Luraghi et al., Supplementary Experimental Procedures

**Tumor dissociation and xenosphere generation**

All procedures involving animal experimentation were approved by the Italian Ministry of Health and by the internal Ethical Committee for Animal Experimentation (FPRC-CESA). Xenopatients were obtained as described (1) and their tumors were mechanically dissociated, digested with Type-I Collagenase (1 mg/ml, Life Technologies-Invitrogen), filtered through a 70 µm cell strainer (BD Falcon), and cleared with histopaque-1077 (Sigma), according to manufacturer instructions. Cells were grown in ultra-low attachment plastics (Sigma-Corning) in standard stem cell medium. This included human recombinant epidermal growth factor (EGF, 20 ng/ml; Sigma), basic fibroblast growth factor 2 (bFGF, 10 ng/ml; Peprotech), added to basal stem cell medium, i.e. DMEM/F-12 (Sigma) supplemented with 2 mM glutamine (Sigma), penicillin-streptomycin (1:100, EuroClone), N-2 supplements (Life Technologies-GIBCO), 0.4% BSA (Sigma), 4 µg/ml heparin (Sigma), CD Lipid Concentrate (1:100, Life Technologies-GIBCO). Cells were kept in humidified incubators at 37°C, with 5% O₂ and 5% CO₂. To obtain differentiated cells, dissociated xenospheres were resuspended in DMEM (Sigma) supplemented with 2 mM glutamine (Sigma), penicillin-streptomycin (1:100, EuroClone), 10% Fetal Bovine Serum (FBS) (Lonza) and plated in pro-adhesive tissue culture-dishes.

**Spheropatient generation and therapy**

To generate spheropatients, xenospheres were dissociated (5mM EDTA; Trypsin 8mM) to obtain single-cell suspensions, resuspended in a 1:1 mixture of basal stem-cell medium (w/o GFs) and matrigel (BD Bioscience) at a density of 10⁶ cells/ml. 10⁵ cells were subcutaneously injected into 6-weeks old NOD/SCID female mice (Charles River Laboratories) or human HGF knock-in SCID mice, generated by AVEO as follows. Homologous recombination in H12C23 (a 129SvEv strain ES cell line developed at AVEO) mouse embryonic stem cells was performed by electroporation with a targeting vector designed to replace exons 3-6 of the mouse hepatocyte growth factor (Hgf) locus with a cDNA sequence encoding exons 2-18 of the human hepatocyte growth factor (HGF), a polyA signal, and a frt-flanked PGK-neo cassette. A correctly targeted clone was identified, (JB26A10) and the selectable marker was removed by transient transfection with aFLPe expressing plasmid (pCAGGS-FLPe; Genebridges). Correctly targeted ES cells were then injected into recipient blastocysts and chimeric mice were bred with C57BL/6 mice to establish the hHGFki colony (Hgf<sup>am1.1(HGF)Aveo</sup>). These mice were then bred with C3H<sup>scid</sup> (C3SnSmn.CB17-Prkd<sup>esid</sup>/J; see Stock No. 001131) to generate the hHGFki;SCID double mutant mice. The hHGFki allele was then
backcrossed to C.B-17 SCID mice (Taconic) for 8 generations followed by sib mating to make both the hHGFki and SCID alleles homozygous. The mice are maintained as double homozygous hHGFki/hHGFki; SCID/SCID C.B 17 N8 inbred strain.

When tumors reached an average volume of 400 mm$^3$, mice were randomized, and treated with 20 mg/kg cetuximab (Merck) twice-weekly, or with 50 mg/Kg of JNJ-38877605 (2) daily, or with both inhibitors, or with vehicle. Tumor size was measured once-weekly by caliper, and volume was calculated using the formula $4/3(d/2)^2D/2$, where $d$ is the minor and $D$ is the major tumor axis.

