



PI3K and MAPK Signaling Nodes Serve as Divergent Drivers of Phenotypic Plasticity in Cancer-Associated Fibroblasts in Colorectal Cancer

Zihan Xia^{1,2}, Felix De Vuyst^{1,2}, Sam Ernst^{1,2}, Ujjwal Suwal³, Amélie Vander Cruyssen^{1,2}, Pekka Rappu³, Jyrki Heino³, Sándor Dedeyne^{1,2}, Wim Ceelen^{2,4}, Ligia Craciun⁵, Pieter Demetter⁵, An Hendrix^{1,2}, and Olivier De Wever^{1,2}

ABSTRACT

Cancer-associated fibroblasts (CAF) exhibit phenotypic heterogeneity with each functional state playing critical roles in tumor progression. Notably, subtypes like inflammatory CAFs (iCAF), characterized by increased chemokine/cytokine secretion, and myofibroblast-like CAFs (myCAF), characterized by enhanced extracellular matrix (ECM) deposition and increased actomyosin contractility, can undergo phenotypic switching in response to cues from the tumor microenvironment and therapeutic interventions. Elucidation of the signaling pathways associated with the diverse phenotypes could enable development of strategies to therapeutically reprogram CAFs. Through the analysis of single-cell RNA sequencing data from patients with colorectal cancer, we identified that the phosphatidylinositol-3-kinase (PI3K)/mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathways, among other pathways, are linked to the formation of myCAF and iCAF subtypes, respectively. Unbiased pharmacologic interference of 12 distinct signaling pathways using three-dimensional (3D) human colorectal cancer-derived CAF cultures, *ex vivo* patient-derived tumor

fragments, and mouse models further revealed the significance of PI3K/mTOR and MAPK/ERK signaling in CAF plasticity and functional behavior. PI3K/mTOR inhibition drove iCAF formation through compensatory FGF2 release and FGFR1–JAK2–STAT3 activation, leading to chemokine/cytokine secretion that promoted tumor spheroid growth and neutrophil infiltration. Conversely, MEK inhibition induced a myCAF phenotype via interferon-dependent ROCK and JAK1 signaling, resulting in ECM production that enhanced tumor colony formation. In summary, these findings reveal a functional significance of PI3K/mTOR and MAPK/ERK signaling pathways in CAF plasticity and underscore how standard-of-care targeted therapies can directly influence CAF phenotypes in colorectal cancer.

Significance: The PI3K/mTOR and MAPK/ERK signaling pathways regulate the formation of CAF subtypes in colorectal cancer, providing insights into how fibroblasts adapt to cancer-targeted therapies and potential strategies to harness stromal plasticity.

Introduction

Cancer-associated fibroblasts (CAF) have emerged as pivotal actors of the tumor microenvironment (TME), driving processes such as extracellular matrix (ECM) accumulation and remodeling, cytokine and growth factor secretion, and extracellular vesicle release (1–3). Using a wide range of preclinical models, these processes have been shown to actively promote tumor growth (4),

metastasis (5), immune evasion (6), and therapy resistance (7). Due to their pro-oncogenic functions, CAFs have long been considered a promising therapeutic target. However, despite positive outcomes in preclinical studies, clinical trials targeting CAFs or their signaling pathways have had limited efficiency, with some even showing worsened outcomes (8–11).

Over the past decade, advancements in single-cell RNA sequencing (scRNA-seq) and innovative mouse models have significantly deepened our understanding of the remarkable heterogeneity among CAFs, revealing diverse origins, spatial distributions, phenotypes, and functions—features consistently observed across various tumor types (12). In pancreatic ductal adenocarcinoma (PDAC), scRNA-seq analyses of mouse and human tumors identified three distinct CAF subsets: myofibroblastic CAFs (myCAF), inflammatory CAFs (iCAF), and antigen-presenting CAFs (apCAF; ref. 13). myCAFs exhibit enhanced contractility and are characterized by α -smooth muscle actin (α SMA)-positive stress fibers, as well as ECM deposition and remodeling. In contrast, iCAFs exhibit lower α SMA expression but enhanced secretion of inflammatory mediators such as interleukin 6 (IL6), leukemia inhibitory factor (LIF), and CXCL12 (13, 14). apCAFs expressing major histocompatibility complex (MHC) class II genes and CD74 have been associated with antigen presentation to CD4⁺ T cells and immunomodulatory functions (13). Notably, these distinct CAF phenotypes are not fixed but can transit into

¹Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University, Ghent, Belgium. ²Cancer Research Institute Ghent, Ghent, Belgium. ³Department of Life Technologies and InFLAMES Flagship, University of Turku, Turku, Finland. ⁴Department of Gastro-Intestinal Surgery, Ghent University Hospital, Ghent, Belgium. ⁵Division CMP, Cerba Path, Brussels, Belgium.

Z. Xia and F. De Vuyst contributed equally to this article.

Corresponding Author: Olivier De Wever, Laboratory of Experimental Cancer Research, Department of Human Structure and Repair, Ghent University Hospital, The Core, Entry 37A, C. Heymanslaan 10, Ghent B-9000, Belgium. E-mail: Olivier.dewever@ugent.be

Cancer Res 2026;86:3040–59

doi: 10.1158/0008-5472.CAN-25-0766

This open access article is distributed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) license.

©2026 The Authors; Published by the American Association for Cancer Research

one another depending on TME-derived cues (15) and therapeutic regimens (15–17), suggesting that phenotypic plasticity is governed by unique signaling pathways (12).

To further understand the mechanisms driving the formation of distinct CAF phenotypes, we aimed to identify differentially enriched signaling pathways between myCAFs and iCAFs, taking colorectal cancer as our main tumor type of interest. Colorectal cancer causes more than 0.9 million deaths annually worldwide and is marked by significant interpatient genetic heterogeneity and patient-specific therapeutic responses (18, 19). CAFs are among the most abundant cell types in the colorectal cancer TME, with their high abundance linked to poor overall survival (OS) and altered responses to targeted therapies in patients (20, 21). Within the TME, growth factors such as fibroblast growth factor 2 (FGF2) and TGF β , together with inflammatory cytokines, including IL6, IL1 β , as well as direct ECM interactions, activate diverse downstream signaling cascades that contribute to fibroblast reprogramming (22). Although the mechanisms underlying CAF activation are relatively well characterized, how these signaling pathways integrate to regulate CAF plasticity remains incompletely understood. In this study, we found that the phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway is enriched in myCAFs, whereas mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling is more abundant in iCAFs across five colorectal cancer scRNA-seq datasets. Moreover, analyses of CAF phenotype-related functions, secretome, proteome, and transcriptome have shown that CAFs derived from patient cultures, patient-derived tumor fragments (PDTF), and mouse cancer models undergo a phenotypic switch upon pharmacologic inhibition of PI3K/mTOR or MAPK signaling. These results highlight the crucial role of these pathways in shaping CAF phenotypes and warrant further investigation into the impact of targeted therapies on TME elements such as CAFs.

Materials and Methods

Ethics statement

The collection of peripheral blood from healthy donors was conducted in accordance with the Declaration of Helsinki guidelines and approved by the Ethical Committee (EC/2014/0655) of Ghent University Hospital. Collection of tissue samples from patients was conducted in accordance with the Declaration of Helsinki guidelines and approved by the Ethical Committee (BC-06978) of Ghent University Hospital. All participants provided written informed consent prior to participation.

Cell culture

Isolation and culture of human colorectal CAFs have been described previously (23, 24). Briefly, tissue samples were washed with PBS, cut into small pieces ($\pm 2 \text{ mm}^3$), and placed on tissue culture plastic in a droplet of fetal bovine serum (FBS, #P30-330, Pan Biotech) for 24 hours to facilitate adherence. Fresh DMEM high-glucose (HG) medium (#41965039, Gibco) supplemented with 10% FBS and 100 $\mu\text{g/mL}$ penicillin/streptomycin (P/S, #15070063, Thermo Fisher Scientific) was then added, and cultures were maintained for 10 to 14 days until CAFs surrounding the tissue reached confluence. CAF cultures from five patients (four primary colorectal cancer and one peritoneal metastasis of colorectal cancer) were included; from one patient, two independent cultures were

established. One CAF culture was immortalized by human telomerase reverse transcriptase (hTERT) transduction (25). CAFs were characterized by brightfield microscopy, immunocytochemistry, flow cytometry, and Western blotting.

Human colorectal cancer cell lines (HCT116, RRID: CVCL_0291; HT29, RRID: CVCL_A8EZ; SW480, RRID: CVCL_0546; and HCT8/E11, RRID: CVCL_5679) as well as the murine colorectal cancer cell line CT26-Luc (RRID: CVCL_7254) were obtained from the American Type Culture Collection (ATCC). All cultures were maintained in DMEM HG supplemented with 10% FBS and 100 $\mu\text{g/mL}$ P/S, and experiments were initiated under these conditions. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. All cell lines were routinely tested for *Mycoplasma* contamination using the MycoAlert Mycoplasma Detection Kit (#LT07-318, Lonza). For all experiments, cells between passages 5 and 9 were used.

Spheroid formation

Single-cell suspensions were prepared at concentrations of 1×10^5 cells/mL for CAFs, 1×10^4 cells/mL for HCT116, SW480, and HCT8/E11, and 2.5×10^4 cells/mL for HT29. Cells were seeded into U-shaped, 384-well ultra-low attachment (ULA) plates (#MS-9384UZ, S-bio) with 80 μL of cell suspension per well, as previously described (26). To prevent evaporation, plates were sealed with Breath-Easy semipermeable tape (#BEM-1, Diversified Biotech) and incubated at 37°C in a humidified atmosphere. Treatments were initiated 48 hours after spheroid seeding.

Ex vivo patient-derived tumor fragments

Establishment and culture

PDTFs derived from a peritoneal metastasis of colorectal cancer were obtained with informed written patient consent, in accordance with the regulatory guidelines of the Ethics Committee of Ghent University Hospital. Tumor material was immediately processed by semi-automated cutting into small fragments (0.125 mm³) and placed on ice (27). Individual fragments were then seeded in prewarmed (37°C) DMEM low-glucose (LG, #31885023, Thermo Fisher Scientific) supplemented with 10% FBS and 100 $\mu\text{g/mL}$ P/S. After 24 hours, fragments were treated with trametinib (Tram; 200 nmol/L) or everolimus (Eve; 1 $\mu\text{mol/L}$) for 4 days. Tumor fragments were cultured at 37°C in a humidified atmosphere with 5% CO₂ for up to 5 days.

PDTF scRNA-seq sample preparation

PDTFs ($n = 192$ per condition) were dissociated as described previously (28). The resulting filtered single-cell suspensions were processed using the Gel bead-in-Emulsion (GEM)-X Flex Sample Preparation v2 Kit (#PN-1000781, 10X Genomics) following the manufacturer's instructions. Based on DAPI staining, cells were separated from debris by BD FACSymphony S6/S8. Fixed and sorted cells were multiplexed prior to GEM generation, barcoding, and library preparation following the 10X Genomics User Guide (CG000787), using the Chromium GEM-X Flex Hybridization and Wash Kit (PN-1000789) and GEM-X FLEX GEM and Library Kit (PN-1000782). Pooled samples were loaded on a Chromium X platform (10x Genomics) to generate single-cell GEMs. Library preparation was performed by the VIB Single Cell Core (Gent-Leuven, Belgium) and sequenced on an Illumina NovaSeq 6000.

Compound and recombinant cytokine treatments

CAFs cultured under 2D or 3D spheroid conditions were treated with 16 inhibitors for functional assay, proteomics, and Lumines analysis. All compounds used in screening experiments were tested in triplicate, using a single dose commonly used in the literature. Compounds were diluted to a final working concentration of 0.1% DMSO. CAFs were also treated with various recombinant cytokines and specific pathway inhibitors. Details on these compounds, recombinant cytokines, and respective doses are provided in Supplementary Table S1.

Functional assays

Collagen contraction assay

The collagen gel solution was prepared using 3.7 mg/mL of collagen type I from rat tail (# sc-136157, Santa Cruz Biotechnology), mixed at a 1:3 ratio with CMF-HBSS in culture medium. The pH was subsequently adjusted to 7.4 using a 1 mol/L NaOH solution. CAFs at a density of 2×10^5 cells/mL were added into 500 μ L of the collagen gel solution and seeded onto a 24-well plate. Once the gel solidified, it was overlaid with 500 μ L of medium with or without various inhibitors and recombinant cytokines. After 24 hours, gel areas were photographed and measured using ImageJ software. The percentage of gel contraction was calculated as follows: (well area – gel area)/well area $\times$ 100%.

Collagen gel invasion assay

The collagen gel solution was prepared as described previously. Two layers of 250 μ L solution were added to each well of a 24-well plate. After the lower gel layer solidified, an additional layer of collagen gel containing the spheroids was added on top. Once the upper gel layer had solidified, 500 μ L of medium, with or without various inhibitors, was added to each well. After 3 days, spheroids were imaged, and the maximum invasion distance was measured using ImageJ software.

Scratch wound assay

CAFs were cultured to 100% confluence, manually scratched with a 200 μ L sterile pipette tip to create a wound in the cell monolayer. After washing three times with PBS, CAFs were treated with or without various inhibitors. The scratch gap area at 48 hours in each treatment group was measured at four different positions and compared with the gap area at 0 hours. The migration rate was calculated as follows: (scratch area at 0 hours – scratch area at 48 hours)/scratch area at 0 hours $\times$ 100%.

Outgrowth assay

Forty-eight hours after seeding, CAF spheroids were collected and transferred to a flat bottom 96-well plate. Then, 100 μ L of medium, with or without inhibitors was added to each well. After 3 to 5 days, spheroids were imaged, and the outgrowth area was measured using ImageJ software.

Cell death

The single-cell suspension of CAFs (1×10^5 cells/mL) was mixed with 1.5 μ g/mL propidium iodide (PI; #P4170, Sigma-Aldrich) and seeded into U-shaped, 384-well ULA plates. After 48 hours of spheroid formation, the medium was replaced with medium containing different inhibitors for 3 days. The average PI fluorescence intensity across the spheroid area was captured and quantified using the Incucyte ZOOM (Sartorius).

ATP assay using CellTiter-Glo

For 3D-cultured CAFs and colorectal cancer spheroids, each spheroid was transferred from the culture medium into white 96-well plates (#236108, Thermo Fisher Scientific). An equal volume of CellTiter-Glo 3.0 Reagent (#G9683, Promega) was added to each well. For 2D-cultured cells, an equivalent volume of CellTiter-Glo 2.0 Reagent (#G9243, Promega) was added to each well. The plates were shaken on an orbital shaker for 5 minutes and then incubated at room temperature for 10 to 20 minutes to allow the luminescent signal to stabilize. Luminescence readings were recorded using Gen5 Data Analysis Software (v3.08.01).

Luminex

The supernatant of CAF spheroids treated for 5 days with DMSO or different type of inhibitors was collected and passed through a 0.2 μ m filter (#A37111, Novolab). For #5.3hTERT cultures, the supernatant was processed with the *Human Cytokine/Chemokine Array 71-Plex Panel* by Eve Technologies. For other primary CAF cultures, the supernatant was quantified using the *ProcartaPlex Human Cytokine/Chemokine/Growth Factor Panel 1, 45-plex* (#EPX450-12171-901, Thermo Fisher Scientific), following the manufacturers' instructions. For additional experiments, the supernatant of CAF spheroids treated with DMSO or Tram (100 nmol/L) for 3 days was processed with the *Human MMP 9-Plex/TIMP 4-Plex Panel* and *Human Cytokine Array 65-Plex Panel*. Readout was performed on the Luminex 100/200 System (Merck Millipore) with Luminex xPonent v3.1 software. All analyses were performed on a minimum of three biological replicates per condition, with background correction by subtracting the cytokines concentrations from the medium without cells.

