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Supplementary Information for:

Mice Expressing Fluorescent PAR_2 Reveal That Endocytosis Mediates Colonic Inflammation and Pain

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SI Materials and Methods

Animals. Male C57BL/6J (#00064 Jax®, wild-type), *$Par_2^{-/-}$* (#0004993 Jax®), *$Par_2Na_v1.8$* (1) and *Par_2-mugfp* mice (8-10 weeks) were maintained in a light (12-h light/dark cycle) and temperature (22°C) controlled environment with free access to food and water. The New York University Institutional Animal Care and Use Committee approved all studies.

***Par_2-mugfp* mice.** Knockin mice were generated in which muGFP was fused to the C-terminus of mouse PAR_2. A 7.9 Kb segment of genomic DNA containing exon 2 of the mouse *Par_2* gene (*F2rl1*) was sub-cloned from the C57 mouse genomic BAC clone RP23-143N17 into a high copy plasmid. The *Par_2-mugfp* gene was synthesized by fusing muGFP to the 3' end of exon2 of the *Par_2* gene, removing the stop codon (GeneArt). A PAR_2-muGFP-loxP-PGK-neomycin-loxP cassette was used to create the PAR_2 targeting construct. The targeting construct comprised *Par_2-mugfp*, a phosphoglycerine kinase neomycin cassette flanked by loxP sites (floxed PGK neomycin cassette) downstream of the stop codon of *Par_2-mugfp*, and a

downstream KpnI site for southern screening of the ES cells. The targeting vector was linearized with AscI, and the digest was purified using phenol/chloroform and electroporated into Bruce4 (C57BL/6J) ES cells. Clones were selected in G418 (150 µg/ml) for 7 days. On day 8, 400 clones were screened by Southern blotting. Extracted DNA was digested with KpnI overnight. Screening with 3' and 5' probes identified 5 positive clones (1.25% recombination). The 5 clones were expanded, digested with KpnI and AseI, and analyzed by Southern blotting both at 3' and 5' arm of homology for reconfirmation of correct targeting. The clones were also digested with BamHI and analyzed using an internal probe annealing on the NEO cassette, which excluded random integration or insertion of a concatemer. Clones were sequenced to verify the presence and the integrity of LoxP sites and insertion of *Par*$_2$*-mugfp*. Two clones were injected into BalbC mouse blastocysts. Chimeras (black and white) were generated from both clones and were bred with wild-type C57/BL6J female mice to generate black F1 progeny, indicating germ line transmission. Expression of *Par*$_2$*-mugfp* was confirmed by Southern blotting and PCR.

qRT-PCR. RNA was isolated from snap-frozen tissues or cells using Direct-zol RNA Kit (#R2050, Zymo Research). cDNA was synthetized from 50 ng of DNase-treated RNA using a High-Capacity cDNA Reverse Transcriptase Kit (#4374966, Applied Biosystems). cDNA (50 or 100 ng) was amplified for 40 cycles by qRT-PCR using the QuantStudio 3 Real-Time PCR System and TaqMan Fast Advanced PCR Mastermix (#4444556, Applied Biosystem). Primers to *F2rl1* (Mn00433160_m1), *β-actin* (Mn02619580_g1), *Dnm1* (Mn00802468_m1), *Dnm2* (Mn00514582_m1), *Dnm3* (Mn00554098_m1), *TNF-α* (Mm00443258_m1), *IL-1β* (Mm00434228_m1), *CXCL1* (Mm04207460_m1) or *Gapdh* (Mn99999915_g1) were used (Table S1). The relative abundance of mRNA was calculated as described (2, 3).

Immunofluorescence. Tissues were fixed (4% paraformaldehyde, PFA in PBS, 2 h, 4°C), cryoprotected (30% sucrose, PBS, 48 h, 4°C), and embedded in tissue freezing medium (TFM, #TFM-5, General Data). Frozen sections (8 µm) were prepared. Sections were blocked in 10% normal donkey serum (NDS), 0.05% Triton X-100 in PBS (1 h, room temperature, RT). Sections were incubated with rabbit anti-GFP (1:400, overnight, 4°C; #600-401-215L, Rockland Immunochemicals). Slides were washed and incubated with donkey anti-rabbit Alexa Fluor® 488 (1:1000, 45 min, RT; Invitrogen). To detect early or late endosomes, sections were incubated with rabbit anti-EEA1 (1:300; #AB2900, Abcam) or rabbit anti-Rab7a (1:600; #AB137029, Abcam), respectively, and GFP Booster Alexa Fluor® 488 (1:400; #gb2AF488, Chromotek) (overnight, 4°C). Slides were washed and incubated with donkey anti-rabbit Alexa Fluor® 647 (1:1000, 45 min, RT; #A21206, Invitrogen). To identify the plasma membrane, slides were incubated with goat anti-human and -mouse E-cadherin (1:200, overnight, 4°C; #AF648, R&D Systems). Slides were washed and incubated with donkey anti-goat Alexa Fluor® 568 (1:1000, 45 min, RT; #A11057, Invitrogen). Slides were incubated with DAPI (1 µg/ml, 5 min) and mounted in ProLong® Gold Antifade (Thermo Fisher). Sections were observed using a Leica SP8 confocal microscope with HCX PL APO 40x (NA 1.30) or 63x (NA 1.40) oil objectives (Leica-Microsystems). Images were processed using Adobe Photoshop and Illustrator.

RNAScope® *in situ* hybridization. The RNAScope® system was used according to the manufacturer's directions for fresh-frozen tissue, except for omission of the initial on-slide fixation step (RNAScope® Multiple Fluorescent Reagent Kit, #323146, Advanced Cell Diagnostics). Probes to *F2rl1* (#417541-C1), *Gfp* (409011-C2), *Dnm1* (#446931-C3), *Dnm2* (#451831) and *Dnm3* (#451841-C2) were used (Table S1). Sections were incubated with Opal 620 (#FP1495001KT, Akoya Biosciences) and Opal 520 (#FP1487001KT) reagents (1:1000) for fluorescence detection. To detect neurons, hybridized slides were blocked and incubated with guinea pig anti-NeuN antibody (1:400; #N90, EMD Millipore) or rabbit anti-PGP9.5 antibody (1:500; # Z511601-2, Agilent) (overnight, 4°C). Slides were washed and incubated with goat anti-guinea pig Alexa Fluor® 647 (1:1000, 45 min, RT; #A21450, Invitrogen) or goat anti-rabbit Alexa Fluor® 488 (1:1000, 45 min, RT; #A21206, Invitrogen). Slides were mounted and imaged as described for immunofluorescence.

***Ex-vivo* agonist-induced endocytosis of PAR$_2$-muGFP.** The colon was excised, rinsed and opened along the mesenteric line. The colon was divided into four 1 cm segments that were pinned flat with the mucosa uppermost. Segments were equilibrated in oxygenated Dulbecco's Modified Eagle's Medium (DMEM; 30 min, 37°C). Tissue was incubated with trypsin (140 µM), 2-Furoyl-LIGRLO-NH_2 (2F, 100 µM), or vehicle (60 min, 37°C). Tissue was processed for localization of PAR$_2$-muGFP, EEA1, Rab7a and E-cadherin by immunofluorescence.

