Deletion of *CUL4B* in gut epithelium promotes *Apc*$^{Min/+}$ adenoma formation by impacting the microenvironment

Beibei Guo[1,2], Yawen Zheng[2,5], Yujia Fan[1,2], Yang Yang[1,2], Yuxing Wang[2], Liping Qin[2], Yachun An[1,2], Xiaoran Xu[1,2], Xiyu Zhang[2], Gongping Sun[3], Hao Dou[2], Changshun Shao[4], Yaoqin Gong[2], Baichun Jiang[2], Huili Hu[1,2]

[1] The Key Laboratory of Experimental Teratology, Ministry of Education, Department of Systems Biomedicine, School of Basic Medical Sciences, Shandong University, Jinan, China

[2] The Key Laboratory of Experimental Teratology, Ministry of Education, Department of Medical Genetics, School of Basic Medical Sciences, Shandong University, Jinan, China

[3] The Key Laboratory of Experimental Teratology, Ministry of Education and Department of Histoembryology, Shandong University Cheeloo Medical College, Shandong University School of Medicine, Jinan, China.

[4] The First Affiliated Hospital of Soochow University and State Key Laboratory of Radiation Medicine and Protection, Institutes for Translational Medicine, Soochow University, Suzhou, China.

[5] Department of Obstetrics & Gynecology, Qilu Hospital of Shandong University, Jinan, Shandong, PR China

Correspondence:

Huili Hu, School of Basic Medical Sciences, Shandong University, 250012 Jinan, China. Phone: +86-531-88382236; E-mail: huhuili@sdu.edu.cn

Baichun Jiang, School of Basic Medical Sciences, Shandong University, 250012 Jinan, China. Phone: +86-531-88382043; E-mail: jiangbaichun@sdu.edu.cn

SUPPLEMENTARY MATERIALS AND METHODS

LOH analysis

Adenoma tissue were excised carefully, and DNA was extracted. LOH at *Apc* locus was determined by sequencing the production of PCR with mismatched pairs of primers. Primers used for sequencing were listed in the Table S2.

Cell lines culture and viral infection

Cultured HCT116 and CT26 were purchased from cell bank of the Chinese Academy of Sciences and GeneCopoeia (Rockville, MD, USA) respectively. HCT116 were cultured in McCoy's 5A medium (Sigma-Aldrich, M4892) supplemented with 20% fetal bovine serum (AusGeneX, FBSSA500-S), penicillin (100 mg/ml, BBI, A600135) and streptomycin (100mg/ml, BBI, A100382). CT26 were cultured in Roswell Park Memorial Institute RPMI 1640 medium (Gibco, C11875500) supplemented with 10% fetal bovine serum (Gibco, 10099-141C), penicillin (100 mg/ml) and streptomycin (100 mg/ml). All cell lines were identified and were regularly tested for Myco-Blue Mycoplasma Detector (Vazyme, D101-02).

Cul4b knockdown (shCUL4B) and the control cells (shNC) were generated by transfecting with virus (the ORF of human CUL4B was cloned into the lentiviral expression vector pLKO.1-EGFP-Puro by Dr. Yang Yang) after 24 h plated in 6-mm dishes, and changed into culture medium after 24 h post transfection, and screened by puromycin (Gibco, A1113803). For siRNA experiments, three independent siRNA sequences were tested and the one with the highest efficiency (CAGGCTCTATCGGGTATTT) was used with LipofectamineTM 3000 (Invitrogen, L3000001) according to the manufacturer's instructions.

RNA-Seq and RT-qPCR

RNA isolation of organoids was performed with RNeasy Mini Kit (QIAGEN, 74104) following the manufacturer's instructions. Other samples, including tissues and 2D cell lines, were lysed in Trizol (Invitrogen, 15596018). For RNA sequencing, the samples were processed using Illumina nova 6000 platform (Novogene, Beijing, China) after quality evaluation and sequencing data were generated with 150-bp paired-end for each sample. RNA was reversely transcribed with Recombinant M-MuLV Reverse Transcriptase, RNase H Minus (Thermo, ep0442). qPCR analysis was performed with SYBR Green Mixture

(Roche, 4729692001) in qTOWER3G. The primers for qPCR were designed using Primer3 (RRID: SCR_003139) and NCBI-BLAST listed in Table S3. Gene expression was normalized to the referee *Gapdh* and to control.

