Supplementary information, Figure S1

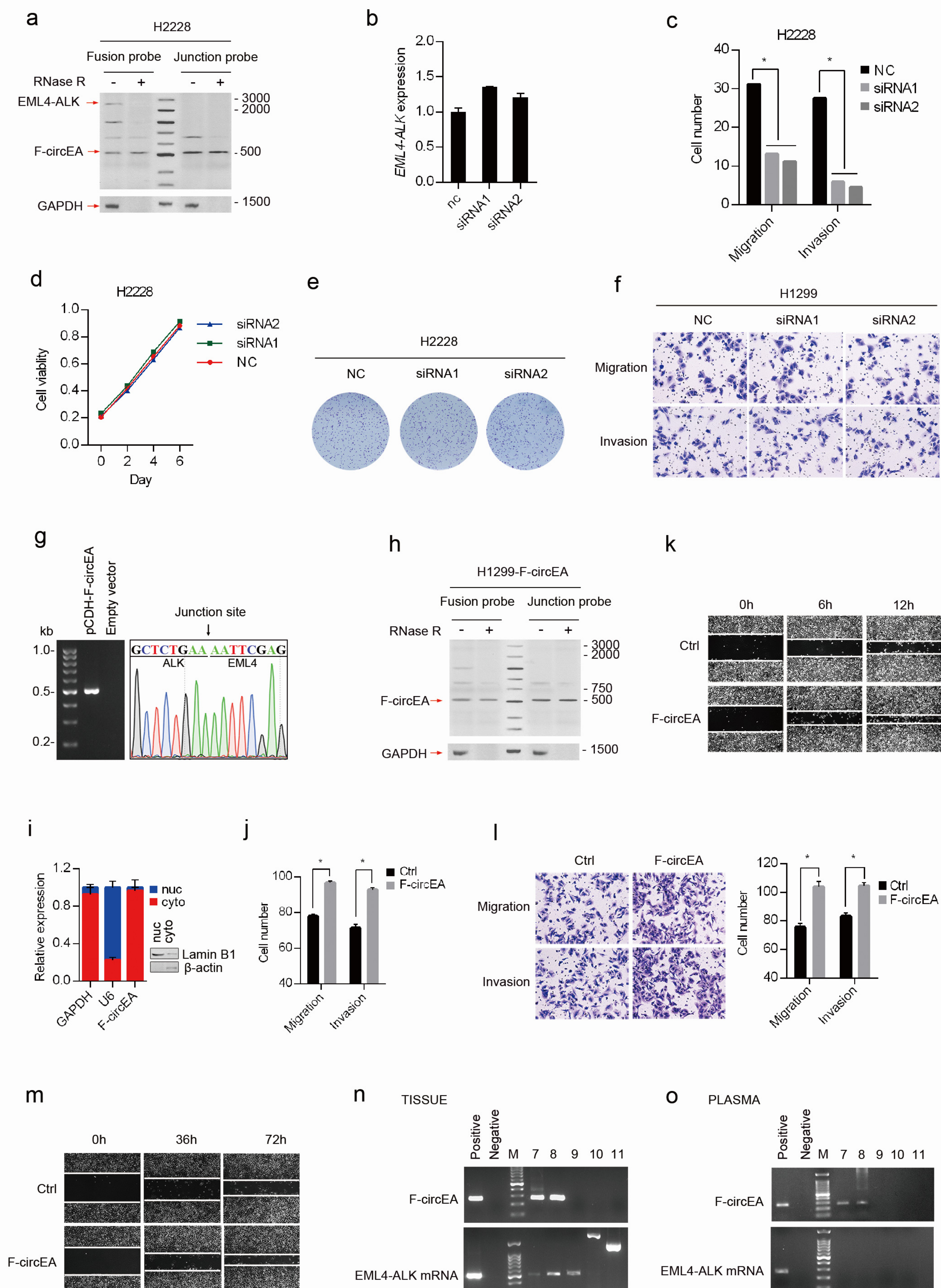

Figure S1 **a** RNA solution hybridization assays for F-circEA and *EML4*-ALK mRNA detection in H2228 cells using ^{32}P-labled oligonucleotide probes. **b** RT-qPCR analyses of *EML4-ALK* mRNA expression in H2228 cells transfected with F-circEA siRNAs (siRNA1 and siRNA2) or scramble negative control (NC). Data were shown as the mean ± SD. **c** Quantification of Transwell assays in H2228 cells transfected with F-circEA siRNAs or scramble negative control (NC). $^*P < 0.05$. **d, e** MTT **(d)** and colony formation assays **(e)** of H2228 cells transfected with F-circEA siRNAs or scramble negative control (NC). **f** Transwell assays of H1299 cells transfected with F-circEA siRNAs or scramble negative control (NC). **g** Agarose gel electrophoresis and Sanger sequencing of RT-PCR products from F-circEA-overexpressing H1299 cells. **h** RNA solution hybridization assays for F-circEA and *EML4-ALK* mRNA in F-circEA-overexpressing H1299 cells. **i** Cell nucleus/cytoplasm fractionation and RT-qPCR analyses showed the cytoplasmic distribution of F-circEA in the F-circEA-overexpressing A549 cells. Western blot analysis indicated good nucleus/cytoplasm isolation. Data was shown as the mean ± SD. **j** Quantification of Transwell assays in empty vector-transfected (Ctrl) or F-circEA-overexpressing (F-circEA) H1299 cells. $^*P < 0.05$. **k** Representative images of wound healing assays in empty vector-transfected (Ctrl) or F-circEA-overexpressing (F-circEA) H1299 cells. **l** Representative images (left) and quantification (right) of Transwell assays in empty vector-transfected (Ctrl) or F-circEA-overexpressing (F-circEA) A549 cells. $^*P < 0.05$. **m** Representative images of wound healing assays in empty vector-transfected (Ctrl) or F-circEA-overexpressing (F-circEA) A549 cells. **n, o** Agarose gel electrophoresis of RT-PCR products from tumor tissues **(n)** or plasma **(o)** of NSCLC patients with *EML4-ALK* variant 3b (patients 7-9), variant 1 (patient 10) and variant 6 (patient 11).

Supplementary information, Data S1

Materials and Methods

Plasmid construction

To construct the F-circEA-overexpressing plasmid, the sequence including partial exon 4-6 of EML4 and the partial exon 20-22 of ALK was amplified from the cDNAs of H2228 cells, and inserted into the vector pCDH-CMV-circRNA-EF1-copGFP, which was constructed by placing the reverse repeat of cirR-7 regions plus up- and down-stream flanking introns of SLC34a2-ROS1 fusion gene into the multiple cloning sites of pCDH-CMV-circRNA-EF1-copGFP (System Biosciences).

Cell culture and transfection

