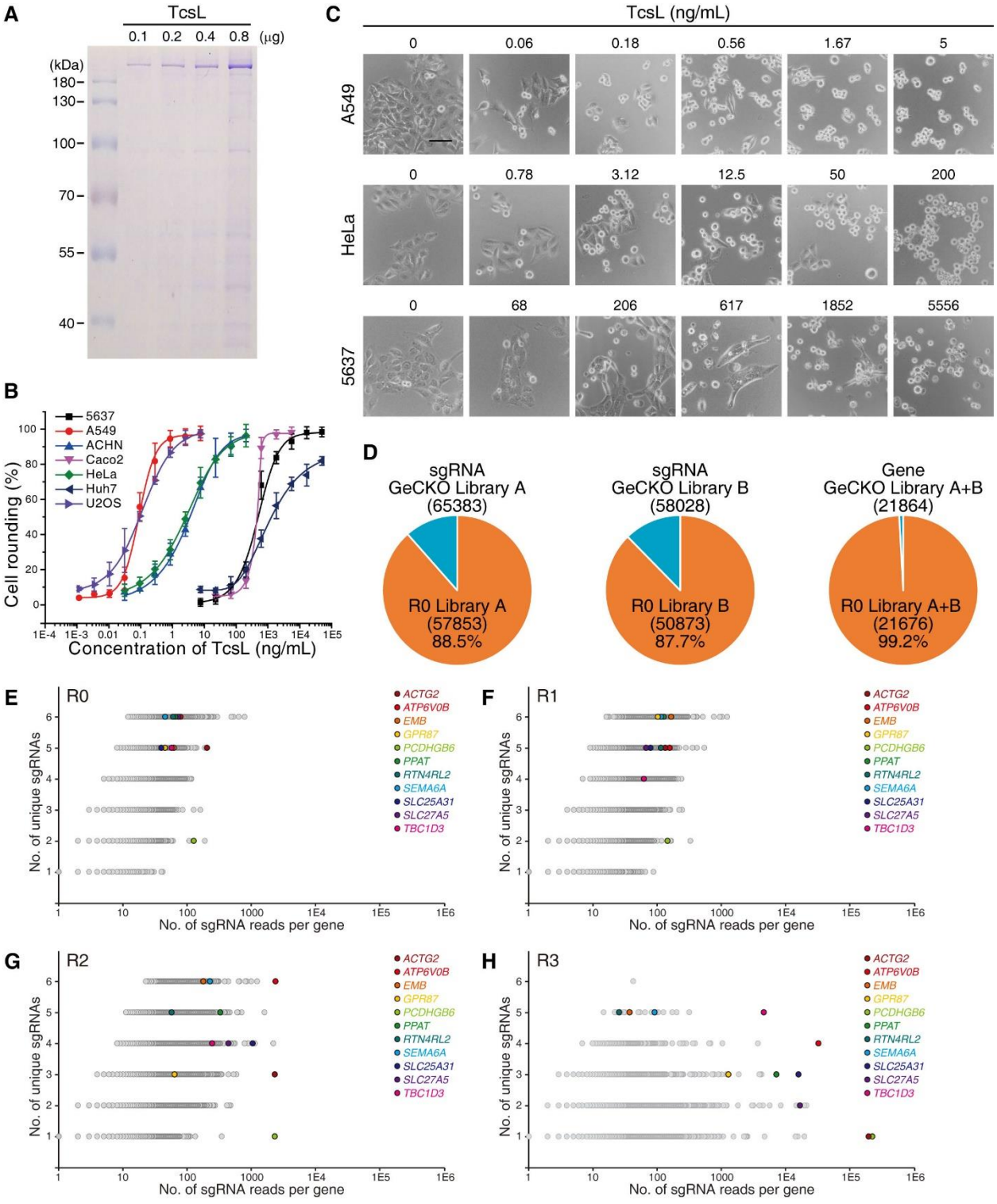

A
TcsL
0.1 0.2 0.4 0.8 (μg)
(kDa)
180
130
100
70
55
40
B
5637
A549
ACHN
Caco2
HeLa
Huh7
U2OS
Cell rounding (%)
Concentration of TcsL (ng/mL)
C
TcsL (ng/mL)
A549
0 0.06 0.18 0.56 1.67 5
HeLa
0 0.78 3.12 12.5 50 200
5637
0 68 206 617 1852 5556
D
sgRNA GeCKO Library A (65383)
R0 Library A (57853) 88.5%
sgRNA GeCKO Library B (58028)
R0 Library B (50873) 87.7%
Gene GeCKO Library A+B (21864)
R0 Library A+B (21676) 99.2%
E
R0
F
R1
G
R2
H
R3
No. of unique sgRNAs
No. of sgRNA reads per gene
ACTG2
ATP6V0B
EMB
GPR87
PCDHGB6
PPAT
RTN4RL2
SEMA6A
SLC25A31
SLC27A5
TBC1D3

Figure S1. CRISPR-cas9 mediated genome-wide screen for TcsL. Related to Figure 1.

(A) SDS-PAGE and Coomassie blue staining showing purified TcsL. Recombinant His6-tagged TcsL was expressed in *B. megaterium* and purified through Ni-NTA column and gel filtration column.

(B) A range of human cell lines were exposed to TcsL for 24 h and the percentages of rounded cells were plotted over toxin concentrations. A549 cells and U2OS cells are the most sensitive ones to TcsL. Error bars indicate mean ± s.d., $N = 3$.

(C) Representative images showing the cell rounding effect in A549, HeLa, and 5637 cells after incubation with the indicated concentrations of TcsL for 24 h. Scale bar, 50 μm.

(D) The recovery rates of sgRNAs and genes identified in R0 compared with the original GeCKO-V2 library.

(E-H) Genes identified in each round (**E** for R0, **F** for R1, **G** for R2, **H** for R3) are plotted based on the number of unique sgRNAs (*y* axis) and total number of sgRNA reads (*x* axis). The top hits investigated here are marked and color-coded. They are enriched from R0 to R3.

