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Figure S1 (related to Figure 1). Silencing of β arr1-2 and other signaling molecules in hCMEC/D3 cells

(A) hCMEC/D3 cells mostly express β arr2. Immunoblot experiments with the A1CT antibody on 20 μ g of cell lysate indicate that hCMEC/D3 cells predominantly express β arr2. Lysates of HEK-293T cells (\approx 1 μ g) expressing either exogenous β arr1 or β arr2 were used as markers for determining the migration of β arr isoforms.

(B, C) RNA interference experiments to selectively inhibit expression of β arr1 and β arr2 (β arrs), GRK-2 kinase, β 2-adrenoceptor (β 2-AR) and the α -subunit of the heterotrimeric Gs protein ($G\alpha_s$) (see Figure 1C and D). **(B)** The efficacy of β arr1+2 (45 \pm 5% residual protein), GRK-2 (38 \pm 3% residual protein) and $G\alpha_s$ (23 \pm 6% residual protein) gene silencing were determined by immunoblots using total cell lysates, appropriate antibodies and actin as loading control. **(C)** β 2-adrenoceptor silencing (16.3 \pm 7.0 residual cDNA) was assessed by RT-PCR experiments using GAPDH as control.

Figure S2 (related to Figure 2). Characterization of isoproterenol effects on bacteria and hCMEC/D3 cells

(A-C) Absence of direct effect of isoproterenol on *N. meningitidis* growth, aggregation and static adhesion. Bacterial growth curves **(A)** and aggregate formation (counted under 4x objective) under shearing forces **(B)**, in the absence and presence of 100 μ M isoproterenol. See methods for the experimental details. **(C)** Static adhesion of bacteria on hCMEC/D3 cells pre-treated or not with isoproterenol (iso, 10 μ M). Static adhesion was determined by infecting hCMEC/D3 monolayers at a MOI of 100 for 30min. The total number of cell-associated colony forming units (CFU) was determined after washing of the cell monolayer followed by scraping the cells from the dish before plating on agar plates.

(D) Production of cAMP in hCMEC/D3 cells assessed by a cAMP reporter assay in response to bacterial adhesion or β -adrenergic ligands. The assay is described in Supplemental Experimental Procedures. WTNm, wild-type 2C43 bacteria; WTNm propra, wild-type 2C43 bacteria in the presence of 100nM propranolol; Pile- Nm, 2C43 Δ PilE bacteria devoid of type IV pili. iso, 10 μ M isoproterenol; iso - propra, 10 μ M isoproterenol and 100nM propranolol; ***(p <0.001).

(E) β arr C-ter-GFP inhibits β 2AR endocytosis. HEK-293 cells transfected with the indicated plasmids, were harvested, treated or not (basal) with 10 μ M isoproterenol for 30 min at 37°C, chilled on ice, fixed and labeled with the anti-myc 9E10 monoclonal antibody (1h on ice). After incubation with a cy5-labeled anti-mouse secondary antibody (2h on ice), cells were fixed again and submitted to FACS analysis. Surface receptor (labeled by the myc antibody) was measured in GFP positive cells (expressing control GFP or β arr C-ter GFP). Data are expressed as the percentage (\pm SEM of triplicates) of untreated control cells normalized to 100%. * (p <0.05).

(F) Images representative of the experiment shown in Figure 2D.

Cells transfected with the indicated GFP-containing plasmids were infected, fixed and immunostained for ezrin (Red) and host cell or bacterial DNA stained with DAPI. Arrows indicate colonies that adhere to GFP-expressing cells. Asterisks indicate colonies that adhere to cells negative for GFP expression. Bar: 10 μ M.

(G) Images representative of the experiment shown in Figure 2E.

Cells were fixed and immunostained for ezrin and host cell or bacterial DNA stained with DAPI. Arrows indicate Nm colonies. Iso : Isoproterenol. Bar: 10 μ M.

Figure S3 (related to Figure 5). Characterization of *Nm*-induced gaps in hCMEC/D3 cell monolayers. Cells were stained for VE-cadherin and bacteria labeled with anti-pili antibodies. Cell nuclei and bacterial DNA are stained with DAPI. The area surrounding a gap between two adjacent cells, identified by the interruption in VE-cadherin staining of plasma membranes, is enlarged in top panels and the gap itself circled. The enlarged area corresponds to rectangles in middle panels. Images in z (bottom panels) have been acquired at the level of the arrowhead. The position of the gap is indicated by the dotted line. DAPI and staining for type-IV Pili demonstrate the presence of bacteria above and through the gap.

Figure S4 (related to Figure 6). Multiple roles of β -arrestins downstream of *Nm*-activated β -adrenoceptors

(A,B) Recruitment of Src, actin and activated (phospho-) Src under *Nm* colonies in hCMEC/D3 cells. HCMEC/D3 cells were infected with the 2C43 strain, fixed, immunostained for Src and actin (A) or phospho-Src (P-Src, B) and bacterial DNA stained with DAPI Bar: 10 μ m.

(C) Dominant negative effect of myc- β arr2- Δ Src on Src activation in hCMEC/D3 cells. Immunoblot with anti-phospho-Src and anti-Src antibodies on lysates of hCMEC/D3 cells expressing myc- β arr2 or myc- β arr2- Δ Src, and incubated or not with *Nm*.

