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ORIGINAL ARTICLE

Cyr61 activates retinal cells and prolongs photoreceptor survival in rd1 mouse model of retinitis pigmentosa

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Abstract

Subretinal injections with glial cell line-derived neurotrophic factor (GDNF) rescue morphology as well as function of rod cells in mouse and rat animal models of retinitis pigmentosa. At the same time, it is postulated that this effect is indirect, mediated by activation of retinal Müller glial (RMG) cells. Here, we show that Cyr61/CCN1, one of the secreted proteins up-regulated in primary RMG after glial cell line-derived neurotrophic factor stimulation, provides neuroprotective and pro-survival capacities: Recombinant Cyr61 significantly reduced photoreceptor (PR) cells death in organotypic cultures of Pde6b^{rd1} retinas. To identify stimulated pathways in the retina, we treated Pde6b^{rd1} retinal explants with Cyr61 and observed an overall increase in activated Erk1/2 and Stat3 signalling molecules characterized by activation-site-specific phosphorylation. To identify Cyr61 retinal target cells, we isolated primary porcine PR, RMG and retinal pigment epithelium (RPE) cells and exposed them separately to Cyr61. Here, RMG as well as RPE cells responded with induced phosphorylation of Erk1/2, Stat3 and Akt. In PR, no increase in phosphorylation in any of the studied proteins was detected, suggesting an indirect neuroprotective effect of Cyr61. Cyr61 may thus act as an endogenous pro-survival factor for PR, contributing to the complex repertoire of neuroprotective activities generated by RMG and RPE cells.

Keywords: neuroprotection, retinal degeneration, RMG, RPE.

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Retinitis pigmentosa (RP) refers to a group of inherited retinal degenerations (RD) in which mutations that perturb critical physiological functions of photoreceptors (PR) or retinal pigment epithelial (RPE) cells lead to progressive visual loss. The genes affected by mutations leading to RP cover very diverse functions in the retina including components of the visual transduction cascade, renewal and shedding of the rod outer segments, visual cycle and retinol metabolism (van Soest *et al.* 1999). As a result of the high heterogeneity in genes causative of RP, gene-specific therapy of RD may be restricted

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Abbreviations used: BCA, bicinchoninic acid; BDNF, brain-derived neurotrophic factor; BSA, bovine serum albumin; CNS, central nervous system; CNTF, ciliary neurotrophic factor; CX3CL1, fractalkine; Cyr61/CCN1, cysteine-rich protein 61; DHA, docosahexaenoic acid; DM, n-Dodecyl β-D-maltoside; DPBS, Dulbecco's phosphate-buffered saline; FGF-1, fibroblast growth factor 1; FGF-2, fibroblast growth factor 2; GDNF, glial cell line-derived neurotrophic factor; GM-CSF, granulocyte–macrophage colony-stimulating factor; GS, glutamine synthetase; HSPG, heparan sulphate proteoglycans; ICC, immunocytochemistry; IGFBP-3, insulin-like growth factor-binding protein 3; IL-2, interleukin 2; IL-6, interleukin 6; JAK, Janus kinase; LIF, leukaemia inhibitory factor; MAPK, mitogen-activated protein kinase; mRMG, mouse RMG; NGS, normal goat serum; NPD1, neuroprotectin D1; NSI, normalized signal intensity; ONL, outer nuclear layer; Pde6b^{rd1}, C3H/HeJ mice; PFA, paraformaldehyde; PGF, placenta growth factor; PN, post-natal; PR, photoreceptor cells; RD, retinal degenerations; RGC, retinal ganglion cells; RMG, retinal Müller glial cells; ROP, retinopathy of prematurity; RPE, retinal pigment epithelium; RP, retinitis pigmentosa; TF, tissue factor; VEGF, vascular endothelial growth factor.

to only a subset of affected patients. In contrast to gene therapy, trophic factor delivery can hardly ever be curative, but offers a very important advantage. Trophic factor application may provide PR protection irrespective of the underlying gene defect. Neuroprotective and trophic factors are generated endogenously and, as a critical part of central nervous system microenvironments, on a life-long basis, securing the long lifespan of central nervous system neurons. If given exogenously, they may have the potential to slow down PR death in RD and may therefore preserve residual vision. Glial cell line-derived neurotrophic factor (GDNF) has been shown to support PR survival in various rodent experimental models of RD (Frasson *et al.* 1999; McGee Sanftner *et al.* 2001; Buch *et al.* 2006; Dong *et al.* 2007; Gregory-Evans *et al.* 2009; Dalkara *et al.* 2011; Lipinski *et al.* 2011; Touchard *et al.* 2012). However, our previous work indicates that PR do not possess receptors to directly bind GDNF molecules, and the major GDNF-receptive cells in porcine retina are retinal Müller glial (RMG) cells, which then transmit the neuroprotective effect of GDNF to PR (Hauck *et al.* 2006). As monitored by changes in cellular RNA levels, GDNF-stimulated RMG cells produce and release many potentially neuroprotective factors, among them potentially those that transduce GDNF-mediated protection of PR.

To systematically define RMG-derived molecules that support PR functional rescue, we previously applied a transcriptome approach to screen for GDNF-induced transcripts in mouse retinal tissue (Del Rio *et al.* 2011). This led to the finding that expression of only a few transcripts increases after GDNF stimulation, including osteopontin, which promotes PR survival *in vitro* as well as *ex vivo* in Pde6b^{rd1} (*rd1*) retinal explants (Del Rio *et al.* 2011). As a survey of transcriptional changes from the whole retinal tissue may not be sensitive enough to reflect changes happening in a single retinal cell type, where RMG transcriptional response to GDNF may be masked by a large amount of transcripts from other cell types, we chose a targeted approach based on a protein array and tested for a pre-defined panel of 53 candidate proteins to detect differences in secretion after GDNF stimulation. Here, we show that cysteine-rich heparin-binding protein 61 (Cyr61, synonyms: insulin-like growth factor-binding protein 10 or CCN family member 1) features pro-survival activity on PR in retinal explants of *rd1* mouse model for RP.

Materials and methods

Animals

Human glial fibrillary acidic protein-enhanced green fluorescent protein transgenic mice of C57BL6 background (kindly provided by Magdalena Götz, Institute of Stem Cell Research, Helmholtz Zentrum Muenchen, Germany) were employed for RMG isolation and cultures. Retinal explants were performed from C3H/HeJ mice (Pde6b^{rd1},*rd1*) (Jackson Laboratories, Bar Harbor, ME, USA). The day of birth was defined as post-natal day (PN) 0. Animals were maintained with free access to water and food in an air-conditioned room on a 12-h light–dark cycle. All experiments were carried out in accordance with applicable German laws, with the European Council Directive 86/609/EEC, and with ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Photoreceptors were isolated from adult porcine eyes, provided by a local slaughterhouse.

Materials

Calcein (Invitrogen, Carlsbad, CA, USA), Cyr61 (R&D Systems Inc., Minneapolis, MN, USA), complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), DM (n-Dodecyl β-D-maltoside; Sigma, Munich, Germany), GDNF (Peprotech, Hamburg, Germany), Hoechst 33342 (Molecular Probes, Leiden, The Netherlands), papain (EC 3.4.22.2; Worthington, Lakewood, NJ, USA), phosphatase inhibitor cocktails 2 and 3 (Sigma), propidium iodide (Life Technologies, Carlsbad, CA, USA), R16 serum-free medium (Invitrogen Life Technologies, Paisley, UK), Tissue Tek (Sakura Finetek, Zoeterwoude, Netherlands), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Roche Diagnostics, Mannheim, Germany).

