Supplementary Information

*Desmoglein 2 is a receptor for adenovirus serotypes 3, 7, 11, and 14.*

Hongjie Wang¹², ZongYi Li¹, Ying Liu¹, Jonas Persson¹, Ines Beyer¹, Thomas Möller³, Dilara Koyuncu⁴, Max R. Drescher¹, Robert Strauss¹, Xiao-Bing Zhang⁵, James K. Wahl III⁶, Nicole Urban⁷, Charles Drescher⁷, Akseli Hemminki², Pascal Fender⁸, André Lieber¹
**Fig S1. Tools for Ad receptor identification and competition studies for Ad attachment.**

a) Amino acid alignment of N-termini of Ad pentons. The protease cleavage site at aa 37/38 is marked in orange. b) Scheme of viral particles and particle components used in this study. PtDd possess 12 units containing penton base and trimeric fiber. BsDd only contain penton base. c) Competition of 3H-labeled Ad14, Ad14a, Ad11 and Ad35 virus attachment to HeLa cells after pre-incubation with Ad3 BsDd, PtDd, or antiCD46 antibodies (aCD46). Attachment in PBS-treated cells was taken as 100%. N=5. Notably, the finding that PtDd partially blocks Ad35 could be due to the physical proximity of DSG2 and CD46 in HeLa cells. d) Ad3 attachment studies as in c) after preincubation with both Ad3 fiber knob and BsDd (BsDd+Ad3K). The molar concentration of Ad3K was equal that of fiber knob within PtDd used for competition. e) Scheme of Ad3-GFP vector. The vector is based on wild-type Ad deleted for nt 29,892-30,947 to accommodate the CMV-GFP-polyA expression cassette. The lower panel shows restriction enzyme analyses of wt Ad3 and Ad3-GFP with the expected fragments. M: DNA size marker. f and g) Validation of polyclonal rabbit antibody against recombinant Ad3 fiber knob. f) Western blot. Ad knobs (Ad3K, Ad5K, 14K, 14aK, Ad11K, Ad35K) or PtDd were separated in PAG cells with (B) and without (UB) sample denaturation. Filters were incubated with antiAd3K antiserum and anti-rabbit-HRP antibodies. g) Inhibition of Ad attachment: 3H-labeled Ad3, Ad14 and Ad5 virus were incubated with PBS (white bars) or rabbit anti-Ad3K serum (grey bars) for one hour on ice, then added to HeLa cells for attachment studies.
**Fig. S2.** Binding of PtDd to immobilized DSG2 in the absence and presence of soluble DSG2 competitor protein.
Fig. S3. Receptor validation studies in cell lines.
a) DSG2 flow cytometry of HeLa cells at 48 hours after incubation with 37.5ng DSG2 siRNA (red) or control siRNA (green). The MFI of DSG2 in control and DSG2-siRNA treated cells are 169 and 23.8, respectively. b to d) Studies on 293 cells. b) 293 cells were transfected with 37.5ng or 1,000ng control or DSG2 siRNA and DSG2 protein levels were analyzed 48 hours later. c) siRNA treated 293 cells were infected with Ad vectors at an MOI of 20pfu/cell and GFP levels were measured 18 hours later. d) Competition of Ad infection by recombinant DSG2 protein. (e-g) Transduction of human cell lines. Human Burkitt’s B-lymphoma Raji cells (e), human T-lymphoma HH cells (f) and human mantle cell lymphoma Mino cells (g) were infected with Ad3-GFP and Ad5/35-GFP at increasing MOI and GFP expression was measured. N=3. Standard deviation was less than 10% for all data points. The upper panels show DSG2 levels in the corresponding cell lines.
Fig. S4. Confocal immunofluorescence analysis of epithelial cells.