Quantification of endocytosis of PAR_2-muGFP. PAR_2-muGFP fluorescence <2 pixels (0.2 μm) of the plasma membrane (identified by E-cadherin immunoreactivity) of colonocytes was defined as plasma membrane-associated receptor. PAR_2-muGFP fluorescence >0.5 pixels (0.5 μm) of the plasma membrane was defined as internalized receptor. The plasma membrane : cytosolic PAR_2-muGFP fluorescence ratio was determined to quantify endocytosis, as we have described (4). A total of 6 images (63X magnification) were analyzed for each mouse (N=5 mice for control and treatment groups; 30 images analyzed per experimental group).

Colitis-induced endocytosis of PAR_2-muGFP. For DSS-induced colitis, mice received 3% DSS (#0216011050, MP Biomedicals) in drinking water for 7 d. Controls received plain water. Weight, stool consistency and signs of rectal bleeding were recorded daily. Body weight loss (0, none; 1, 1-5%; 2, 5-10%; 3, 10-20%; 4, >20%), stool consistency (0, normal; 2, loose stools; 4, diarrhea), and stool blood (0, negative; 2, positive hemoccult test; 4, gross bleeding) were used as parameters for the Disease Activity Index (DAI). After 24 h recovery, the colon was removed, the length was measured, and tissues were processed for localization of PAR_2-muGFP and EEA1. For TNBS-induced colitis, mice received a single intracolonic injection of 150 μl TNBS (4 mg/ml in 0.9% NaCl, 50% ethanol; #P2297, Millipore Sigma) or vehicle (0.9% NaCl/ 50% ethanol), *via* a polyethylene catheter inserted 3 cm from the anus under isoflurane (5%) anesthesia. The colon was removed at 72 h post-TNBS and was processed for hematoxylin and eosin staining and for localization of PAR_2-muGFP and EEA1.

PAR_2-evoked colonic and somatic nociception. Investigators were blinded to treatments and genotypes. Mice were acclimatized to the room, apparatus and investigator for 2 h per day for 2 days before study. To assess colonic pain, 2F (100 μM) or vehicle (0.9% NaCl, control) was injected into the colon of anesthetized mice (5% isoflurane) by enema (150 μl, 3 cm from anus). To assess colonic nociception, the abdominal area was divided into 9 equal quadrants; von Frey filaments of increasing force were applied to the central quadrant, which corresponds to the area of the colon (5). Responses to von Frey filament were arching of the back, jumping and raising the rear legs. Responses were measured hourly for 6 h. Results are expressed as percent baseline. To assess somatic pain, trypsin (140 nM) or vehicle was injected subcutaneously into the plantar surface of the left hind paw (10 μl) of anesthetized mice. Nociception was assessed by measuring withdrawal thresholds to stimulation of the plantar surfaces of the ipsilateral (injected) and contralateral (non-injected) hindpaws with calibrated von Frey filaments (1). Responses were measured hourly for 4 h. Results are expressed as percent baseline.

***In vivo* shRNA knock-down of *Dnm2*.** Mouse *Dnm2* cDNA clone no. TL513405 (locus ID 13430) and non-effective 29-mer scrambled shRNA cassette in pGFP-C-shLenti Vector were from OriGene (Table S1). The *Dnm2*-knock-down construct was mixed with polyethyleneimine-based transfection reagent (*in vivo*-jetPEI®, 201-50G; Polyplus) in a 7:1 N:P ratio (polyethyleneimine nitrogen to DNA phosphate ratio) (6). Food was withheld overnight from *Par_2-mugfp* mice. The next morning, scrambled control cDNA or *Dmn2* cDNA *in vivo*-jetPEI® mixture was injected into the colon of anesthetized mice (5% isoflurane) by enema (100 μg, 150 μl, 3 cm from anus). Scrambled control cDNA or *Dnm2* cDNA was administered again the same evening to increase transfection efficiency. After 48 h (0 h), 2F (100 μM) was injected into the colon by enema (150 μl, 3 cm from anus). Colonic nociception was measured from 0-6 h post-2F, and PAR_2-evoked colonic inflammation was assessed collecting the terminal colon (3 cm from anus) to measure cytokine expression. *Dnm2* was localized in the colon by RNAScope® and PAR_2-muGFP was localized by immunofluorescence.

Quantification of *Dnm2* knockdown. *Dnm2* was localized in colon sections by RNAScope®. Confocal images were analyzed using Fiji ImageJ (NIH) according to ACD Bio-Techne Technical Note. Regions of interest were defined by applying a threshold with the moments setting (Min & Max) and analyzing particles with size range from 0 to infinity. Regions of interest were overlaid on the original micrograph and the number of dots per area and the number of particles were quantified. Results are expressed as dots/mm^2 tissue. A total of 6 images (63X magnification) were analyzed for each mouse (N=5 mice for control and treatment groups; 30 images analyzed per experimental group).

Cell lines. T84 cells (ATCC® CCL-248™) were cultured in DMEM F-12 Medium (ATCC, #30-2006) supplemented with heat inactivated fetal bovine serum (FBS, 5%), penicillin (100 U/ml) and streptomycin

(100 mg/ml). Human kidney epithelial HEK293T cells (#CRL-3216™, American Type Culture Collection) and Kirsten murine sarcoma virus-transformed rat kidney epithelial cells (KNRK) were cultured in DMEM supplemented with FBS (10%), L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were maintained at 37 °C in 5% CO_2 and 95% O_2.

PAR_2-muGFP signaling. KNRK were transiently transfected with cDNA encoding mouse PAR_2 fused to C-terminal muGFP, human PAR_2 fused to N-terminal Flag and C-terminal HA11, or muGFP alone using polyethylenimine (PEI, Polysciences; 1:6 DNA:PEI). After 24 h, cells were plated in poly-D-lysine-coated 96-well plates. After 48 h, cells were incubated in Hanks Balanced Salt Solution (HBSS) with graded concentrations of trypsin or 2F for 40 min (5% CO_2, 37°C). Inositol monophosphate (IP-1) was measured using an IP-One HTRF® assay kit (#40451 Cisbio, PerkinElmer).

PAR_2-muGFP trafficking. HEK293T cells in a 10 cm dish were transfected with PAR_2-muGFP (5 µg) using PEI. Cells were incubated overnight and were then plated (6 x10^5 cells) on 12 mm glass coverslips pre-coated with poly-D-lysine in 24-well plates. Cells were transduced with CellLight fluorescent protein markers (3 µl/well) to identify early endosomes, late endosomes or the Golgi apparatus. After 24 h, cells were incubated in HBSS with 2F (10 µM) or vehicle (control) for 30 min (37°C). Cells were fixed (4% PFA, 20 min, 4°C), washed in PBS and mounted on glass slides with ProLong Glass antifade mountant (ThermoFisher). Cells were imaged using a SP8 confocal microscope with HCX PL APO 63x (NA1.40) oil objective (Leica-Microsystems). Images were processed using ImageJ (NIH) and Adobe Illustrator.

Trafficking of PAR_2, βARR and $G\alpha_q$. HEK293T cells stably expressing human Flag-PAR_2-HA were transfected with YFP-βARR1 (2.5 µg) plus YFP-βARR2 (2.5 µg) using PEI and were plated as described above. Cells were incubated with 2F (10 µM) or vehicle (30 min) and were fixed. Cells were blocked in PBS containing 5% normal horse serum (NHS) and 0.2% Triton X-100 (1 h, RT). Cells were incubated with rat anti-HA (1:1000; #12158167001, Roche), mouse anti-$G\alpha_q$ (1:500; #MABS1135, Millipore) and rabbit anti-EEA1 (1:500; #ab2900, Abcam) in PBS containing 3% NHS and 0.2% Triton X-100 (overnight, 4°C). Slides were washed and incubated with donkey anti-rat Alexa Fluor® 568, donkey anti-mouse Alexa Fluor® 405 and donkey anti-rabbit Alexa Fluor® 647 (1:1,000, Thermo Fisher Scientific; 1 h, RT). Coverslips were mounted and imaged as described above.

