

Supplement Material & Methods:

Cell culture:

HL-1 cells, a murine atrial cardiomyocyte cell line, was kindly provided by William C. Claycomb (New Orleans, USA).¹ Cell culture reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany). Cells were kept in Claycomb Medium (#51800C) supplemented with 10% fetal bovine serum (#F2442, batch 058K8426), 100 µM norepinephrine, penicillin, streptomycin, and 2 mM L-glutamine at 37°C, 5% CO₂ and 100% humidity. For immunostaining and liberase assays cells were seeded at 25.000 cells/cm² and incubated for seven days with daily medium exchange on glass coverslips or 24-well-plates coated with 0.02% gelatin and 25 µg/ml fibronectin. 24 h before fixation or lysis, cells were treated with 1.8 mM Ca²⁺ to provide for adequate desmosomal cohesion.

Exogenous Dsg2 expression:

Vector encoding for human Dsg2 and various mutations of Dsg2 (D154E, D264E, N266S, D494N, A517V, G812C, G812S, C813R, V920G) were kindly provided by Dr. Katja Gehmlich. Human cDNA was cloned into the pEGFP-N1 vector using *XhoI* and *BamHI* restriction sites and verified by sequencing. Transfection of HL-1 cells with endotoxin-free vector DNA at 2 µg/ml for 24 h was performed using Lipofectamine® 2000 (Life Technologies, Darmstadt, Germany) following manufacturer's instructions. Successful transfection was confirmed by Western blot analysis and fluorescence imaging making use of the EGFP-tag situated C-terminally of the Dsg2 constructs.

siRNA-mediated Dsg2 knockdown:

ON-TARGET plus SMARTpool mouse Dsg2-siRNA was purchased from Dharmacon/Thermo Fisher Scientific (Lafayette, USA, #L-042514-01-0005) dissolved in siRNA buffer at 20 µM and stored at -80°C. Non-target siRNA (Dharmacon, #D-001810-10-05) was dissolved at 100 µM.

siRNAs were applied at 60 nM final concentrations. siRNA was transferred into HL-1 cells seeded on 24-well-plates using RNAimax (Life Technologies) following manufacturer's instructions. siRNA-incubation was performed for 24 h. Transfections with non-target siRNA were performed in parallel on the same plate. Knockdown efficiency was confirmed by Western blot analysis.

Antibodies & reagents:

The following primary antibodies were used for immunofluorescence and Western blot analyses: polyclonal rabbit anti-Dsg2-rb 5 for immunofluorescence and as an inhibitory antibody for liberase assay (Progen Biotechnik, Heidelberg, Germany, #610121); monoclonal mouse anti-Dsg1/2 DG 3.10 for Western blot and as a control antibody for liberase assay (Progen, #61002); monoclonal mouse anti-N-Cad for immunofluorescence (BD Biosciences, Heidelberg, Germany, #610921); monoclonal mouse anti-GAPDH-Peroxidase for Western blot without secondary antibody (Sigma-Aldrich, #G9295); monoclonal mouse anti- α -Tubulin DM1A for Western blot (Abcam, Cambridge, UK, #ab7291). The inhibitory and control antibodies used in the liberase assay were dialyzed against phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and applied for 24 h incubation of HL-1 cells.

As secondary antibodies for Western blot analyses we used peroxidase-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) or goat anti-mouse IgG+IgM (Dianova) antibodies. For immunofluorescence staining Cy2- or Cy3-conjugated goat anti-rabbit IgG (Dianova) and Cy3-conjugated goat anti-mouse IgG (Dianova) were used.

