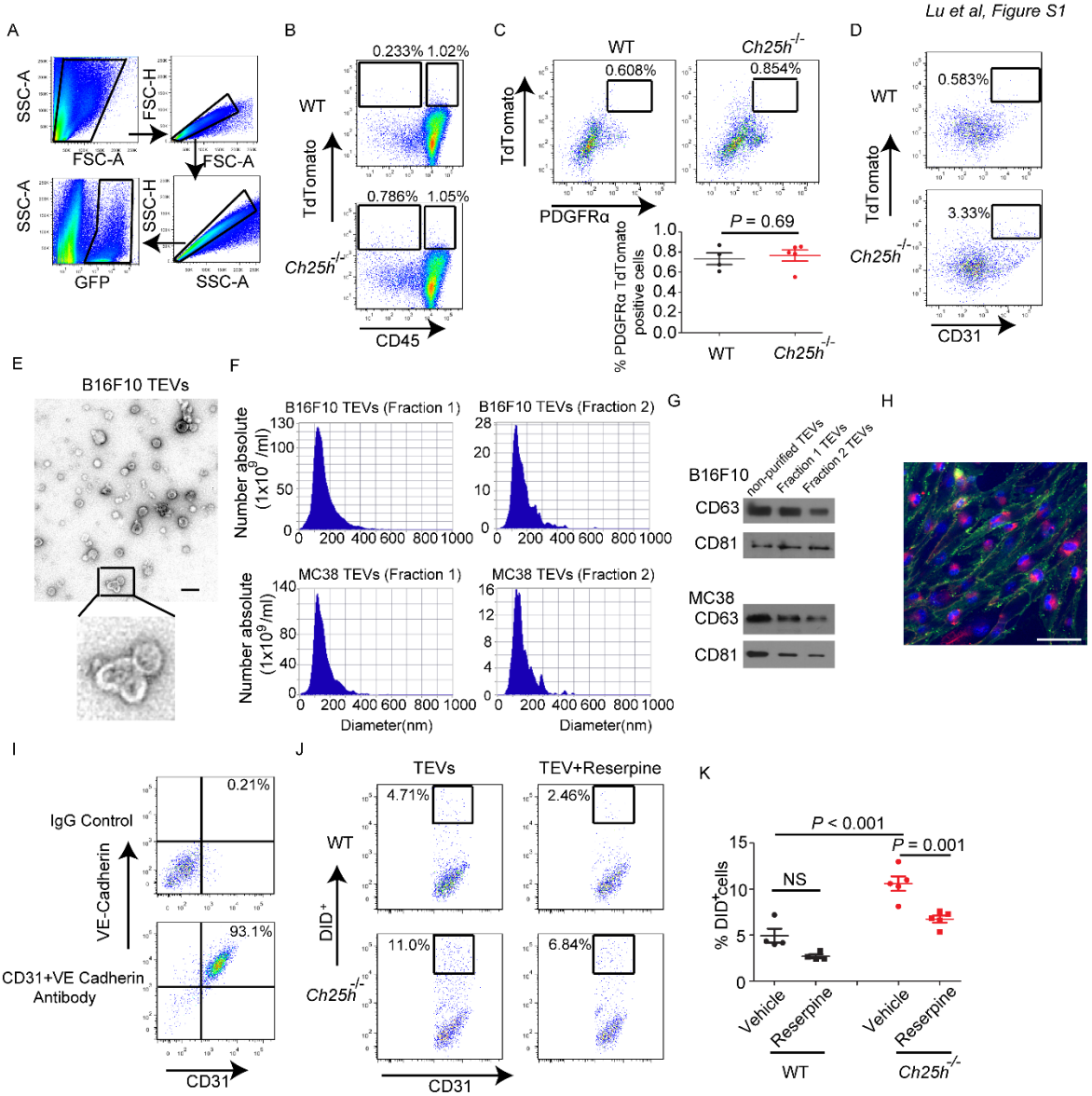
A
SSC-A
FSC-A
FSC-H
FSC-A
SSC-A
GFP
SSC-H
SSC-A
B
WT
0.233% 1.02%
TdTomato
Ch25h-/-
0.786% 1.05%
CD45
C
WT
Ch25h-/-
0.608%
0.854%
TdTomato
PDGFRα
P = 0.69
% PDGFRα TdTomato positive cells
WT
Ch25h-/-
D
WT
0.583%
TdTomato
Ch25h-/-
3.33%
CD31
E
B16F10 TEVs
F
B16F10 TEVs (Fraction 1)
B16F10 TEVs (Fraction 2)
Number absolute (1x10^9 /ml)
MC38 TEVs (Fraction 1)
MC38 TEVs (Fraction 2)
Diameter(nm)
G
non-purified TEVs
Fraction 1 TEVs
Fraction 2 TEVs
B16F10
CD63
CD81
MC38
CD63
CD81
H
I
IgG Control
0.21%
VE-Cadherin
93.1%
CD31+VE Cadherin Antibody
CD31
J
TEVs
TEV+Reserpine
WT
4.71%
2.46%
DID+
Ch25h-/-
11.0%
6.84%
CD31
K
P < 0.001
P = 0.001
NS
% DID+cells
Vehicle
Reserpine
Vehicle
Reserpine
WT
Ch25h-/-

Figure S1: CH25H and reserpine control ICBT between malignant and endothelial cells (related to Figure 1)

A. Flow cytometry gating strategy for ICBT analysis in tumor microenvironment
B. Flow cytometry analysis of TdTomato^{+}CD45^{-}and TdTomato^{+}CD45^{+}in tumors from GFP^{+}WT and GFP^{+}*Ch25h*$^{-/-}$ mice (n=4-5 for each group)
C. Flow cytometry analysis and quantification of TdTomato^{+}PDGFRα^{+}in tumor from GFP^{+}WT and GFP^{+}*Ch25h*$^{-/-}$ mice (n=4-5 for each group)
D. Flow cytometry analysis of TdTomato^{+}CD31^{+}positive cells in tumor from GFP^{+}WT and GFP^{+}*Ch25h*$^{-/-}$ mice (n=4-5 for each group)
E. Analysis of morphology of TEVs from B16F10 by electron microscopy. Scale bar, 200μm.
F. Nanoparticle tracking analysis (NTA) of diameter of B16F10 and MC38 TEVs purified from iodixanol gradient ultracentrifugation.
G. Western blot analysis of protein markers in TEVs and additionally purified TEVs F1 and F2 fractions (obtained from the 1.11-1.12 g/ml iodixanol density layer).
H. Immunofluorescent analysis of CD31 and VE-cadherin in primary ECs isolated from the lungs of naïve mice. Scale bar, 100μm.
I. Flow cytometry characterization of primary ECs isolated from the lungs of naïve mice
J. Flow cytometry analysis of % of DID^{+} CD31^{+} cells upon incubation of indicated ECs with DID-labeled TEVs (5 μg/ml) in the presence or absence of reserpine (10 μM) for 12 hours.
K. Quantification of data presented in panel "**J**". n=4-5 for all groups.

Data are represented as mean±S.E.M; Statistical analysis was performed using two-tailed Student's t test (**C**) or One-way ANOVA and Tukey's multiple comparison (**K**). NS, not significant. Experiments were performed independently at least 3 times.

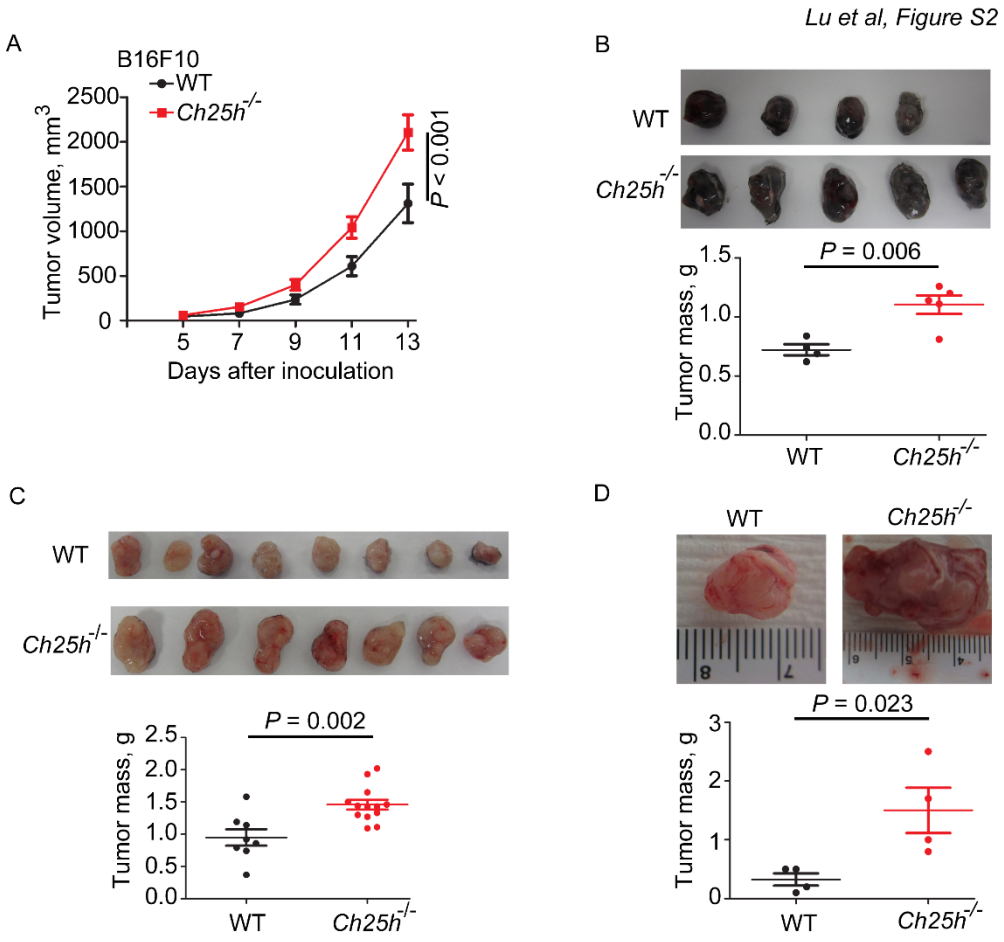

Figure S2: Stromal CH25H restricts growth of solid tumors (related to Figure 2)

