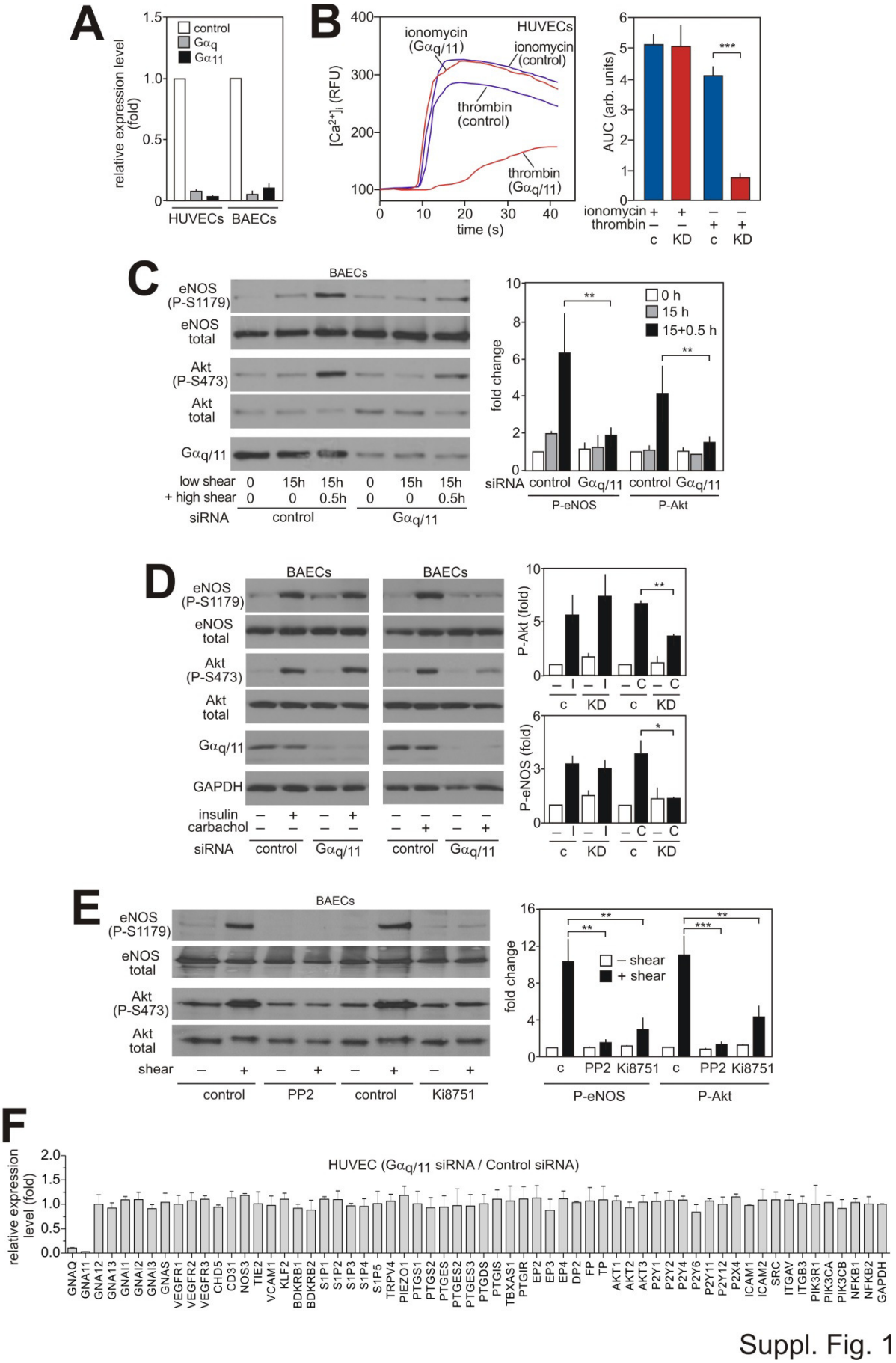
Supplementary Information (Ms Wang et al.)

Supplementary Data

Supplementary Figure 1. $G\alpha_q/G\alpha_{11}$ mediated effects in endothelial cells *in vitro*.

