Supplemental material

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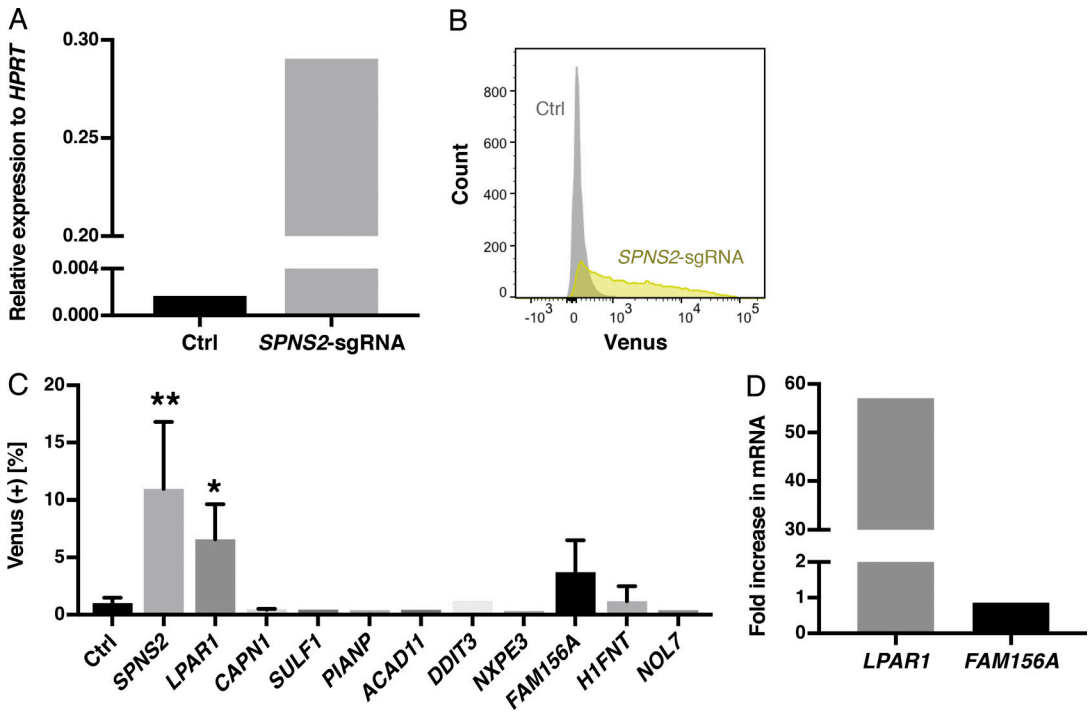

Figure S1. **SPNS2 and LPAR1 SAM sgRNAs activate target genes and Venus expression. (A)** *SPNS2* mRNA level was measured with quantitative PCR in the cells transduced with SAM sgRNA targeting the *SPNS2* gene or empty vector (Ctrl). **(B)** Flow cytometric analysis of Venus-expressing cells after induction of *SPNS2* expression with SAM sgRNA. **(C)** Individual SAM sgRNA of top 10 hits or *SPNS2* was transduced and the number of Venus-positive cells was counted with a flow cytometer. n = 1–9 for each group; data are expressed as mean ± SD. P values were determined by one-way ANOVA followed by Sidak's multiple comparisons test; **, P = 0124; *, P ≤ 0.0001. **(D)** Fold increase of mRNA expression by SAM sgRNA targeting *LPAR1* or *FAM156A* was measured by quantitative PCR. n = 1. Ctrl, control.