A. Growth of B16F10 tumors (inoculated s.c. at $1x10^6$) in WT and *Ch25h*$^{-/-}$ mice. n=4-5 for each group.
B. Representative images and quantification of tumor size and weight at day 15 from the experiment described in **panel A**.
C. Representative images and quantification of MC38 tumor size and weight at day 15 from the experiment described in **Figure 2D**.
D. Representative images and quantification of mass of prostate tumors formed in WT and *Ch25h*$^{-/-}$ mice (n=4 each) at 50 days after inoculation of TRAMP-C2-luc cells.

Data are shown as mean±S.E.M; Statistical analysis was performed using two-tailed Student's t test (**B, C, D**) or two-way ANOVA and Tukey post-hoc analysis (**A**). Experiments were conducted independently at least 3 times.

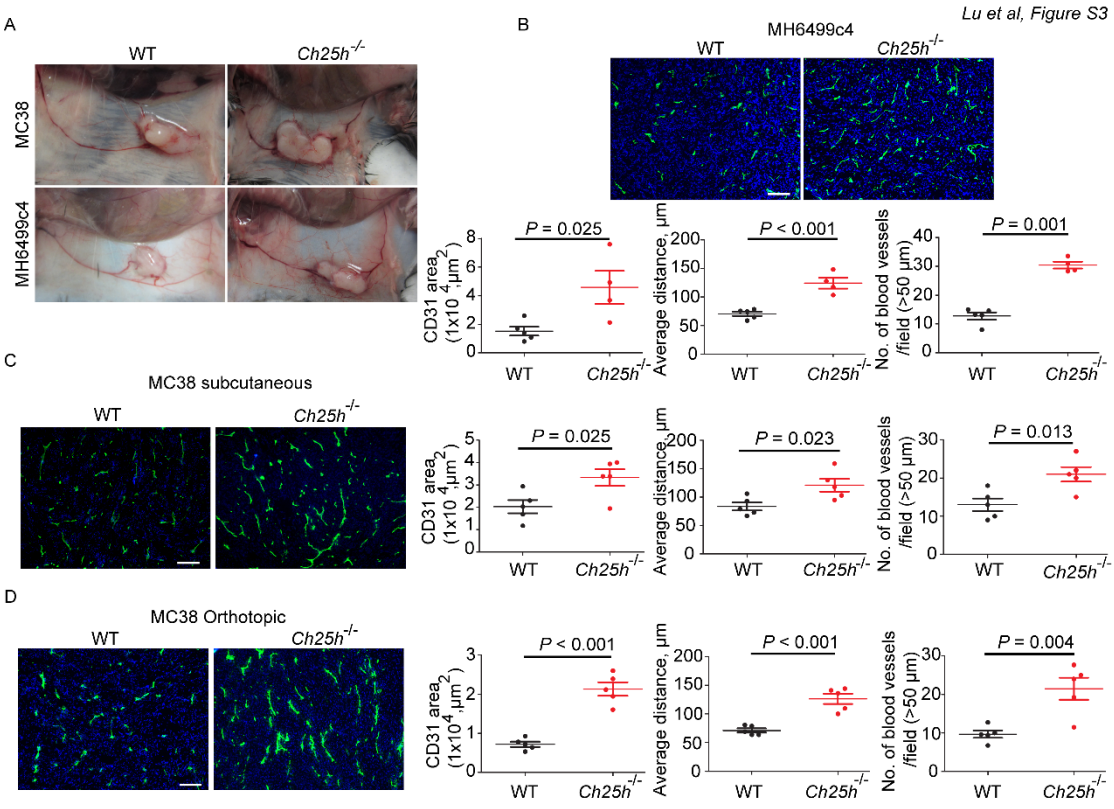

Figure S3: The angiostatic role of CH25H in tumor microenvironment (related to Figure 3).

A. A representative image of MC38 colon adenocarcinoma and MH6499c4 pancreatic ductal adenocarcinoma tumors and surrounding blood vessels in WT and *Ch25h*$^{-/-}$ mice.

B. Analysis of CD31^{+} ECs in MH6499c4 tumors (s.c., 1x10^{5}) of comparable size grown in WT (n=5) and *Ch25h*$^{-/-}$ (n=4) mice. Scale bar, 100μm.Quantification of CD31 positive area, number and average distance of blood vessels longer than 50 μm averaged from five random fields in sections from each of 4-5 animals are shown.

C. Analysis of CD31^{+} ECs in MC38 tumors (s.c., 1x10^{6}) of comparable size grown in WT (n=5) and *Ch25h*$^{-/-}$ (n=5) mice. Scale bar, 100 μm. Quantification of CD31 positive area, number and average distance of blood vessels longer than 50 μm averaged from five random fields in sections from each of 5 animals are shown.

D. Analysis of CD31^{+} ECs in MC38 tumors (intra-cecal inoculation, 5x10^{5}) grown in WT (n=5) and *Ch25h*$^{-/-}$ (n=5) mice. Scale bar, 100 μm. Quantification of CD31 positive area, number and average distance of blood vessels longer than 50 μm averaged from five random fields in sections from each of 5 animals are shown.

Data are shown as mean±S.E.M; Statistical analysis was performed by two-tailed Student's t test (**B, C, D**). Experiments were conducted independently at least 3 times.

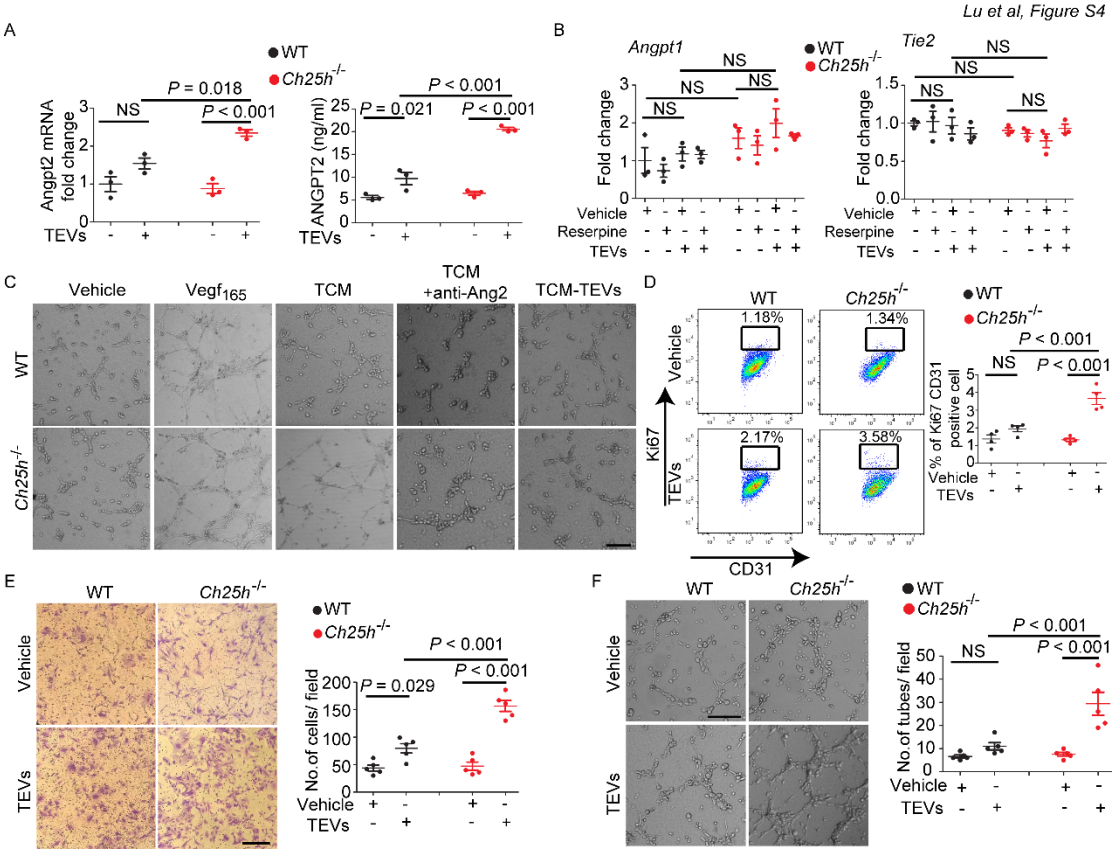
A
WT
Ch25h-/-
Angpt2 mRNA fold change
P = 0.018
NS
P < 0.001
TEVs
ANGPT2 (ng/ml)
P < 0.001
P = 0.021
P < 0.001
B
Angpt1
Tie2
Fold change
NS
Vehicle
Reserpine
TEVs
C
Vehicle
Vegf165
TCM
TCM +anti-Ang2
TCM-TEVs
WT
Ch25h-/-
D
1.18%
1.34%
2.17%
3.58%
Ki67
CD31
% of Ki67 CD31 positive cell
E
No.of cells/ field
P = 0.029
F
No.of tubes/ field