(D) Co-immunoprecipitation study of myc- β arr2 in HEK-293 cells transfected with the indicated plasmids. Experiments were performed with 250 μ g of cell lysates. 10 μ g of the input were co-migrated for comparison.

(E) Schematic model depicting the involvement of β -adrenoceptors and β -arrestins in *N. meningitidis*-induced cell signaling. Activation of Src by β -adrenoceptors–recruited β -arrestins leads to cortactin phosphorylation and cortactin-dependent formation of actin-rich cellular protrusions, which stabilize the bacterial colony. The β -arrestin-dependent recruitment of junctional proteins beneath the colony eventually results in opening a paracellular route for bacteria

Figures S5 (Related to Figure 7) Both *Nm* and *Ng* recruit and activate the β 2AR- β arr2 signaling pathway likely via their closely related pilins.

(A) Recombinant pilins bound to staphylococci. MBP and MBP fused to the indicated pilins were produced as described in Supplemental Experimental Procedures. Purification of each recombinant protein was assessed by Coomassie-blue staining. Recombinant proteins were then incubated with anti-MBP antibodies and staphylococci. After centrifugation and several washes, coated bacteria were lysed and binding of recombinant proteins was assessed by immunoblot experiments using antibodies for Pile, PilV and compP. Asterisks indicate the heavy chain of the anti-MBP antibody.

(B) *Neisseria gonorrhoeae* colonies recruit β arr2, β 2AR and cortical plaque components. HCMEC/D3 cells were transfected with β 2AR-GFP or β arr2-GFP plasmids, infected with *Ng*, fixed and immuno-stained for ezrin (top) or stained with DAPI to visualize the *Ng* colony (bottom). Arrows indicate *Ng* colonies. scale bar: 10 μ m.

(C,D) Pre-treatment of hCMEC/D3 cells with isoproterenol inhibits ezrin recruitment under *Neisseria gonorrhoeae* colonies. Pictures: hCMEC/D3 cells were pre-incubated with 10 μ M isoproterenol (Iso) for 1h or left untreated, infected with *Ng* and fixed (same experiment as in Fig 2 for *Nm*). Cells were labeled with anti-ezrin antibodies and phalloidin and DAPI to visualize actin and *Ng* colonies (arrowed), respectively. Arrows indicates *Ng* colonies. Scale bar: 10 μ m. Quantitative aspects of the effect of isoproterenol pretreatment on ezrin recruitment by *Ng* are shown by the histograms: bars indicate SEM from 3 experiments in duplicate. ** (p<0,001).

(E) Dominant negative effect of β arr2- Δ Src-GFP on *Ng*-induced recruitment of actin in hCMEC/D3 cells. Experiments were conducted as in the Figure 6B for *Nm*. Bars indicate SEM from 1 experiment in triplicate ** (p<0,02).

Movie S1 (related to Figure 3). 3D reconstitution of the cortical plaque beneath a *N. meningitidis* colony in reconstituted HEK-293 cells. Cells transfected with plasmids for CEACAM-1, β 2AR and β arr2 were infected with the 2C43 SiaD- Opa+ strain, fixed, stained with anti-ezrin and both bacterial DNA and host-cell nuclei were stained with DAPI. Several cross sections were obtained using a confocal microscope and processed using IMARIS software to obtain 3D reconstruction of the area. Ezrin (in red) is enriched into microvilli-like structures surrounding bacteria (small blue dots) adhering onto the apical domain.

Movie S2 (related to Figure 3). 3D reconstitution of the cortical plaque beneath *N. meningitidis* colonies in hCMEC/D3 cells. hCMEC/D3 cells were infected with the 2C43 strain, fixed and immuno-stained for ezrin. DNA of both bacteria and cell nuclei were stained with DAPI. Several cross sections were obtained using a confocal microscope and processed using IMARIS software to obtain 3D reconstruction of the area. Ezrin staining (in red) is over-exposed to delineate the cell body. Ezrin is enriched into microvilli-like structures that surround bacteria (small blue dots) adhering onto the apical domain (compare to **Movie S1**).

Movie S3 (related to Figure 4). Downregulation-endocytosis of the β 2adrenoceptor impairs *N meningitidis* resistance to shear stress. Adhesion and growth of GFP-expressing 2C43 *Nm* on confluent hCMEC/D3 cells under laminar flow was recorded for 90 min (1 frame every 20 sec) in the absence of pre-treatment. Arrowed colonies (both large or small) resist to the lamina flow.

Movie S4 (related to Figure 4). Downregulation-endocytosis of the β 2adrenoceptor impairs *N meningitidis* resistance to shear stress (continued). Experiment conducted as in **Movie S3** but after 1h pre-treatment with 10 μ M isoproterenol. Meningococci are detached by flow from the arrowed growing colonies.