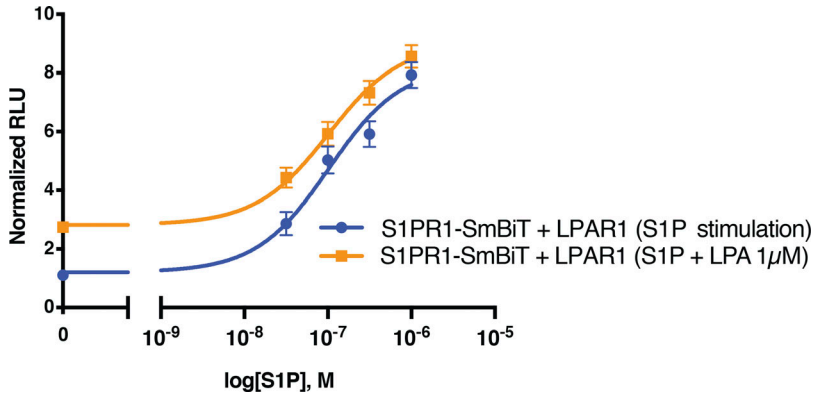

Figure S2. **LPAR1 stimulation with LPA causes an additive effect in S1P-stimulated S1PR1-SmBiT/β-arrestin coupling.** S1PR1-SmBiT and LPAR1 were cotransfected with LgBiT-β-arrestin1 into HEK293 cells, and luminescence was measured ∼15 min after S1P stimulation in the absence (blue line) or presence (orange line) of 1 µM LPA. n = 3 independent experiments in triplicate; data are expressed as mean ± SEM. RLU, relative light units.

