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Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☒	☐	A description of all covariates tested
☒	☐	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☐	☒	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Immunofluorescence images were acquired with Leica Application Suite X.3.5.2.18963 Flow cytometry data were acquired with BD FACSDiva Software v8.0.2. qRT-PCR data were acquired with QuantStudio Real-Time PCR Software v1.3. RNA sequencing data acquired with NextSeq500 (Illumina) scRNA-seq data were acquired with NextSeq500 (Illumina) ATAC-seq data were acquired with NovaSeq (Illumina)
Data analysis	Immunofluorescence images were analysed with Imaris Version 7.2.3 Flow cytometry data were analysed with FlowJo Version 10 qRT-PCR data and statistical analysis were analysed with GraphPad Prism Version 8.0 RNA sequencing data were primarily analysed with bcl2fastq version 2.20.0.422, BOWTIE2 Version 2.2.6, TOPHAT Version 2.1.0, SAMTOOLS Version 1.3 and HTSeq Version 0.6.1, and then visualized with gplots Version 3.0.3 and GSEA version 4.0.3 scRNA-seq data were analysed with CellRanger Version 2.1.0, Seurat Version 2.3.2 and pySCENIC Version 0.10.3 ATAC-seq data were analysed with esATAC Version 1.8.0, HOMER 4.11, Bedtools Version 2.26.0, Deeptools Version 3.5.0 and intervene 0.6.5 ATAC-seq and scRNASeq data integration were performed with iRegulon 1.3

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All raw sequencing data that support the findings of this study have been deposited in the Sequence Read Archive (SRA) with the accession code PRJNA595166. All processed data that support the findings of this study have been deposited in the Gene Expression Omnibus (GEO) GSE156245. All code used for data visualization of the scRNA-seq and ATAC-seq data can be found at https://github.com/sun758426-china/Intestinal-Mesenchymal-Stromal-Cell.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	Sample size were chosen based on prior knowledge from previous experiments. Key experiments were repeated by 3 independent researchers. Colon tissues and stromal cells in this study from at least 3 animals per genotype were analysed to be sure differences were reproducible. Variability in the ex vivo assays used in this study tends to be very low, so n=3 is the accepted norm in the field.
Data exclusions	No data were excluded.
Replication	Both in vitro and in vivo experiments were performed with at least 3 biological replicates.
Randomization	Age- and sex-matched mice were randomized during separation from breeding cages after weaning.
Blinding	For quantifications that were done with FACS, blinding was performed by labeling the test tubes numerically without prior knowledge of the genotype/treatment of the sample. Body weight, stool score and histology score were assessed blindly before genotypes were known.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☐	☒ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used	Western blot: Anti-MEKK2 (MAP3K2) antibody (clone EP626Y) Abcam Cat#ab33918 (1:2000) Anti-GAPDH antibody Cat#10494-1-AP Proteintech (1:5000) Phospho-Erk5 (Thr218/Tyr220) Antibody Cell Signaling Technology Cat#3371S (1:1000) Phospho-SAPK/JNK (Thr183/Tyr185) Rabbit mAb (clone 81E11) Cell Signaling Technology Cat#4668S (1:1000) Phospho-p38 MAPK (Thr180/Tyr182) Antibody Cell Signaling Technology Cat#9211S (1:1000) Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody Cell Signaling Technology Cat#9101S (1:1000) Flow Cytometry: FITC-anti-mouse CD45.1 (clone A20) Thermo Fisher Scientific Cat#11-0453-81 (1:200)

