Supplemental materials and methods

Patients and cell lines

All CRC tissue specimens, including 78 paired CRC primary tumor samples and adjacent normal tissue samples and 48 pairs of non-recurrence versus recurrence CRC tissues for qPCR analysis, 383 pairs of CRC tissue specimens for IHC staining analysis, 24 paired CRC primary tumor samples and adjacent normal tissue samples for MeRIP-qPCR assays, were collected after obtaining written informed consent and in accordance with the guidelines of Institutional Review Board and the Declaration of Helsinki (Table S1b) in Sun Yat-sen University Cancer Center (SYSUCC, Guangzhou, China). The human CRC cell lines HCT116 and HT29, and the mouse CRC cell line CT26 were purchased from Shanghai Institute of Cell Biology (Chinese Academy of Medical Sciences, Shanghai, China). Another mouse CRC cell line MC38 was purchased from iCell (Shanghai, China). These cells were characterized before use via short tandem repeat DNA fingerprinting and were negative for mycoplasma contamination.

Methionine restriction treatment

L-Methionine (Macklin, Shanghai, China) was added to methionine-deficient DMEM culture medium (Rigorous Scientific, Guangzhou, China) containing 10% dialyzed FBS (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin–streptomycin. CRC cells were seeded with DMEM overnight and re-treated with methionine-restricted culture medium or control medium for 12 h.

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For an *in vivo* methionine restriction diet, 0.86% methionine and 0.12% methionine diets were purchased from Dyets Company (Wuxi, China).

Immunoblotting and IHC

Immunoblotting and IHC were conducted as previously reported[1]. Blotted membranes were incubated overnight at 4°C with the primary antibody. Quantification of immunoblot was analyzed by ImageJ. Stained IHC sections were incubated for 2 hours at 37°C with the primary antibody, and then they were reviewed and scored independently by two expert pathologists. Next, a final score was calculated by multiplying the proportion of positively stained tumor cells (0–100%) by the staining intensity (0, 1, 2, or 3). Detailed information on the antibodies used is listed in Table S2.

RNA isolation and qPCR analysis

Total RNA was isolated using TRIzol reagent. Next, cDNA was synthesized using the Prime Script RT Master Mix Kit (Takara, Tokyo, Japan) and served as a template for real-time PCR using GoTaq qPCR Master Mix (Promega, Madison, USA) according to the manufacturer's instructions. All the data were analyzed and normalized to GAPDH data. The used primers are listed in Table S3.

Cell viability assay

CRC cells (2×10^3 cells/well) were seeded in 96-well plates overnight, and cell viability was quantified by measuring the absorbance at 490 nm on a Synergy Multi-Mode Microplate Reader (Biotek, Winooski, USA).

Cell transfection, treatment and lentiviral-based gene transduction

Small interfering RNAs (siRNAs) targeting METTL3 and METTL14 (human or mouse) were synthesized by RiboBio (Guangzhou, China). Transfection experiments were performed using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, USA). Lentiviruses for human YTHDF1 overexpression, human shYTHDF1 expression and mouse shYthdf1 expression were synthesized by OBiO Technology (Shanghai, China). Cells were infected with lentiviruses and selected using puromycin for one week. The target sequences are listed in Table S3.

m^{6}A dot blot

Total RNA was isolated with TRIzol reagent following the manufacturer's protocol. Briefly, RNA samples were denatured at 95°C for 3 min and then chilled on ice immediately. Quantified RNA samples were loaded onto an Amersham Hybond-N+ membrane (GE Healthcare, Little Chalfont, UK). After UV cross-linking, the membrane was blocked with 5% defatted skim milk in PBST buffer for 1 hour and incubated with an anti-m^{6}A antibody for 2 hours, while the loading control was stained with 0.02% methylene blue in 0.3 M sodium acetate at room temperature. After three washes with PBST, the experimental membrane was incubated with HRP-conjugated goat anti-rabbit IgG for 1 hour at room temperature and detected with Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Waltham, USA).

DNA dot blot

Total DNA was isolated with TIANamp Genomic DNA Kit (TIANGEN Biotech, Beijing, China) following the manufacturer's protocol. Briefly, DNA samples

were diluted by 0.1 M NAOH and denatured at 95°C for 10 min, and then chilled on ice immediately. The rest of the steps are similar to the m^6A dot blot assay except with an anti-5-mC antibody.