Figure S4: The ICBT-driven activation of endothelial cells is controlled by CH25H (related to Figure 4)

A. qPCR (left panel) and ELISA (right panel) analyses of levels of ANGPT2 mRNA (left) or protein (right) in WT and *Ch25h*$^{-/-}$ primary lung ECs (n=3) treated with MC38 TEVs (20 μg/ml) for 12 hr in vitro.
B. qPCR analysis of relative mRNA levels of Angpt1 and Tie2 (n=3 for each group) in WT and *Ch25h*$^{-/-}$ ECs treated with vehicle or reserpine (10μM for 8 hr) followed by MC38 TEVs (20μg/ml) or PBS treatment for 12 hr in vitro.
C. Representative images illustrating the tube formation assay described in **Figure 4F**. Scale bar,100μm.
D. Analysis of Ki67^{+} WT and *Ch25h*$^{-/-}$ ECs after treatment with or without MC38 TEVs (20μg/ml for 6 days). Flow cytometry data (left) and their quantification (right, n=4 for each group) are shown.
E. Analysis of migration of WT and *Ch25h*$^{-/-}$ ECs treated or not with MC38-derived TEVs (20μg/ml for 12 hr). Representative images (left) and quantification (n=5 for each group) are shown. Scale bar, 100μm.
F. Representative images (left) and quantification (right, n=5 for each group) of tube formation assay in WT and *Ch25h*$^{-/-}$ ECs treated with B16F10-derived TEVs (20μg/ml for 12 hr). Scale bar, 100μm.

Data are shown as mean±S.E.M; Statistical analysis was performed One-way ANOVA and Tukey's multiple comparison (**A, B, D, E, F**). NS, not significant. Experiments were conducted independently at least 3 times.

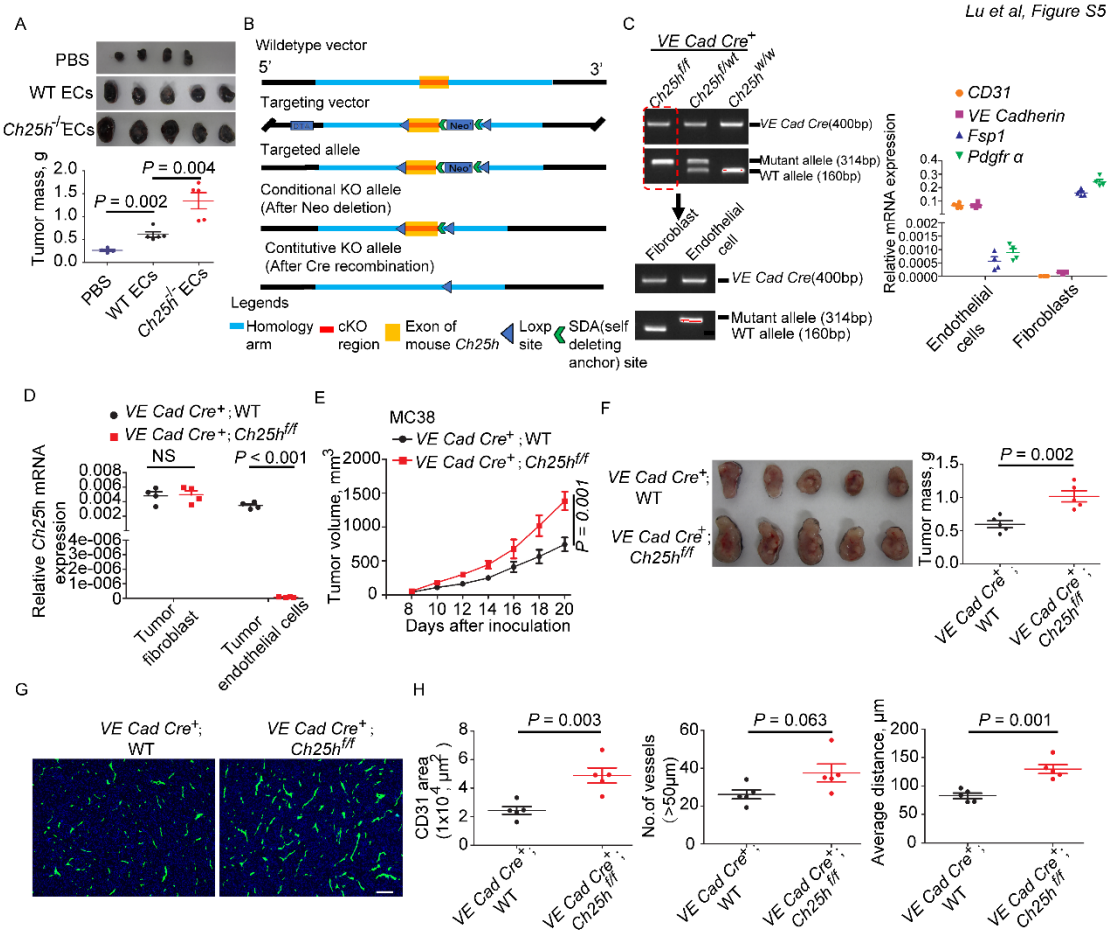
A
PBS
WT ECs
Ch25h-/- ECs
Tumor mass, g
P = 0.004
P = 0.002
PBS
WT ECs
Ch25h-/- ECs
B
Wildetype vector
5'
3'
Targeting vector
Targeted allele
Conditional KO allele
(After Neo deletion)
Contitutive KO allele
(After Cre recombination)
Legends
Homology arm
cKO region
Exon of mouse Ch25h
Loxp site
SDA(self deleting anchor) site
C
VE Cad Cre+
Ch25hf/f
Ch25hf/wt
Ch25h w/w
VE Cad Cre(400bp)
Mutant allele (314bp)
WT allele (160bp)
Fibroblast
Endothelial cell
VE Cad Cre(400bp)
Mutant allele (314bp)
WT allele (160bp)
CD31
VE Cadherin
Fsp1
Pdgfr α
Relative mRNA expression
Endothelial cells
Fibroblasts
D
VE Cad Cre+; WT
VE Cad Cre+; Ch25hf/f
Relative Ch25h mRNA expression
NS
P < 0.001
Tumor fibroblast
Tumor endothelial cells
E
MC38
VE Cad Cre+; WT
VE Cad Cre+; Ch25hf/f
Tumor volume, mm3
P = 0.0001
Days after inoculation
F
VE Cad Cre+; WT
VE Cad Cre+; Ch25hf/f
Tumor mass, g
P = 0.002
G
VE Cad Cre+; WT
VE Cad Cre+; Ch25hf/f
H
CD31 area (1x10-4, μm2)
P = 0.003
No.of vessels (>50μm)
P = 0.063
Average distance, μm
P = 0.001

Figure S5: Endothelial expression of CH25H drives its angiostatic and anti-tumorigenic function in vivo (related to Figure 5)

A. Representative images (upper) and quantification of masses of tumors (bottom) developed from B16F10 melanoma cells co-injected with PBS, WT or *Ch25h*$^{-/-}$ ECs as described in **Figure 5B**. Tumors were harvested 15 days after inoculation.
B. Schematic of conditional targeting of the single exon of the *Ch25h* gene (as described in Methods).
C. Genotyping analysis(left) of *VE-Cadherin Cre*$^{+}$ *Ch25h*$^{f/f}$ mice demonstrating the status of *Ch25h* in primary lung endothelial cells and fibroblasts. In the right panel we show the results of the qPCR analysis of ECs' (*CD31* and *VE cadherin*) and fibroblasts' (*Fsp1* and *Pdgfrα*) marker genes in cells isolated from *VE cadherin*;*Ch25h*$^{f/f}$ mice lung using CD31 positive select microbeads kit (n=4 for each group).
D. qPCR analysis of Ch25h relative mRNA level in tumor fibroblast and endothelial cells from *VE Cadherin*$^{+}$; WT and *VE Cadherin*;*Ch25h*$^{f/f}$ mice(n=4 for each group).
E. Analysis of growth of MC38 tumors (s.c., $1x10^6$) in *VE-Cadherin Cre*+ WT and *VE-Cadherin Cre*$^{+}$ *Ch25h*$^{f/f}$ mice (n=5 for each group).
F. Representative images and MC38 tumor mass analysis from experiment described in **panel E**. Tumors were harvested on day 21 after inoculation.
G. Representative immunofluorescence images of CD31 staining of MC38 tumors from *VE-Cadherin Cre*$^{+}$ WT and *VE-Cadherin Cre*$^{+}$ *Ch25h*$^{f/f}$ mice. Scale bar, 100μm.
H. Quantification of CD31-positive area, average length and number of blood vessels (> 50 μm) from experiment shown in **panel G**. Data averaged from five random fields in sections from each of five animals are shown.