L-tryptophan (L-Tryp, Sigma-Aldrich) and L-phenylalanine (L-Phen, Sigma-Aldrich) were dissolved in PBS at 10 mM, pH was adjusted to 7.4 and aliquots stored at -20°C. Both amino acids were used in working concentrations of 20 μ M to 2 mM as indicated. EGTA (AppliChem, Darmstadt, Germany) was dissolved at 0.5 M in PBS and used in working concentrations of 5 mM. Production of the cyclic single peptide Ac-cyclo-(CLNSMGQDC)-NH₂ was commissioned to

Bachem (Bubendorf, Switzerland). This peptide was modeled to interfere with desmoglein-trans-interaction by occupation of the binding pocket of a single desmoglein. A corresponding tandem peptide (Bachem) combining two cyclic peptides with a linker is able to bind two desmogleins on neighboring cells.² Both peptides were dissolved at 10 mM concentration in PBS and stored at -20°C. Tandem peptide biotinylation was performed using sulfo-NHS-biotin according to manufacturer's instructions (Pierce, Rockford, USA) and the resulting product (TP-Bio) was extensively dialysed against PBS to remove unconjugated biotin. Working concentrations for peptides were 20 or 40 µM as indicated. (-)Isoproterenol hydrochloride (Iso, Sigma-Aldrich) was dissolved at 10 mM in PBS and applied at a concentration of 2 µM. Phenol Red (Sigma-Aldrich) was added to L-Phen, L-Tryp or Iso for Langendorff applications at a concentration of 0.1 mg/ml. All solutions used for cell culture were sterile filtrated before use.

Immunostaining:

HL-1 cells were grown on coated glass coverslips for 7 days. Cells were washed with PBS, fixed in 2% paraformaldehyde in PBS for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min, following blocking of unspecific binding sites with 3% bovine serum albumin and 10% normal goat serum in PBS for 30 min. Primary antibodies diluted 100-fold in PBS were incubated at 4°C in a humid chamber over night. Species-matched Cy2- or Cy3-conjugated secondary antibodies were applied at 300- to 600-fold dilution in PBS at room temperature in a humid chamber for 1 h. Streptavidin-AlexaFluor488 (Life Technologies) was applied at 20 µg/ml for 1 h for visualization of TP-Bio. Glass coverslips were mounted using 1.5% of the anti-fading reagent n-propyl gallate (Sigma-Aldrich) and 60% glycerol in PBS. Staining was evaluated using a Leica SP5 confocal microscope (Leica, Mannheim, Germany) equipped with a x63 oil objective using LAS-AF software for image capturing and ImageJ software (NIH, Bethesda, USA) for image analysis.

Atomic force microscopy measurements of Dsg2 and N-Cad interactions

Homophilic binding of recombinant Dsg2 and N-Cad was tested by application of force spectroscopy using a Nanowizard III atomic force microscope (JPK Instruments, Berlin, Germany) mounted on an optical microscope (Axio Observer.D1, Carl Zeiss Microscopy, Jena, Germany). Dsg2-Fc was expressed in CHO cells and purified from cell culture supernatant as described before.³ N-Cad-Fc was purchased from R&D Systems (Abingdon, UK). Recombinant proteins were coupled to flexible Si₃N₄ AFM cantilevers (MLCT probes, spring constants 0.01 and 0.03 N/m, Bruker, Calle Tecate, CA, USA) and mica sheets (SPI supplies, West Chester, PA, USA) via flexible polyethylene glycol spacers (acetal-PEG-NHS) essentially as described before.^{4, 5} In brief, cantilevers and mica were functionalized with amino groups by ethanolamine treatment and then coupled to the *N*-hydroxysuccinimide ester group of the heterobifunctional linker. The linker's acetal-function was then converted to an aldehyde group by citric acid treatment to allow reaction with Dsg2 or N-Cad. Functionalized samples were stored at 4°C and used within five days. Measurements were performed with tips and mica from at least 2 different coating procedures. To detect possible homophilic interactions of Dsg2 and N-Cad, the AFM tip was lowered onto the mica surface and retracted again, and binding events were detected by constantly measuring the deflection of the cantilever as outlined before.⁵ 500 approach-retract cycles at 25 different positions on the mica were recorded for each of 4-6 cantilever/mica combination, yielding >2,000 force curves per condition. The AFM cantilever was moved in constant force mode with speed of 2 μm/s in a z-range of 600 nm and 0.1 s delay time on the mica. Measurements were performed in Hank's buffered saline solution (HBSS) at 37°C. The number of curves containing binding events was determined and divided by the total number of force curves obtained (termed binding frequency). Binding frequency before addition of L-Tryp, L-Phen, SP or TP was set to 100% and compared to the binding frequency after treatment.