a) Polarized colon cancer T84 cells. The XY planes (XY-1 and XY-2) on the left side were taken at different depths in the cell monolayer and show that DSG2 is localized at the distal end of tight junctions. b) Polarized colon cancer CaCo-2 cells. The lower right panel shows cells incubated with Cy3-Ad3 particles. The arrow marks an Ad3 particle (red) bound to DSG2 (green). c and d) Confocal microscopy of DSG2 (red) and the adherence junction protein E-cadherin (green) in polarized T84 (c) and breast cancer BT474 cells (d). e) Stacked XY sections of SAEC cells incubated with 1 µg/ml Cy5-PtDd for 15 minutes at room temperature. PtDd signals are purple. The thin arrow marks cell surface bound PtDd. The thick arrow labels cytoplasmic PtDd. All scale bars are 20 µm.
Fig. S5. DSG2 expression and Ad3 binding/infection in human platelets and granulocytes. a) Immunofluorescence analysis of human blood smears. Smears were stained for the platelet marker CD41. The left panel shows ~50 clustered platelets with CD41 (green) signals. A large fraction of platelets is also positive for DSG2 (red). Middle panel: For analysis of Ad binding, non-fixed blood smears were incubated with Cy3-labelled Ad3 at an MOI 400pfu/cell for 20 min at room temperature, washed with PBS, and then fixed for CD41 antibody staining. Ad3 appears as red dots associated with platelets. Right panel: Flow cytometry of platelets purified from peripheral blood shows that 12% of platelets (CD41+) are positive for DSG2. Further characterization of this platelet subfraction is required.

b) Immunofluorescence analysis of cytospins of human peripheral blood mononuclear cells (PBMC). Left panel: staining for the pan-leukocyte marker CD45 and DSG2. Double positive cells are labeled by arrows. Middle panel: PBMC were infected with Ad3-GFP at an MOI of 25pfu/cell and stained for DSG2 24 hours later. DSG2-positive, GFP-expressing cells are marked by arrows. Right panel: Granulocytes were isolated from Ad3-GFP transduced PBMCs using Magnetic Labeled Bead Cell Separation (MACS) for CD15-positive cells and subjected to flow cytometry for DSG2 and GFP. The graphs show that 33.2% of granulocytes express DSG2 and that this correlates with Ad3-GFP transduction, i.e. 30.5% of granulocytes were GFP positive.
**Fig. S6. Analysis of pathways triggered by PtDd.**

**a**) Pathways found to be deregulated at 12 hours after incubation of BT474 cells with PtDd compared to PBS (left panel) or BsDd (right panel) treatment. Genome-wide mRNA expression array data were analyzed by Pathway Express software. Shown are the numbers of deregulated genes in a given pathway as well as the predicted impact that deregulation of these genes has for the pathway. The impact factor of the entire pathway includes a probabilistic term that takes into consideration the proportion of differentially regulated genes on the pathway and gene perturbation factors of all genes in the pathway. (The gene perturbation factor reflects the relative importance of each gene for the pathway.) A pathway is considered activated when the expression of crucial stimulating genes is upregulated and/or the expression of inhibitory genes is downregulated.

**b**) Example of an activated pathway upon PtDd treatment (Phosphatidylinositol signaling). Genes that were found to be upregulated in arrays studies are highlighted in blue.
**Fig.S7. Effect of PtDd on mAb therapy.**

**a)** Ad3 and PtDd do not enhance killing of Her2/neu-negative MDA-MB-231 breast cancer cells by Herceptin. MDA-MB-231 breast cancer cells were incubated with 0.5µg/ml of BsDd or PtDd, or 2x10⁸ viral particles/ml of uv-inactivated Ad5 or Ad3 for 12 hours, followed by an incubation with Herceptin (15µg/ml) for 30min. Cell viability was measured 2 hours later by WST-1 assay from Roche Biosciences. Viability of PBS-treated cells was taken 100%.  

**b)** Ad3 and PtDd enhance killing of EGFR-positive colon cancer cells by Erbitux (anti-EGFR). LoVo cells (EGFR-positive) were incubated with 0.5µg/ml of PtDd for 12 hours, followed by incubation with Erbitux (15µg/ml) for 30min. Cell viability was measured 2 hours later by WST-1 assay. Viability of PBS-treated cells was taken 100%.  

**c)** Effect of DSG2 siRNA on adherence junctions of BT474 cells. Shown is claudin 7 staining of BT474 cells at day 2 after treatment with PBS or DSG2 siRNA. The scale bar is 40µm.

* p<0.05.
Supplementary Material and Methods

**Recombinant proteins:** Recombinant human Desmoglein-1 (DSG1) and Desmoglein-2 (DSG2) protein were from Leinco Technologies, Inc. (St. Louis, MO). Both rhDSG1 and rhDSG2 protein contain the full extracellular domain fused with human IgG1 on its C-terminal. The GST-tagged desmocollin 2 (DSC2) protein was purchased from Novus Biologicals, LLC (Littleton, CO).

**Inhibitors of metabolic pathways.** Wortmannin (PI3K inhibitor) and UO126 (ERK1/2 inhibitor) were purchased from Merck (Darmstadt, Germany). The following concentrations were used based on previous studies: Wortmannin (2.5 µM) and UO126 (5 µM). Controls were dilution buffers. Cells were incubated with inhibitors o/n.