PerCP/Cy5.5-anti-mouse CD45.2 (clone 104) Thermo Fisher Scientific Cat#45-0454-80 (1:200)
PE-anti-mouse CD24 (clone M1/69)Thermo Fisher Scientific Cat#12-0242-82 (1:200)
PE/Cy7-anti-mouse CD44 (clone IM7) Thermo Fisher Scientific Cat#25-0441-82 (1:200)
FITC Rat Anti-Mouse CD45 (clone 30-F11) BD Biosciences Cat#553079 (1:200)
PerCP/Cy5.5 anti-mouse CD45 (clone 30-F11) BD Biosciences Cat#550994 (1:200)
BV421 Rat Anti-Mouse CD31 (clone MEC13.3) BD Biosciences Cat#562939 (1:200)
Alexa Fluor® 488 anti-mouse CD31 Antibody (clone 390) BioLegend Cat#102414 (1:200)
APC/Cy7 Syrian Hamster anti-mouse Podoplanin (clone 8.1.1) BioLegend Cat#127418 (1:200)
BV605 Rat anti-mouse CD90.2 (clone 30-H12) BD Biosciences Cat#740334 (1:200)
BV421 Rat anti-mouse CD34 (clone MEC14.7) BioLegend Cat#119321 (1:200)
PerCP/Cy5.5 Armenian Hamster anti-mouse/rat CD81 (clone Eat-2) BioLegend Cat#104912 (1:200)
APC anti-mouse/rat CD81 Antibody (clone Eat-2) BioLegend Cat#104910 (1:200)
PE/Cy7 Rat anti-mouse CD138 (Syndecan-1) (clone 281-2) BioLegend Cat#142514 (1:200)
APC Rat Anti-Mouse CD326 (EpCAM) (clone G8.8) Thermo Fisher Scientific Cat#17-5791-82 (1:200)
Alexa Fluor® 488 anti-mouse CD326 (Ep-CAM) Antibody (clone G8.8) BioLegend Cat#118210 (1:200)
PE Rat Anti-Mouse CD31 (PECAM-1) (clone 390), Thermo Fisher Scientific Cat#12-0311-82 (1:200)
PE anti-mouse CD146 Antibody (clone ME-9F1) BioLegend Cat#134704 (1:200)
Immunofluorescence:
BV421 Rat anti-mouse CD34 (clone MEC14.7) BioLegend Cat#119321 (1:50)
AF647 Rat anti-mouse CD90.2 (clone 30-H12) BioLegend Cat#105318 (1:100)
AF594 Rat anti-mouse CD326 (Ep-CAM) (clone G8.8) BioLegend Cat#118222 (1:1000)
AF488 Rat anti-mouse CD31 (clone MEC13.3) BioLegend Cat#102514 (1:200)
AF594 Syrian Hamster anti-mouse Podoplanin (clone 8.1.1) BioLegend Cat#127414 (1:200)
AF488 Rat Anti-Mouse LYVE1 (clone ALY7) Thermo Fisher Scientific Cat#53-0443-82 (1:200)
Armenian hamster Anti-Mouse CD81 (clone Eat2) Thermo Fisher Scientific Cat#MA1-80209 (1:600)
AF647 Goat anti-Hamster IgG (H+L) Cross-Adsorbed Secondary Antibody Thermo Fisher Scientific Cat#A-21451
Others
Anti-KLF2 Antibody Merck Millipore Cat#09-820
Rabbit (DA1E) mAb IgG XP® Isotype Control Cell Signaling Technology Cat#3900S
Mouse R-Spondin 1 Antibody R&D Systems Cat#MAB3474 (1:500)

Validation

Anti-MEKK2 (MAP3K2) antibody (clone EP626Y) is validated for WB using MEKK2 KO mouse tissues.
Validation data for commercial antibodies are available on vendor websites.
Anti-MEKK2 (MAP3K2) antibody (clone EP626Y) Abcam Cat#ab33918 (1:2000)
(App: FC, ICC/IF, IHC-P, IP, WB; React: Mouse, Rat, Human)

Anti-GAPDH antibody Cat#10494-1-AP Proteintech (1:5000)
(App:WB,IP, IHC, IF, FC, CoIP,ELISA; React: Human, Mouse, Rat, Pig, Arabidopsis, Corn)

Phospho-Erk5 (Thr218/Tyr220) Antibody Cell Signaling Technology Cat#3371S (1:1000)
(App:WB; React: Human, Mouse, Rat, Monkey)