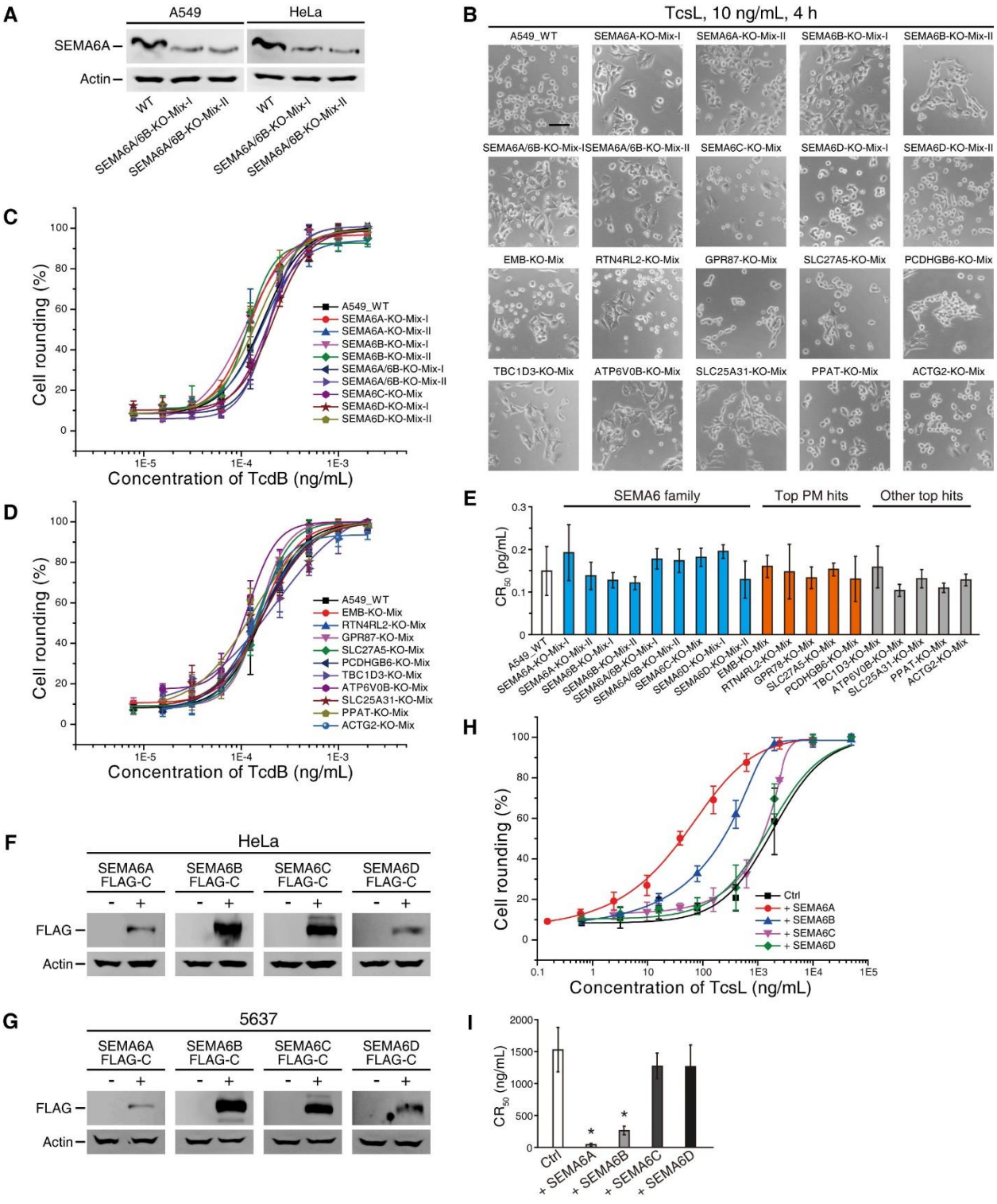

A
A549
HeLa
SEMA6A
Actin
WT
SEMA6A/6B-KO-Mix-I
SEMA6A/6B-KO-Mix-II
B
TcsL, 10 ng/mL, 4 h
A549_WT
SEMA6A-KO-Mix-I
SEMA6A-KO-Mix-II
SEMA6B-KO-Mix-I
SEMA6B-KO-Mix-II
SEMA6A/6B-KO-Mix-I
SEMA6A/6B-KO-Mix-II
SEMA6C-KO-Mix
SEMA6D-KO-Mix-I
SEMA6D-KO-Mix-II
EMB-KO-Mix
RTN4RL2-KO-Mix
GPR87-KO-Mix
SLC27A5-KO-Mix
PCDHGB6-KO-Mix
TBC1D3-KO-Mix
ATP6V0B-KO-Mix
SLC25A31-KO-Mix
PPAT-KO-Mix
ACTG2-KO-Mix
C
Cell rounding (%)
Concentration of TcdB (ng/mL)
D
Cell rounding (%)
Concentration of TcdB (ng/mL)
E
SEMA6 family
Top PM hits
Other top hits
CR50 (pg/mL)
F
HeLa
SEMA6A FLAG-C
SEMA6B FLAG-C
SEMA6C FLAG-C
SEMA6D FLAG-C
FLAG
Actin
G
5637
H
Cell rounding (%)
Ctrl
+ SEMA6A
+ SEMA6B
+ SEMA6C
+ SEMA6D
Concentration of TcsL (ng/mL)
I
CR50 (ng/mL)

Figure S2. Testing the sensitivity of TcsL and TcdB on mixed KO cells and SEMA6A/6B overexpression cells. Related to Figure 2.

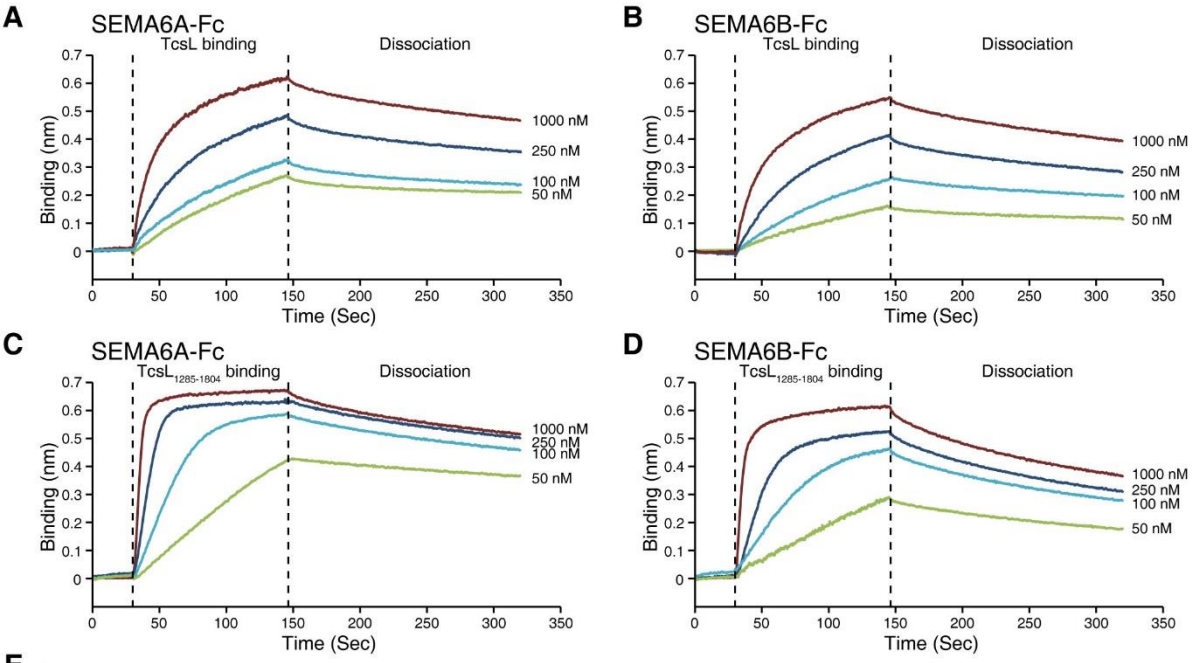
(A) The KO efficiency of SEMA6A in A549 and HeLa cells were confirmed via immunoblot detecting endogenous SEMA6A. Actin was used as a loading control. Representative images were shown from three independent experiments.

(B) Experiments were carried out as described in Figures 2A and 2B. Representative images showing the cell rounding effect in A549 WT and the indicated mixed KO cells after 4 h incubation with TcsL (10 ng/mL). Scale bar, 50 μm.

(C-E) The indicated mixed A549 KO cell lines were exposed to TcdB for 24 h and the percentages of rounded cells were plotted. Their CR_{50} are plotted in a bar-chart (**E**). PM, plasma membrane. Error bars indicate mean ± s.d., $N = 3$.

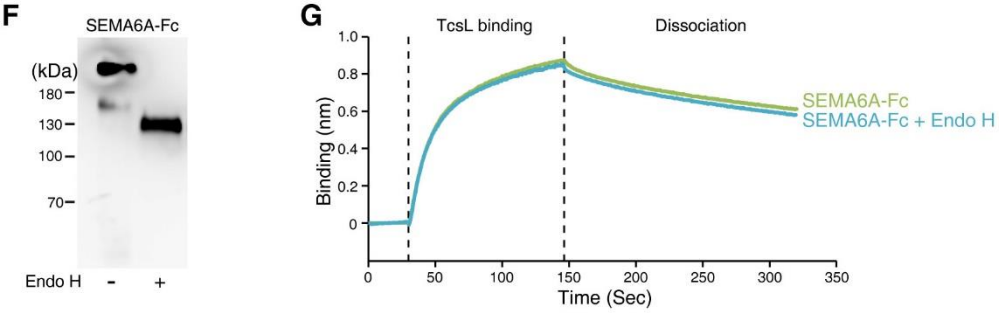
(F-G) SEMA6A, 6B, 6C, and 6D were expressed in HeLa (panel **F**) or a bladder carcinoma cell line 5637 (panel **G**) via lentiviral transduction. Expressed exogenous SEMA6 proteins in cells were confirmed via immunoblot detecting the triple FLAG tag fused to their C-termini. Actin was used as a loading control. Representative images were shown from two independent experiments.

(H-I) 5637 cells overexpressing SEMA6 family proteins via lentiviral transduction were exposed to TcsL for 24 h and the percentages of rounded cells were plotted (panel **H**). Their CR_{50} were determined and shown in a bar-chart (panel **I**). Ctrl: Control. Error bars indicate mean ± s.d., $N = 3$, *, $p < 0.01$ (one-way ANOVA).



E

	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
TcsL vs SEMA6A-Fc	$4.71 \times 10^4 \pm 1.12 \times 10^3$	$1.87 \times 10^{-3} \pm 4.76 \times 10^{-5}$	$3.97 \times 10^{-8} \pm 1.95 \times 10^{-9}$
TcsL vs SEMA6B-Fc	$3.61 \times 10^4 \pm 7.01 \times 10^2$	$2.18 \times 10^{-3} \pm 4.19 \times 10^{-5}$	$6.04 \times 10^{-8} \pm 2.34 \times 10^{-9}$
$TcsL_{1285-1804}$ vs SEMA6A-Fc	$2.45 \times 10^5 \pm 6.34 \times 10^3$	$1.58 \times 10^{-3} \pm 8.08\times 10^{-5}$	$6.45 \times 10^{-9} \pm 4.97 \times 10^{-10}$
$TcsL_{1285-1804}$ vs SEMA6B-Fc	$1.51 \times 10^5 \pm 2.49 \times 10^3$	$3.48 \times 10^{-3} \pm 6.27 \times 10^{-5}$	$2.30 \times 10^{-8} \pm 7.94 \times 10^{-10}$



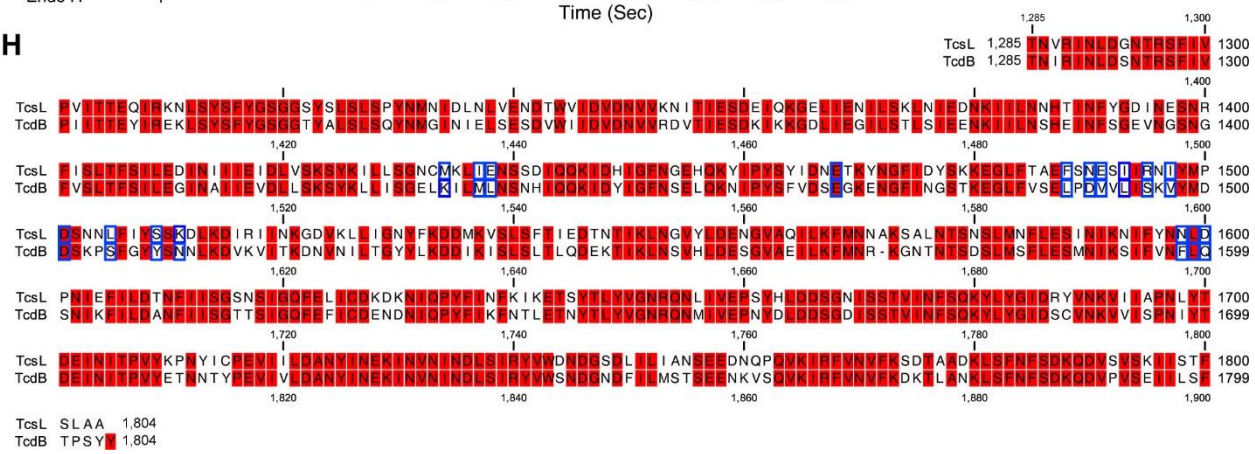


Figure S3. Characterizing TcsL-SEMA interactions. Related to Figure 3.

(A-B) The binding kinetics and affinity were determined using BLI assays for interactions between TcsL and SEMA6A-ECD (**A**), and between TcsL and SEMA6B-ECD (**B**). These parameters are summarized in panel **E (**mean ± s.d.). Representative sensorgrams from one of two independent experiments are shown.

(C-E) The binding kinetics and affinity were determined using BLI assays for interactions between $TcsL_{1285\text{-}1804}$ and SEMA6A-ECD (**C**), and between $TcsL_{1285\text{-}1804}$ and SEMA6B-ECD (**D**). These parameters are summarized in panel **E (**mean ± s.d.). Representative sensorgrams from one of two independent experiments are shown.

(F) SEMA6A-ECD was treated with Endo H and analyzed by immunoblot, which showed a reduced molecular weight compared with untreated SEMA6A-ECD.

(G) Binding of SEMA6A-ECD treated with Endo H to TcsL was characterized using BLI assays, which showed similar levels of binding comparable with untreated SEMA6A-ECD.

(H) Sequence alignment of TcsL and TcdB at TcdB-FBD region. The conserved residues are marked in red. The key residues involve in TcdB-CRD2 interactions are highlighted in blue and the majority of them are different between TcsL and TcdB.

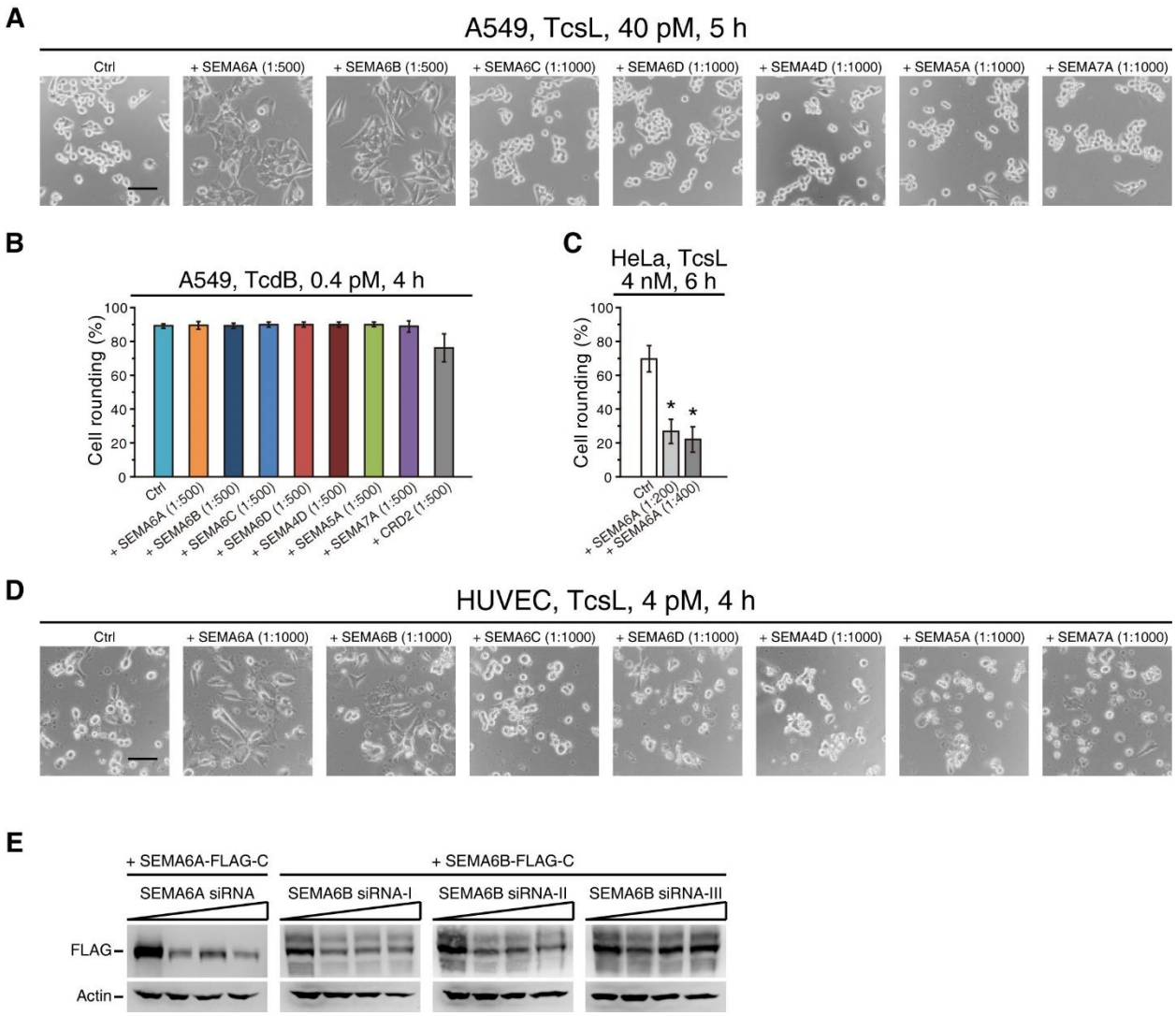

A
A549, TcsL, 40 pM, 5 h
Ctrl
+ SEMA6A (1:500)
+ SEMA6B (1:500)
+ SEMA6C (1:1000)
+ SEMA6D (1:1000)
+ SEMA4D (1:1000)
+ SEMA5A (1:1000)
+ SEMA7A (1:1000)
B
A549, TcdB, 0.4 pM, 4 h
Cell rounding (%)
100
80
60
40
20
0
Ctrl
+ SEMA6A (1:500)
+ SEMA6B (1:500)
+ SEMA6C (1:500)
+ SEMA6D (1:500)
+ SEMA4D (1:500)
+ SEMA5A (1:500)
+ SEMA7A (1:500)
+ CRD2 (1:500)
C
HeLa, TcsL
4 nM, 6 h
Cell rounding (%)
100
80
60
40
20
0
*
*
Ctrl
+ SEMA6A (1:200)
+ SEMA6A (1:400)
D
HUVEC, TcsL, 4 pM, 4 h
Ctrl
+ SEMA6A (1:1000)
+ SEMA6B (1:1000)
+ SEMA6C (1:1000)
+ SEMA6D (1:1000)
+ SEMA4D (1:1000)
+ SEMA5A (1:1000)
+ SEMA7A (1:1000)
E
+ SEMA6A-FLAG-C
+ SEMA6B-FLAG-C
SEMA6A siRNA
SEMA6B siRNA-I
SEMA6B siRNA-II
SEMA6B siRNA-III
FLAG
Actin

Figure S4. SEMA6A and 6B mediate TcsL entry into cells. Related to Figure 4.

(A) Experiments were carried out as described in Figures 4B and 4C. Representative images of the cell rounding effect in A549 cells are shown. Scale bar, 50 μm.

(B) A549 cells were exposed to either TcdB alone (0.4 pM, 4 h) or TcdB pre-incubated with the indicated proteins at the indicated molar ratio. The percentages of rounded cells at 4 h are shown in a bar chart. Error bars indicate mean ± s.d., $N = 3$.

(C) HeLa cells exposed to either TcsL alone (4 nM, 6 h) or TcsL pre-incubated with SEMA6A-ECD at 1:200 or 1:400 molar ratios. The percentages of rounded cells are shown in a bar chart. Error bars indicate mean ± s.d., $N = 3$, *, $p < 0.01$ (Student's *t*-test).

(D) Experiments were carried out as described in Figures 4D and 4E. Representative images of the cell rounding effect in HUVECs are shown. Scale bar, 50 μm.

(E) Validation of siRNA knockdown efficacy. HEK293T cells were seeded in 24-well plate and transfected with SEMA6A or SEMA6B (with C-terminal 3xFLAG tag). The gradient of siRNA was set up as 0, 1, 2, and 3 μL siRNA (20 μM stock) plus 2, 2, 4, and 6 μL Lipofectamine RNAiMAX reagent, respectively. Cells were harvested 48 h later and subjected to immunoblot assays. Expression of SEMA6A and 6B was detected via their FLAG tags. Actin served as a loading control. Representative blots are shown from two independent experiments. SEMA6A siRNA and SEMA6B siRNA-II were selected for the double knockdown experiment in HUVECs.

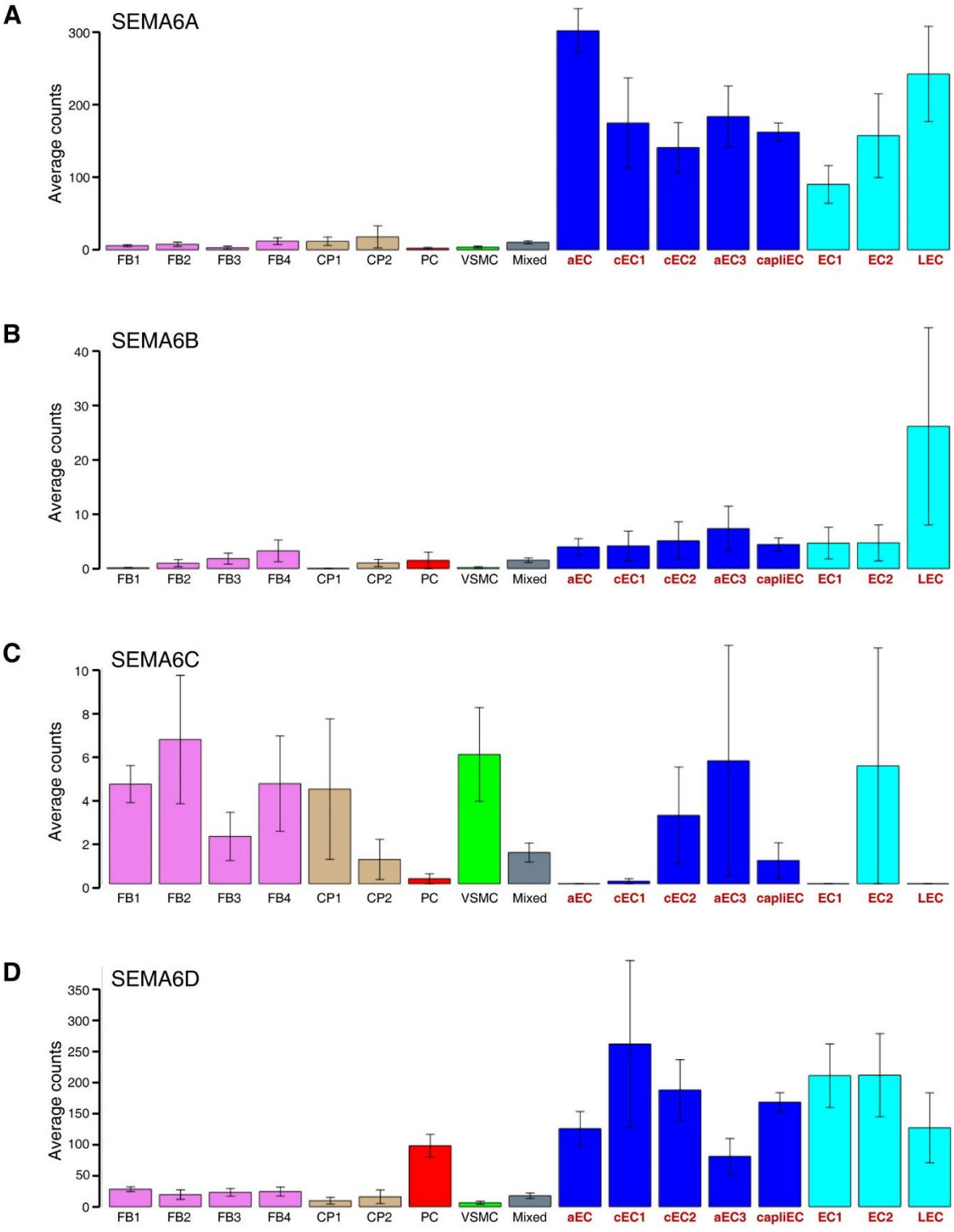

A
SEMA6A
Average counts
300
200
100
0
FB1 FB2 FB3 FB4 CP1 CP2 PC VSMC Mixed aEC cEC1 cEC2 aEC3 capliEC EC1 EC2 LEC
B
SEMA6B
Average counts
40
30
20
10
0
FB1 FB2 FB3 FB4 CP1 CP2 PC VSMC Mixed aEC cEC1 cEC2 aEC3 capliEC EC1 EC2 LEC
C
SEMA6C
Average counts
10
8
6
4
2
0
FB1 FB2 FB3 FB4 CP1 CP2 PC VSMC Mixed aEC cEC1 cEC2 aEC3 capliEC EC1 EC2 LEC
D
SEMA6D
Average counts
350
300
250
200
150
100
50
0
FB1 FB2 FB3 FB4 CP1 CP2 PC VSMC Mixed aEC cEC1 cEC2 aEC3 capliEC EC1 EC2 LEC

Figure S5. Expression of SEMA6 family in different cell types in mouse lung tissues. Related to Figure 5.

Expression of SEMA6A (**A**), 6B (**B**), 6C (**C**), and 6D (**D**) in various lung cells were plotted based on published single cell RNAseq data (http://betsholtzlab.org/VascularSingleCells/database.html) (He et al., 2018; Vanlandewijck et al., 2018). FB: Vascular fibroblast-like cells; CP: Cartilage perichondrium; PC: Pericytes; VSMC: Vascular smooth muscle cells; EC: Endothelial cells (highlighted in red); capil - capillary; a - arterial; c - continuum; L - Lymphatic; 1,2,3,4 - subtypes.

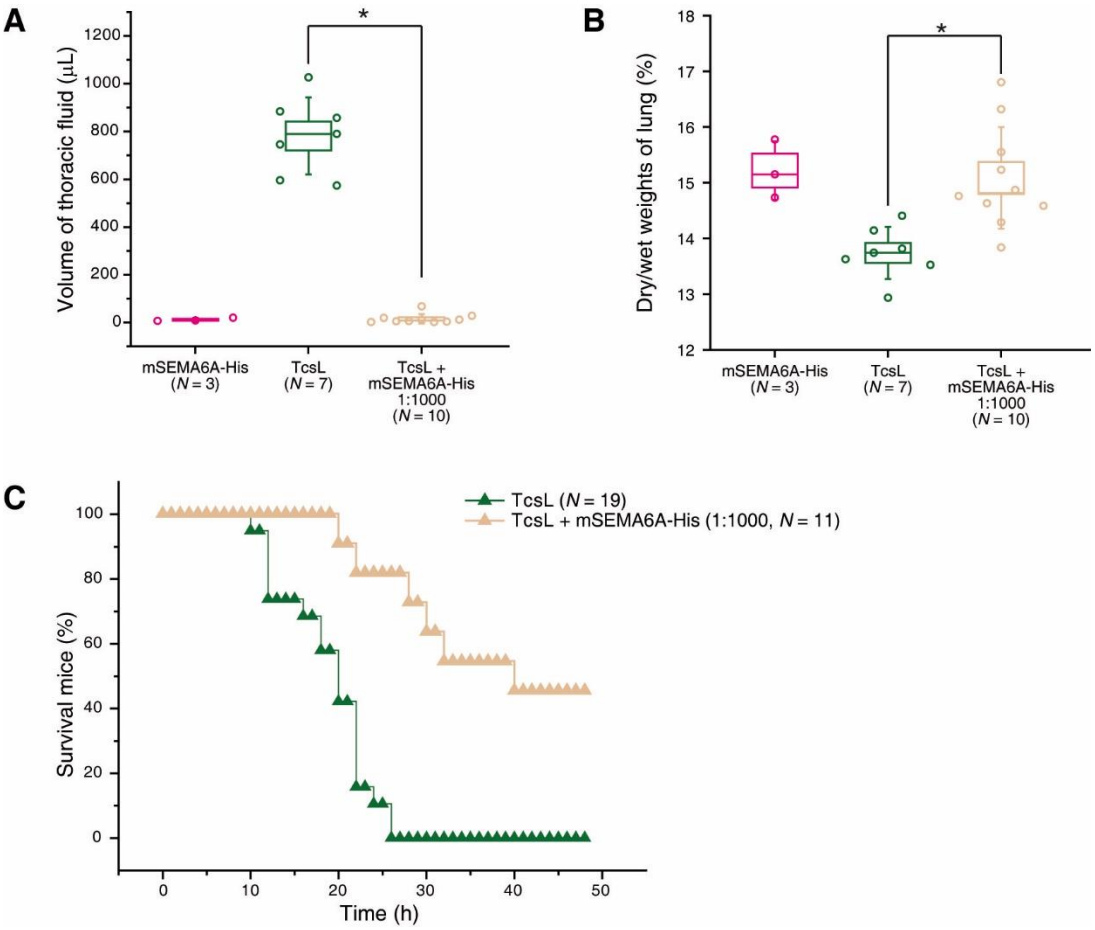

A
Volume of thoracic fluid (μL)
1200
1000
800
600
400
200
0
*
mSEMA6A-His (N = 3)
TcsL (N = 7)
TcsL + mSEMA6A-His 1:1000 (N = 10)
B
Dry/wet weights of lung (%)
18
17
16
15
14
13
12
*
mSEMA6A-His (N = 3)
TcsL (N = 7)
TcsL + mSEMA6A-His 1:1000 (N = 10)
C
TcsL (N = 19)
TcsL + mSEMA6A-His (1:1000, N = 11)
Survival mice (%)
100
80
60
40
20
0
0
10
20
30
40
50
Time (h)

Figure S6. His-tagged SEMA6A-ECD reduces TcsL-induced toxicity in mice. Related to Figure 5 and Figure 6.

(A-B) Co-injection of TcsL (20 ng per 25 g bodyweight) with mouse SEMA6A-His protein (mSEMA6A-His) at 1:1000 (w/w) ratio reduced accumulation of fluid in the thoracic cavity (**A**) and reduced dry-to-wet weight ratio of lung tissue 4 h after the injection (**B**). Injection of mSEMA6A-His alone was examined in parallel. Boxes indicate ± s.e.m., error bars indicate ± s.d., *, $p < 0.01$ (one-way ANOVA).

(C) Five in a total of eleven mice with co-injection of TcsL and mSEMA6A-His (1:1000 ratio) survived, while all mice injected with TcsL alone died ($p < 0.001$, Kaplan-Meier Log-Rank test).

Table S1. **Oligonucleotides used in this study. Related to STAR Methods section.**

Name	Sequence (5'-3')	Purpose
SEMA6A_sg_F	CACCGTTGCCATGCGAAATACTGAT	sgRNA Targeting SEMA6A
SEMA6A_sg_R	AAACATCAGTATTTCGCATGGCAAC	sgRNA Targeting SEMA6A
SEMA6A-II_sg_F	CACCGGGCTTGTGGCCCACAAACAC	sgRNA Targeting SEMA6A
SEMA6A-II_sg_R	AAACGTGTTTGTGGGCCACAAGCCC	sgRNA Targeting SEMA6A
SEMA6B_sg_F	CACCGCGAGTGTCGAAACTTCGTAA	sgRNA Targeting SEMA6B
SEMA6B_sg_R	AAACTTACGAAGTTTCGACACTCGC	sgRNA Targeting SEMA6B
SEMA6B-II_sg_F	CACCGTGGGGGGCTCCAGCTCTACG	sgRNA Targeting SEMA6B
SEMA6B-II_sg_R	AAACCGTAGAGCTGGAGCCCCCCAC	sgRNA Targeting SEMA6B
SEMA6C_sg_F	CACCGTATCCCTTCAGTATCTAACA	sgRNA Targeting SEMA6C
SEMA6C_sg_R	AAACTGTTAGATACTGAAGGGATAC	sgRNA Targeting SEMA6C
SEMA6D_sg_F	CACCGAGTGATAGTCGACAGTATTA	sgRNA Targeting SEMA6D
SEMA6D_sg_R	AAACTAATACTGTCGACTATCACTC	sgRNA Targeting SEMA6D
SEMA6D-II_sg_F	CACCGGAAAGCTGACTGCCCTCAAC	sgRNA Targeting SEMA6D
SEMA6D-II_sg_R	AAACGTTGAGGGCAGTCAGCTTTCC	sgRNA Targeting SEMA6D
EMB_sg_F	CACCGAGTCATAACATATCACTGAC	sgRNA Targeting EMB
EMB_sg_R	AAACGTCAGTGATATGTTATGACTC	sgRNA Targeting EMB
RTN4RL2_sg_F	CACCGATCGAGACAAGATGCTGCCC	sgRNA Targeting RTN4RL2
RTN4RL2_sg_R	AAACGGGCAGCATCTTGTCTCGATC	sgRNA Targeting RTN4RL2
GPR87_sg_F	CACCGGTCTGCGTGTAATGTTTGCC	sgRNA Targeting GPR87
GPR87_sg_R	AAACGGCAAACATTACACGCAGACC	sgRNA Targeting GPR87
SLC27A5_sg_F	CACCGGTCGAACTGCACCAGCTCAA	sgRNA Targeting SLC27A5
SLC27A5_sg_R	AAACTTGAGCTGGTGCAGTTCGACC	sgRNA Targeting SLC27A5
PCDHGB6_sg_F	CACCGTTTCGACCAGACGTCCTACG	sgRNA Targeting PCDHGB6
PCDHGB6_sg_R	AAACCGTAGGACGTCTGGTCGAAAC	sgRNA Targeting PCDHGB6
TBC1D3_sg_F	CACCGGCTTCCGCTTTGATGTGGCA	sgRNA Targeting TBC1D3
TBC1D3_sg_R	AAACTGCCACATCAAAGCGGAAGCC	sgRNA Targeting TBC1D3
ATP6V0B_sg_F	CACCGTTGTTGTTGTAGCTTCGAAA	sgRNA Targeting ATP6V0B
ATP6V0B_sg_R	AAACTTTCGAAGCTACAACAACAAC	sgRNA Targeting ATP6V0B
SLC25A31_sg_F	CACCGCCCTTGAATTGTCGCTCCTC	sgRNA Targeting SLC25A31
SLC25A31_sg_R	AAACGAGGAGCGACAATTCAAGGGC	sgRNA Targeting SLC25A31
PPAT_sg_F	CACCGTTCGTTGTTGAAACACTTCA	sgRNA Targeting PPAT
PPAT_sg_R	AAACTGAAGTGTTTCAACAACGAAC	sgRNA Targeting PPAT
ACTG2_sg_F	CACCGGTGTGACATTGACATCCGTA	sgRNA Targeting ACTG2
ACTG2_sg_R	AAACTACGGATGTCAATGTCACACC	sgRNA Targeting ACTG2
SEMA6A_01F	AGCTTGGTACCGAGCTCGGATCCATGAGGTC AGAAGCCTTGCTGCTAT	Clone SEMA6A into pcDNA3.1-3xFlag-C
SEMA6A_02R	GAACCAGAACCAGAACCGAATTCTGTACAC GCATCATTGGGCTTCATGG	Clone SEMA6A into pcDNA3.1-3xFlag-C

SEMA6B_01F	AGCTTGGTACCGAGCTCGGATCCATGCAGAC CCCGCGAGCGTCCCCTC	Clone SEMA6A into pcDNA3.1-3xFlag-C
SEMA6B_02R	GAACCAGAACCAGAACCGAATTCGGGCACG GGGGGCGCAGTCCT	Clone SEMA6A into pcDNA3.1-3xFlag-C
SEMA6C_01F	AGCTTGGTACCGAGCTCGGATCCATGCCCCG TGCCCCC	Clone SEMA6A into pcDNA3.1-3xFlag-C
SEMA6C_02R	GAACCAGAACCAGAACCGAATTCAAAGTTG AAACGGCCGCCGTTC	Clone SEMA6A into pcDNA3.1-3xFlag-C
SEMA6D_01F	AGCTTGGTACCGAGCTCGGATCCATGAGGGT CTTCCTGCTTTGTGCC	Clone SEMA6A into pcDNA3.1-3xFlag-C
SEMA6D_02R	GAACCAGAACCAGAACCGAATTCGTATGTGT ATTTGTTCAGTGGTCTGACAGATGG	Clone SEMA6A into pcDNA3.1-3xFlag-C
LentiHygro_01F	GGAACCAATTCAGTCGACTGGATCCGCTTGG TACCGAGCTCGGATCC	Transfer gene from pcDNA3.1 to LentiHygro
LentiHygro_01F	TTTGTACAAGAAAGCTGGGTCTAGACTCTAG ACTCGAGCGGCCGC	Transfer gene from pcDNA3.1 to LentiHygro
TcsL_01F	TTTAAGAAGGAGATATACCATGGGATTCACA ACTATAGATGGTAATAAATATTACTTTGACC CAAC	Clone TcsL-1856-2364 into pET28a
TcsL_02R	GCGGCCGCAAGCTTGTCGACTTCACTAACTA CTAATTCAGCTGTATCAGGGTCAAAATAG	Clone TcsL-1856-2364 into pET28a
TcsL_05F	AAGAAGGAGATATACCATGGGAACTAATGT AAGAATAAATCTAGATGGCAATACTAGAAG	Clone TcsL-1285-1804 into pET28a
TcsL_06R	GCGGCCGCAAGCTTGTCGACAGCTGCAAGTG AAAATGTTGAAATAATTTT	Clone TcsL-1285-1804 into pET28a
TcsL_12F	AACATTTTCACTTGCAGCTGTCGACACCGAG CTCGGATCCATG	Clone 3xFLAG tag between TcsL-1285-1804 and His tag
TcsL_13R	CGAGTGCGGCCGCAAGCTTGTCGACGAATTC AGAACCAGAACCAGAACC	Clone 3xFLAG tag between TcsL-1285-1804 and His tag
TcsL_14F	ATAAAATACTTCTTTCTGGTGAATTAAAAAT ATTGATGTTAAATTCAAATCATATTCAACAG AAAATAGATT	Switch TcdB 1431-1600 to TcdL
TcsL_15R	TCTAGTATAAACTCGATATTAGATTGTAAGA AATTAACGAAAATACTTTTTATATTCATACTT TCT	Switch TcdB 1431-1600 to TcdL
TcsL_16F	TCGTTAATTTCTTACAATCTAATATCGAGTTT ATACTAGATACTAATTTCATAATAAGTGGTA GC	Switch TcdB 1431-1600 to TcdL
TcsL_17R	AACATCAATATTTTTAATTCACCAGAAAGAA GTATTTTATAAGATTTTGATACTAAATCAATT TCT	Switch TcdB 1431-1600 to TcdL
TcsL_18F	ATAAATTACTTATTTCTGGCAATTGTATGAA ATTGATAGAAAACTCATCTGATATTCAAC	Switch TcsL 1431-1601 to TcdB
TcsL_19R	TCTAATATAAACTTAATATTAGGGTCTAGAT TATTGTAGAAAATATTTTTTATATTGATACTT TCTAAG	Switch TcsL 1431-1601 to TcdB
TcsL_20F	TCTACAATAATCTAGACCCTAATATTAAGTT TATATTAGATGCTAATTTTATAATAAGTGGT ACTACTTCTATT	Switch TcsL 1431-1601 to TcdB
TcsL_21R	TCTATCAATTTCATACAATTGCCAGAAATAA GTAATTTATATGATTTAGATAATAAATCAAC TTCTATAA	Switch TcsL 1431-1601 to TcdB
lentiGP_01F	AATGGACTATCATATGCTTACCGTAACTTGA AAGTATTTCG	PCR the sgRNA locus

lentiGP_03R	ATGAATACTGCCATTTGTCTCAAGATCTAGT TACGC	PCR the sgRNA locus
SEMA6A_Sense	GCAGUGGAGUAUAACACCA[dT][dT]	siRNA for SEMA6A
SEMA6A_Antisense	UGGUGUUAUACUCCACUGC[dT][dT]	siRNA for SEMA6A
SEMA6B_I_Sense	CCGUGAAACAUGACUCCAA[dT][dT]	siRNA for SEMA6B
SEMA6B_I_Antisense	UUGGAGUCAUGUUUCACGG[dT][dT]	siRNA for SEMA6B
SEMA6B_II_Sense	GGGAUGCUCUUCACAGCUA[dT][dT]	siRNA for SEMA6B
SEMA6B_II_Antisense	UAGCUGUGAAGAGCAUCCC[dT][dT]	siRNA for SEMA6B
SEMA6B_III_Sense	GAGUUUAACUACCUGGAGA[dT][dT]	siRNA for SEMA6B
SEMA6A_Antisense	UGGUGUUAUACUCCACUGC[dT][dT]	siRNA for SEMA6B