

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Patients and tissue samples

Fresh gastric cancer (GC) specimens were obtained from Fujian Medical University Union Hospital (Fuzhou, China) with written informed consent from all patients. For single-cell RNA sequencing (scRNA-seq), we profiled 14 surgically resected GC specimens collected after neoadjuvant PD-1 inhibitor-based immunotherapy, including responders (n = 5) and non-responders (n = 9). For independent validation, 78 post-neoadjuvant resection specimens were analyzed by bulk RNA sequencing, immunohistochemistry (IHC), immunofluorescence (IF). Clinical response was evaluated according to RECIST v1.1 and pathological tumor regression grade (TRG).

Tumor regression grade was centrally assessed using the Becker criteria, based on the percentage of residual viable tumor cells in the tumor bed, as follows: TRG 1a, no residual tumor cells; TRG 1b, <10% residual tumor cells; TRG 2, 10%–50% residual tumor cells; and TRG 3, >50% residual tumor cells. Human studies: The human study protocol was approved by the Ethics Committee of Fujian Medical University Union Hospital (Approval No. 2020KY0150), and written informed consent was obtained from all participants prior to enrollment.

Animals

For in vivo studies, C57BL/6J mice (6–8 weeks old) were purchased from Shanghai Model Organisms Center, Inc. (Shanghai, China) and maintained under specific pathogen-free conditions at the Animal Experiment Center of Fujian Medical University. Mice were housed in a temperature- and humidity-controlled environment ($22 \pm 2^{\circ}\text{C}$; 40%–60% humidity) under a 12-h light/12-h dark cycle with ad libitum access to autoclaved chow and water. Both male and female mice were used. All animal procedures were performed in accordance with institutional and national guidelines and were approved by the Institutional Animal Care and Use Committee of Fujian Medical University (Approval No. IACUC FJMU2023-Y-0207).

Cell culture

Primary CAF isolation and FACS purification

Fresh gastric tumor tissues were minced into approximately 1 mm³ fragments and digested in DMEM containing collagenase A (2.5 mg/mL) and DNase I (0.1 mg/mL) at 37°C for 30 min with gentle agitation. Enzymatic digestion was quenched with an equal volume of DMEM containing 10% fetal bovine serum (FBS), and the cell suspension was passed through a 70- μ m sterile cell strainer to remove undigested material. Cells were pelleted by centrifugation at 300 $\times$ g for 5 min, treated with ACK lysis buffer for 5 min at room temperature to remove erythrocytes, washed, and resuspended in staining buffer (PBS containing 1% FBS).

For flow cytometric sorting, single-cell suspensions were stained with a viability dye and fluorochrome-conjugated antibodies against EpCAM (CD326), CD31, and CD45 to exclude epithelial, endothelial, and hematopoietic cells, respectively. CAFs were defined as live EpCAM-CD31-CD45⁻ cells and sorted using a BD FACS Aria III cell sorter (BD Biosciences). For experiments requiring TRPA1-based stratification, an antibody against an extracellular epitope of TRPA1 was included in the staining panel. Sorted cells were seeded into tissue culture flasks and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO₂. Cells from passages 2–5 were used for all assays.

Cell lines

The human gastric cancer cell line AGS and the human monocytic cell line THP-1 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). STR authentication was performed by the provider. Routine mycoplasma testing was not performed by the authors. AGS cells were cultured in F-12K medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified 5% CO₂ incubator. Cells were passaged at 70%–80% confluence and used within 20 passages after thawing.

THP-1 cells were maintained in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin under the same culture conditions. To induce macrophage-like differentiation, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA, 100 ng/mL) for 24 h, washed 2–3 times with pre-warmed PBS to remove residual PMA, and then

rested in PMA-free medium for an additional 24 h before use. Differentiation was verified by the acquisition of adherent morphology and, where indicated, expression of macrophage markers such as CD68 and CD14.

PBMC isolation and T-cell expansion

Peripheral blood from healthy donors was collected in EDTA-containing tubes and processed within 4 h under approved protocols with written informed consent. Whole blood was diluted 1:1 with PBS containing 2% FBS and 2 mM EDTA, carefully layered over Ficoll-Paque PLUS, and centrifuged at $800 \times g$ for 20 min at room temperature with the brake off. The mononuclear cell layer was collected, washed twice with PBS containing 2% FBS, and resuspended in complete RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. Cell viability was assessed by trypan blue exclusion, and only samples with viability $\geq 90\%$ were used.

For T-cell expansion, PBMCs were seeded at 1×10^6 cells/mL and stimulated with anti-human CD3 (5 $\mu\text{g/mL}$) and anti-human CD28 (2 $\mu\text{g/mL}$) antibodies in the presence of recombinant human IL-2 (200 U/mL). Cultures were maintained at 37°C in a humidified 5% CO₂ incubator, and medium was replenished every 2 days with fresh IL-2-containing medium to maintain a density of approximately 1×10^6 cells/mL. Expanded cells were harvested on days 7–10, washed twice with PBS to remove residual IL-2, and used for downstream assays. In selected experiments, CD8⁺ T cells were enriched from PBMCs by magnetic negative selection (Miltenyi Biotec), and purity was verified by flow cytometry where required.

Syngeneic subcutaneous tumor model

Syngeneic subcutaneous tumors were established in 6–8 weeks old C57BL/6 mice by subcutaneous injection of 1×10^6 YTN3 murine gastric cancer cells into the right flank in a total volume of 100 μL containing 50% Matrigel in ice-cold PBS. Tumor growth was monitored every 3 days using digital calipers, and tumor volume was calculated as follows: volume (mm^3) = (length $\times$ width²)/2.

When the mean tumor volume reached 150–250 mm³, mice were randomized into treatment groups to balance baseline tumor size. Treatment was initiated immediately thereafter. Anti-PD-L1 antibody (selleck Cat#A2004) or isotype control was administered intraperitoneally at the indicated dose and schedule. WNT5A pathway modulation was performed using FOXY-5 or BOX5 at the indicated dose and schedule. A2A receptor blockade was achieved using Ciforadenant. Vehicle controls were included where appropriate. Sample sizes for animal experiments were selected based on prior experience with this model and standard practice in the field. No formal a priori power calculation was performed. Group sizes were generally n = 5–10 mice per arm unless otherwise specified. At study endpoint, tumors were harvested for flow cytometry, IF, and RNA analyses.

METHOD DETAILS

scRNA-seq data processing and analysis

Data sources

The single-cell RNA-sequencing data generated in this study have been submitted to the Genome Sequence Archive for Human (GSA-Human) under submission ID subHRA025030. Public bulk RNA-seq datasets for gastric cancer, including GSE84437 and GSE62254, were obtained from the Gene Expression Omnibus (GEO), and TCGA-STAD data were obtained from the Genomic Data Commons or UCSC Xena portal.

Preprocessing of scRNA-seq data

Raw scRNA-seq data were processed using Cell Ranger (v6.0.1, 10x Genomics) with default parameters and the GRCh38 reference genome. The resulting gene-by-cell matrices were imported into Seurat for downstream analysis. Cells were excluded if they met any of the following criteria: fewer than 200 detected genes, more than 5,000 detected genes, or greater than 20% mitochondrial transcripts. Genes expressed in fewer than 3 cells were removed. After quality control, data were normalized and log-transformed, and Harmony was used for batch correction. Custom scripts used for data processing and visualization are available from the corresponding author upon reasonable request.

Dimension reduction, clustering, and cell-type annotation

Highly variable genes were identified using Seurat, followed by data scaling and principal component analysis (PCA). The top principal components were used for shared nearest-neighbor graph construction and clustering. Cell types were annotated based on canonical marker genes, including EPCAM/KRT18/KRT19 for epithelial cells, CD2/CD3D/CD3E/CD3G for T cells, CD68/C1QA for myeloid cells, PECAM1/VWF for endothelial cells, COL1A2/COL3A1/COL6A1 for fibroblasts, GATA2/TPSB2 for mast cells, MS4A1/BANK1 for B cells, and MZB1/JCHAIN for plasma cells. To remove doublets, each major cell type was re-clustered individually. Clusters co-expressing markers from multiple lineages were identified and excluded. The remaining high-confidence cells were retained for downstream analyses. Fibroblast cells were extracted and re-analyzed to investigate intratumoral heterogeneity. Subclustering was performed using standard Seurat workflows. A distinct CAF subpopulation with high TRPA1 expression (TRPA1⁺ CAFs) was identified, co-expressing fibroblast markers such as *Pdgfra*, *Col1a1*, *Acta2*. Marker genes and differentially expressed genes (DEGs) across CAF subsets were determined using the FindMarkers function with Wilcoxon rank-sum test and Bonferroni correction. Functional enrichment of DEGs was conducted using clusterProfiler.

Differential expression analysis

Differentially expressed (DE) genes were detected by the 'FindMarkers' function, which performs differential expression analysis based on the non-parametric Wilcoxon rank sum test for two cell groups. Genes, with log₂ (fold change, FC) > 0.25 and FDR<0.05, were defined as differentially expressed genes.

Trajectory analysis

Monocle (version 2.12.0) was applied to construct differentiation trajectory of fibroblasts subpopulations. The data from the Seurat object was extracted and loaded into the CellDataSet object, and then differential gene expression analysis was performed using genes expressed in more than 10 cells. Further, significant DEG was selected as sequencing

gene. After dimension reduction using DDRtree method, the default parameters are used for cell sorting and trajectory construction.