BRET cDNAs. Mini (m) Gα proteins coupled to Venus were from N. A. Lambert (Augusta University). For ebBRET studies, constructs were modified to replace Venus with *Renilla*® luciferase 8 (Rluc8). RGFP-CAAX (prenylation CAAX box of KRas), tdRGFP-Rab5a and Rluc2-βARR2 were from M. Bouvier (Université de Montréal). For nbBRET studies, PAR_2-NP was cloned using Gibson Assembly with cDNA encoding human PAR_2 with an N-terminal POMC signal sequence and Flag tag in a pcDNA5/FRT vector. The 13 amino acid natural peptide fragment of nanoluciferase (GVTGWRLCERILA, NP (7)) was appended to the C-terminus of PAR_2 *via* a flexible linker (LRPLGSSGGG). Localization markers CAAX or FYVE tagged on the N-terminus with HA, a short linker (GGSG) and the LgBiT tag were from A. Thomsen (New York University).

BRET transfections. For ebBRET assays of PAR_2 translocation from the plasma membrane to early endosomes, T84 cells were transfected using Lipofectamine 3000 (Thermo Fisher) with human PAR_2-Rluc8 (100 ng/well, 96-well plate) and RGFP-CAAX (150 ng) for plasma membrane localization or tdRGFP-Rab5a (150 ng) for early endosome localization. For BRET1 assays of PAR_2 translocation to different organelles, HEK293T cells were transfected with PEI with human PAR_2-Rluc8 (50 ng) and Venus-KRas (20 ng, plasma membrane), Venus-Rab5a (15 ng, early endosomes), Venus-Rab7a (15 ng, late endosomes), Venus-Giantin (20 ng, cis-Golgi), YFP-TGN38 (20 ng, trans-Golgi) or Venus-Rab11a (20 ng, recycling endosomes). To disrupt Dnm, T84 cells were transfected with DnmK44A (300 ng) or human Dnm2 siRNA (100 nM) or control siRNA (100 nM) and HEK293T cells were transfected with DnmK44A (50 ng). For ebBRET assays of trafficking of mGα proteins to the plasma membrane or early endosomes, T84 cells were transfected using Lipofectamine 3000 with human Flag-PAR_2-HA (100 ng) and Rluc8-$mG\alpha_{si/s/sq}$ (150 ng) (8, 9) and either RGFP-CAAX (150 ng) or tdRGFP-Rab5a (150 ng). For ebBRET assays of recruitment of βARR2 (10) to the plasma membrane or early endosomes, T84 cells were transfected using Lipofectamine 3000 with human Flag-PAR_2-HA (100 ng) and Rluc2-βARR2 (150 ng) and RGFP-CAAX (150 ng) or tdRGFP-Rab5a (150 ng). For equivalent studies in HEK293T cells, cells were transfected using PEI

with human Flag-PAR_2-HA (10 ng) and Rluc8-mG$\alpha_{si/s/sq}$ (10 ng) and either RGFP-CAAX (20 ng) or tdRGFP-Rab5a (15 ng). Alternatively, HEK293T cells were transfected with PEI with human Flag-PAR_2-HA (20 ng) and Rluc2-βARR2 (10 ng) and RGFP-CAAX (20 ng) or tdRGFP-Rab5a (15 ng). For nbBRET studies of signalosome formation, T84 cells were transfected using Lipofectamine 3000 with POMC-Flag-PAR_2-NP (100 ng/well, 96-well plate), HA-LgBiT-CAAX or HA-LgBiT-FYVE (150 ng), and either Venus-mGα_{si}, Venus-mGα_{sq} or YFP-βARR1 (150 ng). Alternatively, HEK293T cells were transfected with PEI with POMC-Flag-PAR_2-NP (10 ng/well, 96-well plate), HA-LgBiT-CAAX or HA-LgBiT-FYVE (10 ng), and either Venus-mGα_{si}, Venus-mGα_{sq}, Venus-mGα_s or YFP-βARR1 (10 ng). To disrupt Dnm, HEK293T cells were transfected with DnmK44A or pcDNA5/FRT negative control (50 ng). Cells were studied 48 h after transfection.

BRET measurements. T84 or HEK293T cells were washed with HBSS containing 10 mM HEPES at pH 7.4. In some experiments, cells were pre-incubated in HBSS containing 0.45 M sucrose or 10 µM GB88 (11) (30 min, 37°C). Prior to BRET measurements, substrate was added for BRET1 (coelenterazine H, 2.5 µM, 5 min), ebBRET (Prolume Purple Coelenterazine, 2.5 µM, 5 min) or nbBRET (furimazine, 10 µM, 15 min). BRET was recorded for up to 25 min in a Synergy Neo2 Microplate reader (BioTek) using BRET1 filters (acceptor filter: 540 ± 25 nm, donor filter: 460 ± 40 nm; for BRET1 and nbBRET) or BRET2 filters (acceptor filter: 515 ± 15 nm, donor filter: 410 ± 40 nm; for ebBRET). Baseline was measured for 2-3 min and cells were then challenged with trypsin (100 nM), 2F (10 or 100 µM) or vehicle. ΔBRET represents the BRET signal in the presence of agonist, minus the BRET signal over time in the presence of vehicle.

Localization of HA-LgBiT markers. HEK293T cells on 12 mm glass coverslips were transfected with HA-LgBiT-CAAX or HA-LgBiT-FYVE (50 ng/well, 24-well plates). After 48 h, cells were fixed (4% PFA, 20 min, 4°C) and blocked in PBS containing 3% normal goat serum and 0.3% saponin (1 h, RT). Cells were incubated with rat anti-HA (1:1000; #11867423001, Roche) (overnight, 4°C). Slides were washed and incubated with goat anti-rat Alexa Fluor® 488 (1:1000, Invitrogen; 1 h, RT). Coverslips were washed, stained with DAPI (5 min, RT), mounted and imaged as described above.

T84 TEER. T84 cells were cultured on 6.5 mm Transwell inserts with 0.4 µm pore size (Corning Incorporated) at a density of 2×10^5 cells/insert. TEER was measured using a dual electrode and an epithelial volt/ohm meter (World Precision Instruments). When the TEER reached ~2000 Ω, trypsin (100 nM; T2600000, Sigma), 2F (10 µM) or vehicle (PBS) was added to the basolateral compartment. Transwells with culture medium only were used as blank controls. TEER was measured in triplicate Transwells after 0 and 5 or 6 h at 37°C. The average value was recorded as the measured TEER. TEER ($\Omega\cdot cm^2$) = (measured TEER–blank control TEER) × area. To assess the role of PAR_2, GB88 (11) (10 µM) or vehicle (0.8% DMSO) was added to the basolateral and apical compartments 30 min before addition of PAR_2 agonists. To evaluate the role of Dnm2, both sides of monolayer were treated with 15 pmol/well of either human Dnm2 siRNA or control siRNA (Dharmacon) and Lipofectamine 3000 overnight before addition of PAR_2 agonists (Table S1).

