Galectin-3 Induces Clustering of CD147 and Integrin-β1 Transmembrane Glycoprotein Receptors on the RPE Cell Surface

Claudia S. Priglinger1,2*, Christoph M. Szober1, Siegfried G. Priglinger2, Juliane Mertl3, Kerstin N. Euler1, Marcus Kernt1, Gabor Gondi4, Jennifer Behler3, Arie Geerlof5, Anselm Kampik1, Marius Ueffing3,6, Stefanie M. Hauck3

1 Department of Ophthalmology, Ludwig-Maximilians-University, Munich, Germany, 2 Department of Ophthalmology, Allgemeines Krankenhaus der Stadt Linz, Linz, Austria, 3 Research Unit Protein Science, Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), Neuherberg, Germany, 4 Research Unit Gene Vectors, Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), Neuherberg, Germany, 5 Protein Expression and Purification Facility, Institute of Structural Biology, Helmholtz Center Munich, German Research Center for Environmental Health (GmbH), Neuherberg, Germany, 6 Centre of Ophthalmology, Institute for Ophthalmic Research, University of Tübingen, Tübingen, Germany

Abstract

Proliferative vitreoretinopathy (PVR) is a blinding disease frequently occurring after retinal detachment surgery. Adhesion, migration and matrix remodeling of dedifferentiated retinal pigment epithelial (RPE) cells characterize the onset of the disease. Treatment options are still restrained and identification of factors responsible for the abnormal behavior of the RPE cells will facilitate the development of novel therapeutics. Galectin-3, a carbohydrate-binding protein, was previously found to inhibit attachment and spreading of retinal pigment epithelial cells, and thus bares the potential to counteract PVR-associated cellular events. However, the identities of the corresponding cell surface glycoprotein receptor proteins on RPE cells are not known. Here we characterize RPE-specific Gal-3 containing glycoprotein complexes using a proteomic approach. Integrin-β1, integrin-α3 and CD147/EMMPRIN, a transmembrane glycoprotein implicated in regulating matrix metalloproteinase induction, were identified as potential Gal-3 interactors on RPE cell surfaces. In reciprocal immunoprecipitation experiments we confirmed that Gal-3 associated with CD147 and integrin-β1, but not with integrin-α3. Additionally, association of Gal-3 with CD147 and integrin-β1 was observed in co-localization analyses, while integrin-α3 only partially co-localized with Gal-3. Blocking of CD147 and integrin-β1 on RPE cell surfaces inhibited binding of Gal-3, whereas blocking of integrin-α3 failed to do so, suggesting that integrin-α3 is rather an indirect interactor. Importantly, Gal-3 binding promoted pronounced clustering and co-localization of CD147 and integrin-β1, with only partial association of integrin-α3. Finally, we show that RPE derived CD147 and integrin-β1, but not integrin-α3, carry predominantly β1,6-N-actyl-D-glucosamine-branched glycans, which are high-affinity ligands for Gal-3. We conclude from these data that extracellular Gal-3 triggers clustering of CD147 and integrin-β1 via interaction with β1,6-branched N-glycans on RPE cells and hypothesize that Gal-3 acts as a positive regulator for CD147/integrin-β1 clustering and therefore modifies RPE cell behavior contributing to the pathogenesis of PVR. Further investigations at this pathway may aid in the development of specific therapies for PVR.

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* E-mail: claudia.alge-priglinger@med.uni-muenchen.de

Introduction

It is well established that ligand binding and cell surface cross-linking of transmembrane proteins can lead to the assembly of large multicomponent protein complexes [1–3]. While in this respect protein–protein interactions have been well studied in the recent years, there is an increasing awareness that ligand binding to information stored in cell surface glycans can also lead to the assembly of large component protein complexes and modulate transmembrane signaling [4,5]. Figuring prominently in deciphering the information stored in the glycan complexes is the protein family of galectins. Galectins belong to the large family of lectins which bind to oligosaccharide complexes specifically via beta (β)-galactoside moieties. Among these the 30 kDa member Galectin-3 (Gal-3) is unique in that it is composed of a C-terminal carbohydrate recognition domain and an N-terminal non-carbohydrate-binding domain that facilitates its multimerization [6]. Gal-3 has been shown to function through both intracellular and extracellular actions. Related to its intracellular functions, Gal-3 has been identified as a component of heterogeneous nuclear ribonucleoprotein (hnRNP) [7], a factor in pre-mRNA splicing [8], and has been found to control cell cycle and prevent T cell apoptosis [9], whereas extracellular Gal-3 has been demonstrated...
Galectin-3 binds to CD147 and Integrin-β1 in RPE

Isolation of Human RPE Cells and Human RPE Cell Culture

RPE cells were isolated from human cadaver eyes obtained from the Eye Bank of the Department of Ophthalmology at the Linz General Hospital (Linz, Austria) and processed within four to 24 h after death. Donor age ranged from 27 to 79 years and none of the donors had a known history of eye disease. Methods for securing human tissue were humane, included proper consent and approval of the relatives, and complied with the Declaration of Helsinki. Human postmortem donor eyes were enucleated by an ophthalmologist according to the institutions standard operating procedures. The isolation of RPE cells from human cadaver eyes for scientific purposes was approved by the ethical committee of the Land Oberoesterreich. Human retinal pigment epithelium (RPE) cells were harvested from twenty post-mortem eyes following the procedure as described previously [35,36]. Primary RPE cells were subcultured and maintained in Dulbecco's modified Eagles medium (DMEM; Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS; Biochrom) at 37°C and 5% CO2. Primary human RPE cells of passage 3 to 7 were used for experiments. When indicated, different concentrations of galectin-3 (Gal-3) were included in the culture medium. For preparation of protein lysates the posterior poles from eyes of seven different donors were prepared as described above. RPE cells were released from Bruch’s membrane by gently pipetting ice cold phosphate buffered saline (PBS, pH 7.4) solution into the eye cup. The suspended RPE cells were transferred to a 35 mm2 petri dish and checked for cross contamination using a microscope.
suspensions were then transferred to a 2.0 ml microcentrifuge tube and centrifuged for 5 min at 8000 rpm. After centrifugation the supernatant was removed and replaced by RIPA cell lysis buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1% NP40; 0.5% deoxycholate; 0.1% SDS) containing an appropriate amount of protease inhibitors (Complete Mini, Roche, Mannheim, Germany). Further protein extraction was carried out as described above. Cell preparations were snap frozen in liquid nitrogen and stored at −70°C for future use.