Cell culture

All experiments were performed using primary cell cultures: mouse retinal Müller glial [mouse RMG (mRMG)] enriched cultures were obtained from adult GFAP-eGFP/C57BL6. For biochemical analysis of Cyr61-activated pathways, primary porcine PR, primary porcine RMG cells and primary porcine RPE cells were used.

Mouse RMG cells were isolated from GFAP-eGFP/C57BL6 eyes, employing a panning method (Hauck *et al.* 2003) with modifications. Shortly, retinas were dissected and treated with 2.2 units of activated papain (EC 3.4.22.2; Worthington) for 15 min. Thereafter, the papain activity was stopped with Dulbecco's Modified Eagle's medium (Dulbecco's modified Eagle's medium (DMEM); GIBCO, Invitrogen, Paisley, UK), containing 10% fetal bovine serum (FBS; GIBCO, Invitrogen, Paisley, UK), and tissue was carefully triturated into single cells. Cells were washed once and then plated on 12 well plates (BD Falcon, Franklin Lakes, NJ, USA) using complete medium: DMEM containing 10% FBS, and 1% penicillin/streptomycin (GIBCO, Invitrogen, Paisley, UK) in 5% CO_2 at 37°C and cultured for 7 days (d7). One day prior to stimulation of mRMG, complete medium was exchanged to serum-free medium. The next day new medium containing GDNF (0.1 μg/mL) was applied for an additional 24 h. Afterwards, supernatants were removed, filtered (cut-off 0.2 μm) to remove residual cells and large cellular debris. Cells were lysed in 100 μL of lysis buffer [1% DM in 1% Tris Buffer Saline, TBS-T; 50 mM Tris-HCl pH7.5, 150 mM NaCl containing 5% of Tween-20 (Sigma)], including complete protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3. Supernatants and cell lysates were stored at −80°C until further analyses.

Porcine PR were isolated and cultured as described before (Hauck *et al.* 2008), with the following modifications. The eyes were enucleated within 5 min after death of the animals and kept on ice in CO_2-independet medium until further use. The eyeballs were disinfected and the retina was isolated. Afterwards, the major blood vessels were cut from the retina and the remaining tissue was cut into small pieces and distributed into 15 mL Falcon tubes (BD Falcon). To dissociate the retina, 2.86 units of activated papain

were added to the tissue and kept in 37°C for 12 min. After this time DMEM-F12 (GIBCO, Invitrogen, Paisley, UK) with 10% FBS and 1 mg/mL DNase (#D4263; Sigma) were added. After remaining tissue pieces settled, supernatant containing released PR were collected to a new 50 mL falcon. PR cells were sedimented by centrifugation (150 *g* for 5 min), re-suspended in DMEM/F-12 containing 10% FBS and plated on 6 cm dishes (Thermo Scientific, Roskilde, Denmark). To control the viability and purity of PR cell fraction, representative aliquots from each PR preparation were additionally plated on a 6 cm dish or 96 well-plate. The day following isolation, PR cells were washed and the cells were starved for 2 h in serum-free medium DMEM/F-12 containing 1% penicillin/streptomycin. Next, cells were stimulated with 1 μg/mL Cyr61 for different time points, or were left in serum-free medium (western blot controls for each time point and immunocytochemistry controls). After experiment the cultures were lysed with radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL pH 7.4, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 0.5% Sodium Deoxycholate, 1% NP-40) supplemented with complete protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3. The total protein content was measured using bicinchoninic acid (BCA) protein assay (bicinchoninic acid, Thermo Scientific, Rockford, IL, USA) and the samples were stored in −80°C until further analysis. To control purity of PR fraction, at the day of the experiment, separately plated aliquots of PR were fixed in 4% paraformaldehyde for 15 min at 24°C, washed with Dulbecco's phosphate-buffered saline (DPBS, GIBCO, Invitrogen, Paisley, UK) and incubated in blocking solution [10% normal goat serum, 1% bovine serum albumin (BSA), 0.5% Triton-X in 0.1 M phosphate buffer (PB; 0.1 M sodium phosphate dibasic, 0.1 M sodium phosphate monobasic, pH 7.4)]. Primary and secondary antibodies were diluted in antibody buffer (3% normal goat serum, 1% BSA and 0.5% Triton-X in 0.1 M PB). The cells were stained for recoverin (AB5585, 1 : 3000, Millipore, Billerica, MA, USA), which in porcine retina is located only in PR (data not shown) (Guduric-Fuchs *et al.* 2009; Ghosh and Arner 2010; Johansson *et al.* 2010). As the presence of RMG in the PR fraction can disturb the result of the experiment, the culture was stained for glutamine synthetase (1 : 1000, BD Transduction Lab, Heidelberg, Germany), a marker for glial cells (Figure S2). After primary antibody, cells were incubated with Alexa Fluor 488-labelled secondary antibody (1 : 1000, Life Technologies) and Hoechst 33342 (1 : 5000) for staining of nuclei. Viability of cells was controlled with calcein (stains living cells; Life Technologies) and propidium iodide (stains dead cells; Life Technologies, Figure S1). PR images were acquired at 100- or 200-fold magnification using Leica DM IRE2 fluorescent microscope.

To isolate porcine RMG, the retinal tissue was dissected as described above and the primary cell culture was prepared as described before (Hauck *et al.* 2008). Briefly, small pieces of the retina were washed with Ringer's solution and incubated with 2.2 units of activated Papain for 40 min in 37°C for tissue dissociation. Enzyme activity was stopped by addition of DMEM containing 10% fetal calf serum. 160 units of DNAse were added and the tissue was further dissociated by gentle trituration. Dissociated cells were centrifuged (150 *g* for 5 min) and plated directly onto cell culture plates. The next day, non-attached cells were removed by gentle agitation and cells were cultured for up to d11 to achieve > 75% confluence. Prior to an experiment, cells were washed and starved in serum-free medium for 3 h. Afterwards, cells were stimulated with 1 μg/mL Cyr61 for indicated periods of time. Next, supernatant was discarded and cells were lysed with RIPA buffer supplemented with complete protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3 and kept on ice for 30 min with occasional vortexing. At the end, cells were centrifuged (16 000 *g* for 30 min) and stored in −80°C for further analysis. Protein content of the lysates was determined with BCA protein assay (Thermo Scientific, Rockford, IL, USA). Samples were stored in −80°C until further analysis.

Porcine primary RPE cells were isolated as described before (Cordeiro *et al.* 2010), with modifications. Briefly, porcine eyes were disinfected, cut around the iris and the vitreous body including the neuroretina was removed. The obtained eyecup with RPE cells still attached to the inner surface was incubated with 1 mM EDTA in DPBS for 15 min at 24°C to discard leftovers of RMG and PR outer segments. Next, the eyecups were incubated with dissociation buffer (1 mM EDTA in DPBS, 260 mM L-Cys, 100 μg/mL BSA, 2.86 units of activated papain) for 25 min in 37°C. RPE cells were released by gentle buffer agitation, collected and centrifuged (150 *g* for 5 min). Re-suspended cells were plated directly onto six well-plates. Cells were cultured up to d10 to achieve > 75% confluence. Prior to an experiment, cells were washed and starved in serum-free DMEM medium for 2 h followed by 1 μg/mL Cyr61 treatment for indicated periods of time. Next, the medium was removed and cells were lysed with RIPA buffer supplemented with complete protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3 and kept on ice for 30 min with occasional vortexing. Eventually, lysates were centrifuged (16 000 *g* for 30 min) and stored in −80°C for further analysis. Protein content of the lysates was determined with BCA protein assay. Samples were stored in −80°C until further analysis.