Proteomics

CAF spheroids were treated in triplicate by the addition of different type of inhibitors or an equivalent of DMSO. After 5 days, the spheroids were collected and washed three times with PBS. Spheroids or decellularized ECM were resuspended in lysis buffer [50 mmol/L triethylammonium bicarbonate and 5% SDS (pH 7.5)], sonicated, and then clarified with centrifugation of 13,000 g for 8 minutes. The subsequent sample preparation process and the parameters for liquid chromatography–tandem mass spectrometry were applied as described previously (29, 30).

Conditioned media from CAFs

CAFs were treated with Eve, either in the presence or absence of pan-FGFR inhibitor (erdafitinib, #HY-18708, MedChem Express) or human FGF2 neutralizing antibody (#AF-233, R&D Systems). After 48 hours of treatment, the medium was replaced with serum-free DMEM-HG, and cells were incubated for an additional 24 hours. CAF conditioned medium (CM) was then collected, centrifuged to remove debris, and filtered through a 0.2- μ m filter.

Polymorphonuclear isolation

Human polymorphonuclears (PMN) were isolated from the peripheral blood of healthy donors using Lymphocyte-poly (#CL5071, Tebubio) density gradient centrifugation (500 g, 30 minutes). After centrifugation (representative image shown in Supplementary Fig. S4F), the peripheral blood mononuclear cells at the upper band and the PMNs at the lower band were resuspended in RPMI 1640 medium (#12004997, Gibco) supplemented with 10% FBS. The PMNs were cultured in 24-well plates at a density of 2.5×10^5 cells/mL at 37°C and 5% CO₂.

CAF supernatant treatment assays

PMN migration

A total of 1×10^5 cells/mL of isolated PMNs were seeded in the upper chamber of IncuCyte Clearview 96-well Cell Migration Plates (#4599, Sartorius) in serum-free medium (DMEM-HG). The chemoattractant of CM from CAF with or without pretreatment are added to the lower chamber. After 4 hours, immune cell migration into the lower chamber is quantified by total cellular adenosine triphosphate (ATP) levels via CellTiter-Glo 2.0.

Cancer cell proliferation

Forty-eight hours after spheroid seeding, 40 μ L of the supernatant of each well was removed, and then 40 μ L of CM of CAFs (1:1 v/v) was added. After 5 days of coculture, total cellular ATP levels were quantified via CellTiter-Glo 3.0.

RNAi studies

Transfection of siRNAs listed in Supplementary Table S2 was performed with jetPRIME DNA/siRNA (co)transfection reagent (#101000001, Sartorius) at a final concentration of 50 nmol/L. Cells were analyzed 48 hours after transfection.

Quantative RT-PCR

Total RNAs were extracted using the miRNeasy Micro Kit (#217084, QIAGEN) according to the manufacturer's instructions and eluted in 14 μ L DNase/RNase-free water. For cDNA synthesis, 1 μ g of RNA was reverse transcribed in a 20 μ L reaction using the iScript cDNA Synthesis Kit (#170-8891, Bio-Rad), following the manufacturer's protocol. Quantitative PCR (qPCR) was performed using the SsoAdvanced Universal SYBR Green Supermix (#1725272, Bio-Rad). Each 5 μ L PCR reaction contained 0.5 μ L each of forward and reverse primers (5 mmol/L), 2.5 μ L of 2x SYBR Green Supermix, and 2 μ L of 1:10 diluted cDNA. The 384-well plate was processed on the Roche LC480 with cycling conditions of 95°C for 5 seconds, 60°C for 30 seconds, and 72°C for 1 second, repeated for 44 cycles. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, with GAPDH as the internal control. Primers are listed in Supplementary Table S2.

Bulk RNA-seq

Spheroids derived from CAFs were treated with Eve, omipalisib (Omp), and uprosertib (Upro) for 24 hours or Tram for 48 hours, with three biological replicates per condition. RNA was extracted using the miRNeasy Micro Kit, and 27.5 ng of RNA per sample was treated with Heat-Labile Double-Strand-Specific DNase (Arctic-zymes) to remove DNA contamination. For PI3K/AKT/mTOR inhibitors, RNA-seq libraries were prepared using the QuantSeq-Pool Sample Barcoded mRNA Seq Library Prep Kit (Lexogen) and sequenced at 1.4 pmol/L with 1% PhiX on a NextSeq 500 system (Illumina) with a high-output 1×75 run. Reads were mapped to the human genome using Tophat, and gene expression counts were generated with HTSeq. For the MEK1/2 inhibitor, libraries were prepared using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina) and sequenced on the NextSeq 500 system with a single-end read length of 75 bp, producing an average of 20 million indexed reads per sample. Sequencing reads were analyzed using the alignment-free Sailfish method. Differential gene expression (DGE) analysis for all conditions was conducted using DESeq2, with significance defined as an adjusted $P < 0.05$ and a fold change (FC) > 1.5 .

Western blotting

Following the indicated treatments, cells or spheroids were washed three times with PBS, and 100 to 200 μ L of Laemmli lysis buffer [0.125 mol/L Tris-HCl (pH 6.8), 10% glycerol, and 2.3% SDS] was added. Cell lysates were then sonicated and centrifuged at 20,000 g for 15 minutes at 4°C, and the supernatants were transferred to new tubes. Protein concentration was measured using the DC Protein Assay Kit (Bio-Rad) following the manufacturer's instructions. Lysates were mixed with reducing sample buffer [0.5 mol/L Tris-HCl (pH 6.8), 43% glycerol, 9.2% SDS, 5% 2-mercaptoethanol, and 5% bromophenol blue] and boiled at 95°C for 5 minutes. Proteins (20–40 μ g per well) were separated by SDS-PAGE, transferred onto 0.2- μ m nitrocellulose membranes (#162-0115, Bio-Rad), blocked with 5% (w/v) milk/BSA in PBS with 0.5% Tween-20, and incubated overnight at 4°C with primary antibodies. After three washes with blocking buffer, membranes were incubated for 1 hour at room temperature with secondary antibodies and then washed six times with PBS containing 0.5% Tween-20. Blots were detected using the Clarity Western ECL Substrate (#1705062, Bio-Rad) and visualized with the iBright CL 750 system (Thermo Fisher Scientific). Antibodies and dilution ratios are listed in Supplementary Table S3.

Decellularized ECM extraction

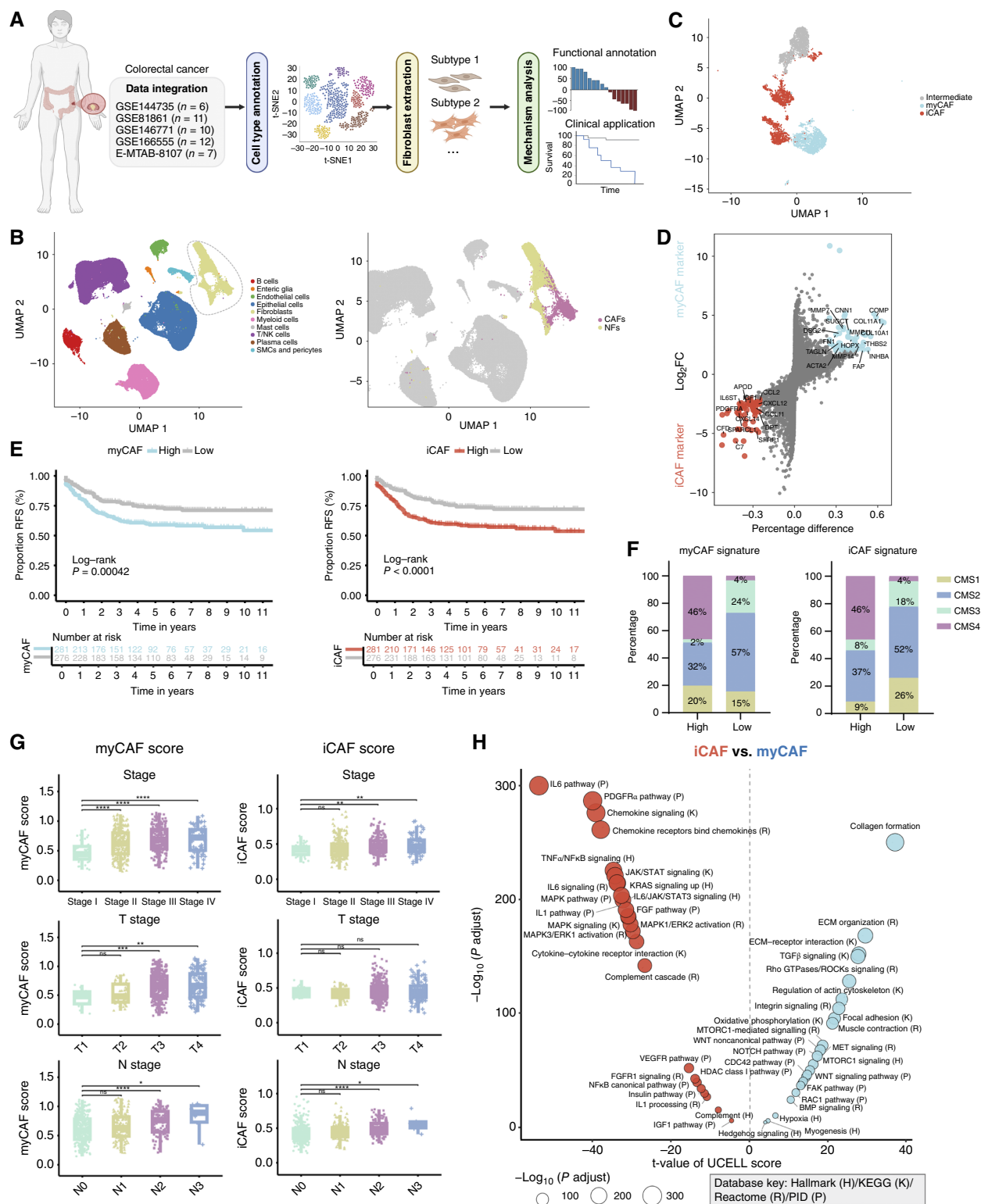
Decellularized ECM was prepared as described previously (31). Briefly, six-well plates or μ -slide eight-well microplates (#80806, Proxylab) were pretreated with 0.2% gelatin solution (#G9391, Sigma-Aldrich) for 1 hour at 37°C, followed by incubation with 1% glutaraldehyde (#G6257, Sigma-Aldrich) for 30 minutes at room temperature. After three washes with PBS, plates were incubated with 1 mol/L ethanolamine (#E9508, Sigma-Aldrich) for 30 minutes at room temperature and washed again three times with PBS. CAFs were seeded at a density of 1×10^6 cells/mL in six-well plates or 2×10^4 cells/mL in eight-well microplates in media containing 50 μ g/mL ascorbic acid (L-ascorbate, #A4034, Sigma-Aldrich), with or without 10 nmol/L Tram. Cells were cultured for 8 days, with media changed every other day. Decellularization was performed by removing cells with an extraction buffer (0.5% Triton X-100 and 20 mmol/L NH₄OH in PBS), followed by multiple PBS washes and treatment with 10 μ g/mL DNase (#9003-98-9, ITW Reagents) for 30 minutes at 37°C. The decellularized ECM was then collected for proteomics analysis or immunofluorescence (IF) staining.

Colony formation assay

Colorectal cancer cells were seeded at 1×10^3 cells/mL on the top of decellularized ECM derived from either CAFs or Tram-treated CAFs and cultured for 7 days to allow colony formation. Colony formation was assessed using crystal violet staining.

In vivo animal studies

Animal experiments were carried out in accordance with the regulatory guidelines of the Ethics Committee of the Ghent University Hospital. Female BALB/cBYJ (RRID: IMSR_JAX: 001026) mice of 5 to 6 weeks old (Charles River Laboratories) were intraperitoneally injected with 1×10^5 CT26-Luc. One week after inoculation, mice were randomized into two groups with equal average bioluminescent signal, and treatment was initiated. Mice received treatments three times per week: Eve (5 mg/kg) or Tram (1 mg/kg; kindly provided by Novartis in nonclinical research collaborations and consortiums) was

**Figure 1.**

scRNA-seq reveals two distinct subtypes of colorectal CAFs and their associations with clinical characteristics. **A**, Overview of the workflow for integration and downstream processing of five public colorectal cancer scRNA-seq datasets. **B**, UMAP plots of the integrated scRNA-seq dataset showing the major cell types (left) and an enlarged view of the fibroblast population separated into NFs and CAFs (right). **C**, UMAP plot of the extracted CAF population showing the annotation of a myCAF, iCAF, and intermediate subset. **D**, DGE between myCAFs and iCAFs (Continued on the following page.)

administered by oral gavage in a vehicle solution (0.5% methylcellulose and 0.2% Tween-80 in distilled water). The control group received the vehicle alone. Bioluminescence imaging (BLI) was performed weekly for follow-up. On day 14 or day 21, mice were sacrificed, and omental and mesenteric metastatic tumor tissues were collected for histologic analysis.

IF staining

Tissue sections

Formalin-fixed, paraffin-embedded (FFPE) mouse tumor sections (5 μ m) were deparaffinized, subjected to heat-mediated antigen retrieval, and then rinsed in PBS. Samples were permeabilized and blocked for 1 hour in blocking buffer (1% BSA, 0.2% Tween-20 in PBS) and incubated overnight at 4°C with primary antibodies. After two washes with PBS containing 0.3% Tween-20, fluorescent secondary antibodies were applied for 1 hour at room temperature, followed by three additional washes with PBS containing 0.3% Tween-20. Nuclei were stained with DAPI for 15 minutes at room temperature. Sections were mounted with Fluoromount (#F4680, Sigma-Aldrich) and coverslipped. Images were acquired on Cytation 5 imaging system (BioTek, Agilent) and Pannoramic 250 Flash III digital slide scanner (3DHISTECH).

Cells and decellularized ECM

CAFs were seeded at 3×10^3 cells/mL in μ -Slide eight-well microplates and cultured overnight. The following day, cells were treated with or without Tram (10 nmol/L) for 48 hours, fixed with 4% paraformaldehyde, washed with PBS, and permeabilized in 0.2% Triton X-100 in PBS for 10 minutes at room temperature. CAFs and decellularized ECM were blocked for 1 hour with blocking buffer [0.5% BSA, 0.02% Triton X-100, and 1:100 goat serum (#X090710-8, Agilent Technologies) in PBS], followed by overnight incubation at 4°C with primary antibodies. After three PBS washes, fluorescent secondary antibodies diluted in blocking buffer were applied for 3 hours at room temperature, followed by three additional PBS washes. Nuclei were stained with DAPI for 15 minutes at room temperature, and samples were mounted in polyvinyl alcohol mounting medium (#10981, Sigma-Aldrich). Confocal images were acquired on a Zeiss LSM 880 with Fast Airyscan. All antibodies and dilutions are listed in Supplementary Table S3.