**Tissue Samples**

Samples of PVR membranes were obtained from patients who were undergoing vitreoretinal surgery for PVR following rhegmatogenous retinal detachment. Before surgery, each participant gave its informed written consent. The procedure was documented in accordance with the guidelines of the institutional review board of the Department of Ophthalmology at the Ludwig-Maximilians-University Munich (Reference Number: IRB-GP-00142009) and was conducted according to the declaration of Helsinki. The collection of PVR membranes for scientific purposes was approved by the ethical committee of the Klinikum of the Ludwigm-Maximilians-University Munich (Permit Number: 331-09) and the ethical committee of the Land Oberoesterreich. Operations (n = 7) were carried out at the Department of Ophthalmology of the Ludwig-Maximilians-University (Munich, Germany) and at the Department of Ophthalmology, AKH Linz (Linz, Austria) by different surgeons, who used conventional three-port vitrectomy. Epiretinal and subretinal membranes were separated from the retina by peeling whole tissues. During the operation membranes were put into phosphate buffered saline (PBS, pH 7.4), then transferred to 4% formaldehyde and processed for embedding in paraffin.

**Human Galectin-3: Expression, Purification, Labeling and Quality Controls**

Human Gal-3 was cloned in the bacterial pETM-11 expression vector using restriction enzymes Ncol/HindIII, resulting in the fusion of the gene to an N-terminal His6-tag. pETM-11/Hgalactin3 was transformed into the *E. coli* strain BL21 (DE3) and cultured at 30°C in 2-L flasks containing 500 ml ZYM 5052 auto-induction medium [37] and 30 μg/ml kanamycin. Cells were harvested by centrifugation after reaching saturation, resuspended in 30 ml lysis buffer (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 10 mM MgSO4, 10 mg/ml DNaseI, 1 mg/ml lysosyme, 10 mM imidazole, 10 mM MgSO4, 0.2% (v/v) NP-40, 1 mg/ml lysosyme, 0.02% (v/v) 1-thioglycerol, pH 8.0), and lysed by sonication. The lysate was clarified by centrifugation (40,000 g) and filtration (0.2 μM). The supernatant was applied to a 5-ml HiTrap Chelating HP column (GE Healthcare, Munich, Germany), equilibrated in buffer A (50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 0.01% (v/v) 1-thioglycerol, pH 8.0) and incubated with 2 mg of purified Gal-3 for 1 hour at RT with 200 μg biotinamidohexanoic acid N-hydroxysuccinimide ester according to the manufacturers instructions (Sigma, Taufkirchen, Germany) followed by dialysis against PBS overnight. Biotinylation was confirmed by western blot analysis by detection with HRP-coupled streptavidin (Roche, Mannheim, Germany).

**Isolation of Galectin-3 Binding Proteins in Human RPE Cells by Co-immunoprecipitation**

Human RPE cells grown in 75 cm² tissue culture flasks were washed twice with ice-cold PBS, collected, and lysed at low stringency conditions in 1% Triton X-100 buffer to avoid dissociation of the protein complexes. For immunoprecipitation experiments used for protein complex identification by mass spectrometry, RPE cells were first washed in PBS reacted with 2 mM DTSSP, (3,3’-dithiobis[sulfosuccinimidylpropionate]; Sigma) followed by addition of 20 mM Tris pH 7.5 for a 15 minute quenching reaction and one wash in PBS before cell lysis. For complete lysis the cellular extracts were allowed to incubate in the presence of an appropriate amount of protease inhibitor cocktail (Complete Mini; Roche) on a rotational shaker for 1 hour at 4°C. After centrifugation for 30 minutes at 16,000 g in the cold the protein content in the supernatant was determined using the Bradford protein assay reagent (Biorad, Munich, Germany). The solubilized protein fraction was then incubated with ProteinG Sepharose beads (ProteinG Sepharose Fast Flow beads, GE Healthcare) in equilibration buffer (50 mM Tris, pH 7.2; 150 mM NaCl) for 1.5 hours at 4°C. The supernatant was then collected and 375 μg of precleared RPE cell lysate was incubated with ProteinG sepharose beads in a total volume of 750 μl of the presence of 2 μg monoclonal anti-human Gal-3 (Novoceastra, Newcastle, UK), or monoclonal anti-human CD147 (Novus Biochemicals, Cambridge, UK), or monoclonal anti-human integrin-α3 (MCA669HT; AbD Serotec, Oxford, UK) for 2 hours on a rotational shaker in the cold. As appropriate negative control in parallel experiments cellular lysates were incubated with ProteinG™ beads in the presence of isotype-matched, pre-immune mouse IgG. The suspension was then transferred to microspin columns (GE-Healthcare), washed several times with 1% Triton X-100 buffer, and bound protein complexes were eluted with 2 μg Cevalmin buffer. The immunoprecipitated complexes were then gently denatured at 70°C for 5 minutes, centrifuged at 1,000 x g for 2 minutes and the supernatant was further processed for protein identification using mass spectrometry or subjected to SDS PAGE. Each co-immunoprecipitation was repeated at least eight times using RPE cell lines derived from different human donors.

**Mass Spectrometric Protein Identification and Quantification**

For mass spectrometry, eluates were precipitated with chloroform/methanol, dissolved in ammonium bicarbonate buffer supplemented with Rapigest (Waters, Milford, MA, USA), followed by reduction using dithiothreitol (Merck, Darmstadt, Germany), and alkylation in iodoacetamide (Merck). Proteins were
then subjected to trypic digest as described before [38] and peptide samples were acidified with TFA to a final concentration of 5% to precipitate the Rapigest surfactant. For LC-MS/MS analysis peptides were loaded automatically on a trap column and separated by HPLC on an UltiMate nano-LC-system (LC Packings, Bensheim, Germany) on an analytical column with a 120 min gradient at 300 nL/min directly into the mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific, Schwerte, Germany). From the MS prescans, ten most abundant peptide ions were selected for fragmentation and during fragment analyses, high resolution MS spectra were acquired with a mass range from 200 to 1500 m/z.