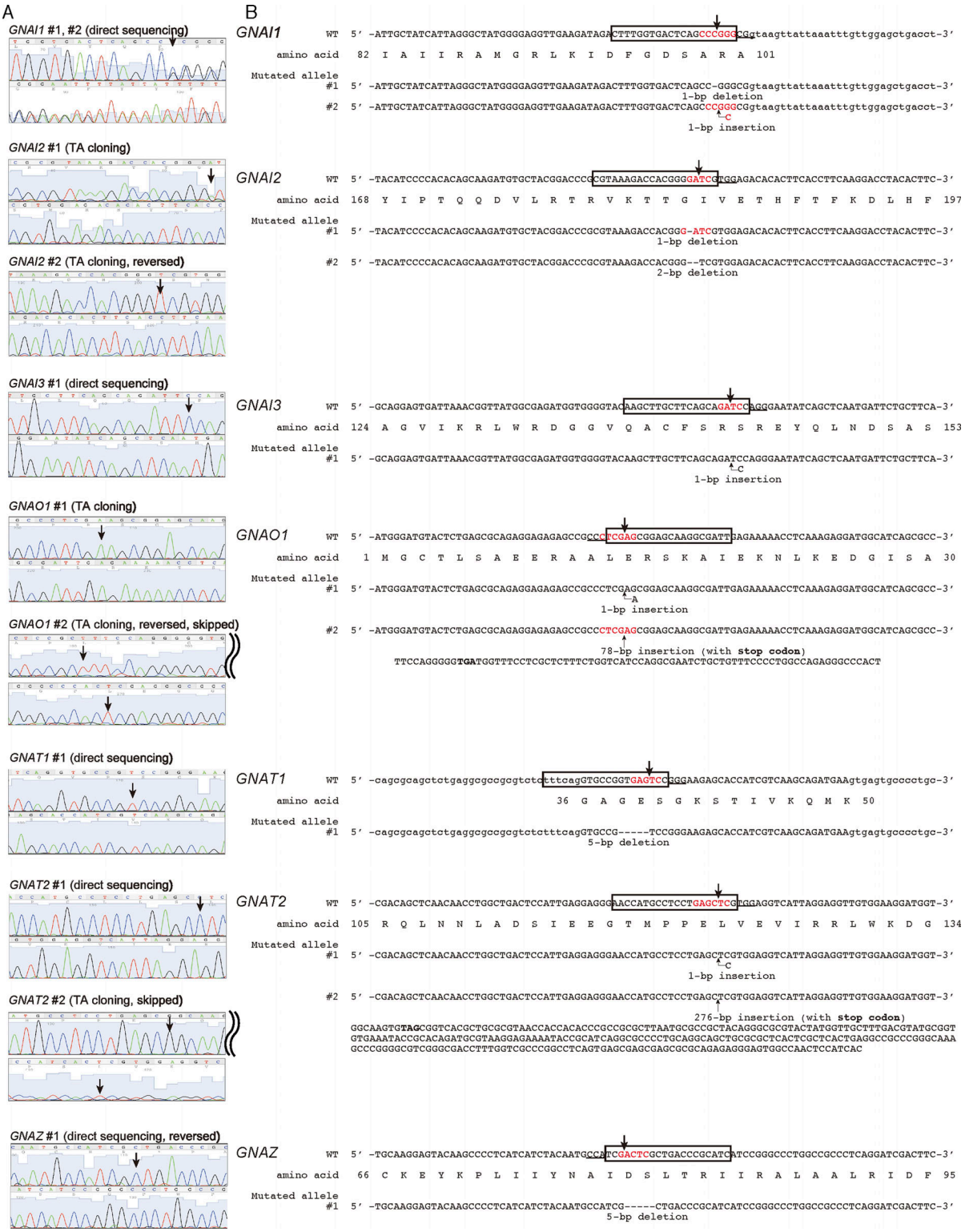
A
B
GNAI1 #1, #2 (direct sequencing)
GNAI2 #1 (TA cloning)
GNAI2 #2 (TA cloning, reversed)
GNAI3 #1 (direct sequencing)
GNAO1 #1 (TA cloning)
GNAO1 #2 (TA cloning, reversed, skipped)
GNAT1 #1 (direct sequencing)
GNAT2 #1 (direct sequencing)
GNAT2 #2 (TA cloning, skipped)
GNAZ #1 (direct sequencing, reversed)
GNAI1
WT 5′ -ATTGCTATCATTAGGGCTATGGGGAGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGgtaagttattaaatttgttggagctgacct-3′
amino acid 82 I A I I R A M G R L K I D F G D S A R A 101
Mutated allele
#1 5′ -ATTGCTATCATTAGGGCTATGGGGAGGTTGAAGATAGACTTTGGTGACTCAGCC-GGGCGgtaagttattaaatttgttggagctgacct-3′
1-bp deletion
#2 5′ -ATTGCTATCATTAGGGCTATGGGGAGGTTGAAGATAGACTTTGGTGACTCAGCCCGGGCGgtaagttattaaatttgttggagctgacct-3′
C
1-bp insertion
GNAI2
WT 5′ -TACATCCCCACACAGCAAGATGTGCTACGGACCCGCGTAAAGACCACGGGATCGTGGAGACACACTTCACCTTCAAGGACCTACACTTC-3′
amino acid 168 Y I P T Q Q D V L R T R V K T T G I V E T H F T F K D L H F 197
Mutated allele
#1 5′ -TACATCCCCACACAGCAAGATGTGCTACGGACCCGCGTAAAGACCACGGG-ATCGTGGAGACACACTTCACCTTCAAGGACCTACACTTC-3′
1-bp deletion
#2 5′ -TACATCCCCACACAGCAAGATGTGCTACGGACCCGCGTAAAGACCACGGG--TCGTGGAGACACACTTCACCTTCAAGGACCTACACTTC-3′
2-bp deletion
GNAI3
WT 5′ -GCAGGAGTGATTAAACGGTTATGGCGAGATGGTGGGGTACAAGCTTGCTTCAGCAGATCCAGGGAATATCAGCTCAATGATTCTGCTTCA-3′
amino acid 124 A G V I K R L W R D G G V Q A C F S R S R E Y Q L N D S A S 153
Mutated allele
#1 5′ -GCAGGAGTGATTAAACGGTTATGGCGAGATGGTGGGGTACAAGCTTGCTTCAGCAGATCCAGGGAATATCAGCTCAATGATTCTGCTTCA-3′
C
1-bp insertion
GNAO1
WT 5′ -ATGGGATGTACTCTGAGCGCAGAGGAGAGAGCCGCCCTCGAGCGGAGCAAGGCGATTGAGAAAAACCTCAAAGAGGATGGCATCAGCGCC-3′
amino acid 1 M G C T L S A E E R A A L E R S K A I E K N L K E D G I S A 30
Mutated allele
#1 5′ -ATGGGATGTACTCTGAGCGCAGAGGAGAGAGCCGCCCTCGAGCGGAGCAAGGCGATTGAGAAAAACCTCAAAGAGGATGGCATCAGCGCC-3′
A
1-bp insertion
#2 5′ -ATGGGATGTACTCTGAGCGCAGAGGAGAGAGCCGCCCTCGAGCGGAGCAAGGCGATTGAGAAAAACCTCAAAGAGGATGGCATCAGCGCC-3′
78-bp insertion (with stop codon)
TTCCAGGGGGTGATGGTTTCCTCGCTCTTTCTGGTCATCCAGGCGAATCTGCTGTTTCCCCTGGCCAGAGGGCCCACT
GNAT1
WT 5′ -cagcgcagctctgaggcgccgcgtctctttcagGTGCCGGTGAGTCCGGGAAGAGCACCATCGTCAAGCAGATGAAgtgagtgcccctgc-3′
amino acid 36 G A G E S G K S T I V K Q M K 50
Mutated allele
#1 5′ -cagcgcagctctgaggcgccgcgtctctttcagGTGCCG-----TCCGGGAAGAGCACCATCGTCAAGCAGATGAAgtgagtgcccctgc-3′
5-bp deletion
GNAT2
WT 5′ -CGACAGCTCAACAACCTGGCTGACTCCATTGAGGAGGGAACCATGCCTCCTGAGCTCGTGGAGGTCATTAGGAGGTTGTGGAAGGATGGT-3′
amino acid 105 R Q L N N L A D S I E E G T M P P E L V E V I R R L W K D G 134
Mutated allele
#1 5′ -CGACAGCTCAACAACCTGGCTGACTCCATTGAGGAGGGAACCATGCCTCCTGAGCTCGTGGAGGTCATTAGGAGGTTGTGGAAGGATGGT-3′
C
1-bp insertion
#2 5′ -CGACAGCTCAACAACCTGGCTGACTCCATTGAGGAGGGAACCATGCCTCCTGAGCTCGTGGAGGTCATTAGGAGGTTGTGGAAGGATGGT-3′
276-bp insertion (with stop codon)
GGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAATGCGCCGCTACAGGGCGCGTACTATGGTTGCTTTGACGTATGCGGT
GTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCCCTGCAGGCAGCTGCGCGCTCACTCGCTCACTGAGGCCGCCCGGGCAAA
GCCCGGGGCGTCGGGCGACCTTTGGTCGCCCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAACTCCATCAC
GNAZ
WT 5′ -TGCAAGGAGTACAAGCCCCTCATCATCTACAATGCCATCGACTCGCTGACCCGCATCATCCGGGCCCTGGCCGCCCTCAGGATCGACTTC-3′
amino acid 66 C K E Y K P L I I Y N A I D S L T R I I R A L A A L R I D F 95
Mutated allele
#1 5′ -TGCAAGGAGTACAAGCCCCTCATCATCTACAATGCCATCG-----CTGACCCGCATCATCCGGGCCCTGGCCGCCCTCAGGATCGACTTC-3′
5-bp deletion