Data are shown as mean±S.E.M. Statistical analysis was performed using One-way ANOVA and Tukey's multiple comparison (**A, D**) or two-tailed Student's t test (**F, H**) or two-way ANOVA and Tukey post-hoc analysis (**E**). Experiments were conducted independently at least 3 times.

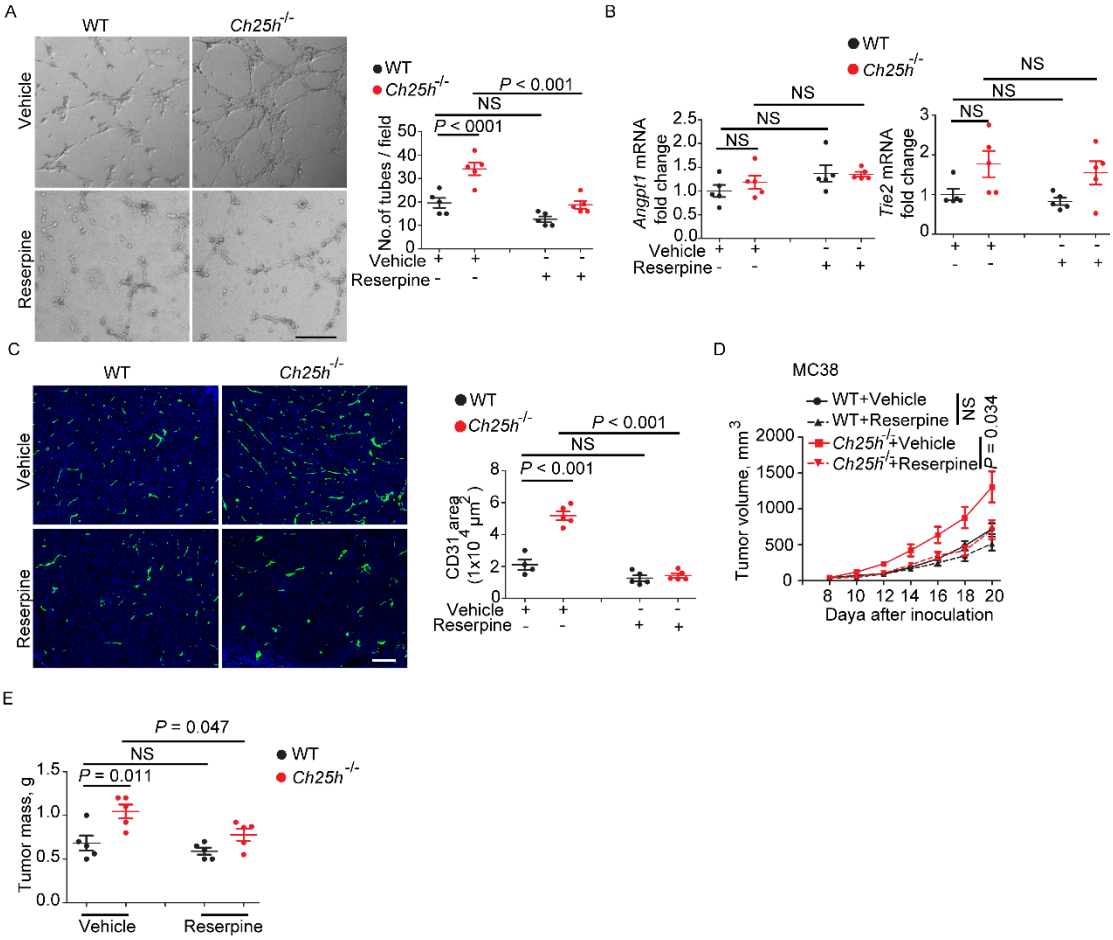
A
WT
Ch25h-/-
Vehicle
Reserpine
WT
Ch25h-/-
P < 0.001
NS
P < 0001
No.of tubes / field
Vehicle
Reserpine
B
WT
Ch25h-/-
NS
Angpt1 mRNA fold change
Tie2 mRNA fold change
Vehicle
Reserpine
C
WT
Ch25h-/-
Vehicle
Reserpine
P < 0.001
NS
P < 0.001
CD31 area (1x10^4 μm^2)
D
MC38
WT+Vehicle
WT+Reserpine
Ch25h-/-+Vehicle
Ch25h-/-+Reserpine
NS
P = 0.034
Tumor volume, mm^3
Daya after inoculation
E
P = 0.047
NS
P = 0.011
WT
Ch25h-/-
Tumor mass, g
Vehicle
Reserpine

Figure 6S: Angiostatic and anti-tumorigenic effects of reserpine in solid tumors (related to Figure 6).

A. Analysis of tube formation by WT or *Ch25h*$^{-/-}$ primary lung ECs pre-treated or not with reserpine (10μM for 8 hr) before being exposed to B16F10-derived TEVs (20μg/ml for 12 hr). Representative images (left) and quantified data (n=5 for each group) averaged from three random fields in each well of five wells are shown. Scale bar, 100μm.
B. qPCR analysis of *Angpt1* (left) or *Tie2* (right) mRNA level in B16F10 tumors from WT and *Ch25h*$^{-/-}$ mice treated with vehicle or reserpine (1mg/kg, i.p. every other day for 4 days). n=5 for each group.
C. Representative immunofluorescence images and quantification of CD31 positive areas in MC38 tumors from WT and *Ch25h*$^{-/-}$ mice (n=5 for each group) treated with vehicle or reserpine as described in **Figure 6A**. Scale bar, 100μm.
D. Analysis of MC38 tumor growth (inoculated s.c. at $1x10^6$) in WT and *Ch25h*$^{-/-}$ mice (n=5 for each group) followed by vehicle or reserpine treatment (1mg/kg, i.p. every other day).
E. Analysis of MC38 tumor mass on day 20 of the experiment described in **panel D**.

Data are shown as mean±S.E.M; Statistical analysis was performed using One-way ANOVA and Tukey's multiple comparison (**A, B, C, E**) or two-way ANOVA and Tukey post-hoc analysis (**D**). NS, not significant. Experiments were performed independently at least 3 times.

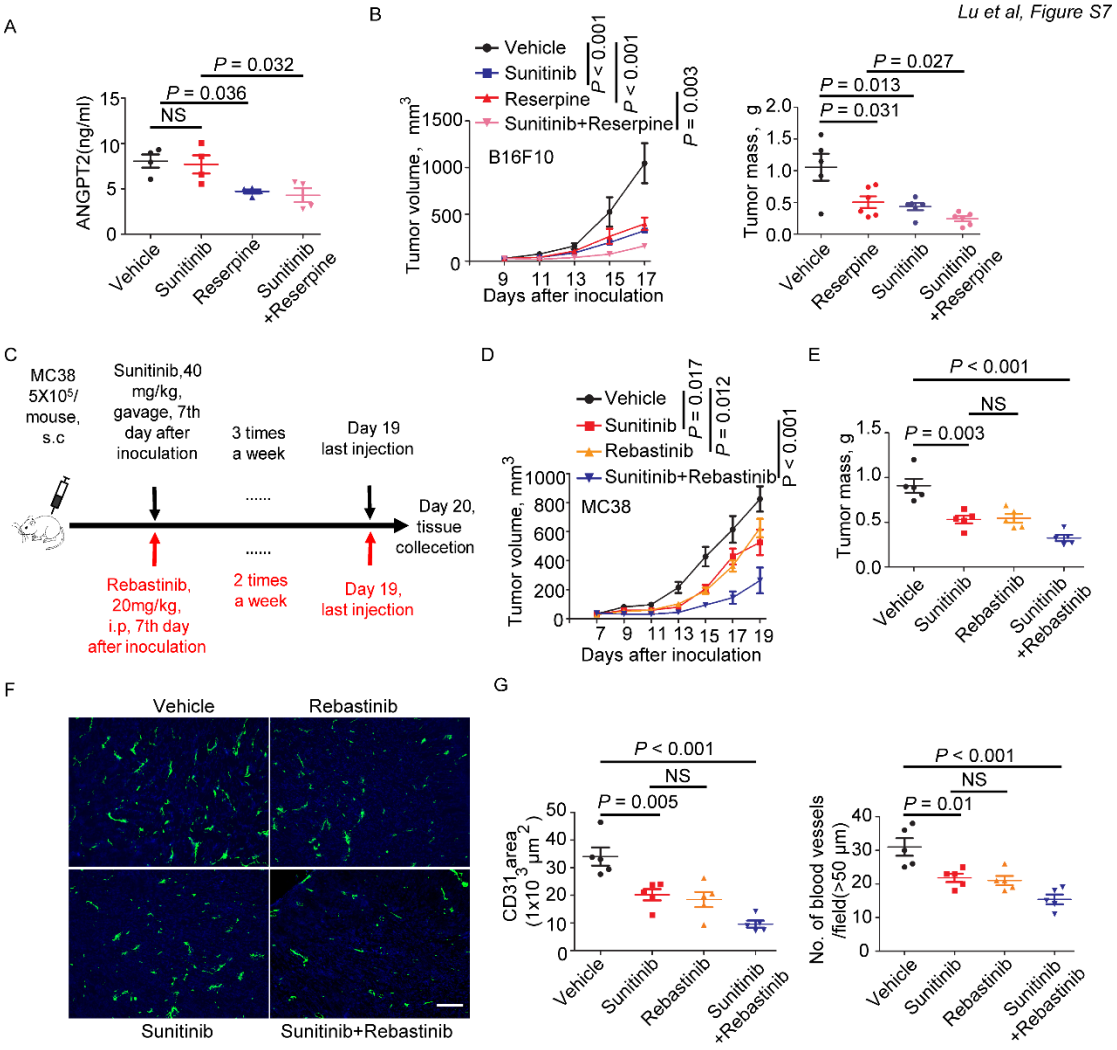
A
ANGPT2(ng/ml)
P = 0.032
P = 0.036
NS
Vehicle
Sunitinib
Reserpine
Sunitinib +Reserpine
B
Vehicle
Sunitinib
Reserpine
Sunitinib+Reserpine
P < 0.001
P < 0.001
P = 0.003
B16F10
Tumor volume, mm3
Days after inoculation
Tumor mass, g
P = 0.027
P = 0.013
P = 0.031
C
MC38 5X10^5/ mouse, s.c
Sunitinib,40 mg/kg, gavage, 7th day after inoculation
3 times a week
Day 19 last injection
Day 20, tissue collecetion
Rebastinib, 20mg/kg, i.p, 7th day after inoculation
2 times a week
Day 19, last injection
D
Vehicle
Sunitinib
Rebastinib
Sunitinib+Rebastinib
P = 0.017
P = 0.012
P < 0.001
MC38
Tumor volume, mm3
Days after inoculation
E
P < 0.001
NS
P = 0.003
Tumor mass, g
F
Vehicle
Rebastinib
Sunitinib
Sunitinib+Rebastinib
G
P < 0.001
NS
P = 0.005
CD31 area (1x10^3 μm^2)
P < 0.001
NS
P = 0.01
No. of blood vessels /field(>50 μm)