Immunohistochemistry

IHC was performed on 5 μ m sections of FFPE peritoneal metastatic tumor tissues from CT26-Luc xenografts. Deparaffinization and antigen retrieval were carried out by heat treatment. The IHC procedure was performed using the ultraView Universal DAB Detection Kit (#760-500, Ventana) on an automated staining instrument (BenchMark ULTRA IHC/ISH System, Roche). Primary antibodies and dilution ratios are listed in Supplementary Table S3.

scRNA-seq analysis

scRNA-seq data were analyzed from both publicly available datasets and PDTFs. PDTF raw sequencing data were processed with Cell Ranger multi v7.2.0 (10x Genomics) using the probe set v1.0.1 for GRCh38-2024-A. All datasets were analyzed in R v4.2.2 (RRID: SCR_002394) using the Seurat package v5.3.0 (RRID: SCR_016341). For publicly available data, cells were filtered using dataset-specific thresholds: Qian and colleagues (32), 500 to 6,000 detected genes, $\leq 40,000$ counts, and mitochondrial content $\leq 15\%$; Lee and colleagues (33), 300 to 5,200 genes, $\leq 35,000$ counts, and mitochondrial content $\leq 10\%$; Uhlitz and colleagues (34), 500 to 4,500 genes, $\leq 35,000$ counts, and mitochondrial content $\leq 10\%$; Li and colleagues (35), 500 to 6,500 genes, $\leq 2,500,000$ counts, and mitochondrial content $\leq 10\%$; and Zhang and colleagues (36), 500 to 6,500 genes, $\leq 35,000$ counts, and mitochondrial content $\leq 10\%$. For PDTFs, cells with < 200 genes or outliers (> 5 MADs for counts/genes or > 15 MADs for mitochondrial proportion) were excluded. After quality control, data were log-normalized, variable genes were identified, and expression values were scaled. Batch effects across patients and datasets were corrected using the Harmony R package (RRID: SCR_022206). Dimensionality reduction was performed by principal component analysis, followed by Uniform Manifold Approximation and Projection (UMAP) embedding (first 26 corrected PCs for public datasets, 11 PCs for PDTFs). Clustering was performed with a nearest-neighbor approach, and cell types were annotated using canonical marker genes (37).

Fibroblasts were extracted from both public datasets and PDTFs for further analysis. In the public datasets, fibroblasts were sub-clustered into myCAFs and iCAFs based on characteristic markers and expression profiles. For PDTFs, fibroblasts were sub-clustered by treatment versus control conditions. Differentially expressed genes between fibroblast subsets were identified with the Seurat “FindMarkers” function ($\log_2$ FC > 2 , percentage difference > 0.2), and the top 30 upregulated and downregulated genes defined myCAF and iCAF signatures. Pathway activity was assessed using the UCell package (RRID: SCR_027109) with Kyoto Encyclopedia of Genes and Genomes (KEGG), HALLMARK, PID, and REACTOME gene sets from the Molecular Signatures Database (MSigDB; <https://www.gsea-msigdb.org/gsea/msigdb>). Pathway activity scores were compared using generalized linear models, with results visualized as heatmaps, volcano plots, or violin plots.

Dataset analysis

The microarray and RNA-seq STAR count data were converted to $\log_2$ (TPM + 1) data. myCAF and iCAF scores were calculated using the myCAF and iCAF gene sets defined in this article and applying the ssGSEA algorithm from the GSVA R package (RRID: SCR_021058; ref. 38). Kaplan–Meier survival analyses were conducted using the survival and survminer R packages (RRID: SCR_021094), with continuous variables in the survival data split at the median. *P* values were calculated using the log-rank test.

(Continued.) iCAFs across five different datasets. The upregulated and downregulated genes, characterized by an absolute $\log_2$ (FC) > 2 and a percentage difference > 0.2 , define the myCAF (blue) and iCAF (red) gene signatures. **E**, Kaplan–Meier curves showing the prognostic value of myCAF and iCAF scores on relapse-free survival in patients with colorectal cancer ($n = 557$, GSE39582 dataset). The *P* value was calculated using the log-rank test. **F**, Bar graphs displaying the distribution of the four CMS subtypes, categorized by high and low myCAF and iCAF signatures in the GSE39582 dataset. **G**, Box plots comparing the myCAF and iCAF scores in different tumor stages in the GSE39582 dataset. The *P* value was determined using the Wilcoxon test *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, nonsignificant. **H**, Pathway enrichment analysis comparing iCAFs (red) vs. myCAFs (blue). The *x*-axis shows the *t* value of the UCell score derived from a linear model, and the *y*-axis and circle size represent the $-\log_{10}$ adjusted *P* value. Pathways were annotated from Hallmark (H), KEGG (K), Reactome (R), and PID (P) databases. **A**, Created in BioRender. Xia, Z. (2025) <https://BioRender.com/53ee380>.

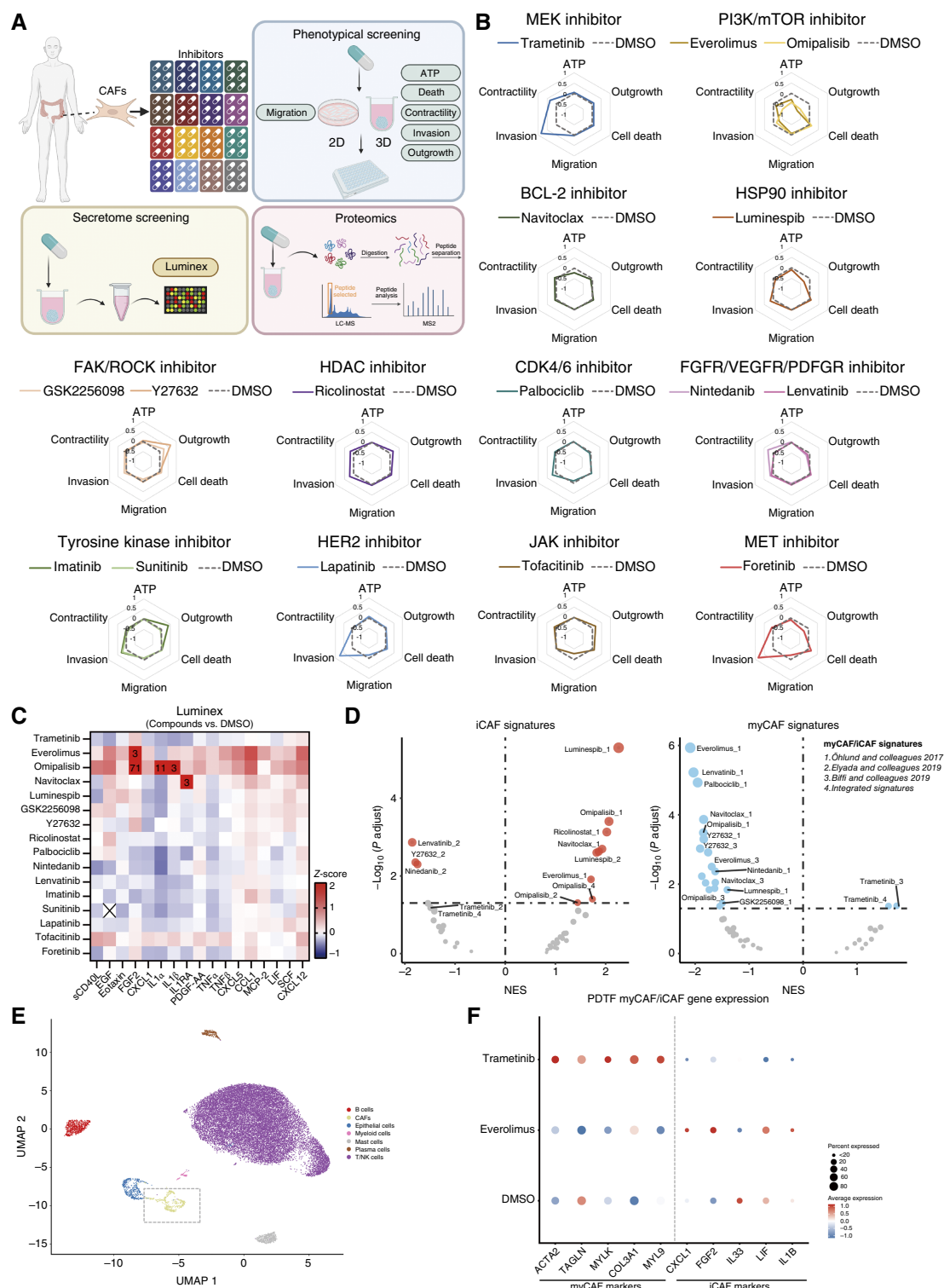


Figure 2.

CAF phenotypic switching by pharmacologic targeting of crucial signaling nodes. **A**, A schematic summary of screening experiments. Colorectal CAF were exposed to 16 targeted treatments and evaluated by functional assays, Luminex analysis, and proteomics. **B**, Spider plots illustrating six functional phenotype metrics in CAFs with and without inhibitors treatment. Axes represent Z-scores for cell death, ATP content, contractility, migration, and outgrowth. A higher Z-score indicates a higher metric value. **C**, Heatmap displaying Z-scores of iCAF-related cytokines in the supernatant of CAFs treated with various inhibitors for 5 days, measured by Luminex analysis. **D**, GSEA of iCAF and myCAF signatures using proteomics data from CAFs treated (Continued on the following page.)

Gene set enrichment analyses

Gene set enrichment analysis (GSEA) was performed using the org.Hs.eg.db (RRID: SCR_024739) and clusterProfiler (RRID: SCR_016884) R packages (39). Preranked list of genes from the DGE analyses were used with gene sets defined based on previous studies (e.g., myCAFs, iCAFs, and IFN⁺CAFs; refs. 13–15, 40–43) or obtained from the MSigDB database.

Statistical analysis

Z-scores were calculated in Excel (v.2510) for the phenotypic screening results and the Luminex data analysis using the formula $Z = (x - \mu)/\sigma$, in which x is the value of each replicate, μ is the mean of the control condition, and σ is the standard deviation of the control condition. Experimental data were analyzed using GraphPad Prism 9 software (RRID: SCR_002798), whereas bioinformatic analyses were conducted in R (version 4.2.2). Statistical comparisons were performed using unpaired Student *t* test or ANOVA tests for comparisons among three or more groups. The Wilcoxon test was used to compare UCell signature scores between myCAFs and iCAFs, and Spearman correlation analysis was performed to assess the correlation between different variables. $P < 0.05$ was considered statistically significant.

Results

scRNA-seq reveals distinct subtypes of colorectal CAFs and their associations with clinical characteristics

To investigate the role of CAF subtypes in colorectal cancer progression, we integrated five recently published scRNA-seq datasets (GSE144735, GSE81861, GSE146771, GSE166555, and E-MTAB-8107; Fig. 1A; Supplementary Table S5 for the number of cells analyzed, and Supplementary Table S6 for associated metadata; refs. 32–36). UMAP cluster annotation according to the original datasets was performed to validate the batch correction (Supplementary Fig. S1A). Major cell types were identified and clustered based on specific marker expressions. Fibroblasts were further divided into normal fibroblasts (NF) and CAFs, with CAFs subsequently subclustered according to their distinct gene expression profiles (Fig. 1B and C; Supplementary Fig. S1B and S1C; Supplementary Table S7). Current data indicate that certain CAF subpopulations exhibit similar features and gene-expression signatures across various cancer types. Based on the expression of established gene sets (4, 13–15, 40–42, 44, 45), CAF subclusters were classified as myCAFs, among others marked by *ACTA2*, *TAGLN*, *ACTG2*, *MYL9*, and *MYLK* expression; iCAFs, among others marked by *CFD*, *DPT*, *C7*, *PDGFRA*, and *CXCL12* expression; and “intermediate” CAFs with an intermediate expression of the specific myCAF and iCAF markers (Supplementary Fig. S1D and S1E). DGE analysis between myCAFs and iCAFs was performed (Fig. 1D; Supplementary Table S8). The top 30 upregulated and downregulated genes, selected based on log₂ FCs and differences in the percentage of expressing cells, were used to establish the myCAF and iCAF gene signature sets (Supplementary Table S8).

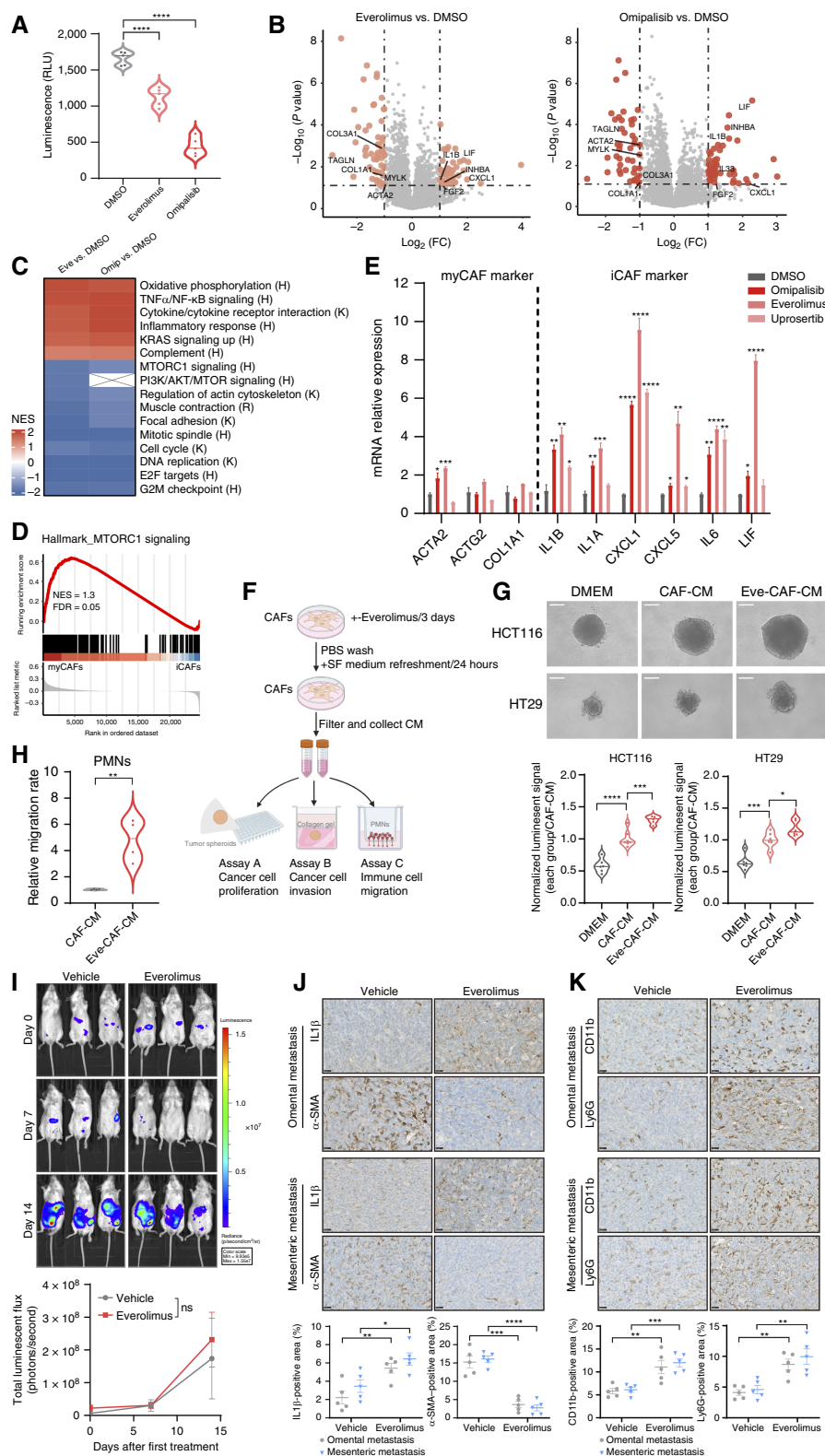
To assess the clinical relevance of CAF subtypes, we used the myCAF and iCAF gene signature sets to stratify patients into high and low myCAF/iCAF groups. Kaplan–Meier analysis of the GSE39582 dataset ($n = 557$) showed that patients with colorectal cancer with low scores for either myCAF or iCAF exhibited significantly higher relapse-free survival rates (Fig. 1E). In the Cancer Genome Atlas (TCGA)-COAD ($n = 288$) and GSE17536 ($n = 176$) datasets, only high myCAF accumulation was associated with poorer disease-specific survival and OS rates (Supplementary Fig. S1F–S1I). Furthermore, we found that patients classified under consensus molecular subtype 4 (CMS4), which is characterized by high stromal infiltration and the lowest OS rates (46), exhibited a higher abundance of both myCAFs and iCAFs compared with those in the CMS1–3 subtypes (Fig. 1F). Moreover, both myCAF and iCAF scores were higher in patients with poor prognostic features, such as advanced American Joint Committee on Cancer stage and nodal stage. However, only the myCAF signature showed a significant correlation with advanced tumor stage (Fig. 1G).