For relative quantification, the acquired spectra were loaded into the Progenesis LC-MS software (version 2.5, Nonlinear Dynamics, Newcastle Upon Tyne, UK) for label free quantification and analyzed as described previously [38,39]. Briefly, after feature detection and exclusion of features with only one charge or more than eight charges, all raw abundances were normalized across samples to minimize variation introduced by experimental variations and then MS/MS spectra were exported as Mascot generic file (mgf). Peptide identification was performed with Mascot search engine (version 2.2, Matrix Science Inc., Boston, MA, USA) in the Ensembl Human database (Release 69, October 2012; 100607 sequences) with 10 ppm mass tolerance for peptides and 0.6 Da mass tolerance for fragments ions, carbamidomethylation set as fixed modification and oxidation of methionines and deamidations of asparagines and glutamines allowed as variable modifications. A Mascot-integrated decoy database search calculated a false discovery rate of <1.5%, using an ion score cut-off of 30 and a significance threshold of p<0.01 for all searches. Peptide assignments were re-imported into the Progenesis software. After summing up the abundances of all peptides allocated to each protein, the ratios of each protein as compared to the non-immune mouse IgG negative controls were calculated based on these abundances. All identified proteins with their respective raw and normalized abundances are listed in table S1.

Western Blot Analysis
Immunoprecipitated proteins were separated on SDS-PAGE gels. After wet blotting onto a polyvinyl difluoride membrane (GE Healthcare), membranes were blocked in 5% nonfat dry milk and then incubated with mouse monoclonal anti-human Gal-3 (Novocasta, 1:250), or mouse monoclonal anti-human CD147 (Novus Biochemicals, 1:1000), or a goat polyclonal anti-human integrin-β3 (SantaCruz, 1:500), or a mouse anti-human integrin-β1 (BD Biosciences, 1:1000) in 5% skim milk in TBS-T (0.1% Tween 20, 50mM Tris pH 7.4, 5mM NaCl) and allowed to react overnight at 4°C. Appropriate secondary horseradish peroxidase-coupled antibodies (JacksonImmuNoResearch, Newmarket, UK) were used in a dilution of 1:15000. Protein signals were then visualized using ECL Plus enhanced chemiluminescence kit (GE Healthcare) and signals were captured on Hyperfilm ECL (GE Healthcare). All experiments have been repeated at least six times using RPE cells derived from different donors.

Immunocytochemistry and Immunohistochemistry of RPE Cell Cultures
RPE cells were grown on glass coverslips and maintained in DMEM supplemented with 10% FCS until they reached subconfluence. When indicated, cells were then treated with galectin-3 (40 μg/mL) in DMEM for 30 minutes at 37°C. After three washes in PBS, cells were fixed in 4% paraformaldehyde for 5 minutes on ice. After another three washes in PBS, specimens were blocked with 1% BSA in PBS for 1 hour at room temperature, and incubated with polyclonal rabbit anti-human Gal-3 (dilution 1:100; Santa Cruz, Heidelberg, Germany), and monoclonal mouse anti-human CD147 (dilution 1:100; Novus Biochemicals), and either monoclonal rat anti-human integrin-β3 (dilution 1:100; dilution 1:100; clone 9G7, see below), or a monoclonal rat anti-human integrin-β1 (dilution 1:50; clone A1B2; developed by C.H. Damsky and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, USA) for 2 hours at RT. After three washes in PBS nuclei were counterstained with 1 μg/mL Hoechst 33342 (Sigma) and cells were incubated with Alexa-Fluor 488™-conjugated anti-mouse IgG, or Alexa-Fluor 568™-conjugated anti-rabbit IgG, or Alexa-Fluor 647™-labelled anti-rat IgG, or the respective combinations as secondary antibodies for 1 hour at RT in the dark (diluted 1:2,000; -all obtained from Invitrogen, Karlsruhe, Germany). All antibodies were diluted in PBS containing 1% BSA. Cells incubated with non-related isotype IgG or secondary antibodies alone served as negative controls. Images were generated on a fluorescence microscope using the apotome mode (Axio Imager Z1Zeiss, Göttingen, Germany) and the Axioscope 4.6 software (Zeiss). Monoclonal antibody 8G7 (rat IgG2a) against integrin-β3 (CD49c) was generated by injecting cell membrane particles (exosomes) of the human lung cancer cell line A549 into Lou/c rat. The fusion was performed using standard procedures. Hybridoma supernatants were tested by flow cytometry on A549 cells. The specificity of the mAb was determined by immunoprecipitation followed by mass spectrometry.

Flow Cytometry
Measurement of RPE cell surface expression of candidates was performed on FACS Canto II with FACS Diva 6.1.3 software (both BD Biosciences, Heidelberg, Germany). Cell staining was performed in 96 well round-bottom plates with 2x10⁵ cells per well. Cells were either incubated with biotinylated recombinant Gal-3 (50 μg/mL) or a monoclonal anti-human CD147 antibody (dilution 1:10; Novus Biochemicals) or a monoclonal anti-human integrin-β3 antibody (clone 9G7, dilution 1:5) or a monoclonal anti-human integrin-β1 (clone J1B1A, Chemicon, dilution 1:50) for 1 hour at 4°C. Biotinylated Gal-3 was then stained with avidin-FITC (dilution 1:200; Southern Biotech, Birmingham, Alabama, USA), CD147 and integrin-β1 with secondary goat anti-mouse IgG1 PE antibody (dilution 1:300; Southern Biotech) and integrin-β3 with secondary mouse anti-rat IgG2A FITC-antibody (dilution 1:200; TIB173; ATCC, Wesel, Germany) in staining buffer for 30 min at 4°C. Cells were kept at 4°C in staining buffer with 1% PFA until processing. 10000 cells were measured per staining. For blocking experiments, cells were first incubated with anti-human CD147, anti-human integrin-β1 or anti-human integrin-β3 antibody as described above to block CD147, integrin-β1 and integrin-β3 sites on the cell surface, followed by incubation with 50 μg/mL biotinylatedGal-3 for 1 hour at 4°C and avidin-FITC as described. Isotype controls were performed for Gal-3 and all antibodies used, respectively. In order to evaluate the carbohydrate dependence of Gal-3, binding cells were pre-incubated with 100 mM β-lactose (Sigma), the haptenic sugar to block carbohydrate-dependent galectin-binding, or 100 mM of the non-specific sugar sucrose for 20 minutes before Gal-3 was added.