IL-8 secretion from human colonic mucosa. Non-cancerous regions of human colon, collected from patients undergoing resection for treatment of colon cancer, were obtained from UCLA Surgical Pathology Institutional Review Board approval was waived since no patient-identifiable information was obtained. Samples of mucosa were cut into 3 mm^3 segments and were placed in serum-free RPMI1640 medium with 1% penicillin/streptomycin (15140122, Thermo Fisher) (12). Explants were preincubated with dyngo4a (30 µM) (13) (Dnm inhibitor, ab120689, Abcam) or vehicle (0.8% DMSO) for 30 min at 37°C, and were then incubated with 2F (10 µM) or vehicle (PBS) for 4 h at 37°C. IL-8 was measured in the medium by ELISA (DY208, R&D Systems).

Statistics. Data are presented as mean±SEM. Differences were assessed using Student's t test for two comparisons and 1- or 2-way ANOVA and Tukey's, Dunnett's or Šidák's *post-hoc* test for multiple comparisons. $P<0.05$ was considered significant at the 95% confidence level.

Materials and data availability. All of the data and protocols are in the paper or the supporting appendix.

Supplementary Information Figures

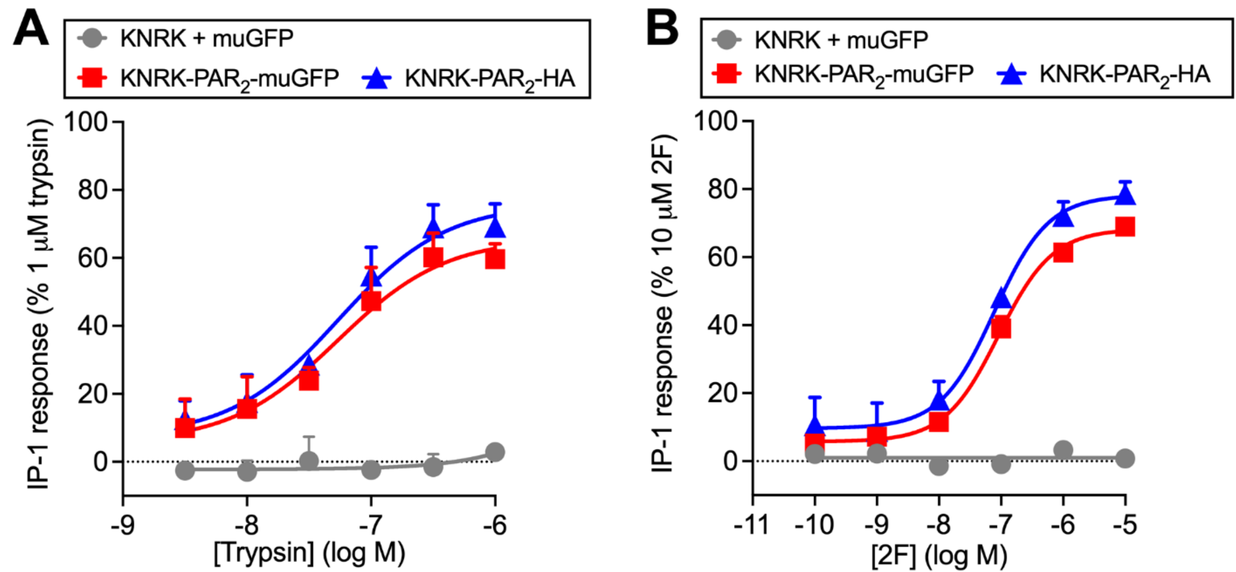


Figure S1. PAR_2-muGFP signaling and trafficking. A, B. Trypsin- (**A**) and 2F- (**B**) stimulated IP-1 formation in KNRK cells transiently expressing PAR_2-muGFP, PAR_2-HA11 or muGFP alone. Mean±SEM. N=3 independent experiments.

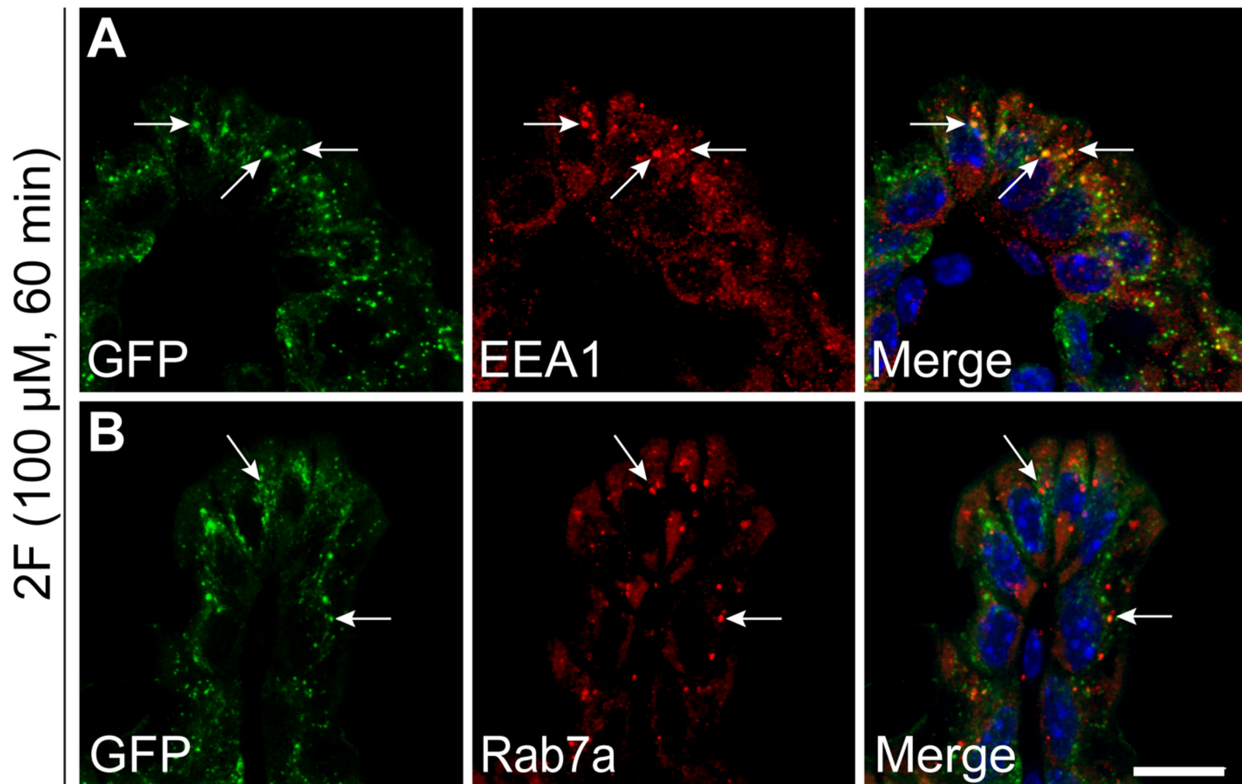


Figure S2. **Agonist-evoked trafficking of PAR_2-muGFP to early and late endosomes of colonocytes**. **A, B.** Localization of GFP immunoreactivity in isolated segments of colon from *Par_2-mugfp* mice incubated with 2F (10 µM, 60 min). **A.** Colocalization (arrows) of GFP (green) and EEA1 (red). **B.** Colocalization (arrows) of GFP and Rab7 (red). Representative images, independent experiments, N=5 mice. Scale bar, 10 µm.

