Supporting Information

Supplementary Experimental Procedures

Cell culture

MKN28 cells were purchased from JCRB Cell Bank (Japanese Collection of Research Bioresources Cell Bank), and SGC7901 cells were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. MKN28-M, SGC7901-M, MKN28-NM and SGC7901-NM sub-lineage cells were screened and maintained by our laboratory.[1] GES-1 cells were obtained from the Beijing Institute for Cancer Research. HeLa and 293T cells were purchased from the Cell Bank at the Shanghai Institute for Biological Sciences (Chinese Academy of Sciences). 293T cells were cultured in high-glucose DMEM, while all other cells were cultured in RPMI1640 (GIBCO) medium with 10% fetal calf serum at 37°C and 5% CO_2 as well as under saturated humidity and static conditions.

Western blot analysis

Western blot analysis was performed using standard procedures. The following antibodies were used in the experiments: anti-SLIT2 antibody (OriGene, EPR2771, USA), anti-β-Catenin antibody (Millipore, ABE208, USA), anti-Vimentin antibody (Cell Signaling Technology, 3932, USA), anti-E-Cadherin antibody (BD Biosciences, 610182, USA), anti-MMP-2 antibody (Novus Biologicals, NB200-114AF647, USA) and anti-MMP-9 antibody (Novus Biologicals,

NBP2-13173R, USA). The other antibodies used in the western blot analyses were the same as those used in the immunohistochemistry analyses.

Immunohistochemistry

Tissue staining was performed according to the VECTASTAIN Elite ABC system protocol (Vector Laboratories, PK-6101, USA). The primary antibodies used were as follows: anti-POU2F2 antibody (LifeSpan Biosciences, LS-C105600, USA), anti-ROBO1 antibody (Abcam, ab7279, UK) and anti-P65 antibody (Abcam, ab7970, UK). POU2F2-positive and P65-positive cells were stained brown in the nucleus, and ROBO1-positive cells were stained brown in the cytoplasm and cell membrane. Both the immunohistochemical staining score of positive cells and the intensity of the positive cells on each slide were calculated using the semi-quantitative scoring method.[2] The scores were graded, and the final results were obtained with the sum of the two scores. A total score of 0 - 3 was considered negative, and the total score of 4 - 7 was considered positive. The histological evaluation was performed at the Department of Pathology at the Xijing Hospital by two independent senior pathologists without knowing any information about the patient's clinical features.

Immunofluorescence

Cells were plated in 8-well chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System, 154534, USA). Sub-confluent cells were rinsed with 1×PBS, fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.2% Triton X-100 for 10 minutes and blocked with 20%

sheep serum for 60 minutes. Cells were then incubated with primary antibody (used in western blot analysis) overnight at 4°C in a humidified chamber followed by incubation with the Alexa Fluor 594-conjugated secondary antibody (Donkey Anti-Rabbit IgG, Proteintech, SA00006-8; and Donkey anti-Mouse IgG, Proteintech, SA00006-7, USA) for 1 hour at room temperature in the dark. The nuclei were stained with DAPI (5 ng/ml in PBS), and the slides were mounted with ProLong® antifade Reagents (Invitrogen, P36935, USA). Slides were examined by the Fluoview FV1200 Laser Scanning Confocal Microscope (Olympus Corp.)

qRT-PCR

Total RNA of cells was extracted with TRIzol according to the manufacturer's instructions (Invitrogen Life Technologies). One microgram of RNA was used as the template, and a thermal cycler (Hema 9600, Zhuhai, China) was used for the reverse transcription of cDNA. The related gene expression of each cDNA template was detected by the BIO-RAD CFX96TMOptics Module using the SYBR green PCR Master Mix (DRR081, TaKaRa). The primer sequences for each gene are shown in Table S3. All experiments were repeated three times, and each sample was assayed in triplicate. GAPDH was used as the internal control, and the relative mRNA expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method as follows: $\Delta Ct = Ct_{\text{Target gene}} - Ct_{\text{GAPDH}}$ and $\Delta\Delta Ct = \Delta C_{\text{experimental group}} - \Delta Ct_{\text{control group}}$. Statistical analyses were conducted using Student's t test where $p < 0.05$ was considered significant. The detection of miR-218 and the internal reference, U6, has been described in previous studies.[1]