Purity of all cell fractions (RMG, PR, RPE) was additionally proved with western blot technique. Representative aliquots from isolated primary cell fractions were blotted on one membrane and stained for recoverin (PR marker, AB5585, 1 : 2000, Millipore, Billerica, MA, USA), vimentin (RMG marker, 1 : 500, V6630, Sigma) and RPE65 (RPE marker, sc-32893, 1 : 400, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (data not shown).

Mouse cytokine membrane array

The detection of expression differences in GDNF-treated mRMG d7 supernatants and lysates were performed with Proteome Profiler™ Mouse Angiogenesis Array (cat. nr ARY015, R&D Systems, Wiesbaden- Nordenstadt, Germany) according to the manufacturers' protocol. Supernatants and lysates from mRMG were mixed with a cocktail of biotinylated detection antibodies, and incubated overnight on nitrocellulose membranes with antibodies spotted in duplicates for each protein and on nitrocellulose membranes. Signal intensities at each capture spot directly correspond to the amount of captured protein. Array image files were analysed using the image analysis software -Image J (NIH Image, National Institutes of Health, Bethesda, MD, USA). The pixel density of each spot was determined resulting in a protein value (Pv). One number was assigned to each protein (p = 1, 2,. . .) on an array. Each protein was represented by two spots (a,b) per array (Pv_n, Pv_m where n = 1a, 2a,. . ., m = 1b, 2b,. . .). Background value (Bcv) was subtracted from positive control (Pc) (mean value from three pairs of control spots in three corners of an array) as well as from each Pv.

Normalized signal intensity (NSI) for each spot was determined from calculation NSIn = (Pv_n-Bcv)/(Pc-Bcv) and NSIm = (Pv_m-Bcv)/(Pc-Bcv). Then for each protein the ratio GDNF/ctr was calculated from mean value (averaging NSIn and NSIm values per protein) on arrays incubated with experimental samples, divided by the respective mean values of NSI on control arrays. The experiment was performed in 3 (for supernatant) and 2 (for lysate) independent biological repetitions.

Organotypic retinal cultures

Organotypic retinal cultures were performed as described before (Del Rio *et al.* 2011) with slight modifications. Briefly, two types of experiments were conducted: short-time [explanted post-natal day (PN) 5 and cultivated 6 days *in vitro* (DIV6)] as well as long-term (PN5 DIV12) retinal explant cultures. Retinas with RPE attached obtained from 5-day-old (PN5) Pde6b^{rd1} animals were isolated as described previously (Caffe *et al.* 2001; Del Rio *et al.* 2011). Shortly, eyes of PN5 animals were enucleated, transferred into an aseptic environment and incubated in R16 serum-free medium containing 36 units of proteinase K (EC 3.4.21.14; MP Biomedicals, Santa Ana, CA, USA) at 37°C for 15 min. Then the eyes were transferred to R16 medium containing 20% of FBS for 5 min to stop proteinase K activity. Thereafter, the anterior segment, lens, vitreous, sclera and choroids were removed, the retina was cut at four edges and explants were flattened on a polycarbonate membrane (Corning Incorporated, Corning, NY, USA). Explants were incubated in 5% CO_2 at 37°C in R16 nutrient medium which was replaced every second day (first application of Cyr61 was DIV2 for both types of experiments). Experimental explants were exposed to 0.5 μg/mL (short-term retinal explant culture) or 1 μg/mL (long-term retinal explant culture) of human recombinant Cyr61. Untreated retinal explants served as a control. Explants were fixed in 4% paraformaldehyde and then cryoprotected with increasing gradient of sucrose diluted in 0.1 M PB (pH 7.4): for 1 h in 10% sucrose at 24°C, 1 h in 20% sucrose in at 24°C followed by overnight incubation in 30% sucrose in 4°C. Explants were embedded in Tissue Tek (Sakura Finetek, Zoeterwoude, Netherlands) and cryosectioned using a cryotome (CM 180, Leica, Wetzlar, Germany).

Explants used for western blot analyses were incubated in R16 serum-free medium containing 1 μg/mL Cyr61 for the indicated time periods. The tissue was collected, put on ice and homogenized with sonification in lysis buffer (50 mM Tris, pH 7.4, 250 mM NaCl, 25 mM EDTA, 1% NP-40, 10% glycerol) and centrifuged (16 000 *g* for 30 min). Cleared lysates were stored in −80°C for further analysis. Experiment was performed two times. In each experiment, for each time point 2–5 retinas were pooled together.

Western blots

To evaluate protein changes in retinal stimulated explant tissue, as well as from PR and RMG, 10, 15 or 20 μg total protein of lysates were separated on 10% dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (GE Healthcare, Munich, Germany). Unspecific binding was blocked for 1 h with 5% milk in TBS-T at 24°C. Blots were incubated overnight at 4°C with primary antibodies [Cell Signaling Technology, Leiden, The Netherlands: phosphorylated (p)Akt (#4060, 1 : 2000), phosphorylated extracellular-signal-regulated kinase 1 and extracellular-signal-regulated kinase 2 (pErk1/2) (#4370, 1 : 2000) and phosphorylated signal transducer and activator of transcription 3 (pStat3) (#9131 or #9145, 1 : 1000)] diluted in 5% milk in TBS-T, washed and incubated with horseradish peroxidase-coupled secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA). Signal was developed with the ECL+-enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK). Blots were stripped in a stripping buffer (100 mM 2-Mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl pH 6.7) for 30 min in 50°C and re-probed with appropriate antibodies [Cell Signaling: Stat3 (# 4904, 1 : 1000), Erk1/2 (#9102, 1 : 1000), Akt (#9272, 1 : 2000)] to verify equivalent protein loading. Results in graphs represent three independent repetitions of each type of experiment.

TUNEL assay

Sections from retinal explants were assayed for detection of cell death by a TUNEL assay according to the manufacturer′s instructions. For negative control, slides were incubated only in labelling solution and without terminal transferase. For positive control, sections were incubated for 30 min with DNase I (3U/mL, Roche Diagnostics, Mannheim, Germany) in 50 mM Tris-HCl, pH 7.5, 1 mg/mL BSA (PAA Laboratories GmbH, Pasching, Austria) to induce DNA strand breaks. No labelling was observed in the negative control sections, whereas in the positive control sections, all retinal layers were stained (data not shown). To label all cell nuclei, Hoechst was added at the end of the TUNEL assay procedure. A region of interest of the central retina with a width of approximately 120 μm per section was evaluated.