Transcription factor regulon analysis

SCENIC (Single-Cell regulatory Network Inference and Clustering) is an analytical tool for single-cell RNA sequencing data designed to infer transcription factor regulatory networks and cell states. It reveals intracellular regulatory mechanisms by identifying gene expression patterns, constructing gene regulatory networks, and evaluating the activity of transcription factors. It combines modular analysis based on gene coexpression to effectively identify regulatory signatures across cell types and states.

Public dataset analysis and survival correlation

Marker genes from CAF subclusters were identified using the FindMarkers function in Seurat and defined as cluster-specific gene signatures. To assess their clinical relevance, survival analysis was performed using bulk RNA-seq datasets from gastric cancer cohorts, including TCGA-STAD and GEO datasets (GSE84437, GSE62254). TCGA data were obtained from UCSC Xena (<https://xena.ucsc.edu/>), and GEO expression matrices and clinical metadata were downloaded from the GEO database. Patients were stratified into high and low expression groups based on the median expression level of each CAF signature. Overall survival (OS) was compared using Kaplan–Meier curves, and statistical significance was assessed by log-rank test. Analyses were performed in R using the survival and survminer packages.

Cell-cell communication analysis

we used CellChat to infer the number of cell-cell interaction between different cell types. CellChat is based on a comprehensive database of signaling molecular interactions, including polymeric receptor-ligand complexes, soluble agonists and antagonists, and stimulant and inhibitory membrane-binding co-receptors, to infer biologically significant cell-to-cell communication from the scRNA-seq data.

Spatial transcriptomics

Spatial transcriptomic (ST) data from four gastric cancer patients were collected and analyzed using the Seurat software package (version 3.0. 1) for the gene-spot matrix generated after ST data processing of ST and Visium samples. The signature score obtained from scRNA-seq (TRPA1_CAFs, Macro_SPP1 and Treg_CD25) was calculated using the AddModuleScore function with default parameters in Seurat. Spatial feature plots were generated by the SpatialFeaturePlot function in Seurat.

Immunohistochemistry

Tumor tissues were fixed in 10% neutral-buffered formalin, paraffin-embedded, and sectioned at 4 µm. Sections were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval in citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide, and nonspecific binding was blocked with 5% normal goat serum. Sections were incubated overnight at 4°C with primary antibodies against the indicated markers, followed by HRP-conjugated secondary antibodies. Signal was developed using DAB, and sections were counterstained with hematoxylin.

Quantification was performed using ImageJ. For markers evaluated by chromogenic IHC, staining intensity was quantified using an H-score calculated as: (% weak staining × 1) + (% moderate staining × 2) + (% strong staining × 3), yielding a score from 0 to 300.

Immunofluorescence

Paraffin-embedded tissue sections (4 µm thick) were baked at 60 °C for 1 hour, deparaffinized in xylene (3 × 10 min), and rehydrated through graded ethanol (100%, 95%, 85%, 75%, 50%, 5 min each). Antigen retrieval was performed in citrate buffer (10 mM, pH 6.0) at 95 °C for 20 min.

Sections were permeabilized with 0.3% Triton X-100 in PBS for 15 min and blocked with 5% goat serum for 1 hour at room temperature. Slides were incubated overnight at 4 °C with primary antibodies including anti-α-SMA, anti-SPP1, anti-TRPA1, anti-WNT5A, and anti-CD68 (see Supplementary Table S2).

After PBS washing, sections were incubated with Alexa Fluor–conjugated secondary antibodies for 1 hour at room temperature in the dark. Nuclei were counterstained with DAPI and mounted with anti-fade mounting medium.

Images were acquired using a confocal microscope (e.g., Leica SP8), and fluorescence signals were analyzed using ImageJ software.

Flow cytometry

Single-cell suspensions were prepared following standardized protocols. For fresh tumor tissues, samples were first minced into 1–2 mm³ pieces and incubated with a digestion solution prepared in RPMI 1640 medium containing 1% FBS. The mixture was incubated in a 37°C constant temperature shaker (150 rpm) for 30 min to achieve complete tissue dissociation. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus density gradient centrifugation (400 × g, 30 min at room temperature). Cultured cells were collected after digestion with 0.25% trypsin-EDTA. All cell suspensions were filtered through a 70 µm cell strainer to remove undigested tissue debris and cell clumps. The filtrate was centrifuged at 1500 rpm for 5 min, the supernatant was discarded, and cells were resuspended in PBS containing 1% fetal bovine serum (FBS) and adjusted to a concentration of 1×10^6 cells/mL.

Dead cell exclusion was performed using Zombie UV Fixable Viability dye (BioLegend). The dye was added to the cell suspension at a ratio of 1 µL per 1×10^6 cells and incubated for 15 min at room temperature in the dark. Cells were then washed twice with PBS containing 1% FBS (1500 rpm, 5 min) to remove unbound dye, ensuring that subsequent fluorescent signals originated exclusively from viable cells.

Tumor-infiltrating immune cells were profiled by staining for CD45, CD3, CD4, CD25, and FOXP3 to identify regulatory T cells (Tregs). Myeloid populations were analyzed using CD68, CD11b, F4/80, and SPP1 to assess macrophage phenotypes and inflammatory states. For intracellular markers such as FOXP3 and SPP1, cells were fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set (Invitrogen) according to the manufacturer's instructions (see Supplementary Table S2).

Flow cytometry data acquisition was performed using an LSRFortessa X-20 instrument (BD Biosciences). A uniform acquisition threshold was set for all samples, and at least 1×10^5 viable cell events were collected per sample to ensure statistical power. Post-acquisition data analysis was conducted using FlowJo software (v10.8.1) following a strict standardized gating strategy: first, forward scatter (FSC) and side scatter (SSC) parameters were used to define single-cell populations, excluding debris and doublets; second, viable cell populations were delineated based on viability dye exclusion; finally, target cell subsets were identified and quantified based on fluorescence intensity of surface and intracellular markers.

To validate the accuracy of gating boundaries, isotype controls matching the subtype and fluorescent label of the detection antibodies were included. These controls simulate background signals generated by non-specific antibody binding, providing a key reference for defining positive/negative populations in each fluorescence channel and effectively eliminating false-positive results caused by antibody cross-reactivity or Fc receptor binding.

For quantitative comparisons between experimental groups (e.g., responders vs. non-responders), statistical analysis strategies were selected based on experimental design. For comparisons between two independent samples, an unpaired two-tailed Student's t-test was used, with the Shapiro–Wilk test to verify data normality and the F-test to assess homogeneity of variances. For comparisons involving three or more groups, one-way analysis of variance (ANOVA) was performed, followed by Tukey's HSD test for post-hoc multiple comparisons. A p-value < 0.05 was considered statistically significant for all analyses, and results were expressed as mean $\pm$ standard deviation (Mean $\pm$ SD).

Fluorescence-activated cell sorting

To isolate distinct subpopulations of cancer-associated fibroblasts (CAFs), fluorescence-activated cell sorting (FACS) was employed to obtain TRPA1⁺ CAFs and TRPA1⁻ CAFs. Single-cell suspensions were prepared from fresh tumor tissues via enzymatic digestion using a digestion solution containing collagenase IV and DNase I, incubated at 37°C for 30 min. The cells were then resuspended in PBS containing 1% fetal bovine serum (FBS) and adjusted to

a concentration of 1×10^6 cells/mL. A mixture of fluorochrome-conjugated antibodies (including anti-CD31, anti-CD45, anti-CD326, and anti-TRPA1 antibodies) was added to the cell suspension and incubated in the dark at 4°C for 30 min. After incubation, cells were washed twice with PBS containing 1% FBS and centrifuged ($300 \times g$, 5 min) to remove unbound antibodies.

Cell sorting was performed using a BD FACSAria™ III cell sorter, with target cells defined by the following gating strategy: first, CD31⁺ (endothelial cells), CD45⁺ (immune cells), and CD326⁺ (epithelial cells) populations were excluded. Subsequently, based on differential TRPA1 expression, the CD31⁻CD45⁻CD326⁻ cell population was further divided into TRPA1⁺ CAFs (CD31⁻CD45⁻CD326⁻TRPA1⁺) and TRPA1⁻ CAFs (CD31⁻CD45⁻CD326⁻TRPA1⁻).

The purity of the sorted populations was validated by post-sort flow cytometry, with a required purity of $\geq 90\%$ for the target subpopulation. Qualified cells were used for subsequent functional experiments and molecular mechanism analyses.

Co-culture experiments

For indirect co-culture, primary cancer-associated fibroblasts (CAFs, 1.5×10^5 cells/well) were seeded in the upper chamber of a 6-well Transwell system (0.4 μm pore size; Corning), and THP-1-derived macrophages (5×10^4 cells/well) were seeded in the lower chamber. Cells were cultured in RPMI-1640 medium supplemented with 10% FBS. After 5 days of co-culture, cells were harvested for downstream flow cytometry, qPCR, or cytokine analysis.