Western Blot

Total proteins were extracted with Trizol or protein lysis following the manufacturer's instructions. 40 μg of protein were separated on a 10% SDS-PAGE and transferred onto PVDF membrane. The membranes were blocked with 5% fat-free milk in tris buffered saline containing 0.1% Tween-20 (BBI, 9005-64-51) for 1 h at room temperature and incubated with primary antibodies (anti-CUL4B: Sigma-Aldrich, Cat# C9995; anti-CUL4A: Boster, A01579-1; anti-G-CSF: Abcam, ab181053; anti-GAPDH: Cell Signaling Technology, Cat# 5174) overnight at 4°C. The membranes were washed in TBST and incubated with HRP-labeled secondary antibody (anti-rabbit: Jackson ImmunoResearch, 111-005-003; anti-mouse: Jackson ImmunoResearch, 115-005-003) for 1 h at room temperature. After incubation, membranes were washed and proteins were detected using a SuperSignal Chemiluminescence kit (Thermo, 34580).

Single-cell assay

Organoids were removed from Matrigel and dissociated into single cells using Trypsin-EDTA for 5-15 min at 37°C. After washing and counting, cells were resuspended in Matrigel and seeded into 24-well plate. After 4 days culture, organoids formation and size were recorded using BioTek CYTATION5 Image Reader and Invitrogen FL image platform, respectively.

Transwell migration assay

MDSCs were placed in the upper Transwell with $5x10^4$ per well after isolating MDSCs according to the above method, and the 600 μl conditioned medium of 10^5 organoids for 3 days were added in the lower Transwell. The cells were counted in the lower Transwell for 3 h.

ELISA assays

The supernatant of cultured organoids was collected, and residual cells were removed by centrifugation. G-CSF in supernatant was determined by mouse G-CSF ELISA kit (4A Biotech, CME0030) according to the manufacturer's instructions. The absorbance of the samples was measured at 450 nm using a