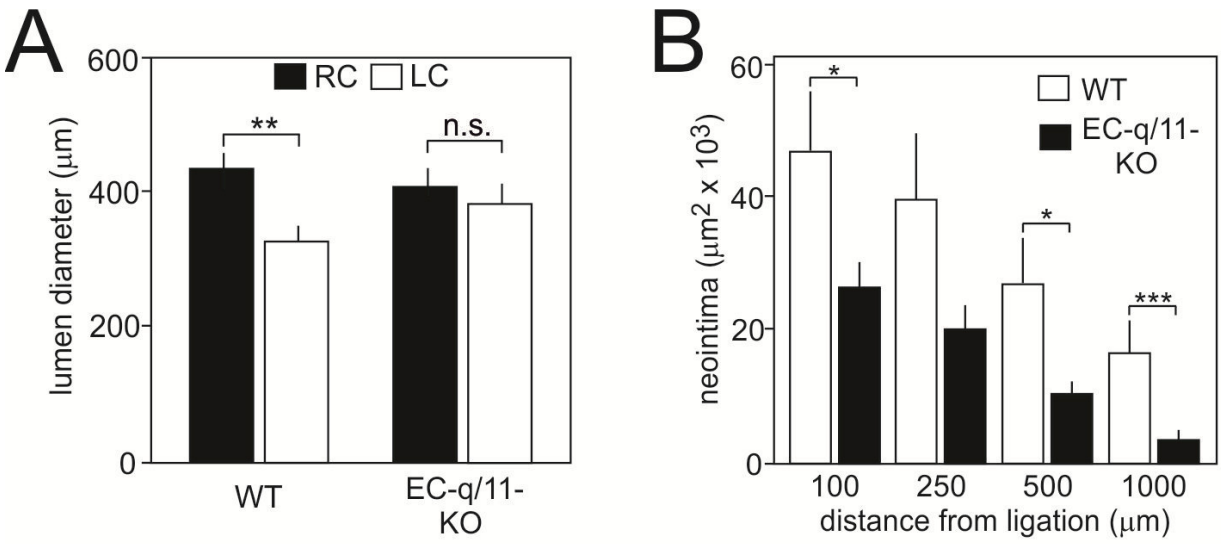
The indicated cells were transfected with scrambled siRNA (control) or siRNA directed against $G\alpha_q$ and $G\alpha_{11}$ (A-D) or were incubated in the absence (-) or presence of 10 μM PP2 or 500 nM Ki8751 (E). (**A**) Quantitative RT-PCR analysis of the efficiency of siRNA-mediated knock-down of $G\alpha_q$ and $G\alpha_{11}$ compared to control treated cells (control was set as 1) (n=3). (**B**) Fluo-4-loaded cells (n=27 (ionomycin control), n=19 (ionomycin $G\alpha_{q/11}$), n=16 (thrombin control), n=18 (thrombin $G\alpha_{q/11}$); 3 independent experiments) were exposed to 10 μM ionomycin or 10 U/ml thrombin, and $[Ca^{2+}]_i$ was determined as fluorescence intensity (RFU, relative fluorescence units). Bar diagram (B) shows area under the curve (AUC). Shown are means -/+ s.e.m.; ***, P≤0.001 (two-tailed Student's *t* test). (**C-E**) BAECs were exposed to low and high shear (3 and 35 dynes/cm^2, respectively, C), to 1 μM insulin (I) or 10 μM carbachol (C) (D) or to fluid shear at 20 dynes/cm^2 (E) for the indicated time; KD, knock-down. Akt and eNOS activation was determined by Western blotting for phosphorylated Akt and eNOS. Knock-down of $G\alpha_q/G\alpha_{11}$ was verified by anti-$G\alpha_q/G\alpha_{11}$ immunoblotting. Bar diagrams show the densitometric evaluation (n=3). (**F**) Expression of the indicated genes in HUVECs. Cells were treated with control siRNA or with siRNA directed against $G\alpha_q/G\alpha_{11}$, and the expression of the indicated genes was determined by qRT-PCR (for primer sequences, see below). Shown is the expression level in cells after knock-down of $G\alpha_q/G\alpha_{11}$ divided by the expression level under control conditions. Shown in C-F are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01; ***, P≤0.001 (two-way ANOVA and Bonferroni's *post hoc* test).