To assess the immunomodulatory effect of CAF-macrophage interactions on T cells, peripheral blood mononuclear cells (PBMCs) were added to the lower chamber in parallel experiments following 5 days of CAF-macrophage co-culture and incubated for an additional 48 h. At the end of the co-culture period, PBMCs were collected and stained for Treg-associated markers, including CD45, CD3, CD4, CD25, and FOXP3. Cells were first stained for surface antigens (CD45, CD3, CD4, CD25), followed by fixation, permeabilization, and intracellular staining for FOXP3 using the FOXP3/Transcription Factor Staining Buffer Set (eBioscience). Flow cytometric acquisition was performed on an LSRFortessa X-20 system,

and data were analyzed using FlowJo software (v10.8.1). The proportion of CD4⁺CD25⁺FOXP3⁺ Treg cells was quantified and compared across experimental conditions.

To investigate the role of PD-L1 blockade in regulating MDK expression, immune-tolerant AGS cells were further co-cultured with freshly activated PBMCs in a Transwell system (0.4 µm pore size; Corning) for an additional 4 days. During this period, cells were treated with either anti-PD-L1 antibody (10 µg/mL) or an isotype control. PBMCs in the upper chamber were stimulated with anti-CD3/CD28 antibodies to maintain activation throughout the co-culture period. After 5 days, AGS cells were harvested from the lower chamber for MDK expression analysis by qPCR and western blot, and culture supernatants were collected for cytokine detection by ELISA. This experimental setup allowed us to assess whether anti-PD-L1 treatment modulates MDK expression in tumor cells indirectly through immune cell-mediated signaling.

Generation of immune-tolerant AGS cells

To establish immune-tolerant gastric cancer cells, AGS cells were co-cultured with activated PBMCs in a Transwell system (0.4 µm pore size; Corning) for 14 days in RPMI-1640 medium supplemented with 10% FBS. During this period, PBMCs were stimulated with anti-CD3/CD28 antibodies to maintain activation. After co-culture, AGS cells were collected, washed, and re-plated for subsequent experiments. These cells are referred to as “immune-tolerant AGS cells” and were used to assess MDK expression, resistance to immune-mediated killing, or signaling pathway activation. The immune-tolerant phenotype was confirmed by reduced sensitivity to T-cell cytotoxicity and altered expression of immunomodulatory molecules.

Apoptosis analysis

After isolation of TRPA1⁺ cancer-associated fibroblasts (CAFs) by flow cytometry sorting, cells at different passage stages were selected for apoptosis level analysis. The specific procedure was as follows: Cells were harvested by digestion with 0.25% EDTA-free trypsin, then washed twice with PBS (centrifuged at 1500 rpm for 5 minutes) to remove residual

trypsin. According to the instructions of the Annexin V-FITC/PI Apoptosis Detection Kit, an appropriate amount of staining reagent was added to the cell suspension, followed by incubation at room temperature for 15 minutes in the dark. Data were acquired using a CytoFLEX LX flow cytometer and analyzed with FlowJo software (v10.8.1).

Western blot

Total protein was extracted from cultured cell using RIPA lysis buffer supplemented with protease and phosphatase inhibitors. Protein concentrations were determined using a BCA assay. Equal amounts of protein (20–40 µg per lane) were separated by SDS–PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% non-fat dry milk in TBST for 1 hour at room temperature and incubated overnight at 4 °C with primary antibodies at 1:1000 dilution. The following antibodies were used: GAPDH, TRPA1, SPP1, Tubulin, WNT5A, LEF1, SDC2, PI3K-p110, phospho-AKT (p-AKT), phospho-GSK3β, and β-catenin (detailed information in Supplementary Table S2).

Quantitative real-time PCR

Total RNA was extracted using TRIzol reagent and reverse-transcribed into cDNA. Quantitative PCR was performed using SYBR Green Master Mix on a Bio-Rad real-time PCR system. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method with GAPDH as the internal control. Primer sequences are listed in Supplementary Table S1.

Luciferase reporter assay

Indicated CAFs were seeded in 12-well plates at ~70% confluence and cultured for 12 hours prior to transfection. Cells were co-transfected with a pGL4.10 luciferase reporter plasmid containing either the TRPA1 or LEF1 promoter region, together with a LEF1 expression vector or control vector (pGL3.1), and the phRL-TK Renilla luciferase vector as an internal control. Transfections were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

After 24 hours of transfection, cells were treated with indicated reagents or vehicle control. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System

(Promega) following lysis in 1× passive lysis buffer for 15 minutes. Firefly luciferase activity was normalized to Renilla luciferase activity in each well. Relative promoter activity was calculated and compared between experimental and control conditions.

Chromatin immunoprecipitation (ChIP)-qPCR

ChIP assays were performed using a commercial magnetic ChIP kit according to the manufacturer's instructions. Briefly, cells were crosslinked with formaldehyde, lysed, and sonicated to generate chromatin fragments of 200–500 bp. Immunoprecipitation was performed using anti-LEF1 antibody or control IgG. Purified DNA was analyzed by qPCR using primers targeting the relevant promoter regions. Enrichment was normalized to input and IgG controls. Primer sequences are listed in Supplementary Table S3.

CRISPR genome editing and gene overexpression

For gene knockout, sgRNAs targeting the indicated genes were designed and cloned into a Cas9-expressing vector. Following transfection, cells were selected with puromycin, and knockout efficiency was verified by western blotting. For overexpression studies, full-length cDNAs of the indicated genes were cloned into expression vectors or lentiviral vectors. Overexpression was validated by qRT-PCR and western blotting.

siRNA transfection

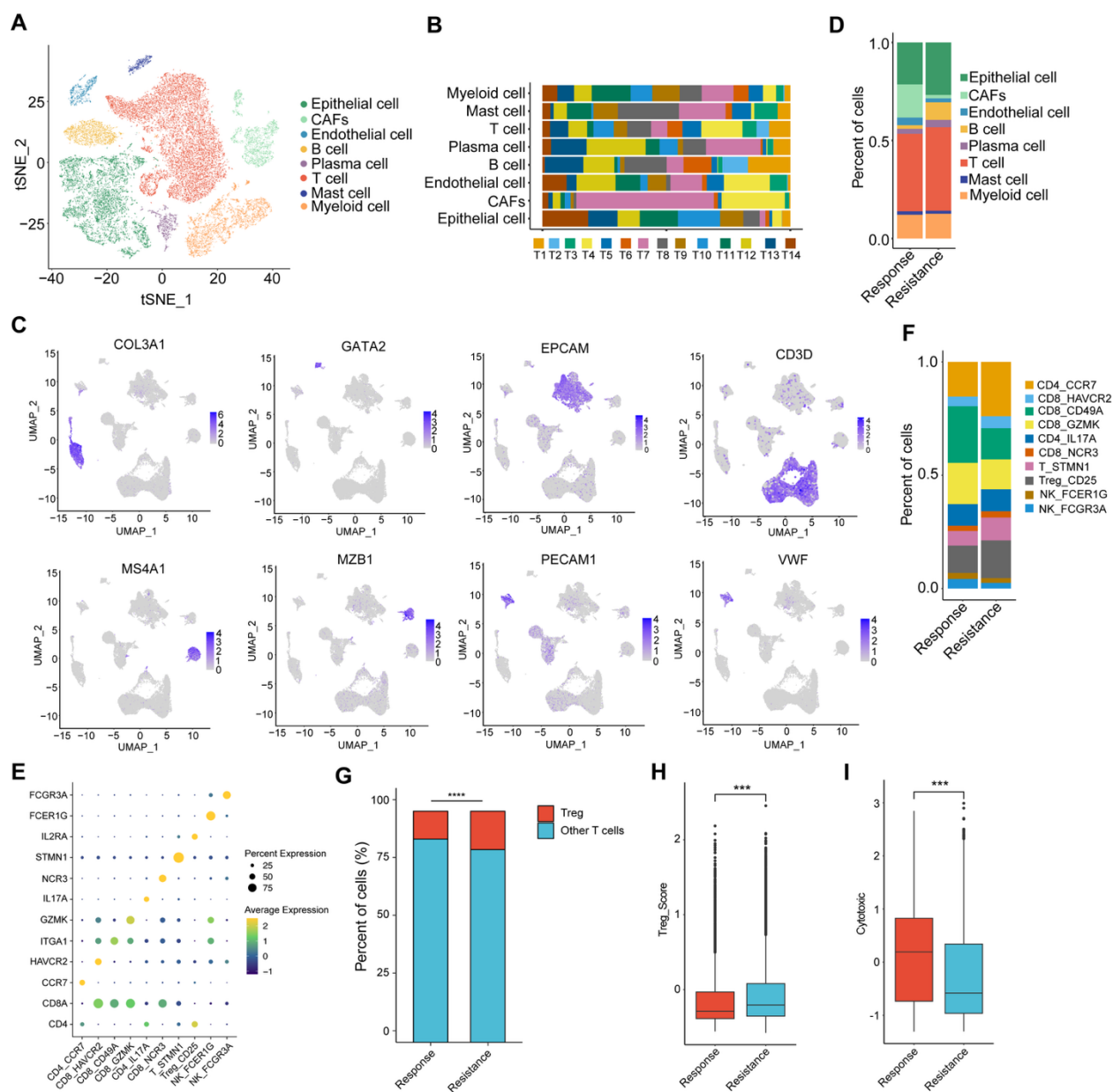
siRNAs targeting the indicated genes were synthesized commercially and transfected using Lipofectamine RNAiMAX according to the manufacturer's instructions. Cells were seeded in six-well plates 24 h prior to transfection to achieve 60–70% confluence at the time of transfection. siRNA duplexes and Lipofectamine RNAiMAX were diluted separately in Opti-MEM, combined, and incubated for 20 min at room temperature to form complexes, which were then added to the cells. Cells were harvested 48–72 h after transfection for downstream analyses. Knockdown efficiency was validated by western blotting, with protein levels normalized to GAPDH or tubulin loading controls.