Figure S3. Continued

Figure S3. **DNA sequences of the Gα genomic loci in mutant HEK293 cells. (A)** Genomic DNA sequences near the sgRNA-target sites were analyzed by a direct sequencing method or TA-cloning method. Arrows indicate positions of sequences that mismatch those of the wild-type allele. **(B)** Nucleotide sequences of mutant clones. sgRNA target sequences are boxed, and PAM sequences (NGG) are underlined. Arrows indicate putative double-stranded break sites. Restriction enzyme sites are shown in red letters. Sequences in capital and lower letters indicate exons and introns, respectively. Note that all mutant alleles carried frameshift mutations or harbor a premature stop codon within the insertional sequences. **(C–I)** Alignment of deduced amino acids of the G-protein α subunit mutant HEK293 cells. **(C)** Gαi1 subunit encoded by the *GNAI1* gene. **(D)** Gαi2 subunit encoded by the *GNAI2* gene. **(E)** Gαi3 subunit encoded by the *GNAI3* gene. **(F)** Gαo subunit encoded by the *GNAO1* gene. **(G)** Gαt1 subunit encoded by the *GNAT1* gene. **(H)** Gαt2 subunit encoded by the *GNAT2* gene. **(I)** Gαz subunit encoded by the *GNAZ* gene. Note that all alleles were introduced by a frameshift mutation or an insertional sequence carrying a premature stop codon and did not produce functional Gα subunits owing to lack of the C-terminal residues critical for interaction with GPCRs.