Supplemental Experimental Procedures

Cell culture

The immortalized human brain micro-vascular endothelial cell line hCMEC/D3, which retains the main characteristics of primary brain endothelial cells, was described previously (Weksler et al., 2005). hCMEC/D3 cells were grown at a density of 25×10^3 cells per cm^2 in flasks coated with $5 \mu\text{g}/\text{cm}^2$ rat tail collagen type I (BD Bioscience), in EBM-2 medium (Lonza) supplemented with 5% of Fetal Calf Serum, 1% of Penicillin-Streptomycin, $1.4 \mu\text{M}$ hydrocortisone, $5 \mu\text{g}/\text{ml}$ ascorbic acid, 10mM HEPES and 1ng/ml b-FGF, at 37°C in a humidified incubator in 5% CO_2 . Human Embryonic Kidney 293 (HEK-293) cells were grown in flasks coated with 0.01% poly-L-lysine (Sigma), containing DMEM medium supplemented with 10% Fetal Calf Serum and 1% Penicillin-Streptomycin, in 5% CO_2 -enriched humidified atmosphere. For immunofluorescence assays, cells were grown on 12-mm diameter glass coverslips with appropriate coating and media.

The ability of bacteria to transmigrate through hCMEC/D3 cells monolayers was assayed with Millicell® inserts of indicated pore size. Cells were seeded at a density of 5×10^4 cells per cm^2 on inserts pre-coated with rat tail collagen type I (thick coating, modified from Bornstein and Murray (Bornstein and Murray, 1958)) for 12-15 days. The medium was replaced every 3-4 days and 24h before the experiment.

Bacterial strains, growth and infection conditions

A piliated capsulated Opa⁻ variant of serogroup-C meningococcal strain 8013, designated 2C43, was used throughout the study (Nassif et al., 1993). A non-capsulated derivative of this strain was engineered by introduction of a *siaD* mutation. Isogenic non-piliated and piliated Opa⁺ strains of this non-encapsulated 2C43 derivative were designated SiaD⁻ Opa⁺ PilE⁻ and SiaD⁻ Opa⁺, respectively (Coureuil et al., 2009). Isogenic piliated and non-capsulated Opa⁺ mutant for the *pilV* gene was designated SiaD⁻ Opa⁺ PilV⁻ (Mikaty et al., 2009). *N. meningitidis* expressing the green fluorescent protein (GFP) were obtained by introducing in bacteria the pAM239 plasmid by conjugation (Mairey et al., 2006). MS11 (Merz et al., 1999), a piliated strain of *Neisseria gonorrhoeae* (Ng), was used for the experiments shown in the figures S5b-d.

The day before the infection, hCMEC/D3 cells were serum-starved in EBM-2 medium containing 0.1% Bovine Serum albumin (BSA). The day of infection, bacteria from an overnight culture were adjusted to $\text{OD}_{600}=0.05$ and incubated for 2h at 37°C in pre-warmed starvation medium. Bacteria were added to cells at a multiplicity of infection (MOI) of 100 and allowed to adhere for 30 minutes. Unbound bacteria were washed with fresh medium and infection was allowed to proceed for 2h. When needed, drugs were added to cell culture 1h before infection.

Bacteria were tested for growth and aggregation. Bacterial colonies harvested from GCB agar plates were grown to $\text{OD}_{600}=0.05$ in EBM2, 0.1% BSA in the presence or absence of $100 \mu\text{M}$ isoproterenol and incubated at 37°C in a 5% CO_2 atmosphere under constant shaking. The OD_{600} was then measured after 1, 2, 3, 4 and 8h. Aggregation was determined after 2h of incubation in EBM2, 0.1% BSA in the presence or absence of $100 \mu\text{M}$ isoproterenol at 37°C in a 5% CO_2 atmosphere under constant shaking. A cone and plate assay was then performed as described previously (Mikaty et al., 2009) using a cone and plate rheometer (Brookfield Engineering Laboratories Inc., Middleboro, MA, USA).

Plasmids and transfections

Plasmids coding for β arr1-GFP, β arr2-GFP (Scott et al., 2002a), β arr2-Cherry (Boullaran et al., 2007), myc- β arr1 and myc- β arr2 (Storez et al., 2005), the carboxyterminal

tail of β arr2- fused to GFP (Scott et al., 2002b) the human β 2AR-YFP (β 2AR-YFP) and myc- β 2AR-Renilla luciferase fusion protein (myc- β 2AR-Rluc) (Angers et al., 2000), the AT1R (Scott et al., 2006), CCR5 and CXCR4 (Achour et al., 2009) were described previously. Plasmids coding for dominant negative Eps15 (Eps15-DIII-GFP) and control Eps15-DIII Δ 2-GFP (Benmerah et al., 1999) were generous gifts of Dr. Alexandre Benmerah (Institut Cochin, Paris, France). The plasmid encoding CEACAM-1 (Virji et al., 1996) was kindly provided by Dr. Mumtaz Virji (Department of Cellular and Molecular Medicine, School of Medical Sciences, University of Bristol, Bristol, UK). The 4xCRE-Luciferase reporter plasmid was purchased from Stratagene. Plasmids for VE-cadherin-GFP and p120-catenin-GFP expression were kindly provided by Dr. David Cheresh (Moore's University of California-San Diego Cancer Center, La Jolla, California, USA) and Dr. René-Marc Mège (INSERM U839, Paris, France), respectively. β arr2- Δ Src (β arr2 P91G, P93D, P94R, P97L) was prepared by site directed mutagenesis using β arr2-GFP or myc- β arr2 as templates and the QuikChange (Stratagene) protocol. The chimeric Nter β 2AR-AT1R receptor was constructed using a 2-step procedure. First a unique Kpn1 site was introduced by site-directed mutagenesis in the human AT1R immediately before the beginning of the first transmembrane domain (position F28). The N-terminus of the human β 2AR, amplified by PCR, was then subcloned in phase upstream of the codon for F28 using the Kpn1 site. The constructs for β arr2- Δ Src and Nter β 2AR-AT1R were sequenced to verify the phases and the absence of undesired mutations (DNA sequence facility, Institut Cochin).

