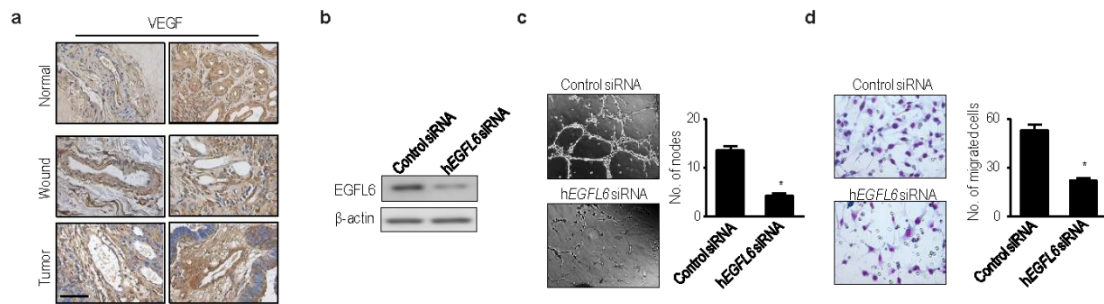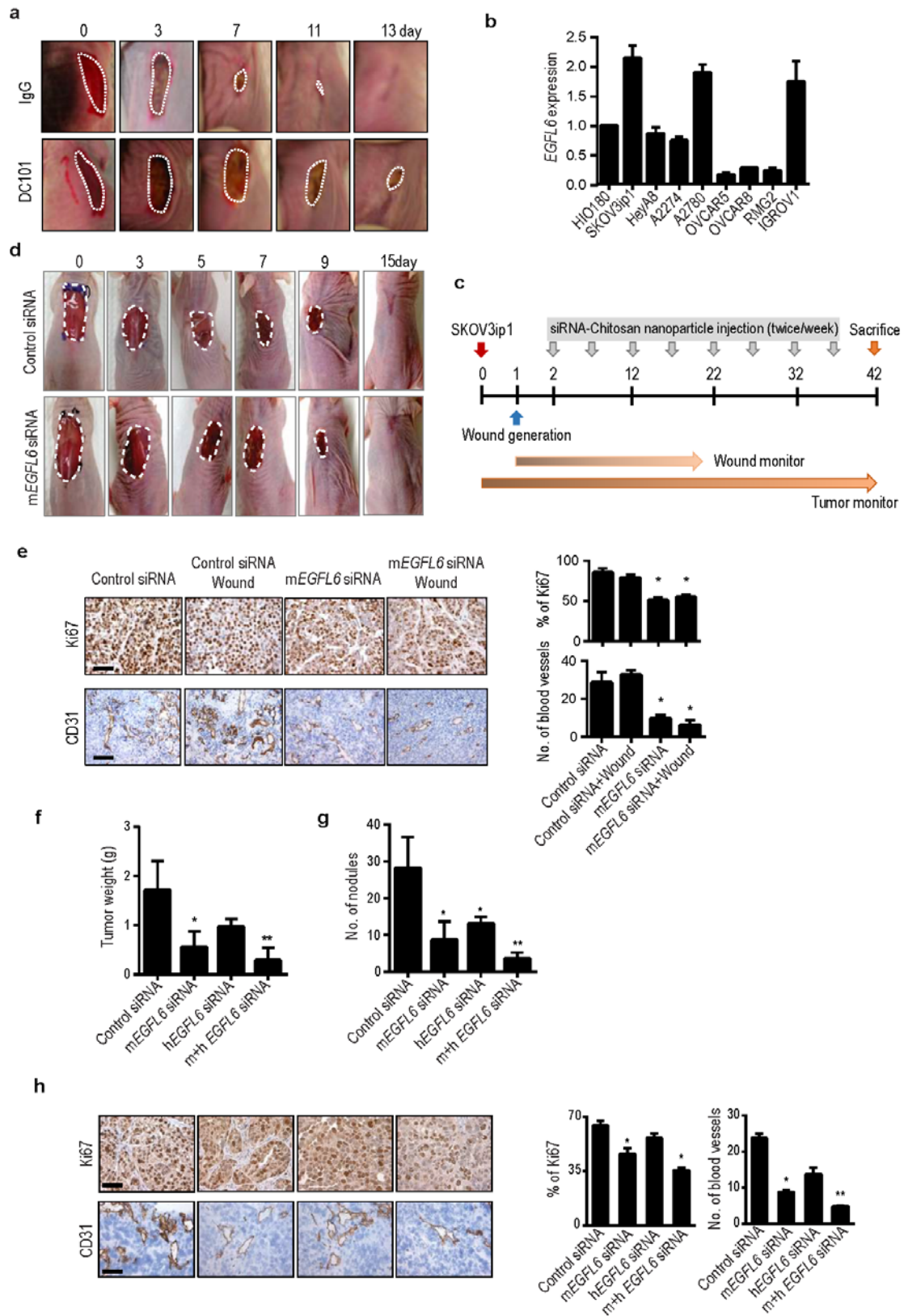Supplementary Figures