The NSCLC cell lines (H2228, H1299 and A549) were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were transfected with F-circEA-overexpressing plasmid or siRNAs by Lipofectamine® 2000 Reagent (Invitrogen) according to the manufacture's instructions. The siRNAs targeting the junction region of F-circEA were synthesized by GenePharma (Shanghai, China) and listed in the Supplementary Information, Table S1.

Cellular nucleus/cytoplasm fractionation

Cells were resuspended in the RLN buffer (50 mM Tris-HCl, pH 7.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40), and incubated on ice for 1 min. Then the cell lysates were centrifuged at 300 g for 3 min to collect the supernatants as the cytoplasmic fractions. The remaining pellets were washed with RLN buffer twice and then resuspended in RLN buffer again as nucleic fractions.

RNA isolation, F-circEA identification and quantitative RT-PCR

The lung tissues and plasma of NSCLC patients were collected from West China Hospital (China), which was approved by the Ethics Committee of West China Hospital of Sichuan University. Written informed consent for research purposes was provided for the patients. Total RNAs were extracted from cells or patient tissues by TRIzol reagent (Life Technologies) according to the manufacture's instructions. Total RNAs were extracted from patient plasma using TRIzol® LS Reagent (Life Technologies).

To detect circular RNAs, total RNAs (5 μg) were incubated with 15 units of Ribonuclease R (RNase R, Epicentre Technologies) at 37 °C for 15 min to digest the linear RNAs, then precipitated by ammonium acetate and glycogen. The resulting RNAs were subjected to reverse transcription using the RETRO script® kit (Life Technologies) with random primers according to the manufacture's instructions. Then PCR reactions were performed using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme) and divergent primers, which were listed in Supplementary information, Table S1. F-circEA was detected from patients' samples using nested PCR (first round PCR primers: F2/R2; nested PCR primers: F3/R3).

For quantitative real-time PCR, cDNAs were generated by M-MLV Reverse Transcriptase Kit (Life Technologies), and the qPCR was performed with SYBR Green Master Mix using StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA). U6 or GAPDH mRNAs were used as controls.

RNA solution hybridization

Oligonucleotide probes were end-labeled by T4 polynucleotide kinase (NEB) with [γ-^{32}P]-ATP (PerkinElmer). RNA samples with or without RNase-R digestion were hybridized with

oligonucleotide probes at 42 °C for 3 hours. Hybridized RNA samples were subjected to native agarose electrophoresis and transferred onto nitrocellulose membranes, which were detected by Typhoon FLA 7000 Phosphor Imager. The oligonucleotide probes were listed in the Supplementary information, Table S1.