Image analysis and statistics

Retinal explant images were acquired at 200-fold magnification (Axio Imager Z1, Zeiss, Munich, Germany). Subsets from each retinal section were defined and analysed with Definiens Developer XD 2 software (Definiens AG, Munich, Germany). A specific rule set was developed to analyse the images as described previously (Del Rio *et al.* 2011). The total number of cells in outer nuclear layer (ONL) was determined by measurement of the ONL area and dividing it through the average cell size. For calculation of percentage of TUNEL positive cells the number of TUNEL positive cells was divided by the total number of cells in ONL. Statistical comparisons among different experimental groups were done using the one-tailed Mann–Whitney U-test implemented in GraphPad Prism (V6.03, La Jolla, CA, USA). Error bars indicate standard error of the mean (SEM); levels of significance: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

GDNF induces changes in secreted and intracellular primary mouse RMG protein profile

GDNF had been found to delay PR cells death upon injections into the subretinal space of *rd1* mice (Frasson *et al.* 1999). We could show, that this pro-survival effect on the neurons is mediated by stimulation of retinal RMG cells (Hauck *et al.* 2006). It was hypothesized that GDNF binds to its receptors on RMG, which in turn results in an increased secretion of neuroprotective molecules potentially prolonging PR survival (Hauck *et al.* 2006). To identify the GDNF-induced protein expression profile changes in RMG cells, we

screened protein response profiles with antibody-based arrays (Fig. 1). To confirm purity of primary RMG cell culture, we used eyes from GFAP-eGFP/C57BL6 mice, whose RMG can be distinguished from other cells by their green fluorescence. In our investigations, we analysed total cell lysates from 0.1 μg/mL GDNF treated mRMG as well as supernatants containing proteins secreted in response to the treatment (Fig. 1a,f) and compared them to untreated controls (Fig. 1b,g). In mRMG supernatants, seven proteins were significantly induced: tissue factor, Cyr61, endostatin, fibroblast growth factor 1, fractalkine (CX3CL1), insulin-like growth factor-binding protein 3 and placenta growth factor, when defining a threshold of at least twofold expression change (for details see Table 1 and Fig. 1c). Increase in abundance of these seven proteins is illustrated as a comparison of normalized signal intensities (Fig. 1d). To correlate the induced changes in secreted proteins to their intracellular levels, total cell lysates from mRMG were analysed: All seven proteins displayed greater than twofold abundance relative to controls in response to GDNF treatment (Table 1 and Fig. 1h). Changes in protein quantity between control and GDNF-stimulated samples are additionally presented as normalized signal intensities for a representative experiment (Fig. 1i).

Among the proteins, Cyr61 was significantly induced by GDNF. This extracellular matrix protein is known for its pro-survival effect in breast cancer cells (Menendez *et al.* 2005), oral squamous cell carcinoma (Kok *et al.* 2010), osteosarcoma (Sabile *et al.* 2012) and many other malignant tumour cells (Babic *et al.* 1998; Tang *et al.* 2011). On the basis of these previous studies pointing to a potential pro-survival activity and as a result of the strong and robust induction of Cyr61 in mRMG supernatant (Table 1 and Fig. 1c–e) and in cell lysate (Table 1 and Fig. 1h–j), we selected this molecule for further functional studies.

Cyr61 reduces cell death in Pde6b^{rd1} retinal explant cultures

To validate the hypothesis that Cyr61 may mediate neurotrophic support to retinal PR in response to GDNF treatment of RMG, we investigated the direct effect of Cyr61 application to organotypic retinal cultures and quantitatively assessed PR survival rates as described before (Del Rio *et al.* 2011). The influence of Cyr61 on PR survival was examined on explant cultures prepared from eyes of *rd1* mice – a well-known animal model for human RP. These mice carry a mutation in the PDE6b gene that results in fast degenerating PR (Bowes *et al.* 1990; Pennesi *et al.* 2012). Retinas were explanted at PN5 and cultured for 6 or 12 days with serum-free medium (control samples, Fig. 2b) or with Cyr61 supplemented serum-free medium for 4 and 10 days (experimental samples, 0.5 μg/mL and 1 μg/mL, respectively; Fig. 2a). Results of TUNEL analysis of *rd1* short-term retinal explants (Fig. 2c) indicated decreased PR death rates in explants treated with Cyr61 (mean 2.3%; ± 0.42 of TUNEL positive cells in the ONL) as compared to untreated controls (mean 5.1%; ± 0.94 of TUNEL positive cells in the ONL). After 12 days *in vitro,* the reduced amount of TUNEL positive cells under Cyr61 treatment is still evident (mean 4.7%; ± 0.9 of TUNEL positive cells in the ONL) (Fig. 2d) compared to control (mean 10.3%; ± 1.14 of TUNEL positive cells in the ONL). This decreased death rate translated into a significantly higher numbers of residual PR in long-term retinal explants (mean 682; ± 66) in comparison to control samples (403; ± 19) (Fig. 2e). As the average PR size was comparable between control versus stimulated explants for both, short-term and long-term retinal explants (Figure S4, a and b respectively), we conclude that the observed difference was solely the result of decreased cell death in Cyr61 treated explants in comparison to controls.

Cyr61 activates MAPK/Erk and JAK/Stat pathways in retinal explants from Pde6b^{rd1} mice

As Cyr61 has been shown to stimulate phosphoinositide 3-kinase (PI3K) PI3K/Akt and MAPK/Erk prosurvival pathways in cancer cells (Menendez *et al.* 2005; Yoshida *et al.* 2007; Sabile *et al.* 2012; Zhang *et al.* 2012), and the janus kinase (JAK)/Stat signalling cascade was reported to be induced in many studies on retinal degenerations (Adamus *et al.* 2003; Maier *et al.* 2004; Joly *et al.* 2008; Chollangi *et al.* 2009; Schallenberg *et al.* 2012) we tested for activation of all three pathways. To identify potential responsive signalling pathways in a model as close as possible to the *in vivo* situation, we established short-term organotypic tissue cultures using retinas from *rd1* mice as disease model. Mouse retinal explants were treated with Cyr61 (1 μg/mL) and activation of MAPK/Erk, PI3K/Akt pathways as well as activation of the JAK/Stat pathway was screened by western blots using phospho-peptide antibodies directed against activated forms of critical components in these pathways (Fig. 3a). Treatment with Cyr61 resulted in an increase of phosphorylated Erk1/2, equivalent to increased MAPK activity, which peaked at 20 min (ratio 3.3) and slowly declined thereafter (2 h: ratio 1.8) (Fig. 3b). Likewise, Cyr61 treatment resulted in phosphorylation of Stat3, a transcriptional activator down-stream of the JAK, which increased after 20 min (ratio 1.7) sustaining for at least 60 min (ratio 1.8). After 2 h pStat3 decreased to control levels (Fig. 3c). In contrast, we did not find any differences in Akt phosphorylation in Cyr61-stimulated explants (Fig. 3a).