Supplementary Fig. 1.

Supplementary Figure 1. Related to Figure 1. ; EGFL6 regulates tumor angiogenesis. a, Expression of VEGF in human normal ovary, wound, and ovarian tumor samples. Representative images stained from different samples. Scale bar = 50 µm. In **b**, Control siRNA- and *EGFL6* siRNA-treated RF24 cells characterize EGFL6 expression. **c-d**, Silencing of *EGFL6* using specific siRNA decrease tube formation (**c**) and migration (**d**). Migrated cells were examined at 400X magnification.

Supplementary Fig. 2.

Supplementary Figure 2. Related to Figure 2. ; Silencing of EGFL6 decreases tumor proliferation. a, Pictures of representative wounds on mice treated with either control IgG antibody or DC101 (anti-VEGFR2). **b**, Expression of *EGFL6* mRNA in human ovarian cancer cells. **c,** Schematic diagram of the SKOV3ip1 tumor model, wound generation, and treatment regimen. **d**, Pictures of wounds on mice injected with SKOV3ip1 tumor cells and then wound was created on the dorsal side of the mice. Animals were treated with either Control siRNA-CH or m*EGFL6* siRNA-CH. Representative pictures of healing wounds in Control siRNA- and m*EGFL6* siRNA-treated mice. **e,** Animals were treated with either Control siRNA-CH or m*EGFL6* siRNA-CH with or without wound. Harvested tumors were stained for Ki67 (proliferation) and CD31 (microvessel). Error bars indicate SEM. *p<0.05 vs. Control siRNA. **f** and **g**, Effects of endothelial cell (m*EGFL6* siRNA) or tumor (h*EGFL6* siRNA) targeted *EGFL6* siRNA on tumor weight and tumor nodules in SKOV3ip1 orthotopic mouse model of ovarian cancer. Seven days after tumor cell injection, mice began receiving therapy: (1) Control siRNA, (2) m*EGFL6* siRNA, (3) h*EGFL6* siRNA, and (4) m*EGFL6* siRNA + h*EGFL6* siRNA. Mice were euthanized when any animals in a control or treatment group became moribund. **h**, Effect of targeted *EGFL6* siRNAs on proliferation and microvessel density. Harvested tumors were stained for Ki67 (proliferation) and CD31 (microvessel). Scale bar = 50 µm. The bars in the graphs correspond sequentially to the labeled columns of images on the left. Error bars indicate SEM. *p<0.05 vs. Control siRNA