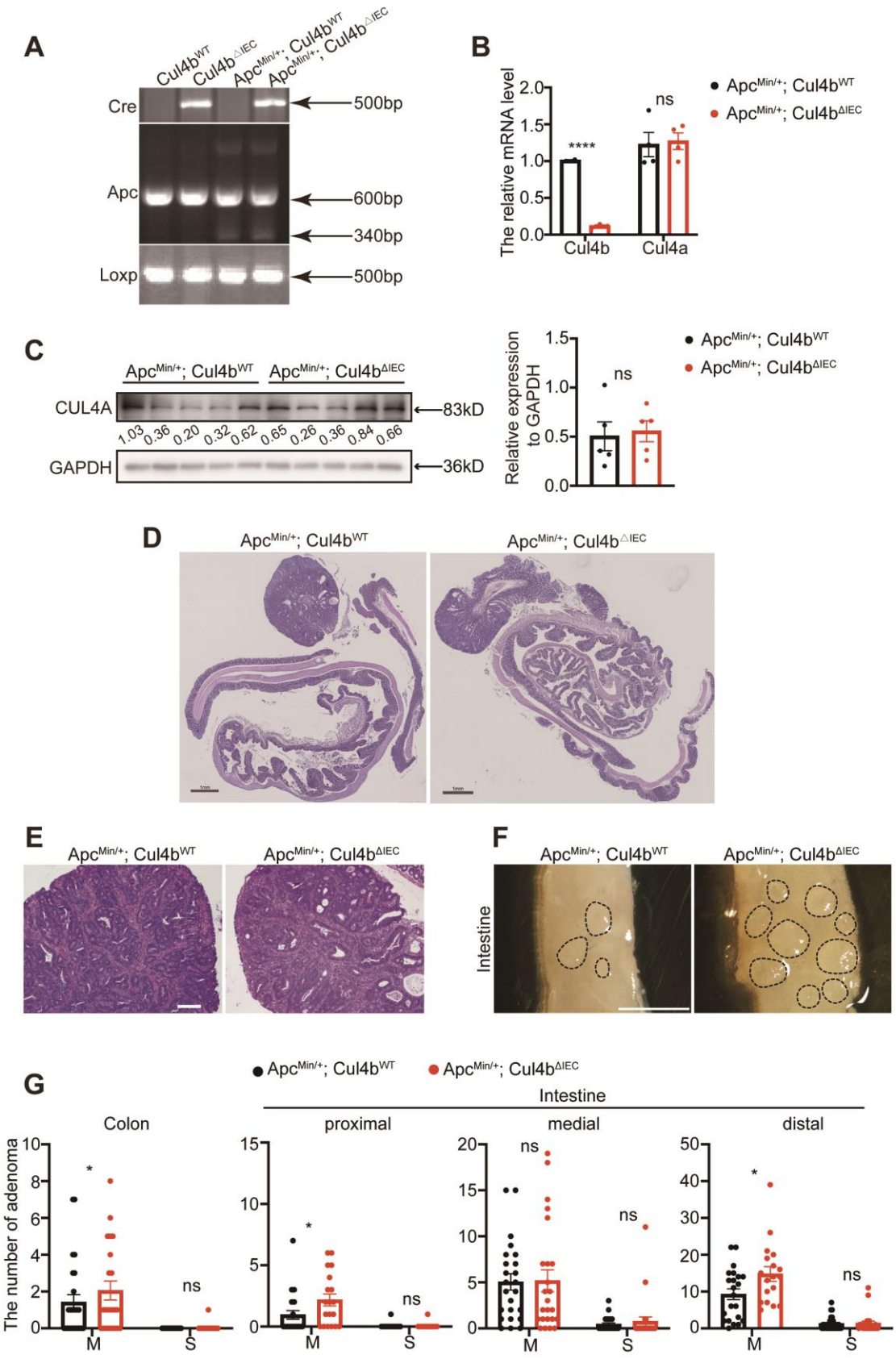
microplate spectrophotometer. A standard curve was generated and obtained as the control.

Database analysis

The Spearman correlation analysis of CUL4B and CSF3 in COAD, LUAD and PRAD from TCGA and the survival analysis were performed using TIMER2.0 (http://timer.comp-genomics.org). The enrichment analysis of genes related to MDSCs in GDS2974 was executed using Bioinformatics (http://www.bioinformatics.com.cn).