hCMEC/D3 cells were transfected using the Amaxa Nucleofactor Kit for Huvec (Amaxa Biosystem) or jetPEI transfection reagent (Polyplus Transfection) according to manufacturer instructions. HEK-293 cells were transfected using Lipofectamine 2000 (Invitrogen) following manufacturer instructions.

Inhibition of protein expression by RNA interference

Small interfering RNAs targeting human β arrestin-1 (5'-AAAGCCUUCUGCGCGGAGAAU-3'), β arrestin-2 (5'-GGCUUGUCCUCCGCAAAGAC-3'), (Scott et al., 2006) and GRK2 (5'-AAGAAGUACGAGAAGCUGGAGUU-3') were synthesised by Eurogentec. To silence the expression of $G\alpha_s$ and of the β 2-adrenoceptor, a pool of four siRNA duplexes (ON-Target plus SMARTpool, NM_080426 and NM_000024 from Dharmacon, respectively) were used. The efficacy of knockdown was assessed three days after transfection by immunoblotting or by RT-PCR assays using whole cell lysates. An off-target siRNA (5'-GATAAGGTGCTGCGTGGACCC-3') and the siCONTROL siRNA (Dharmacon) were used as controls. SiRNAs were transfected using Amaxa Nucleofactor Kit for Huvec according to manufacturer's instructions. The infections with *Nm* were performed three days after transfection.

Antibodies, drugs and receptor ligands

The following monoclonal antibodies (mAb) or polyclonal antibodies were used for immunofluorescence labeling or immunoblotting: anti-ezrin rabbit antibody (generously provided by Dr. P Mangeat CNRS, UMR5539, Montpellier, France), anti- β arr rabbit A1CT antibody (kindly provided by Dr Robert Lefkowitz, Duke University, Durham, USA), rabbit monoclonal anti- β arr2 antibody (C16D9, Cell Signaling), anti- $G\alpha_s$ rabbit antibody (generously provided by Dr. Ralf Jockers, Institut Cochin, Paris, France), anti-GRK2 mouse mAb (clone C5/1.1, Upstate), anti-src rabbit polyclonal antibody (SC-18, Santa Cruz), anti-phosphorylated Src (PY418) rabbit monoclonal antibody (Bioscience, #44-660G), anti-actin rabbit polyclonal antibody (Sigma-Aldrich), anti-VE-cadherin rabbit polyclonal antibody (Bender Medsystems), anti-p120-catenin mouse mAb (BD Transduction Laboratories), anti-myc mouse mAb or rabbit polyclonal antibody (Cell Signaling), anti-type IV pili mouse mAb

20D9 and anti-GFP mouse mAb 3E6 (Invitrogen). Following goat secondary antibodies were used for immunofluorescence labelling or immunoblotting: anti-mouse IgG (H+L) and anti-rabbit IgG (H+L) coupled to Horse Radish Peroxidase (Jackson ImmunoResearch Laboratories) and anti-mouse IgG (H+L) and anti-rabbit IgG (H+L) coupled to Alexa-488 or Alexa-546 or Alexa-633 (Invitrogen).

Isoproterenol, propranolol, angiotensin II (Ang II) forskolin and PP2 Src inhibitor were purchased from Sigma-Aldrich. SDF-1 (agonist of CXCR4 receptor), MIP-1 β (agonist of CCR5 receptor), IL8 were all from Preprotech.

Immunofluorescence experiments

Cells were fixed for 20min in PBS 4% PFA and permeabilized for 5min in PBS containing 0.1% Triton X-100. Cells were incubated with primary antibodies at the recommended concentration in PBS containing 0.3% BSA for 1h. After 3 washings in the same buffer, Alexa-conjugated phalloidin and DAPI (0.5 μ g/ml) were added to Alexa-conjugated secondary antibodies for 1h. After additional washings, coverslips were mounted in mowiol. Image acquisition was performed on a laser-scanning confocal microscope (Zeiss LSM 510). Images were collected and processed using the Leica Application Suite AF lite (Leica) software. 3D reconstruction was performed using the IMARIS software.

Quantitative analysis of ezrin (and other proteins) recruitment under bacterial colonies

After infection, cells were treated for immunofluorescence analysis with appropriate antibodies and observed under a light fluorescent microscope. For each *Nm* strain, the recruitment efficiency was estimated by determining the proportion of colonies that efficiently recruited the protein of interest in a “honeycomb shape” just under the colonies. Importantly, in uninfected cells, no ezrin, actin or Src were found in such honeycomb-shaped clusters at the plasma membrane. At least 50 colonies were scrutinized per coverslip. Each experiment was repeated several times (at least 3). Data was examined for significance using Student’s t-test.