a

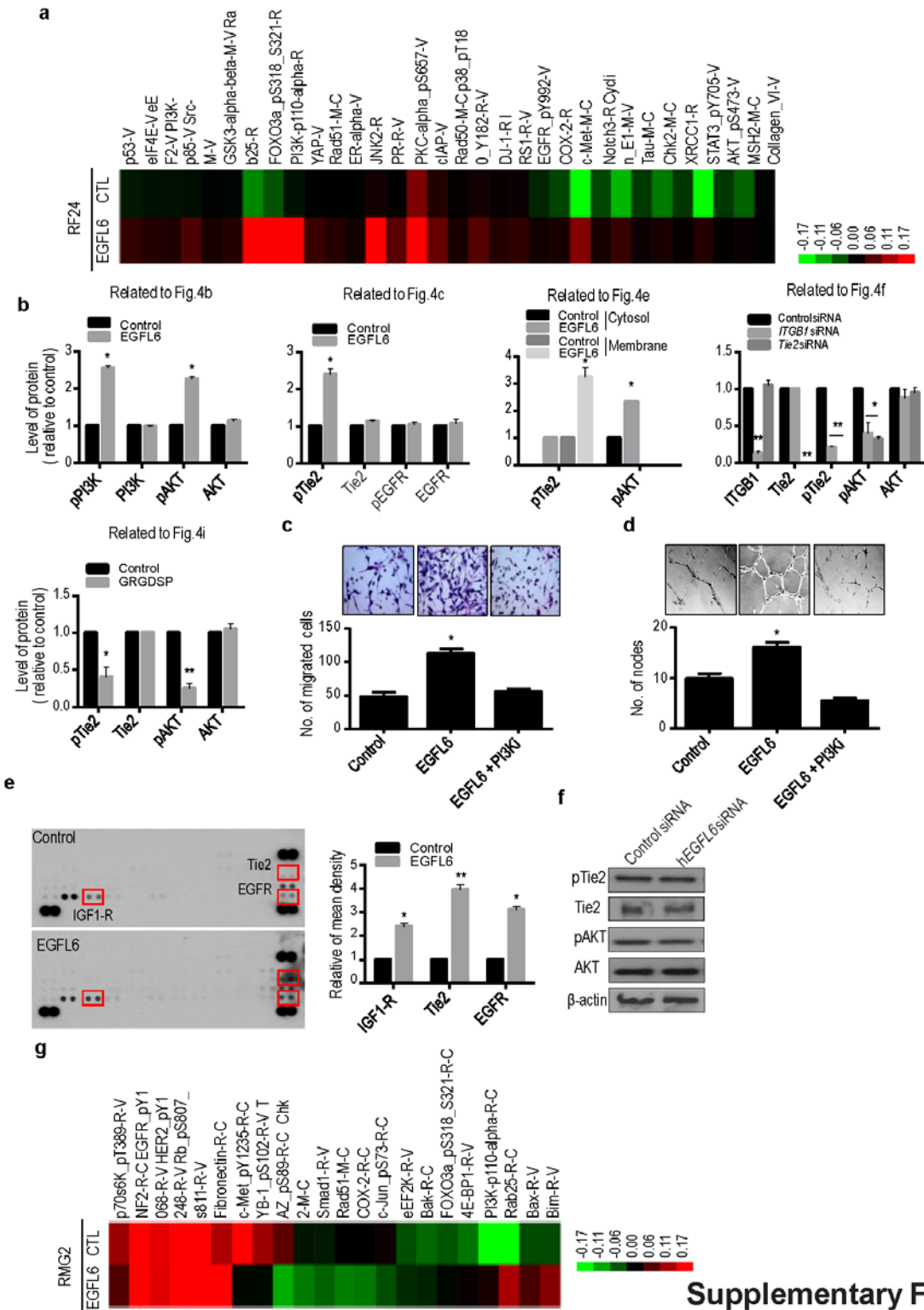TWIST binding motif in EGFL6 promoter
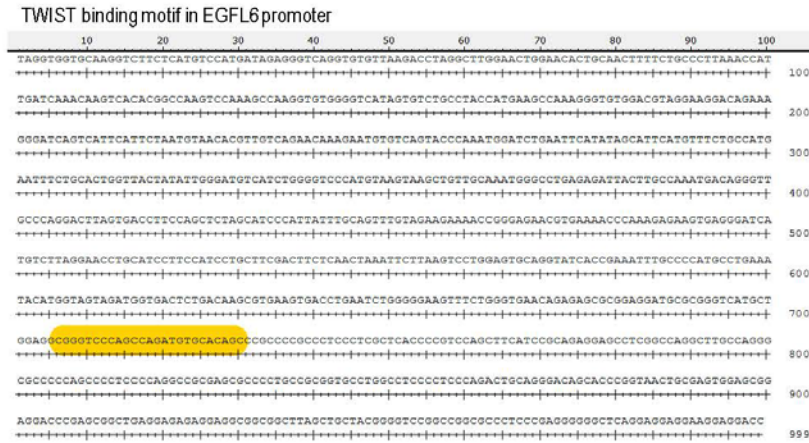