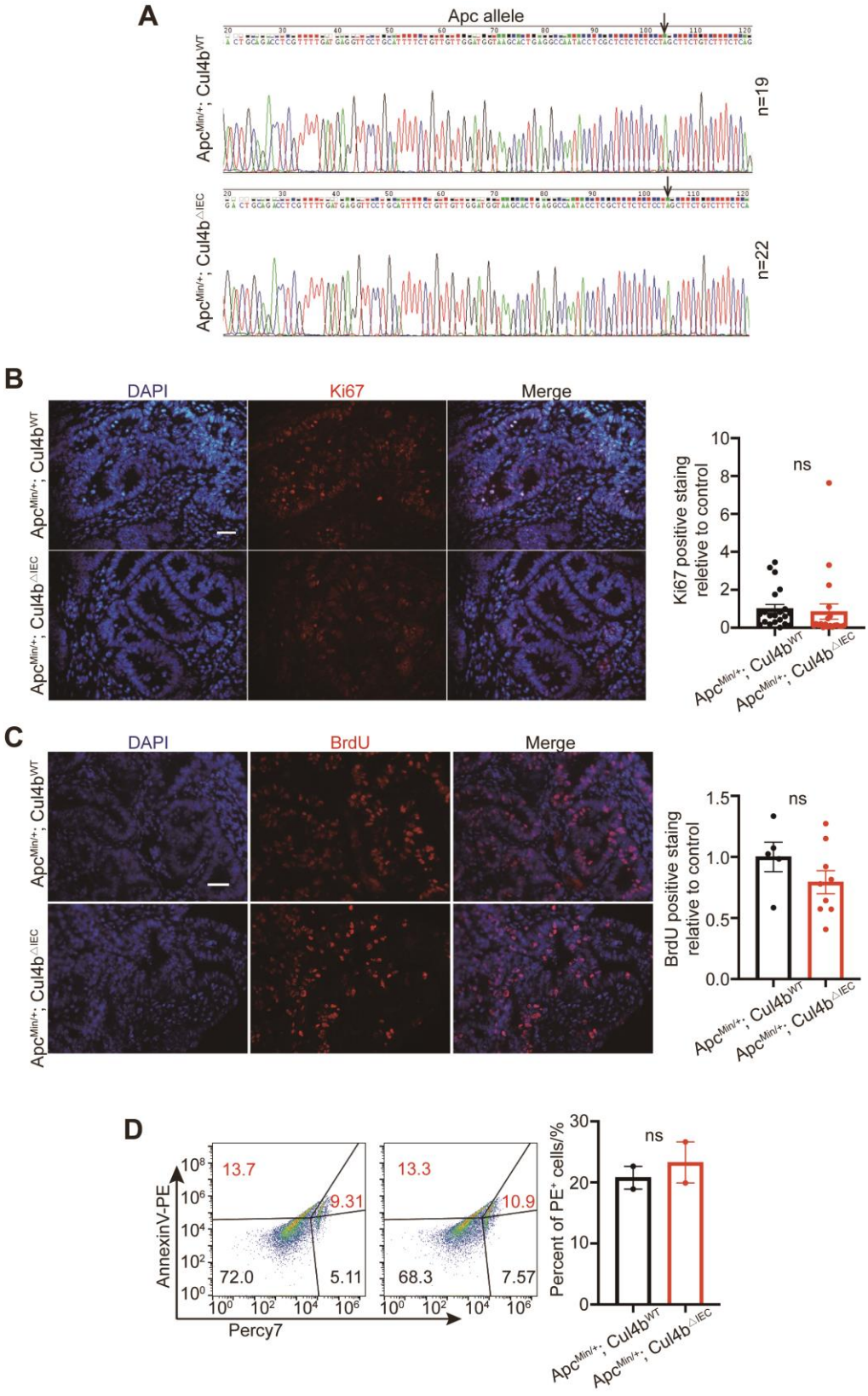
SUPPLEMENTARY FIGURES

Figure S1. Generation and characterization of *Apc$^{Min/+}$*; *Cul4b$^{\Delta IEC}$* mice.



(A) Genotyping of *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ and control *Apc*$^{Min/+}$; *Cul4b*^{WT} mice. **(B-C)** No difference was found between the adenoma of *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ mice and control *Apc*$^{Min/+}$; *Cul4b*^{WT} mice in the mRNA and protein expression of CUL4A. p-values ****<0.0001, ns, not significant. **(D)** The representative image of the panoramic scan of the adenomas from *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ and control *Apc*$^{Min/+}$; *Cul4b*^{WT} mice. Scale Bar=1 mm. **(E)** The representative images of the sections stained by hematoxylin and eosin (H&E) of adenomas from *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ and *Apc*$^{Min/+}$; *Cul4b*^{WT} mice. Scale Bar=100 μm. **(F)** The representative images of the adenomas from *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ and *Apc*$^{Min/+}$; *Cul4b*^{WT} mice. Scale Bar=5 mm. Dotted lines indicate adenomas in intestine. **(G)** Total number of the medium (1-2 mm, M) and small (<1 mm, S) adenomas in proximal, medial and distal segments of small intestine and colon from *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ and *Apc*$^{Min/+}$; *Cul4b*^{WT} mice (n=9). All mice were 5-month-old. p-values *<0.05, ns, not significant.