CRE-Luciferase assay

Luciferase activity in cell lysates was measured using the Luciferase Reporter Assay System (Promega) according to manufacturer instructions. Briefly, hCMEC/D3 cells were transfected with the 4xCRE-Luciferase plasmid (Stratagene) the day before the experiment. Cell infection was performed as described above at a MOI of 100. After 2h of infection or incubation with drugs or ligands, cells were washed twice with ice-cold PBS, and lysed using the Luciferase Cell Culture Lysis Reagent. Lysates were centrifuged at 12,000 \times g for 2min at 4°C. Supernatants were tested for luciferase expression using a VICTOR³ multilabel counter (Perkin-Elmer).

Nm adhesion under shear stress

Shear stress assays were performed as described previously (Mairey et al., 2006). Briefly, cells were grown on disposable flow chambers (15 μ -Slide VI, IBIDI) coated with 5 μ g/cm² rat tail collagen type I. Bacteria grown to OD₆₀₀=0.05 in EBM2, 0.1% BSA medium were incubated for 2h at 37°C under 5% CO₂ atmosphere. GFP expression in bacteria was induced by adding 1 mM IPTG in EBM2, 0.1% BSA. For experiments aimed at determining the growth of colonies under shear stress, 10⁷ bacteria were then introduced into the flow chamber and allowed to adhere for 20 min without flow. Subsequently, a flow corresponding to a shear stress of 0.2 dyn/cm² was applied using a syringe pump (Harvard Apparatus). For adhesion assays under shear stress, 10⁷ bacteria were introduced into the flow chamber and allowed to adhere for 20 min under a shear stress of 0.04 dyn/cm², subsequently the chamber

was washed 1 min under a shear stress of 1 dyn/cm². Fluorescent bacteria in the chambers were observed using an Olympus CKX41 inverted microscope with a 20x objective, equipped with a shutter for the fluorescence lamp and a Hamamatsu ORCA285 CCD camera. Images were collected and processed using the Openlab software (Improvision). The field under observation corresponded to a surface of 425 μm by 320 μm with a resolution of 0.63 μm per pixel. Images were analyzed using the ImageJ software.

Nm transmigration assay

Cell permeability to *Nm* was determined as described previously (Coureuil et al., 2009). Briefly, hCMEC/D3 cells were grown on 3 μm pore size MilliCell® inserts and infected at a MOI of 10 (10⁶ bacteria per insert). Four hours after infection, inserts were placed in a well with fresh medium. After 30min, the number of bacteria, which had crossed the monolayer of endothelial cells, was then measured by plating the content of dilutions of the lower compartment on agar plates and counting the number of colony forming units (CFU). The effect of isoproterenol on *Nm* transmigration was determined by comparing the number of migrating bacteria through the monolayer of endothelial cells per hour in the presence and absence of the agonist.

To estimate the surface and number of macroscopic cell-cell junction openings, MilliCell® inserts were fixed at the end of the experiments in 4% PFA and immunostained for VE-cadherin and actin. Several baso-lateral cross-sections of each insert were acquired using a Leica SP5 confocal microscope. The surface and number of gaps within the hCMEC/D3 cell monolayer was determined using the Leica Application Suite-AF (LAS-AF) software. Data were expressed as the number of gaps per 4.10⁵ μm² of hCMEC/D3 cell monolayer surface. The size of gaps was determined using the size of a *Nm* diplococcus as surface unit (approximately 1.7 μm²). The total surface occupied by the colonies was determined using an anti-pili staining and analyzed using ImageJ software. This total surface was similar with or without isoproterenol.

Expression and purification of MBP-pilin recombinant proteins

Recombinant MBP-pilin fusion proteins were produced as described before (Helaine, 2007). Fragments of pilE, pilV and comP genes lacking the region coding for the amino acid residues 1 to 28 of the full-length proteins were amplified by PCR and subcloned in pMAL-p2X vector (New England Biolabs). The resulting plasmids, pMAL-pilV and pMAL-comP, contain in frame the malE gene, which encodes the maltose-binding protein (MBP), followed by the Factor Xa protease recognition site and the truncated pilin coding regions. MBP-pilin fusion proteins are directed into *E.coli* periplasm, where disulfide bond formation can occur. These plasmids were transformed in the PAP5198 *E.coli* strain (generously provided by Dr. Olivera Francetic, Institut Pasteur, Paris, France), deleted for the periplasmic enzymes: OmpT Prt and DegP. The fusion proteins were purified on amylose resin (New England Biolabs). The sequence of forward (F) and reverse (R) primers used in the PCR reactions are listed below (restriction sites used for cloning are in parentheses):

pilEp2XF (EcoR1) 5'-GAATTCGCCCGCGCACAAGTTTCC-3'
pilEp2XR (Pst1) 5'-CTGCAGTTAGCTGGCAGATGAATCATCG-3'
pilVp2XF (EcoR1) 5'-GAATTCGGCGCGTCCGCCTGTTCGG-3'
pilVp2XR (Pst1) 5'-CTGCAGTTAGTCGAAGCCGGGGCAGG-3'
comPp2XF (EcoR1) 5'-GAATTCGAGAAAGCAAAGATAAATGC-3'
comPp2XR (Pst1) 5'-CTGCAGCTACTTAAATAACTTGCAGTCC-3'