A
relative expression level (fold)
control
Gαq
Gα11
HUVECs
BAECs
B
HUVECs
ionomycin (Gαq/11)
ionomycin (control)
thrombin (control)
thrombin (Gαq/11)
[Ca2+]i (RFU)
time (s)
AUC (arb. units)
ionomycin
thrombin
c
KD
C
BAECs
eNOS (P-S1179)
eNOS total
Akt (P-S473)
Akt total
Gαq/11
low shear
+ high shear
siRNA
control
Gαq/11
0 h
15 h
15+0.5 h
fold change
P-eNOS
P-Akt
D
BAECs
GAPDH
insulin
carbachol
P-Akt (fold)
P-eNOS (fold)
E
shear
PP2
Ki8751
– shear
+ shear
F
HUVEC (Gαq/11 siRNA / Control siRNA)
relative expression level (fold)
GNAQ
GNA11
GNA12
GNA13
GNAI1
GNAI2
GNAI3
GNAS
VEGFR1
VEGFR2
VEGFR3
CHD5
CD31
NOS3
TIE2
VCAM1
KLF2
BDKRB1
BDKRB2
S1P1
S1P2
S1P3
S1P4
S1P5
TRPV4
PIEZO1
PTGS1
PTGS2
PTGES
PTGES2
PTGES3
PTGDS
PTGIS
TBXAS1
PTGIR
EP2
EP3
EP4
DP2
FP
TP
AKT1
AKT2
AKT3
P2Y1
P2Y2
P2Y4
P2Y6
P2Y11
P2Y12
P2X4
ICAM1
ICAM2
SRC
ITGAV
ITGB3
PIK3R1
PIK3CA
PIK3CB
NFKB1
NFKB2
GAPDH

Suppl. Fig. 1

Supplementary Figure 2. Effect of endothelium-specific $G\alpha_q/G\alpha_{11}$ deficiency on remodeling of the common carotid artery

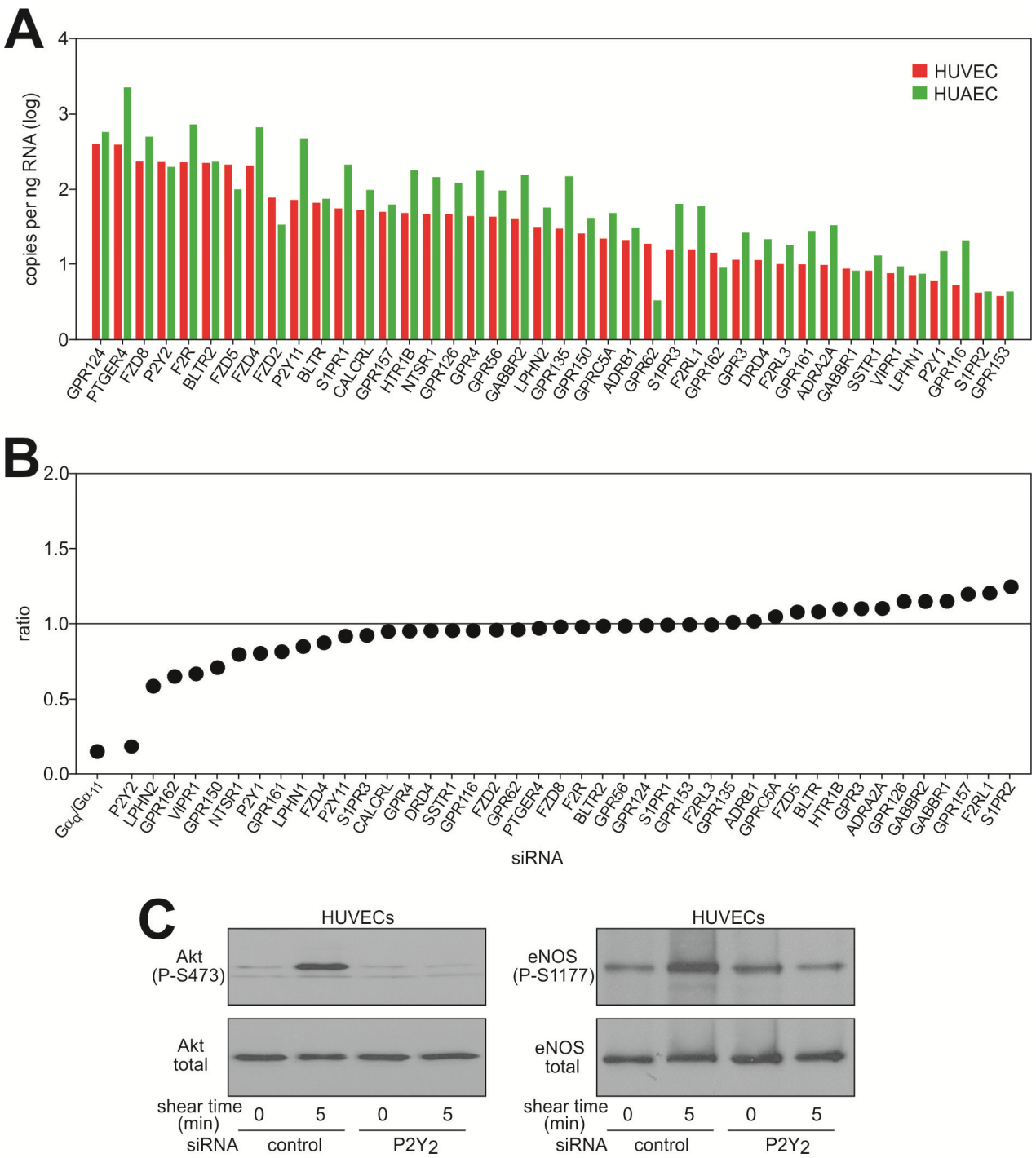
(**A**) In wild-type (WT) and $G\alpha_q/G\alpha_{11}$-deficient mice (EC-q/11-KO), the left external artery was ligated, and two weeks later the left and right common carotid arteries were histologically analyzed. The diameter of the vessels was calculated by measuring the circumference of the vessels in histological sections. Bar diagrams show lumen diameter of the left and right common carotid artery 2 weeks after ligation of the external carotid artery (n=8). (**B**) The left common carotid artery of wild-type (WT) and endothelium-specific $G\alpha_q$/ $G\alpha_{11}$-deficient mice (EC-q/11-KO) was ligated, and 4 weeks later, formation of a neointima in the common carotid artery was analyzed. Bar diagrams show the neontima area 4 weeks after the ligation of the common carotid artery in wild-type (WT; n=9) and EC-q/11-KO mice (n=8). Shown are means ± s.e.m.; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; n.s., not significant (two-tailed Student's *t*-test).