Transwell invasion assay

The membrane of the upper chamber of 24-well transwell plates (Corning, # 3422) was coated with 60 μl of serum-free medium with diluted Matrigel (BD, Cat. 356243). The plates were placed in a 37°C incubator and incubated for 4 to 5 hours for the polymerization of Matrigel into a gel. The gel was then hydrated with 200 μl of serum-free medium containing 10 g/L BSA. The upper chamber was then seeded with 200 μl of cell suspension (containing 1×10^5 cells), and 500 μl of medium containing 10% FBS was added to the lower chamber. The chamber was removed after 24 hours of culture. A swab was used to wipe off the Matrigel and the cells in the upper chamber, and the transmembrane cells were fixed with formaldehyde at room temperature for 30 minutes, stained with crystal violet for 20 minutes, washed three times with clean water and counted under a microscope.

Lipopolysaccharide (LPS; Sigma, L2880, USA) treatment: If it was necessary, 12 hours after cell seeding, LPS was added into the upper chamber with a final concentration of 10 μg/ml, while the same amount of PBS was added to the upper chamber of the control group.

Visceral metastasis experiments via tail vein injection in nude mice

Various cells in the logarithmic growth phase were trypsinized, washed twice with PBS and pelleted by centrifugation. The cells were re-suspended in serum-free RPMI1640 culture medium. Each nude mouse was injected with 2.5×10^6 cells (0.1 ml suspension) via the tail vein.

In vivo bioluminescence imaging (BLI). Anesthetized mice were injected intraperitoneally with filter-sterilized d-luciferin solution (150 mg/kg body weight, Xenogen, Caliper Life

Sciences, Hopkinton, MA, USA). Bioluminescence images were acquired by using a small animal imaging system (IVIS Kinetics; Caliper Life Science, USA). Ten minutes after d-luciferin injection, BLI was conducted. Regions of interest (ROIs) were drawn, and luminescence intensity was quantified and processed using Living Image Software (Version 4.2; Caliper Life Science). Bioluminescent intensity is presented as photons/s/cm2/sr. The imaging parameters were as follows: binning = 8 and f/stop = 1.

Histology of lung metastatic tumors. After six weeks, the mice were sacrificed by cervical dislocation. Nude mouse lung paraffin sections were prepared and analyzed by H&E staining.

LPS treatment. If necessary, animals were injected i.p. with 10 μg of LPS in PBS or PBS alone after 7 days of tumor cell injection once a week. After 7 days of the last treatment with LPS, mice were sacrificed, and their lungs were removed, photographed, formalin fixed, sectioned and H&E stained.

All animal experimental protocols were approved by the Animal Research Committees of Xijing Hospital.

ChIP-PCR analysis

Briefly, approximately 1×10^7 cells in the logarithmic growth phase were seeded into a 10-cm dish. When the cells reached 80-90% confluence, they were cross-linked with 1% formaldehyde, and 1 ml of cell lysate was added and incubated in an ice bath for 10 min. The chromatin DNA was sonicated, and the samples were centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was then collected. Three samples (20 μl) of fragmented DNA were used as the

experimental group, the control group and the input. Anti-transcription factor antibody (1-10 μg) was added to the experimental group, and the same amount of IgG was added to the control group. The samples were incubated overnight at 4°C. Protein A-coated beads were used to precipitate the DNA-protein-antibody complex, which was eluted with the elution buffer and then treated with proteinase K at 45°C for 2 hours. ChIP and input DNA fragments were purified and recovered (Qiagen, 28006), and they were used as the templates for PCR amplification (the primers for the ROBO1 and POU2F2 promoters are listed in Table S3). The PCR products were applied to a 1.5% agarose gel electrophoresis and sequenced after the recovery of the target fragment. The antibodies used in the experiments were as follows following: anti-POU2F2 antibody (sc-377475 X, Santa Cruz), anti-NF-κB p65 antibody (sc-8008, Santa Cruz), anti-NF-κB p50 antibody (sc-8414, Santa Cruz) and normal mouse IgG (sc-2025, Santa Cruz).