Transwell migration and invasion assay

Cell migration assays were performed using Transwell chamber (Millipore), and cell invasion assays were done with chambers uniformly covered with Matrigel (BD Biosciences) diluted with RPMI-1640 (1:7). Cells were suspended in RPMI-1640 medium containing 5% BSA and seeded into the top chamber, while RPMI-1640 medium supplemented with 10% FBS was added into the bottom chambers as chemoattractant. After incubation at 37 °C for 24 hours, cells that did not migrate or invade through the pores of the Transwell inserts were removed with a cotton swab. Cells present at the lower surface of the membrane were fixed by 4% paraformaldehyde for 20 min, stained with 1% crystal violet (Sigma) for 15 min. The cells were counted in at least three randomly selected microscopic fields under an inverted phase-contrast Microscope. The experiment was repeated in 3 independent experiments.

Wound healing assay

Cells were cultured in the 6-well plate and wounded using a sterilized pipet tip to make a straight scratch. After being rinsed with physiological saline gently, the cells were incubated in RPMI-1640 medium containing 0.5% FBS and 1% penicillin/streptomycin. Pictures were taken by an Olympus digital camera for every 6 hours.

MTT and colony formation assays

Cells were seeded into the 96-well plate for MTT assay. At the indicated time points, the cells were incubated with 100 μL sterile MTT (Beyotime, Shanghai, China) at 37 °C for 4 hours, then the media were removed and replaced with 150 μL DMSO. The absorbance was measured at 570 nm. For colony formation assay, cells were seeded into the 6-well plate and cultured for 5 days, then the cells were stained with crystal violet solution (0.5%) for 20 minutes. After removing crystal violet solution, the cells were washed with water and colony formation was recorded by high-resolution scanner. All experiments were performed in triplicates.

Statistical analysis

Statistical analyses were performed using Student's t-test in GraphPad Prism 6.0. $P < 0.05$ was considered a statistically significant.

Supplementary information, Table S1

Primers and oligonucleotides used in this study:

Primers for *EML4-ALK* fusion gene (AB374362.1):

F1: TACCAGTGCTGTCTCAATTGCAG

R1:TGCCAGCAAAGCAGTAGTTG

Primers for F-circEA:

F2: GCAGAGCCCTGAGTACAAGC

R2: GCTTGGTTGATGATGACATCTTTATG

Nested primers for F-circEA:

F3: CAACTACTGCTTTGCTGGCA

R3: GCATTCTTGCTTTCTGGAGTTT

Primers for the establishment of F-circEA-overexpressing plasmid:

F4: AGCTGTTTTCTCATCCACAGAATTCGAGCATCACCTTCTCCCCAG

R4: TTCTGAAGAATCAAACTTACTTCAGAGCACACTTCAGGCAGCGT

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qPCR primers for F-circEA:

F5: CTGCAAGTGGCTGTGAAGA

R5: TCTGTGTATTTGGAGAGGTTGTG

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qPCR primers for *EML4-ALK* mRNA*:*

F6: CTGCAGACAAGCATAAAGATGTC

R6: CATGGCTTGCAGCTCCT

Oligo-nucleotide fusion probe in RNA solution hybridization:

CCGGCGGTACACTTGGCTGTTTTTTCGCG

Oligo-nucleotide junction probe in RNA solution hybridization:

GTGATGCTCGAATTTTCAGAGCACACTTCAGG

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siRNAs for F-circEA knockdown:

siRNA1: GTGTGCTCTGAA AATTCGA

siRNA2: GCTCTGAA AATTCGAGCAT

F-circEA sequence:

AATTCGAGCATCACCTTCTCCCCAGCCCTCTTCACAACCTCTCCAAATACACAGACAAA
CTCCAGAAAGCAAGAATGCTACTCCCACCAAAAGCATAAAACGACCATCACCAGCTGA
AAAGTCACATAATTCTTGGGAAAATTCAGATGATAGCCGTAATAAATTGTCGAAAATAC
CTTCAACACCCAAATTAATACCAAAAGTTACCAAAACTGCAGACAAGCATAAAGATGTC
ATCATCAACCAAGCAAAAATGTCAACTCGCGAAAAAAACAGCCAAGTGTACCGCCGGA
AGCACCAGGAGCTGCAAGCCATGCAGATGGAGCTGCAGAGCCCTGAGTACAAGCTGAG
CAAGCTCCGCACCTCGACCATCATGACCGACTACAACCCCAACTACTGCTTTGCTGGCA
AGACCTCCTCCATCAGTGACCTGAAGGAGGTGCCGCGGAAAAACATCACCCTCATTCGG
GGTCTGGGCCATGGCGCCTTTGGGGAGGTGTATGAAGGCCAGGTGTCCGGAATGCCCAA
CGACCCAAGCCCCCTGCAAGTGGCTGTGAAGACGCTGCCTGAAGTGTGCTCTGAA