Suppl. Fig. 2

Supplementary Figure 3. Identification of $P2Y_2$ as a receptor mediating fluid shear stress-induced Akt phosphorylation in HUVECs

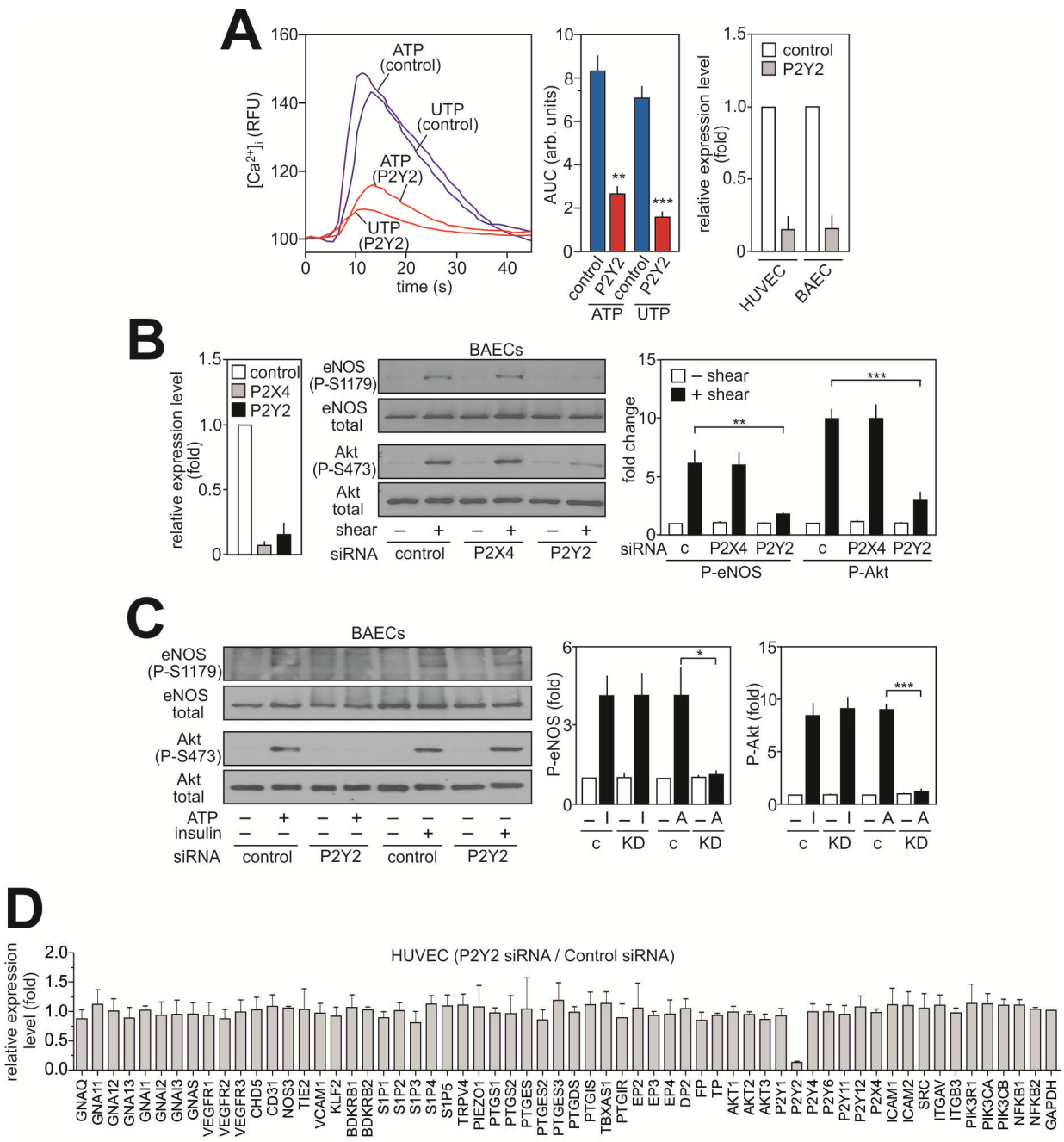
(**A**) Expression of non-olfactory GPCRs found both in HUVECs and human umbilical artery endothelial cells (HUAECs). Expression was determined by quantitative RT-PCR. (**B**) SiRNA screen to identify GPCRs mediating fluid shear stress-induced increases in Akt phosphorylation in HUVECs. Shown is the ratio of the fluid shear stress effect on Akt phosphorylation in cells transfected with an siRNA pool against a particular human GPCR and cells transfected with control siRNA. The plot shows the ranked average ratios of one out of two experiments performed with 42 siRNA pools directed against GPCRs found to be expressed in HUVECs and HUAECs (a). siRNAs directed against $G\alpha_q$ and $G\alpha_{11}$ served as a positive control. (**C**) HUVECs were transfected with scrambled (control) siRNA or siRNA directed against $P2Y_2$, and