Phospho-SAPK/JNK (Thr183/Tyr185) Rabbit mAb (clone 81E11) Cell Signaling Technology Cat#4668S (1:1000)
(App: WB, IP, IHC; React: Human, Mouse, Rat, Drosophila, Yeast)

Phospho-p38 MAPK (Thr180/Tyr182) Antibody Cell Signaling Technology Cat#9211S (1:1000)
(App: WB, IP, IF; React: Human, Mouse, Rat, Monkey, Drosophila, Pig, Yeast)

Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody Cell Signaling Technology Cat#9101S (1:1000)
(App:WB, IP, IF, FC, React: Human, Mouse, Rat, Hamster, Monkey, Mink, Drosophila, Zebra fish, Bovine, Pig, C.elegans)

FITC Rat Anti-Mouse CD45 (clone 30-F11) BD Biosciences Cat#553079 (1:200)
(App: FC React: Mouse)

BV421 Rat Anti-Mouse CD31 (clone MEC13.3) BD Biosciences Cat#562939 (1:200)
(App:FC, IF React: Mouse)

Alexa Fluor® 488 anti-mouse CD31 Antibody (clone 390) BioLegend Cat#102414 (1:200)
(App:FC React: Mouse)

APC/Cy7 Syrian Hamster anti-mouse Podoplanin (clone 8.1.1) BioLegend Cat#127418 (1:200)
(App: FC React: Mouse)

BV605 Rat anti-mouse CD90.2 (clone 30-H12) BD Cat#740334 (1:200)
(App: FC React: Mouse)

BV421 Rat anti-mouse CD34 (clone MEC14.7) BioLegend Cat#119321 (1:200)
(App: FC, ICC React: Mouse)

PerCP/Cy5.5 Armenian Hamster anti-mouse/rat CD81 (clone Eat-2) BioLegend Cat#104912 (1:200)
(App: FC React: Mouse, Rat)

APC anti-mouse/rat CD81 Antibody (clone Eat-2) BioLegend Cat#104910 (1:200)
(App: FC React: Mouse, Rat)

PE/Cy7 Rat anti-mouse CD138 (Syndecan-1) (clone 281-2) BioLegend Cat#142514 (1:200)
(App: FC React: Mouse)

Alexa Fluor® 488 anti-mouse CD326 (Ep-CAM) Antibody (clone G8.8) BioLegend Cat#118210 (1:200)
(App: FC, IHC-F React: Mouse)

PE anti-mouse CD146 Antibody (clone ME-9F1) BioLegend Cat#134704 (1:200)
(App: FC React: Mouse)

FITC-anti-mouse CD45.1 (clone A20) Thermo Fisher Scientific Cat#11-0453-81 (1:200)
(App: FC ICC React: Mouse, Zebra fish)

PerCP-Cy5.5-anti-mouse CD45.2 (clone 104) Thermo Fisher Scientific Cat#45-0454-80 (1:200)
(App: FC React: Mouse, Zebra fish)

APC Rat Anti-Mouse CD326 (EpCAM) (clone G8.8) Thermo Fisher Scientific Cat#17-5791-82 (1:200)
(App: FC, IF, IHC-F, ChIP, IHC, Biological Function React: Human, Mouse)

PE Rat Anti-Mouse CD31 (PECAM-1) (clone 390), Thermo Fisher Scientific Cat#12-0311-82 (1:200)
(App: FC, IHC, ICC, IF, IHC-F React: Mouse, Human, Zebra fish, Hamster)

BV421 Rat anti-mouse CD34 (clone MEC14.7) BioLegend Cat#119321 (1:50)
(App: FC ICC React: Mouse)

AF647 Rat anti-mouse CD90.2 (clone 30-H12) BioLegend Cat#105318 (1:100)
(App: FC IHC-F React: Mouse)

AF594 Rat anti-mouse CD326 (Ep-CAM) (clone G8.8) BioLegend Cat#118222 (1:1000)
(App: ICC, IHC-F, 3-D IHC React: Mouse)

AF488 Rat anti-mouse CD31 (clone MEC13.3) BioLegend Cat#102514 (1:200)
(App: FC React: Mouse)