**Antibodies.** The following antibodies were used for immunocytochemistry and flow cytometry studies: FITC conjugated mouse anti-CD46 (BD Biosciences, San Jose, CA), FITC conjugated rabbit anti-E-cadherin (BD Biosciences), polyclonal goat anti-DSG2 (R&D Systems, Inc, Minneapolis, MN), mouse mAb anti-DSG2 (clone 6D8) (Cell Sciences, Canton, MA), mouse mAb anti-DSG2 (clone AH12.2) (Santa Cruz Biotechnology Inc, Santa Cruz, CA), rabbit anti-Claudin 7 (abcam, Cambridge, MA), goat anti-Lipocalin 2 (abcam), mouse mAb anti-Her2/neu (abcam), mouse mAb anti-Vimentin (Sigma, St Louis, MO), mouse mAb anti-CD41 (abcam), rabbit anti-Laminin (DAKO, Denmark), FITC conjugated goat anti-adenovirus (Millipore Billerica, MA). Rabbit anti-phospho-P13K (p85/p55), anti-P13K (p85), anti-phospho-p44/42 MAPK, and anti-p44/42 MAPK were from Cell Signaling Technology (Boston, MA). Mouse mAb anti-GAPDH was from abcam. Anti-DSG2 mAbs 20G1, 7H9, 13B11, 10D2 and 8E5 were purified from hybridoma culture supernatant using HiTrap protein G (GE Healthcare, Piscataway, NJ) following the manufacturer’s protocol. IgG protein concentration was measured spectrophotometrically.

**Cell lines.** 293 (Microbix, Toronto, Ontario, Canada), HeLa (American Type Culture Collection, ATCC) were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine (Glu), 100 units/mL penicillin, and 100 µg/mL streptomycin (P/S). Human retinoblastoma line Y79 (ATCC HTB18) and human lymphoma cell lines Ramos (CRL-1596), Raji (ATCC CCL-86), Mino (ATCC CRL-3000), U937 (ATCC CRL-1593.2), Karpos-299 (DSMZ ACC31), HH (ATCC CRL-2105), and BJAB cells (obtained from Edward A. Clark, University of Washington) as well as erythroleukemia K562 cells (ATCC CCL-243) were cultured in RPMI 1640 supplemented with 10% FBS, Glu and P/S. Colon cancer T84 cells (ATCC CCL-248)) were cultured in a 1:1 mixture of Ham’s F12 medium and DMEM, 10% FBS, Glu and P/S. CaCo-2 (ATCC HTB-37) were cultured in DMEM 20% FBS, Glu and P/S. BT474 (ATCC HTB-20) is a Her2/neu-positive breast cancer cell line with epithelial cell features. BT-474-M1 is a tumorigenic subclone of BT474 (provided by Mien-Chie Hung, MD Andersen, Houston). Primary ovarian cancer cells from tumor biopsies were cultured in mammary epithelial growth medium (MEGM), containing 3µg/L hEGF, 5µg/L insulin, 5µg/L hydrocortisone, 26µg/L bovine pituitary extract, 25µg/L amphotericin B) (Lonza), supplemented with 10% FBS, 10µg/L ciprofloxacin, 5µg/L plasmocin, Glu, and P/S. SAEC cells were cultured in SABM pus supplements as recommended by Lonza (Walkersville, MD).

**Tissues.** Paraffin sections of normal human tissue were provided by the Department of Pathology, University of Washington. Peripheral blood mononuclear cells (PBMCs) from healthy donors were provided by Nora Disis (Tumor Vaccine Group, Department of Oncology, University of Washington).
**Ad3-GFP construction.** To construct Ad3-GFP, the genome of wild-type Ad3 (GB strain) was first rescued into a modified cosmid vector. The multiple cloning site between EcoRI and NheI in pWE15 (Stratagene, La Jolla, CA) was modified as follows: EcoRI- FseI- PmeI- XbaI- PmeI- FseI- HindIII- EcoRV (pWEA). Ad3 virus genomes were isolated from 50 µl purified wt Ad3 virus (2x10¹¹pfu/ml) and subjected to pronase digestion, phenol-chloroform purification, and ethanol precipitation. Virus DNA was dissolved in TE buffer and ligated with pWEA which was digested with PmeI. Ligation products were packaged into phages using Gigapack III plus Packaging Extract (Stratagene, La Jolla, CA) and propagated (pWEA-Ad3). The restriction enzyme digestion maps (HindIII and BglII) and sequencing data (using primers specific to the 5’ITR, 3’ITR and fiber) were in agreement with data from the Genbank (accession no. DQ086466). A ~2kb CMV-GFP-pA transgene cassette was inserted into the E3 regions of the Ad3 genome in front of the fiber gene by PCR cloning. As a result of this, the regions from 29,892 to 30,947 of the Ad3 genome (DQ086466) was replaced with the transgene cassette. The recombinant Ad3-GFP viral genome was released by FseI digestion and transfected into 293 cells using a standard calcium phosphate method. After a first round of rescuing on 293 cells, HeLa cells were infected with the cell lysate containing Ad3-GFP virus for further virus propagation.