Lectin Affinity Analyses
Protein (600 μg) from RPE cell lysates prepared as described above in RIPA buffer (50 mM Tris-HCl pH 7.4; 150 mM NaCl; 0.1%(w/v) SDS; 0.5%(w/v) sodium deoxycholate; 1%(v/v) NP-
40) was precleared for 1 hour with 15 μl of ProteinG agarose beads (SantaCruz Biotechnology) to reduce non-specific binding, and then incubated with 15 μl agarose-conjugated PHA-L, or agarose-conjugated ConA, or beads alone (all obtained from Vector Laboratories, Burlingame, CA, USA) for 1 hour at 4°C on a rotational shaker. The beads were washed 3 times in RIPA cell lysis buffer and once in PBS. Bound proteins were eluted by boiling in SDS-PAGE sample buffer without β-mercaptoethanol, separated by 12% SDS-PAGE, transferred to PVDF membranes, and analyzed by western blot analysis using the same antibodies as described above.

**Immunohistochemical Staining of Tissue Sections**

For immunohistochemistry PVR membranes were fixed in 4% formaldehyde, embedded in paraffin and cut into sections of 8 μm each. Sections were then set onto glass slides and incubated at 42°C for 48h to prevent washing off the sections during the process of immunohistochemical staining. In order to remove the paraffin and to rehydrate the sections, they were first rinsed with xylene (once for 5 min) and isopropanol (twice for 5 min), followed by ethanol (96% and 70% - each for 5 min) and finally millipore water (5 min). Heat antigen retrieval of Gal-3 was performed by boiling the sections in citrate buffer pH 6.1 (Dako, Hamburg, Germany) for 15 min. Prior to incubation with the primary antibody, sections were blocked for 40 min in a humid environment with 1% BSA in phosphate buffered saline-tween (PBS +0.1% Tween20), in order to prevent non-specific staining. Sections were then incubated with biotinylated Gal-3 (5 μg/ml diluted in PBS-T +1% BSA) or anti-human CD147 (1:100, Novus Biochemicals), or anti-human integrin-β1 (dilution 1:50; Developmental Studies Hybridoma Bank, clone A1B2) overnight at 4°C followed by incubation with Avidin-FITC diluted in PBS-T +1% BSA (dilution 1:200, Invitrogen, Darmstadt, Germany) or the respective secondary antibodies as described above for one hour at RT in the dark. Cell nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI; dilution 1:1000, Invitrogen). Finally, the sections were mounted with glass coverslips using fluorescence mounting medium (Dako). Fluorescent images were recorded with an Axio Imager M1 or Z1 and the Axio Vision 4.6 software (both from Zeiss, Göttingen, Germany) at a total magnification of 40-fold.

**Results**

**CD147 and Integrin-β1 are Major Gal-3 Binding Proteins in Cultured RPE Cells**

In order to identify cell surface ligands specific for RPE cells we used cultured human RPE cells as a model system for early PVR. When human RPE cells are cultured on plastic, they escape growth arrest and fail to maintain a differentiated morphology. For this reason this provides a well-accepted in vitro model for the fibroblast-like phenotype of RPE cells as found in PVR [40]. Potential Gal-3 interacting proteins were isolated by co-immunoprecipitation experiments followed by identification with liquid chromatography coupled to mass spectrometry (LC-MS/MS).

In an attempt to find a receptor for exogenous Gal-3 on the cell surface we in a first step lysed RPE cells with prior addition of 12.5–25.0 μg/mL Gal-3. These concentrations are just above the bottom level of the dose response curve for inhibition of RPE attachment as determined in previous experiments [29] and were chosen in order to avoid oversaturation of ligands and non-specific binding. Despite of thorough washing before incubation with anti-Gal-3 coupled beads and a series of low stringency washes to avoid dissociation of the Gal-3 containing protein complexes subsequent mass-spectrometry detected an excess of Gal-3 in the eluate with qualitatively unreliable peptide identifications for candidate protein interactors (data not shown). For this reason we next co-immunoprecipitated endogenous Gal-3 from whole RPE cell lysates without exogenously added Gal-3. Mass spectrometric analysis of solubilized proteins that bound with high affinity to the sepharose-bound monoclonal anti-Gal-3 antibody resulted in the unequivocal identification of twenty five proteins (Table 1). Gene ontology (GO) annotations assigned thirteen (52%) to a nuclear or cytoplasmic location (hnRNP A3, 40s ribosomal protein S27-like protein, 60S ribosomal protein L8, UPF0027 protein C22orf28, nucleosome assembly protein 1-like 4, protein LSM12 homolog, nuclear fragile X mental retardation-interacting protein 2, interleukin enhancer-binding factor 3, nucleophosmin, POTE ankyrin domain family member E, RNA binding motif protein X-linked-like 1, histone H1.3, RNA-binding protein FUS), five (20%) to the cytoskeleton or structural components (septin-11, myosin-11, keratin 3, tropomyosin β-chain, gelsoilin), two (8%) proteins were predominantly secreted (α2 macroglobulin, galectin-3 binding protein), and five (20%) proteins were attributed to the cell membrane (CD147, integrin α3, integrin β1, monocarboxylate transporter-4, galectin-3). The data are compiled in Table 1 (subcellular localization; biological functions of the candidate ligands are also included.

As expected we in this approach predominantly found ligands for exogenously expressed Gal-3, consistent with the majority of the receptor proteins identified being assigned to the nucleus or cytoplasm. Based on our previous observation that exogenous Gal-3 binds to the RPE cell surface in a carbohydrate-dependent manner and thereby appears to influence RPE-cell adhesion our interest was focused on RPE-specific Gal-3 glycoprotein receptors on the RPE cell surface. Therefore proteins attributed to cytoskeleton, nucleus, or cytoplasm were excluded from further studies. Of the remainder all except for MCT-4 were described glycoproteins (Table 1) and therefore potential galectin-3 interacting proteins. Out of these all except for α2-macroglobulin, which is essentially located in the extracellular matrix (ECM), have been reported to play a role in cellular adhesion. Integrin-α3 and integrin-β1 have previously been shown to be binding partners of Gal-3 [19], validating the suitability of our approach.

The highest enrichment factor in the Gal-3 immunoprecipitation, however, together with a high Mascot confidence score (104) was observed for CD147, a transmembrane glycoprotein which has not previously been described in context with Gal-3. CD147 plays a role in several biological processes, including cell adhesion, plasma membrane integration of monocarboxylate transporters, inflammation, or angiogenesis [41,42], among others. It is heavily glycosylated and may therefore be a reasonable ligand for Gal-3.