cells were exposed to fluid shear (12 dynes/cm^2) for the indicated time. Akt and eNOS activation was determined by Western blotting for phosphorylated Akt, eNOS and total Akt and eNOS (n=2).


A
copies per ng RNA (log)
HUVEC
HUAEC
GPR124 PTGER4 FZD8 P2Y2 F2R BLTR2 FZD5 FZD4 FZD2 P2Y11 BLTR S1PR1 CALCRL GPR157 HTR1B NTSR1 GPR126 GPR4 GPR56 GABBR2 LPHN2 GPR135 GPR150 GPRC5A ADRB1 GPR62 S1PR3 F2RL1 GPR162 GPR3 DRD4 F2RL3 GPR161 ADRA2A GABBR1 SSTR1 VIPR1 LPHN1 P2Y1 GPR116 S1PR2 GPR153
B
ratio
Gαq/Gα11 P2Y2 LPHN2 GPR162 VIPR1 GPR150 NTSR1 P2Y1 GPR161 LPHN1 FZD4 P2Y11 S1PR3 CALCRL GPR4 DRD4 SSTR1 GPR116 FZD2 GPR62 PTGER4 FZD8 F2R BLTR2 GPR56 GPR124 S1PR1 GPR153 F2RL3 GPR135 ADRB1 GPRC5A FZD5 BLTR HTR1B GPR3 ADRA2A GPR126 GABBR2 GABBR1 GPR157 F2RL1 S1PR2
siRNA
C
HUVECs
Akt (P-S473)
Akt total
shear time (min) 0 5 0 5
siRNA control P2Y2
HUVECs
eNOS (P-S1177)
eNOS total
shear time (min) 0 5 0 5
siRNA control P2Y2

Suppl. Fig. 3

Supplementary Figure 4. $P2Y_2$ mediates effects on endothelial cells *in vitro*.