Cyr61 does not stimulate primary PR cells

To obtain a sufficient amount of protein for further western blot analysis, we prepared primary PR cells from porcine retina. These cultures were used to test for direct Cyr61-mediated neuroprotection. Highly purified PR with low level of RMG contamination (see Materials and Methods, Figure S2) were stimulated with 1 μg/mL Cyr61 for 10, 20 or 120 min and their protein lysates analysed by western blotting. PR show basal level of phosphorylation of Erk1/2,

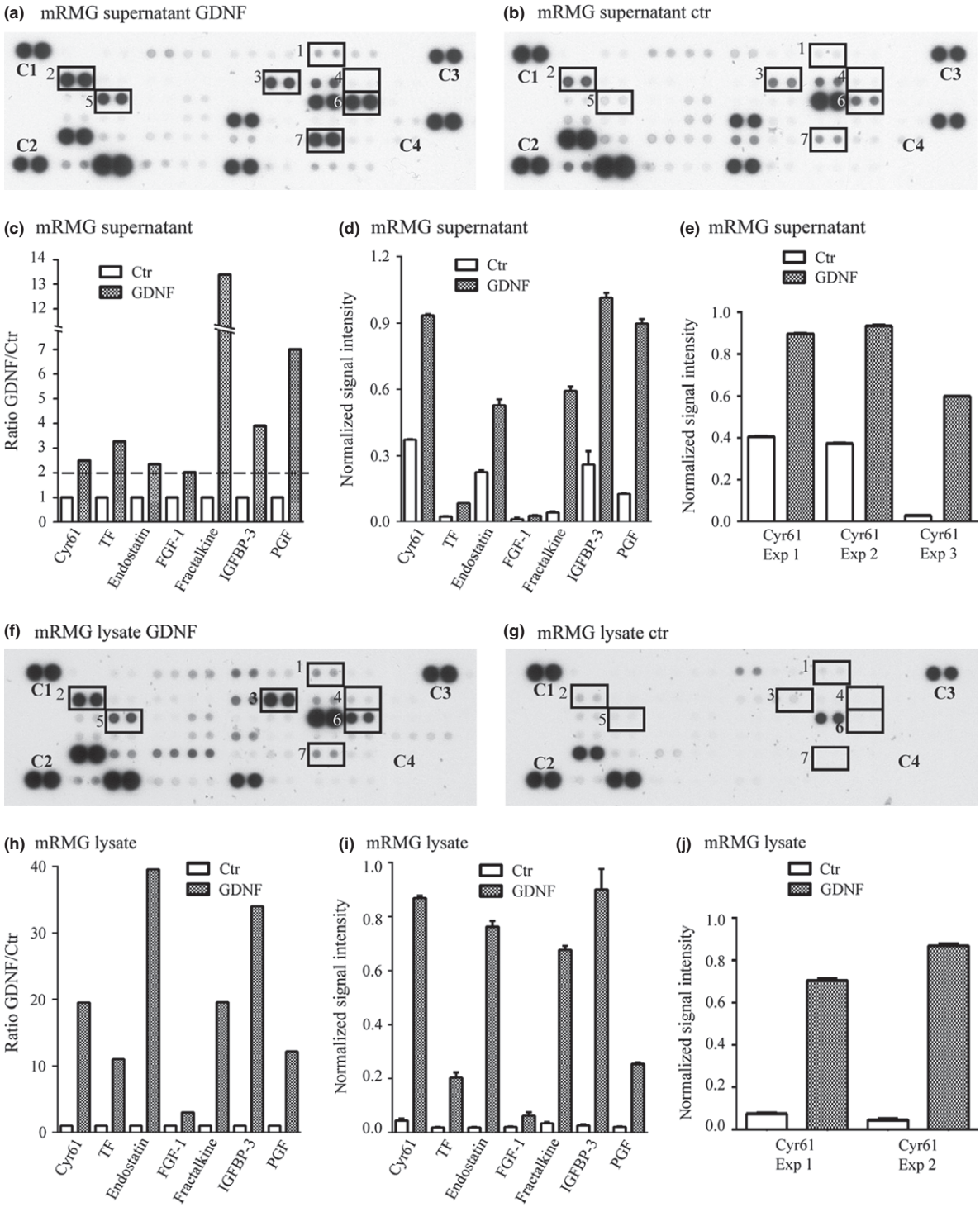

Fig. 1 Cyr61 is secreted by glial cell line-derived neurotrophic factor (GDNF) stimulated Müller glial cells. Mouse retinal Müller glial cells (RMG) (mRMG) were incubated with GDNF (0.1 μg/mL) for 24 h, and the conditioned medium as well as the cell lysate were analysed via Proteome Profiler™ Mouse Angiogenesis protein arrays. Analysis of two representative experiments [one for supernatant (a–e) and one for lysate (f–j)] is shown. The seven induced proteins of a total of 53 probed are boxed on the control (a, f) and experimental membranes (b, g), and numbered according to Table 1. Results of the experiments were quantified and visualized (c, h) comparing normalized signal intensity between control and experimental samples. The relative increase after GDNF stimulation is shown in panels D and I. Changes in Cyr61 expression for each experiment in the conditioned medium supernatant (e) and lysate (j) are displayed as a comparison of normalized signal intensity. C1, C2, C3 – positive controls; C4 – negative controls.

Table 1 List of mRMG-derived proteins induced in supernatant and lysate after GDNF stimulation.

		Supernatant[c]			Lysate[d]	
Number[a]	Name of the protein[b]	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2
1	Tissue factor (Coagulation factor III, TF)	3.27	5.47	2.25	4.43	11.02
2	Cystein-rich protein 61 (Cyr61, CCN1)	2.51	2.20	20.44	9.39	19.55
3	Endostatin, Colagen XVIII	2.34	2.22	3.82	14.02	39.55
4	Fibroblast growth factor (FGF-1)	2.02	7.29	4.05	6.16	3.03
5	Fractalkine (CX3CL1)	13.39	2.03	13.29	12.34	19.59
6	Insuline-like growth factor-binding protein (IGFBP-3)	3.9	14.94	10.79	39.36	34.03
7	Placenta growth factor (PGF)	7.00	5.25	82.19	16.11	12.20

[a]Number associated with the protein in the membrane array (Fig. 1).
[b]Name of proteins according to the mouse cytokine membrane array protocol.
[c]Ratio treated/untreated for supernatant for three independent experiments.
[d]Ratio treated/untreated for lysate for two independent experiments.

Akt and Stat3, but without any significant increase after Cyr61 medium supplementation (Figure S3). As Cyr61 has previously been described to act via activation of these proteins this suggests a lack of Cyr61-mediated activation of these three neuroprotective signalling pathways in PR and strengthens the hypothesis of an indirect mechanism of protection.

Cyr61 activates JAK/Stat, PI3K/Akt and MAPK/Erk pathways in primary RMG cells

As Cyr61 failed to stimulate PR directly, we tested whether Cyr61 can activate the above signalling pathways in RMG. Primary RMG cells were prepared from porcine retina and cultured for up to 11 days (Fig. 4a). Müller glia displayed a very characteristic morphology with rounded cell bodies containing nuclei and multiple long, spindly protrusions. Porcine RMG cultures were treated with 1 μg/mL Cyr61 for different time points, and activation of signal transduction pathways was monitored by western blots. After 10 min of Cyr61 treatment, we could detect a very strong phosphorylation of Stat3 (ratio 4.4) that then decreased after 20 min (ratio 2.2) dropping below control level after 60 min (ratio 0.6) and not rising even after 120 min (ratio 0.6, Fig. 4b). When analysing PI3K/Akt signalling pathway, we observed a peak in phosphorylation of Akt after 10 min (ratio 2.6) after which the effect was slowly diminishing (20 min: ratio 1.8) decreasing below control level after 60 min (ratio 0.4) and slowly rising again with 0.5 ratio after 120 min (Fig. 4c). Evaluation of changes in phosphorylation levels of Erk1/2 presented significant increase in phosphorylation after 30 min (ratio 2.1) after which pErk1/2 started to slowly decrease reaching ratio 1.9 and 1.8 after 1 and 2 h of treatment respectively (Fig. 4d).