ELISA

Cell-free culture supernatants were collected and centrifuged to remove debris. Secreted proteins or cytokines were quantified using commercial ELISA kits according to the manufacturer's instructions. Absorbance at 450 nm was measured using a microplate reader, and concentrations were determined from standard curves. Values were normalized to cell number or total protein where appropriate.

Quantification and statistical analysis

Statistical details for individual experiments are provided in the figure legends or main text. Analyses were performed using GraphPad Prism and R unless otherwise specified. Data are presented as mean $\pm$ SD, as indicated in the figure legends. Unless otherwise stated, comparisons between two groups were performed using two-tailed unpaired Student's t-tests, and comparisons among multiple groups were performed using one-way ANOVA followed by appropriate post hoc testing. A P value < 0.05 was considered statistically significant. No blinding was performed during group allocation, treatment administration, or outcome assessment.



Extended Data Figure 1. Cellular composition and transcriptional features of single-cell transcriptomes from ICB-treated gastric cancer

(A) t-SNE visualization of 28,612 single cells from 14 gastric cancer (GC) specimens collected after immune checkpoint blockade (ICB), colored by major cell lineages.

(B) Sample-wise composition of major cell lineages across all 14 patients, showing inter-patient heterogeneity in stromal and immune populations.

(C) Feature plots of representative marker genes used for cell-type annotation: COL3A1 (CAFs), GATA2 (mast cells), EPCAM (epithelial cells), CD3D (T cells), MS4A1 (B cells), MZB1 (plasma cells), PECAM1 (endothelial cells), and VWF (endothelial cells).

(D) Comparison of major cell-type proportions between ICB-responsive and ICB-resistant tumors.

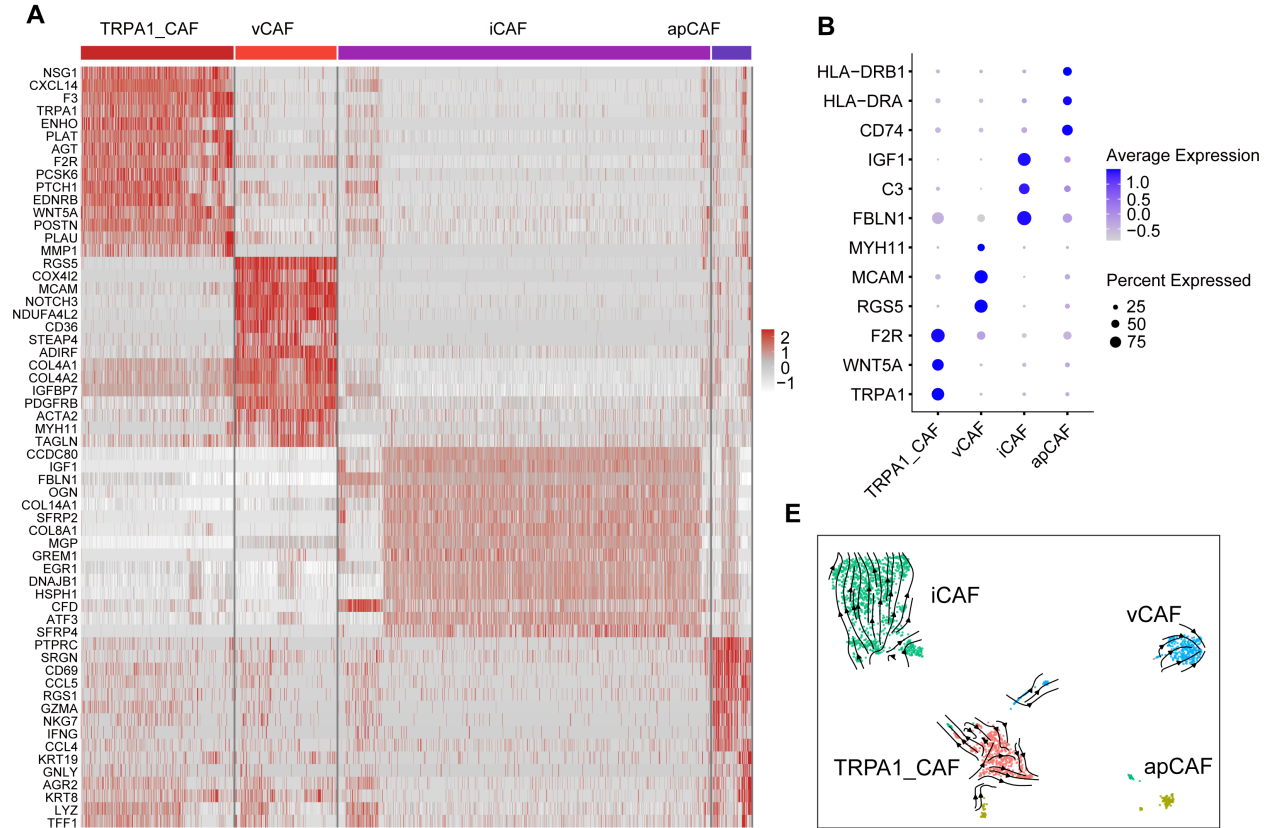
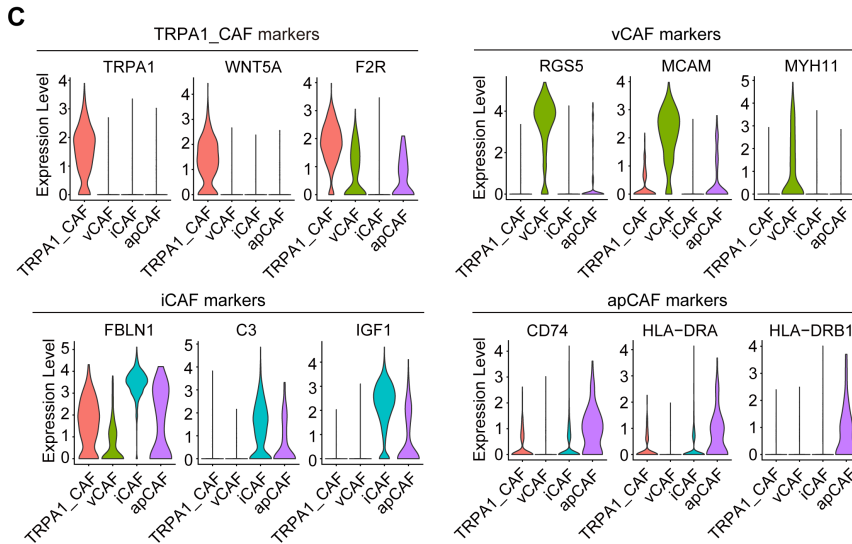
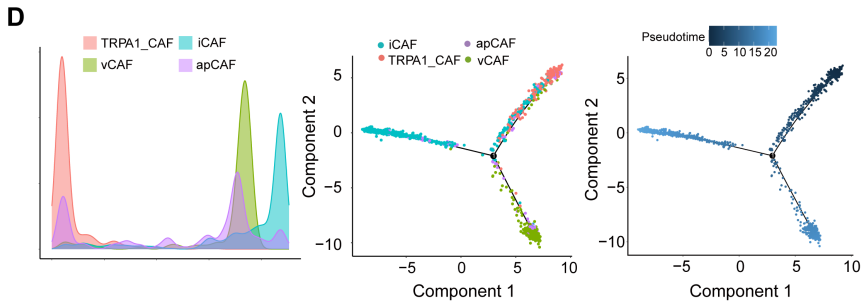
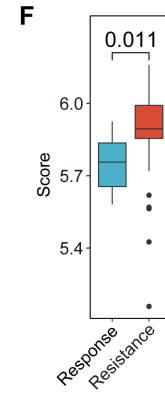
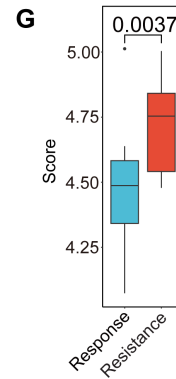
(E) Dot plot showing expression of canonical marker genes used to annotate T/NK cell subsets, including naïve/memory CD4⁺ T cells (CCR7), cytotoxic CD8⁺ T cells (GZMK, NCR3), exhausted CD8⁺ T cells (HAVCR2), regulatory T cells (IL2RA/CD25), and NK cells (FCGR3A, NKG7).

(F) Relative abundance of annotated T/NK cell subsets in responsive and resistant tumors.

(G) Quantitative comparison of regulatory T cells (Tregs) versus non-Treg T cells between ICB-responsive and -resistant tumors. Statistical significance was assessed using Pearson's χ^2 test

(H–I) Gene signature–based functional scoring of T-cell states at the single-cell level, showing enrichment of Treg-associated transcriptional programs (H) and a reduction in cytotoxic CD8⁺ T-cell signatures (I) in resistant tumors. Module scores were calculated using predefined gene signatures and compared between groups using the Wilcoxon rank-sum test.

Significance levels are defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant.