Coating of Staphylococcus aureus with MBP-pilin fusion proteins

S. aureus (ATCC 25923) expressing specific receptors for the Fc domain of IgG immunoglobulins were used for this experiment. Bacteria from an overnight culture were adjusted to OD₆₀₀=0.05 and incubated for 2h at 37°C in pre-warmed LB medium. About 10⁸ bacteria were incubated with 2µg of anti-MBP antibody for 20min at room temperature. Bacteria were then centrifuged at 4000rpm for 5min, washed with warm LB 3 times, incubated with 2.5µg of each recombinant fusion protein for 20 min at room temperature. After several washes using DMEM medium supplemented with 10% Fetal Calf Serum, bacteria were used in adhesion assays (incubations of 30 min) or lysed in modified RIPA buffer (50mM Tris pH 7.5, 2mM EDTA, 150mM NaCl, 0.1 % (w/v) SDS, 0.5 % (w/v) Na deoxycholate) containing 1X protease inhibitors cocktail for immunoblots (Sigma-Aldrich).

Reverse-PCR

To determine the efficacy of the siRNA-mediated inhibition of the β2AR, total RNA were extracted using a Rneasy Mini Kit (Qiagen) according to the manufacturer protocol. DNA contaminants were removed using Turbo DNase (Ambion). Equal amounts of RNA from control siRNA- or β2AR siRNA-transfected cells were used as templates for reverse transcription with the Superscript II Reverse Transcriptase (Invitrogen) and an oligo-dT primer, following manufacturer instructions. PCR reaction mix was prepared containing 1XPCR Buffer (Takara), 1µM dNTP Mix (Roche), 1µM of each primer, 0.5 units of Takara Taq polymerase and 5 µL of cDNA. Primers used for β2AR sequence amplification were: (5'-ATTGAGACCCTGTGCGTGAT-3', forward) and (5'-CTGAAAGACCCTGGAGTAGA-3', reverse); primers for GAPDH sequence amplification were: (5'-TTCCTACCCCAATGTGTCC-3', forward) and (5'-TCTTGCTCAGTGTCCTTGCT-3', reverse). The amplified products were subjected to electrophoresis on 1.5 % agarose gels and stained with ethidium bromide.

Immunoprecipitation and Immunoblotting

For the experiments aimed at determining the efficacy of siRNA-dependent knockdown, whole cell lysate of hCMEC/D3 cells were washed with ice-cold PBS and lysed in modified RIPA buffer containing 1X protease inhibitors cocktail (Sigma-Aldrich). Whole cell lysates were then boiled and analyzed by SDS-PAGE.

For immunoprecipitation studies, HEK-293 cells were transfected with appropriate plasmids using lipofectamine 2000. Two days after transfection dishes were lysed in immunoprecipitation buffer (10mM Hepes, 50mM NaCl, 5mM EDTA, 1mM CaCl₂, 1mM MgCl₂ and 0,5% Triton X100) containing a protease inhibitor cocktail (Sigma-Aldrich), on ice for 1h. After centrifugation at 13000xg for 20min, the supernatant was collected and protein concentration determined (Bio Rad protein assay). Supernatants were incubated with the anti-myc antibody for 2h at room temperature and then incubated with protein G-Sepharose beads for 1h at 4°C. After centrifugation, the immunobead-bound material was washed 3 times with lysis buffer. The immunoprecipitates were resolved in 10% SDS-PAGE gels.

After transfer on nitrocellulose (Optitran BA-S 83, Millipore), blots were probed with the appropriate antibodies. Immunoblots were revealed by luminescence (ECLplus; GE Healthcare Life Sciences).

