Supplemental Materials and Methods

Real-time PCR analysis. The primers for amplifying human EphB3 gene were as follows: forward, 5′-TCGTGGTCATCGCTATCGTCT-3′, and reverse, 5′-AAACTCCCGAACAGCCTCATT-3′. The primers for β-actin were: forward, 5′-GATCATTGCTCCTCCTGAGC-3′, and reverse, 5′-ACTCCTGCTTGCTGATCCAC-3′. The relative mRNA level of target genes to that of β-actin in either clinical samples or cultured cell lines was calculated according to the methods described by Wang et al. (1). The statistical results were considered significant at $p<0.05$ and highly significant at $p<0.01$. All data analyses were done using SPSS 13.0 statistical software.

Plasmid construction and transfection. Full length cDNA of EphB3 was subcloned from pBluescript, which was kindly provided by Dr. Strebhardt, into pcDNA3-HA (kindly provided by Dr. Xiangjun Tong). The EphB3-HA-pcDNA3 and the empty plasmid were transfected into H520 using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA). The transfected cells were selected with G418 (400 ug/ml) and after 2 weeks of selection, surviving clones were picked and further confirmed by Western blotting. Luciferase full length cDNA was cloned into FG12 expressing construct (kindly provided by Dr. Yingying Le) and used for lentivirus package. A549 cells were then infected with luciferase lentivirus for metastasis assay.

RNAi-mediated knockdown of EphB3. Two sequences for EphB3 small interfering

RNA and one scrambled sequence were synthesized. The sequences were as follows: B3siRNAcon 5’-ACCGATGTTGTCAACGACTAGTTTCAAGAGAACTAGTCGTTGACAA CATCTTTTTGGATCCC-3’ and 5’-TCGAGGGATCCAAAAAGATGTTGTCAACGACTAGTTC TCTTGAAACTAGTCGTTGACAACAT-3’; B3siRNA2# 5’-ACCGCTGCAGCAGTACATTGCTTTCAAGAGAAGCAATGTACTGCTGCAG CTTTTTGGATCCC-3’ and 5’-TCGAGGGATCCAAAAAGCTGCAGCAGTACATTGCTTCTCTTGAAAGCAA TGTACTGCTGCAG-3’; B3siRNA4# 5’-ACCGTGTGCCGTGGTCGACTGATTCAAGAGATCAGTCGACCACAACAC ACTTTTTGGATCCC-3’ and 5’-TCGAGGGATCCAAAAAGTGTGCCGTGGTCGACTGATCTCTTGAATCAGT CGACCACAACACA-3’. FG12 lentiviral vector was used to produce small double-stranded RNA (small interfering RNA) to inhibit target gene expression. The information and usage of this vector system has been previously described in detail by Deng et al. (2).

Cell migration assay. The migration assay was carried out using a twelve-well Transwell polycarbonate filter (Neuro Probe) with an 8-um pore size. 1×10^5 cells were seeded into upper wells of the Transwell and incubated for 6 h at 37°C in medium containing 1 % FBS. Medium with 10 % FBS was used as a chemoattractant

in the bottom wells. Cells that did not migrate through the pores of the Transwell were manually removed with a rubber swab. Cells that migrated to the lower side of the membrane were stained with eosin and photographed using an inverted microscope.

MTT assay. Cells were plated in 96-well plates with a concentration of 1000 cells per well. 20 ul 5 mg/ml MTT was added into the media and cultured at 37°C. After 4 h, the medium was removed and 200 ul DMSO was added to dissolve the generated deposits and the absorbance at 550 nm was measured by an automatic microplate reader. The measurement process was performed every 24 hour for 4 or 5 days to generate a cell growth curve.

BrdU Incorporation Assay. Control cells and EphB3 silencing cells were grown to 50 % confluence. BrdU labeling reagent (Zymed Laboratories Inc.) was added into medium and incubated for 1 h before cells were processed for immunostaining. The BrdU medium was removed, and then the cells were rinsed with PBS, fixed and permeabilized with 2 N HCl. After washing three times with PBS, cells were stained with anti-BrdU primary antibody followed by goat anti-murine secondary antibody. Slides were analyzed using a fluorescent microscope, and the mean ± s.d. of cells with BrdU incorporation was calculated.