In order to confirm the presence of Gal-3 in CD147 containing protein complexes reciprocal experiments were performed. Gal-3 immunoblotting confirmed the presence of the 30 kDa band representing Gal-3 (Fig. 1A, lane 2) in anti-CD147 immunoprecipitates, but not in the control immunoglobulin immunoprecipitates (Fig. 1A, lane 3). In parallel experiments integrin-β3 protein complexes were precipitated. To our surprise, western blot analysis failed to detect anti-Gal-3 reactive components in the eluted fraction from the integrin-β3 immunoprecipitation (Fig. 1B, lane 2), but Gal-3 could be recovered from re-immunoprecipitation experiments with an antibody specifically recognizing the integrin-β1 subunit (Fig. 1C, lane 2). These data suggest a probable interaction of Gal-3 with CD147 and integrin-β1 in cultured RPE cells, whereas the interaction with integrin-α3 may be rather indirect or less stable. This is also supported by the use of mild crosslinking before cell lysis in the mass spectrometry approach.
Table 1. Isolation of RPE cell proteins with high affinity binding to Gal-3.

| Accession number | ratio Gal-3/ neg control | Gene name | GO term “Plasma membrane or extracellular region” | Glycosylation | Protein name | Subcellular localization | Biological process |
|-------------------|---------------------------|-----------|------------------------------------------------|---------------|--------------|--------------------------|--------------------|
| ENSP00000376309   | Infinity                  | HNRNPA3   | Heterogeneous nuclear ribonucleoprotein A3 (hnRNP A3) | nucleus       | HNRNPA3      | nucleus                  | RNA trafficking and splicing |
| ENSP00000331019   | Infinity                  | RPS27L    | 60S ribosomal protein S27-like protein              | nucleus       | RPS27L       | cytoplasm                | DNA repair, apoptosis |
| ENSP00000333769   | Infinity                  | BSG       | CD147 (Basigin)                                     | Golgi membrane, cell membrane, endoplasmatic reticulum | CD147 | Golgi membrane, cell membrane, endoplasmatic reticulum | Tissue invasion, MMP-production, angiogenesis, targets MCTs to plasma membrane |
| ENSP00000264893   | Infinity                  | SEPT11    | Septin-11                                           | cytoskeleton  | SEPT11       | cytoskeleton             | Cytokinesis         |
| ENSP00000262584   | 363.2                     | RPL8      | 60S ribosomal protein L8                            | cytoplasm     | RPL8         | cytoplasm                | RNA binding, translation |
| ENSP00000215038   | 279.6                     | C22orf28  | UPF0027 protein C22orf28                            | cytoplasm     | C22orf28     | cytoplasm                | RNA-binding         |
| ENSP0000007722    | 124.3                     | ITGA3     | Integrin alpha-3                                     | cell membrane | ITGA3        | cell membrane            | Adhesion, migration, signalling |
| ENSP00000300356   | 54.2                      | MYH11     | Myosin-11                                           | cytosol, myosin filament | MYH11 | cytosol, myosin filament | Stress fiber component, smooth muscle contraction |
| ENSP000002154301  | 48.5                      | LGAL3     | Galectin-3                                           | nucleus, cytoplasm, plasma membrane, secreted | LGAL3 | nucleus, cytoplasm, plasma membrane, secreted | Adhesion, migration, chemotaxis, differentiation |
| ENSP00000303351   | 39.2                      | ITGB1     | Integrin beta-1                                      | cell membrane | ITGB1        | cell membrane            | Adhesion, migration, signalling |
| ENSP00000436488   | 26.9                      | NAP1L4    | Nucleosome assembly protein 1-like 4                | cytoplasm     | NAP1L4       | cytoplasm                | Nucleosome assembly |
| ENSP00000252242   | 26.0                      | KRT5      | Keratin, type II cytoskeletal 5                      | intermediate filament | KRT5 | intermediate filament | Structural component |
| ENSP00000354219   | 25.9                      | TPM2      | Tropomyosin beta chain                               | cytoskeleton  | TPM2         | cytoskeleton             | Actin-filament binding, motility |
| ENSP00000293406   | 15.9                      | LSM12     | Protein LSM12 homolog                               | nucleus, cytoplasm | LSM12 | nucleus, cytoplasm | RNA metabolism |
| ENSP0000040888    | 12.5                      | GS8       | Gelsolin                                            | cytoplasm, cytoskeleton | GS8 | cytoplasm, cytoskeleton | Actin-modulating protein |
| ENSP00000376150   | 11.0                      | SLC16A3   | Monocarboxylate transporter 4                        | cell membrane | SLC16A3      | cell membrane            | Energy metabolism |
| ENSP00000253388   | 10.9                      | NUFIP2    | Nuclear fragile X mental retardation-interacting protein 2 | cytoplasm, nucleus | NUFIP2 | cytoplasm, nucleus | RNA-binding |
| ENSP00000250241   | 10.7                      | ILF3      | Interleukin enhancer-binding factor 3               | cytoplasm, nucleus | ILF3 | cytoplasm, nucleus | Transcription |
| ENSP00000323929   | 8.9                       | A2M       | Alpha-2-macroglobulin                               | cytosol, secreted | A2M | cytosol, secreted | Protease inhibitor |
| ENSP00000296930   | 7.5                       | NPM1      | Nucleophosmin                                       | nucleus, nucleolus | NPM1 | nucleus, nucleolus | RNA-binding, centrosome cycle |
| ENSP00000439819   | 7.2                       | POTEE     | POTE ankyrin domain family member E                 | cytoplasm     | POTEE | cytoplasm | ATP-binding |
| ENSP00000318415   | 5.2                       | RBMXL1    | RNA binding motif protein, X-linked-like 1          | cytoplasm     | RBMXL1 | cytoplasm | RNA-binding |
| ENSP00000244534   | 5.0                       | HIST1H1D  | Histone H1.3                                        | nucleus       | HIST1H1D | nucleus | Transcription, nucleosome condensation |
| ENSP00000254108   | 4.7                       | FUS       | RNA-binding protein FUS                              | nucleus       | FUS | nucleus | DNA synthesis |
| ENSP00000262776   | 4.1                       | LGALS3BP  | Galectin-3-binding protein                           | secreted      | LGALS3BP | secreted | Cell adhesion, ECM component |

Galectin-3 Binds to CD147 and Integrin-b1 in RPE
while the reverse immunoprecipitation experiments were performed without crosslinking.

