Supplementary Materials and Methods

Cell lines and culture conditions

The PC-9 and A549 cell lines were obtained from RIKEN Cell Bank (Ibaraki, Japan). The Calu-3 and NCI-H2342 cell lines were obtained from the American Type Culture Collection (ATCC). The PC-9 cells were cultured in Roswell Park Memorial Institute Medium-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). The Calu-3 cells were cultured in Eagle's Minimum Essential Medium (EMEM) with L-glutamine and phenol red (Wako, Osaka, Japan) medium supplemented with 10% FBS. The A549 cells were cultured in Dulbecco's modified Eagle medium/F12 (DMEM/F12) (Thermo Fisher Scientific) supplemented with 10% FBS. The NCI-H2342 cells were cultured in DMEM/F12 (Thermo Fisher Scientific) supplemented with 5% FBS, 0.005 mg/ml human insulin (Wako), 0.01 mg/ml transferrin (Sigma-Aldrich), 30 nM sodium selenite (Sigma-Aldrich), 10 nM hydrocortisone (Wako), 10 nM beta-estradiol (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich).

Antibodies List:

Antibodies	Dilation ratio	Commercial sources
Anti-ECT2	1:500	Millipore, Billerica, MA
Anti-FAK	1:1000	Thermo Fisher Scientific
Anti-Phos-FAK (Tyr397)	1:1000	Abcam, Cambridge, UK
Anti-Phos-FAK (Tyr925)	1:1000	Cell Signaling, Danvers, MA
Anti-Phos-FAK (Tyr576/577)	1:500	Cell Signaling
Anti-Src	1:10 000	Abcam
Anti-Phos-Src (Tyr416)	1:500	Abcam
Anti-p130Cas	1:500	Biosciences Transduction Laboratories, Lexington, KY
Anti-Phos-p130Cas (Tyr410)	1:1000	Cell Signaling
Anti-Crk	1:500	BD Biosciences Transduction Laboratories
Anti-Phos-Crk (Tyr221)	1:500	Cell Signaling
Anti-integrin β1	1:1000	Abcam
Anti-Phos-integrin β1(Thr788/789)	1:500	Abcam
Anti-paxillin	1:500	Abcam
Anti-Phos-paxillin (Tyr118)	1:1000	Abcam
Anti- Myc-tag (9B11) mouse mAB	1:1000	Cell Signaling
Anti-DYKDDDDK tag(9A3) mouse mAB (binds to the same epitope as Sigma Anti-FLAG M2 Antibody)	1:500	Cell Signaling
Anti-Rac1	1:1000	Thermo Fisher Scientific
Anti-Cdc42	1:1000	Cell Signaling
Anti-β-actin	1:5000	Sigma-Aldrich
Anti- polyclonal goat anti-rabbit immunoglobulin horseradish peroxidase (HRP)	1:200 000	Dako, Glostrup, Denmark
Anti-polyclonal goat anti-mouse immunoglobulin	1:150 000	Dako

Primers pair used for RT-PCR

ECT2-forward 5′-CAAATGGATGCCCGAGCTG-3′,

ECT2-reverse 5′-ACGTCGTTTGCGATTGCTG TTA-3′,

FAK-forward 5′-ACGAAATGCTGGGCCTATGAC-3′,

FAK-reverse 5′-GACTCCATCCTCATGCGCTCT-3′,

CRK-forward 5′- CTCCCACTACATCATCAACAGCA-3′,

CRK-reverse 5′-CTGGATCTGGAAACTGGTTCTATCA-3′,

ITGB1-forward 5′-GGTTTCACTTTGCTGGAGATGG-3′,

ITGB1-reverse 5′-CAGTTTCTGGACAAGGTGAGCAATA-3′.

siRNAs targeting *ECT2* or FAK

ECT2#1-forward 5′-CAGAUAUCUUUGAUGUACACACUAA-3′,
ECT2#1-reverse 5′-UUAGUGUGUACAUCAAAGAUAUCUG-3′,
ECT2#2-forward 5′-GGUCCAUCACAUGGGUGGAGUUAUU-3′,
ECT2#2-reverse 5′-AAUAACUCCACCCAUGUGAUGGACC-3′,
FAK#1-forward 5′-GGAGAUGUACAUCAAGGCAUUUAUA-3′,
FAK#1-reverse 5′-UAUAAAUGCCUUGAUGUACAUCUCC-3′,
FAK#2-forward 5′-CGGACAGCGUGAGAGAGAAAUUUCU-3′,
FAK#2-reverse 5′-AGAAAUUUCUCUCUCACGCUGUCCG-3′.