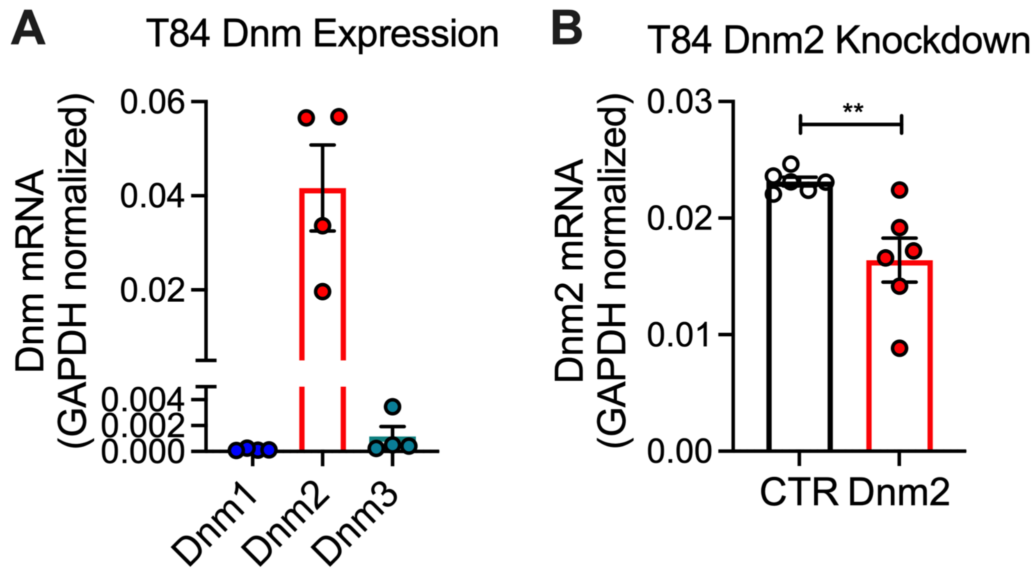


Figure S3. Dnm isoforms in T84 cells. A. Expression of *Dnm1*, *Dnm2* and *Dnm3* mRNA in T84 cells. **B.** siRNA knockdown of *Dnm2* in T84 cells assessed 48 h after treatment with Dnm2 or control (CTR) siRNA. N=4 or 6 experiments. ***P*<0.01, Student's t-test.

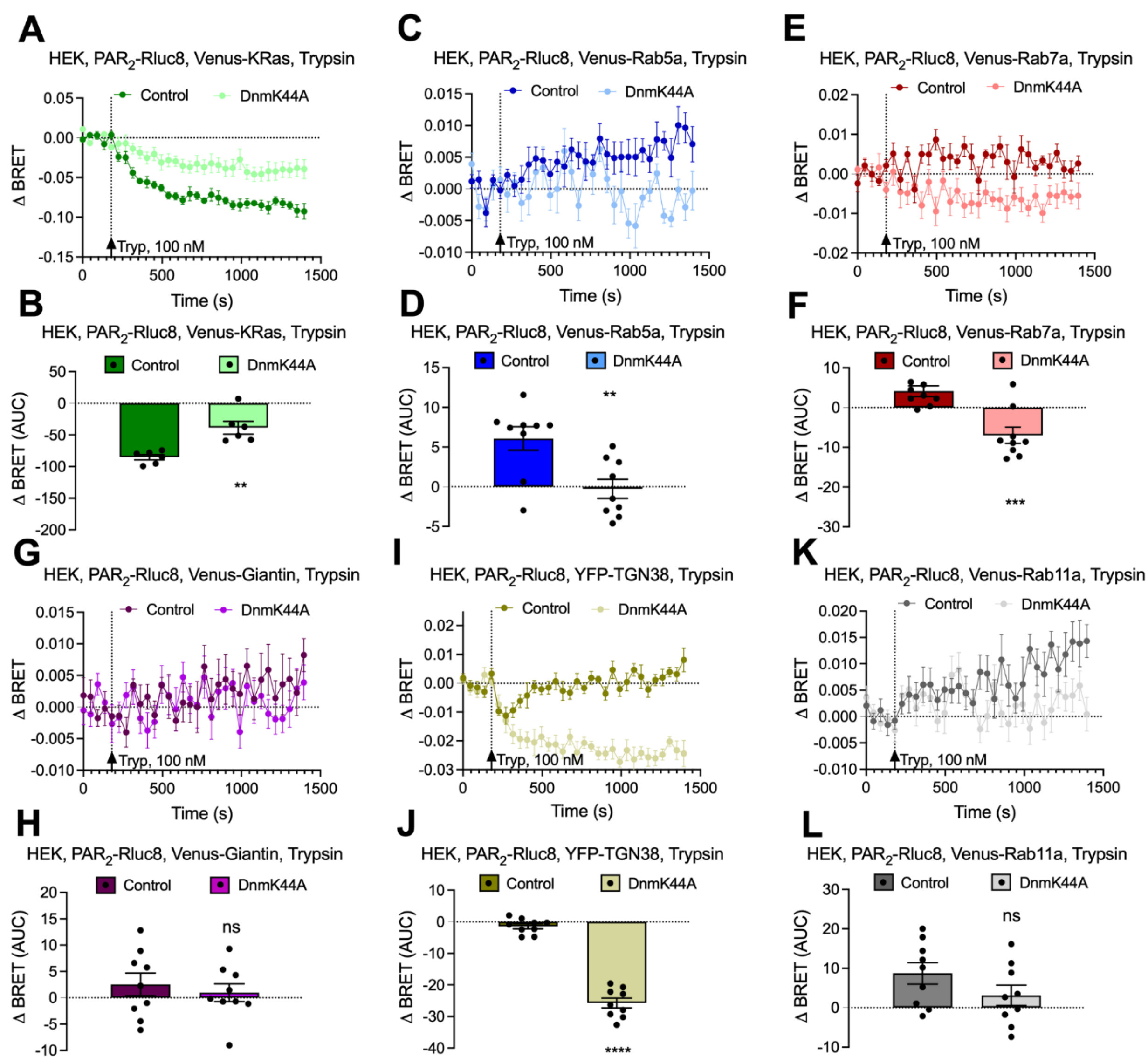


Figure S4. PAR$_2$ trafficking to endosomes. Effects of trypsin (100 nM) on translocation of PAR$_2$-Rluc8 from the plasma membrane (Venus-KRas, **A, B**) to early endosomes (Venus-Rab5a, **C, D**), late endosomes (Venus-Rab7a, **E, F**), cis-Golgi (Venus-Giantin, **G, H**), trans-Golgi (YFP-TGN38, **I, J**) and recycling endosomes (Venus-Rab11, **K, L**) in HEK293T cells. Cells were transfected in the absence (control) or presence of DnmK44A. AUC, area under curve. N=6-11 independent experiments. Mean±SEM. ***P*<0.01, ****P*<0.001, *****P*<0.0001. 1-way ANOVA, Tukey's test.