CD147, but not Integrin-α3, Serves as Major Galectin-3 Counter-receptor in Dedifferentiated RPE

In order to further verify our findings from the co-immunoprecipitation experiment and to validate our novel Gal-3 binding protein CD147, flow cytometry as a measure for the nature of the interaction of exogenous Gal-3 and CD147 on the RPE cell surface was performed. This experiment provided us with five important results: first, we confirmed that both CD147 and exogenous Gal-3 are detected on the surface of dedifferentiated RPE cells (Fig. 2A). Second, co-incubation of cells with β-lactose, an inhibitor of carbohydrate-dependent Gal-3 binding but not with sucrose (used as osmolarity control), reduced Gal-3 binding to the RPE cell surface, confirming a carbohydrate-dependent Gal-3 cell surface interaction (Fig. 2A, right panel). Third, in support of CD147 being a direct Gal-3 interaction partner, binding of Gal-3 to RPE cells was substantially reduced when cells were pre-incubated with a neutralizing antibody to CD147 (Fig. 2B, left panel), but not by a non-relevant control IgG (Fig. 2B, right panel). Fourth, pre-incubation with a neutralizing antibody to integrin-β1 partially blocked binding of Gal-3 to the RPE cells (Fig. 2C, left panel) while blocking of integrin-α3 binding sites with neutralizing anti-integrin-α3 failed to reduce binding of Gal-3 (Fig. 2C, right panel). These findings further support the notion that CD147 is a major counter-receptor for Gal-3 on RPE cells, and that binding is also promoted by integrin-β1, while integrin-α3 may be rather an indirect receptor for Gal-3. Fluorescence micrographs illustrating the blocking of the Gal-3 binding sites by anti-CD147 are also presented (Fig. 2D).

CD147 Co-localizes with Gal-3 and Integrin-β1 on RPE Cells in Culture

To corroborate the presence and distribution of CD147 on the surface of dedifferentiated RPE, cells were stained with an antibody against CD147. Fluorescence microscopy revealed prominent reactivity of the plasma membrane, consistent with even distribution all over the RPE cell surface (Fig. 3B,C). Staining of cultured RPE cells with an antibody against Gal-3 showed that the expression of the lectin was abundant intracellular and predominantly perinuclear, which is in agreement with findings in other human cell types [34,44] (Fig. 3A). Consistent with this, the merged image of CD147 and Gal-3 reveals only very weak co-localization (yellow) of the two proteins at the RPE cell surface (Fig. 3C). Thus, in dedifferentiated RPE, CD147 is evenly distributed over the RPE cell surface, which is in clear contrast to the polarized distribution found in native RPE [34].

To determine whether CD147 also co-localizes with exogenously added Gal-3 cultured RPE cells were treated with 40 μg/ml Gal-3 for 15 minutes before fixation and immunostaining. In addition to a prominent staining for CD147 at the plasma membrane cells exposed to Gal-3 exhibited a distinct punctuate localization (yellow) of the two proteins at the RPE cell surface (Fig. 3D). Further, co-incubation of cells with α3 integrin, Gal-3, and β1 on RPE cells was substantially reduced when cells were pre-incubated with integrin-β1 neutralizing antibody (Fig. 2C, left panel). These findings further support the notion that CD147 is a major counter-receptor for Gal-3 on RPE cells, and that binding is also promoted by integrin-β1, while integrin-α3 may be rather an indirect receptor for Gal-3. Fluorescence micrographs illustrating the blocking of the Gal-3 binding sites by anti-CD147 are also presented (Fig. 2D).

Figure 1. Gal-3 associates specifically with CD147 and integrin-β1 in cultured RPE cells. Whole cellular protein lysates were prepared by treatment of RPE cells with 1% Triton X-100 in PBS. (A) CD147 containing immunocomplexes were precipitated from 375 μg of proteins using 2 μg of a monoclonal anti-human CD147 antibody (lane 2), or a non-related isotype control (lane 3). Equal amounts of immunoprecipitation eluates, or whole cellular lysate (lane 1) were separated by SDS-PAGE and analyzed by immunoblotting using a monoclonal anti-Gal-3 antibody. (B, C) Cell lysates were processed as described for CD147 and precipitated using 2 μg of a monoclonal antibody recognizing specifically the integrin-α3 (B, lane 2) and integrin-β1 (C, lane 2) subunits, respectively. Note that the eluates precipitated with anti-CD147 (A; lane 2) and anti-integrin-β1 (C; lane 2) as well as the total cell extract (A; lane 1) contain the anti-Gal-3 reactive band at 30 kDa. In contrast, no anti-Gal-3 reactive component was recovered from cell extracts precipitated with anti-integrin-α3 (B; lane 2) and with the isotype control (A,B,C; lane 3). Experiments have been repeated at least eight times using cultured human RPE cells from different donors. Representative blots are shown. doi:10.1371/journal.pone.0070011.g001
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**A**

![Graph showing the binding of Gal-3 to CD147 and positional shift](image1)

**B**

![Graph showing the binding of Gal-3 to CD147 and positional shift](image2)

**C**

![Graph showing the binding of Gal-3 to integrin β1 and positional shift](image3)

**D**

![Immunofluorescence images](image4)

Galectin-3 (Gal-3) interacts with CD147 and integrin-β1 in retinal pigment epithelial (RPE) cells, as demonstrated by flow cytometry and immunofluorescence studies. The graphs illustrate the changes in cell-surface-bound Gal-3 upon interaction with these molecules, and the images show the localization of Gal-3 and the antibodies used in the experiment.
cells, staining for integrin-β1 was similarly evenly distributed as integrin-α3 staining (data not shown), while in Gal-3 treated cells the staining pattern for integrin-β1 presented in a pronounced punctuate pattern indicating clustering (Fig. 3P). This pattern was similar to that of Gal-3 (Fig. 3G,M) and CD147 (Fig. 3H,N) with complete signal co-localization between exogenous Gal-3 and CD147 (Fig. 3O, yellow), as well as between Gal-3 and integrin-β1 (Fig. 3R, yellow), and between integrin-β1 and CD147 (Fig. 3Q, white). In accordance with the results from reciprocal immunomunoprecipitation as well as from blocking experiments in FACS analyses, this may indicate that Gal-3 associates with αβ3β1 integrin via binding to integrin-β1 or CD147 and to a lesser extent via direct interaction with the α3 subunit. Controls with secondary antibody alone were negative for fluorescent signal (data not shown).