Multiple complex signals contribute to the phenotypic heterogeneity and plasticity of CAFs. To gain a better understanding of the mechanisms driving the formation of these two distinct CAF populations in colorectal cancer, we focused on identifying the signaling pathways enriched in their respective phenotypes. Consistent with previous findings (13), the analysis of signaling-related gene signatures revealed a striking contrast between iCAFs and myCAFs (Supplementary Table S9). Several well-known pathways, including focal adhesion, smooth muscle contraction, and ECM-related pathways, were significantly enriched in myCAFs. In contrast, inflammatory pathways such as NF- κ B, IL6/JAK/STAT3, chemokine/cytokine signaling, and the complement cascade were more prevalent in iCAFs (Fig. 1H). Additionally, myCAFs were enriched for pathways involving activation of integrin, TGF β , WNT, and PI3K/AKT/mTOR signaling. They also showed higher representation in pathways related to mechanical signal transduction, such as focal adhesion kinase (FAK), Rho-Rho-kinase (ROCK), and CDC42. Conversely, we found that iCAFs were enriched in pathways associated with FGF/FGFR1, IL1, and MAPK/ERK activation (Fig. 1H).

CAF phenotypic switching by pharmacologic targeting of crucial signaling nodes

To better understand the signaling pathways involved in CAF phenotypic modulation, pharmacologic screening was performed by targeting major signaling pathways in human CAFs from colorectal cancer tissues. In total, we collected four CAF cultures from primary colorectal cancer (#2, #5.1, #5.3, and #6), as well as one CAF culture from colorectal cancer peritoneal metastasis (#16). Mesenchymal origin was confirmed by assessing morphotype, α SMA, and vimentin expression (Supplementary Fig. S2A and S2B). One early-passage patient-derived CAF culture (#5.3) was immortalized with hTERT, as previously described (25), to enable faithful long-term experimentation and prevent the emergence of a senescence-associated secretory phenotype (47). The hTERT-immortalized CAFs retained fidelity with their parental line

(Continued.) with 16 inhibitors. Volcano plots display the normalized enrichment score (NES) against the log₁₀ of adjusted *P* values, with adjusted $P < 0.05$ indicating significant enrichment. **E**, UMAP plot of the combined data of PDFTs derived from a colorectal cancer peritoneal metastasis treated with among Tram, Eve, or DMSO control. The CAF population is highlighted and extracted for further analysis. **F**, Dot plot illustrating the expression of myCAF/iCAF markers across different treatment conditions in the CAF cluster of the PDFT scRNA-seq dataset. Dot color represents the average expression level, and dot size indicates the percentage of cells within the cluster expressing the gene. **A**, Created in BioRender. Xia, Z. (2025) <https://BioRender.com/d66m742>.

**Figure 3.**

PI3K/mTOR inhibition mediates a phenotypic switch toward an iCAF subtype. **A**, Violin plot showing the proliferation of CAFs cultured in 3D spheroids following exposure to Omp and Eve for 5 days. Statistical significance was assessed by unpaired Student *t* test, comparing each treatment group with the DMSO control. **B**, Volcano plots of differentially abundant proteins in CAFs treated with either Eve or Omp for 5 days. myCAF or iCAF (Continued on the following page.)

(#5.3 vs. #5.3hTERT), as demonstrated by comparable morphology, vimentin expression, and expression of common CAF markers (Supplementary Fig. S2B–S2D). This hTERT CAFs was used for subsequent studies, whereas early-passage patient-derived cultures were used to confirm the major findings (Supplementary Fig. S2E–S2H).

In vivo, CAFs are arranged in a multilayered network pattern. To replicate this organization *in vitro*, we utilized CAF cell aggregates formed into spheroids based on our previous expertise (26). To assess the impact of various kinase and nonkinase inhibitors on CAF phenotypes (Fig. 2A), we conducted a series of functional assays measuring ATP content, outgrowth, cell death, type I collagen contractility, and invasion. Inherent to the conditions of the *in vitro* scratch assay, migration was evaluated under 2D culture conditions. Metrics were visualized as a spider plot (transformed into Z-scores), enabling direct comparison of phenotypic changes in CAFs under different inhibitor treatments (Fig. 2B; Supplementary Table S10). CAF metabolic activity, assessed by measuring cellular ATP content, was notably reduced by the PI3K/mTOR inhibitor Omip and the mTOR inhibitor Eve. CAF metabolic activity was also slightly inhibited by the BCL-2 inhibitor navitoclax (Nav) and the HSP90 inhibitor luminespib (Lum). Furthermore, the outgrowth of CAFs from spheroids was restricted by PI3K/mTOR inhibitors and the HSP90 inhibitor. CAFs exhibited a notable increase in contraction and invasion capacity, significant phenotypic traits of myCAF, when treated with Tram, a potent allosteric inhibitor of mitogen-activated extracellular signal-regulated kinase (MEK)1/2, a crucial intermediate of the MAPK/ERK pathway. Notably, the HER2 inhibitor lapatinib and the MET inhibitor foretinib also enhanced the invasive ability of CAFs but did not stimulate contraction. Interestingly, none of the inhibitors induced cell death, indicating that CAF exhibit an intrinsic survival program to targeted (kinase) inhibitors.

iCAFs are characterized by secretion of various inflammatory cytokines and growth factors that promote cancer cell growth and modulate the immune landscape within the TME (13, 14, 37). The supernatant from CAF spheroids treated with different inhibitors was collected and analyzed using Luminex cytokine profiling on a panel of 71 human cytokines and chemokines. iCAF-related cytokine concentrations were converted to Z-scores and visualized in a heatmap. Interestingly, FGF2 secretion from CAFs significantly increased following treatment with Omip and Eve. Furthermore, the levels of several inflammatory factors, including IL1 β , IL1 α , and CXCL12, were elevated in the CM from CAFs following treatment with PI3K/mTOR inhibitors (Fig. 2C; Supplementary Fig. S2F).

CAF spheroids were treated in triplicate with various inhibitors and subsequently collected for proteomic analysis. Among these inhibitors, Tram significantly increased the presence of proteins implicated in contractility, including ACTA2, TAGLN, MYL9, and MYLK and the matrix protein COL3A1 (Supplementary Fig. S3A). In contrast, Eve and Omip treatment reduced these contractile and matrix proteins while increasing the abundance of inflammatory cytokines and growth factors such as IL1 β , IL33, LIF, CXCL1, and FGF2. Additionally, Nav and Lum also led to increased expression of several inflammatory proteins. Similarly, immunoblot analysis of α SMA expression (encoded by ACTA2) in CAFs treated with seven different inhibitors confirmed a significant reduction in α SMA levels with PI3K/AKT/mTOR inhibitors, whereas levels increased dramatically with the MEK inhibitor (Supplementary Fig. S3B). To further validate the phenotypic alterations in CAFs following treatment with inhibitors, we compared the proteomics data of CAFs with the integrated iCAF/myCAF gene signatures established in Fig. 1D, as well as signatures derived from three previous studies (Fig. 2D; refs. 13–15). GSEA revealed that Omip, Eve, Lum, and Nav significantly induced an iCAF phenotype. Each of these four inhibitors exhibited inhibitory effects on either CAF metabolic activity or outgrowth (Fig. 2B). In contrast, CAFs treated with Tram exhibited positive enrichment for myCAF signatures, whereas the ROCK inhibitor Y-27632 seemed to inhibit the myCAF phenotypes. Interestingly, nintedanib and lenvatinib, broad-spectrum tyrosine kinase inhibitors with activity against PDGFRs, FGFRs, and VEGFRs, suppress both the myCAF and iCAF phenotypes.

To validate the key findings obtained with MEK and PI3K/mTOR inhibition in the hTERT culture, we used the early-passage patient-derived cultures (Supplementary Fig. S2A and S2B). These experiments reproduced the patterns observed in the hTERT culture: treatment with Eve or Omip consistently induced iCAF marker expression and enhanced secretion of proinflammatory cytokines (Supplementary Fig. S2E and S2F). In contrast, Tram promoted the expression of myCAF markers and stimulated a contractile phenotype (Supplementary Fig. S2G and S2H), thereby reinforcing the distinct functional outcomes of PI3K/mTOR versus MEK inhibition. Further proof-of-concept confirmation was obtained by an *ex vivo* treatment of PDTFs, in which CAFs maintain a spatial configuration embedded within their native TME architecture. Peritoneal metastases from colorectal cancer are particularly stroma-rich, containing higher levels of CAFs than primary tumors (48), making them an ideal model to assess the impact of pharmacologic interventions on CAF phenotypes in colorectal cancer. PDTFs were established from resected peritoneal metastatic lesions of one patient with

(Continued.) phenotype associated proteins are labeled. Differentially abundant proteins were determined based on an absolute log₂ (FC) value >1 and $P < 0.05$ between the two groups. **C**, Heatmap depicting the GSEA of significantly enriched gene sets in CAFs treated with Eve or Omip. Significant enrichments were identified based on normalized enrichment score (NES) with an absolute value >1 and FDR <0.05. **D**, GSEA plots illustrating the Hallmark_mTORC1 signaling pathway, comparing myCAF and iCAF populations in integrated scRNA-seq datasets. **E**, qRT-PCR analysis of myCAF and iCAF marker expression in CAFs treated with Omip (100 nmol/L), Eve (1 μ mol/L), and uprosertib (1 μ mol/L) for 24 hours. Data were analyzed using unpaired Student *t* test, comparing each treatment group with the DMSO control. **F**, Experimental layout of the tumor spheroid growth (assay A), collagen gel invasion (assay B), and neutrophil transwell migration (assay C). **G**, Two colorectal cancer spheroids treated with control medium (DMEM), CM from CAFs (CAF-CM), or CM of CAFs pretreated with Eve (Eve-CAF-CM). Representative images and the corresponding quantification of spheroid proliferation under each condition. **H**, Evaluation of neutrophil migration toward CAF-CM or Eve-CAF-CM. Each dot corresponds to results using neutrophils from one healthy donor. Statistical significance was established using unpaired Student *t* test, with each group normalized and then compared with the CAF-CM group. **I**, Longitudinal BLI monitoring tumor growth in a CT26-Luc peritoneal metastasis mouse model treated with vehicle or Eve (5 mg/kg). Representative BLI images are shown, along with the quantification of total luminescent radiance (photons/second/cm²/steradian) emitted from the peritoneal cavity ($n = 5$ per group). Data were analyzed using unpaired Student *t* test on day 14. **J** and **K**, Representative IHC images and quantification of IL1 β and α SMA staining (**J**), as well as CD11b and Ly6G staining (**K**), of vehicle- and Eve-treated tumor sections from omentum and mesentery metastasis (Scale bar, 25 μ m). Data were analyzed using unpaired Student *t* test. The results are presented as the mean $\pm$ SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, nonsignificant. **F**, Created in BioRender. Xia, Z. (2025) <https://BioRender.com/y43s102>.

colorectal cancer and *ex vivo* treated with vehicle, Tram, or Eve. PDTFs from each condition were harvested for scRNA-seq (Supplementary Fig. S3C). Resulting UMAP clusters were annotated to cell types using canonical markers (Fig. 2E), and the CAF cluster was extracted for further analysis. Hallmark pathway analysis confirmed that Tram significantly downregulated MAPK activity in CAFs, whereas Eve selectively reduced PI3K/mTOR and mTORC1 signaling and upregulated MAPK signaling (Supplementary Fig. S3D). Consistent with results from CAF aggregate cultures, scRNA-seq demonstrated induction of myCAF markers following Tram treatment, whereas iCAF markers showed increased abundance in response to Eve (Fig. 2F).

PI3K/mTOR inhibition mediates a phenotypic switch toward an iCAF subtype

Dysregulation of the PI3K/AKT/mTOR signaling pathway is seen in 40% to 60% of patients with colorectal cancer and presents an attractive therapeutic target (49, 50). Additionally, the PI3K/AKT/mTOR pathway primarily promotes the transition of various precursor cell types into CAFs (22). A significant inhibition of CAF metabolic activity was observed upon blocking the PI3K/mTOR pathway without induction of cell death (Figs. 2B and 3A). Volcano plot analysis of CAF spheroids treated with Eve and Omip revealed a reduction in myCAF-associated proteins and an induction of iCAF-related inflammatory factors, which support the Luminex data (Figs. 2C and 3B; Supplementary Fig. S3A). Consistently, GSEA revealed strong enrichment of signatures associated with inflammatory pathways—including TNF α /NF- κ B, inflammatory response, cytokine activity, and the complement cascade—in Eve- and Omip-treated CAFs, all of which were also upregulated in iCAFs. In contrast, myCAF-related pathways, such as muscle contraction and focal adhesion, along with cell cycle and DNA replication pathways, as well as PI3K/AKT/mTOR and mTORC1 signaling, were significantly downregulated (Fig. 3C; Supplementary Fig. S4A). In agreement with Fig. 1H, analysis of CAF from five integrated scRNA-seq datasets of human colorectal cancer revealed that PI3K/AKT/mTOR and mTORC1 signaling were selectively elevated in myCAF compared with iCAF (Fig. 3D; Supplementary Fig. S4B). qRT-PCR analysis of CAFs further confirmed that blockade of the PI3K/AKT/mTOR pathway led to increased levels of iCAF markers (*IL1A*, *IL1B*, *CXCL1*, *CXCL5*, *IL6*, and *LIF*), with no change in myCAF markers (*ACTG2* and *COL1A1*; Fig. 3E). Moreover, GSEA of bulk RNA-seq data confirmed that PI3K/AKT/mTOR inhibition in CAF cultures significantly enriched iCAF signatures while downregulating myCAF signatures (Supplementary Fig. S4C).

We next investigated whether Eve-treated CAFs affect colorectal cancer cell behavior by assessing the growth and invasion of colorectal cancer cells in 3D spheroid assays (Fig. 3F). Consistent with previous studies, CM from CAFs (CAF-CM) alone significantly promoted 3D colorectal cancer spheroid growth (Supplementary Fig. S4D). Tumor spheroids exposed to CM from CAFs pretreated with Eve (Eve-CAF-CM) grew even larger (Fig. 3G) and showed more invasion into type I collagen (Supplementary Fig. S4E) compared with those treated with CAF-CM. CXCL1, known to be a crucial chemokine implicated in the recruitment of neutrophils, was observed to be dramatically upregulated in CAFs following PI3K or AKT or mTOR inhibitor treatment (Fig. 3B and E). We then examined the impact of Eve-CAF-CM on neutrophil migration *in vitro* (Fig. 3F). The attraction of human PMN leukocytes (PMNs or neutrophils) was significantly enhanced by CAF-CM (Supplementary Fig. S4F and S4G), and this effect was further markedly increased by Eve-CAF-CM (Fig. 3H).