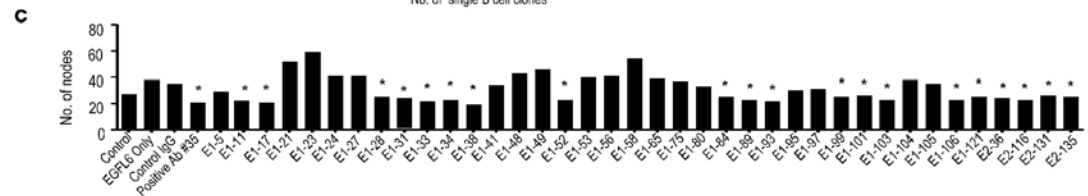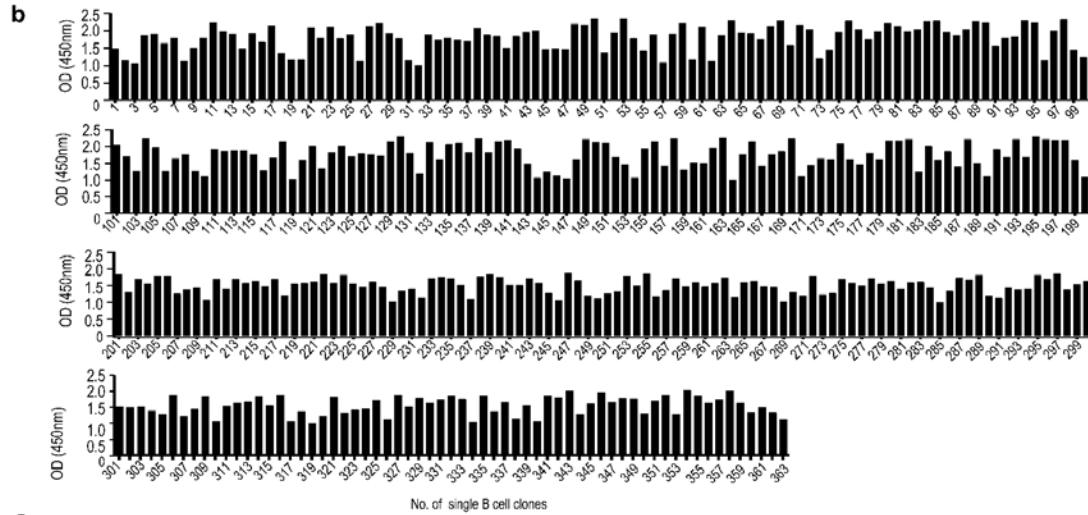