**Mass-spectroscopy** was performed by Martin Sadilek at the University of Washington, Department of Chemistry MS Core as described earlier ².

**Competition attachment studies.** Adherent cells were detached from culture dishes by incubation with Versene and washed with PBS. A total of 1.8x10⁵ cells / tube was resuspended in 100 µl of ice-cold adhesion buffer containing ³H-labeled Ad at a multiplicity of infection (MOI) of 8,000 VP per cell. After 1 h of incubation at 4°C, cells were pelleted and washed twice with 0.5 ml of ice-cold wash-buffer (PBS, 1%FBS). After the last wash, the supernatant was removed and the cell-associated radioactivity was determined with a scintillation counter. The number of viral particles (VP) bound per cell was calculated by using the virion specific radioactivity and the number of cells. For competition studies, the 4.5 µg competitor (BsDd, PtDd, fiber knobs, fibers, antibodies) were allowed to attach for 60 min at 4°C in attachment buffer and non-bound knob removed by washing cells twice with PBS before cells were resuspended in attachment buffer containing ³H-labeled Ad. For competition studies with antibodies, HeLa cells were detached from culture plates by versene. A total of 1.8 x 10⁵ cells were incubated with 50 µg/ml of monoclonal antibody against DSG2 on ice for one hour, then 8,000 vp/cell of ³H-Ad3 were added.

**siRNA studies:** A total of 1x10⁵ HeLa were transfected with 1 µg DSG2 siRNA or control siRNA. Forty eight hours after siRNA transfection, cells were collected with versene and the attachment of ³H-Ad3 or ³H-Ad35 virus was analyzed as described above. Alternatively, forty eight hours after siRNA transfection, cells were infected with Ad3-GFP, Ad35-GFP and the first-generation vectors Ad5/35-GFP and Ad5-GFP at an MOI of 50 pfu/cells and GFP expression was analyzed 18 hours later.

**Cy3-labelling of Ads.** Purified Ad was labeled with Cy3 fluorochromes (Amersham CyTM Bis-Reactive Dye, GE Healthcare, Little Chalfont, UK) following the manufacturer’s protocol without modifications. The ratio between the volume of virus and labeling reagent was 1:9. To remove unbounded virus, the labeled virus was dialyzed against 10mM Tris-HCl pH7.5, 10mM MgCl₂, and 10% glycerol at 4°C overnight.

**Immunofluorescence/Confocal analyses.** Cells were cultured in 8 chamber glass slides (BD Falcon), washed twice with ice-cold PBS and then fixed with methanol/aceton (1:1 vol/vol) for 15 min at 4°C or with 4% paraformaldehyde for 30 min at 4°C. After fixation, cells were washed with PBS twice and
blocked with 500 µl PBS/2% dry-milk powder for 20 min at room temperature. Antibody staining was performed in 100 µl PBS for 90 min at 37°C or 4°C overnight. If needed, secondary antibodies directed against the appropriate host, were applied after 3 washes with PBS for 45 min at room temperature. After 3 washes with PBS, glass slides were mounted using VECTASHIELD with DAPI (Vector Labs). Photographs were taken with a Leica DFC300FX digital camera. Confocal images were taken on a Zeiss META confocal microscope using 40x or 100x oil lenses and Zeiss 510 software (Zeiss MicroImaging, Thornwood, NY).

For attachment studies with Cy3 labelled Ads, cells were mounted to the slide by cytopsin and incubated with Cy3 labeled Ad3 at an MOI of 100 pfu/cell for 20 min at room temperature, washed with PBS, then fixed with cold acetone and methanol. Studies with BT474 cells were performed in chamber glass slides. Blood smears were fixed with cold acetone and methanol.

Polarized cells in Transwell inserts were fixed with cold acetone/methanol for 15 min., blocked with 20% non fat milk and then stained with indicated antibodies.