To further evaluate the role of mTORC1 signaling in CAFs *in vivo*, we administered the mTOR inhibitor Eve to tumor-bearing mice using the syngeneic CT26-Luc colorectal cancer peritoneal metastasis model. Treated mice exhibited an increased tendency for tumor growth/peritoneal metastasis on day 14, although this was not statistically significant (Fig. 3I). Interestingly, Eve significantly increased IL1 β expression in stromal regions between the epithelial cell nests, whereas α SMA levels were significantly reduced in both omental and mesenteric metastases (Fig. 3J; Supplementary Fig. S4H). This suggests that mTOR inhibition may drive a shift in the CAF population from a myCAF phenotype to a more iCAF state. To confirm that there is a shift in CAF phenotype rather than a specific killing of the myCAF population, co-IF with α SMA and IL1 β was performed. This showed a dual positivity for α SMA and IL1 β in the CAFs, confirming the phenotypic shift (Supplementary Fig. S4I). Consistently, the infiltration of neutrophils was significantly increased in both omental and mesenteric metastases of Eve-treated mice, as shown by the CD11b and Ly6G levels (Fig. 3K). Together, these findings support the conclusion that PI3K/AKT/mTOR pathway inhibitors induce the iCAF phenotype, thereby enhancing neutrophil migration.

PI3K/mTOR inhibition induces iCAF phenotype through FGF2/FGFR pathway activation

The marked increased expression and release of FGF2 (Figs. 2C, F, and 3B) may serve as a compensatory mechanism to counteract PI3K/mTOR inhibitor reduced metabolic activity as measured by ATP content. Interestingly, Chen and colleagues (45) and Ma and colleagues (41) demonstrated increased expression of FGF2 and FGF7 in iCAFs in bladder cancer. Consistent with their findings, we also observed higher expression levels of FGF2 and FGF7 in fibroblasts compared with other cell types in colorectal cancer, particularly in iCAFs. Furthermore, *FGFR1* was more abundantly expressed in iCAFs compared with myCAFs (Fig. 4A; Supplementary Fig. S5A). FGFR1 binds FGF, including iCAF-derived FGF2 and FGF7. In agreement, adding an FGF2-neutralizing antibody to PI3K/mTOR inhibitor-treated CAF further suppressed CAF ATP content, suggesting that PI3K/mTOR inhibitor-induced elevation of FGF2 supports CAF metabolic activity (Supplementary Fig. S5B). Additionally, FGFs have been shown to induce an iCAF phenotype (51, 52). Similarly, we observed that recombinant FGF2 (rhFGF2, 1 ng/mL) increased iCAF marker levels and decreased myCAF marker expression (Fig. 4B). Moreover, FGFR1 phosphorylation and IL1 β levels were significantly increased in CAFs treated with either PI3K/AKT/mTOR inhibitors or varying doses of rhFGF2 (Fig. 4C). Additionally, a strong positive correlation was observed between FGF2 and *IL1B* expressions, as well as the iCAF score in patients with colorectal cancer from TCGA and GSE39582 datasets (Fig. 4D; Supplementary Fig. S5C). Blocking the FGF2/FGFR1 pathway with a neutralizing antibody or the selective pan-FGFR tyrosine kinase inhibitor erdafitinib significantly reduced the PI3K/AKT/mTOR inhibitor-induced upregulation of iCAF markers (Fig. 4E; Supplementary Fig. S5D). This intervention also reversed colorectal cancer spheroid growth and neutrophil migration stimulated by Eve-CAF-CM (Fig. 4F–H; Supplementary Fig. S5E and S5F). Mechanistically, we observed that phosphorylation of FGFR1 at Tyr653/654 and its downstream JAK2/STAT3 signaling, a pathway critical for iCAF formation, were both increased by PI3K/AKT/mTOR inhibitors. Blocking FGFR significantly inhibited

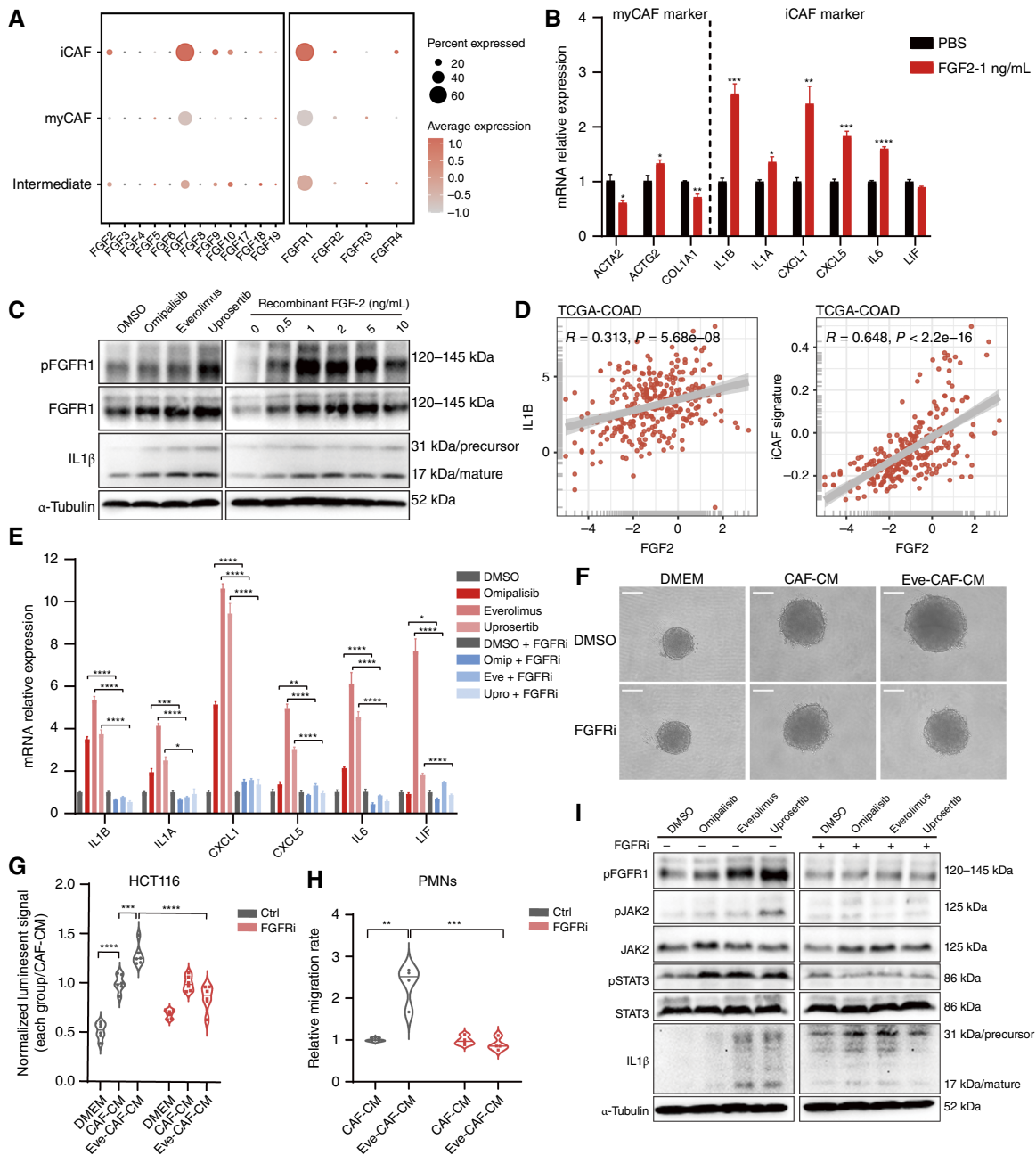
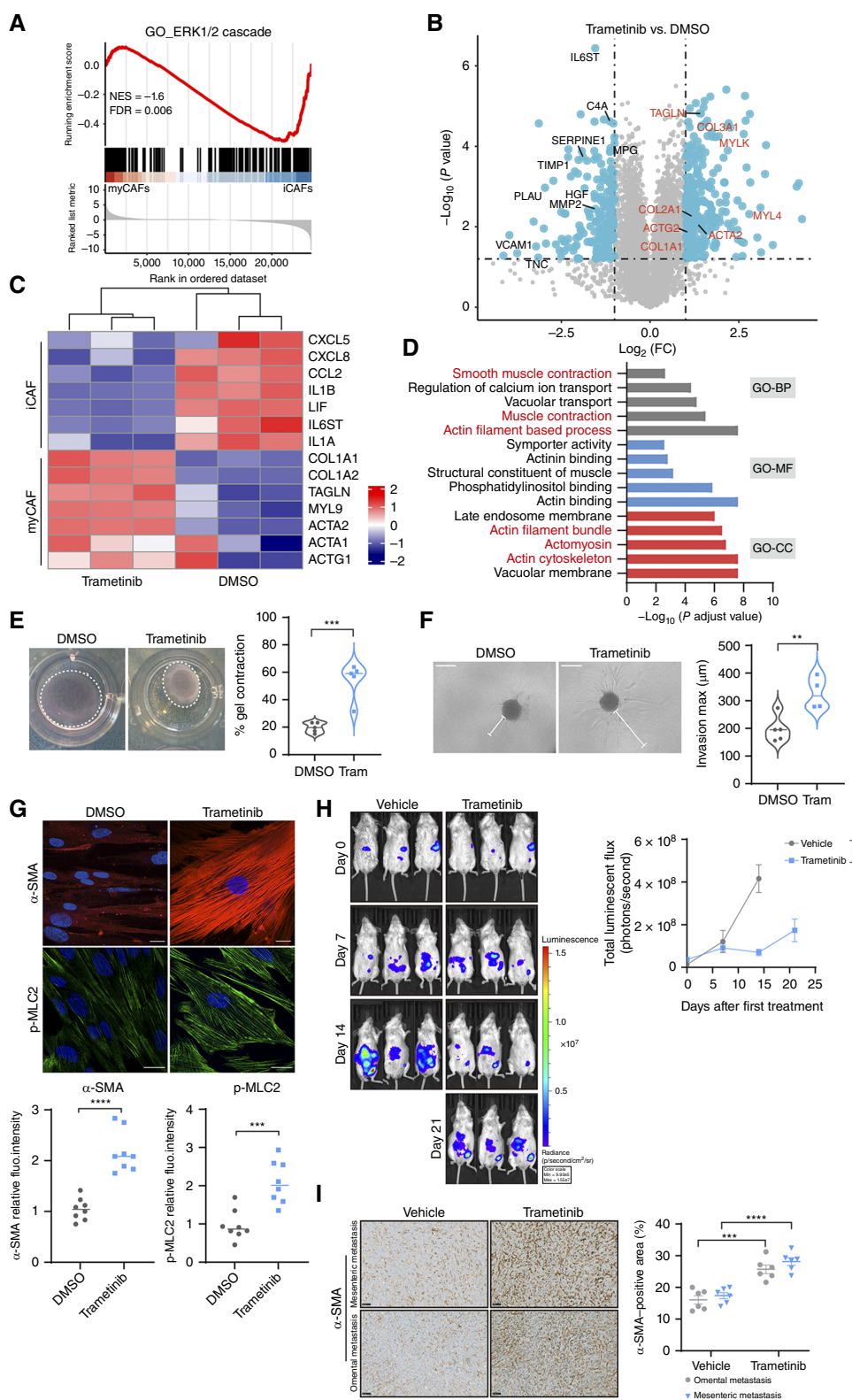


Figure 4.

PI3K/mTOR inhibition induces iCAF phenotype through FGF2/FGFR pathway activation. **A**, Dot plot illustrating the expression levels of *FGFs* and their receptors (*FGFRs*) across the different CAF subtypes identified in the integrated scRNA-seq dataset. Each dot's color represents the gene's average expression level across the cluster, whereas its size indicates the percentage of cells within the cluster that express the gene. **B**, mRNA expression of myCAF and iCAF markers in CAFs treated with recombinant FGF2 (rhFGF2, 1 ng/mL) for 24 hours. Data were analyzed using unpaired Student *t* test. **C**, Western blot analysis of Tyr653/654 phosphorylation of FGFR1 (pFGFR1), FGFR1, and IL1 β in CAFs treated with Omp, Eve, Upro, or varying doses of rhFGF2 for 24 hours. α -Tubulin was implemented as a loading control. **D**, Correlation analysis between *FGF2*, *IL1B* expression, and iCAF scores in patients with colorectal cancer from the TCGA-COAD dataset. Red plotting symbols represent individual samples. Correlation coefficients (*R*) and *P* values were calculated using Spearman's correlation analysis. **E**, qRT-PCR analysis of iCAF markers expression in CAFs treated with Omp, Eve, and uposertib (Upro), with or without the pan-FGFR inhibitor erdafitinib (500 nmol/L) for 24 hours. **F** and **G**, HCT116 cells were cultured as spheroids and treated with either control medium, or CAF-CM/Eve-CAF-CM, both in the presence and absence of pan-FGFR inhibitor for 5 days. Representative images (**F**) and the corresponding quantification (**G**) of spheroid proliferation under each condition. **H**, Evaluation of neutrophil migration toward CAF-CM or Eve-CAF-CM in the presence and absence of pan-FGFR inhibitor. Each dot corresponds to results using neutrophils from one healthy donor. Statistical significance was established using unpaired Student *t* test, with each group normalized and then compared with the CAF-CM group. **I**, Western blot analysis of pFGFR1, pJAK2/JAK2, pSTAT3/STAT3, and IL1 β in CAFs treated with Omp, Eve, and uposertib in the presence or absence of pan-FGFR inhibitor for 24 hours. α -Tubulin was implemented as a loading control. Error bars represent mean $\pm$ SEM from at least triplicate. *, *P* $\leq$ 0.05; **, *P* $\leq$ 0.01; ***, *P* $\leq$ 0.001; ****, *P* $\leq$ 0.0001.

**Figure 5.**

MEK1/2 inhibition mediates a phenotypic shift toward a myCAF subtype. **A**, GSEA plots illustrating the GO_ERK1/2 cascade, comparing myCAF and iCAF populations in the integrated scRNA-seq dataset. **B**, Volcano plots of differentially abundant proteins in CAFs treated with Tram for 5 days. myCAF phenotype associated proteins are labeled with red color. Differentially abundant proteins were determined based on an absolute $-\log_{10}(P \text{ value})$. (Continued on the following page.)

FGFR1 phosphorylation and the JAK2/STAT3 pathway, leading to disruption of mature IL1 β production (Fig. 4I). Collectively, these data strongly support the notion that the formation of iCAFs induced by PI3K/mTOR inhibitors is dependent on the activation of the FGF2/FGFR1 pathway (Supplementary Fig. S5G).

MEK1/2 inhibition mediates a phenotypic shift toward a myCAF subtype

Dysregulation and overactivation of the MAPK pathway, also known as the RAS–RAF–MEK–ERK pathway, are implicated in approximately one thirds of all cancers, including colorectal cancer. Targeting this pathway has emerged as a promising therapy (53). Interestingly, the MAPK/ERK signaling pathway is differentially enriched in iCAFs compared with myCAFs (Figs. 1H and 5A; Supplementary Fig. S6A). Following MEK1/2 inhibition by Tram, CAF spheroids exhibited an increase in myCAF-associated proteins (Fig. 5B; Supplementary Fig. S3A). Bulk RNA-seq analysis further confirmed the induction of myCAF marker genes and the down-regulation of iCAF marker genes in Tram-treated CAFs (Fig. 5C). Additionally, GSEA exhibited a strong enrichment of myCAF-related signatures, including smooth muscle contraction, actomyosin, and actin cytoskeleton, in Tram-treated CAFs (Fig. 5D; Supplementary Fig. S6B). Collagen gel contraction and invasion assays demonstrated that CAFs treated with Tram displayed a significantly enhanced ability to contract and invade the ECM (Fig. 5E and F). IF staining revealed significantly higher presence of α SMA and phospho-myosin light chains 2 (pMLC2; actomyosin contractility) organized stress fibers, following Tram treatment (Fig. 5G; Supplementary Fig. S6C). Corroborating with pharmacologic targeting of MEK1/2, immunoblot analysis shows that RNA interference-mediated knockdown of MEK1, MEK2, or both dramatically increased α SMA expression (Supplementary Fig. S6D).