b
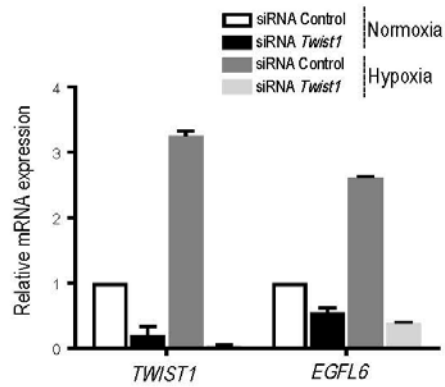
Supplementary Fig.3.

Supplementary Figure 3. Related to Figure 3. ; TWIST1 binds to the EGFL6 promoter. a, TWIST1 binding motif in EGFL6 promoter sequence. **b,** Expression of *TWIST1* and *EGFL6* mRNA in Control siRNA- or *TWIST1* siRNA-treated RF24 cells.

Supplementary Fig. 4.

Supplementary Figure 4. Related to Figure 4. ; Treatment of endothelial cells with EGFL6 activates PI3K/AKT signaling. a, Heat map representation of RPPA analysis showing protein expression change in Control and EGFL6-treated RF24 endothelial cells. **b**, Bar graphs show quantification of protein expression. Error bars indicate SEM. **c** and **d**, EGFL6-mediated migration and tube formation (lower panel) was reduced by PI3K inhibition in endothelial cells. **e**, Human phosphorylated RTK arrays were used to examine EGFL6-induced RTK phosphorylation levels in RF24 cell lysates. **f**, Effect of *EGFL6* siRNA on Tie2/AKT activation in SKOV3ip1 ovarian cancer cells. **g**, Heat map representation of RPPA analysis showing protein expression change in vehicle and EGFL6-treated RMG2 ovarian cancer cells. ******$p<0.005$, *$p<0.05$

a Section of EGFL6 monoclonal antibody flow chart

Identification from single B cell clones 363 clones → Screening of biological function in tube formation 38 clones → Cloning and sequencing 18 mAbs → Recombinant expression of mAbs 5 mAbs → *in vivo* study 2 mAbs

b

OD (450nm)

No. of single B cell clones

c

No. of nodes

d

mAb	KD(M)	kon(1/Ms)	kdis(1/s)	Rmax	Full CHI^2	Full R^2
#93mAb	1.89E-09	1.11E+05	2.09E-04	0.249	0.0486	0.9976
#135mAb	2.19E-09	2.20E+05	4.82E-04	0.315	0.0955	0.9975

e Related to Fig.5b

Level of protein (relative to control)

Control ab / #93 ab / #135 ab

pTie2 Tie2 pAKT AKT

f

Control IgG   mAb#93   mAb#135

Tumor volume (mm^3)

Control IgG   mAb#93   mAb#135

C control IgG
mAb #93
mAb #135

Tumor volume (mm^3)

Weeks after tumor challenge

g

Control IgG   mAb #93   mAb #135

Ki67

CD31

% of Ki67

IgG #93 #135

No. of blood vessels

IgG #93 #135
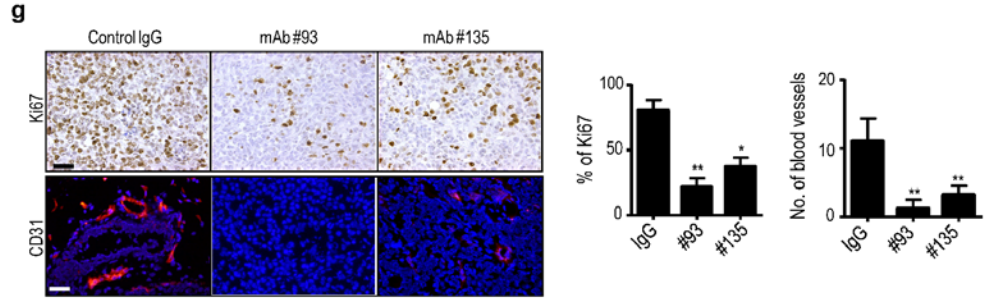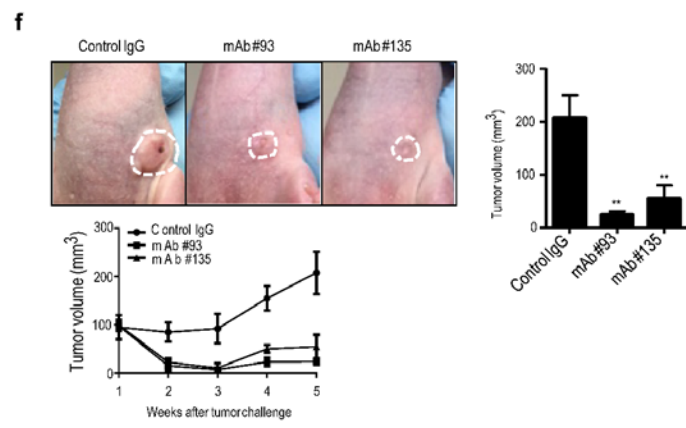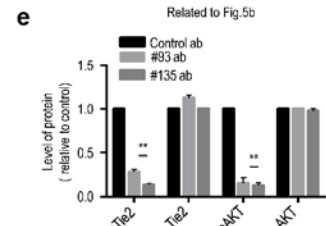
Supplementary Fig.5.