Supplemental References

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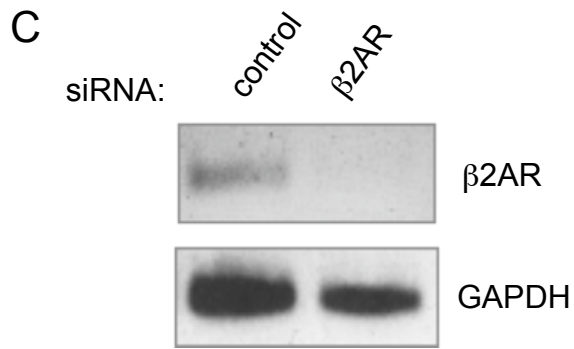
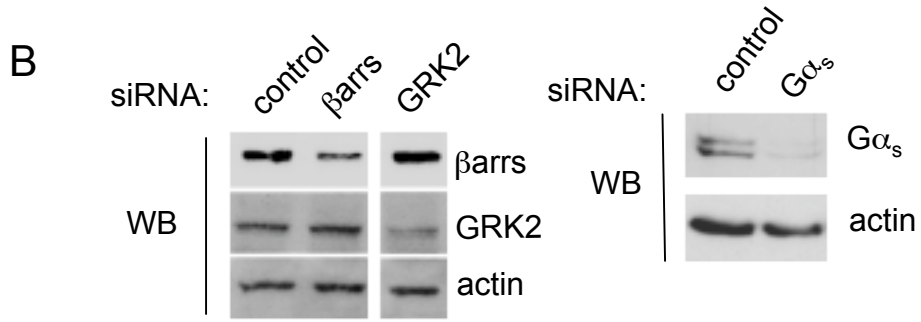
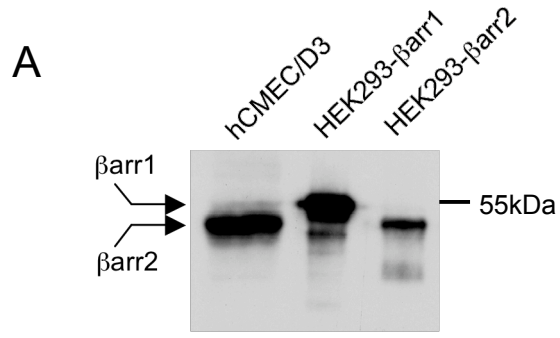
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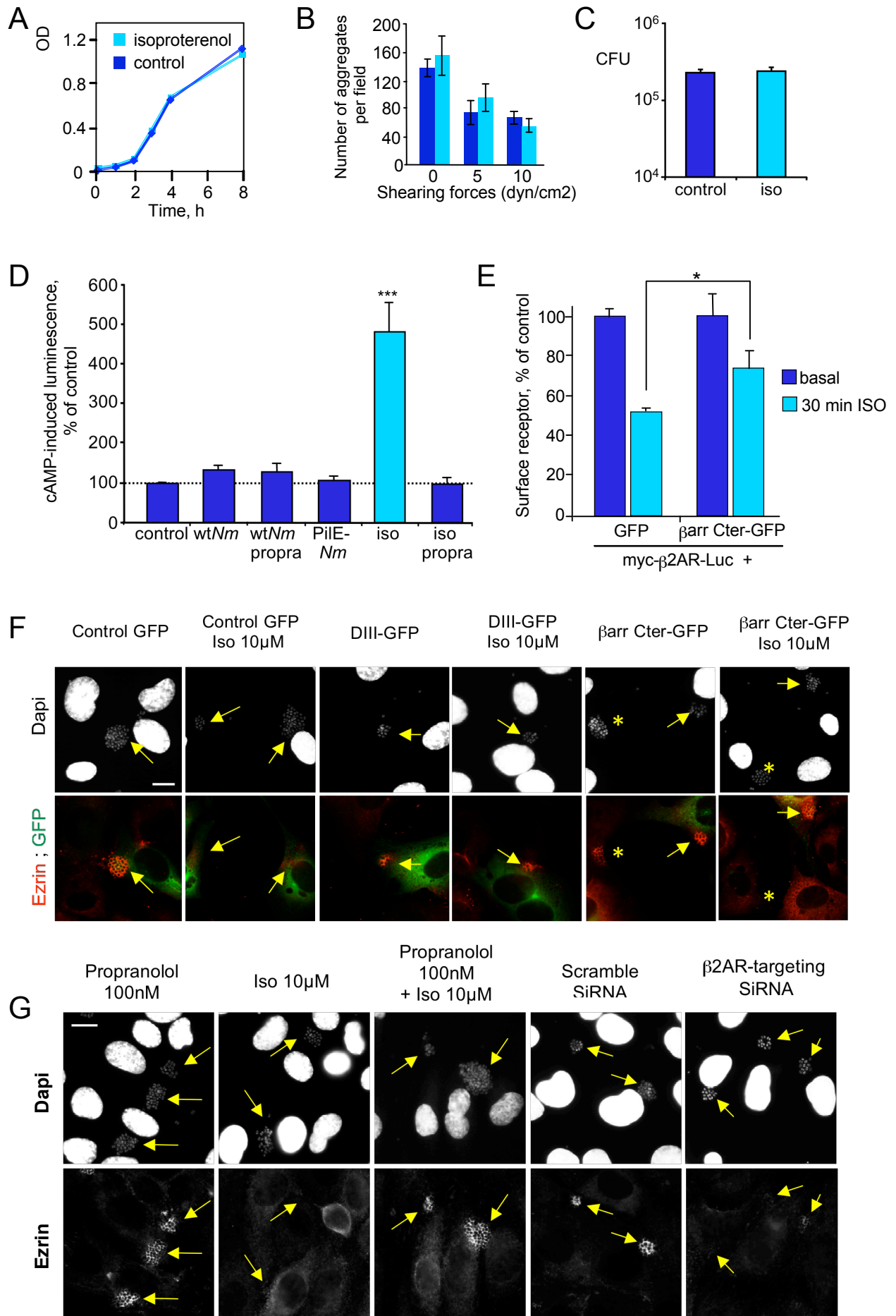