To determine the role of MAPK signaling in CAFs *in vivo*, we treated tumor-bearing mice with Tram for 20 days using the CT26-Luc peritoneal metastasis model. Contrary to what was observed in Eve, BLI showed a significant reduction in tumor growth/peritoneal metastasis following treatment with Tram compared with vehicle, although a slight acceleration in growth was observed after 14 days treatment (Fig. 5H). Histologically, this treatment led to significantly increased levels of α SMA in both omental and mesenteric metastatic niches (Fig. 5I), suggesting that MEK inhibition promotes the induction of myCAFs *in vivo*.

MEK1/2 inhibition enhances collagen deposition and ECM remodeling

MyCAFs are characterized not only by actomyosin-mediated contractility but also by increased ECM remodeling and deposition

(13). Indeed, treatment of CT26 peritoneal metastasis bearing mice with the MEK1/2 inhibitor resulted in a substantial increase in collagen deposition as revealed by Trichrome and Sirius red staining (Fig. 6A). Gene Ontology (GO) analysis and GSEA revealed that the upregulated genes in Tram-treated CAF cultures were associated with collagen-containing ECM, ECM organization, and extracellular structure organization (Fig. 6B; Supplementary Fig. S7A). Utilizing the ECM knowledge database, MatrisomeDB (54), we found that 58 matrisome-associated genes were upregulated and 40 were downregulated in the Tram-treated group (Fig. 6C). Tram upregulated 26 core matrisome genes in CAFs, including nine collagens (e.g., COL1A1, COL1A2, COL3A1, and COL4A1), 11 glycoproteins (e.g., MGP, NTN1, CRISPLD2, MFAP4, and IGFBP5), and six proteoglycans (e.g., DCN, LUM, and PODN). ECM-affiliated genes regulated by Tram were linked to cytoskeleton regulation, including members of the semaphorin–plexin system (e.g., SEMA3A, SEMA3B, and SEMA6A). Moreover, 19 upregulated ECM regulators induced by Tram play prominent roles in ECM degradation, including members of the matrix metalloproteinase (MMP) family and their regulators. Collectively, these data indicate that Tram can significantly alter the transcriptional profile associated with collagen deposition and ECM remodeling in CAFs.

To further investigate the role of Tram in CAFs involved in matrix production and remodeling, we extracted and collected decellularized ECM (Fig. 6D). Mass spectrometry analysis revealed an overall increase in ECM component deposition in the Tram-treated group, in concordance with the RNA-seq data (Fig. 6E). The N-hydroxysuccinimide (NHS)-tagged dye binds ECM proteins via amine-reactive cross-linking. IF staining with NHS ester shows that Tram altered matrix alignment, shifting the ECM structure from anisotropic to isotropic (Fig. 6F). IF staining and immunoblot analysis of ECM labeled with antibodies against COL1A1, COL3A1, COL4A1, and fibronectin confirmed a significant increased deposition of collagens in the Tram-treated group, also marked by a disorganized pattern with larger void spaces (Fig. 6E and F; Supplementary Fig. S7B and S7C). These ECM structural alterations may be attributed to force-mediated matrix remodeling (55, 56), driven by Tram-induced enhancement of actomyosin contractility. In addition, Luminex MMP profiling of a panel of nine MMPs and four tissue inhibitors of metalloproteinases (TIMP) revealed a significant increased secretion of MMP10 and TIMP2 in the supernatant of CAFs treated with Tram for 3 days (Supplementary Fig. S7D). Lastly, to further explore whether the ECM remodeled by Tram (Tram-ECM) influenced the behavior of colorectal cancer cells, colorectal cancer cells were seeded on top of the decellularized ECM (Fig. 6D). The colony formation assay showed no major change in the number of colonies but

(Continued.) log₂ FC value > 1 and $P < 0.05$ between the two groups. **C**, Heatmap showing the mRNA expression of myCAF and iCAF markers in CAF treated with Tram for 48 hours. The color of each cell represents the relative abundance of the gene. **D**, Bar graph showing the top 5 significantly enriched GO terms across biological process (BP), cellular component (CC), and molecular function (MF) categories in Tram-treated CAFs, as identified through GSEA of the proteomics data. Significant enrichments were determined based on NES >1 and FDR <0.05. **E** and **F**, Representative images and quantification of collagen gel contractility (**E**) and invasion (**F**) in CAFs treated with DMSO or Tram. Scale bar, 200 μ m. Statistical significance was determined using unpaired Student *t* test. **G**, Representative images and quantification of the cellular cytoskeleton in CAFs following treatment with Tram or DMSO, visualized by α SMA and pMLC2 staining. Nuclei are counterstained with DAPI. Statistical significance was determined using unpaired Student *t* test. Scale bar, 20 μ m. **H**, Longitudinal BLI monitoring tumor growth in a CT26-Luc peritoneal metastasis model treated with either vehicle or Tram (1 mg/kg). Representative BLI images are shown, along with the quantification of total luminescent radiance (photons/second/cm²/steradian) emitted from the peritoneal cavity ($n = 6$ per group). Data were analyzed using unpaired Student *t* test on day 14. **I**, Representative IHC images and quantification of α SMA staining in vehicle- and Tram-treated tumor sections from omental and mesenteric metastases (Scale bar, 50 μ m). Data were analyzed using unpaired Student *t* test. The results represent mean $\pm$ SEM. **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$.

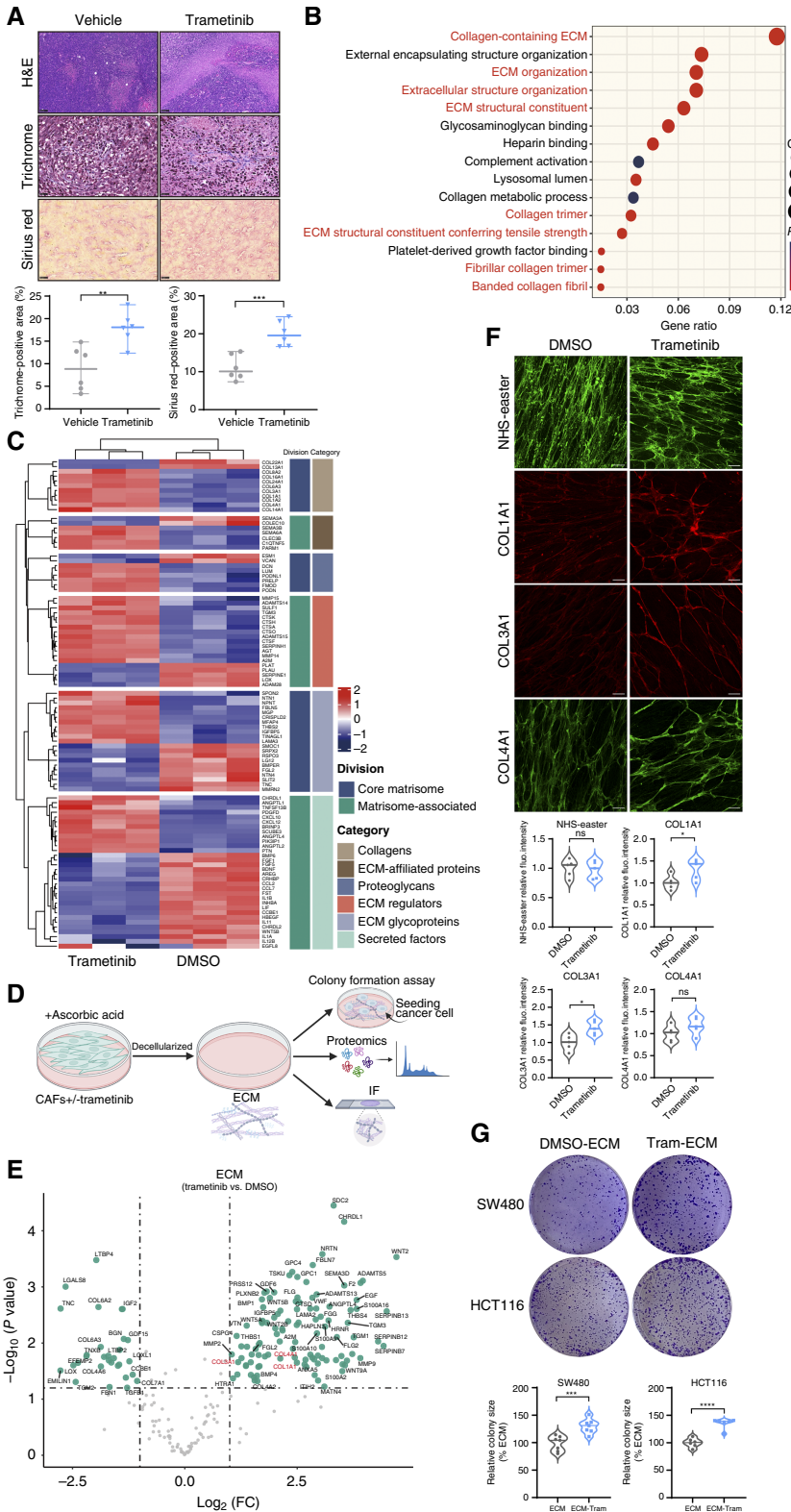
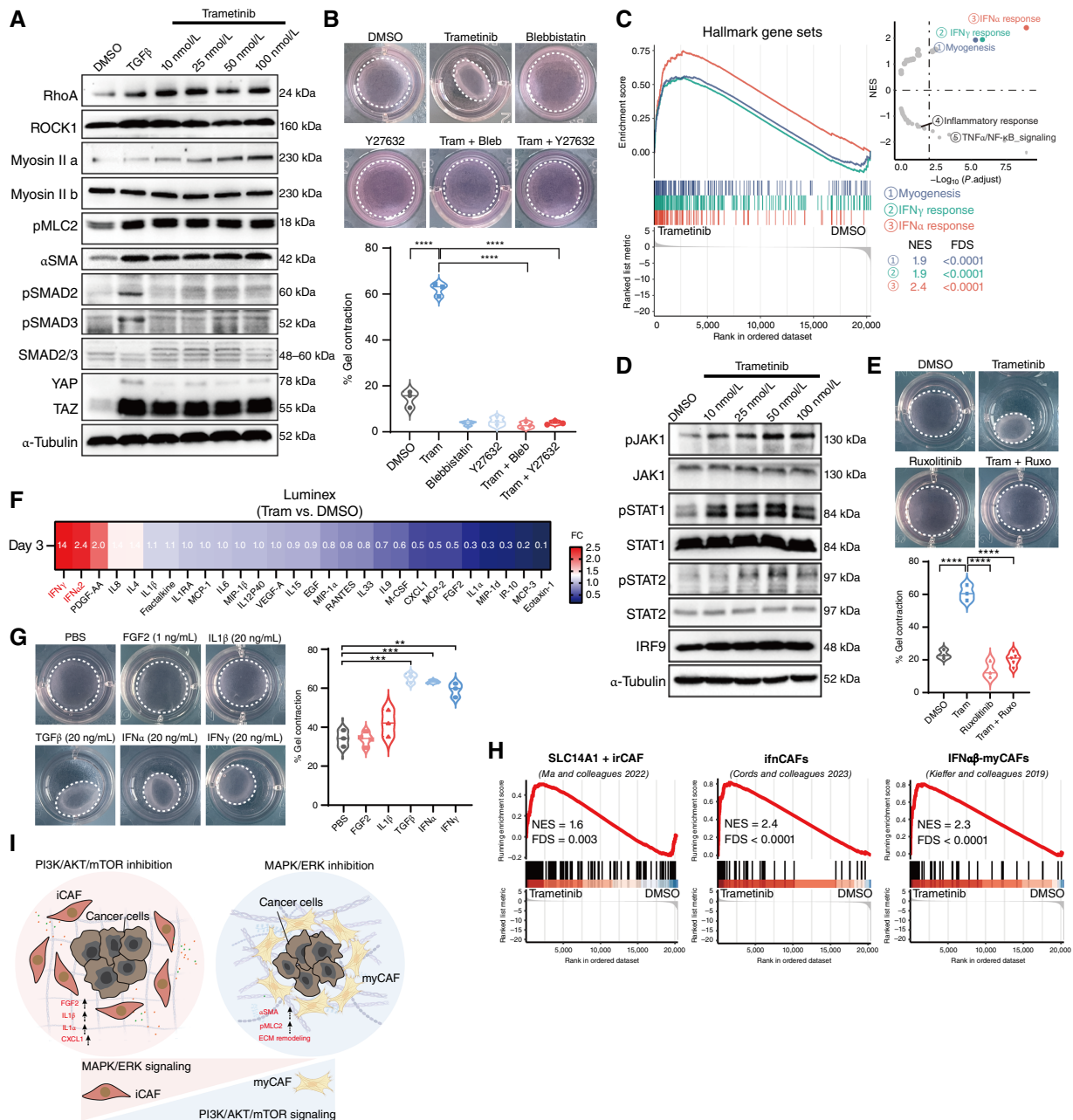


Figure 6. MEK1/2 inhibition enhances collagen deposition and ECM remodeling. **A**, Representative images and quantification of hematoxylin and eosin (H&E), Masson's trichrome, and picosirius red staining of CT26-Luc tumor sections from vehicle- and Tram-treated groups ($n = 6$ per group). Scale bar, 100 μm for hematoxylin and eosin, 25 μm for trichrome and Picosirius red. **B**, Dot plot showing the top 15 GO enrichment terms in Tram-treated CAFs based on bulk RNA-seq data. Dot size indicates the number of genes overlapping each GO term, whereas color represents the adjusted P values. **C**, Heatmap illustrating the mRNA expression of ECM-related genes in CAFs treated with Tram or DMSO. The color of each cell represents the relative abundance of the gene. **D**, Experimental layout for the extraction and identification of decellularized ECM derived from CAFs. **E**, Volcano plots of differential ECM-related proteins in decellularized ECM from CAFs treated with or without Tram for 8 days. Differentially abundant proteins were determined based on an absolute $\log_2 FC$ value > 1 and $P < 0.05$ between the two groups. Red-marked proteins are further analyzed for spatial organization in the next panel. **F**, High-resolution confocal images and quantification of the decellularized ECM extract from DMSO- and Tram-treated CAFs, labeled with Alexa Fluor 488 NHS Ester and antibodies against COL1A1, COL3A1, and COL4A1. Statistical significance was determined using unpaired Student t test. Scale bar, 20 μm . **G**, Representative images and quantification of colony formation assay for SW480 and HCT116 cells seeded on decellularized ECM derived from DMSO- or Tram-treated CAFs. Statistical significance was assessed using unpaired Student t test, with results presented as the mean $\pm$ SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. **D**, Created in BioRender. Xia, Z. (2025) <https://BioRender.com/a69u780>.

instead an increased size of the colonies, most probably due to increased colorectal cancer cell proliferation on Tram-ECM compared with DMSO-ECM (Fig. 6G). Overall, these experiments

demonstrated that the MEK1/2 inhibitor enhances matrix production and remodeling, with ECM-derived signals supporting colorectal cancer cell growth.