Figure S7: Combination of Reserpine with anti-angiogenic therapy. (related to Figure 7).

A. ELISA analysis of ANGPT2 levels in plasma from MC38 tumor-bearing mice treated as described in **Figure 7A**; n=4 for each group.
B. Analysis of B16F10 tumor volumes and masses in WT mice treated as indicated. n=5-6 for each group.
C. Schematic of treatment of MC38 tumors-bearing mice with Sunitinib, Rebastinib or their combination.
D. Growth of MC38 tumors in *Ch25h*$^{-/-}$ mice treated with Vehicle, Sunitinib, Rebastinib or their combination (n=5 for each group).
E. Representative the tumor weight at day 20 from experiment described in **panel C**.
F. Representative immunofluorescence image of CD31 staining of MC38 tumors from *Ch25h*$^{-/-}$ mice treated with Vehicle, Sunitinib, Rebastinib and their combination. Scale bar, 100μm. (n=5 for each group).
G. Quantification of CD31 positive area(left) and number of blood vessels (right) from **panel F**. Quantifications averaged from five random fields in sections from each of five animals are shown. (n=5 for each group)

Data are shown as mean±S.E.M; Statistical analysis was performed using One-way ANOVA and Tukey's multiple comparison (**A, B, E, G**) or two-way ANOVA and Tukey post-hoc analysis (**B, D**). NS, not significant. Experiments were performed independently at least 3 times.

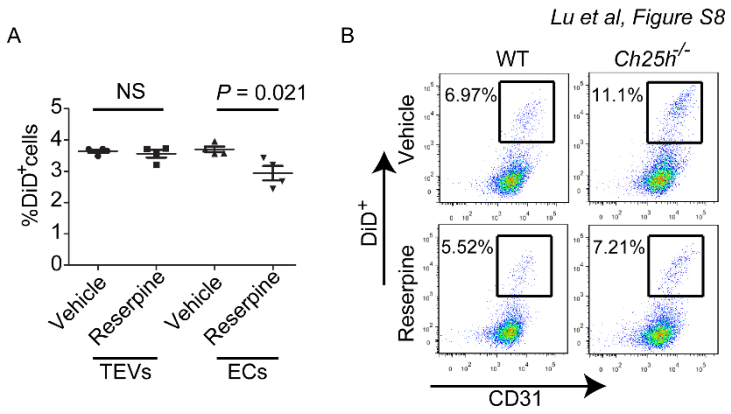

Figure S8: Mechanism of Reserpine mediated TEVs inhibition (related to Figure 8).

A. Analysis of % DiD^+ cells upon incubation of either Reserpine treated TEVs (10µM for 4 hr) with Vehicle treated ECs or Vehicle treated TEVs with Reserpine treated ECs (10µM for 4 hr).
B. Flow cytometry analysis of % of DID^+ $CD31^+$ cells upon incubation of indicated ECs with DID-labeled Liposome (1 µg/ml) in the presence or absence of reserpine (10 µM) for 8 hours.

Data are shown as mean±S.E.M; Statistical analysis was performed using One-way ANOVA and Tukey's multiple comparison(A). Experiments were performed independently at least 3 times.

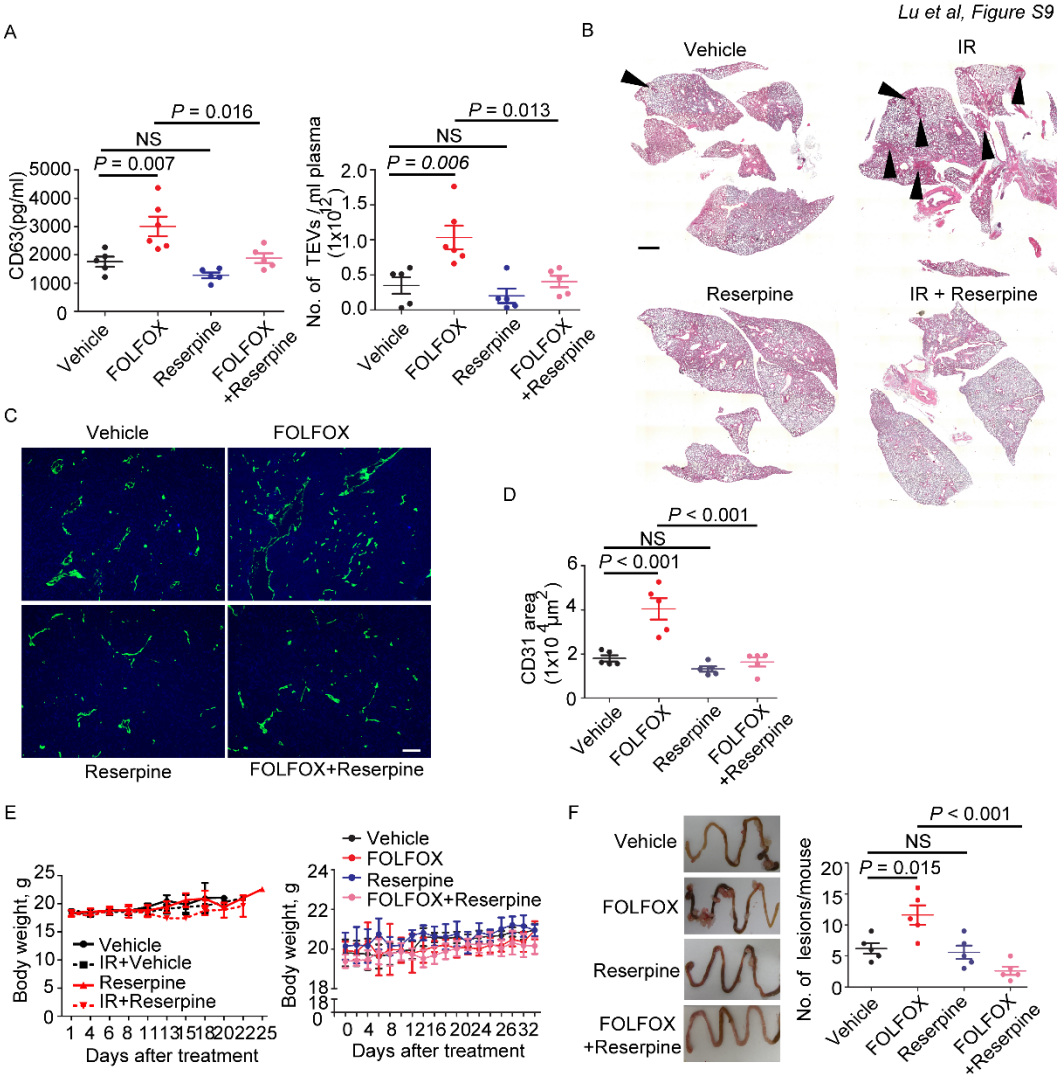
A
CD63(pg/ml)
P = 0.016
NS
P = 0.007
Vehicle
FOLFOX
Reserpine
FOLFOX +Reserpine
No. of TEVs /ml plasma (1x10^2)
P = 0.013
NS
P = 0.006
B
Vehicle
IR
Reserpine
IR + Reserpine
C
Vehicle
FOLFOX
Reserpine
FOLFOX+Reserpine
D
CD31 area (1x10^4 μm^2)
P < 0.001
NS
P < 0.001
E
Body weight, g
Vehicle
IR+Vehicle
Reserpine
IR+Reserpine
Days after treatment
FOLFOX
Reserpine
FOLFOX+Reserpine
F
No. of lesions/mouse
P < 0.001
NS
P = 0.015

Figure S9: Administration of reserpine improves the outcomes of radio/chemotherapies (related to Figure 9).