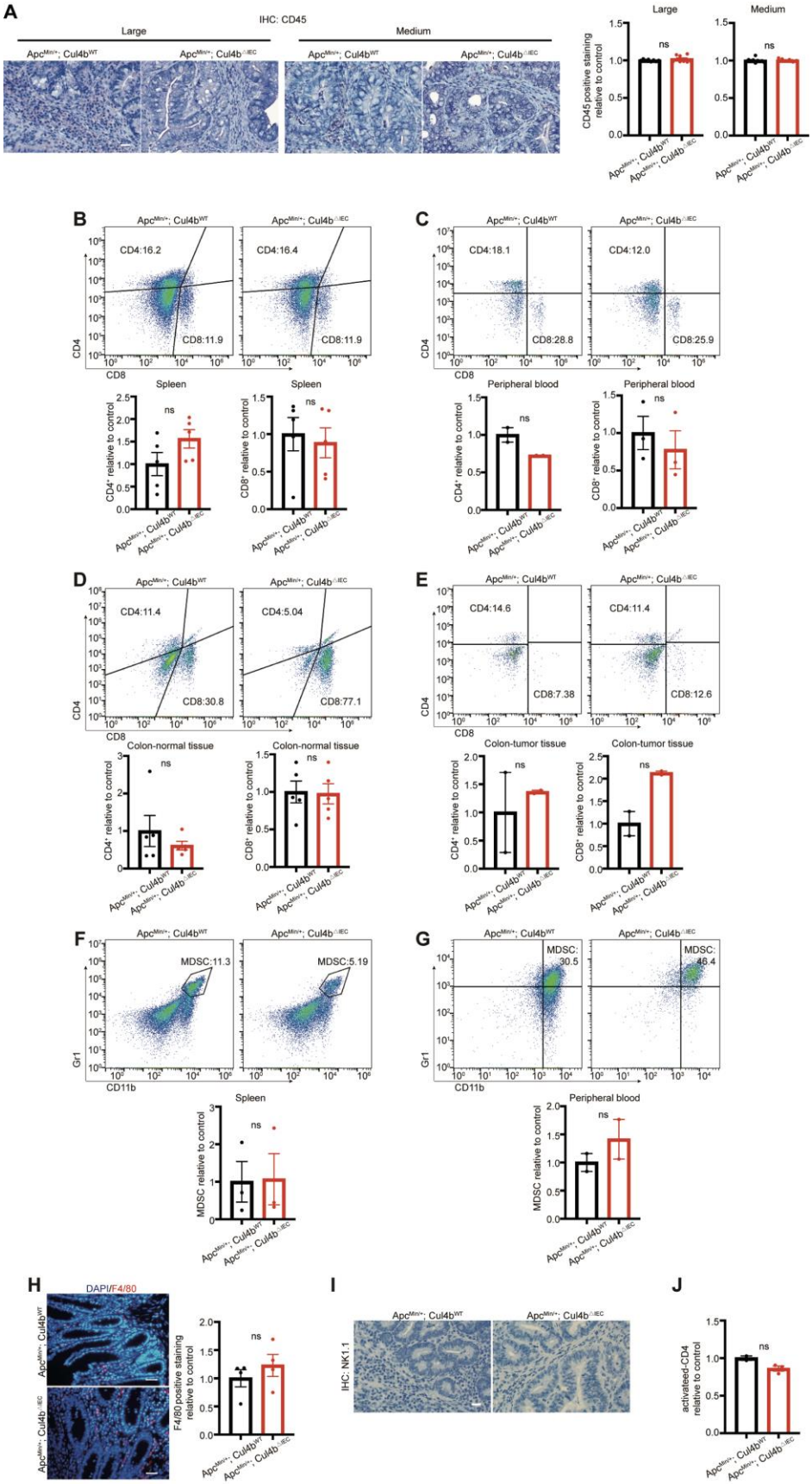
Figure S2. CUL4B ablation had no effect on adenoma cell proliferation and apoptosis in vivo.