Extended Data Figure 2. Characterization and lineage trajectory of CAF subtypes in gastric cancer

(A) Heatmap of differentially expressed genes across four CAF subtypes—TRPA1⁺ CAF, vascular CAF (vCAF), inflammatory CAF (iCAF), and antigen-presenting CAF (apCAF)—showing distinct transcriptional signatures.

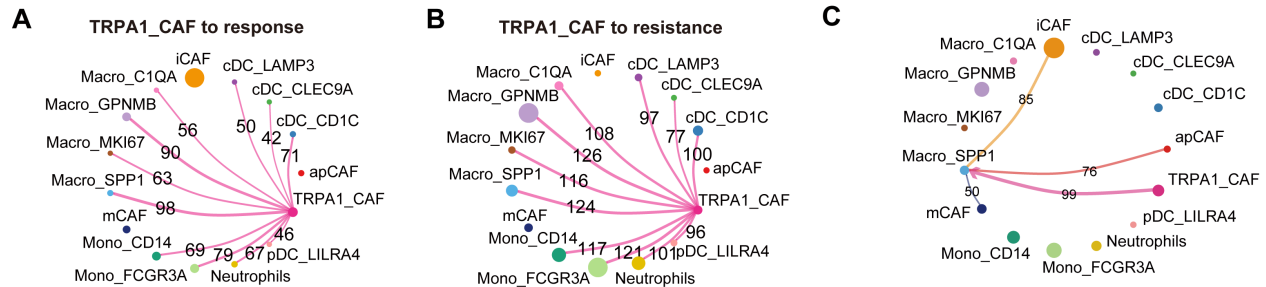
(B) Dot plot highlighting representative marker genes for each CAF subtype: TRPA1, F2R, and WNT5A for TRPA1⁺ CAFs; RGS5, MCAM, and MYH11 for vCAFs; FBLN1, C3, and IGF1 for iCAFs; and CD74, HLA-DRA, and HLA-DRB1 for apCAFs.

(C) Violin plots displaying the expression levels of subtype-defining genes across CAF clusters.

(D) Pseudotime trajectory analysis using Monocle2 showing that TRPA1⁺ CAFs are positioned at the origin of fibroblast lineage progression, indicating an early and transcriptionally plastic state.

(E) RNA velocity plot confirming directional transitions among CAF subtypes.

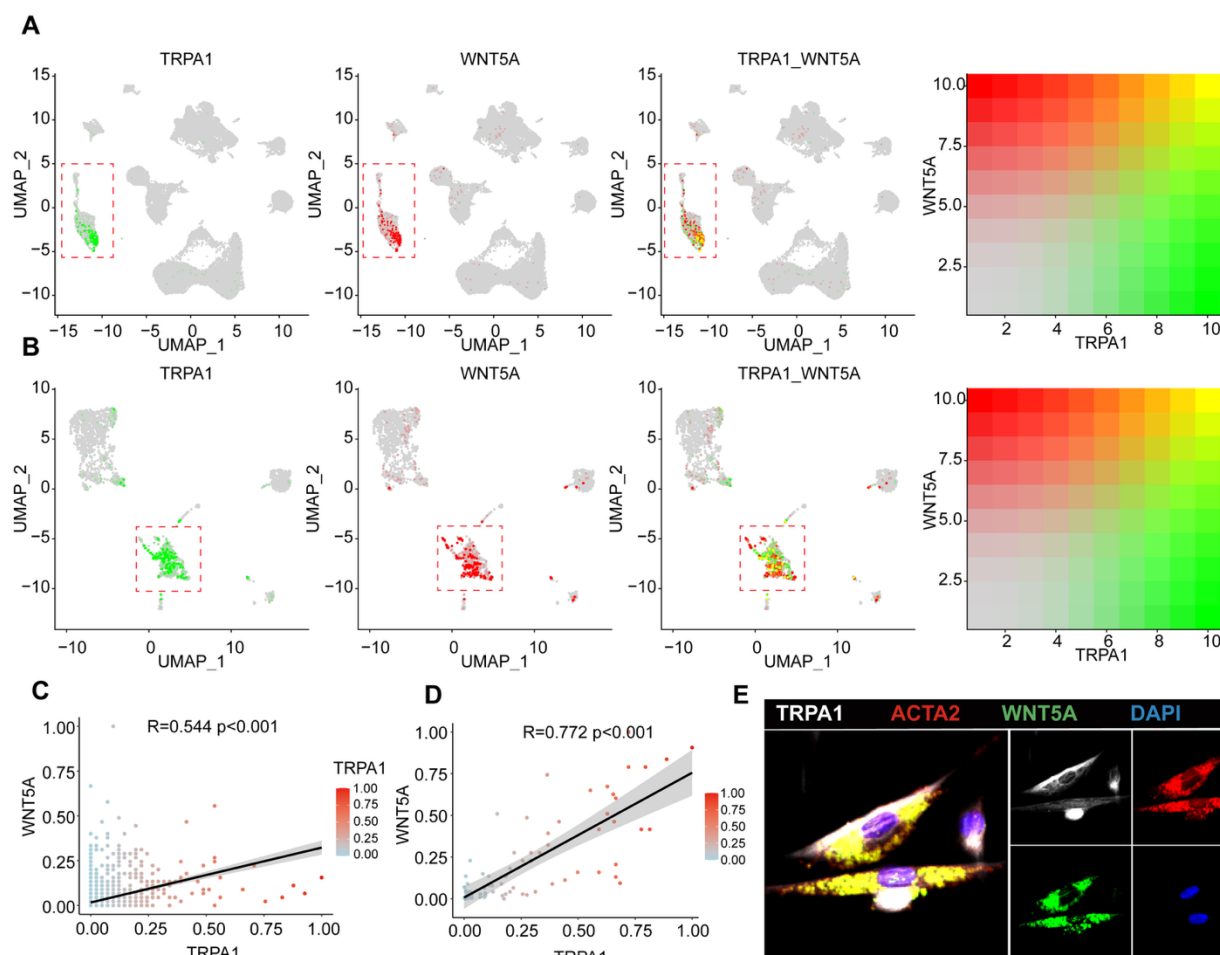
(F, G) Box plots showing significantly higher enrichment scores of the TRPA1⁺ CAF signature in ICB-resistant tumors compared with responsive tumors in independent bulk RNA-seq datasets of patients treated with anti-PD-1 therapy. Statistical significance was assessed using the Wilcoxon rank-sum test.



Extended Data Figure 3. Enhanced TRPA1⁺ CAF–myeloid cell communication in immunotherapy-resistant gastric cancer

(A–B) CellChat network analysis showing predicted ligand–receptor interactions between TRPA1⁺ CAFs and individual myeloid subsets in ICB-responsive (A) and ICB-resistant (B) tumors. Compared with responsive tumors, resistant samples exhibit increased communication strength and connectivity between TRPA1⁺ CAFs and multiple macrophage clusters—particularly SPP1⁺ macrophages.

(C) Comparative network analysis among CAF subtypes demonstrating that TRPA1⁺ CAFs exhibit the strongest predicted communication with SPP1⁺ macrophages compared to other CAF populations (iCAF, vCAF, apCAF).



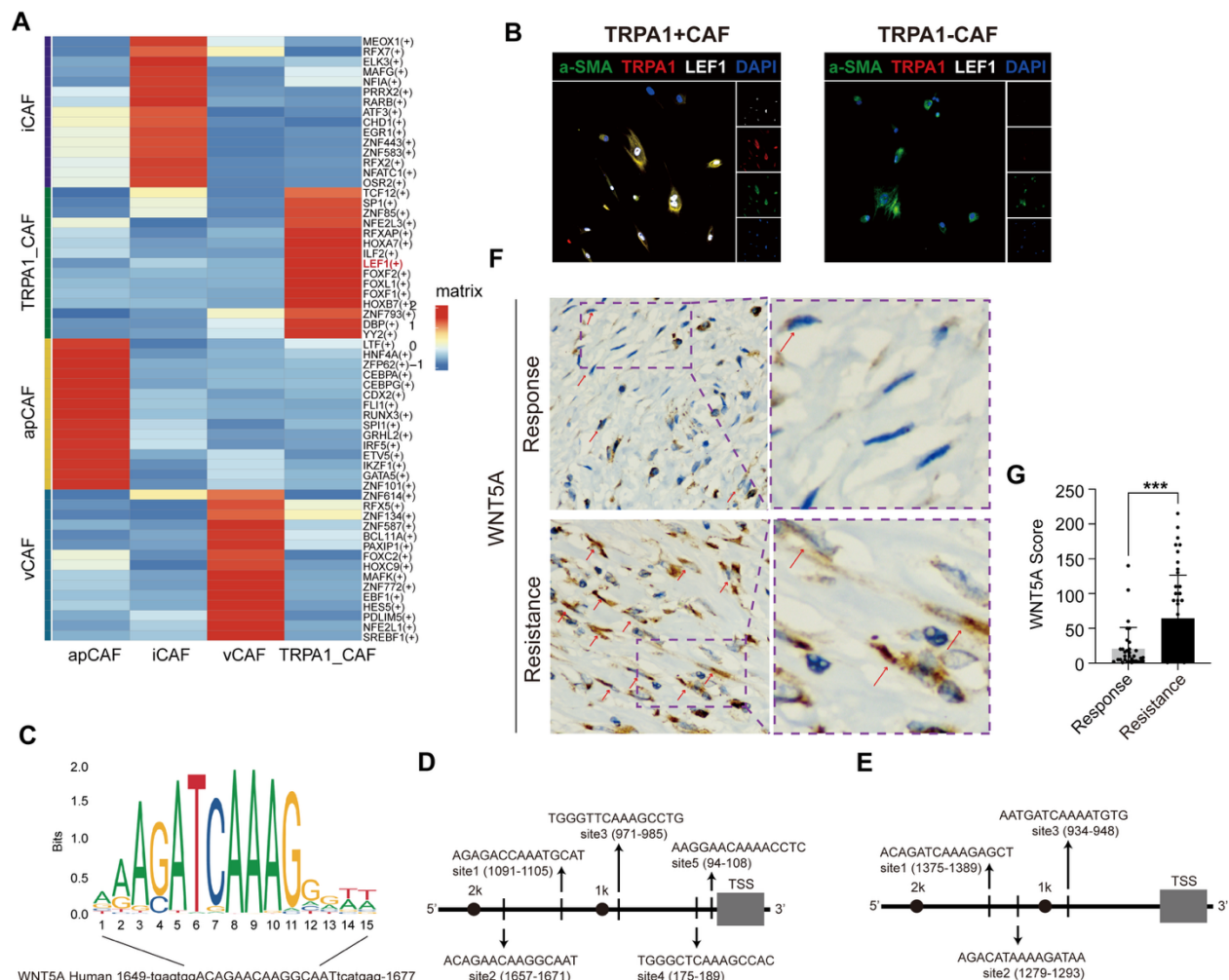
Extended Data Figure 4. TRPA1⁺ CAFs are the predominant stromal source of WNT5A and show a strong positive correlation with WNT5A expression