**Figure 7.**

ROCK and IFN signaling cooperate to regulate MEK1/2 inhibitor-mediated actomyosin contractility. **A**, Western blot analysis of RhoA/ROCK, myosin IIa/b, pSMAD2/3, αSMA, and YAP/TAZ expression in CAFs treated with various doses of Tram, with TGFβ (20 ng/mL) as a positive control. α-Tubulin was used as a loading control. **B**, Representative images and quantification of collagen gel contraction assays of CAFs treated with Tram, with or without blebbistatin (10 μmol/L) or Y27632 (10 μmol/L). Statistical significance was assessed using ANOVA test. **C**, GSEA of Tram-treated vs. DMSO-treated CAFs using MSigDB hallmark gene sets. Left shows the running enrichment scores of the top three significant pathways, whereas right displays a volcano plot of the normalized enrichment score (NES) against $\log_{10}$ of the adjusted *P* value. **D**, Western blot analysis of IFNs response signaling pathways in CAFs treated with increasing doses of Tram. α-Tubulin was used as a loading control. **E**, Representative images and quantification of collagen gel contraction assays of CAFs treated with Tram, with or without the JAK1/2 inhibitor ruxolitinib (10 μmol/L). Statistical significance was assessed using ANOVA. **F**, Luminex analysis of human cytokine/chemokine profiles in supernatants from CAF spheroids treated with or without Tram for 3 days. The heatmap displays the FC ranking of cytokines between the Tram- and DMSO-treated groups. **G**, Representative images and quantification of collagen gel contraction assays of CAFs treated with recombinant TGFβ (20 ng/mL), IL1β (20 ng/mL), FGF2 (1 ng/mL), IFNα (20 ng/mL), and IFNγ (20 ng/mL). Statistical significance was assessed using ANOVA. **H**, GSEA plots for SLC14A1+ irCAF, IFNCAF, and IFNαβ-myCAF signatures in Tram-treated vs. DMSO-treated CAFs. **I**, Schematic summary of the phenotypic switch in colorectal CAFs induced by PI3K/mTOR and MEK1/2 inhibitors. The results represent mean ± SEM. **, *P* ≤ 0.01; ***, *P* ≤ 0.001; ****, *P* ≤ 0.0001.

ROCK and interferon signaling cooperate to regulate MEK1/2 inhibitor-mediated actomyosin contractility

Actomyosin contractility in CAFs is driven by the phosphorylation of MLC2 and the activity of myosin II, downstream of the Rho/ROCK signaling pathway (55). In this study, immunoblot analysis showed a significant increase in the expression of RhoA, ROCK1, myosin IIa, pMLC2, and α SMA in CAFs treated with TGF β (as a positive control) or varying doses of Tram (Fig. 7A). However, no increase in phospho-SMAD2/3, the downstream effector of canonical TGF β signaling, was observed in CAFs treated with Tram. Additionally, the interaction between actin and myosin II transmits mechanical cues to the nucleus, with YAP/TAZ transcriptional pathways functioning downstream of RhoA/ROCK-mediated actin cytoskeletal rearrangements (56, 57). Notably, we observed increased expression of YAP/TAZ, as well as activation of the FAK pathway in Tram-treated CAFs (Fig. 7A; Supplementary Fig. S8A). Furthermore, the increased contractility driven by Tram was inhibited by the ROCK inhibitor Y-27632 or the myosin II inhibitor blebbistatin, but not by the TGF β receptor inhibitor SB-431542 (Fig. 7B; Supplementary Fig. S8B), suggesting that Tram induces the myCAF phenotype independently of TGF β signaling.

GSEA revealed strong enrichment of pathways associated with interferon (IFN) α and IFN γ responses, accompanied by downregulation of inflammatory pathways in Tram-treated CAFs (Fig. 7C). Consistent with these findings, we confirmed the upregulation of IFN-inducible genes (e.g., *IFIT1*, *IFIT3*, *GBP2*, and *CXCL10*), along with activation of the downstream JAK/STAT signaling cascade in Tram-treated CAFs (Fig. 7D; Supplementary Fig. S8C). Additionally, STAT1, a central transcription factor in the IFN response pathway, exhibited increased activation and nuclear translocation in Tram-treated CAFs (Supplementary Fig. S8D). ROCK and JAK1 kinase cooperate to induce actomyosin contractility in CAFs (58). Consistently, Tram-mediated collagen gel contraction was strongly inhibited by the JAK1 inhibitor ruxolitinib (Fig. 7E), or by RNA interference-mediated depletion of *JAK1*, *STAT1*, and *STAT2*, but not by depletion of *JAK2* (Supplementary Fig. S8E and S8F). These findings indicate that ROCK and JAK1 mediate Tram-induced actomyosin contractility.

To further confirm the direct role of IFNs in Tram-treated CAFs, we observed a significant increased secretion of IFN α 2 and IFN γ in the supernatant of CAFs treated with Tram for 3 days, as measured by Luminex cytokine profiling and further validated by qRT-PCR analysis (Fig. 7F; Supplementary Fig. S8G). In contrast, some inflammatory cytokines, including IL1 α , IL1 β , IL6, and IL8, were either inhibited or showed no change in the Tram-treated groups (Fig. 7F). scRNA-seq analysis revealed that the receptors for IFN α 2 (*IFNAR1* and *IFNAR2*) and IFN γ (*IFNGR1* and *IFNGR2*) were broadly expressed in both myCAF and iCAF, with *IFNAR2* and *IFNGR2* selectively higher in myCAF (Supplementary Fig. S8H). Additionally, similar to TGF β , IFN α 2 and IFN γ effectively increased CAF contractility via activation of the RhoA/ROCK pathway, whereas IL1 β and FGF2 had no effect (Fig. 7G; Supplementary Fig. S8I). Lastly, we observed an increase in phosphorylated STING, TBK1, and IRF3 in CAFs following Tram treatment, core molecules that play a critical role in driving IFN responses (Supplementary Fig. S8J). Notably, pharmacologic inhibition of STING/TBK1 blocked the Tram-mediated activation of JAK/STAT signaling and collagen gel contraction (Supplementary Fig. S8K and S8L). Several studies have reported the existence of a subpopulation of IFN-licensed CAFs characterized by a strong response to IFNs (40, 41, 43). In

support, we found that IFN $^{+}$ CAF signatures from previous studies were strongly enriched in Tram-treated CAFs (Fig. 7H), suggesting that MEK1/2 inhibition regulates this phenotypic switch (Supplementary Fig. S8M).

Discussion

Despite advances in characterizing CAF heterogeneity in PDAC (13), breast cancer (40, 59), and lung cancer (60), a comprehensive analysis of colorectal CAFs remains lacking. In colorectal cancer, two main CAF subtypes have been described—ECM-related CAF-A and myofibroblast-like CAF-B. However, the relatively small number of CAFs (30–800 cells) analyzed in prior study may hamper the detection of the full spectrum of CAF subpopulations (35). In this study, we analyzed CAF heterogeneity in human colorectal cancer by integrating five public datasets (>12,000 fibroblasts) into a batch-corrected atlas, identifying distinct myCAF and iCAF clusters with divergent signaling. apCAFs were not detected, as CD74 and HLA-DR were broadly expressed across all CAFs, consistent with known MHC II expression by colonic fibroblasts in the lamina propria that enables antigen presentation and Treg induction (61, 62). Our findings suggest that these resident mesenchymal cells of the colon may be co-opted by cancer cells to evolve into CAFs. Additionally, consistent with previous studies (20, 63), we found that both myCAFs and iCAFs were associated with poor prognostic features in patients with colorectal cancer. IL1/STAT3 and TGF β signaling have been identified as two opposing pathways driving the formation of iCAFs and myCAFs, respectively (15). Our findings confirm the activity of these pathways in colorectal cancer myCAF and iCAF subpopulations, further suggesting that the key characteristics of these CAF subsets are conserved across various organs, cancer types, and species. Additionally, we observed a significant increased abundance of PI3K/mTOR signaling in myCAFs and marked elevation of MAPK/ERK signaling in iCAFs. Although PI3K/mTOR and MAPK/ERK signaling drive tumor progression and are therapeutic targets in cancer cells, their roles, and drug responses in CAFs remain unclear.

Early studies show that mTOR inhibition with SOM230 in pancreatic CAFs reduces cytokine secretion such as IL6 (64). In this study, we observed that PI3K/mTOR inhibitors significantly induced the secretion of FGF2 and inflammatory cytokines, including IL1 β , IL1 α , and CXCL12, in CAFs (Figs. 2C and 7I). GSEA revealed enrichment of the iCAF signature and inflammatory response pathways in CAFs treated with PI3K/mTOR inhibitors. In agreement, treatment with Eve led to a significant reduction in α -SMA levels, along with downregulation of the myCAF signature.

The activation of FGF and TGF β pathways was shown to regulate opposing key effectors in CAFs: TGF β activation drives the myCAF phenotype, whereas FGF activation promotes the iCAF phenotype, enhancing inflammation (51). Consistently, we confirmed the critical role of FGF in iCAF formation via the FGFR1/JAK2/STAT3 pathway. Specifically targeting the FGF2/FGFR pathway in Eve-treated CAFs reduced tumor spheroid growth and neutrophil infiltration. Although these findings are based on pharmacologic inhibition, we acknowledge the potential for genetic loss-of-function as an important complementary strategy. Although 40% to 60% of patients with colorectal cancer exhibit dysregulation of the PI3K/AKT/mTOR pathway, the clinical efficacy of Eve in treating advanced colorectal cancer has been limited (49). Our findings provide a strong rationale for further investigation of

combined FGFR inhibition and Eve treatment in both preclinical and clinical settings.

BRAF^{V600E} mutations are present in approximately 10% of colorectal cancer and confer poor prognosis in the metastatic setting (65). However, unlike BRAF-mutant melanoma, metastatic colorectal cancer with the same mutations has demonstrated a significant lack of sensitivity to BRAF or MEK inhibitor monotherapy in clinical trials (66). The BRAF inhibitor PLX4720 activates CAFs, enhancing ECM remodeling and FAK-dependent survival signaling in melanoma. Coinhibition of BRAF and FAK effectively blocks ERK reactivation in melanoma (67). MEK inhibition with Tram in CAFs induces a contractile, myofibroblast-like ECM-remodeling phenotype (Fig. 7I). In the KRAS^{G12C}-mutant CT26-luc peritoneal metastasis model, Tram significantly enhanced collagen deposition and αSMA expression in peritoneal metastatic niches. Mechanistically, the increased actomyosin contractility induced by Tram is dependent on ROCK and IFN-stimulated JAK/STAT signaling. scRNA-seq of CAF-S1 isolated from primary breast cancer has revealed eight distinct subclusters. Notably, within the myCAF, a subpopulation termed IFNαβ myCAF was identified, distinguished by high expression of genes associated with IFNα/β signaling (40). Similarly, a comparable Tram-regulated IFN signaling subtype was observed in CAFs consistent with colorectal cancer cell lines exhibiting resistant to MEK inhibition (68). In PDAC, combining MEK and JAK inhibitors reshapes stromal plasticity by suppressing iCAFs and enriching mesenchymal stem cell-like CAFs, helping overcome immunotherapy resistance (69). The combination with anti-PD-1 is currently in a phase I trial for chemorefractory metastatic PDAC (NCT05440942; ref. 70). Consistent with findings in PDAC, our study provides a strong scientific rationale for evaluating MEK and JAK cblockade in colorectal cancer.

In conclusion, our study highlights the remarkable plasticity of CAFs, enabling them to sense and adapt to stress induced by cancer-targeted therapies, resulting in phenotypic shifts between distinct states. These results raise the possibility that the efficacy of standard-of-care drugs could be enhanced by understanding their impact on the tumor stroma.

Data Availability

The microarray data analyzed in this study were obtained from the Gene Expression Omnibus (GEO) repository at (<https://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE39582 and GSE17536. The RNA-seq gene expression and clinicopathologic data for colorectal cancer (TCGA-COAD) analyzed in this study were obtained from TCGA repository at (<https://portal.gdc.cancer.gov/>). The

scRNA-seq data analyzed in this study were obtained from the GEO repository under accession numbers GSE166555, GSE81861, GSE146771, and GSE144735 and from ArrayExpress repository at (<https://www.ebi.ac.uk/arrayexpress/>) under accession number E-MTAB-8107. The proteomics data supporting the findings of this study have been deposited in the ProteomeXchange Consortium via the PRIDE repository at the European Bioinformatics Institute (<https://www.ebi.ac.uk/pride/>) under the dataset identifiers PXD050005 and PXD058691. The count matrix of bulk RNA-seq data for Tram-treated CAFs is provided in Supplementary Table S4. The raw bulk RNA-seq data for PI3K/AKT/mTOR inhibitor-treated CAFs generated in this study have been deposited in the NCBI GEO repository under accession code GSE309233. The raw scRNA-seq data for PDTFs generated in this study have been deposited in the NCBI GEO repository under accession code GSE309346. The code used in this article is freely available at Code Ocean (<https://codeocean.com/capsule/4252620/tree/v1>). All other raw data generated in this study are available upon request to the corresponding author.

Authors' Disclosures

O. De Wever reports grants from China Scholarship Council, Kom op tegen Kanker, Stichting tegen Kanker, and Concerted Research Action of Ghent University during the conduct of the study, as well as nonfinancial support from Novartis, Boehringer Ingelheim, GSK, and Eisai outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

Z. Xia: Conceptualization, formal analysis, methodology, writing—original draft, project administration. F. De Vuyst: Validation, methodology, writing—review and editing. S. Ernst: Investigation, methodology. U. Suwal: Methodology. A. Vander Cruyssen: Methodology. P. Rappu: Methodology. J. Heino: Software, formal analysis, methodology. S. Dedeyne: Conceptualization, software, formal analysis, writing—review and editing. W. Ceelen: Conceptualization, visualization, methodology, writing—review and editing. L. Craciun: Visualization, methodology. P. Demetter: Conceptualization, visualization, methodology, writing—review and editing. A. Hendrix: Conceptualization, supervision, writing—review and editing. O. De Wever: Conceptualization, supervision, methodology, writing—review and editing.

Acknowledgments

This work was supported by Kom op tegen Kanker, Stichting tegen Kanker, the China Scholarship Council (202006160045), and the Concerted Research Actions of Ghent University. Mass spectrometry analyses were performed at the Turku Proteomics Facility, supported by Biocenter Finland.

Note

Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Received February 19, 2025; revised October 7, 2025; accepted March 10, 2026; posted first March 12, 2026.