Supplementary Figure 5. Related to Figure 5. ; Screening, binding affinity, and functional activity of EGFL6 antibodies. a, Flowchart of generation and selection of EGFL6 monoclonal antibodies. **b**, Bar graphs represent EGFL6 binding signal of monoclonal antibody clones by ELISA. **c**, Bar graph is representative for tube formation of selected monoclonal antibodies. **d**, The kinetic constants of EGFL6 monoclonal antibodies (#93 and #135) were determined by Octet. Full CHI^2 (sum of squared deviations; deviation measures the differences between the fitting curve and the actual data points) and the full R^2 (coefficient of the determination; 1.0 is for perfect curve fit) suggest good fit for the determined kinetic constants. **e**, Bar graph shows inhibition of pTie2 and pAKT by the two lead EGFL6 antibodies. **f**, Effect of EGFL6 blocking antibodies on MDA-MB-231 tumor-bearing mice tumor volumes. Seven days after tumor cell injection in mammary fat pad, mice were randomly divided into three groups (10 mice/group) to receive therapy: (1) Control Ab IgG, (2) EGFL6 #93, and (3) EGFL6 Ab #135 (5 mg/kg). Antibody was given once a week. Representative images of breast cancer model treated with EGFL6 antibodies. **g**, Effect of targeted EGFL6 on proliferation (Ki67) and microvessel density (CD31). Scale bar = 100µm. Error bars indicate SEM. *p<0.05 vs. Control IgG.

a

EGFL6 allele

Targeted allele

EGFL6 fl/fl allele

EGFL6 fl/fl;Tie2 cre allele

◁ LoxP   F FRT

b EC isolation using CD31 beads

WT   KO

Counts

CD31 positive

c RNA level in EC

WT   KO

EGFL6

GAPDH

d

WT   KO

WT ☐   KO ☐

Weight (g)

Body Lung Liver Kidney Spleen Heart

e

Lung Liver Kidney Spleen Heart

WT

KO

f

Pre-ligation 24h-post-ligation 96h-post-ligation

WT

KO

g

Tumor volume (mm³)

Days after tumor challenge

WT ●   KO ■

h

HYPOXIA

EGFL6

α5β1   Tie2 P

PI3K P

AKT P

Angiogenesis

TWIST   EGFL6

Tumor Endothelial cell

Supplementary Fig.6.