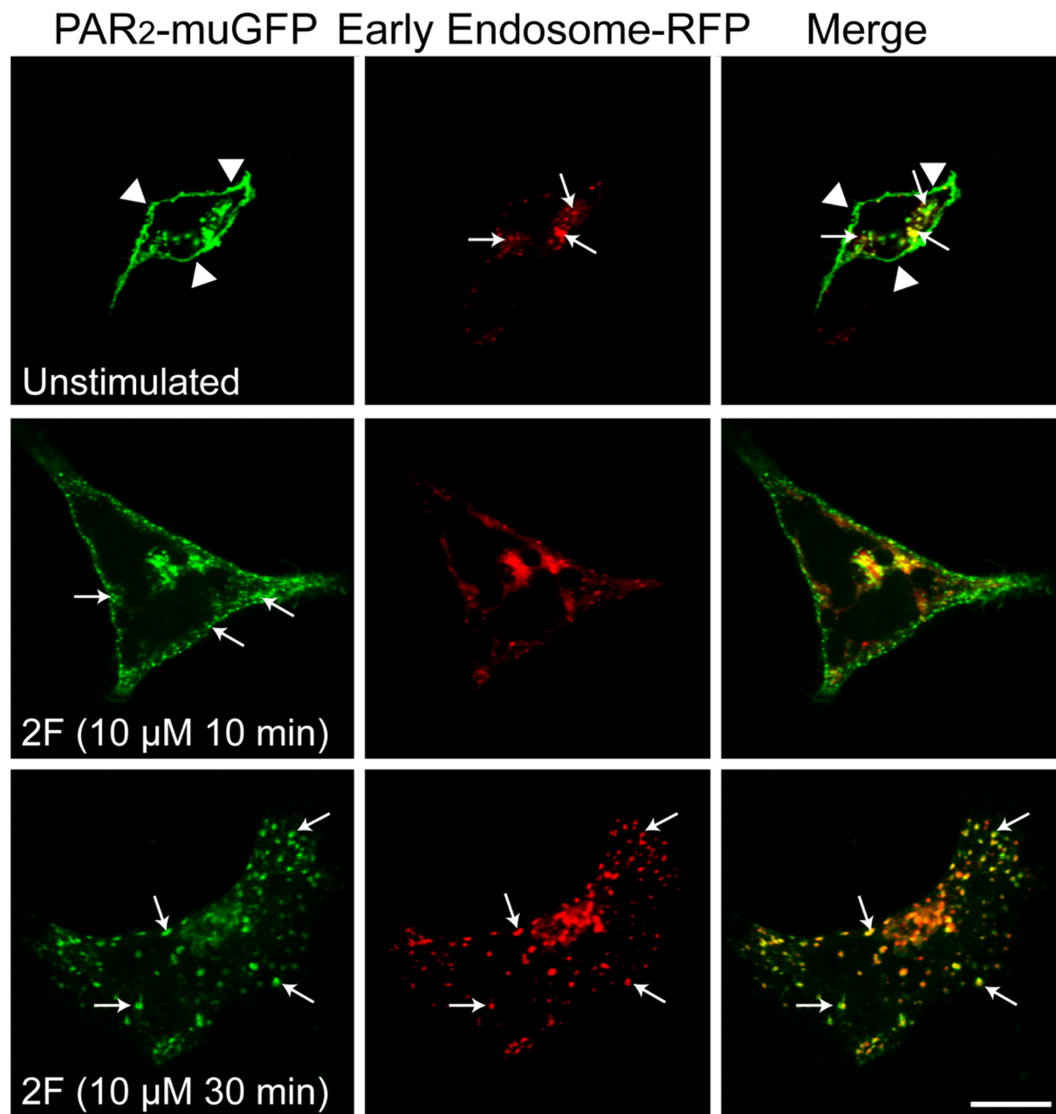
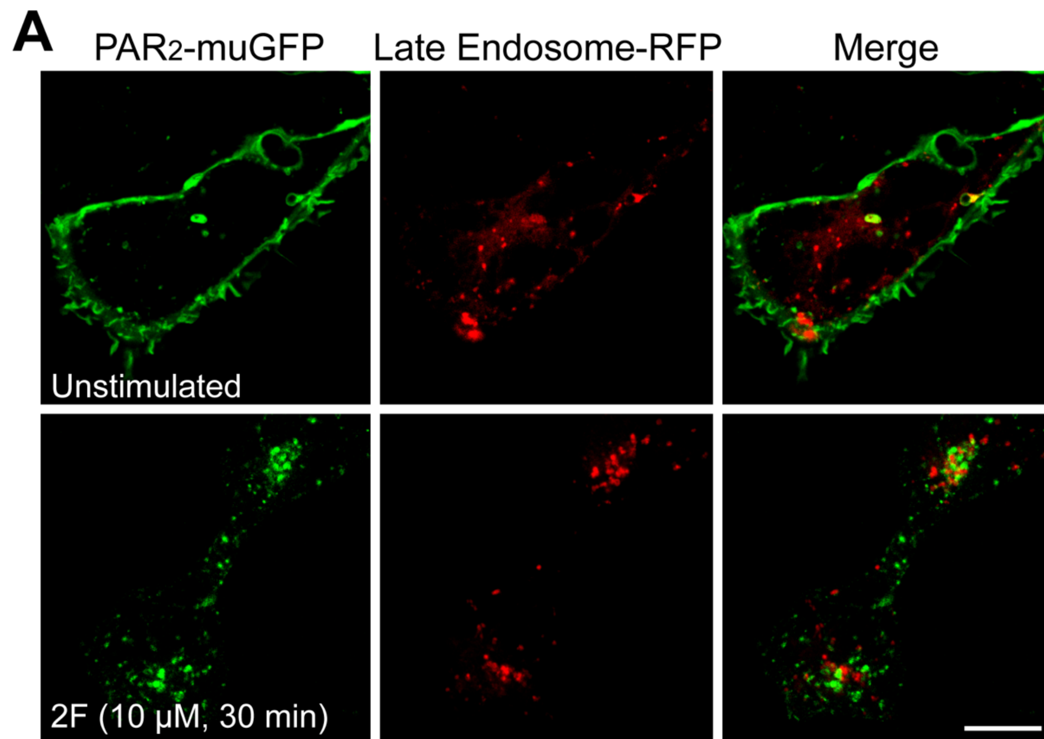


Figure S5. Agonist-evoked trafficking of PAR_2-muGFP in HEK293T cells. HEK293T cells expressing PAR_2-muGFP and CellLight early endosome marker tagged with red fluorescent protein (RFP) were incubated with 2F (10 μM, 10 or 30 min) or vehicle (unstimulated control). Arrow heads, plasma membrane; arrows, colocalization of PAR_2-muGFP and early endosomes. Representative images. N=5 experiments. Scale bar, 10 μm.