(A–B) UMAP and co-expression plots showing overlapping spatial distribution of TRPA1 and WNT5A expression in gastric cancer single-cell RNA-seq datasets, where TRPA1 and WNT5A are co-expressed. Heatmaps (right) display positive co-expression patterns between TRPA1 and WNT5A across individual cells.

(C) Correlation analysis from single-cell RNA sequencing showing a significant positive association between TRPA1 and WNT5A transcript levels (Pearson correlation; $R = 0.544$, $p < 0.001$).

(D) Correlation between TRPA1 and WNT5A protein expression scores based on immunohistochemistry (IHC) analysis of neoadjuvant immunotherapy-treated gastric cancer specimens (Pearson correlation; $R = 0.772$, $p < 0.001$).

(E) Representative immunofluorescence images of primary cultured human CAFs showing colocalization of TRPA1 (white) and WNT5A (green) within ACTA⁺ CAFs (red). Images are representative of three independent biological replicates, using CAFs derived from distinct patient donors.



Extended Data Figure 5. LEF1 is enriched in TRPA1⁺ CAFs and transcriptionally regulates WNT5A expression

(A) Heatmap showing inferred transcription factor activity across CAF subtypes derived from single-cell RNA-sequencing of immune checkpoint blockade-treated gastric cancer samples (n = 14 patients; 5 responders, 9 non-responders).

(B) Representative immunofluorescence staining of primary cultured CAFs showing nuclear LEF1 localization in TRPA1⁺ CAFs but minimal expression in TRPA1⁻ CAFs. Images are representative of three independent biological replicates derived from distinct patient CAF donors.

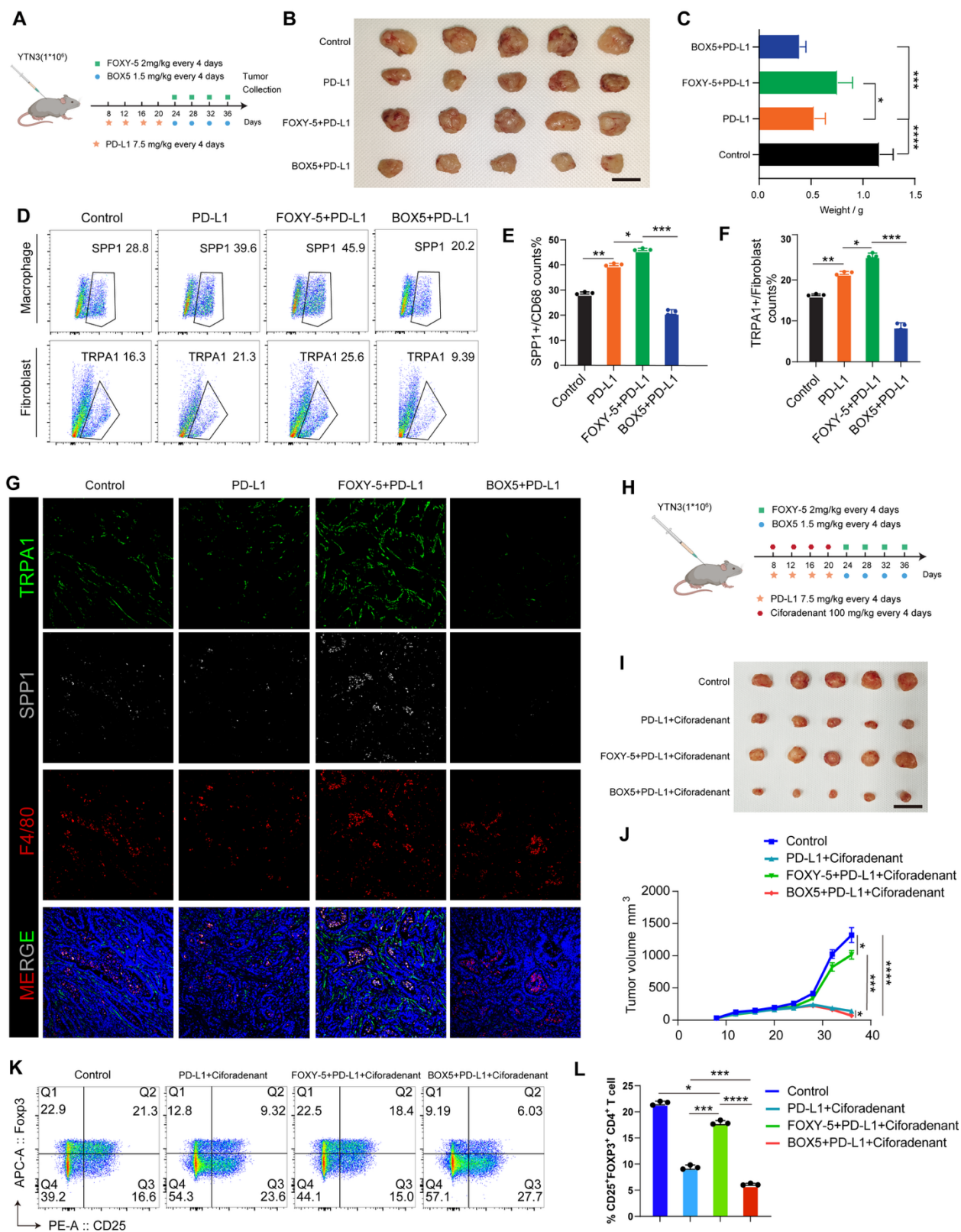
(C) Motif analysis showing the consensus LEF1-binding sequence identified within the WNT5A promoter region.

(D–E) Schematic representation of predicted LEF1-binding sites within the human WNT5A (D) and TRPA1 (E) promoter regions based on in silico analysis.

(F) Representative immunohistochemistry (IHC) images of gastric cancer specimens showing higher WNT5A expression in tumors from immunotherapy-resistant patients compared to responders.

(G) Quantification of WNT5A IHC staining scores across an independent cohort of 78 ICB-treated gastric cancer patients (responsive, $n = 33$; resistant, $n = 45$), demonstrating significantly elevated WNT5A expression in resistant tumors ($p < 0.001$). Statistical significance was assessed using a two-tailed Mann–Whitney U test;

Significance levels are defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; ns, not significant.



