Supplementary Materials and Methods

Antibodies and reagents. The antibodies used for this study were as follows: rabbit anti-RIG-I (Cell Signaling Technology, #3743, USA), mouse anti-PD-L1 (Proteintech, #66248-1-Ig, CN), rabbit anti-PD-L1 (Proteintech, #28076-1-AP, CN), rabbit anti-SPOP (Proteintech, #16750-1-AP, CN), rabbit anti-CUL1 (Proteintech, #12895-1-AP, CN), rabbit anti-Ub (Proteintech, #10201-2-AP, CN), rabbit anti-P27 (Proteintech, #25614-1-AP, CN), rabbit anti-P53 (Cell Signaling Technology, #9282S, USA), rabbit anti-VISTA (Proteintech, #24849-1-AP, CN), rabbit anti-PD-L2 (Proteintech, #18251-1-AP, CN), rabbit anti-B7H3 (Zenbio, #R381871, CN), rabbit anti-GAPDH (Servicebio, #GB15004, CN), rabbit anti-HA (Proteintech, #51064-2-AP, CN), rabbit anti-Myc (Proteintech, #16286-1-AP, CN), rabbit anti-His (Proteintech, #10001-0-AP, CN), rabbit anti-FLAG (Proteintech, #80010-1-R, CN), anti-rabbit IgG-HRP (Servicebio #G1214, CN) and anti-mouse IgG-HRP (Servicebio #G1214, CN), APC anti-mouse CD3a (BD, #553066, USA), APC/Cyanine7 anti-mouse CD3 (Biolegend, #100222, USA), PerCP-Cy™5.5 anti-mouse CD8a (BD, #551162, USA), APC anti-mouse CD8a (Biolegend, #100712, USA), FITC anti-mouse CD274/PD-L1 (Elabscience, #E-AB-F1132C, CN), APC anti-human CD274/PD-L1 (Elabscience, #E-AB-F1133E, CN). Cycloheximide (CHX) (concentration, 200μg/mL) and MG132 (concentration, 20μM) were purchased from MedChemExpress (#HY-12320, #HY-13259, USA).

Plasmids and transfection. HA-RIG-I, His-Ub, FLAG-PD-L1, Myc-SPOP, and Myc-CUL1 were cloned into pcDNA3.1, and pLVX-U6-DDX58-shRNA-EGFP (Sequence shRNA#1: TAGTAATGCTGGTGTAATT; shRNA#2: CCGGCACAGAAGTGTATAT) for human and mouse plasmids was obtained from the MiaoLing Plasmid Platform (Wuhan, CN). The psPAX2 and pMD2.G plasmids were obtained from our laboratory. The mouse siRNA sequences to Ddx58 were obtained from Co., Ltd. (Sequence siRNA#1: GCAGGTTACTGTGGACTTT; siRNA#2: GCACATCATTGAAGACAAT). px459-Ddx58-mouse (Sequence sgRNA1: CGAGCCAGGAACTCATGTAG; sgRNA2: GCCAGGAACTCATGTAGCTG) was purchased from Tsingke Biotechnology (Beijing, CN). Recombinant Ddx58-mouse (GV707) and negative control lentiviruses were purchased from the Shanghai GeneChem Company (Shanghai, CN). HT29 cells were transiently transfected with the human HA-RIG-I plasmid using Lipofectamine 2000. A CRISPR/Cas9

approach was used for RIG-I knockout in CT26 cells using Lipofectamine 2000 transfection reagent (Invitrogen, #11668019, USA). After incubation for 48 h, the medium was replaced with fresh medium containing 9 μg/mL puromycin (BioFroxx, #58-58-2, GER) for one week for selection. Nontargeting sgRNAs were added as a control. RIG-I-knockdown HT29 cells were constructed as follows: lentivirus was produced by the cotransfection of 293T cells with the pMD2.G, psPAX2 and PLVX-shDDX58 expression vectors and harvested virus after 48 h and 72 h. HT29 cells were infected with the virus and 6 ng/mL polybrene (Beyotime, #C0351, CN) for 48 h. Control cell lines were generated using the same protocol with viruses containing the empty vector.

RT–PCR and real-time PCR. Total RNA was extracted from cells or tissues with TRIzol reagent (Invitrogen). Reverse transcription (RT) was conducted with the ReverTra Ace® qPCR RT Master Mix (TOYOBO). Real-time PCR was performed using UltraSYBR Mixture (Cwbio) on a Roche LightCycler 96 (Roche). The primers used are shown in **Supplementary Table S2**.

Animal experiments. WT or RIG-I-KO CT26 or MC38 cells (2×10^6) in 100 μl of PBS mixed with 100 μl of Matrigel (Corning, USA) were subcutaneously injected into BALB/c, C57BL/6 and nude mice (n=8). The tumor volume was calculated as follows: tumor volume = (length * width^2)/2. The same CT26 cells were injected into the tail vein of BALB/c mice to generate a lung metastasis model. Vector or RIG-I-overexpressing CT26 cells (5×10^5) in 50μl of PBS were directly inoculated into the subserous membrane of the cecum of BALB/c mice to generate an orthotopic colon cancer model. Mice in the orthotopic model were randomly divided into four groups and intraperitoneally injected with anti-PD1 mouse mAb (Clone: RMP1-14, #BE0146, BioXCell, USA) or IgG isotype control (Clone: 2A3, #BE0089, BioXCell, USA) at 200μg per mouse 3 times every week for 2 weeks.

