 (68.6)	
pT stage (%)				0.205
T2	12 (17.1)	8 (22.9)	4 (11.4)	
T3/T4	58 (82.9)	27 (77.1)	31 (88.6)	
Gleason score (%)				**0.027***
⩽7	27 (38.6)	18 (51.4)	9 (25.7)	
＞7	43 (61.4)	17 (48.6)	26 (74.3)	
Tissue grade (%)				0.324
＜3	11 (15.7)	7 (20.0)	4 (11.4)	
⩾3	59 (84.3)	28 (80)	31 (88.6)	
Bone metastasis (%)				**0.031***
No	35 (50.0)	22 (62.9)	13 (37.1)	
Yes	35 (50.0)	13 (37.1)	22 (62.9)	
Lymphatic metastasis (%)				**0.048***
No	44 (62.9)	26 (74.3)	18 (51.4)	
Yes	26 (37.1)	9 (25.7)	17 (48.6)	
Pathological stage (%)				0.356
＜IIIA	5 (7.1)	4 (11.4)	1 (2.9)	
⩾IIIA	65 (92.9)	31 (88.6)	34 (97.1)	

[#]*P* value was analyzed by chi-sequared test; [*] indicates $P < 0.05$ with statistical significance.

Supplementary Materials and Methods

Antibodies and reagents

Anti-RNF41 antibody (Cat No. 17233-1-AP), anti-β-actin antibody (Cat No. HRP-66009), anti-HA antibody (Cat No. HRP-81290), anti-Flag antibody (Cat No. 66008-4-Ig) and anti-Myc antibody (Cat No. 16286-1-AP) were purchased from Proteintech (Wuhan, China). Anti-MYO1C antibody (ab154498) and anti-Ki67 antibody (ab15580) were purchased from Abcam (Cambridge, United Kingdom). All were used according to the manufacturers' recommendations.

Plasmids and cloning

Flag-RNF41 (G25067-9) and Myc-MYO1C (P33176) expression plasmid was generated by subcloning the into pcDNA3 vector and were obtained from MiaoLingBio, China. RNF41 different domains were subcloned into the pcDNA3-Flag vector. RNF41 mutant was generated by using the QuikChange II Site-Directed Mutagenesis Kit. Primers used for cloning are available upon request.

shRNA lentiviral vector packaging and transduction

shRNA lentiviral vector packaging and transduction were described as previously[1]. lentiviral vectors shRNA-RNF41, shRNA-MYO1C and shRNA-GFP pLKO1 (control vector) were purchased from QEgene (Shanghai, China). Lentiviral vector encoding shRNA was packaged in 293T cells by calcium phosphate transfection. The supernatants that contained lentiviral particles was collected 48h after transfection. The indicated PCa cells were then transduced with the supernatant in the presence of polybrene (8 μg/mL) for 24 h before replacement with fresh growth media. Cells were analyzed at 48 or 72 h post transduction.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was described as previously[1]. Primers for qPCR analysis of human gene transcripts were:

RNF41:

Forward Primer: 5'-AGA CAC GCA TCG CAG AG-3'

Reverse Primer: 5'-GAA GGT TGG GGT TGA CAC T-3'

β-actin：

Forward Primer: 5’-TCT CCC AAG TCC ACA CAC GG-3’

Reverse Primer: 5’-GGC ACG AAG GCT CAT CA-3’

Primers for qPCR analysis of mouse gene transcripts were:

Ptch1:

Forward Primer: 5’- CTCTGGAGCAGATTTCCAAGG-3’

Reverse Primer: 5’- TGCCGCAGTTCTTTTGAATG-3’

Gli1:

Forward Primer: 5’-GGAAGTCCTATTCACGCCTTGA-3’

Reverse Primer: 5’-CAACCTTCTTGCTCACACATGTAAG-3’

Hhip:

Forward Primer: 5’-CCTGTCGAGGCTACTTTTCG-3’

Reverse Primer: 5’-TCCATTGTGAGTCTGGGTCA-3’

RANKL:

Forward Primer: 5’-CAGTGGGAGATGTTAGACTCATG-3’

Reverse Primer: 5’-GAAGGGGCACATGACCAGGGACCAAC-3’

BMP2:

Forward Primer: 5’-GGTCACAGATAAGGCCATTGC-3’

Reverse Primer: 5’-GCTTCCGCTGTTTGTGTTTG-3’

BMP4:

Forward Primer: 5’-GAGGAGGAGGAAGAGCAGAG-3’

Reverse Primer: 5’-TGGGATGTTCTCCAGATGTT-3’

GAPDH:

Forward Primer: 5’-TCCCACTCTTCCACCTTCGATGC-3’

Reverse Primer: 5’-GGGTCTGGGATGGAAATTGTGAGG-3’

Western blotting

Proteins were prepared as previously described[2]. Briefly, protein extracts were separated by gradient SDS-PAGE gel and then electroblotted onto a PVDF membrane (Cytiva, catalog number: 10600021). The membranes were incubated with the indicated primary antibodies at 1: 1000 at 4℃ overnight, respectively, followed by incubation with corresponding secondary antibodies at 1: 10000 at room temperature for 1 h.