References

- Kalluri R. The biology and function of fibroblasts in cancer. *Nat Rev Cancer* 2016;16:582–98.
- Chen Y, McAndrews KM, Kalluri R. Clinical and therapeutic relevance of cancer-associated fibroblasts. *Nat Rev Clin Oncol* 2021;18:792–804.
- Biffi G, Tuveson DA. Diversity and biology of cancer-associated fibroblasts. *Physiol Rev* 2021;101:147–76.
- Affo S, Nair A, Brundu F, Ravichandra A, Bhattacharjee S, Matsuda M, et al. Promotion of cholangiocarcinoma growth by diverse cancer-associated fibroblast subpopulations. *Cancer Cell* 2021;39:866–82.e11.
- Zhong B, Cheng B, Huang X, Xiao Q, Niu Z, Chen Y, et al. Colorectal cancer-associated fibroblasts promote metastasis by up-regulating LRG1 through stromal IL-6/STAT3 signaling. *Cell Death Dis* 2021;13:16.
- De Jaeghere EA, Denys HG, De Wever O. Fibroblasts fuel immune escape in the tumor microenvironment. *Trends Cancer* 2019;5:704–23.
- Su S, Chen J, Yao H, Liu J, Yu S, Lao L, et al. CD10⁺GPR77⁺ cancer-associated fibroblasts promote cancer formation and chemoresistance by sustaining cancer stemness. *Cell* 2018;172:841–56.e16.
- De Jesus-Acosta A, Sugar EA, O'Dwyer PJ, Ramanathan RK, Von Hoff DD, Rasheed Z, et al. Phase 2 study of vismodegib, a hedgehog inhibitor, combined with gemcitabine and nab-paclitaxel in patients with untreated metastatic pancreatic adenocarcinoma. *Br J Cancer* 2020;122:498–505.
- Hofheinz R-D, al-Batran S-E, Hartmann F, Hartung G, Jäger D, Renner C, et al. Stromal antigen targeting by a humanised monoclonal antibody: an early phase II trial of sibtrotuzumab in patients with metastatic colorectal cancer. *Onkologie* 2003;26:44–8.
- Chen Y, Kim J, Yang S, Wang H, Wu C-J, Sugimoto H, et al. Type I collagen deletion in αSMA⁺ myofibroblasts augments immune suppression and accelerates progression of pancreatic cancer. *Cancer Cell* 2021;39:548–65.e6.
- Hakim N, Patel R, Devoe C, Saif MW. Why HALO 301 failed and implications for treatment of pancreatic cancer. *Pancreas (Fairfax)* 2019;3:e1–4.

12. Xia Z, De Wever O. The plasticity of cancer-associated fibroblasts. *Trends Cancer* 2025;11:770–89.
13. Elyada E, Bolisetty M, Laise P, Flynn WF, Courtois ET, Burkhardt RA, et al. Cross-species single-cell analysis of pancreatic ductal adenocarcinoma reveals antigen-presenting cancer-associated fibroblasts. *Cancer Discov* 2019;9:1102–23.
14. Öhlund D, Handly-Santana A, Biffi G, Elyada E, Almeida AS, Ponz-Sarvisé M, et al. Distinct populations of inflammatory fibroblasts and myofibroblasts in pancreatic cancer. *J Exp Med* 2017;214:579–96.
15. Biffi G, Oni TE, Spielman B, Hao Y, Elyada E, Park Y, et al. IL1-induced JAK/STAT signaling is antagonized by TGF β to shape CAF heterogeneity in pancreatic ductal adenocarcinoma. *Cancer Discov* 2019;9:282–301.
16. Grauel AL, Nguyen B, Ruddy D, Laszewski T, Schwartz S, Chang J, et al. TGF β -blockade uncovers stromal plasticity in tumors by revealing the existence of a subset of interferon-licensed fibroblasts. *Nat Commun* 2020;11:6315.
17. Wang H, Li N, Liu Q, Guo J, Pan Q, Cheng B, et al. Antiandrogen treatment induces stromal cell reprogramming to promote castration resistance in prostate cancer. *Cancer Cell* 2023;41:1345–62.e9.
18. Morgan E, Arnold M, Gini A, Lorenzoni V, Cabaasag CJ, Laversanne M, et al. Global burden of colorectal cancer in 2020 and 2040: incidence and mortality estimates from GLOBOCAN. *Gut* 2023;72:338–44.
19. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008;135:1079–99.
20. Calon A, Lonardo E, Berenguer-Llargo A, Espinet E, Hernando-Mombona X, Iglesias M, et al. Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nat Genet* 2015;47:320–9.
21. Woolston A, Khan K, Spain G, Barber LJ, Griffiths B, Gonzalez-Exposito R, et al. Genomic and transcriptomic determinants of therapy resistance and immune landscape evolution during anti-EGFR treatment in colorectal cancer. *Cancer Cell* 2019;36:35–50.e9.
22. Wu F, Yang J, Liu J, Wang Y, Mu J, Zeng Q, et al. Signaling pathways in cancer-associated fibroblasts and targeted therapy for cancer. *Signal Transduct Target Ther* 2021;6:218.
23. De Boeck A, Hendrix A, Maynard D, Van Bockstal M, Daniëls A, Pauwels P, et al. Differential secretome analysis of cancer-associated fibroblasts and bone marrow-derived precursors to identify microenvironmental regulators of colon cancer progression. *Proteomics* 2013;13:379–88.
24. De Wever O, Nguyen Q-D, Van Hoorde L, Bracke M, Bruyneel E, Gespach C, et al. Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. *FASEB J* 2004;18:1016–8.
25. De Vlieghere E, Gremontprez F, Verset L, Mariën L, Jones CJ, De Craene B, et al. Tumor-environment biomimetics delay peritoneal metastasis formation by deceiving and redirecting disseminated cancer cells. *Biomaterials* 2015;54:148–57.
26. Peirsman A, Blondeel E, Ahmed T, Anckaert J, Audenaert D, Boterberg T, et al. MISpheroID: a knowledgebase and transparency tool for minimum information in spheroid identity. *Nat Methods* 2021;18:1294–303.
27. Horowitz LF, Rodriguez AD, Au-Yeung A, Bishop KW, Barner LA, Mishra G, et al. Microdissected “cuboids” for microfluidic drug testing of intact tissues. *Lab Chip* 2021;21:122–42.
28. Blondeel E, Ernst S, De Vuyst F, Diósi Á, Pinheiro C, Estêvão D, et al. Sequential orthogonal assays for longitudinal and endpoint characterization of three-dimensional spheroids. *Nat Protoc* 2025;20:2899–941.
29. Herrera JA, Mallikarjun V, Rosini S, Montero MA, Lawless C, Warwood S, et al. Laser capture microdissection coupled mass spectrometry (LCM-MS) for spatially resolved analysis of formalin-fixed and stained human lung tissues. *Clin Proteomics* 2020;17:24.
30. Xia Z, Vermeulen S, Suwal U, Rappu P, Heino J, De Vuyst F, et al. Cancer-associated fibroblasts mediate resistance to neoadjuvant therapy in breast cancer. *Clin Transl Med* 2024;14:e1779.
31. Castelló-Cros R, Cukierman E. Stromagenesis during tumorigenesis: characterization of tumor-associated fibroblasts and stroma-derived 3D matrices. *Methods Mol Biol* 2009;522:275–305.
32. Qian J, Olbrecht S, Boeckx B, Vos H, Laoui D, Etioglu E, et al. A pan-cancer blueprint of the heterogeneous tumor microenvironment revealed by single-cell profiling. *Cell Res* 2020;30:745–62.
33. Lee H-O, Hong Y, Etioglu HE, Cho YB, Pomella V, Van den Bosch B, et al. Lineage-dependent gene expression programs influence the immune landscape of colorectal cancer. *Nat Genet* 2020;52:594–603.
34. Uhlitz F, Bischoff P, Peidli S, Sieber A, Trinks A, Lüthen M, et al. Mitogen-activated protein kinase activity drives cell trajectories in colorectal cancer. *EMBO Mol Med* 2021;13:e14123.
35. Li H, Courtois ET, Sengupta D, Tan Y, Chen KH, Goh JLL, et al. Reference component analysis of single-cell transcriptomes elucidates cellular heterogeneity in human colorectal tumors. *Nat Genet* 2017;49:708–18.
36. Zhang L, Li Z, Skrzypczynska KM, Fang Q, Zhang W, O'Brien SA, et al. Single-cell analyses inform mechanisms of myeloid-targeted therapies in colon cancer. *Cell* 2020;181:442–59.e29.
37. Koncina E, Nurmik M, Pozdeev VI, Gilson C, Tsenkova M, Begaj R, et al. IL1R1+ cancer-associated fibroblasts drive tumor development and immunosuppression in colorectal cancer. *Nat Commun* 2023;14:4251.
38. Hänzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* 2013;14:7.
39. Yu G, Wang L-G, Han Y, He Q-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 2012;16:284–7.
40. Kieffer Y, Hocine HR, Gentric G, Pelon F, Bernard C, Bourachot B, et al. Single-cell analysis reveals fibroblast clusters linked to immunotherapy resistance in cancer. *Cancer Discov* 2020;10:1330–51.
41. Ma Z, Li X, Mao Y, Wei C, Huang Z, Li G, et al. Interferon-dependent SLC14A1⁺ cancer-associated fibroblasts promote cancer stemness via WNT5A in bladder cancer. *Cancer Cell* 2022;40:1550–65.e7.
42. Galbo PM, Zang X, Zheng D. Molecular features of cancer-associated fibroblast subtypes and their implication on cancer pathogenesis, prognosis, and immunotherapy resistance. *Clin Cancer Res* 2021;27:2636–47.
43. Cords L, Tietscher S, Anzeneder T, Langwieder C, Rees M, de Souza N, et al. Cancer-associated fibroblast classification in single-cell and spatial proteomics data. *Nat Commun* 2023;14:4294.
44. Wu SZ, Roden DL, Wang C, Holliday H, Harvey K, Cazet AS, et al. Stromal cell diversity associated with immune evasion in human triple-negative breast cancer. *EMBO J* 2020;39:e104063.
45. Chen Z, Zhou L, Liu L, Hou Y, Xiong M, Yang Y, et al. Single-cell RNA sequencing highlights the role of inflammatory cancer-associated fibroblasts in bladder urothelial carcinoma. *Nat Commun* 2020;11:5077.
46. Guinney J, Dienstmann R, Wang X, de Reyniès A, Schlicker A, Soneson C, et al. The consensus molecular subtypes of colorectal cancer. *Nat Med* 2015;21:1350–6.
47. Coppé J-P, Desprez P-Y, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* 2010;5:99–118.
48. Ong C-AJ, Zhao JJ, Liu Y, Srivastava S, Chia DKA, Quek YE, et al. Spatial heterogeneity, stromal phenotypes, and therapeutic vulnerabilities in colorectal cancer peritoneal metastasis. *Clin Cancer Res* 2025;31:2515–29.
49. Ng K, Tabernero J, Hwang J, Bajetta E, Sharma S, Del Prete SA, et al. Phase II study of everolimus in patients with metastatic colorectal adenocarcinoma previously treated with bevacizumab-fluoropyrimidine-oxaliplatin and irinotecan-based regimens. *Clin Cancer Res* 2013;19:3987–95.
50. Glaviano A, Foo ASC, Lam HY, Yap KCH, Jacot W, Jones RH, et al. PI3K/AKT/mTOR signaling transduction pathway and targeted therapies in cancer. *Mol Cancer* 2023;22:138.
51. Bordignon P, Bottoni G, Xu X, Popescu AS, Truan Z, Guenova E, et al. Dualism of FGF and TGF- β signaling in heterogeneous cancer-associated fibroblast activation with ETV1 as a critical determinant. *Cell Rep* 2019;28:2358–72.e6.
52. Li C, Chen T, Liu J, Wang Y, Zhang C, Guo L, et al. FGF19-induced inflammatory CAF promoted neutrophil extracellular trap formation in the liver metastasis of colorectal cancer. *Adv Sci (Weinh)* 2023;10:e2302613.
53. Kun E, Tsang YTM, Ng CW, Gershenson DM, Wong KK. MEK inhibitor resistance mechanisms and recent developments in combination trials. *Cancer Treat Rev* 2021;92:102137.
54. Shao X, Taha IN, Clauser KR, Gao YT, Naba A. MatrisomeDB: the ECM-protein knowledge database. *Nucleic Acids Res* 2020;48:D1136–44.
55. Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K, et al. Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. *Nat Cell Biol* 2007;9:1392–400.
56. Calvo F, Ege N, Grande-García A, Hooper S, Jenkins RP, Chaudhry SI, et al. Mechanotransduction and YAP-dependent matrix remodeling is required for the generation and maintenance of cancer-associated fibroblasts. *Nat Cell Biol* 2013;15:637–46.
57. Dupont S, Morsut L, Aragona M, Enzo E, Giulitti S, Cordenonsi M, et al. Role of YAP/TAZ in mechanotransduction. *Nature* 2011;474:179–83.
58. Sanz-Moreno V, Gaggioli C, Yeo M, Albrengues J, Wallberg F, Virois A, et al. ROCK and JAK1 signaling cooperate to control actomyosin contractility in tumor cells and stroma. *Cancer Cell* 2011;20:229–45.

59. Costa A, Kieffer Y, Scholer-Dahirel A, Pelon F, Bourachot B, Cardon M, et al. Fibroblast heterogeneity and immunosuppressive environment in human breast cancer. *Cancer Cell* 2018;33:463–79.e10.
60. Lambrechts D, Wauters E, Boeckx B, Aibar S, Nittner D, Burton O, et al. Phenotype molding of stromal cells in the lung tumor microenvironment. *Nat Med* 2018;24:1277–89.
61. Saada JI, Pinchuk IV, Barrera CA, Adegboyega PA, Suarez G, Mifflin RC, et al. Subepithelial myofibroblasts are novel nonprofessional APCs in the human colonic mucosa. *J Immunol* 2006;177:5968–79.
62. Pinchuk IV, Beswick EJ, Saada JI, Boya G, Schmitt D, Raju GS, et al. Human colonic myofibroblasts promote expansion of CD4⁺ CD25^{high} Foxp3⁺ regulatory T cells. *Gastroenterology* 2011;140:2019–30.
63. Herrera M, Berral-González A, López-Cade I, Galindo-Pumariño C, Bueno-Fortes S, Martín-Merino M, et al. Cancer-associated fibroblast-derived gene signatures determine prognosis in colon cancer patients. *Mol Cancer* 2021;20:73.
64. Duluc C, Moatassim-Billah S, Chalabi-Dchar M, Perraud A, Samain R, Breibach F, et al. Pharmacological targeting of the protein synthesis mTOR/4E- BP 1 pathway in cancer-associated fibroblasts abrogates pancreatic tumour chemoresistance. *EMBO Mol Med* 2015;7:735–53.
65. Barras D. BRAF mutation in colorectal cancer: an update. *Biomark Cancer* 2015;7:9–12.
66. Kopetz S, Desai J, Chan E, Hecht JR, O'Dwyer PJ, Maru D, et al. Phase II pilot study of vemurafenib in patients with metastatic BRAF-mutated colorectal cancer. *J Clin Oncol* 2015;33:4032–8.
67. Hirata E, Girotti MR, Viros A, Hooper S, Spencer-Dene B, Matsuda M, et al. Intravital imaging reveals how BRAF inhibition generates drug-tolerant micro-environments with high integrin β 1/FAK signaling. *Cancer Cell* 2015;27:574–88.
68. Wagner S, Vlachogiannis G, De Haven Brandon A, Valenti M, Box G, Jenkins L, et al. Suppression of interferon gene expression overcomes resistance to MEK inhibition in KRAS-mutant colorectal cancer. *Oncogene* 2019;38:1717–33.
69. Datta J, Dai X, Bianchi A, De Castro Silva I, Mehra S, Garrido VT, et al. Combined MEK and STAT3 inhibition uncovers stromal plasticity by enriching for cancer-associated fibroblasts with mesenchymal stem cell-like features to overcome immunotherapy resistance in pancreatic cancer. *Gastroenterology* 2022;163:1593–612.
70. Hosein PJ, Datta J, Ezenwajiaku N, Zhou Z, Yow MV, Boone M, et al. A phase 1 trial of combined MEK, STAT3 and PD-1 inhibition in metastatic pancreatic ductal adenocarcinoma (PDAC). *J Clin Oncol* 2024;42(3_suppl):TPS713.