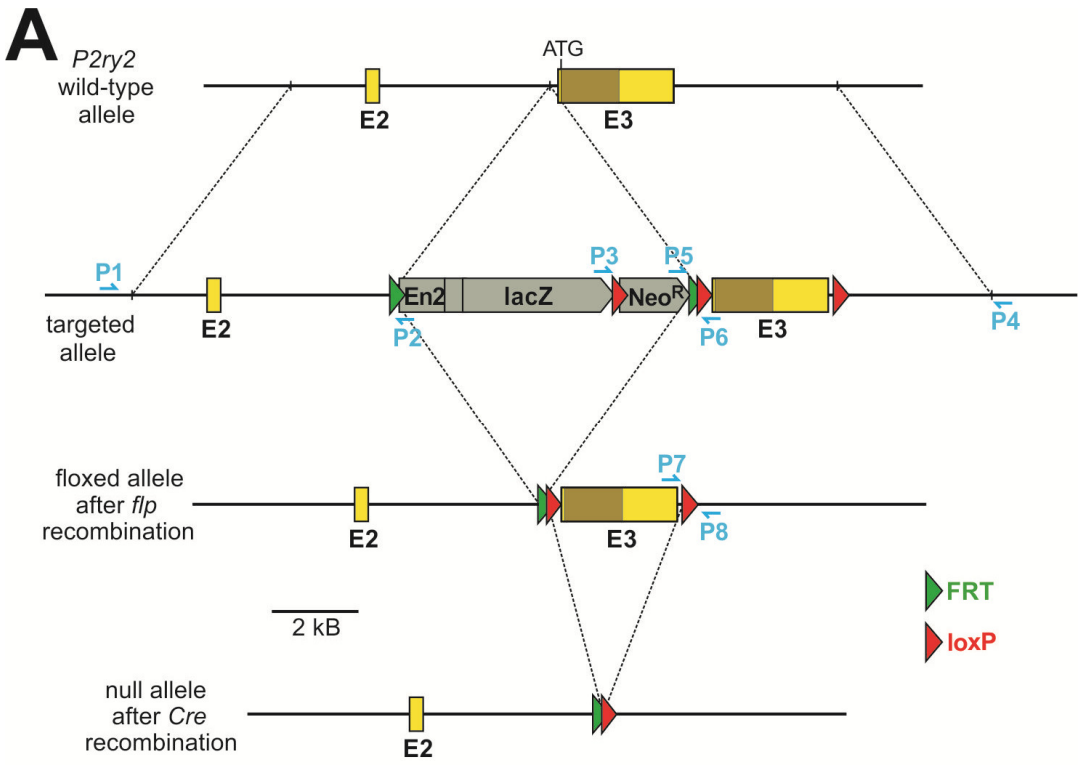
BAECs were transfected with scrambled siRNA (control) or siRNA directed against $P2Y_2$ or P2X4. (**A**) Fluo-4-loaded cells (n=22 (ATP control), n=17 (ATP $P2Y_2$), n=18 (UTP control), n=23 (UTP $P2Y_2$); 3 independent experiments) were exposed to 10 μM ATP or UTP, and $[Ca^{2+}]_i$ was determined as fluorescence intensity (RFU, relative fluorescence units). Bar diagram shows area under the curve (AUC). Right panel in (A) shows efficiency of siRNA-mediated knock-down of $P2Y_2$ compared to control treated cells (control was set as 1) (n=3). Shown are means -/+ s.e.m.; ***, P≤0.001 (two-tailed Student's *t* test). (**B,C**) Cells were exposed to flow (20 dynes/cm^2) for 5 min (B) or to 1 μM insulin (I) or 100 μM ATP (A) (C) for 1 min, and Akt and eNOS activation was determined by Western blotting for phosphorylated Akt and eNOS. Bar diagrams show the densitometric evaluation (n=3); KD, knock-down. (**D**) Expression of the indicated genes in HUVECs. Cells were treated with control siRNA or with siRNA directed against $P2Y_2$, and the expression of the indicated genes was determined by qRT-PCR (for primer sequences, see below). Shown is the expression level in cells after knock-down of $P2Y_2$ divided by the expression level under control conditions. Shown are means -/+ s.e.m.; *, P≤0.05; **, P≤0.01; ***, P≤0.001 (two-way ANOVA and Bonferroni's *post hoc* test).