Focal adhesion disassembly assay. Serum-starved cells were seeded on glass coverslips and treated with 10 μM nocodazole. After 4 h the drug was washed out

with serum-free media and focal adhesions were monitored at different intervals of time. For immunofluorescence, cells were fixed in –20°C methanol for 3 min, rehydrated in PBS, blocked with PBS containing 2 % BSA for 1 h at room temperature, and incubated with primary antibody at 4°C overnight. Secondary antibody was performed at 1:1000 dilution. Fluorescence was monitored by using an inverted confocal laser microscopy.

Apoptosis analysis. Cells were plated in 60-mm dishes and trypsinized when they reached 70 % confluence. After washing with PBS, cells were fixed in 70 % ice-cold ethanol overnight. After staining with propidium iodide (50 ug/ml) and digesting with RNase (100 ug/ml) for 1 h at 37°C, samples were analyzed by flow cytometry (FACSAria, BD).

Supplemental references:

1. Wang Y, Liu DP, Chen PP, Koeffler HP, Tong XJ, Xie D. Involvement of IFN regulatory factor (IRF)-1 and IRF-2 in the formation and progression of human esophageal cancers. Cancer Res 2007 Mar 15;67(6):2535-43.

2. Deng YZ, Chen PP, Wang Y*, et al.* Connective tissue growth factor is overexpressed in esophageal squamous cell carcinoma and promotes tumorigenicity through beta-catenin-T-cell factor/Lef signaling. J Biol Chem 2007 Dec 14;282(50):36571-81.

Supplemental Figure Legends

Fig. S1. Effects of EphB3 silencing in H520/B3 stable clones. A, H520/B3-1 and H520/B3-2 was infected with the indicated lentivirus (B3siRNAcon, B3siRNA2# and 4#), respectively, and the knockdown efficiency was evaluated by Western blotting. B, the effect of EphB3 silencing on cell migration in H520/B3 stable clones was tested by Boyden chamber assay. C, EphB3 silencing decreased the number of viable cells of H520/B3 stable clones as measured by MTT assay. D, attenuated clonogenic growth in soft agar after EphB3 was knocked down in H520/B3 stable clones.

Fig. S2. Phosphorylation level of endogenous EphB3 in A549 and H23. Cell lysate was immunoprecipitated with anti-EphB3 antibody or mouse IgG and probed with pY100. Membrane was stripped and reprobed with anti-EphB3 antibody. Stable clone H520/B3-1 was used as a positive control.

Fig. S3. Immunofluorescent staining of FAK in A549 and H23 cells after EphB3 was stably silenced. A, A549 and H23 cells were infected with indicated lentivirus (B3siRNAcon, B3siRNA2# and 4#), respectively. Focal adhesions were examined by immunofluorescent staining of FAK. B, number of focal adhesions was quantified.

Fig. S4. A, protein levels of FAK (pY 397), FAK, paxillin (pY 118), paxillin and actin were examined by Western blotting in A549 and H23 cells which either were control or EphB3 silencing cells. B, serum-starved control and EphB3 silencing cells of A549 and H23 were treated with 10 μM nocodazole for 4 h. At the indicated time after nocodazole washout, cells were stained with anti-paxillin antibody. B3siRNAcon cells exhibited few punctuate focal adhesions around the cell periphery. Exposure to nocodazole increased the number of focal adhesion (0 min) followed by a rapid disassembly and then reassembly (30 and 60 min) after nocodazole washout. In contrast, EphB3 silencing cells exhibited much slower loss of paxillin staining, which suggested prominent and more stable focal adhesions (30 and 60 min).

Fig. S5. A, RNA level of Bax and Bim were examined by real time PCR in control and EphB3 silencing cells. B, the interaction between TRAF2 and EphB3. Flag-tagged TRAF2 and HA-tagged EphB3 were cotransfected into 293T cells. Cell lysate was incubated with anti-HA, anti-flag antibody or mouse IgG, respectively, and then precipitates were detected for the interaction between TRAF2 and EphB3.