EMSA

NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, 78833) were used to extract the nuclear proteins of SGC7901-M cells. The BCA method was used to measure protein concentration. Based on bioinformatic analysis of the promoter region-binding sites, binding probes of transcription factors were synthesized, biotin-labeled and purified. The sequences for the probes were as follows: probe for POU2F2 (upstream), 5`-CCTTGTTATGCCAATTTGCATTACTAATGAACTTGC-3`; mutation probe for POU2F2 (upstream), 5`-CCTTGTTATGCCAACCCGCATTACTAATGAACTTGC-3`; probe for NF-κB (upstream), 5`-GGCGCAGGGCTTGCTTGATCTTCCCTGCCGCGGCGT-3`; and mutation

probe for NF-κB (upstream), 5`-GGCGCAGGGCTTGCTGCAACTGCCCTGCCGCGGCGT-3`. The synthesized single-stranded probe was annealed into double-stranded DNA. Gel electrophoresis was performed using 6.5% polyacrylamide. PIERCE EMSA kit was used for the different groups of binding reactions of the labeled probe, cold probe and mutated probe. The DNA was transferred to a membrane and cross-linked. The membrane was then exposed and analyzed.

Plasmid construction and cell transfection

Four POU2F2 (NM_002698) and ROBO1 (NM_002941) siRNA interference plasmids were designed. SGC7901-M cells were transfected, and the interference efficiency was measured. Using GV112 (GENCHEM, China) as the backbone vector, the POU2F2- and ROBO1-specific shRNA lentiviral vectors (shPOU2F2 and shROBO1) were constructed with the aim of the highest interference efficiency. The shRNA lentiviral vector, shIKK-β (AF080158.1), was constructed according to a previous study.[3] SLIT2 Human shRNA (OriGene, TG309262) was purchased from OriGene (USA). The interference targets of each gene are shown in Table S4**.** pBabe-Puro-IKBalpha-mut (super repressor) was a gift from William Hahn (Addgene plasmid # 15291).[4]

The following expression plasmids were constructed using GV287 (GENCHEM, China) as the backbone vector: POU2F2 and ROBO1 lentiviral vectors containing full-length cDNA sequences; IKK-β Δ3'UTR, POU2F2Δ3'UTR and ROBO1Δ3'UTR expression vectors lacking the 3'UTR; and shRNA-resistant expression vectors for POU2F2Δ (CDS area 417 A > T, 420 G >

C, 423 A > T, 426 A > T, 429 T > C and 432 A > T) and ROBO1Δ (section 1323 A> T, 1326 T > C, 1329 G > A, 1342 A > G, 1345 T > A and 1348 A > T). A miR-218 overexpression lentiviral vector was constructed, and the lentiviral inhibition vectors of miR-218 were constructed using the methods reported in published papers.[5, 6]

Luciferase assay

pNF-kB-Luc and the control plasmid (Clontech, Mountain View, CA, USA) were used to detect NF-κB activity. We generated the ROBO1 promoter reporter gene construct containing 2000 bp of sequence upstream of the transcription start site, which included a potential POU2F2-binding site (-880 to -873) in the ROBO1 promoter region. We used the pGL3-Basic vector to generate the ROBO1 promoter reporter gene construct, which was named pGL3-ROBO1-p-wt (-2000 ¬ +158). In the ROBO1 promoter region, the binding site of POU2F2, ATTTGCAT, was mutated to CGTAAATA. The ROBO1 promoter reporter gene containing the POU2F2-binding site mutation was constructed and named pGL3-ROBO1-p-mt.

To generate the POU2F2 promoter reporter genes, we had to consider four potential NF-κB-binding sites in the POU2F2 promoter region as follows: -1621 to -1612; -703 to -692; -531 to -522; and -75 to -64. In accordance with the putative binding sites, the gene was truncated sequentially from the 5' end. POU2F2-truncated bodies containing 4, 3, 2 and 1 binding sites of NF-κB were constructed and were named as follows: pGL3-POU2F2-p1 (-2000 to +62); pGL3-POU2F2-p2 (-1000 to +62); pGL3-POU2F2-p3 (-600 to +62); and pGL3-POU2F2-p4 (-300 to + 62). The -531 to -522 binding site sequence (TGATCTTCCC) in

pGL3-POU2F2-p1 was mutated to ACTCTCGTCT. The POU2F2 promoter reporter gene of the NF-κB-binding site mutation was constructed and named pGL3- POU2F2-p1-mt.

The 3'-UTR reporter genes containing the miR-218 binding sites for IKK-β and ROBO1 as well as the reporter genes containing the binding site mutation were constructed similarly to previously reports.[1, 7]

The 3'-UTR reporter of POU2F2 for miR-218 was constructed using the chemically synthesized DNA oligos of POU2F2 3'-UTR and the complementary sequence as follows:

wild type,

CTAGATTTCTAGAGGAGAAGCAGGGTCGCTGCTGCTTCTAGGGTGGGGAGCGGCACC
CCAGTTATGTTGGCAGGTCCCTGCCCCTGCTAATGCCTCTGCTTTGCCTCTTGCAGAA
GCACAATGGTGGGGTTGAGCTCCGGGCTGAGTCCAGCCCTCATGAGCAACAACCCTT
TGGCCACTATCCAAGGTGCGTGCTGCCTCATGTCACACCCATCGTCACCTCTAGACCT;

and mutation,

CTAGATTTCTAGAGGAGAAGCAGGGTCGCTGCTGCTTCTAGGGTGGGGAGCGGCACC
CCAGTTATGTTGGCAGGTCCCTGCCCCTGCTAATGCCTCTGCTTTGCCTCTTGCAGCTT
GTATCTGGTGGGGTTGAGCTCCGGGCTGAGTCCAGCCCTCATGAGCAACAACCCTTT
GGCCACTATCCAAGGTGCGTGCTGCCTCATGTCACACCCATCGTCACCTCTAGACCT.

Chemically synthesized DNA oligos were annealed to form double stranded DNA inserts with sticky ends compatible with XbaI-cut vectors. GV306 vectors were digested with XbaI, gel purified, ligated with the inserts and transformed into DH5a cells. Desired clones were confirmed by sequencing.

The target cells were transfected with the above plasmids and the internal reference plasmid pRL-TK. The luciferase reporter gene assay was then performed. All experiments were repeated three times.

Supplemental References

1 Tie J, Pan Y, Zhao L, *et al.* MiR-218 inhibits invasion and metastasis of gastric cancer by targeting the Robo1 receptor. *PLoS Genet* 2010;6:e1000879.

2 Goethals L, Perneel C, Debucquoy A, *et al.* A new approach to the validation of tissue microarrays. *J Pathol* 2006;208:607-14.

3 Yeramian A, Santacana M, Sorolla A, *et al.* Nuclear factor-kappaB2/p100 promotes endometrial carcinoma cell survival under hypoxia in a HIF-1alpha independent manner. *Lab Invest* 2011;91:859-71.

4 Boehm JS, Zhao JJ, Yao J, *et al.* Integrative genomic approaches identify IKBKE as a breast cancer oncogene. *Cell* 2007;129:1065-79.

5 Scherr M, Venturini L, Battmer K, *et al.* Lentivirus-mediated antagomir expression for specific inhibition of miRNA function. *Nucleic Acids Res* 2007;35:e149.

6 Gentner B, Schira G, Giustacchini A, *et al.* Stable knockdown of microRNA in vivo by lentiviral vectors. *Nat Methods* 2009;6:63-6.

7 Song L, Huang Q, Chen K, *et al.* miR-218 inhibits the invasive ability of glioma cells by direct downregulation of IKK-beta. *Biochem Biophys Res Commun* 2010;402:135-40.