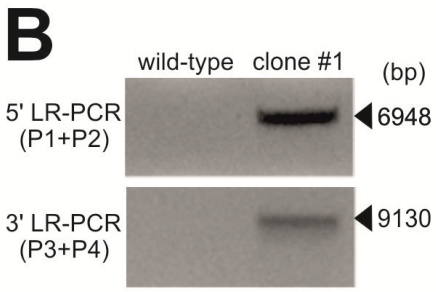

A
ATP (control)
UTP (control)
ATP (P2Y2)
UTP (P2Y2)
[Ca2+]i (RFU)
time (s)
AUC (arb. units)
control
P2Y2
ATP
UTP
relative expression level (fold)
HUVEC
BAEC
B
control
P2X4
P2Y2
relative expression level (fold)
BAECs
eNOS (P-S1179)
eNOS total
Akt (P-S473)
Akt total
shear
siRNA
control
P2X4
P2Y2
– shear
+ shear
fold change
P-eNOS
P-Akt
C
BAECs
ATP
insulin
siRNA
control
P2Y2
P-eNOS (fold)
P-Akt (fold)
KD
D
HUVEC (P2Y2 siRNA / Control siRNA)
relative expression level (fold)
GNAQ
GNA11
GNA12
GNA13
GNAI1
GNAI2
GNAI3
GNAS
VEGFR1
VEGFR2
VEGFR3
CHD5
CD31
NOS3
TIE2
VCAM1
KLF2
BDKRB1
BDKRB2
S1P1
S1P2
S1P3
S1P4
S1P5
TRPV4
PIEZO1
PTGS1
PTGS2
PTGES
PTGES2
PTGES3
PTGDS
PTGIS
TBXAS1
PTGIR
EP2
EP3
EP4
DP2
FP
TP
AKT1
AKT2
AKT3
P2Y1
P2Y2
P2Y4
P2Y6
P2Y11
P2Y12
P2X4
ICAM1
ICAM2
SRC
ITGAV
ITGB3
PIK3R1
PIK3CA
PIK3CB
NFKB1
NFKB2
GAPDH

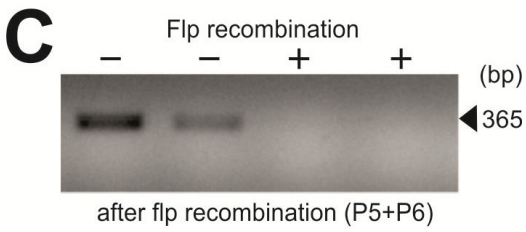
Suppl. Fig. 4

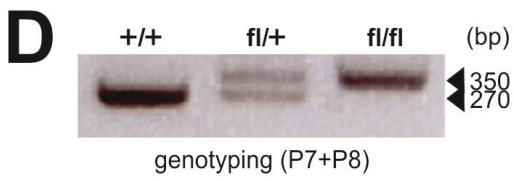
Supplementary Figure 5. Generation of mice carrying a floxed allele of the gene encoding $P2Y_2$

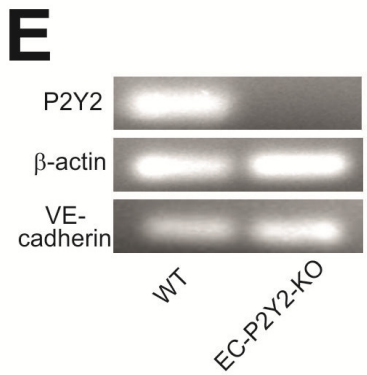
(**A**) JM8A1.N3 mouse embryonic stem (ES) cells generated from the strain C57BL/6N carrying the shown targeted allele of the gene encoding $P2Y_2$ (*P2ry2*) were obtained from EUCOMM (ES cell clone number: HEPD0557 7 B07). ES cells were used to generate chimeric mice, and germ-line transmission was verified after crossing high-percentage chimeras with a Flp deleter mouse line[1]. Mice carrying the indicated floxed allele were then used to generate inducible endothelium-specific $P2Y_2$-deficient mice (Tie2-CreER^{T2};*P2ry2*$^{flox/flox}$). Shown are exon 2 and 3 of the *P2ry2* gene (E2 and E3) with the coding region in exon 3 shaded. Blue arrows indicate position of primers used to verify the alleles and to genotype mice. (**B**) Verification of ES cell clone HEPD0557 7 B07. (**C**) Verification of Flp-mediated recombination. (**D**) Genotyping of mice without or with one or two floxed $P2Y_2$ alleles. (**E**) Expression of $P2Y_2$ in endothelial cells isolated from skeletal muscle derived from wild-type and induced EC-$P2Y_2$-KO mice as analyzed by RT-PCR. Skeletal muscle was minced into small pieces and digested with collagenase II, elastase, dispase and DNAse. Endothelial cells were isolated using CD31-labelled dynabeads and further purified by CD144-PE labelled FACS sorting. RNA was prepared from sorted cells using Qiagen micro kit, and cDNA was preamplified using Nugen Pico WTA kit for expression analysis by RT-PCR.