**Immunohistochemistry on paraffin sections:** Paraffin sections of foreskin and colon were deparaffinized and rehydrated. Antigen retrieval was performed with Vector Antigen Unmasking solutions pH6.0 (Vector laboratories, Inc Burlingame, CA). Mouse anti-DGS2 antibody (3G132) (abcam) was diluted 1:10. Immunohistochemistry was performed using the Polink-2 HRP Broad Kit (Golden Bridge International, Inc. Mukilteo, WA) and DAB as a substrate.

**mRNA expression arrays and data analysis** were performed by the Functional Genomics Core at Center for Ecogenetics and Environmental Health, School of Public Health University of Washington. Total RNA was prepared using RNAeasy columns (Qiagen) and its quality assessed by Agilent 2100 bioanalyzer. Affymetrix Genechip® Human Gene 1.0 ST arrays were processed according to the manufacturer’s instruction. mRNA microarray data were processed and analyzed with Raw microarray data were pre-processed and normalized with Affymetrix® Expression Console™ Software using RMA normalization (http://affymetrix.com). Gene array data has been deposited at NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE24138.

**Pathway analysis:** Pathway-Express (PE) software first calculates a perturbation factor PF(g) for each input gene. This perturbation factor takes into account the (i) normalized fold change of the gene and (ii) the number and amount of perturbation of genes downstream from it. This gene perturbation factor reflects the relative importance of each differentially regulated gene. The impact factor of the entire pathway includes a probabilistic term that takes into consideration the proportion of differentially regulated genes on the pathway and gene perturbation factors of all genes in the pathway. The corrected p value is p value corrected for multiple comparisons. For pathways analysis Log2 fold changes of the array list were used.

**FITC-Dextran diffusion through monolayers of BT474 cells.** BT474 cells were seeded into PET track-etched membrane tissue culture inserts with 0.4 µm pore size (Falcon) and grown to confluence. After rinsing with PBS, cells were incubated with fresh DMEM without phenol red. FITC-dextran (4 kDa) (Fluka) at a concentration of 10 mg/ml in the presence of 0.5 µg/ml BsDd, PtDd or 2x10⁸ Ad particles/ml (added to the apical compartment). Paracellular flux was assessed in aliquots from the apical and basal chambers after 2h of incubation. The tracer partitioning was measured by fluorescence at excitation 492 nm and emission 520 nm. Permeability coefficients (PE) were calculated using the following formula:

\[ PE = \frac{[(\Delta C/A)/\Delta t]*VA}{S*\Delta CL} \]

where PE = diffusive permeability (cm/s), \( \Delta C/A \) = change of FITC- dextran concentration, \( \Delta t \) = change of time, VA = volume of the abluminal medium, S = surface area, \( \Delta CL \) = constant luminal concentration.
**Herceptin cytotoxicity assays.** 5x10⁴ BT474 cells/well were plated in triplicate in 96 well plates and grown to confluence. UV-inactivated Ad particles (2x10⁸ vp/ml = 56 ng virions/ml) or recombinant Ad proteins (500 ng/ml) were added to the medium. Twelve hours later, Herceptin (15 µg/ml) was added and cell viability was measured 2 hours later by an assay, which uses WST-1, a substrate that measures the metabolic activity of viable cells (Roche, San Francisco, CA). Three independent studies were performed. For the studies shown in Fig.5g, metabolic inhibitors were added o/n before incubation with Ad particles or recombinant proteins.

**Erbitux cytotoxicity assays:** 5 x 10⁴ LoVo cells/well were plated in triplicate in 96 well plates and grown to confluence. Recombinant BsDd or PtDd (500ng/ml) were added to the medium. Twelve hours later, Erbitux (15µg/ml) was added and cell viability was measured 2 hours later by WST-1 assay (Roche, San Francisco, CA). Three independent studies were performed.

**Statistical analysis:** All results are expressed as mean +/- SD. Statistical significance was evaluated using GraphPad Prism version 4.00c for Macintosh (GraphPad Software, San Diego, CA). Student’s T-test or ANOVA for multiple testing were applied when applicable. A p-value <0.05 was considered significant.

**References:**
1. Strauss, R., et al. Epithelial phenotype of ovarian cancer mediates resistance to oncolytic adenoviruses. *Cancer Research* **15**, 5115-5125 (2009).
2. Gaggar, A., Shayakhmetov, D. & Lieber, A. Identifying functional adenovirus-host interactions using tandem mass spectrometry. *Methods Mol Med* **131**, 141-155 (2007).