RIP-qPCR assay

The RIP assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore, Billerica, USA) according to the manufacturer's protocol. Briefly, prepared cell lysates were incubated with magnetic beads conjugated with 5 μg of specific antibodies against YTHDF1 or control normal IgG overnight at 4°C. Next, ice-cold washed RNA-protein complexes were digested with proteinase K digestion buffer. Finally, RNA was purified according to phenol-chloroform RNA extraction methods. The relative interactions between YTHDF1 and PD-L1 or VISTA transcripts were determined by qPCR. Relative enrichment was normalized to the input, and IgG was used to test the specificity of RNA-protein interactions. The used primers are listed in Table S3.

CLIP-qPCR

CLIP was performed following a previously reported protocol with some modifications[2]. HCT116 cells were seeded in four 15 cm plates and treated with 100 μM 4-thiouridine for 16 hours. Next, the cells were crosslinked with 150 mJ/cm^2 of 365 nm UVA light and scraped in 5 mL of ice-cold PBS. The cell pellets were resuspended in NP-40 lysis buffer for 15 min at 4°C and then digested with 1 U/μL of RNase T1 (Thermo Fisher Scientific) at 22°C for 12 min. Five micrograms of anti-YTHDF1 or IgG was conjugated to protein A/G magnetic beads and incubated with the digested cell lysates in RIP buffer at

4°C overnight. After 3 washes and digestion with proteinase K, the ice-cold washed RNA-protein complexes were extracted by phenol-chloroform RNA extraction. Further CLIP enrichment in every sample was calculated by qPCR with standardization to the input. The primers used are listed in Table S3.

MeRIP-qPCR

The m^6A enrichment of PD-L1 and VISTA transcripts was determined using the MeRIP-qPCR assay. First, 100 μg of total RNA was sheared to approximately 100 nt in length using an RNA fragmentation reagent (Invitrogen). Next, RNA was incubated using anti-m^6A antibody-coated or rabbit IgG-coated beads in 500 μL of RIP buffer at 4°C overnight with rotation. After 6 washes and digestion with proteinase K, the methylated mRNAs were precipitated using phenol-chloroform RNA extraction methods. One-tenth of the fragmented RNA was saved as an input control, and further m^6A enrichment in each sample was analyzed by qPCR and normalized to the input. The used primers are listed in Table S3.

Polysome profile

Polysome profiling was performed following a procedure reported previously[3]. HCT116 cells were treated with 100 μg/mL of cycloheximide (CHX) for 10 min at 37°C. After washing three times with 100 μg/mL of ice-cold CHX/PBS, the cells were gently collected from dishes using a cell scraper, lysed on ice for 10 min with lysis buffer (20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 300 mM NaCl, 100 μg/mL of CHX, 1% Triton X-100, 1 mM 1,4-dithiothreitol (DTT), 0.5% (w/v) sodium deoxycholate, 1× RNase inhibitor, and 1× EDTA-free protease inhibitor cocktail) and centrifuged at 12000 g and 4°C for 10 min. The supernatant was

loaded onto a 5–50% w/v sucrose gradient, centrifuged at 260808 g and 4°C, fractioned into 15 fractions (0.5 mL per fraction) and analyzed by Gradient Station (BioCamp, New Brunswick, Canada). Samples from each fraction were subjected to qPCR analysis of the PD-L1 or VISTA transcripts. The used primers are listed in Table S3.

m^{6}A motif prediction and an m^{6}A probe

The m^{6}A motifs of PD-L1 and VISTA transcripts were predicted using the SRAMP tool (http://www.cuilab.cn/sramp). Biotinylated PD-L1 and VISTA transcript RNA probes (approximately 20 nt) containing the corresponding m^{6}A motif were synthesized by Tsingke Biotechnology (Beijing, China), and methylated, unmethylated adenosine and adenosine-mutated probes were used.

RNA pull-down assay

The *in vitro* RNA pull-down assay was conducted using the Pierce Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific). Briefly, up to 50 pmol of biotinylated PD-L1 and VISTA RNA probes with methylated or unmethylated adenosine was mixed with 2 mg of protein extract and 50 µL of washed streptavidin beads. After incubation for 1 hour at room temperature and three washes, the streptavidin beads were boiled in SDS buffer and used for a western blotting. For the *in vivo* RNA pull-down assay, 50 pmol of biotinylated PD-L1 and VISTA RNA probes with unmethylated or mutated adenosine was transfected into HCT116 cells overnight. Next, the HCT116 cells were lysed and mixed with 50 µL of washed streptavidin beads. After incubation for 1 hour at room temperature and three washes, the streptavidin beads were boiled in

SDS buffer and used for western blotting. The RNA probe sequences are listed in Table S3.