Electron microscopy:

Cells were grown in 24-well plates and fixed with 1% glutaraldehyde in PBS for 1 h at room temperature. After three washing steps with PBS, cells were incubated with 2% osmiumtetroxid-solution for 1 h at 4°C. Afterwards samples were dehydrated through an ethanol series from 20 to 100% and finally embedded with epon. After trimming, ultrathin sections (60-80 nm) were cut with a diamond knife. Staining was performed with a saturated solution of uranyl acetate for 20 min and lead citrate for 6 min. Pictures were taken with the transmission electron microscope Libra 120 (Zeiss, Oberkochen, Germany).

Western blot analysis:

HL-1 cells grown for seven days in coated 24-well plates were washed with PBS and scratched on ice in SDS-lysis buffer (25 mM HEPES, 2mM EDTA, 25 mM NaF, 1% sodium dodecyl sulfate, pH 7.4). Lysates were sonicated, boiled at 95°C for 5 min and stored at -80°C until further use. Protein content was estimated using the BCA-method (Thermo Scientific, Waltham, USA, #23225). Protein samples were denatured in Laemmli buffer prior to separation on 10% SDS-polyacrylamide gels. Proteins were transferred on nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany) using the wet-blot method and blocked in 5% non-fat milk in TBS-T (20 mM Tris-base, 137 mM NaCl, 0.0475% Tween, pH 7.6) buffer. Primary antibody incubation was performed in 5% non-fat milk or bovine serum albumin in TBS-T according to manufacturer's instructions at 4°C over night. Species-matched peroxidase-conjugated secondary antibodies were incubated at room temperature for 2 h in 5% non-fat milk in TBS-T and developed using the ECL-method (0.5% luminol, 0.25% paracumaric acid, 0.03% hydrogen peroxide, 0.1 M Tris-HCl) for chemiluminescence protein band visualization. Band densitometry was performed using ImageJ software.

Langendorff-heart preparation:

Animal handling and sacrifice was in accordance with guidelines of the European Commission and local university regulations. Adult Balb/c or C57BL/6 wild-type mice were obtained from the institutes' animal facilities and sacrificed by cervical dislocation and the hearts were harvested. Hearts were immersed in filtered carbogen-gassed ice-cold modified Krebs-Henseleit buffer (118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 1.18 mM KH₂PO₄, 1.64 mM MgSO₄, 1.85 mM CaCl₂, 0.5 mM EDTA, 2 mM pyruvic acid, 5.55 mM glucose) supplemented with 2.75 KU/ml heparin. Heart beats stopped immediately and lung tissue was trimmed. The aorta was cannulated quickly and secured by a suture. The preparation procedure was performed in less than 5 min. Hearts were perfused in the retrograde Langendorff mode with heparin-free carbogen-gassed Krebs-Henseleit buffer supplemented with 18.8 nM (-)-norepinephrine at 37°C with 60 mmH₂O constant pressure using an ADInstruments (Spechbach, Germany) Langendorff apparatus equipped with LabChart7 software. Hearts resumed beating and continued to beat in a regular rhythm. Two-point electrocardiogram measurements were performed and chemicals were applied in phenol red-stained buffer. Coronary effluate was collected in one minute intervals and analyzed spectrophotometrically for phenol red staining at 485 nm to estimate the concentrations of L-Phen, L-Tryp or Iso, respectively. SP was applied with a perfusor adding a 0.8 mM solution at a pump speed corresponding to a twentieth of coronary flow, yielding a final concentration of 40 µM. Aortic pressure was measured with the help of a pressure transducer linked to a side arm of the aortic cannula. Exclusion criteria were preparation duration of more than 10 min before cannulation, sustained baseline arrhythmia, and survival time of the preparation of less than one hour.

Supplement Figures

Figure S1: Disruption of HL-1 monolayers induced by Dsg2-interference.

Fragmentation of the HL-1 monolayers in the liberase assay was monitored in an inverted microscope. L-Tryp caused a dose-dependent increase of fragment number, whereas L-Phen

treatment showed no difference compared with control. SP likewise induced fragmentation. TP had no effect on fragmentation on its own, however, rescued L-Tryp effects during co-incubation. Differences in illumination are caused by the meniscus of the enzyme solution in the cell culture well-plate.