A. ELISA analysis of CD63 levels (left panel) and NTA analysis of numbers of TEVs (right panel) in mice bearing MC38 orthotopic tumors treated as described in **Figure 9E**.
B. Representative H&E staining of lungs from mice bearing B16F10 subcutaneous tumors of approximately 2000 mm3 following vehicle or reserpine (1 mg/kg) treatment three times a week with or without irradiation (described in **Figure 9B**). Scale bar, 1mm.
C. Immunofluorescence images of angiogenesis of MC38 orthotopic tumor from WT mice treated with vehicle, FOLFOX, reserpine and FOLFOX+reserpine as described in **Figure 9E**. Scale bar: 100 μm.
D. Quantification of the CD31 positive areas from experiment described in panel C; n=5 for each group.
E. Body weight of mice from experiments described in **Figure 9A** (left) and **Figure 9E** (right).
F. Representative images and quantitative analysis of local intestinal metastatic lesions found in mice bearing orthotopically implanted MC38 tumors and treated as indicated (as described in **Figure 9E**).

Data are shown as mean±S.E.M; statistical analysis was performed using One-way ANOVA and Tukey's multiple comparison (**A, D, F**). NS, not significant. Experiments were performed independently at least 3 times.

MC38 cell line TEVs

Fraction	Refractive Index	Density (g/mL)
1	1.369	1.11*pure EV fraction
2	1.37	1.12*pure EV fraction
3	1.372	1.125
4	1.373	1.127
5	1.373	1.127
6	1.373	1.127
7	1.374	1.13
8	1.374	1.13
9	1.374	1.13
10	1.375	1.136

B16F10 Cell line TEVs

Fraction	Refractive Index	Density (g/mL)
1	1.363	1.11*pure EV fraction
2	1.367	1.11*pure EV fraction
3	1.37	1.12
4	1.37	1.12
5	1.372	1.125
6	1.372	1.125
7	1.372	1.125
8	1.374	1.13
9	1.375	1.136
10	1.378	1.136

Table 2

Primers	Sequences (5’-3’)
Ang1 Forward	*CACGTGGAGCCGGATTTCT*
Ang1 Reverse	*ATCTGGGCCATCTCCGACTT*
Ang2 Forward	*CCAACTCCAAGAGCTCGGTT*
Ang2 Reverse	*CGGTGTTGGATGACTGTCCA*
Vegfα Forward	*GGAGATCCTTCGAGGAGCACTT*
Vegfα Reverse	*GGCGATTTAGCAGCAGATATAAGAA*
Fgf1 Forward	*CTGGCTTCTAAGTGTGTTAC*
Fgf1 Reverse	*GAAGAAACAGTATGGCCTTC*
Tie2 Forward	*AAGCAACCCAGCCTTTTCTC*
Tie2 Reverse	*TGAGCATTCTCCTTTGGAC*
Glut1 Forward	*GCAGGAGTGTCCGTGTCTTC*
Glut1 Reverse	*CCTGTCTCTTCCTACCCAACC*
Mmp9 Forward	*GCCCTGGAACTCACACGACA*
Mmp9 Reverse	*TTGGAAACTCACACGCCAGAAG*
gfp Forward	*ACTACCTGAGCACCCAGTCC*
gfp Reverse	*CTTGTACAGCTCGTCCATGC*

Detailed Materials and Methods

Human colorectal carcinoma specimens and their analyses

Human colorectal carcinoma tissue microarrays, consisting of formalin-fixed, paraffin-embedded tissue cores were stained for CH25H. Cohort 1 consisted of 99 colorectal colon cancer and 59 normal adjacent colorectal specimens (US Biomax, Inc; Rockville, MD). Patient age at diagnosis ranged from 33 to 86 (median age=64) with 79% of cases being low grade (Grade 1 or 2) and 21% being high grade (Grade 3). Forty-two percent of the population was female and 58% was male. Cohort 2 contained 68 cases of colorectal cancer with outcome data obtained from the Medical College of Wisconsin pathology tissue archives. Median time of follow-up was 56 months (range=0.77-128 months). Median age of diagnosis was 69 (range=22-91). Eighty-five percent of the cases were low grade (Grade 1 or 2) and 15% were high grade (Grade 3). Fifty-five percent of the population was female and 45% were male. Cohort 3 represented 188 colorectal cancer cases, of which, 150 cases had matched normal adjacent colon tissue (US Biomax, Inc). Age of diagnosis ranged from 22 to 90 (median=70). Sixty-five percent of the cases were low grade (Grade 1 or 2) while 35% were high grade (Grade 3). Forty-five percent of the population was female and 55% was male. Cohort 4 consisted of 59 colorectal colon cancer and 59 normal adjacent colorectal specimens (US Biomax, Inc; Rockville, MD). Patient age at diagnosis ranged from 33 to 86 (median age=64) with 79% of cases being low grade (Grade 1 or 2) and 21% being high grade (Grade 3). Forty-two percent of the population was female and 58% was male.

CH25H, CD31 and cytokeratin detection was performed using immunofluorescence-immunohistochemistry as previously described (1). Antigen retrieval was performed using citrate buffer pH 6.1 and biomarker detection was achieved using mouse monoclonal CH25H antibody (LSBio, LS-B14159/71037; 1:1000), mouse monoclonal CD31 antibody (DAKO, M0823; 1:200) and rabbit polyclonal anti-pan-cytokeratin (DAKO, Z0622; 1:100). High-resolution, whole-slide digital images were captured using the Pannoramic 250 Flash II slide scanner (3DHISTECH Ltd., Budapest, Hungary). Quantitative biomarker analysis was performed as previously described using Tissue Studio image analysis software (Definiens, Munich, Germany) to identify epithelial or stromal regions, facilitated by DAPI-stained cell nuclei and cytokeratin-stained cancer cells (2).

Animal Studies

NSG mice were purchased from the Jackson Laboratory. All other mice were on the C57BL/6J background. WT, *Ch25h*$^{-/-}$, *VE-Cadherin-Cre* (3) and the EGFP-transgenic mice (*TgN(ActbEGFP)1Osb/J*) were purchased from the Jackson Laboratory. The conditional *Ch25h* allele was created by flanking the single exon of the Ch25h gene with the loxP sites inserted into the non-conservative regions (~ 1.8 kb upstream of the exon 1 and ~ 0.5 kb downstream of exon 1). Targeting vector, homology arms and cKO region were generated by PCR using BAC clones RP23-392N3 and RP24-61K11 from the C57BL/6J library as the templates. In the

targeting vector (**Figure S5B**), the Neo cassette was flanked by the self-deletion anchor sites and DTA was used for negative selection. C57BL/6 ES cells were used for gene targeting. Other mouse strains were generated by intercrossing; the littermates of 6-8 weeks of age of both sexes were used in the all experiments.

Cell culture

Human 293T and HCT116 cells and mouse B16F10 cells were purchased from ATCC. Mouse MC38 colon adenocarcinoma (from S. Ostrand-Rosenberg, University of Maryland), TRAMP-C2-luc prostate neuroendocrine tumor cells (from Dr. L. Languino, Thomas Jefferson University) and MH6499c4 pancreatic ductal adenocarcinoma (from B. Stanger, University of Pennsylvania) were kindly gifted. All cells were maintained at 37°C with 5% CO2 in DMEM supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100U/ml penicillin-streptomycin and L-glutamine.

Quantitative Real-time PCR

Total RNA from endothelial cells or tumors was extracted using Trizol reagent and analyzed by quantitative real-time PCR using SYBR Green Master Mix. Primers of indicated genes are listed in the **Supplemental Table 2**.

TEVs isolation, characterization and assessment of uptake and production was carried out as previously described (4, 5). Briefly, TEVs were collected from (EV-free) media (Gibco, cat#11965-084) supernatants by ultracentrifucation and additional purification was carried out using the discontinuous iodixanol gradient.

TEVs were spun over 10%-20%-30% iodixanol layers at 350,000g (52,000 rpm for SW 55 Ti rotor), 4°C, for 120 min. Then 10 fractions of 260µL were collected starting from top of the tube, diluted with 1mL PBS and re-sedimented 100,000g (53,000 rpm), at 4°C for 70 min (BECKMAN Optima Ultracentrifuge and TLA-100.2 rotor). Pellets in each fraction were re-suspended in PBS and characterized for density by measuring the weight of each fraction (g/ml, indicated in **Supplemental Table 1**). Fractions 1-2 (1.11-1.12 g/ml) were the only ones containing TEVs.