Cell proliferation, clony formation and soft agar colony-formation assays

Cell proliferation, clony formation and soft agar colony-formation assays were described as previously[3].

Wound healing

The indicated PCa cells were plated onto 6-well plates in triplicate and incubated 24 h at 37 ℃ with 5% CO_2 overnight to achieve 100% confluence of adherent cells. Using a 200 μL pipette tip, a linear scratch was made, running the length of each well. The plate was imaged using a Celigo Imaging Cytometer at indicated timepoints. Each image was quantified using ImageJ. The non-confluent portion of each well was calculated as a mean percentage area for three wells and graphed ±SD. The experiment is representative of three experiments.

Migration and invasion

Migration and invasion results were performed as previously described[3], using transwell assays with Matrigel (invasion) and without Matrigel (migration) according to the manufacturer's directions (Corning Inc., Corning, NY). The indicated PCa cells (1×10^5) were plated in the top well in no FBS RPMI 1640 or DMEM medium. In the bottom well, control media, CM or different concentrations of recombinant GDF15 (Cat.8944-GD-025, R&D Systems Minneapolis, MN) and 10% FBS were added as a chemoattractant. Cells underneath the inserts were analyzed using light microscopy and photographed. The numbers of cells were counted in five random fields for each insert. Each data point represents the average number from three wells.

BrdU assay

BrdU assays were performed as described previously[4]. Briefly, cells were cultured in a 6-well plate overnight followed by a 20-min incubation with 10 μg/ml BrdU. Then, cells were washed twice with PBS and fixed with 70% ethanol at − 20 °C overnight.

Next, cells were denatured in 2 N HCL for 45 min and stained with FITC secondary antibody for 1 h at room temperature. Cells were further incubated with 40 g/ml RNase A and 200 g/ml PI for 30 min and finally analysed by flow cytometry.

Protein immunoprecipitation (IP) and Liquid Chromatography-MS Analysis

Co-IP or IP was described as previously[1]. Cell lysates were captured on Flag-M2 beads or protein A/G agarose beads (Santa Cruz, USA). The complexes were then separated, and the gels were stained with silver or were detected by Western blotting. For Liquid Chromatography-MS Analysis, immunoprecipitation of RNF41 antibodies was performed as described above. The precipitated proteins were eluted 3 times with lysis buffer. The eluted samples were subjected to in-solution trypsin digestion, followed by liquid chromatography-MS analysis and Protein identification was performed using the Mascot (v2.3.02) program and compared against the Uniprot human protein database (released Dec 2014).

Ubiquitination assay

Ubiquitination assay was described as previously[1]. In brief, the cells were transfected with indicated plasmids and lysed with the immunoprecipitation buffer. For immunoprecipitation, 2 mg of protein was incubated with indicated antibodies at 4℃ overnight before Flag-M2 beads or protein A/G beads were added for 2 h. Beads were washed once with TBS, 1% Triton X-100, 1% SDS, twice with 0.5 M LiCl, TBS buffer and again in PBS 1% Triton X-100 containing buffer. Proteins were loaded onto 8% SDS-PAGE gels and immunoblotted with the indicated antibodies.

Immunofluorescence

The indicated cells were fixed in 4% paraformaldehyde for 15 min. After washing with PBS, the cover slides were blocked in PBS containing 0.1% Triton-X100, 2% BSA, and 1% normal goat serum for 1 h at room temperature and then incubated the slides with 1:500/1:1000 dilution of the indicated antibody for 2 h at room temperature. The Alexa 488-conjugated anti-rabbit/anti-mouse antibody or Rhodamine-conjugated anti-rabbit/anti- mouse antibody was added and incubated for 1 hr at room temperature. The slides were then washed three times with PBS and counterstained with 4,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. The slides were captured by using an inverted microscope under a x 60 oil-immersion objective and scanned with a laser confocal system.

Supplemental References

1. Xu, S. *et al.* p300-Mediated Acetylation of Histone Demethylase JMJD1A Prevents Its Degradation by Ubiquitin Ligase STUB1 and Enhances Its Activity in Prostate Cancer. *Cancer research* **80**, 3074-3087 (2020).
2. Xu, S.H. *et al.* ACK1 promotes gastric cancer epithelial-mesenchymal transition and metastasis through AKT-POU2F1-ECD signalling. *The Journal of pathology* **236**, 175-185 (2015).
3. Tang, D.E. *et al.* Targeting the KDM4B-AR-c-Myc axis promotes sensitivity to androgen receptor-targeted therapy in advanced prostate cancer. *The Journal of pathology* **252**, 101-113 (2020).
4. Hu, F. *et al.* Histone demethylase KDM4D promotes gastrointestinal stromal tumor progression through HIF1beta/VEGFA signalling. *Molecular cancer* **17**, 107 (2018).