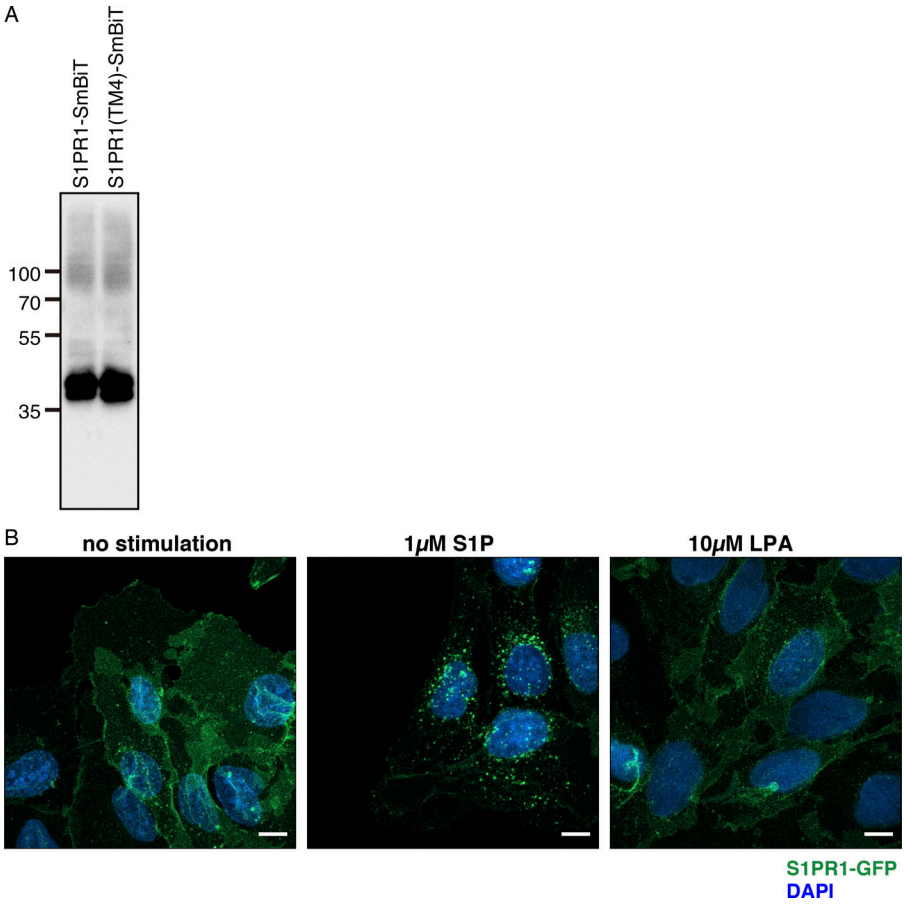

Figure S4. **S1PR1 protein expression and localization. (A)** 1 d after transfection of S1PR1-SmBiT or S1PR1(TM4)-SmBiT plasmid into HEK293A cells, the cells were harvested, and Western blotting was performed using anti-S1PR1 antibody. **(B)** S1PR1 polypeptide is not endocytosed after LPA stimulation. U2OS cells expressing S1PR1-GFP (green) and LPAR1 were stimulated with 1 µM S1P or 10 µM LPA for 60 min after 3-h starvation with 0.1% BSA. Nuclei were stained with DAPI (blue). Bars, 10 µm.