These TEVs were characterized in size and number by nanoparticle tracking analysis (NTA) at the Extracellular Vesicle Core Facility at the University of Pennsylvania using the ZetaView instrument Particle Metrix. TEVs pellets from the ultracentrifugation protocol taken prior to density gradient purification were diluted 1:4000 in 0.2 µm filtered H_2O while TEVs further purified via density gradient were diluted 1:1000 before NTA. There were between 100-500 particles per field on the screen and 11 different fields of view per sample were analyzed at a frame rate of 30 frames per second. Minimum brightness was set to level 25 and minimum tracelength was set to 15.

The TEVs protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, cat#23225). TEVs images were captured by negative stain transmission electron

microscopy (TEM JEOL 100CX II scope). Among the markers of non-purified and purified Fractions 1-2 TEVs we detected CD63 (Thermo Fisher, cat#PA5-92370) and CD81 (Thermo Fisher, cat#MA-32333) by immunoblot analysis. Extracellular vesicles were stored at -80^{o}C for long term storage and thawed on ice before use.

TEV uptake in vitro was examined in the WT and *Ch25h*$^{-/-}$ ECs cultured with TEVs-free media and pre-treated with Vehicle or Reserpine (Sigma-Aldrich, Cat#83580, diluted as previously described in (4), 10μM for 8 hr) followed by TEVs (20μg/ml for 12 hr) that were either labeled with DiD as described in (4) or derived from B16F10 cells stably expressing GFP. Uptake of DiD was monitored by flow cytometry as described (4). Uptake of GFP mRNA expression was analyzed after total RNA was isolated from ECs using Trizol Reagent and chloroform. RNA concentration and purity were determined by using NanoDrop spectrophotometer. Applied Biosystems High-Capacity RNA-to-cDNA Kit was used to make cDNA. The GFP mRNA level measured by quantitative real-time PCR.

For assessment of **TEVs production** by tumor cells in vitro, 6x10^{6} cells were plated in 15cm tissue culture dishes. Upon attachment of all cells, the media was removed and replaced with fresh media that contained 10% of extracellular vesicle-free FBS. The cells were treated with vehicle (DMSO) or Reserpine (10 μM). After two days, the conditioned media was collected for extracellular vesicle (EV) isolation and the total number of cells were counted for each condition. 10 μL of isolated EVs were submitted for NTA analysis, 10 μL were used for protein concentration. The number of vesicles per μL and the amount of protein per vesicle were calculated correspondingly.

For assessment of **TEVs absolute number in plasma from tumor-bearing mice** undergoing chemotherapy, 100 μl plasma was harvested and TEVs were isolated with exosomes isolation kit (Invitrogen, cat#4485229). Pellets were resuspended in 50-80 μl PBS and samples were analyzed by NTA.

Assessment of DiD labeled Liposome uptake in vitro

DiD labeled liposome (FormuMax, cat#F60103F-DD) uptake in vitro was tested in WT and *Ch25h*$^{-/-}$ ECs pre-treated with Vehicle or Reserpine (10μM for 8 hr) followed by DiD labeled liposome (1μg/ml) treatment for 8 hr. Uptake of DiD was tested by using flow cytometry.

Flow cytometry analysis of the intercellular transfer and other immune-techniques

1X10^{6} B16F10-TdTomato cells were inoculated into GFP^{+}WT and GFP^{+}*Ch25h*$^{-/-}$ mice. Tumors were measured by caliper and volume was calculated as width * width * length * 0.5. Mice were euthanized when the tumor volume reached ~ 1500 mm^{3}. Tumor tissues were dissected and digested with 1 mg/ml Collagenase D (Roche, cat#11088882001) plus with 100 μg/ml DNase I (Roche, cat#10104159001) in RPMI medium with 2% FBS for 1 hr with continuous agitation at 37 °C. Digestion mixture was passed through 70 μm cell strainer to prepare single cell suspension and washed with PBS supplemented with 2mM EDTA and 1% FBS. Single cells

were stained with cell surface antibodies: anti-CD45-APC-cy7 (Biolegend, cat#103115), anti-CD31-PE-cy7 (Biolegend, cat#102417) and anti-PDGFRα-APC (Biolegend, cat#135907) and acquired by LSRFortessa flow cytometer (BD Biosciences), and data were analyzed with FlowJo software.

Kits from Boster Bio, LLC (for Angiopoietin 1 and Angiopoietin 2) or (Cusabio Technology, LLCfor CD63) for the Enzyme-linked immunosorbent assay (ELISA) were used to carry out to analyze of levels of respective proteins the blood or the supernatants of ECs cultures or tumor homogenates according to the manufacture instructions.

For the immunofluorescent analyses, tumor tissues were harvested, embeded with frozen O.C.T and cryosectioned into 7μm using Leica CM3050 S Cryostats. Tumor sections were fixed in acetone, washed with PBS, and then blocked with PBS containing 5% goat serum and 1% BSA. Then, the sections were incubated with CD31 primary antibody (BD Bioscience, cat#553370) diluted in PBS at 1:200 for 12 hr at 4°C and followed by wash three times with PBS. Next, samples were incubated goat anti-rabbit secondary antibody (Invitrogen, cat#A-11006 or Invitrogen, cat#A-11007) diluted in PBS 1:500 at room temperature for 1 hr. ProLong Gold Antifade Reagent with DAPI was added after washing. Immunofluorescence images were captured with Olympus BX51 microscopy. CD31 staining area, distance and number of blood vessel were analyzed with Metaphor software.

Isolation, culture and analyses of primary endothelial cells (ECs)

Lungs were collected from 3 weeks-old WT and *Ch25h*$^{-/-}$ mice, washed with ice cold PBS, cut into small pieces using dissection scissors, and incubated with the digestion solution which contains 2 mg/ml Collagenase II (MP Biomedicals, cat#100502) and 100μg/ml DNase I solution for 1 hr with continuous agitation at 37℃. Digestion was stopped by adding pre-warmed FBS followed by centrifugation at 1500 rpm for 5 min. The cell pellet was re-suspended into 5 mL cold DPBS and washed for 2 times. After that, cells were seeded into 15 cm dish and placed into the cell incubator for 1 hr. Nonattached cells were collected and washed with DPBS twice, collected by centrifugation and re-suspended into 190 μl incubation solution (HBSS with 0.5% Fraction V BSA) and incubated with CD31 Microbeads (Miltenyi Biotec, cat#130-097-418) and Fc blocking antibody (Biolegend, cat# 101302) for 15 min. After incubation, cells were added into LS column (Miltenyi Biotec, cat#130-042-401) equilibrated with MACS buffer (PBS with 0.5% BSA and 2 mM EDTA) After 5 washes, column was removed from magnet and CD31^{+} cells were eluted with MACS buffer by positive pressure and spun down at 1500 rpm for 3 min. Finally, CD31^{+} cells were seeded in the dishes coated with 0.2% gelatin (Sigma, cat#G1393) and cultured with ECs media (Advanced DMEM reduced serum media contained 15% FBS, 1% glutamine, 1% penicillin-streptomycin, 0.1 mg/ml endothelial cell growth factor and 0.5 mg/ml heparin sodium) for around 14 days before use. Primary endothelial cells were planted on cover glass pre-coated with gelatin and cultured for 24 hr, fixed with 4% paraformaldehyde solution (Thermo Scientific, cat#19943-K2), and stained with CD31 (BD Bioscience, cat#553370, 1:100) and VE-Cadherin (Invitrogen, cat#36-1900,1:50) primary antibodies. Next, samples were

incubated with goat anti-rabbit (Invitrogen, cat#A11006) and goat anti-rabbit (Invitrogen, cat#A11072) diluted in PBS 1:300 at room temperature for 1 hr. ProLong Gold Antifade Reagent with DAPI was added after washing. Immunofluorescence images were captured with Olympus BX51 microscopy.

Tube formation analysis

25-Hydroxycholesterol (25HC, Sigma, cat#H1015) was dissolved with 100% Ethanol to make a 4 mM working stock solution. Reserpine and Vegf165 (Sigma, cat#SRP4364) were dissolved in DMSO and water, respectively. For Reserpine and 25HC treatment, ECs were pre-treated with Reserpine at the concentration of 10 μM for 8 hr and 25HC at the concentration of 4 μM for 4 hr followed by tumor condition media or TEVs (20 μg/ml) treatment for additional 12 hr. For anti-Angpt2 antibody treatment, WT and *Ch25h*$^{-/-}$ ECs were treated with TEVs in presence or absence of anti-Angpt2 (AlipoGene, cat#AG-27B-0016PF) antibody at concentration of 60 ng/ml for 12 hr. After treatment, ECs were washed with PBS twice and seeded into 24 well plates ($8x10^4$/well) coated with Cultrex RGF BME, type2 (R&D, cat#3533-005-02). The images of tube formation were captured 4 hr after seeding by using contrast phase microscopy and analyzed by image J software.

For Ch25h expression, lentivirus expressing Ch25h (Lenti-Ch25h) and the control virus (Lenti-empty) were produced by human 293T cells and concentrated using high speed centrifugation. *Ch25h*$^{-/-}$ ECs were transduced with lentivirus for 12 hr and transduced cells were cultured with fresh complementary media for extra 48 hr. After that, RNAs were extracted from *Ch25h*$^{-/-}$ and transduced ECs and *Ch25h* gene expression level was measured by q-PCR. Successful transduced cells were applied to conduct tube formation experiment as described above.