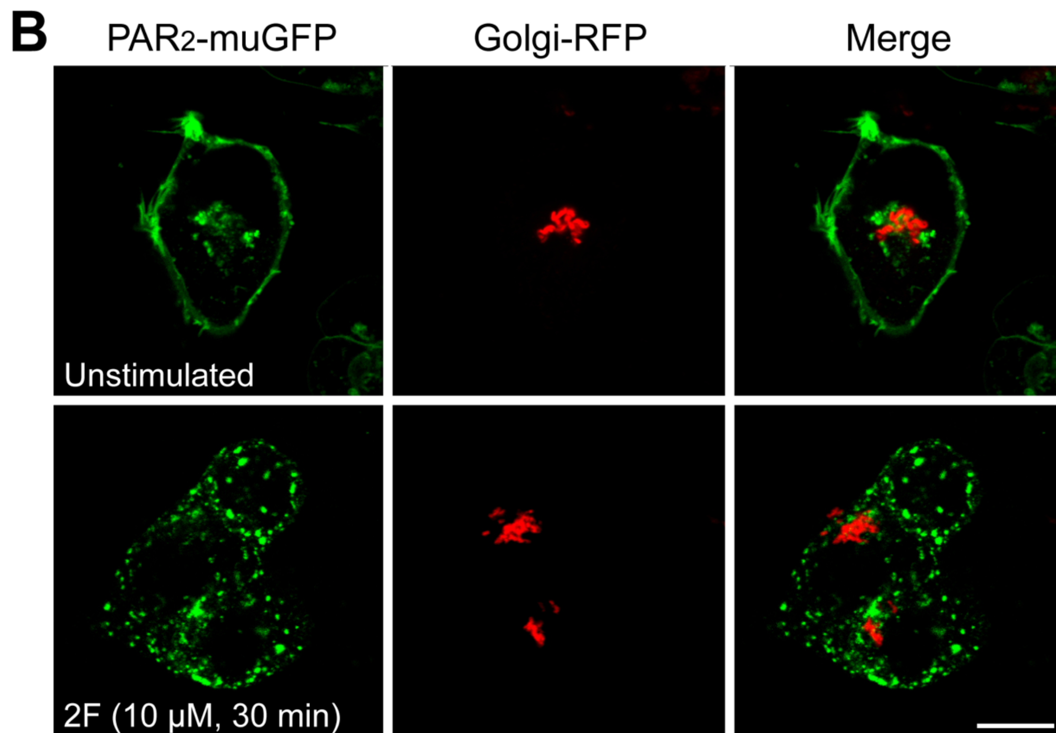


Figure S6. Agonist-evoked trafficking of PAR_2-muGFP in HEK293T cells. HEK293T cells expressing PAR_2-muGFP and CellLight late endosome (**A**) or Golgi apparatus (**B**) markers tagged with red fluorescent protein (RFP) were incubated with 2F (10 μM, 30 min) or vehicle (unstimulated control). Representative images. N=5 experiments. Scale bar, 10 μm.

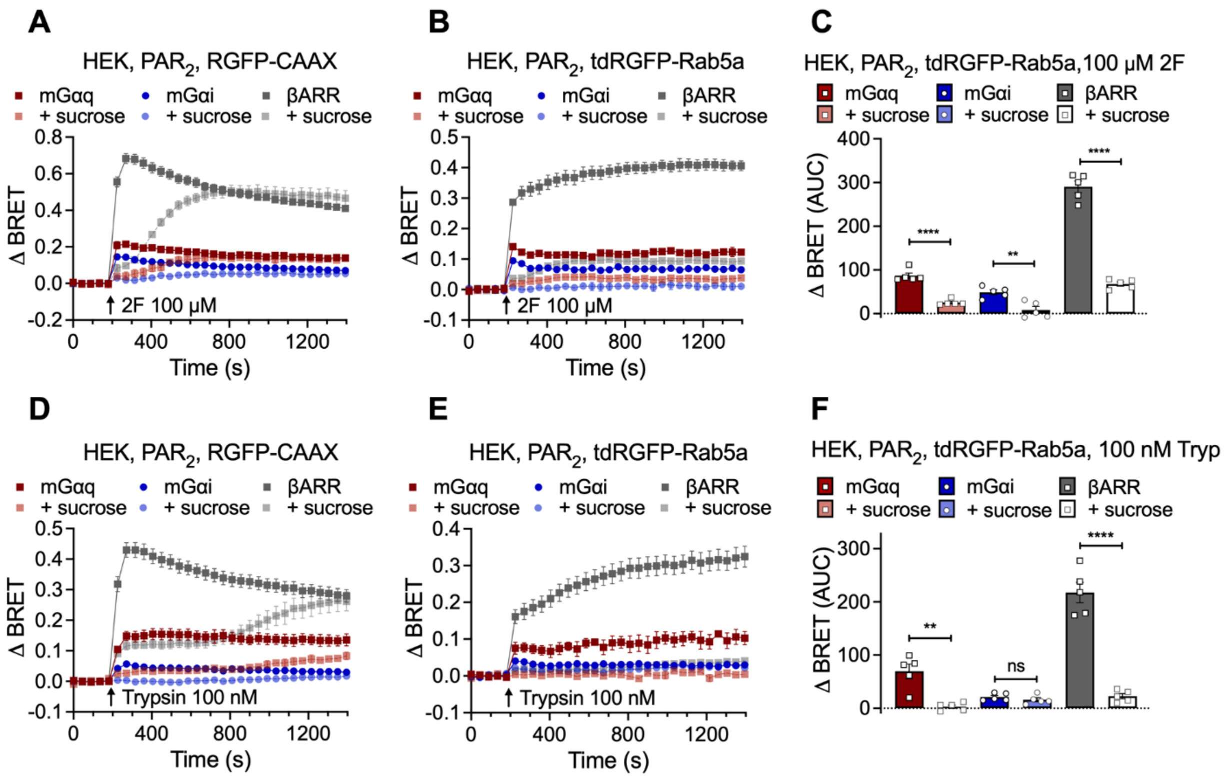


Figure S7. Effects of hypertonic sucrose on ebBRET assays of signalosome assembly. A-C. Effects of 2F (100 μM) on recruitment of mGα_{sq}, mGα_{si} or βARR2 to the plasma membrane (**A**, RGFP-CAAX) or early endosomes (**B, C**, tdRGFP-Rab5a) of HEK293T cells. **D-F.** Effects of trypsin (100 nM) on recruitment of mGα_{sq}, mGα_{si} or βARR2 to the plasma membrane (**D**) or early endosomes (**E, F**) of HEK293T cells. Cells were pre-incubated with vehicle or 0.45 M sucrose (30 min). AUC, area under curve. Mean±SEM. N=5 independent experiments. **P<0.01, ****P<0.0001, ns not significant. **C, F.** Student's t-test.