Cyr61 activates PI3K/Akt and MAPK/Erk pathways in primary RPE cells

To analyse whether RPE cells are responsive to Cyr61, we prepared primary RPE cell cultures from porcine retina (Fig. 5a). This yielded sufficient amount of protein for further western blot analysis. Stimulation of RPE cells with 1 μg/mL Cyr61 resulted in an increase in phosphorylation of Akt and Erk1/2 signalling molecules (Fig. 5), but not Stat3 (data not shown). Activation of Akt was visible after 10 min (ratio 2.7) with a peak at 20 min (ratio 3.4) and this elevation continued until 2 h after stimulation with Cyr61 (ratio 2.8, Fig. 5b). Analysis of phosphorylation of Erk1/2 revealed robust increase in pErk1/2 at 10 min after treatment (ratio 3.8) that stayed at the same level of activation after 20 min (ratio 3.8) and started to drop 2 h after Cyr61 medium supplementation (ratio 1.8, Fig. 5c).

Discussion

Müller glial cells are the main glial cells within the retinal tissue, and thus crucial to the maintenance of homeostasis and metabolic support of retinal neurons (Reichenbach and Bringmann 2013). The protective response involves different mechanisms including production and release of cytokines, neurotrophic and growth factors (Limb *et al.* 2002; Joly *et al.* 2008; Bringmann *et al.* 2009).

The neuroprotective effect of GDNF has been previously discussed as indirect and RMG-associated, as RMG, but not PR, possess GDNF receptors (Hauck *et al.* 2006; Xia *et al.* 2011). Similar to GDNF, indirect prosurvival effect on rod PR cells mediated through RMG was described for brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) (Adamus *et al.* 2003; Wen *et al.* 2006, 2008; Saito *et al.* 2009) as well as for oncostatin M, a member of the interleukin 6 family of cytokines (Xia *et al.* 2011).

Cyr61 as a novel neuroprotective molecule in the retina

Although not the only one found to be up-regulated in response to GDNF treatment, we focused on Cyr61 as a molecule with a documented pro-survival potential in different cancer cells (Dunn *et al.* 1996; Kok *et al.* 2010;

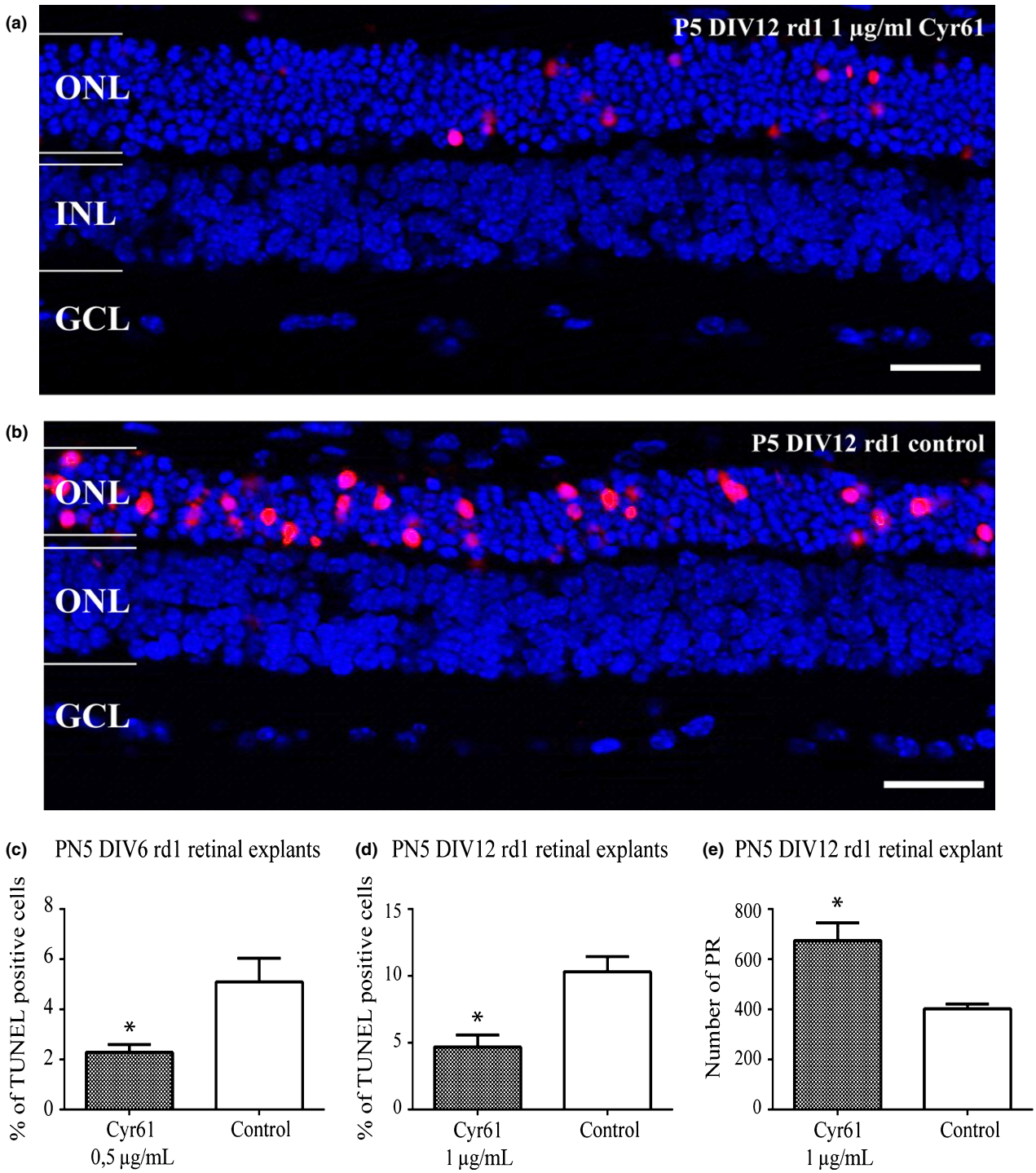

Fig. 2 Cyr61 prolongs photoreceptor survival in *rd1* mouse retinal explants. Mouse *rd1* retinal explants were cultured for 4 days (short term explants) and 10 days (long term explants) in medium supplemented with 0.5 µg/mL and 1 µg/mL Cyr61 respectively. Explants not treated with the factor served as a control. Retinas treated for 10 days with 1 µg/mL Cyr61 (a) exhibit lower amount of TUNEL positive cells as well as thicker outer nuclear layer (ONL) comparing to control (b). The percentage of TUNEL positive cells was analysed and is presented for short-term (PN5 DIV6, c) and long-term (PN5 DIV12, d) retinal explants. The differences in total ONL cells were analysed for long-term retinal explants and the quantitative results showing higher amounts of photoreceptor cells (PR) in Cyr61 treated explants are displayed on a graph (e). Scale bar – 50 µm. $n = 3$ for post-natal (PN)5 DIV6 ctr and PN5 DIV12 Cyr61, $n = 4$ for PN5 DIV6 Cyr61 and PN5 DIV12 ctr. $*p < 0.05$ Mann–Whitney *U*-test.