In vitro flow cytometric analysis

CRC cells were harvested, resuspended in PBS containing Fc Block (BD Biosciences, San Jose, USA) and then incubated for 30 min with primary antibodies or isotype controls. Cell-surface staining was performed for 30 min at 4°C. For the IFN-γ stimulation assay, CRC cells were treated with 10 μg/mL of human or mouse IFN-γ (PeproTech, Rocky Hill, USA) dissolved in PBS for 6 hours. Detailed information on the antibodies used is listed in Table S2.

CE-TOF/MS-based metabolite measurement

Tissue and serum metabolites were analyzed by capillary electrophoresis-time-of-flight mass spectrometry (CE-TOF/MS)-based metabolomics on a CE instrument (Agilent Technologies, Santa Clara, USA) coupled to a time-of-flight mass spectrometer (Agilent Technologies). Serum preparation was performed as described previously[4]. Briefly, serum samples were thawed on ice, and 50 μL of serum was mixed with 450 μL of methanol containing internal standard 1 (IS1; Human Metabolome Technologies (HMT), Tsuruoka, Japan; used to standardize the metabolite intensity). Subsequently, 500 μL of chloroform was added to the above mixture and vortexed for 30 seconds, 200 μL of ultrapure water was added and vortexed for 20 seconds, and then the mixture was centrifuged at 13000 g and 4°C for 15 min. Next, 400 μL of supernatant was transferred and filtered through a 5 kDa cutoff filter (Merck Millipore) to remove proteins by centrifugation at 13000 g and 4°C for 3 hours. The filtered solution was lyophilized and stored in a −80°C freezer. Freshly resected tumor tissues

were weighed and ground in 1 mL of methanol with IS1 (grinding apparatus: Scientz-48, homogenized for 2 min at 40 Hz). The lysates were mixed with 1 mL of chloroform and 400 μL of water for 30 seconds by vortexing. After centrifugation at 16,000 g and 4°C for 15 min, 400 μL of aqueous phase was collected and then filtered through a 5 kDa cutoff filter (Merck Millipore) by centrifugation at 13000 g and 4°C for 4 hours. The filtered aqueous phase was freeze-dried in a vacuum concentrator.

Next, the dried serum and tissue samples were dissolved in ultrapure water containing 50 μM internal standard 2 (IS2; HMT; used to adjust the migration time). A fused silica capillary (50 μm i.d. × 80 cm, HMT) was used for sample separation. Detailed CE-TOF/MS methods were performed as described previously[4]. Qualitative analysis was performed based on a preanalyzed metabolite standard library (HMT). Peak extraction and identification were performed using Quantitative Analysis Software (Agilent Technologies) based on the accurate mass and corrected migration time of preanalyzed standards. Before statistical analysis, the quantity of metabolites was normalized by the peak area of the internal standard and the dry weight of the tissue sample. The serum samples were normalized to the area of the internal standard for each sample.

RIP-seq assay and data analysis

The RIP assay was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Merck Millipore). An anti-YTHDF1 antibody was applied for RIP. Both input samples and RIP samples were prepared for next-generation sequencing (NGS) by Novogene Biotech Company (Beijing, China).

NGS library preparation was performed using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, USA), quantified using a BioAnalyzer High Sensitivity DNA chip, and deep sequenced on a 19Illumina HiSeq 2500. The reads of the mRNA input (RNA-seq) and RIP (RIP-seq) sequencing libraries were aligned to the hg38 reference genome using STAR[5]. MACS/MACS2 was used to call peaks based on the RNA-seq and RIP-seq bam files and Homer[6]. The RNA-seq reads and RIP-seq reads were quantified at the gene level using the RSEM method[7] and normalized to fragments per kilobase of exon model per million mapped fragments (FPKM).

RNA-seq assay and data analysis

Fresh tumors from mice fed the CD or MRD were harvested on day 10. Total RNA was isolated with TRIzol reagent and then sent to Novogene Biotech Company for NGS. The raw reads of RNA-seq data were aligned to the mm10/hg38 reference gene using STAR and assigned to genes by RSEM[5, 7]. Gene expression is represented as FPKM. TCGA RNA-seq data represented as FPKM were downloaded from UCSC Xena (https://xenabrowser.net/datapages/). Differentially expressed genes were called using the edgeR package[8], and genes with an expression level lower than 1 FPKM in more than half of the samples were filtered from the differential expression analysis. GSEA of hallmark gene sets and the effector $CD8^+$ T cell signature in immunologic signature gene sets (c7) was conducted by employing GSEA software for Windows (http://www.gsea-msigdb.org/gsea/downloads.jsp) with ranked gene lists (by fold change). Leukocyte infiltration was quantified using CIBERSORT. The cytotoxic lymphocyte score was calculated using the

single-sample GSEA (ssGSEA) method with the GSVA package from the expression levels of CD8A, CD8B, PRF1, GZMA and GZMB[9]. The TCR shannon entropy in the TCGA CRC dataset was collected from Thorsson *et al*.[10].