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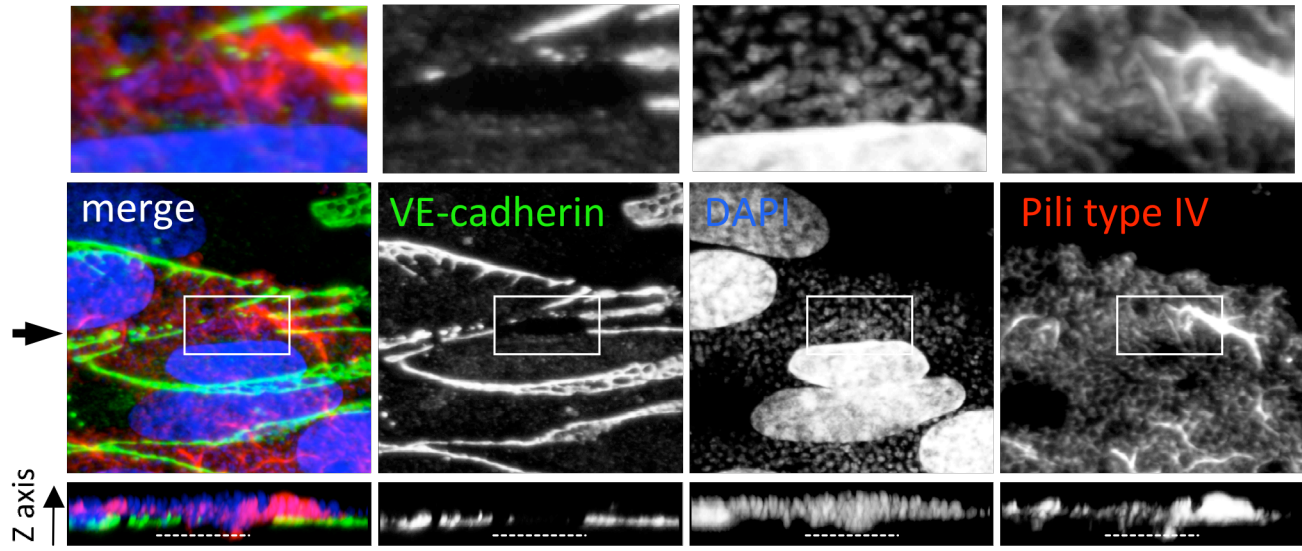
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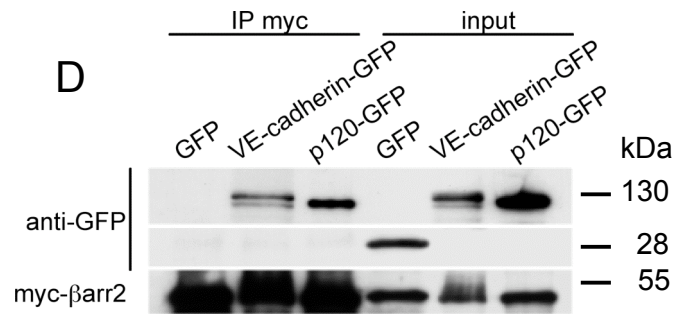
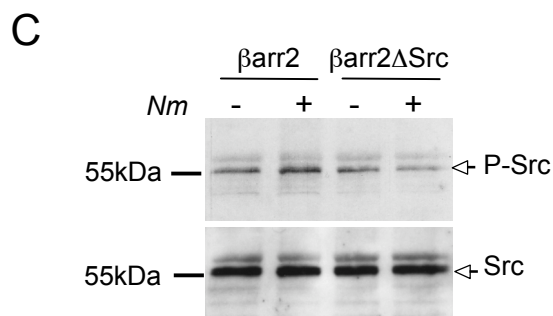
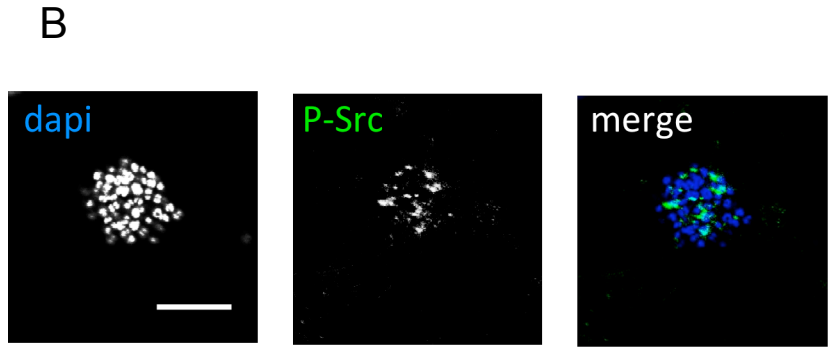
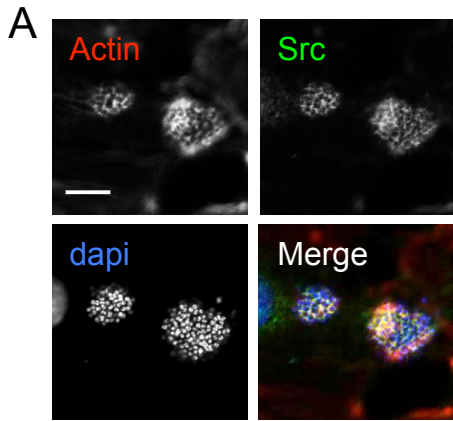
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