RPE-derived CD147 and Integrin-β1 Contain a High Amount of β1,6-branched N-Glycans

Since our experiments point to CD147 and integrin-β1 as the major Gal-3 interacting proteins at the surface of dedifferentiated RPE cells, we next sought to investigate the state of N-glycosylation state of these proteins, because binding of Gal-3 critically depends on the presence of β1,6-N-acetyl-D-glucosamine (GlcNAc) branched glycans [13–15]. In order to determine whether RPE-derived CD147 and integrin-β1 contain the respective N-glycosylation patterns, pull down experiments were conducted using agarose-bound plant lectins. Previous studies have shown that leukoagglutinin of Phaseus vulgaris (PHA-L) specifically interacts with β1,6-GlcNAc-branched N-glycans [15]. Concanavalin A (ConA), which binds high mannose-type oligosaccharides, was used as a control lectin. As shown in Figure 4A, reactivity for CD147 was prominent in glycoprotein complexes precipitated with PHA-L, while the binding to ConA was absent (Fig. 4A). A similar reactivity was found for integrin-β1, the other major Gal-3 binding protein. (Fig. 4C), while western blots with anti-integrin-α3 recovered both, a high-mannose type and a β1,6-GlcNAc-branched N-glycan fraction (Fig. 4B). These data suggest that CD147, integrin-α3, and integrin-β1 in RPE cells carry the preferred N-glycans for Gal-3 binding. The predominance of β1,6-branched complex N-glycans on CD147 and integrin-β1 makes them therefore suitably glycosylated for carbohydrate-dependent interaction with Gal-3.

Localization of Exogenous Gal-3, CD147 and Integrin-β1 in Human PVR Membranes

PVR membranes typically contain a predominance of proliferative and migratory RPE cells as well as other cell types at various stages of epithelial-to-mesenchymal transition (transdifferentiation) [20]. Having found a functional interaction of exogenous Gal-3 with CD147 and integrin-β1 in dedifferentiated RPE cells in vitro, we were interested whether cells in specimen of PVR may encounter exogenous Gal-3. For this purpose, paraffin sections of human PVR membranes were treated with recombinant Gal-3 and subsequently stained with an antibody specific for Gal-3. Immunohistochemistry revealed specific staining in all PVR membrane sections studied (Fig. 5A), indicative of binding of Gal-3 to the fibronectin-rich ECM found in PVR membranes. Staining for Gal-3 was present throughout the ECM of the PVR membrane, occurred in a patchy pattern, and appeared to encircle the cell bodies, so that in this setting RPE cells in PVR membranes could encounter exogenous Gal-3. In order to determine, whether the RPE-specific transmembrane counter-receptors for Gal-3, CD147 and integrin-β1, are found within PVR membranes, sections were stained for CD147 and integrin-β1. In correspondence to our in vitro findings, immunohistochemistry revealed a diffuse staining of the cell bodies for CD147 (Fig. 5B), whereas integrin-β1 appeared in a distinct and punctuate pattern (Fig. 5C). All control sections incubated without the primary antibody were unstained (data not shown). These findings suggest that an interaction between exogenously added Gal-3 and CD147 and integrin-β1 may also occur under in vivo conditions.

Discussion

The overall goal of the present study was to define and validate Gal-3 interacting proteins on the surface of dedifferentiated RPE cells. This study identified CD147 and integrin-β1 as major Gal-3 interacting proteins on RPE cells and provides evidence that CD147 is a novel functional RPE transmembrane receptor for Gal-3. CD147 was isolated together with integrin-β1 by co-immunoprecipitation with Gal-3, and its identity was determined by mass spectrometry and confirmed by western blot of co-immunoprecipitation experiments. Evidence of a Gal-3/CD147 and Gal-3/integrin-β1 interaction at the RPE cell surface was further obtained from inhibition studies, where pre-incubation with anti-CD147 antibodies as well as anti-integrin-β1 antibodies abrogated binding of Gal-3 to the RPE cells. Exposure of RPE cells to exogenous Gal-3 lead to a redistribution of CD147 and integrin-β1 via interaction with Mga5-modified N-glycans on the protein backbones, indicative of receptor clustering.

Searching for RPE-specific Gal-3 ligands by a combined approach of co-immunoprecipitation with quantitative mass spectrometry yielded a number of proteins comprising intracellular as well as extracellular located proteins. Identification of intracellular proteins most likely correlates to the abundant expression of Gal-3 in the cytoplasm and the nucleus of proliferating cells and may reflect its contribution to mRNA splicing or cell-cycle control (reviewed by Liu et al. [46]. However, with respect to the cell surface binding of exogenous Gal-3 and its interference with ERK-MAPK activation in dedifferentiated RPE cells [29], ligands allocated to the plasma membrane were the focus of our study.
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- exogeneous Gal-3

Gal-3

CD147

Gal-3 + CD147

Integrin α3

Integrin α3 + CD147

Gal-3 + Integrin α3

+ exogeneous Gal-3

Gal-3

CD147

Gal-3 + CD147

Integrin α3

Integrin α3 + CD147

Gal-3 + Integrin α3

Integrin β1

Integrin β1 + CD147

Gal-3 + Integrin β1
Various co-immunoprecipitation experiments in different cell types have shown previously that Gal-3 associates with several cell surface glycoproteins, including MUC-1, LAMPS, the receptors for EGF, TGF-β, and VEGF, but also CD98, CD45 and CD71 [2,12,14,17,18,47], among others. As described elsewhere in more detail, galectins can interact with integrins, namely Gal-3 was reported to interact with αβ3, α1β1, α5β1, or αβ3 integrin in other cell types [10–13,19]. The finding that in our study Gal-3 was pulled down together with integrin-α3 and integrin-β1 would fit well into the overall theme of a Gal-3/αβ3β1-integrin interaction [19]. However, although we used low stringency conditions in the re-immunoprecipitation experiments to avoid dissociation of the protein complexes, we could not detect significant amounts of Gal-3 in integrin-α3 eluates, and in addition we observed only partial co-localization of integrin-α3 with Gal-3 in immunocytochemistry experiments. Finally, blocking of integrin-α3 binding sites by specific antibodies failed to prevent Gal-3 binding to the RPE cell surface in flow cytometry. This was in contrast to the experimental observations for the integrin-β1 subunit: Gal-3 could be recovered from the subunit-specific integrin-β1 immunoprecipitates, blocking of integrin-β1 on RPE cell surface partially abrogated Gal-3 binding and integrin-β1 completely co-localized with exogenous Gal-3 on the RPE cell surface, suggesting that in RPE cells Gal-3 directly associates with the integrin-β1 subunit of the αβ3 integrin heterodimer, whereas the association with integrin-α3 appears to be indirect or weaker. These findings are in some contrast to a recent report by Saravanan et al. [19] showing that in a different cell type, corneal epithelial cells, Gal-3 induces lamellipodia formation via interaction with the α2 subunit of the αβ3β1-integrin heterodimer, exclusively. However, these discrepancies could derive from differences in the experimental approaches as well as be due to the different cell types studied. Saravanan et al. did not validate the Gal-3/integrin-α3 association by reciprocal experiments and the role of integrin-β1 in the interaction was not investigated, thus not necessarily excluding a direct interaction between Gal-3 and integrin-β1. Furthermore, it is well established, that Gal-3 influences cellular events in a cell-type specific manner, most likely dependent on varieties in the glycan profile [16].