Cell proliferation assay. Cell growth was measured using the Cell Counting Kit-8 (CCK8) proliferation assay. Briefly, pretreated HT29 or CT26 cells (1×10^3 cells/well) were seeded in 96-well plates for 24 h. At a specific time (0-96 h), 10 μl of CCK8 solution was added to each well and incubated for 2 h before the absorbance at 450 nm was measured.

Enzyme-linked immunosorbent assay (ELISA). IFN-α concentrations (pg/mL) in the cell culture supernatant were measured by ELISA (Heng-yuan Biotechnology Co., Shanghai, China) following the manufacturer's instructions. Briefly, samples (50 μL/well) or prepared standards were added to the wells and incubated at room temperature for 1 h, after which the samples were washed and treated with streptavidin-HRP for 30 min. Finally, the samples were incubated with a TMB substrate solution, and

the absorbance at 450 nm was measured with a microplate spectrophotometer.

Cell and membrane PD-L1 detection. HT29 and CT26 cells were fixed and permeated with 100 μl of 90% MeOH for 15 min, blocked with 3% BSA for 30 min, incubated with anti-human PD-L1 antibody for 60 min at 4°C, washed and incubated with FITC-conjugated secondary antibody for 60 min at 4°C in the dark. Alternatively, cells were directly incubated with FITC- or APC-conjugated PD-L1 antibody for surface staining. The cells were then analyzed by fluorescence-assisted cell sorting (FACS) (BD Biosciences).

***In Vitro* T-cell-killing assay.** $CD8^+$ OT-1 T cells isolated from the spleens of OT-1 mice using $CD8^+$ MACS beads (Miltenyi) according to the manufacturer's instructions were cultured in and stimulated with RPMI 1640 medium containing 5% serum, 55μM β-mercaptoethanol (#PB180633, Pricella, CN), 1000 U/ml IL-2 (#PCK025, Pricella, CN), and 2 μg/ml OVA peptide (#RP10611, GenScript, CN) for 48h. MC38-OVA cells were transfected with the RIG-I plasmid or siRNA, and stimulated OT-1 cells were subsequently cocultured at a 2:1 or 5:1 target:effector ratio for 48 h. An Annexin V-FITC/PI apoptosis detection kit (APPLYGEN, CN) was used to determine the apoptosis ratio.

Flow cytometry analysis. Tumor tissues were dissected, minced, and digested with collagenase I (Biosharp, BS165, CN) and 1 mg/mL DNase I (BioFroxx, 1121MG010, GER) in RPMI 640 medium for 40 min at 37℃. For PBMCs, peripheral blood was taken from the mouse orbit, diluted 1:1 with RPMI 1640 medium, slowly added to Ficoll, and centrifuged at 500 × g for 30 min. The lymphocyte layer was collected. After washing and a second filtration step, the single-cell suspension was labeled with fluorescently tagged antibodies for surface staining. After surface staining, intracellular cytokine staining was performed with a fixation/permeabilization kit (BD Biosciences, USA), and the cells were incubated with specific fluorescently tagged antibodies. The cells were isolated by flow cytometry (Beckman Coulter, USA), and the results were analyzed by FlowJo software.

Immunohistochemical (IHC) and immunofluorescence (IF) analysis. Paraffin-embedded tumor sections were dewaxed, rehydrated, antigen retried, and blocked in 5% BSA for approximately 40 minutes. Then, the cells were incubated with primary antibodies (RIG-I, PD-L1, CD3, CD8) at 4°C overnight. Next, the sections were incubated with anti-rabbit secondary antibody labeled with HRP at room temperature for 2 hours. To demonstrate the binding between RIG-I and PD-L1, cells were washed with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-X, and blocked in 1% BSA. Then, the cells were incubated with RIG-I and PD-L1 antibodies for 1 hour, washed with

PBS, and incubated with FITC-conjugated anti-mouse secondary antibody (Servicebio, GB22301, CN) or CY3-conjugated anti-rabbit secondary antibody (Servicebio GB21303, CN) for 45 minutes. Finally, nuclei were stained with DAPI (Servicebio GDP1024, CN). Cell membrane staining was performed using the Cell Plasma Membrane Staining Kit with DiI (Red Fluorescence, Beyoime, #C1991S, CN) according to the instructions. Fluorescence images were observed under a confocal microscope (Leica-LCS-SP8-STED, Germany) or a fluorescence microscope (Olympus, Japan) and analyzed by ImageJ version 2.1.0.

Analysis of interacting protein sequences by LC–MS/MS. Immunoprecipitation of endogenous human PD-L1 from HT29 cells was performed with an anti-PDL1 antibody or control antibody using lysis buffer. Immunocomplexes were pulled down with protein A/G-Sepharose beads. One-eighth of each immunoprecipitant was boiled in loading buffer and resolved by SDS–PAGE. Following Coomassie brilliant blue staining, the rest of the beads were sent for LC–MS/MS analysis by SpecAlly (Wuhan, CN). The search results were filtered with a 1% FDR at both the protein and peptide levels.