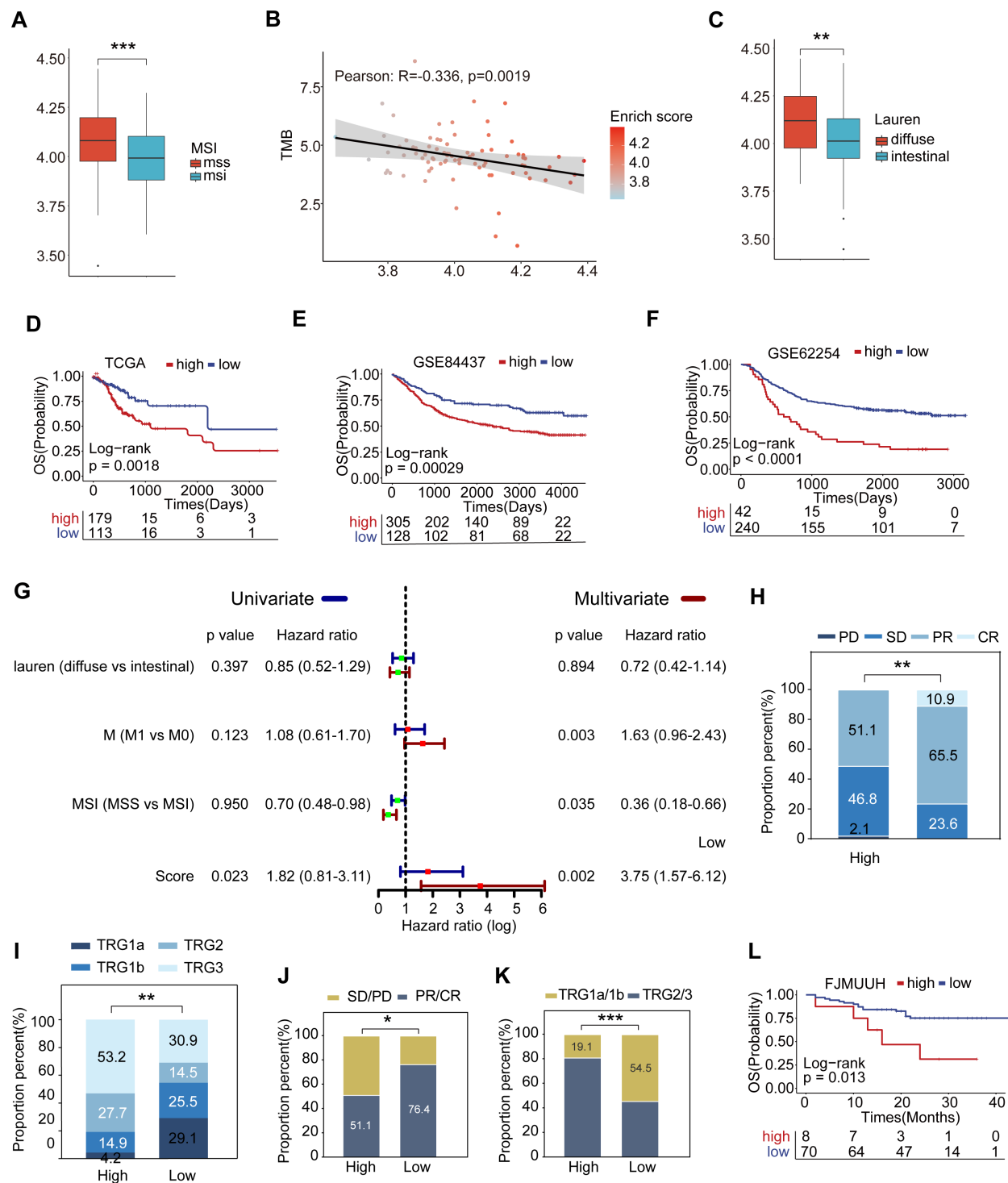
Extended Data Figure 6. Spatial correlation and colocalization of TRPA1+ CAFs,

SPP1⁺ macrophages, and Treg_CD25⁺ cells in immunotherapy-responsive gastric cancer specimens

(A) Spatial transcriptomic correlation matrix and corresponding feature maps showing positive correlations and colocalization among TRPA1⁺ CAFs, SPP1⁺ macrophages (Macro_SPP1), and CD25⁺ regulatory T cells (Treg_CD25) in immunotherapy-responsive gastric cancer tissues. Data are derived from one independent spatial transcriptomic dataset representing a single patient.

(B) Schematic of the in vitro co-culture system combining CAFs, THP-1–derived M0 macrophages, and PBMC-derived CD4⁺ T cells to model stromal–myeloid–T-cell interactions.

(C) Representative gating strategy for CD3⁺ CD4⁺ T cells.



Extended Data Figure 7. A TRPA1⁺ CAF-based gene signature predicts poor survival and resistance to immunotherapy in gastric cancer

(A) Boxplot showing higher TRPA1⁺ CAF signature scores in microsatellite-stable (MSS) compared with microsatellite-unstable (MSI) gastric cancers. Statistical significance was assessed using the two-sided Wilcoxon rank-sum tests.

(B) Scatter plot showing a negative correlation between TRPA1⁺ CAF signature score and tumor mutational burden (TMB) (Pearson correlation; $R = -0.336$, $p = 0.0019$).

(C) Boxplot showing higher TRPA1⁺ CAF signature scores in Lauren diffuse-type compared with intestinal-type gastric cancers. Statistical significance was assessed using the two-sided Wilcoxon rank-sum tests.

(D–F) Kaplan–Meier analyses across three independent cohorts (TCGA, GSE84437, GSE62254) showing significantly worse overall survival in patients with high versus low TRPA1⁺ CAF signature scores. Statistical significance was assessed using the two-sided log-rank test.

(G) Univariate (blue) and multivariate (red) Cox proportional hazards regression analyses evaluating the prognostic significance of TRPA1⁺ CAF signature score together with clinicopathological variables, including Lauren classification, metastatic status (M stage), and MSI status.

(H) Distribution of RECIST v1.1 clinical response categories in the neoadjuvant immunotherapy cohort, shown as proportions of complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD), stratified by TRPA1⁺ CAF signature risk group. Evaluated by Pearson's χ^2 test.

(I) Distribution of Becker tumor regression grade (TRG) categories (TRG1a, TRG1b, TRG2, TRG3) in high- and low-risk groups. Evaluated by Pearson's χ^2 test.

(J) Grouped comparison of objective clinical response, with CR and PR defined as responders and SD and PD defined as resistant. Evaluated by Pearson's χ^2 test.

(K) Grouped comparison of pathological response, with TRG1a/1b defined as major pathological response and TRG2/3 defined as resistant.

(L) Kaplan–Meier analysis of overall survival in the institutional neoadjuvant immunotherapy cohort (FJMUUH), stratified by TRPA1⁺ CAF signature risk group.

Significance levels are defined as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant.

Supplementary Table S1

Primer Name	Sequence (5' → 3')	Length (bp)
TCF12-F(homo)	GAACCATGCTGTGGGACCTT	20
TCF12-R(homo)	AGACTGACAGAGTCTTCCCGA	21
SP1-F(homo)	GCTACAGGGGTCTGATGCTC	20
SP1-R(homo)	AAGGTCTGCCCTGACAATGG	20
ZNF85-F(homo)	CCTTTGGCCGGAGCAGAAT	19
ZNF85-R(homo)	TGCTCTGGGTAGCTGTGAGA	20
LEF1-F(homo)	GAATGTCGTTGCTGAGTGTACT	22
LEF1-R(homo)	GCTGTCTTTCTTTCCGTGCTAAT	23
RFXAP-F(homo)	ACCCTGCTAGTGATGCAACC	20
RFXAP-R(homo)	AAGTGTCTAACAGGTCCGCC	20
HOXA7-F(homo)	TGAGGCCAATTTCCGCATCT	20
HOXA7-R(homo)	CGTCAGGTAGCGGTTGAAGT	20
ILF2-F(homo)	CAGAGTGCCTTAGCAGCCAT	20
ILF2-R(homo)	GGTCAAGGATCCAGGGTGTG	20
NFE2L3-F(homo)	CGCAGAACTGTCGTAAACGC	20
NFE2L3-R(homo)	TTGACTGGCCTACCTTGCTC	20
FOXF2-F(homo)	CTCCAGCATGTCCTCCTACT	20
FOXF2-R(homo)	CTCTGGTGGTGATGGTGATAATAC	20
FOXF1-F(homo)	CCACAACCTCTCGCTCAACG	20
FOXF1-R(homo)	AAGCCGAGCCCGTTCATCAT	20
FOXL1-F(homo)	CTCGCCCATGCTGTATCTGT	20
FOXL1-R(homo)	TGAGCGCGATGTAGCTGTAG	20
HOXB7-F(homo)	CGAGTTCCTTCAACATGCACT	21
HOXB7-R(homo)	CGTTTGCGGTCAGTTCCTGAG	21
ZNF793-F(homo)	ATAGCAACCTCGTCTCAGTGG	21
ZNF793-R(homo)	GATGTCTTCCCAACAGTGGC	20
DBP-F(homo)	GACACCGTGGAGGTGTTGAT	20
DBP-R(homo)	AAGCGATGTCTTCGAGGGTC	20
YY2-F(homo)	ACCACGACCAGGAAATGCTT	20
YY2-R(homo)	GGTCATCTGGAAGTGCTCGT	20
TRPA1-F(homo)	ACAGAAGACAAGTCCTGCCG	20
TRPA1-R(homo)	GAGGGCTGTAAGCGGTTTCA	20
F3-F(homo)	TGACCTCACCACGAGATTG	20
F3-R(homo)	GCTGTCCGAGGTTTGTCTCC	20
Postn-F(homo)	ACGGTGCGATTACATATTCC	21
Postn-R(homo)	ATGTCCAGTCTCCAGGTTGT	20
PLAU-F(homo)	GGTCGCTCAAGGCTTAAGTC	20
PLAU-R(homo)	GGATCTTCAGCAAGGCAATGT	21
WNT5A-F(homo)	GCCAGTATCAATTCCGACATCG	22
WNT5A-R(homo)	TCACCGCGTATGTGAAGGC	19