(A) LOH analysis by DNA sequencing of mutant *Apc* gene in adenoma isolated from *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ (n=22) and control *Apc*$^{Min/+}$; *Cul4b*^{WT} mice (n=19). **(B,**

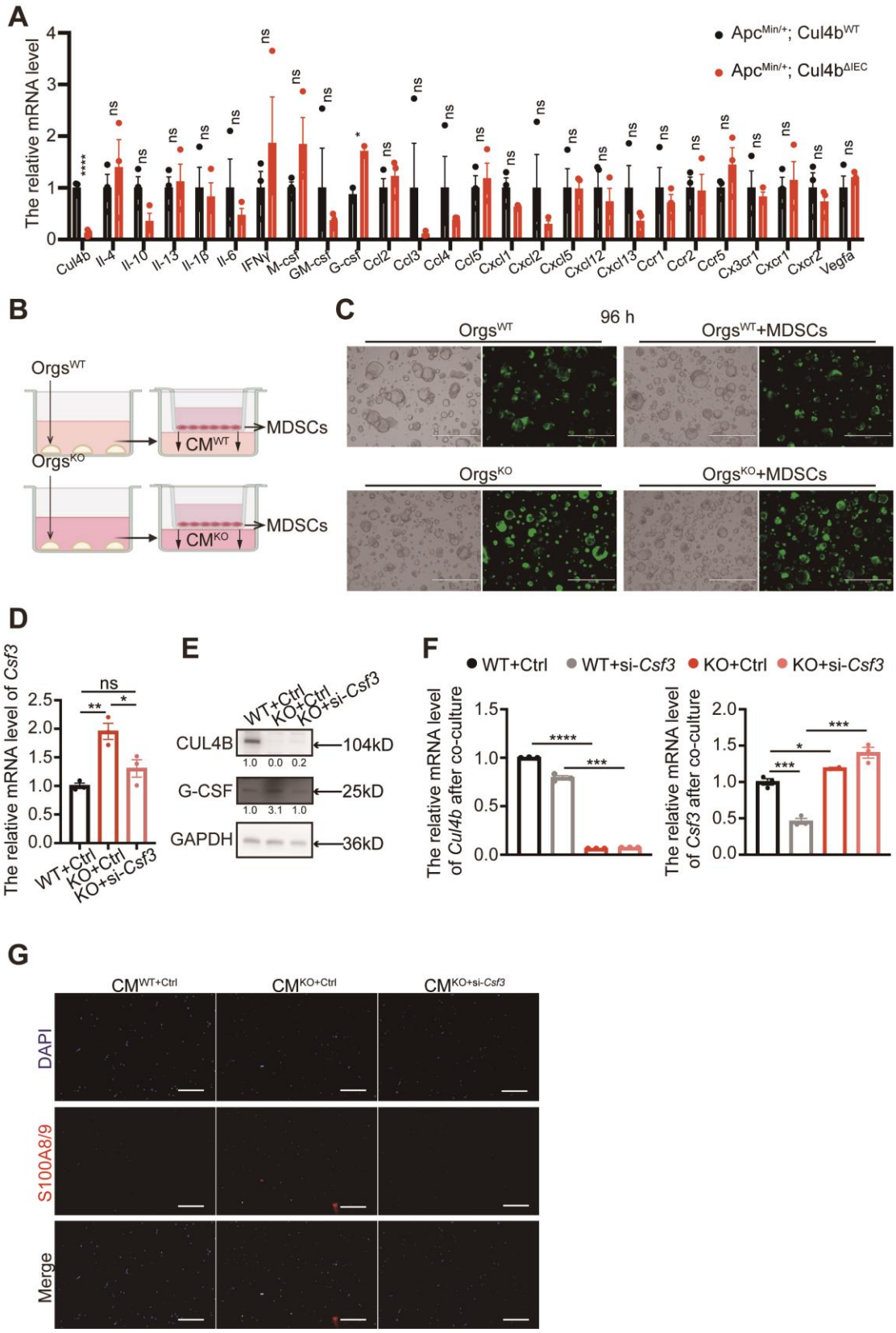
C) Immunofluorescent staining and analysis of cell proliferation markers of Ki67 (**B**) and BrdU (**C**) in *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ and *Apc*$^{Min/+}$; *Cul4b*^{WT} mice. Blue, DAPI. Scale Bar=20 μm. ns, not significant. **(D)** The flow cytometry analysis of Annexin V staining in adenomas derived from *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ and control *Apc*$^{Min/+}$; *Cul4b*^{WT} mice. ns, not significant.