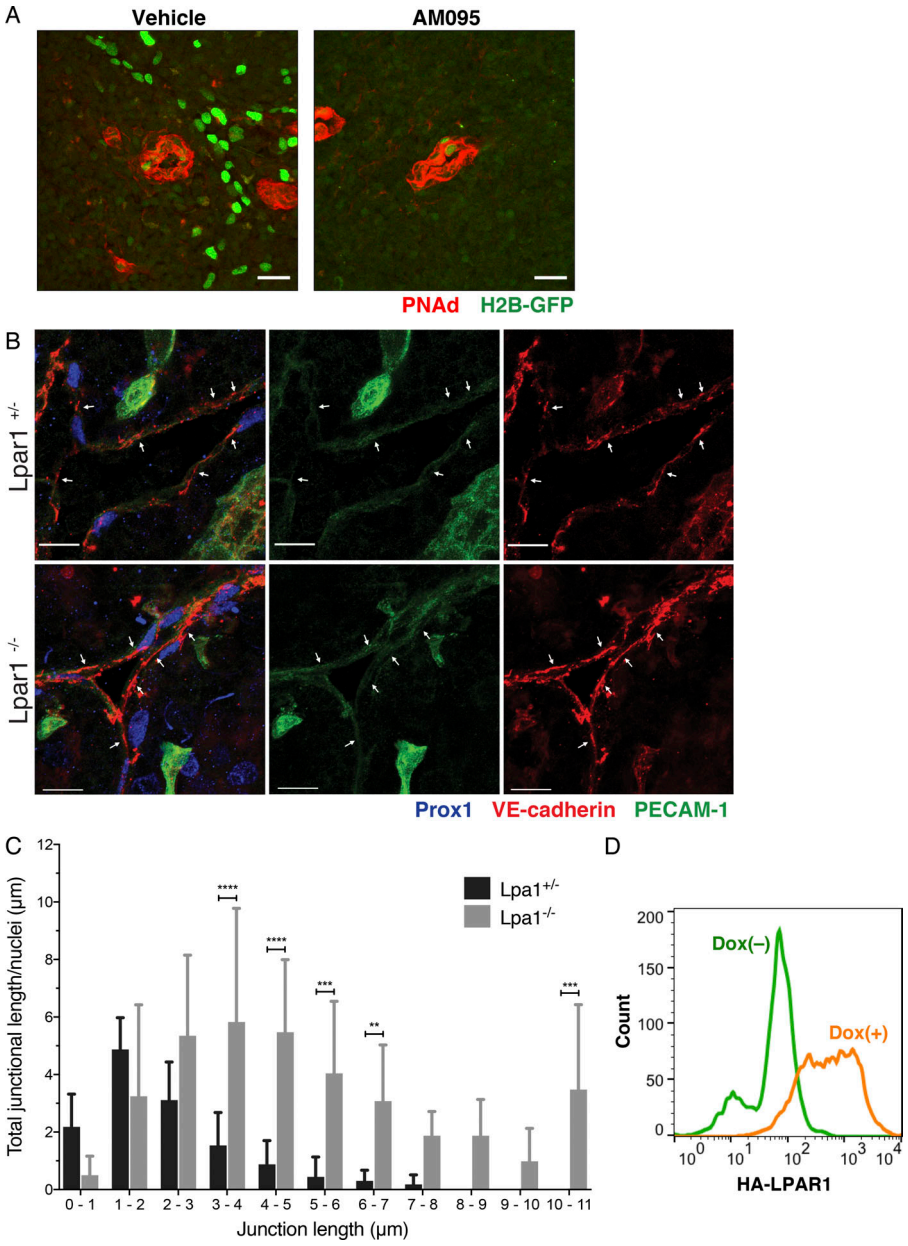

Figure S5. **LPAR1-mediated S1PR1/β-arrestin coupling regulates LEC junctions. (A)** S1PR1/β-arrestin coupling in HEV. Brachial lymph node sections from S1PR1-GFP signaling mice injected with vehicle or AM095 were stained with peripheral node addressin (PNAd; red, HEV). Scale bars, 20 µm. **(B)** 35-µm lymph node sections from *Lpar1*$^{+/-}$ or *Lpar1*$^{-/-}$ mice were stained with Prox1 (blue, lymphatic endothelial nucleus), VE-cadherin (red), and PECAM-1 (green). Arrows indicate VE-cadherin–positive adherens junctions. Bars, 10 µm. **(C)** Quantification of junctional length in confocal images. *n* = 10–12 images each from two mice of each cohort; data are expressed as mean ± SD. P values were determined by Sidak's multiple comparisons test; **, P ≤ 0.0021; ***, P ≤ 0.0002; ****, P ≤ 0.0001. **(D)** The Tet-On system induced LPAR1 expression in HUVECs. Flow cytometric analysis of HA-LPAR1 on HUVEC/pLVX-TetOn-HA-LPAR1 with (orange line) or without (green line) doxycycline (Dox) using anti-HA antibody.