Experimental animals

BALB/c, C57BL/6J and NOG mice (6 weeks) were purchased from Beijing Vital River Laboratory (Beijing, China). *Rag2*$^{-/-}$ mice (4-5 weeks) were purchased from Cavens (Jiangsu, China). Six-week-old intestine-specific *Ythdf1* knockout (*Ythdf1*^{iKO}) mice in the C57BL/6J background were generated by crossing mice carrying a *Ythdf1* exon 3–4 floxed allele with *Villin-Cre-ERT2* mice purchased from Gempharmatech (Jiangsu, China). All mice have been housed in a temperature-controlled room with pathogen-free conditions and a 12 h light–dark cycle.

Subcutaneous tumor model

For the methionine restriction mouse model, 7-week-old female BALB/c, C57BL/6J, and 5-6-week-old *Rag2*$^{-/-}$ mice were used. Control or YTHDF1-knockdown CT26 or MC38 cells (1×10^6 cells suspended in 100 μL of PBS) were subcutaneously injected into mice of the corresponding strain, which were fed a 0.86% or 0.12% methionine diet (Dyets) and treated with IgG or αPD-1 intraperitoneally (10 mg/kg, a gift from Suzhou Junmeng Biosciences, China) beginning on day 6. Tumor size was measured every 3 days using a caliper, and the tumor volume was determined using the following standard formula: volume (mm^3) =0.5* (longer diameter × shorter diameter^2). The tumor volume of up to 2000 mm^3 is considered as the human endpoints. At the end stage,

tumor weight was measured. Tumors and spleens were then collected on day 12 and analyzed by IHC or flow cytometry.

AOM/DSS-induced mouse CRC model

Spontaneous colon tumors were induced in mice as described previously with slight modifications[11]. Briefly, six-week-old mice were intraperitoneally injected with 10 mg/kg of AOM (MP Biomedicals, Irvine, USA) on day 1. After one week, the mice were treated with 1.5% DSS (MP Biomedicals) in the drinking water for 7 days, followed by 14 days of treatment with regular water. This cycle was repeated twice. The mice were fed the CD or MRD for 1 month after the last cycle. For inducible intestinal *Ythdf1* deletion after tumor development, 55 days after AOM, the mice were intragastrically administered tamoxifen (15 mg/kg, Sigma-Aldrich, MO, USA) for 5 days, followed by tamoxifen injection every 5 days until analysis. The animals were sacrificed on day 100 for general inspection and histological analysis. Tumors on the intestine were collected and evaluated to determine the tumor volume using the following formula: volume (mm^3) =0.5* (longer diameter × shorter $diameter^2$). The tumors were then sectioned for H&E staining or IHC.

Human PBMC (HuPBMC) reconstitution mouse tumor model

The huPBMC reconstitution mouse tumor model was induced as described previously with slight modifications[12]. Six-week-old female NOG mice were intravenously injected with 1×10^7 huPBMCs (Xinjin Biotechnology Co., Ltd., Guangzhou, China). Three days after reconstitution of the immune system, the mice were subcutaneously injected with 1×10^6 human CRC cells in the flank region. After three days, the mice were fed a 0.86% or 0.12% methionine diet

(Dyets) randomly. The tumor volume was regularly measured, and the tumor weight was examined when the mice were sacrificed after 3–4 weeks. The tumor volume was estimated using the following formula: volume (mm^3) =0.5* (longer diameter × shorter $diameter^2$). Spleens were collected on day 12 for flow cytometric analysis to detect circulating immune cell percentages. Tumors were collected for IHC to detect $CD8^+$ T cells.

Adoptive transfer model

The adoptive transfer model was induced as described previously with slight modifications[13]. *Rag2*$^{-/-}$ mice were subcutaneously injected with 1×10^6 MC38-OVA cells, which were fed with a 0.86% or 0.12% methionine diet (Dyets) on day 10. After 12 days, tumor-bearing mice were intravenously injected with 1×10^6 OT-1 T cells. Tumors were then collected on day 12 and analyzed by flow cytometry.