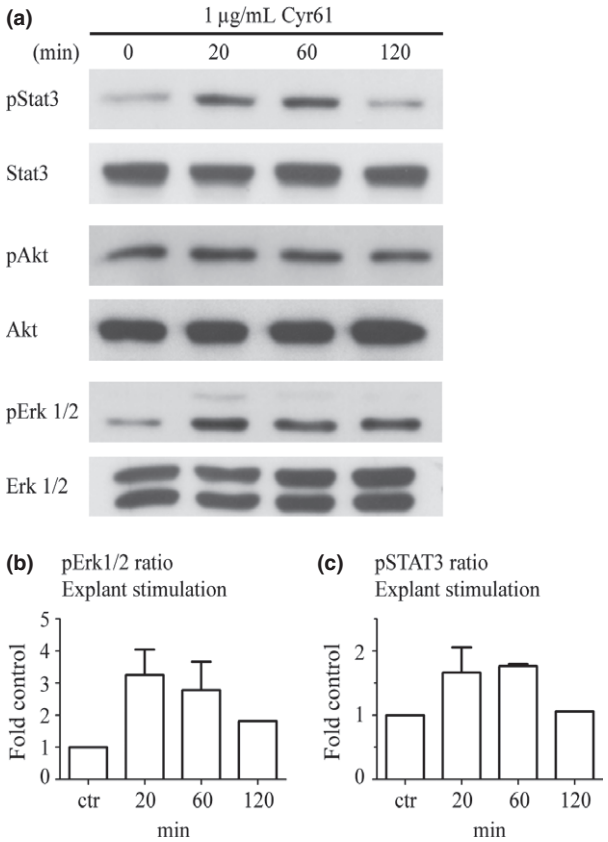

Fig. 3 Cyr61 induce mitogen-activated protein kinase (MAPK)/Erk and Janus kinase (JAK)/Stat activity in rd1 mouse retinal explants. Retinal explants obtained from 6 days old *rd1* mice were stimulated with 1 μg/mL Cyr61 for the indicated times. Western blot results display an increase in pErk1/2 and pStat3, but not pAkt, molecules in explants after 20 min of treatment (a). Quantitative evaluation of changes in the phosphorylation of pErk1/2 and pStat3 are shown in panels b and c respectively.

Tang *et al.* 2011; Sabile *et al.* 2012). Moreover, down-regulation of *cyr61* expression was noticed during retinal neurodegeneration in infantile Batten disease (Haltia 2006; Qiao *et al.* 2007). In our study, Cyr61 significantly increased survival rates of PR in *rd1* retinal explants, which was noticeable after only 4 days of treatment, persisting after 10 days. Next, we aimed at describing signal transduction pathways leading to the observed neuroprotection. We decided to monitor changes in activation of three intracellular signal transduction molecules – Erk1/2, Akt and Stat3 – given that Cyr61 was already reported to provide a survival advantage to cancer cells via pErk1/2 and pAkt (Menendez *et al.* 2005; Yoshida *et al.* 2007; Sabile *et al.* 2012; Zhang *et al.* 2012). Monitoring of pStat3 was chosen in addition, as this transcriptional activator was shown to act down-stream of neuroprotective factors in retinal tissue (Adamus *et al.* 2003; Maier *et al.* 2004; Joly *et al.* 2008; Chollangi *et al.* 2009; Schallenberg *et al.* 2012). In our experiments, Cyr61 increased phosphorylation of Erk1/2 and Stat3, but not Akt in explanted whole retinal tissue. This is in line with previous results suggesting that retinal degeneration induced by light injury may be ameliorated with docosahexaenoic acid, leukaemia inhibitory factor and granulocyte-macrophage colony–stimulating factor (Liu *et al.* 1998; Limb *et al.* 2002; German *et al.* 2006; Schallenberg *et al.* 2012). There, pre-conditioning of the rodent retina with bright light before applying the insult resulted in PR survival accompanied by stimulation of Stat3 and Erk1/2, but not Akt (Liu *et al.* 1998). Following optic nerve injury, up-regulated GDNF and brain-derived neurotrophic factor enhanced activation of Erk1/2 and protected retinal ganglion cells (RGC) from preliminary death (Nakatani *et al.* 2011). Moreover, neuroprotective effect of CNTF on RGC, intravitreal injected in a rat model of multiple sclerosis, was also accompanied by increased phosphorylation of Erk1/2 and Stat3 within the retinal tissue (Maier *et al.* 2004). In addition, neuroprotection of RGC treated with interleukin 2 or fibroblast growth factor 2 (FGF-2) was mediated in an Erk1/2-dependent manner (Rios-Munoz *et al.* 2005; Marra *et al.* 2011). Thus, a wide range of different neuroprotective factors within the retina lead to MAPK/Erk and JAK/Stat activation, but not necessarily PI3K/Akt, which suggests that both pathways are critical for retinal neuron survival.

Cyr61 action in the retina – dissection of cell-type specific responses

In response to external stimuli, RMG cells secrete neuroprotective factors to influence PR fate (Bringmann *et al.* 2009). Cyr61 can influence cell processes via binding to integrins or heparan sulphate proteoglycans (HSPG) at the cell surface (Kular *et al.* 2011). Different mechanisms of action may apply: Cyr61 may stimulate PR directly through yet unknown receptors and/or HSPG, the latter being reportedly expressed on PR (Tawara and Inomata 1987; Tawara *et al.* 1989; Landers *et al.* 1994). Cyr61 may protect PR indirectly through stimulation of RMG by binding to integrins and/or HSPG expressed on RMG (Hering *et al.* 2000; Aricescu *et al.* 2002). Cyr61 may also protect PR indirectly through stimulation of RPE cells by binding to integrins and/or HSPG (Clegg *et al.* 2000; Geraldes *et al.* 2007). Given that none of the intracellular signalling cascades was found induced in PR upon Cyr61 stimulation our data suggest that Cyr61 does not influence PRs survival in a direct way. On the other hand, Cyr61 treatment of primary PR cell cultures may result in activation of other signalling pathways, not monitored in this study.

In contrast, Cyr61 stimulation of primary RMG induced a marked increase in intracellular signalling associated with neuroprotection as well as RMG re-activation in the injured retina, which triggers complex changes on the transcriptome and proteome levels (Kurihara *et al.* 2006; Rhee *et al.* 2007; Kirsch *et al.* 2010). In rat models of retinal degeneration,