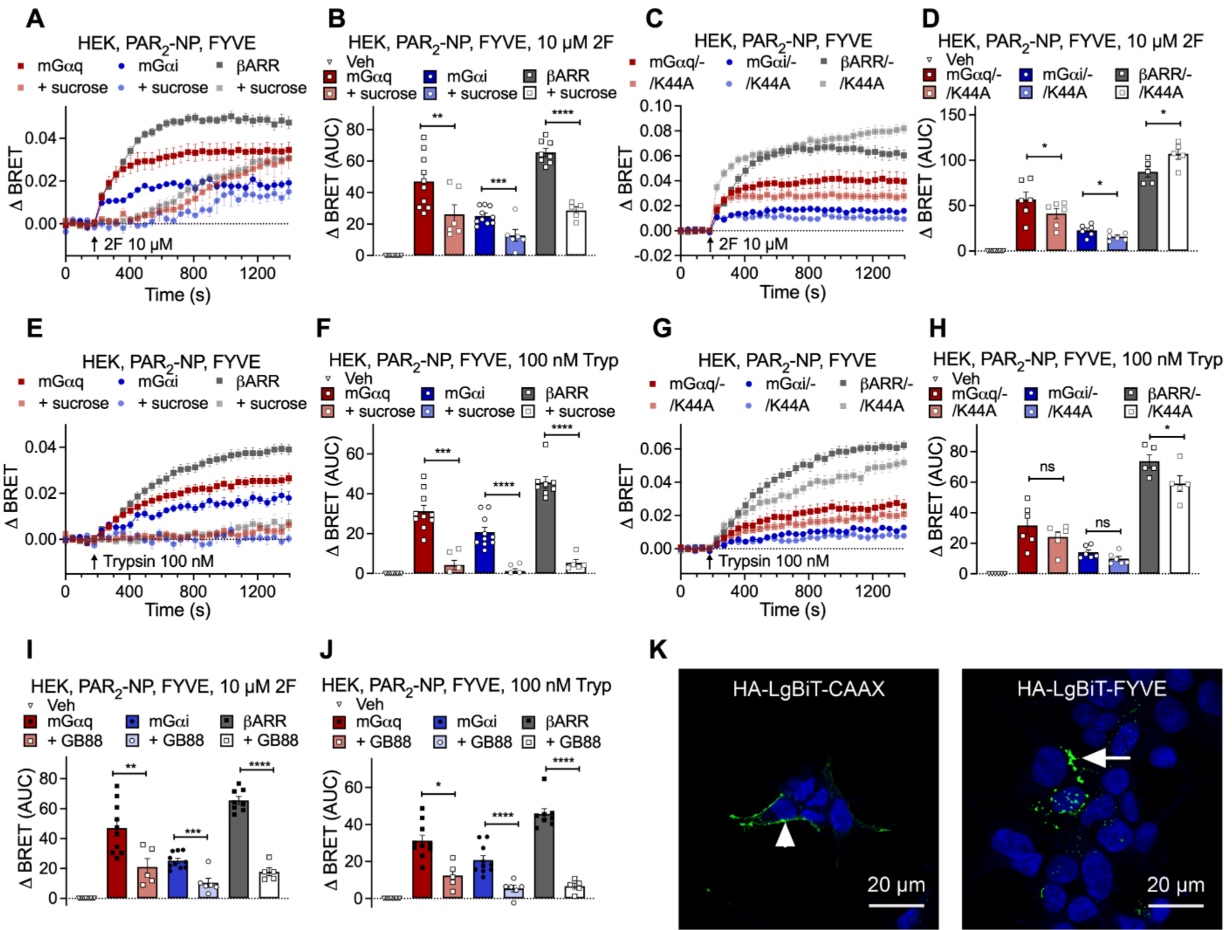


Figure S8. Effects of endocytic inhibitors or PAR_2 antagonist on nbBRET assays of signalosome assembly. A-H. Effects of 2F (**A-D**, 10 μM) or trypsin (**E-H**, 100 nM) on recruitment of $mG\alpha_{sq}$, $mG\alpha_{si}$ or βARR1 to early endosomes (LgBiT-FYVE) in HEK293T cells. Cells were pre-incubated with 0.45 M sucrose (**A-B, E-F**) or were transfected with dominant negative dynamin K44A (**C-D, G-H**). **I-J.** Effects of 2F (**I**, 10 μM) or trypsin (**J**, 100 nM) on recruitment of $mG\alpha_{sq}$, $mG\alpha_{si}$ or βARR1 to the early endosome (LgBiT-FYVE) in HEK293T cells. Cells were preincubated with vehicle or GB88 (10 μM, 30 min). AUC, area under curve. Mean±SEM. N=5 to 10 independent experiments. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, *ns* not significant. **B, F, I, J.** 1-way ANOVA, Holm-Šidák's test. **D, H.** Repeated measures 1-way ANOVA with Šidák's test compared to pcDNA (-) control. **K.** Localization of proteins resident to the plasma membrane (LgBiT-CAAX, arrowhead) and early endosomes (LgBiT-FYVE, arrow) using HA tag immunoreactivity in HEK293T cells. Representative images from 5 experiments.

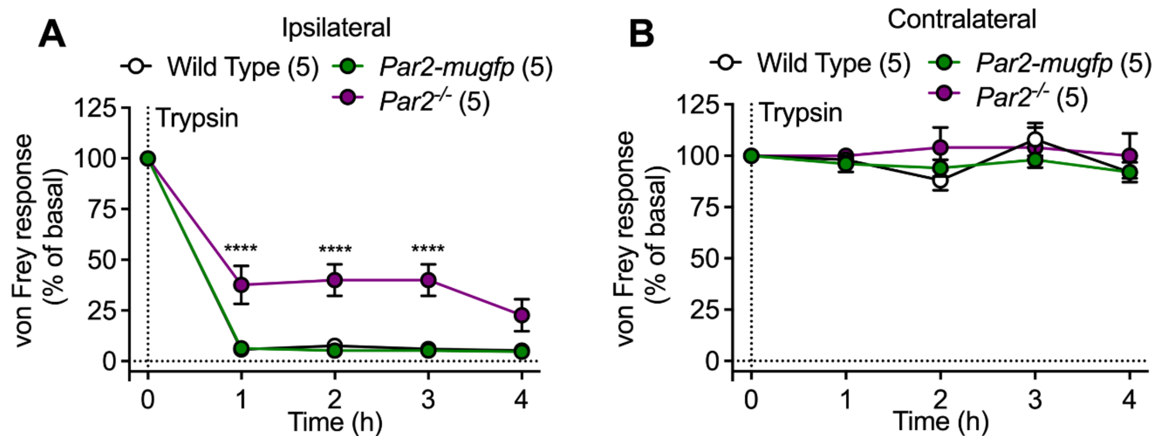


Figure S9. Somatic nociception in *Par$_2$-mugfp*, *Par$_2^{-/-}$* and wild-type mice. A, B. von Frey filament withdrawal responses of the ipsilateral (**A**) and contralateral (**B**) paws of wild-type, *Par$_2$-mugfp* and *Par$_2^{-/-}$* mice after intraplanar injection of trypsin (140 nM, 10 µl). N=5 mice. Mean±SEM. ****P<0.0001 compared to wild-type. 2-way ANOVA, Tukey's test.

Table S1. Taqman, RNAScope® and siRNA probes.

Taqman Probes qRT-PCR

Target	**Thermo Fisher Catalog Number**
Par$_2$ (mouse)	Mn00433160_m1
β-actin (mouse)	Mn02619580_g1
Dnm1 (mouse)	Mn00802468_m1
Dnm2 (mouse)	Mn00514582_m1
Dnm3 (mouse)	Mn00554098_m1
TNF-α (mouse)	Mm00443258_m1
IL-1β (mouse)	Mm00434228_m1
CXCL1 (mouse)	Mm04207460_m1
Gapdh (mouse)	Mn99999915_g1
Dnm1 (human)	Hs01074761_m1
Dnm2 (human)	Hs00974698_m1
Dnm3 (human)	Hs00927946_m1
Gapdh (human)	Hs02758991_m1

Probes RNAScope®

Target	**Advanced Cell Diagnostics Catalog Number**
F2rl1	417541-C1
muGfp	844781-C2
Dnm1	446931-C3
Dnm2	451831-C1
Dnm3	451841-C2

siRNA

Target	**Dharmacon Sequence**
Dnm2	GGCCCUACGUAGCAAACUA, GAGAUCAGGUGGACACUCU, CCGAAUCAAUCGCAUCUUC, GAGCGAAUCGUCACCACUU.
Control	UGGUUUACAUGUCGACUAA, UGGUUUACAUGUUGUGUGA, UGGUUUACAUGUUUUCUGA, UGGUUUACAUGUUUUCCUA.

shRNA

Target	**Origene Sequence**
Dnm2	CGAGAGCAGCCTCATTCTTGCCGTCACAC, CAGATGGTTCAGCAGTTTGGAGTGGACTT, CTGGCTTACCTGTATTCATCAGCAGACCA, AGCAGAGGAATGTCTACAAGGACCTTCGA.

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