**Genomic DNA extraction and mutational screening**

Genomic DNA was extracted with the Wizard® SV genomic DNA purification system (Promega) according to the manufacturer’s instructions. The quality of nucleic acids was verified by measuring the 260/280 absorbance ratio with ND-1000 V3.7.1 Nanodrop (Thermo Scientific). Purified gDNA was analyzed for KRAS, NRAS, BRAF, PIK3CA, ERBB2 and MET as previously described (1, 3). For APC (aa700-1800) specific primers were designed (see Supplementary Table-4). PCR conditions were as follows: 95°C for 3 min; $3 \times [95°C$ for 15 sec, 64°C for 30 sec, and 70°C for 1 min]; $3 \times [95°C$ for 15 sec, 61°C for 30 sec, and 70°C for 1 min]; $3 \times [95°C$ for 15 sec, 58°C for 30 sec, and 70°C for 1 min]; $37 \times [95°C$ for 15 sec, 57°C for 30 sec, and 70°C for 1 min]; and 70°C for 5 min. PCR products were purified using illustra™ ExoStar™ 1-Step (GE Healthcare) according to manufacturer’s instruction. Cycle sequencing was carried out using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing products were purified using AgencourtCleanSeq (Agencourt Bioscience, Beckman Coulter) and analyzed on a 3730 DNA Analyzer ABI capillary electrophoresis system (Applied Biosystems). Sequences were then analyzed using Chromas Lite 2.01 software (http://www.technelysium.com.au/chromas_lite.html) and compared with reference sequences from the Homo sapiens assembly GRCh37 (February 2009). All identified mutations were than compared with those reported in the Catalogue Of Somatic Mutations In Cancer (COSMIC, http://www.sanger.ac.uk/genetics/CGP/cosmic/).

**Flow-cytometric analysis**

Immunophenotype was performed by incubating $2 \times 10^5$ cells with appropriate dilutions of the following antibodies: mouse monoclonal anti-CD133/1 (clone AC133, APC-conjugated, MiltenyiBiotec GmbH); mouse monoclonal anti-CD24 (clone SN3, PE-conjugated, Invitrogen) and mouse monoclonal anti-CD44 (clone MEM-85, FITC-conjugated, Invitrogen), and mouse monoclonal anti-MET (clone 95106, APC-conjugated, R&D Systems Inc.). DAPI was added for dead cell exclusion. Samples were analyzed in a CyAN ADP (DakoCytomation). Data were processed using Summit 4.3 software. (DakoCytomation).
Cell viability and apoptosis assays

Cells were plated at clonal density (10 cells/μl) in ultra-low attachment 96-well plates (Corning-Sigma) in basal stem-cell medium (w/o EGF-bFGF). Growth factors (EGF; bFGF; HGF, Peprotech) were supplied at day 0. When indicated, cetuximab (10 µg/ml), or JNJ-38877605 (0.5 µM), or gefitinib (0.5 µM) were added. ATP production and Caspase 3/7 activity were measured using respectively Cell Titer Glow® (Promega) and Caspase-Glow® 3/7 Assay (Promega), and a GloMax 96 Microplate Luminometer (Promega).

Real time RT-PCR

Total RNA was extracted using miRNAeasy mini Kit according to manufacturer’s instructions (Qiagen). 1 ug of purified RNA was used as a template for cDNA synthesis with random and hexamer primers and the High Capacity Real-time PCR was performed using SYBR Green Master Mix (Applied Biosystems) containing 200 ng of cDNA and FGFR2 QuantiTect® primer 3-5’mix (QT00098560, QIAGEN) or forward and reverse primers for EGF, EGFR, HER2 and HER3 (see Supplementary Table-3) on ABI PRISM 7900HT sequence detection system. Melting curve analysis of PCR products was performed to verify primers specificity. Relative quantification value for target gene was calculated by subtracting scaled CT values from the total number of cycles (i.e. 40) and represented on the histogram y-axis.

Soft Agar assay

Xenospheres were trypsinized and resuspended at concentration of 5x10^3 cells/well in stem cell basal medium with 1% agarose (Seapaque), on a base of 5% agarose. After 12 h, growth factors were added to the culture (20 ng/ml EGF, Sigma; 20 ng/ml HGF, 10 ng/ml bFGF, Peprotech). Medium was replaced twice a week for 3 weeks. Colonies were stained with tetrazolium salts. Images were acquired by a computer scanner and converted to b/w.

Cancer-associated fibroblasts (CAFs) and Fibroblast Conditioned Medium (FCM)

Surgical resection of a primary colorectal cancer was processed as described above. Isolated cells were plated in adhesive dishes in DMEM supplemented with 2 mM glutamine, penicillin-streptomycin, and 10% FBS. Fibroblasts were separated from epithelial cells by serial trypsinization. MRC5 were obtained from ATCC; human hTERT-Fibroblasts (hTERT-F) were kindly provided by R.A. Weinberg (4), and were respectively cultured in MEM or DMEM supplemented with 2 mM glutamine, penicillin-streptomycin and 10% FBS. To obtain FCM, fibroblasts were grown up to confluence and then kept for 24h in basal stem-cell medium (w/o
EGF-bFGF). Collected medium was analyzed with the RayBio® Human Cytokine Antibody Array 5 (RayBiotech, Inc.).