The novel finding of the present study is that Gal-3 can associate with CD147 [48], a widely distributed, highly glycosylated 44–45 kDa cell-surface protein with two immunoglobulin domains. Gal-3/CD147 association was established by means of reciprocal co-immunoprecipitation, and confirmed by FACS analysis and cell surface co-localization experiments.

The human CD147 protein has originally been named for its extracellular matrix metalloproteinase induction (EMMPRIN) activity and is identical to the M6 leukocyte activation antigen [49]. Also, CD147 is highly homologous to a rat molecule named OX-47 [50] or CE9 [51], the mouse basigin [52] or gp42 [53] molecule, and the chicken HT7 [54], neurothelin [55] or 5A11 antigen [56]. CD147 expression is often elevated on tumor cells [57] [58], and it is implicated in various inflammatory disease states, including atherosclerosis, rheumatoid arthritis or chronic liver disease [59,60]. CD147 on tumor cells stimulates MMP production by stromal cells, thereby leading to extracellular matrix degradation [61], elevated tumor growth and metastasis. Stimulation of CD147 promotes angiogenesis through hypoxia-inducible factor (HIF)-2α-mediated upregulation of VEGFR-2 and the soluble isomers of VEGF in endothelial cells [62], but it can also influence angiogenesis indirectly by stimulating fibroblasts to secrete urokinase-plasminogen-activator in a paracrine fashion [63]. CD147 has also been shown to regulate lymphocyte responsiveness and it appears to be essential for cyclophilin-dependent signaling related to chemotaxis of immune cells [42] and adhesion [64]. Interestingly, a monoclonal antibody screen for αβ3 integrin-associated proteins revealed a robust association of the structurally similar αβ3 and αβ3 integrin, but not αβ1 or...
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As a transmembrane glycoprotein, CD147 forms homooligomers in both heterotypic and homotypic cell-cell interactions. These homophilic associations evident as cell surface clustering of CD147 are necessary to induce MMP-production and HIF-2α-mediated regulation of soluble VEGF and VEGFR-2 expression [59,62,66]. The mechanisms ruling over association/dissociation of CD147 homodimers however remain largely elusive. However, there is evidence that association of caveolin-1 with CD147 leads to decreased CD147 cell surface clustering, thus inhibiting CD147-induced MMP production [3]. Caveolin-1 therefore appears to promote dissociation of CD147 homo-oligomers. The existence of such dissociation regulators implies that there might also exist receptors that trigger association of CD147 molecules. Our observation that exogenous Gal-3 induces clustering of CD147 and integrin-β1 may indicate that multivalent Gal-3 triggers cross-linking of CD147 and integrin-β1 and induces the recruitment of the two glycoproteins to receptor microdomains.

CD147 cell surface clustering and the MMP-inducing functions of CD147 are critically dependent on its level of N-glycosylation, namely on its content of complex α1,6-branched N-glycans. It has long been suggested that a lectin-like regulator of CD147 function exploiting its high level of glycosylation may exist [41]. Using PHA-L, a plant lectin with a fine specificity for β1,6-GlcNAc-branched N-glycans, we detected a high level of complex β1,6 branched N-glycans on CD147 and β1-integrin isolated from dedifferentiated RPE cells, supporting the hypothesis that Gal-3 is an inducer of CD147 clustering.

The functional relevance of Gal-3/CD147/integrin-β1 association in RPE cells is unclear. The co-localization with integrin-β1 suggests that Gal-3 may be part of the CD147/integrin-β1 complex [1]. Clustering of CD147 and integrin-β1 together with a complete overlay of the two glycoproteins upon addition of Gal-3 further underscores this assumption. So far we have found that the inhibitory effect of Gal-3 on the adhesion of dedifferentiated RPE cells to extracellular matrix was not altered by anti-CD147 antibodies, while blocking of integrin-β1 reduced both, RPE attachment and binding of Gal-3 to the RPE cell surface (unpublished data). Also anti-CD147 antibodies did not alter the attachment rate of RPE cells when compared to untreated controls, excluding a direct role of CD147 in RPE cell adhesion. Furthermore, preliminary evidence from our study suggests that the Gal-3/CD147/integrin-β1 association is not related to cell adhesion, since we also observed the glycoprotein clustering on adherent cells. This is supported by findings from Berditchevsky et al. [1], who described an interaction between α3β1 integrin and CD147, but were also not able to delineate a functional relevance of this association in cell adhesion.

However, it is perhaps more than a coincidence that Gal-3, CD147 and integrin-β1 have been linked to increased malignancy in a number of tumors [57,58,67–71], and that independently from each other all three have been found to promote angiogenesis.
which the physical association between CD147, integrin-

Clearly, further studies will be needed to determine the extent to

which accompanies profound phenotypic alterations such as
dedifferentiation [29,76], would fit well into the theme of the

pathogenesis of the disease. As compared with other mechanisms

for affinity/avidity regulation, glycan-protein interactions may be

particularly well suited for mediating the prolonged changes in cell

behavior that accompany profound phenotypic alterations such as

those observed during epithelial-to-mesenchymal transition of the

RPE in PVR. Our findings may therefore represent a potential

mechanism for glycosylation-dependent and Gal-3 meditated

clustering of cell-membrane receptors in dedifferentiated RPE

cells and may contribute to explain the cellular processes observed

in the pathogenesis of PVR. Further investigations at this

pathway may lead to a better understanding of PVR and aid in

development of therapeutic agents targeting the glycosylation

pattern of dedifferentiated RPE cells.

**Supporting Information**

**Table S1 Relative abundances of proteins identified by mass spectrometry.**

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**Author Contributions**

Conceived and designed the experiments: CSP SMH AG SGP. Performed

the experiments: CS KE JB JM AG SGP. Analyzed the data: CSP

SMH JM JB CS AG SGP. Contributed reagents/materials/analysis tools:

CSP SMH AG SGP. Wrote the paper: CSP SMH CS.

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