Figure S3. No significance was found in most immune cell populations in *Apc$^{Min/+}$* mice after CUL4B ablation.



(A) The representative images and statistical analysis of immunochemical staining of $CD45^+$ immune cells in medium and large adenoma from $Apc^{Min/+}$; $Cul4b^{\Delta IEC}$ and $Apc^{Min/+}$; $Cul4b^{WT}$ mice. Three independent experiments were performed. Scale Bar=20 μm. ns, not significant. **(B-E)** The flow cytometry analysis of $CD4^+$/$CD8^+$ T cells in spleen (**B**), peripheral blood (**C**), adjacent normal tissues (**D**) and adenomas (**E**) from $Apc^{Min/+}$; $Cul4b^{\Delta IEC}$ and $Apc^{Min/+}$; $Cul4b^{WT}$ mice. ns, not significant. **(F, G)** The flow cytometry analysis of $CD11b^+Gr\text{-}1^+$ MDSCs in spleen (**F**), and peripheral blood (**G**) of $Apc^{Min/+}$; $Cul4b^{\Delta IEC}$ and $Apc^{Min/+}$; $Cul4b^{WT}$ mice. ns, not significant. **(H)** The representative images and statistical analysis of immunofluorescent staining of F4/80 in adjacent tissue sections from $Apc^{Min/+}$; $Cul4b^{\Delta IEC}$ and $Apc^{Min/+}$; $Cul4b^{WT}$ mice. Scale Bar=20 μm. ns, not significant. **(I)** The representative images of immunochemical staining of NK1.1 in adenoma section from $Apc^{Min/+}$; $Cul4b^{\Delta IEC}$ and $Apc^{Min/+}$; $Cul4b^{WT}$ mice. Scale Bar=20 μm. **(J)** The flow cytometry analysis of activated $CD4^+$ T cells within adenoma from $Apc^{Min/+}$; $Cul4b^{\Delta IEC}$ and $Apc^{Min/+}$; $Cul4b^{WT}$ mice. ns, not significant.

Figure S4. CUL4B functioned on MDSCs by G-CSF.



(A) The mRNA level identified by quantitative real-time PCR of the main genes involved in recruiting MDSCs of colon adenomas from *Apc*$^{Min/+}$; *Cul4b*$^{\Delta IEC}$ (n=3)

and *Apc*$^{Min/+}$; *Cul4b*^{WT} (n=3) mice. p-values *<0.05, ****<0.0001, ns, not significant. **(B)** Illustration of MDSC migration assay treated with CM. **(C)** The representative images of co-cultured adenoma organoids at the bright field and fluorescent signals after co-culture 96 h. Scale Bar=400 μm. **(D, E)** The knockdown efficiency of G-CSF using RT-qPCR (**D**) and Western Blot (**E**) of organoids using for collecting CM. p-values, *<0.05, **<0.01, ns, not significant. **(F)** The knockdown efficiency of *Csf3* and the knockdown efficiency of *Cul4b* using RT-qPCR. p-values *P<0.05, ***P<0.001, **** P<0.0001. **(G)** The representative images of immunofluorescent staining of S100A8/9 of MDSCs treated with CM$^{WT+Ctrl}$, CM$^{KO+Ctrl}$ and CM$^{KO+si\text{-}Csf3}$. Scale Bar=20 μm. Red, S100A8/9; Blue, DAPI.