Supplementary Figure 6. Related to Figure 6. ; Generation of *Tie2-cre; EGFL6^{f/f}* knockout mice. a,

Schematic showing the 3 alleles of *EGFL6*. Excision of the neomycin resistance cassette by Flp recombinase

produces the EGFL6^{f/f} floxed allele, which is expressed normally and *Tie2 cre;EGFL6^{fl/fl}* allele. Excision of

exon2 from the EGFL6^{f/f} allele by Cre recombinase produces an *EGFL6 KO* allele. After deletion of exon 2,

there are 4 stop codons predicted in exon 3 alone:

MQPPWGLALPLLLPWVTGGVGTSYV*AQVQVR*VCGTE*M*MLSRIHREDLHS, *=stop codon. **b**, CD31

expression in isolated endothelial cells from *WT* and *KO* mice. **c**, *EGFL6* mRNA level in isolated endothelial

cells. **d**, Representative gross images from *WT* and *KO* mice along with whole body, lung, liver, kidney, spleen,

and heart weights in *WT* and *KO* mice (n=5). **e**, H&E stain of lung, liver, kidney, spleen, and heart tissues from

WT and *KO* mice (n=5). Scale bar = 50 µm **f**, Hind limb ischemia. After arterial ligation,   *WT* and *KO* mice

were assigned to the following groups (n = 5 mice per group): pre-ligated, 24h-post-ligated and 96h-post-

ligated. Blood flow was monitored before and after femoral artery ligation with the use of serial laser Doppler.

g, Tumor growth (volume) in the E0771 breast cancer model. Tumor cell injection was carried out in mammary

fat pad of *WT* or *KO* mice (n = 5 mice per group). **h**, Schematic overview of the EGFL6 signaling axis in tumor

endothelial cells. In hypoxic tumor environment, TWIST1 regulates EGFL6 expression which could bind to

integrin α5β1 and facilitate crosstalk with Tie2 receptor. This triggers downstream signaling to promote tumor

angiogenesis *via* the PI3K/AKT pathway.

Supplementary Table 1. Related to Figure 6. ; Association of clinical and demographic features with vascular EGFL6 in epithelial ovarian carcinoma

	EGFL6 overexpression		
	No	Yes	p-value
Mean age	60.5 yrs (range 35-85 yrs)		
Stage			
Low (I/II)	13	4	
High (III-IV)	42	71	0.002
Grade			
Low	5	4	
High	50	71	0.4
Histology			
Serous	42	64	
Other	13	11	0.19

Supplementary Table 2. quantitative-PCR primer lists

No.	Primers	Sequence (5'-3')
1	Murine EGFL6 F	5'-AGATGTGAATGAGTGTGGAGTC-3'
2	Murine EGFL6 R	5'-AGTTTAGTCTGGCACACGTC-3'
3	Human EGFL6 F	5'-AGAAACAGCAAGGGAGTCTG-3'
4	Human EGFL6 R	5'-GGTTTCATTCCACACTCATTCAC-3'
5	TWIST1 F	5'-5'-CATCCTCACACCTCTGCATT-3'
6	TWIST1 R	5'-GGCCAGTTTGATCCCAGTAT-3'
7	EGFL6 promoter ChIP 1 F	5'-TCATGCTGGAGGCGGGTC-3'
8	EGFL6 promoter ChIP 1 R	5'-GGCTCCTCTGCGGATGAAGC-3'
9	EGFL6 promoter ChIP 2 F	5'-TCCTGGAGTGCAGGTATCAC-3'
10	EGFL6 promoter ChIP 2 R	5'-GAAACTTCCCCCAGATTCAG-3'
11	EGFL6 promoter ChIP negative F	5'-TGGAACTGGAACACTGCAAC-3'
12	EGFL6 promoter ChIP negative R	5'-ACACCTTGGCTTTGGACTTG-3'
13	F1551iNeoS F (for EGFL6 genotyping)	5'-GAGTGGTTGACACTTCACACTAC-3'
14	R1551iNeoS R (for EGFL6 genotyping)	5'-GTCCCTATTCTACTTTGTAGACAGACTACT TG-3'
15	Tie2 Cre transgene F	5'-CGCATAACCAGTGAAACAGCATTGC-3'
16	Tie2 Cre transgene R	5'-CCCTGTGCTCAGACAGAAATGAGA-3'