EC proliferation and migration analysis

For cell proliferation analysis, WT and *Ch25h*$^{-/-}$ were seeded into 24 well plates coated with 0.2% gelatin at the density of $5x10^4$/well. MC38 TEVs (20ug/ml) were added into both of WT and *Ch25h*$^{-/-}$ ECs. Cell numbers were counted at day 3, 6 and 9 days after TEVs treatment. The proliferation marker Ki67 in the treated WT and *Ch25h*$^{-/-}$ ECs on day 6 were stained and analyzed using Flow Cytometry.

For cell migration, WT and *Ch25h*$^{-/-}$ were pre-treated with TEVs (20μg/ml) for 24 hr. Treated cells were seeded into inserted chamber at $5X10^4$/each chamber and cell containing chamber were inserted into 24 well plate with FBS-free fresh MDEM media contained 20ng/ml $VEGF_{165}$ for 24 hr. After that, cell chambers were taken out and residential media were aspirated and washed with PBS for 3 times. The cells were fixed with 4% PFA for 15 min and then 0.2% crystal violet were added into chambers and incubated for 15 min. Cell chambers were washed with PBS for 3 times and cells on the top chamber membrane were swept away followed by washing with PBS. Cell migration images were captured using Olympus BX51 microscopy and analyzed with image J software.

Western blotting analysis of Tie2 Phosphorylation

WT and *Ch25h*$^{-/-}$ ECs were isolated from respective mice and treated with PBS or MC38 derived vesicles (20 µg/ml) for 12 hours. Western blot was carried out using rabbit anti-mouse Tie2 Polyclonal antibody (Thermo Fisher, cat#PA5-80103) and p-Tie2 was tested with rabbit anti-p-Tie2 antibody (Cell signaling Technology, cat#4221S) as described in (6).

Analysis of plasma membrane fluidity was carried out as previously described (7). ECs treated as indicated were suspended in PBS at 4X10^5/ml and incubated with fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma, cat#D208000, 3µM) at 37°C for 20 min and held at 25.0 ±0.5°C for intensity polarization measurement using Tecan Infinit F200 Fluorescence Microplate Reader System (λex = 313 nm; λem = 460 nm). The degree of cell membrane polarization was calculated from p= (F1 -F2)/(F1 + F2), in which the F1 and F2 refers to fluorescence intensity of vertically and horizontally polarized components, respectively, with excitation vertically polarized.

Tumorigenesis studies

For syngeneic subcutaneous tumor model, the B16F10, MC38 or MH6499c4 tumor cells were inoculated into right flank of indicated C57Bl/6J mice. For xenograft studies, human HCT116 (5X10^6) were injected in NSG mice (Jackson). Studies using the orthotopic colon tumor growth, cancer model were carried out as previously described (8). Briefly, after anesthesia, a 1.5cm nick was made in abdominal wall (around) and the cecum was exteriorized and kept moist using PBS. 25µl of MC38 cell suspension (2x10^7/ml) was injected into the cecal wall using 30 G needle and the injection site was covered with cotton swab for 3 min to monitor for leakage. Cecum was gently returned to abdominal wall, then abdominal wall and skin were sutured carefully. For orthotopic prostatic cancer model, 1x10^6 TRAMP-C2-luc/GFP cells were injected into prostate of WT or *Ch25h*$^{-/-}$ mice. Tumor volumes were tracked via detecting Bioluminescence intensity weekly.

FITC-lectin perfusion

MC38 cells (1X10^6) were injected into right flank of WT and *Ch25h*$^{-/-}$ mice. 14 days after injection, mice were anesthetized, injected with FITC-lectin (Thermo Fisher, cat#L32478, 100 µg/mouse, i.v) and allowed to circulate for 10 min. After that, chest was open rapidly and the vasculature was perfused with 30 ml 4% paraformaldehyde (PFA) for 5 min. Tumor tissue were harvested and stored at PFA for overnight before frozen into OCT. Cryosection of tumor was stained with CD31 for the whole blood vessels and FITC positive area was calculated with Metaphor software.

Combination therapies

Reserpine (Sigma-Aldrich, Cat#83580) was administered as previously described in (4). Briefly, Reserpine (dissolved in 0.1% ascorbic acid and diluted in ddH2O) or vehicle (0.1% ascorbic acid

diluted in ddH2O) were administered to B16F10 tumor-bearing mice when the tumors reached 75mm^3 at the dose of 1mg/kg (i.p.; three times a week). Two days later, the tumors were approximately 100-130 mm^3. Matched vehicle and Reserpine mice with similar tumor volumes were chosen to undergo irradiation using the Small Animal Radiation Research Platform (SARRP, Xstrahl Medical & Life Sciences). Briefly, the mice were anesthetized using inhaled isoflurane at a concentration of 2.5% and placed on the stage of the SARRP. Once the tumor isocenter was determined, delivery of the 12 Gy single dose was made using a 1x1 cm collimated beam operating at 175 kV, 15 mA with copper filtration and dose rate at 1.65 Gy/minute. The beam was delivered at such an angle to avoid the spine. Dosimetry was performed using EBT2 gafchromic films.

For the combination of Reserpine with chemotherapy, the ingredients for the FOLFOX regimen (Oxaliplatin, cat#PHR1528; 5-Fluorouracil, cat# F6627; Folinic acid calcium salt hydrate, cat#F7878 – all Sigman Aldrich) were and dissolved in PBS and administered (Folinic Acid: 150 mg/kg, 5- Fluorouracil: 5mg/kg, Oxaliplatin: 1.4mg/kg, all i.p. – with or without Reserpine, 1mg/kg, i.p.; every other day) into mice 10 days after these animals have been inoculated with 5x10^5 MC38 cells injected into the cecum. All mice were sacrificed 45 days after tumor inoculation and tumor, liver and intestine were harvested for the histopathologic analysis.

For combination anti-angiogenic therapy, Sunitinib (BioVision, cat#1611) was dissolved in a vehicle (composed of carboxymethylcellulose sodium (0.5% w/v), NaCl (1.8% w/v), Tween 80 (0.4% w/v), benzyl alcohol (0.9% w/v), in water; the whole formulation was adjusted to pH 6.0). Rebastinib were dissolved in 0.4% hydroxypropyl methylcellulose. MC38 or B16F10 cells (5x10^5) were inoculated into right flank of WT mice. 9 days after tumor inoculation, mice were treated with Sunitinib (40mg/kg, gavage) 3 times a week with or without Reserpine (1mg/kg, i.p.) every other day or Rebastinib (20mg/kg, gavage) twice a week..

RNA Sequencing

Primary lung ECs from *Ch25h*$^{-/-}$ mice were pre-treated with vehicle or Reserpine (10μM for 8 hr) followed by treatment with MC38 TEVs (20μg/ml) or PBS treatment for 12 hr in vitro and total RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN) and analyzed for *Angpt2* mRNA levels by qPCR. These samples were then taken for RNA sequencing (carried out as previously described (9)). Raw reads were mapped to the mouse reference transcriptome (Ensembl) using Kallisto version 0.46.0. Raw sequence data are available on the Gene Expression Omnibus (GEO; **accession no. GSE163941**). All subsequent analyses were carried out using the statistical computing environment R version 4.0.0 in RStudio and Bioconductor version 3.11.1. Briefly, transcript quantification data were summarized to genes using the tximport package and normalized using the trimmed mean of M values (TMM) method in edgeR. Genes with <1 CPM across all groups were filtered out. Normalized filtered data were variance-stabilized using the voom function in limma, and differentially expressed genes were identified with linear modeling using limma (FDR ≤ 0.01; absolute logFC ≥ 1) after correcting for multiple testing using Benjamini-Hochberg.

Quantification and statistical analysis

All these experiments descripted here are representative of at least three times independent experiments (n>5 mice for each group unless specialized explain). For in vitro experiments, cells or tissues from each of these animals were done at least in biological triplicates. All data were showed here as average±S.E.M. Statistical was conducted by using GraphPad Prism 7 software. Comparison between two groups was conducted with Student t test and multiple comparisons was performed by using One-way ANOVA or two-way ANOVA analysis followed by the Bonferroni post-hoc test. Tumor growth curve analysis was conducted with Repeated-measure two-way ANOVA (mixed-model) followed by the Bonferroni post-hoc test. The Kaplan-Meier curves were used to analyze the survival data, and Cox regression was used to compute hazard ratio. P values < 0.05 were considered significant.

Study Approvals

Use of pre-existing human archival de-codified and de-identified CRC tissue arrays, previously collected under informed consent, and samples that could not be directly or indirectly linked to individual human subjects was exempt from institutional review or approved by IRB of the Medical College of Wisconsin.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and were carried out in accordance with the IACUC guidelines. All mice had water ad libitum and were fed regular chow. Mice were maintained in a specific pathogen-free facility in accordance with American Association for Laboratory Animal Science guidelines. Littermate animals from different cages were randomly assigned into the experimental groups. These randomized experimental cohorts were either co-housed or systematically exposed to the bedding of other groups to ensure equal exposure to the microbiota of all groups.

References for Detailed Materials and Methods

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