Supplementary Table S2

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit Anti-Midkine	abcam	Cat#ab52637
Rabbit Anti-LEF1	abcam	Cat#ab137872
Rabbit Anti-CD68	abcam	Cat#ab955
Rabbit Anti-CD274	abmart	Cat#T55980
CD45-APC	BioLegend	Cat#304012,RRID:AB_314400
BD Horizon Fixable Viability Stain 700	Biosciences	Cat#565388,RRID:AB_2869637
Rabbit Anti- β -Catenin	cell signaling	Cat#8480T
Rabbit Anti-Wnt5a/b	cell signaling	Cat#2530S
Rabbit Anti-PI3 Kinase p110 δ	cell signaling	Cat#34050S
Rabbit Anti-Phospho-Akt (Ser473)	cell signaling	Cat#4060S
Rabbit Anti-LEF1 (D6J2W)	cell signaling	Cat#76010s
Rabbit Anti-LEF1 (C12A5)	cell signaling	Cat#2230S
Rabbit Anti-GSK-3 β	cell signaling	Cat#12456S
Rabbit Anti-Akt Antibody	cell signaling	Cat#9272S
Rabbit Anti-Phospho-GSK-3 β (Ser9)	cell signaling	Cat#5558S
Mouse Anti-Wnt5a	GeneTex	Cat#GTX83127
Rabbit Anti-TRPA1	GeneTex	Cat#GTX54765
Mouse Anti-Tubulin	HuaBio	Cat#EM0103
TRPA1-Dylight 488	Invitrogen	Cat#PA5-22833, RRID: AB_11153919
Rabbit Anti-TRPA1	Invitrogen	Cat#PA1-46159, RRID: AB_2209939
Pan-CKAlexaFluor™ 488	Invitrogen	Cat#53-9003-82, RRID: AB_1834350
Rabbit Anti-Osteopontin (OSP)	Invitrogen	Cat#6696-RBM3-P0
Goat anti-Rabbit IgG (H+L) , Alexa Fluor™ 647	Invitrogen	Cat#A-21245, RRID: AB_2535813
Goat anti-Rabbit IgG (H+L) , Alexa Fluor™ 546	Invitrogen	Cat#A-11035, RRID: AB_2534093
Goat anti-Rabbit IgG (H+L) , Alexa Fluor™ 488	Invitrogen	Cat#A-11008, RRID: AB_143165
Goat anti-Mouse IgG (H+L) , Alexa Fluor™ 568	Invitrogen	Cat#A-11004, RRID: AB_2534072
Goat anti-Mouse IgG (H+L) , Alexa Fluor™ 488	Invitrogen	Cat#A-11001, RRID: AB_2534069
FOXP3-APC	Invitrogen	Cat#17-4776-42, RRID: AB_1603280
FOXP3-APC	Invitrogen	Cat#17-5773-82, RRID: AB_469457
F4/80-eFluor™ 450	Invitrogen	Cat#48-4801-82, RRID: AB_1548747
Mouse Anti-F4/80	Invitrogen	Cat#14-4801-82, RRID: AB_467558
Donkey anti-Mouse IgG (H+L) , Alexa Fluor™ 647	Invitrogen	Cat#A-31571, RRID: AB_162542
CD45-APC, eBioscience™	Invitrogen	Cat#17-0459-42, RRID: AB_10667894
CD45-FITC, eBioscience™	Invitrogen	Cat#11-0451-82, RRID: AB_465050
CD4-FITC, eBioscience™	Invitrogen	Cat#11-0049-80, RRID: AB_657742
CD4-FITC, eBioscience™	Invitrogen	Cat#11-0041-82, RRID: AB_464892
CD3e-APC, eBioscience™	Invitrogen	Cat#17-0031-82, RRID: AB_469315
CD326 (EpCAM)-eFluor™ 450, eBioscience™	Invitrogen	Cat#48-5791-82, RRID: AB_10717090
CD326 (EpCAM)-PE.Cyanine7,eBioscience™	Invitrogen	Cat#25-9326-42, RRID: AB_2573542
CD31 (PECAM-1)-eFluor™ 450,eBioscience™	Invitrogen	Cat#48-0319-42, RRID: AB_10854276
CD31 (PECAM-1)-PE-Cyanine7, eBioscience™	Invitrogen	Cat#25-0311-82, RRID: AB_2716949
Mouse Anti-CD3	Invitrogen	Cat#14-0037-82, RRID: AB_467057
CD25-PE, eBioscience™	Invitrogen	Cat#12-0251-82, RRID: AB_465607
CD25-PE, eBioscience™	Invitrogen	Cat#12-0259-42, RRID: AB_1659682
CD11b-PE, eBioscience™	Invitrogen	Cat#12-0112-82, RRID: AB_2734869
Alpha-Smooth Muscle Actin-Alexa Fluor™ 488, eBioscience™	Invitrogen	Cat#53-9760-82, RRID: AB_2574461
Osteopontin Polyclonal antibody	Novus	NB100-1883
Rabbit anti-Osteopontin	Proteintech	Cat#22952-1-AP, RRID: AB_2783651
Rabbit anti-Midkine	Proteintech	Cat#11009-1-AP, RRID: AB_2250619
Mouse Anti-GAPDH	Proteintech	Cat#60004-1-Ig, RRID: AB_2107436
Mouse Anti- α -SMA	Proteintech	Cat#67735-1-Ig, RRID: AB_2918504

Rabbit Anti-ACTA2	Proteintech	Cat#14395-1-AP, RRID: AB_2223009
Mouse Anti-SDC2	Proteintech	Cat#67088-1-Ig, RRID: AB_2882395
Anti- α -SMA	SIGMA	Cat#SAB5500002
DAPI	SouthernBiotech	Cat#0100-20
Goat Anti-Rabbit IgG H&L (HRP)	zen bio	Cat#511203
Goat Anti-Mouse IgG H&L (HRP)	zen bio	Cat#511103
Bacterial and virus strains		
FV034 lentiviral vector	Future Biotechnology	N/A
Biological samples		
Paraffin-embedded gastric cancer sections	Union Hospital Affiliated to Fujian Medical University	N/A
Fresh gastric cancer samples	Union Hospital Affiliated to Fujian Medical University	N/A
Chemicals,peptides,andrecombinantproteins		
Recombinant Human MDK	MCE	Cat#HY-P73295
Box5	MCE	Cat#HY-123071
Ciforadenant	MCE	Cat#HY-101978
5-Fluorouracil	MCE	Cat#HY-90006
anti-PD-L1	selleck	Cat#A2004
Foxy-5	selleck	Cat#S6961
Critical commercial assays		
Annexin V-FITC/PI Apoptosis Detection Kit Annexin V-FITC/PI	yeasen	Cat#40302ES20
(WNT5A) ELISA Kit	JONLNBIO	Cat#JL47455-48T
Human Osteopontin (OPN) ELISA Kit	JONLNBIO	Cat#JL10368-48T
Human Midkine/MDK ELISA Kit	abcam	Cat#ab193761
BeyoFast™ SYBR Green One-Step qRT-PCR Kit	Beyotime	Cat#D7268S
Transcription Factor Buffer Set	Biosciences	Cat#562574
BD Cytotfix/Cytoperm™ Fixation/Permeabilization Kit	Biosciences	Cat#554714
Collagenase A	MCE	Cat#HY-E70005K
Dual-LuciferaseReporterAssaySystem	Promega	Cat#E1910
Pierce Magnetic ChIP Kit	ThermoFisher Scientific	Cat#26157
Experimental models:Cell lines		
AGS	ATCC	CRL-1739
THP-1	ATCC	TIB-202
Mouse YTN-3	Yamamoto et al. ⁷³	N/A
cancer-associated fibroblasts	Union Hospital Affiliated to Fujian Med	N/A
Oligonucleotides		
qRT-PCRprimers,see Supplementary Table S1	This manuscript	N/A
ChIP primers,see Supplementary Table S3	This manuscript	N/A
sgRNA targeting sequences, sgLEF1-1:TCAGGAGCCCTACCACGACA sgLEF1-2:GTACCCGGAATAACTCGAGT sgLEF1-3:CCAACACCCCGGAGACAA	This manuscript	N/A
Software and algorithms		
FlowJo	FlowJoSoftware	www.flowjo.com
GraphPadprism10.0	GraphPad Software	www.graphpad.com
LAS X	Leica Microsystems	https://cn.leica-microsystems.com.cn/service/software-download/las-x-lsr
Image J	Image J Wiki	https://imagej.net/ij/

Supplementary Table S3		
Primer Name	Sequence (5' → 3')	Length (bp)
LEF1-WNT5A-chip-F1	GTCAGACCAGTGAGACATTAGG	22
LEF1-WNT5A-chip-R1	ATGGAAGTCAGTGATGAAGCC	21
LEF1-WNT5A-chip-F2	AGTGGACAGAACAAAGGCAATT	21
LEF1-WNT5A-chip-R2	GCAAGGAAGGAGGAAGACTT	20
LEF1-WNT5A-chip-F3	GGCACTATCATTGACCTCACTT	22
LEF1-WNT5A-chip-R3	CCTCAATACCTGCCCTAAGC	20
LEF1-WNT5A-chip-F4	TCAAAGAAACATTTCTGGGCTCA A	24
LEF1-WNT5A-chip-R4	AAGGCAACAGAATGGAAAGGG	21
LEF1-WNT5A-chip-F5	TGAGAAGGAGAGGAAGGAACA	21
LEF1-WNT5A-chip-R5	GCTTTGAGCCCAGAAATGTTT	21
LEF1-WNT5A-chip-F6	AAGTCCTAGAGCCGCTTGTT	20
LEF1-WNT5A-chip-R6	GCCAAACCACTATCACCTGAG	21
LEF1-WNT5A-chip-F7	CCACGGGAAATCAAGACACAA	21
LEF1-WNT5A-chip-R7	GACTGAGGGCTTGAAGAAATC	22