AF594 Syrian Hamster anti-mouse Podoplanin BioLegend Cat#127414 (1:200)
(App: ICC IHC-F React: Mouse)

AF488 Rat Anti-Mouse LYVE1 (clone ALY7) Thermo Fisher Scientific Cat#53-0443-82 (1:200)
(App: FC ICC IF IHC-F IHC React: Mouse)

Armenian hamster Anti-Mouse CD81 (clone Eat2) Thermo Fisher Scientific Cat#MA1-80209 (1:600)
(App: ELISA FC IHC-F IP WB React: Mouse, Rat)

AF647 Goat anti-Hamster IgG (H+L) Cross-Adsorbed Secondary Antibody Thermo Fisher Scientific Cat#A-21451
(App: ICC IF IHC React: Hamster)

Anti-KLF2 Antibody Merck Millipore Cat#09-820
(App: WB ChIP React: Mouse)

Rabbit (DA1E) mAb IgG XP® Isotype Control Cell Signaling Technology Cat#3900S
(App: IHC IF F ChIP React: None)

Mouse R-Spondin 1 Antibody R&D Systems Cat#MAB3474 (1:500)
(App: Biological Function React: Mouse)

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)	National Collection of Authenticated Cell Cultures Serial No. SCSP-502
Authentication	D5S818: 8,9 ; D13S317: 12,14 ; D7S820: 11 ; D16S539: 9,13 ; vWA: 16,19 ; THO1: 7,9.3 ; Amelogenin: X ; TPOX: 11 ; CSF1PO: 11,12
Mycoplasma contamination	Mycoplasma has been tested negative.
Commonly misidentified lines (See ICLAC register)	None

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals	Map3k2-/- mice have been described previously (Guo, Z. et al. Molecular and cellular biology 22, 5761-5768 (2002)). Map3k2

Laboratory animals	floxed mouse line, Rspo1-tdTomato reporter line and Col1a2-CreERT2 mouse line were generated by Shanghai Model Organisms Center, Inc. (SMOC). Ptprca Pepcb (CD45.1) (Stock No: 002014), Lgr5-EGFP-IRES-CreERT2 (Lgr5-EGFP) (Stock No. 008875), Vil1-Cre (Stock No.021504), Rosa26 LSL tdTomato (Stock No. 007914) and mTmG mice (Stock No. 007676) were obtained from the Jackson Laboratory. All mice were bred and maintained at accredited animal facilities under SPF conditions in individually ventilated cages on a strict 12h day/night cycle with a regular chow diet at 24 degrees celcius and 60% humidity. Unless otherwise indicated, 10-16 weeks old and sex-matched mice were used in all assays.
Wild animals	This study did not involve wild animals.
Field-collected samples	No field collection of samples was conducted in this study.
Ethics oversight	All animal experiments were performed according to local guidelines for the use and care of laboratory animals as provided by Shanghai Jiao Tong University School of Medicine Institutional Animal Care and Use Committees (IACUC).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Mouse colon were surgically excised, flushed with PBS, then opened longitudinally and cut into ~0.5 cm length pieces. The colon tissue was then washed further and twice incubated in HBSS containing 2% FBS, 1 mM EDTA and 1 mM DTT, at 37℃ with constant shaking at 200 rpm for 20 min to release epithelial cells. The mucosal tissues were then washed with DMEM containing 10% FBS and digested in DMEM containing 10% FBS, collagenase type Ⅷ (50units/ml, Sigma) and DNase Ⅰ (50 units/ml, Sigma) at 37℃ for 50 min. After digestion, the remaining tissue fragments were collected into a 50ml tube and vortexed vigorously for 30s and passed through a 100μm cell strainer. The resultant cell suspension was then centrifuged at 1500 rpm, 4℃ for 5 min before discarding the supernatant and re-suspending the cell pellets in 1×PBS containing 2% FBS. Single cell suspensions were stained with fluorophore-conjugated anti-mouse antibodies at the recommended dilutions for 30min at 4℃ in the dark. Cells were then washed once with 1×PBS containing 2% FBS and re-suspended in 1×PBS containing 2% FBS for flow cytometry or cell sorting. For live sorting experiments, antibody-stained cells were immediately sorted into DMEM containing 20% FBS
Instrument	FACS sorting was performed on BD FACSAriaIII. FACS analysis was performed on BD LSRFortessa and BD LSRFortessa X20.
Software	Data was acquired using BD FACSDiva software v8.0.2, and analysed using FlowJo 10.
Cell population abundance	Sorted populations were >90% pure.