Mouse tumor sample dissociation

Fresh isolated tumor samples were collected immediately after euthanasia, minced into small pieces using a scalpel, placed in a 5 mL Eppendorf tube containing the corresponding enzyme D, enzyme R, and enzyme A according to a mouse tumor dissociation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and incubated at 37°C with rotation for 40 min. The samples were passed through 70 μm and 40 μm cell strainers and washed twice with MACS buffer. After the supernatant was removed, the pelleted cells were softly suspended in 40% Percoll (Cytiva, Shanghai, China) and purified via gradient centrifugation. The cell pellets were finally resuspended in sorting buffer (PBS

supplemented with 2% FBS) for further flow cytometry or sorting GFP^+ tumor cells by fluorescence-activated cell sorting.

Mouse tumor draining lymph node dissociation

Tumor draining lymph nodes were crushed in 6 cm dishes, passed through 40 μm cell strainers and washed twice with MACS buffer. The cell pellets were finally resuspended in sorting buffer (PBS supplemented with 2% FBS) and then subjected to flow cytometry.

Mouse spleen samples dissociation

Spleens were crushed in 6 cm dishes, passed through 40 μm cell strainers and washed twice with MACS buffer. The pelleted cells were resuspended in red blood cell lysis buffer (Sigma-Aldrich) and incubated for 5 min. The cell pellets were finally resuspended in sorting buffer (PBS supplemented with 2% FBS) and then subjected to flow cytometry.

Mouse blood dissociation

Whole blood was sampled via the eyes in a tube with heparin sodium, then lysed with lysis buffer (Sigma-Aldrich) with gentle shaking for 15-20 min at room temperature and stopped in PBS. The samples were passed through 40 μm cell strainers and washed with MACS buffer. The cell pellets were finally resuspended in sorting buffer (PBS supplemented with 2% FBS) for further flow cytometry.

***In vivo* flow cytometric analysis**

Single cells from mouse tumor tissues, spleens, tumor draining lymph nodes, blood were prepared as described above, which were stained with Zombie for

15 min at room temperature, washed, and stained with anti-mouse CD45, CD3 and CD8 antibodies.

Single cells from fresh mouse tumor tissues or spleens were treated with Cell Stimulation Cocktail (eBioscience, San Diego, USA) at 37°C for 4 hours and subsequently stained with Zombie for 15 min at room temperature. Washed mouse tumor cells or mouse splenocytes were stained with specific antibodies for 30 min at 4°C; the antibodies included anti-mouse CD45, CD3 and CD8 antibodies. The cells were then washed and fixed and permeabilized with fixation buffer (BD Biosciences) or Foxp3 / Transcription Factor Staining Buffer (Invitrogen) overnight at 4°C. Next, the fixed cells were washed twice with 1× intracellular staining perm and wash buffer (BD Biosciences) and incubated with anti-mouse IFN-γ and/or GZMB and/or Ki67 antibodies for 30 min at 4°C.

The washed splenocytes from huPBMC-reconstituted mice were stained with anti-human CD45, CD3 and CD8 antibodies. The cells were then washed, permeabilized and stained with anti-human IFN-γ. To optimize fluorescence compensation settings for multicolor flow cytometric analysis, compensation beads (eBioscience) were also incubated with the above antibodies. The cells were evaluated by flow cytometry, and the data were analyzed using CytExpert software. Detailed information on the antibodies used is listed in Table S2.

CD8⁺ T cell cytotoxicity

MC38-OVA cells were seeded into the 96-well plates with DMEM complete medium overnight. OT-1 CD8⁺ T cells were isolated from the spleens of OT-1 mice with 10 ng/mL IL-2, 10 nM ova and 100 mM β-mercaptoethanol. Purified OT-1 CD8⁺ T cells were co-cultured with MC38-OVA cells at a ratio of 1:1 with methionine-restricted or control culture medium. After 12 h, the CD8⁺ T cell cytotoxicity efficiency was measured by quantifying the release of endogenous lactate dehydrogenase (LDH) with a LDH Assay Kit (Cytotoxicity) (Abcam, MA, USA).

Statistics

The data are presented as means ± standard deviation (SD). Student's *t* test was used to test the significance of differences between two groups. One-way analysis of variance (ANOVA) and two-way ANOVA was used for multigroup comparisons. Statistical analyses for survival were performed using the log-rank test. The data were analyzed using GraphPad Prism Software. The level of significance was set at $P<0.05$ (*) or $P<0.01$ (**).

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