**Western Blotting**

Xenospheres and their derived differentiated cells protein expression were analyzed on whole-cell lysates, solubilized in boiling Laemmli buffer. For MET and EGFR phosphorylation analysis cells were solubilized with EB-extraction buffer (20 mMTris-HCl pH 7.4, 150 mMNaCl, 10% Glycerol, 1% TritonX-100, 5mM EDTA) in the presence of protease and phosphatase inhibitors. Protein concentration was determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology). Equal amounts of proteins were resolved by SDS-PAGE in reducing conditions and analyzed by immunoblotting with the following antibodies: mouse monoclonal anti-MET (DL21, ref. 5), rabbit monoclonal anti-EGFR (Cell Signaling Technology), goat polyclonal anti-HGF (R&D Systems), rabbit anti-phospho MET (Tyr 1349, Cell Signaling Technology), rabbit anti-phospho ERK 1/2 (Thr202/Tyr204, Cell Signaling Technology), rabbit anti-ERK 1/2, rabbit anti-phosphoAkt (Ser473, Cell Signaling Technology), rabbit anti-Akt (Cell Signaling Technology), rabbit anti-phospho S6 (Ser235/236, Cell Signaling Technology), rabbit anti-S6 (Cell Signaling Technology), rabbit anti-phospho EGFR (Tyr1068, Abcam). Mouse monoclonal anti-Hsp70 antibody, goat polyclonal anti-β-actin (Santa Cruz Biotechnology) and mouse monoclonal anti-Vinculin (Sigma Aldrich) were used as controls of equal protein loading. Antibodies were visualized with appropriate horseradish peroxidase-conjugated secondary antibodies (Amersham), and the enhanced chemiluminescence system (ECL, Amersham). Blot images were captured using the FujiFilm LAS-3000 digital imaging system.

**Immunohistochemistry**

Tumors explanted from mice were formalin-fixed and paraffin-embedded according to standard procedures. 4-μm tissue sections were dried in a 37°C oven overnight. Slides were de-paraffinized in xylene and rehydrated through graded alcohol to water. Endogenous peroxidase was blocked in 3% hydrogen peroxide for 30 minutes. Microwave antigen retrieval was carried out using a microwave oven (750 W for 10 minutes) in 10 mmol/L citrate buffer, pH 6.0. Slides were incubated with individual primary antibodies overnight at 4°C inside a moist chamber. After washings in TBS, anti-rabbit secondary antibody (DakoEnvision+System-horseradish peroxidase–labeled polymer, Dako) was added. Incubations were carried out for 1 hour at room temperature. Immunoreactivities were revealed by incubation in DAB chromogen (DakoCytomation Liquid DAB Substrate Chromogen System, Dako) for 10 minutes. Slides were counterstained in Mayer's hematoxylin, dehydrated in graded alcohol, cleared in xylene, and the coverslip was applied by
using DPX. A negative control slide was processed with secondary antibody, omitting primary antibody incubation. The following antibodies were used: anti-phospho-S6 ribosomal protein rabbit mAb (Ser235/236, clone D57.2.2E; Cell Signaling Technology), anti-phospho-p44/42 MAPK (ERK1/2) rabbit mAb (Thr202/tyr204, clone D13.14.4E; Cell Signaling Technology), anti-CK20 rabbit mAb (Abcam), anti-LGR5 rabbit mAb (Abcam) and anti-beta-catenin mouse mAb (BD Transduction laboratories). Images were captured with the LEICA LAS EZ software with the use of a LEICA ICC50 HD microscope.

Statistical analysis

Numerical results were expressed as means ± standard error of the mean (SEM). Statistical significance was evaluated using two-tailed Student’s t-tests. p< 0.05 were considered statistically significant. Statistical analysis of HGF expression in a publicly available dataset (GEO dataset GSE5851) (ref.6) was performed by Fisher exact test (http://graphpad.com/quickcalc/contingency1/) on data extracted from KRASwt patients, taking the 75th Percentile as threshold and comparing patients with reported Progressive Disease versus those with Complete/Partial Response and Stable Disease.

References