Gating strategy

For detect specific Map3k2 gene deletion and Rspo1 expression in the colon stromal cells, the sort gating strategy is CD326+ IECs, CD45+ leukocytes, CD31+ endothelial cells (CD45-CD326-CD31+), gp38+ intestinal mesenchymal stromal cells (CD45-CD31-CD326-gp38+) CD90-gp38+ stroma (CD45-CD31-CD326-gp38+CD90-) and CD90+gp38+ stroma (CD45-CD31-CD326-gp38+CD90+) (Extended Data Fig.4b, Fig. 3a).
For single cell (sc)RNA-seq,the sort gating strategy is CD326-CD45-CD31-gp38+CD90+ (Fig. 3d).
For sort and analysis cluster 2 and 5 cells, the gating strategy is cluster 2 cells (CD326-CD45-CD31-gp38+CD90medCD81-CD34-CD138+) and cluster 5 cells (CD326-CD45-CD31-gp38+CD90medCD34+CD81+ CD138-) (Fig. 3h, 4a, 4c; Extended Data Fig.6d, 6e, 6f,8a, 8b, 8c, 8d, 8g, 10i).
For ATAC-seq, the sort gating strategy is myofibroblasts (CD45-CD326-CD31-CD146+), CD138+ stromal cells (CD45-CD326-CD31-CD146-gp38+CD34-CD138+), CD34SP stromal cells (CD45-CD326-CD31-CD146-gp38+CD34+CD138-CD81-), MRISC cells (CD45-CD326-CD31-CD146-gp38+CD34+CD138-CD81+) and IECs (CD45-CD326+) (Extended Data Fig. 9a).
For the detection of gene expression Rspo1-tdTomato cell, the sort gating strategy is CD326-CD45-CD31-gp38+tdTomato- (tdTomato-) and CD326-CD45-CD31-gp38+tdTomato+ (tdTomato+) (Extended Data Fig. 7e, 7f).
For the detection of CD90 expression in different cell types of WT mice colon, the gating strategy is CD326+ IECs (CD45-CD326+), CD45+ leukocytes(CD45+CD326-), Lymphatic endothelial cells (CD45-CD326-gp38+CD31+), Blood endothelial cells (CD45-CD326-gp38-CD31+), CD90-gp38+ stroma (CD45-CD31-CD326-gp38+CD90-), CD90+gp38+ stroma (CD45-CD31-CD326-gp38+CD90+) (Extended Data Fig. 5d, 5e).
For the detection of tdTomato+ cell in Rspo1 reporter mice and Col1a2-CreERT2 reporter mice, the gating strategy is CD326+ IECs (CD45-CD326+), CD45+ leukocytes(CD45+CD326-), Lymphatic endothelial cells (CD45-CD326-gp38+CD31+), Blood endothelial cells (CD45-CD326-gp38-CD31+), gp38+ intestinal mesenchymal stromal cells (CD45-CD31-CD326-gp38+) CD90-gp38 + stroma (CD45-CD31-CD326-gp38+CD90-) , CD90+gp38+ stroma (CD45-CD31-CD326-gp38+CD90+), cluster 2 cells (CD326-CD45-CD31-gp38+CD90medCD81-CD34-CD138+) and cluster 5 cells (CD326-CD45-CD31-gp38+CD90medCD34+CD81+ CD138-) (Extended Data Fig. 7g, 7h).
For the detection of intestinal stem cell, the gating strategy is CD326+CD45-CD44-CD24low (Extended Data Fig. 1h).

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.