A
P2ry2
wild-type
allele
ATG
E2
E3
P1
P3
P5
targeted
allele
En2
lacZ
Neo^R
P2
P6
E3
P4
floxed allele
after flp
recombination
P7
E2
E3
P8
FRT
2 kB
loxP
null allele
after Cre
recombination
E2


B
wild-type
clone #1
(bp)
5' LR-PCR
(P1+P2)
6948
3' LR-PCR
(P3+P4)
9130


C
Flp recombination
–
–
+
+
(bp)
365
after flp recombination (P5+P6)


D
+/+
fl/+
fl/fl
(bp)
350
270
genotyping (P7+P8)


E
P2Y2
β-actin
VE-
cadherin
WT
EC-P2Y2-KO

Suppl. Fig. 5

References (Supplementary Data)

1. Rodriguez, C.I.*, et al.* High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat. Genet.* **25**, 139-140 (2000).

qPCR primer sequences (Human gene/Forward primer/Revers primer/Probe; s. Suppl. Figs. 1 and 4)

GNAQ: gactacttcccagaatatgatggac / ggttcaggtccacgaacatc/#27

GNA11: gcatccaggaatgctacgac / ggtcaacgtcggtcaggtag/#53

GNA12: gcgagttcgaccagaagg / catcaacaagaacccttgagc/#67

GNA13: tcgggaaaagacctatgtgaa / caaccagcaccctcatacct/#3

GNAI1: aagtacaattgtgaagcagatgaaa / tggtgttactgtagaccactgctt/#35

GNAI2: ctcaacgactcagctgccta / tgctgtgtggggatgtagtc/#1

GNAI3: tgggacggctaaagattgac / ataattgccgggcatcatc/#60

GNAS: agagacccccagttgaggag / taccccggagagggtacttt/#12

VEGFR1: caggaatgtatacacaggggaag / tttttgttgcagtgctcacc/#65

VEGFR2: gctcaagacaggaagaccaag / ggtgccacacgctctagg/#27

VEGFR3: actgcaagaacgtgcatctg / catagtggccctcgtgct /#10

CDH5: acaaggacactggcgaaaac / cgtcatgaactttgatggtga/#12

PECAM1: ggtggatgaggtccagatttc / cagcacaatgtcctctccag/#77

ENOS: ccgacgctacgaggagtg / ctgctccagcacctccag/#43

TIE2: gcaacttgacttcggtgcta / tgttgactctagctcggacca/#32

VCAM1: agttgctgatgtatacccatttga / aattcctgactcttcatgagatgat/#19

KLF2: cgtgctggacttcatcctg / gggttcggggtaatagaacg/#42

BDKRB1: agaccacagcgctgatcc / caggaaggcaaagaagtggt/#3

BDKRB2: tcaccaacatgctcctgaat / cgtgcagaaggtgatgacac/#4

S1P1: ccgcctcttcctgctaatc / gcagttccagcccatgatag/#3

S1P2: ccactcggcaatgtacctgt / acgcctgccagtagatcg/#61

S1P3: ctcctgatcgggatgtgc / cagtcagggagattgtgcag/#26

S1P4: gcgtctacggcttcatcg / ctggagcagcggtcaaag/#3

S1P5: gagtggctcccagcagtc / gctgaagctcccatcaagg/#27

TRPV4: atgctgagcagcaccaagta / aatgagcatgttgaggagca/#18

PIEZO1: cgtcttcgtggagcagatg / gcccttgacggtgcatac/#41

PTGS1: tccatgttggtggactatgg / cagcaatctggcgagagaa /#81

PTGS2: cctactggaagccaagcact / acagcccttcacgttattgc/#22

PTGES: gatgcacttcctggtcttcc / taggtcacggagcggatg/#60

PTGES2: ctgctacccacgcagagc / ggctccccttacgagctg/#70

PTGES3: atgtatttaccatttactggaatgga / ttcctgtgacattacagcaagc/#6
PTGDS: cacacactgtggatgggact / ctggaagttgggctgcac /#3
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PTGIR: tcttcatccttttccgcaag / acaggcagcagacccaga/#11
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P2Y4: catcacccgcaccatttact / ttcagtactcggcagtcagc/#64
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ICAM2: aggtcatcctgacactgcaa / tgcactcaatggtgaaggac/#76
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