RNA sequencing analysis

Calu-3 and NCI-H2342 cells were cultured on COL1-coated dishes. Calu-3 cells were transfected with siECT2#1 or siCON for 48 h and 72 h, while NCI-H2342 cells were transfected for 48 h. RNA was extracted from six sample defined as one sample for each condition; (siECT2#1, siCON from Calu-3 at 48 h), (siECT2#1, siCON from Calu-3 at 72 h), and (siECT2#1, siCON from NCI-H2342 at 48 h), Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific) from six samples in accordance with the manufacturer's protocol. RNA quality was analyzed using an Agilent Bioanalyzer RNA 6000 Pico Kit (Agilent, Santa Clara, CA). Ribosomal RNA depletion using 500 ng of total RNA was carried out with a NEBNext rRNA-depletion Kit (New England Biolabs, Ipswich, MA), followed by directional library synthesis using a NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs). The library was validated with the Bioanalyzer (Agilent Technologies) to determine size distribution and concentration, then 2×36 base paired-end sequencing was carried out with a NextSeq 500 (Illumina) at Tsukuba i-Laboratory LLP (Tsukuba, Ibaraki, Japan). Reads were mapped on the hg19 human genome and quantified using a CLC Genomics Workbench (QIAGEN, Redwood City, CA). To estimate transcription expression patterns among siECT2#1 and siCON sets, read counts were estimated for each transcript in individual samples as reads per kilobase per million reads (RPKM) and then normalized

using the CLC Genomic Workbench software. To identify the differentially expressed genes, data were filtered according to absolute fold change and a difference of >2 fold. RNA-seq data have been deposited in the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) (accession no. PRJNA646209). For functional enrichment analysis, we assigned Gene Ontology (GO) terms and conducted a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of common genes in Calu-3 and NCI-H2342 cells after excluding histone genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov) with $P < 0.05$ and more than 5 genes. Up-regulated and down-regulated genes were defined by normalized gene expression values of ≤1 (siCON/siECT2#1) or ≥1 (siCON/siECT2#1), respectively.

Immunofluorescence

Calu-3 and NCI-H2342 cells were transfected with siECT2#1 or siCON and cultured for 48 h. The cells were collected and seeded on non-coated coverslips (Matsunami) or COL l-coated coverslips (Iwaki Biosciences, Tokyo, Japan). After 12 h, the cells were fixed with 10% neutral buffered formalin for 10 min and permeabilized with 0.5% Triton™ X-100 (Thermo Fisher Scientific). They were then stained with rhodamine-phalloidin (Thermo Fisher Scientific) for 30 min at room temperature. For staining of focal adhesion, NCI-H2342 cells transfected with siECT2#1 were plated on COL1-coated coverslips, followed by the protocol described above. Samples were then incubated with anti-Phos-paxillin (Tyr118) (primary antibody) at 1:100 for 1 h at room temperature. They were then incubated with anti-rabbit IgG- conjugated Alexa Fluor 568 antibody (Thermo Fisher Scientific) (secondary antibody) at 1:200 for 30 min at room temperature. The fluorescence images for the above experiments were captured and analyzed using a confocal microscope (Leica TCS SP5; Leica Microsystems, Wetzlar, Germany). For validation of the knockdown efficiency of ECT2

using immunofluorescence, three cell lines (Calu-3, PC-9, and NCI-H2342) were transfected with siECT2#1 or siCON and cultured on COL l-coated coverslips (Iwaki Biosciences). The cells were fixed in methanol for 10 min, washed three times with PBS, and then incubated with Tris-buffered saline (TBS) for 30 min. The cells were then reacted with anti-ECT2 (primary antibody) at 1:200 for 1 h at room temperature and washed with PBS three times. They were then reacted with Alexa fluor 555-conjugated anti-rabbit IgG (Thermo Fisher Scientific) (secondary antibody) at 1:1000 for 1 h at room temperature. After being rinsed again with PBS three times, the coverslips were mounted using Vectashield HardSet Mounting Medium with DAPI (Vector Laboratories Inc., Burlingame, CA), and images were captured using a fluorescence microscope (Biorevo BZ-9000).

Pull-down assays for Rac1 and Cdc42

Calu-3 cells were transfected with siECT2#1 or siCON for 72 h. Rac1 and Cdc42 activities were then determined using a Pull-Down Kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol.