Reagents, Antibodies, and plasmids
pcDNA3/epithin, pcDNA3/epithin\textsuperscript{S805A}, pcDNA3/epi\Delta, anti-N and mAb5 antibodies have been described \textsuperscript{36}. pFlag-CMV1/hTie2 was kindly provided by Dr. Gou Young Koh (KAIST). Anti-Tie2 antibody against its cytoplasmic domain and anti-myc antibody were from SantaCruz Biotechnology. Anti-flag (M2), anti-tubulin (tub2.1) and anti-phosphotyrosine (4G10) antibodies were purchased from Sigma. Tie2 mutants (deletion in Tie2\Deltac and Tie2\Deltae are 780\textsuperscript{th} ~ 1124\textsuperscript{th} and 27\textsuperscript{th} ~ 729\textsuperscript{th} amino acids, respectively) in pFlag-CMV1 and epithin mutants (deletion in epithin\DeltaCUB and epithin\DeltaLDLRA are 213\textsuperscript{th} ~ 446\textsuperscript{th} and 447\textsuperscript{th} ~ 604\textsuperscript{th} amino acids, respectively) in pcDNA3 were prepared using PCR. Site-directed mutagenesis was performed using QuickChange\textsuperscript{TM} (Stratagene). To generate pSUPER-epi, a DNA duplex containing a target sequence in epithin, GGTCGCGCTTCAAACTCTTC, was cloned into pSUPER (OligoEngine). For pSUPER-ctl, the target sequence was replaced by a scrambled sequence, GTGCCTTTCAAACTCTTC. Epithin expression lentiviral construct, pSIN/epithin, was generated by cloning epithin gene into a lentiviral vector (pRRLSIN.cPPT.PGK.IRES.GFP.WPRE or pSIN in short) modified from pRRLSIN.cPPT.PGK-GFP.WPRE (Addgene). Tie2 knock down lentiviral constructs were generated by inserting two different hairpin sequences (oligo ID: TRCN0000023556 and TRCN0000023557, Open Biosystems) into pLKO.1 (Addgene).

Cell lines and culture
427.1.86, HEK293T, and 4T1 cells were maintained in DMEM (Life Technologies, Inc.) with 10\% FBS (Life Technologies, Inc.). MS1 cells were maintained in DMEM with 5\% FBS. To generate the epithin knock-down cell lines, 427 Epi-KD, pSUPER-epi was transfected into 427.1.86 cells with pcDNA3 that encodes a neomycin resistant gene. After growing in the presence of 800 \mu g/ml G418 for 10 days, clones derived from single cells in 96 well were tested for their epithin expression in both the protein and RNA level to select a stable knock-down clone. GPF-427 and GFP-427 Epi-KD were generated similarly except using pcDNA3/eGFP instead of pcDNA3. Clones from 4T1 cells were
generated by infecting 4T1 cells with control or epithin shRNA lentivirus (Santa Cruz Biotechnology, INC) followed by growing in the presence of 2 μg/ml puromycin and single cell sorting as above. For transient transfections, Lipofectamine and Lipofectamine Plus reagent (Invitrogen) were used for 427.1.86 and MS1, and a calcium phosphate method for the HEK293T cells. Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from ScienCell Research Laboratories (Carlsbad, CA), and maintained in Endothelial Growth Medium-2 (EGM-2, Lonza) which contains hydrocortisone, hFGF-B, VEGF, R3-IGF-1, ascorbic acid, heparin, FBS, hEGF, and GA-1000 in Endothelial Basal Media-2 (Lonza). The medium was replaced every other day, and cells were split when subconfluent.

Co-immunoprecipitation
427.1.86 cells on a 100-mm dish were transfected with Tie2 or Tie2 mutant cDNA. 24 h after transfection, cells were maintained without serum for 12 h, treated with 10 mM PMA and/or 10 mM leupeptin for various times, and lysed in 50 mM HEPES, 1 % NP-40, 150 mM NaCl, 1 mM EDTA with protease inhibitors. The lysates were subjected to immunoprecipitation as described previously 23.

Surface biotinylation
After transfection of Tie2 cDNA into 427.1.86 cells on a 60-mm dish, cells were treated with PMA. The cells were washed and then incubated with 1 mg/ml sulfo-NHS-biotin (PIERCE) at 4 °C for 30 min. After quenching the biotinylation reaction with 100 mM glycine, cells were lysed with lysis buffer (50 mM HEPES, 1 % NP-40, 150 mM NaCl, 1 mM EDTA with protease inhibitors). The lysates were incubated with immobilized streptavidin beads (PIERCE) at 4 °C for 2 h. After washing three times, the samples were subjected to western blotting using anti-flag antibody.

Transendothelial migration assay
For microscopic analysis of transendothelial migration, MS1 cells were cultured on a gelatin-coated coverslip in a 12-well plate to form a confluent monolayer. GFP-427 and GFP-427-Epi-KD cells were detached using Cellstripper™ (Cellgro) and were
resuspended in growth medium (concentration, $10^5$ cells/ml). 1 ml of the cell suspensions were added to each MS1 monolayer. After 8 h, the cells were washed, fixed, and stained with rhodamine phalloidin, and observed with a fluorescence microscope (Axiovert 200M, Zeiss) equipped with a 10X Planapochromat objective lens. Images were processed using AxioVision RE software (Zeiss). Transmigrated cells were determined by morphology based on the GFP and F-actin pattern. The optical sections were obtained with LSM510 with C-Apochromat 40X/1.2W Korr lens at a 2X digital zoom setting and assembled using the LSM Image Browser (Carl Zeiss). For using Boyden Chamber, $5 \times 10^4$ MS1 cells were grown on 8 $\mu$m pore Chamber (Insert, Falcon) until they reach to confluent. 4T1 cells were stained with CFSE (Molecular Probes) and resuspended into serum-free DMEM. The CFSE-stained $10^5$ 4T1 cells were added onto the MS1 cells and incubated for 4 hr. Cells were fixed with 3.7% formaldehyde, and unmigrated cells were scrubbed and washed. Images of CFSE-positive cell were obtained by using IX-51 microscope (Olympus) with AxioCam MRm (Zeiss), and the cell numbers were analyzed with ImageJ (National Institute of Health).

**Histochemistry**

Cryosections of the thymus were prepared with six-week-old Tie2-GFP mouse (Jackson Laboratory, strain name: STOCK Tg(TIE2GFP)287Sato/J). Sections were incubated with affinity-purified anti-N antibody and then by rhodamine-conjugated anti-rabbit antibody. They were then observed with a fluorescence microscope (Axiovert 200M, Zeiss) equipped with a 10X Planapochromat objective lens. Fluorescence images were acquired using a digital camera with a cooled charge-coupled device (Axio-Cam HRm, Zeiss) and AxioVision software (Zeiss). To obtain images at a higher resolution, the sections were observed using a LSM510 laser scanning confocal microscope (Carl Zeiss) with a C-Apochromat 40X/1.2 W Korr lens. Z-stacks were captured at a 2X digital zoom setting and presented as Z-projected overlay images using LSM510 software (Carl Zeiss). For staining normal thymic sections with $\alpha$-SMA, Axio Imager A1 microscope (Zeiss) was used.

**Tie2 phosphorylation analysis**
Cells expressing Tie2 were lysed with RIPA buffer (1% Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate) containing protease inhibitor cocktail (Roche) and phosphoSTOP (Roche). Cell lysates were clarified by centrifugation at 14,000 g. The clarified cell lysates were incubated with anti-Tie2 antibody raised against Tie2 cytoplasmic tail (Santa Cruz Biotechnology) for overnight, and further incubated with protein G-sepharose for 2 hr. Beads bound proteins were washed, and analyzed by SDS-PAGE and western blotting with anti-phosphotyrosine antibody (4G10).

**Lentivirus preparation and infection**

3 μg of lentiviral vector (pSIN/epithin or pSIN control vector), as well as 2 μg of pCMV delta R8.2 (Addgene), and 1 μg of pMD2.G (Addgene) were co-transfected into HEK293T cells on each 100 mm dish using Lipofectamine (Invitrogen) and Plus reagent (Invitrogen) according to manufacture’s manual. At 3 hr after transfection, the transfection medium was replaced with 7 ml of UltraCULTURE media (BioWhittaker). At 48 hr after transfection, medium from the transfected cells (usually on four 100mm dishes) was harvested and pooled. Harvested medium was clarified by centrifugation at 1,000 rpm for 10 min and filtration with a 0.45 μm filter, and then concentrated using Centricon (Millipore, 30 kD cutoff). HUVECs at 80% confluence were infected with the concentrated virus. At 6 hr after infection, medium was replaced with fresh EGM-2, and cells were further incubated for 24 hour before harvested for immunoprecipitation.
Figure S1. Identification of epithin interacting proteins and MALDI-TOF mass mapping
After deprived of serum for 12 h, 427 cells on thirty 150 mm dishes were treated with 10 mM PMA for 1 h, and then lysed in lysis buffer (50 mM HEPES, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA with protease inhibitors). The lysates were incubated with anti-N antibody (IgG purified) conjugated beads at 4 °C for 2 h and washed with the lysis buffer. The bound proteins were eluted with 150 mM NaCl, 10^{-2} M HCl. The eluates were precipitated with trichloroacetic acid, and then fractioned by SDS-PAGE. After silver staining, candidate protein bands were excised from the gel, de-stained, trypsinized, and extracted. The trypsinized samples were analyzed using a mass spectrometer (voyager DE-TM STR Biospectrometry workstation) to obtain peptide mass fingerprints, and database search was done using MS-FIT proteomic tool (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm). Arrow head indicates captured epithin and arrow indicates Tie2.

Figure S2. Epithin expression localizes near blood vessels.
(A) A cryosection of a mouse thymus was stained with affinity-purified anti-N antibody followed by biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase. They were then visualized using a Metal Enhanced DAB substrate. The arrowhead in (a) indicates the blood vessel. A magnified image of the staining localized at the blood vessel is shown in (b). The staining completely disappeared when the peptide used to generate anti-N antibody was added during immunostaining (c). (B) A cryosection of the thymus from a Tie2-GFP mouse, which express GFP under the control of a Tie2 promoter, was stained with affinity-purified anti-N antibody followed by rhodamine-conjugated anti-rabbit antibody. GFP expression (a, d), anti-N antibody staining (b, e), and merged images (c, f) are shown. As expected, green fluorescence was observed in the major vessels and smaller vessels in the cortex. Anti-N antibody staining showed that epithin is located in the major internal vessels of the corticomedullary junction and in smaller vessels in the cortex (arrows); it was also observed in the thymic
subcapsulary area (arrow heads). The digital enlargement of areas in small rectangle in (c) are shown (c’). For images at a higher resolution (d, e, f), the images from optical sections generated using a confocal microscope were z-projected to obtain the final combined images. Since section was made askew to the blood vessel, GFP signal coming from both outside and inside of the section become broader in the combined images than the epithin signal coming only from the surface of the section (see supplementary movie 1). Therefore, one of the optical sections was also shown (g) to clarify the colocalization issue on a specific focal plane. This higher magnification showed that the epithin staining in the vessel structures did not precisely co-localize but resided in the surrounding area of the GFP-expressing cells. Scale bar in C, 500 μm. Scale bar in F, 20 μm. (C) A cross section of mouse thymus was stained for α-smooth muscle actin antibody (α-SMA) (Millipore) (b) and epithin (H-270, Santa Cruz) (c). A negative control stained with only secondary antibody (a) and the merged image (d) were also shown.

Figure S3. Expression of epithin in HUVEC induces Tie2 cleavage and its phosphorylation

HUVEC cells were infected with lentivirus particles containing epithin gene (Epithin) or control virus (-). At 30 hr after infection cells were lysed, and normal rabbit serum (ctl) or anti-Tie2 antibody (anti-Tie2) raised against Tie2 cytoplasmic tail were added to the lysates for immunoprecipitation. The precipitated proteins were analyzed by western blotting using anti phospho-tyrosine antibody which was detected by secondary antibody conjugated with a fluorescent dye, IR800. The blot was visualized using infrared scanner and the raw image is shown (B). The same membrane was also analyzed for the precipitated Tie2 by western blotting, using anti-Tie2 antibody directly conjugated with HRP (A). Expression of epithin in cell lysates was analyzed in (C). The truncated Tie2 (arrow head) is clearly detected in cells infected with epithin lentivirus (A), showing that epithin expression induces the cleavage in the primary endothelial cell, HUVEC. Phosphotyrosine blot in (B) shows basal levels of Tie2 phosphorylation of HUVEC in the absence of agonist. Our quantification analysis showed that the phosphorylation signal of truncated form increased to 3 fold when epithin is present. In addition, the amount of truncated Tie2 to the amount of intact Tie2 in the lane 3 of Tie2 blot (A) is 15.6 %, or
even less when considering the signal from intact Tie2 band is saturated. However, the phosphorylation level of truncated Tie2 is 34.9% of that of intact Tie2 in the phosphotyrosine blot (B). Thus, cleaved Tie2 is efficiently phosphorylated than intact Tie2 in HUVEC as well as observed in HEK293T (Fig. 5A). Taken together, epithin expression in HUVEC can induce tyrosine phosphorylation of Tie2 by inducing its cleavage.

**Figure S4. Absence of Tie2 extracellular domain enhances tyrosine phosphorylation of Tie2**

(A) Domain structure of Tie2. IG, immunoglobulin like domain; E, EGF like domain; FN, Fibronectin type III domain; T, transmembrane domain. A truncated Tie2 construct, truncTie2, is indicated. (B) Wild type Tie2 (wt), truncated Tie2 construct (trunc), and/or epithin were transfected into HEK293T cells, and Tie2 constructs were immunoprecipitated with anti-Tie2 antibody. The degrees of phosphorylation in each condition were analyzed by anti phosphor-tyrosine antibody (4G10) (upper panel). The precipitates Tie2 constructs were also analyzed by blotting with anti Tie2 antibody (lower panel).

**Figure S5. Knock-down of epithin from 427 cell line decreases the ability of transendothelial migration**

The ability of transendothelial migration of epithin knock-down clone, 427 Epi-KD, was less than that of 427. Asterisks indicate P<0.01.

**Figure S6. Epithin in 4T1 cells degrade Tie2 in endothelial cells in a trans manner.**

Epithin expressing 4T1 clone (D5) and epithin knock downed 4T1 clone (B3) were tested for their ability to degrade Tie2 in MS1 when co-cultured as in Fig. 4A.

**Figure S7. Tie2 knock-down in endothelial cells enhances transendothelial migration.**

(A) Two different hairpin sequences for knocking down mouse Tie2 (oligo ID: TRCN0000023556 and TRCN0000023557, Open Biosystems) as well as scrambled
sequence (scrambled) were ligated into pLKO.1, and lentiviral particles generated by those constructs were infected to MS1 cells. Knock-down efficiencies were tested by western blotting with anti Tie2 antibody. (B) MS1 cells infected with lentivirus encoding TRCN0000023557 (TRCN23557) were used as Tie2 knock-down MS1 cells for transendothelial migration assay. MS1 cells infected with control virus (scrambled) and Tie2 knock-down MS1 cells (Tie2 KD) were plated to transwells, and transendothelial migration of 4T1 cell was analyzed as in Fig. 7B.

**Figure S8. The numbers of tumor nodules are correlated with body weights of mice.**
The development of tumor nodules decreased the body weight of mice. The body weight of A7 and D5-injected mice was less than that of B3, C5 and G3-injected mice. The body weights were measured immediately before sacrificing mice.

**Figure S9. Tie2 knock-down in endothelial cells enhances transendothelial migration.**
(A) Human Tie2 amino acid sequence was analyzed by COILS, a coiled-coil region prediction program (http://www.ch.embnet.org/software/COILS_form.html), to search any coiled-coil region in Tie2. The calculated probabilities that the sequence will adopt a coiled-coil conformation are plotted against amino acid residue number. The predicted coiled-coil region is indicated as yellow box in a Tie2 diagram. IG, immunoglobulin like domain; E, EGF like domain; FN, Fibronectin type III domain; T, transmembrane domain. (B) Human Tie2 amino acid sequence is shown with a putative transmembrane region boxed and the predicted coiled-coil region highlighted with yellow.
Figure S1
Figure S3

A

IP: ctl  Anti-Tie2
IB: anti-Tie2-HRP

B

IP: ctl  Anti-Tie2
IB: anti-pY

C

IB: mAb5
Figure S4

A

![Diagram showing the structure of Tie2 with Truncated Tie2 (truncTie2)]

B

<table>
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<tr>
<td>Epithin:</td>
<td>-</td>
<td>-</td>
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- IB: pY
- IB: anti-Tie2 - HRP
- IP: Anti-Tie2
Figure S5
Figure S6
Figure S7

(A) Western blot analysis showing the expression of Anti-Tie2 and Anti-tubulin in different conditions: scrambled, TRCN23556, and TRCN23557. IB: Immunoblot.

(B) Bar graph representing the number of migrated cells/field for scrambled and Tie2 KD (TRCN23557) conditions.
Figure S9

A

B

1 MDLSLAVLC GVSLLGSGTV EGAMDLILIN SLPLVSDAEI SLTCTASGWR PHEPITRGR
61 FEAIOIQGRO QELEQTVQER ERAKKEVKYK ERIATKINAY FCEGKVRGAE AHTIRMKRGQ
121 QA5FLPAtlIT MTKVQGDNVNV ISFKVRLKE EDAVYRNGS FIHSVFRHEV FDLIEVHLPH
181 AQPQDAQYYS ARIIYGGNLT SATFLIVVRR CEQAQWGGFCN NHTCQACMN GVCHDTEGC
241 TCPGPSPM3RT CECADLEITF GRCFEECSQG QGEGKSVYFC LEPPYQGC3CA TQYKGIQCE
301 ACHEFYQED CIRKCGCNGNG ECMDQREQECGL CSPHEQGQLQC REDEQMTF KYYDKLHHE
361 VNGKPEPCIC FASGQWELPTN EEMTLKPDGD TVLHPPDFNN TDHFSVAFVT IHRILPPDSG
421 VNVCSSVNTVA GMEVEKPRNIS VKLVPKEILNA FNVIDTGHNF AVINISSSEPY FGDPFIKSPE
481 ILKYNVNYHE AQHQTQTVNE IVTNYLEPLL TEYELCVQTV RBSGEGGEGHF GPVPERTTA
541 IGLYPRGGLNL LLPSY5QGINL LTVQPPFPSS EDDFVYEVER RSVKSDQGNN IKVPGNITSV
601 LIINLQPEQF YYVRARINFT AQGEWSEDLT AWTLSDLIPF QPEFMISKNI THSEAVISWT
661 IILGYSISSI TIRYKVQUKN EDQHVDWVIK NMTITQYQLK GLEPETAIQN DIFLNNNGS
721 SNAPFHELIV TLPEQAPAD LGGGRHLILH ILGSAGMTCL TVLAFLLILF QLKRANQVQR3
781 MAQQONVRK IRSVQFNSGOT LIALRRKWNK PDFTYYVVLW WNDIKFQEVER GEGOKFLQVLK
841 ARIKRDGLRM DAIIKPRKKEY ASKDKMRFYDA GELEVLCGLI HPPSRLLLGL AGCERGQYLX
901 AIDYAPGSHL LDFLRQGRVL ETDFAPAFN STASTLSSQQL LHFAADWJAR GMDVLSQGKF
961 IHTRDLARRNI LGVENYARKI ADFGLSRGQEE VYVKTMGRL PVRWMAISB XNYSYTTNSD
1021 VWSYGVLLWE IVSLQGGTPY IGMCTAELYDK LPQGYRLKKH LHCDEVDYDL MRQCWREKPY
1081 ERPSFAQILV SLHRMLEERK TVNTLTLKF EYAGIDCSA EKAA