References:

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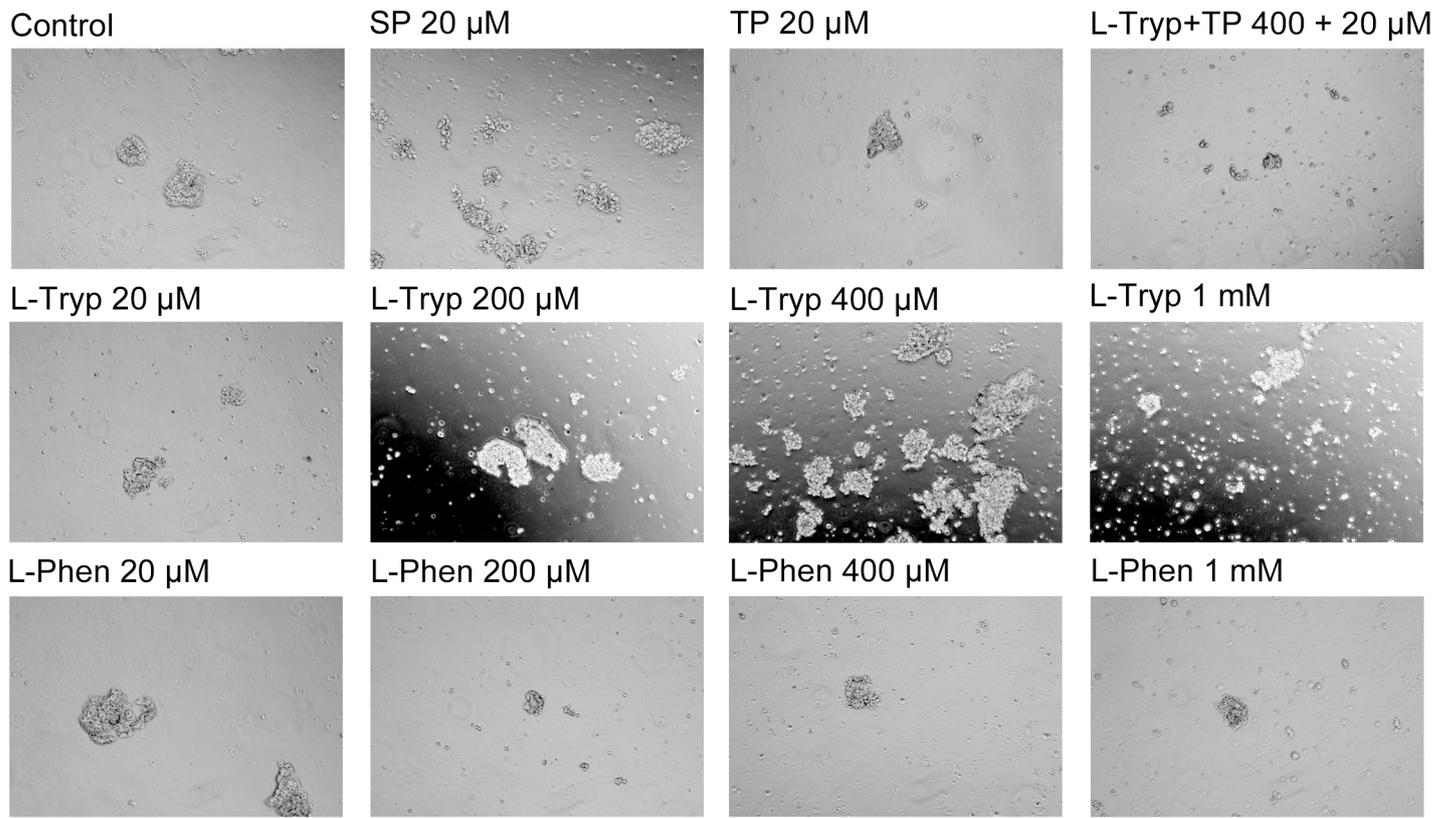


Fig S1