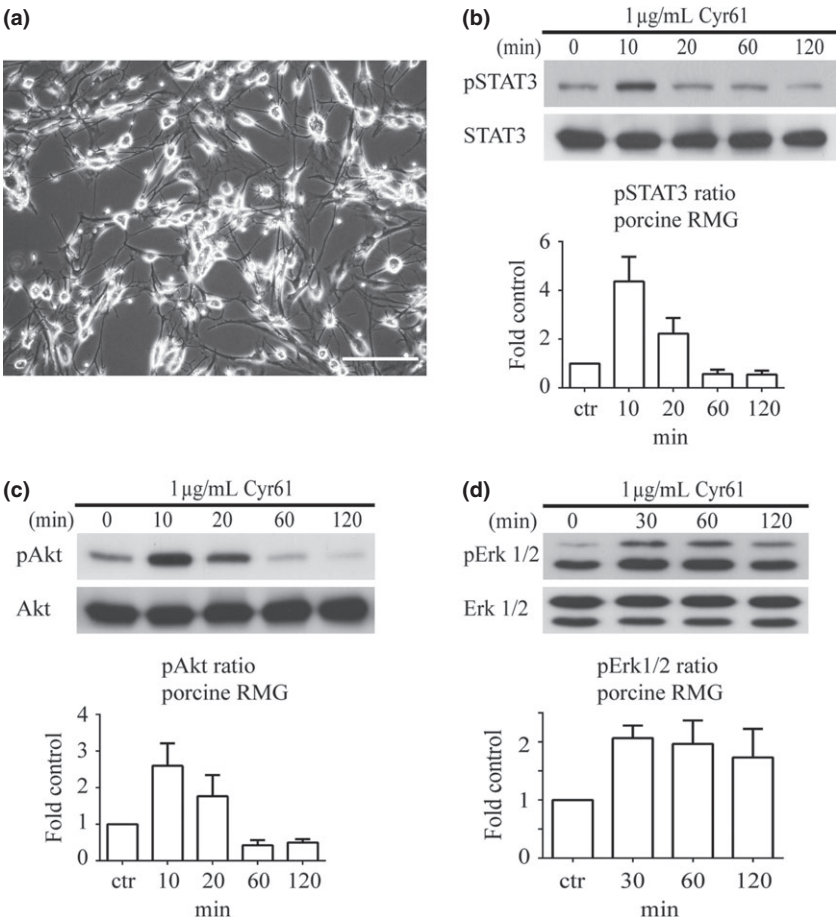

Fig. 4 Cyr61 induces Janus kinase (JAK)/Stat, PI3K/Akt and mitogen-activated protein kinase (MAPK)/Erk activity in primary retinal Müller glial cells (RMG) cells. Primary RMG cells were isolated from porcine eyes, cultured until confluence > 75% was achieved (11 days in culture, phase contrast microscopy, panel a) and stimulated with Cyr61 (1 μg/mL) for the indicated time periods. Western blots display changes in the phosphorylation of Stat3 (b), Akt (c) and Erk1/2 (d) in total cell lysates. Quantitative evaluation of changes in the phosphorylation of Stat3 (b), Akt (c) and Erk1/2 (d) are shown in graphs. Scale bar – 200 μm.

neuroprotective effect on PR through Stat3 phosphorylation in RMG was observed for CNTF (Adamus *et al.* 2003) as well as for oncostatin M (Rhee *et al.* 2007). Moreover, activation of Stat3 was also shown to be induced in retinal regeneration in the injured zebrafish retina (Harada *et al.* 2003).

Cyr61 stimulation of RMG led to a strong and persistent activation of MAPK/Erk signalling. Active pErk1/2 accompanies retinal protection through release of neuroprotective factors such as FGF-2 or CNTF (Liu *et al.* 1998; Hauck *et al.* 2006; Bringmann *et al.* 2009). On the other hand, inhibition of phosphorylation of Erk1/2 in RMG in ischaemia–reperfusion rat model resulted in increased death of RGC (Akiyama *et al.* 2002). Furthermore, Cyr61 treatment led to PI3K/Akt pathway activation in porcine RMG. Akt phosphorylation has also been observed in RMG in response to GDNF (Hauck *et al.* 2006). Taken together, our data imply that GDNF triggers a complex, yet restricted response of transcriptional activity in RMG cells. Cyr61 in turn likely activates autocrine-signalling loops in RMG that contribute to a sustained pro-survival activity in the intact retina.

Cyr61 treatment of primary porcine RPE cells resulted in an increase in Akt and Erk1/2 phosphorylation. Activation of Akt or Erk1/2 by a variety of factors was shown to be crucial to protect primary RPE from oxidative-stress-related injury (Tsao *et al.* 2006; Faghiri and Bazan 2010; Halapin and Bazan 2010; Patel and Hackam 2013). Massive RPE atrophy may follow photoreceptor degeneration in patients with RP (Mitamura *et al.* 2012). The *rd1* (Pennesi *et al.* 2012) and the *rd10* mouse (Pennesi *et al.* 2012; Samardzija *et al.* 2012) RP animal models show a similar pattern. Consequently, activation of Akt and Erk1/2 may also protect RPE cells from degenerative stimuli released by dying PR. This can in turn up-regulate the release of neuroprotective factors like αB-crystallin (Sreekumar *et al.* 2010), pigment epithelium-derived factor (Verity *et al.* 1998; Cayouette *et al.* 1999; Aymerich *et al.* 2001; Cao *et al.* 2001; Murakami *et al.* 2008) or neuroprotectin D1 (Bazan 2006; Halapin and Bazan 2010), supporting photoreceptor survival during development of RP (Gaur *et al.* 1992; Schraermeyer and Heimann 1999; Marc *et al.* 2003; Mukherjee *et al.* 2007; Bazan 2008; Sreekumar *et al.* 2010).

Besides its protective features, Cyr61 can exert strong angiogenic properties resembling those of vascular endothelial growth factor (Brigstock 2002; Yu *et al.* 2008; Harris *et al.* 2012). Both molecules were positively tested for

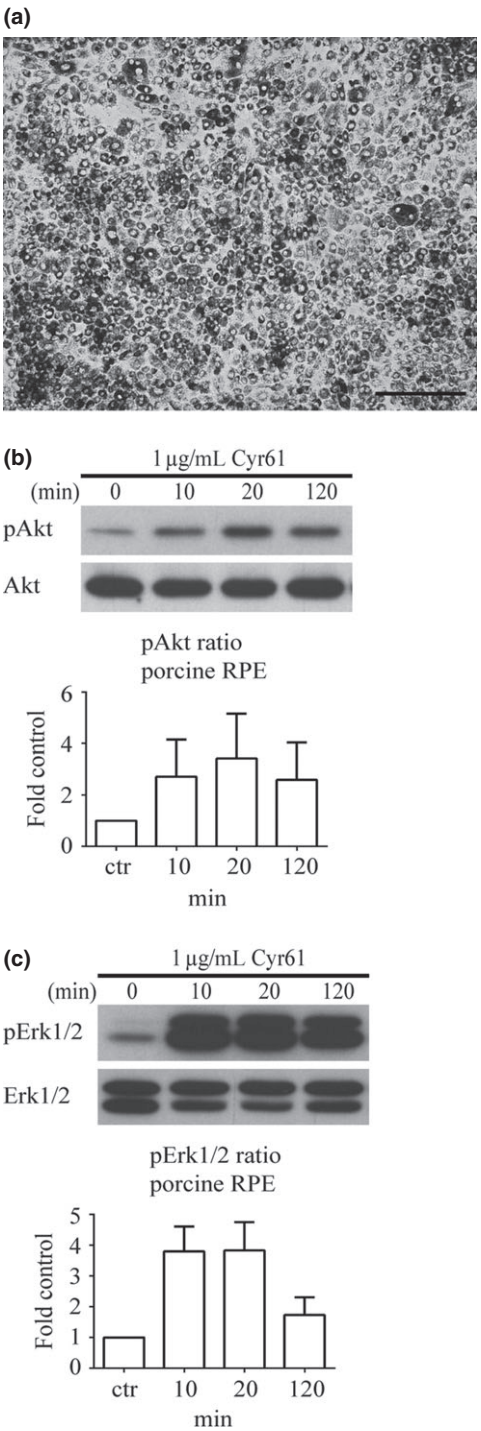

Fig. 5 Cyr61 induces phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/Erk activity in primary retinal pigment epithelium (RPE) cells. Primary porcine RPE cells were cultured up to 10 days (displayed at 5 days in culture, phase contrast microscopy, panel a). For the experiments, the cells were treated with 1 μg/mL Cyr61 for the indicated periods of time. Western blot represent changes in the phosphorylation of Akt (b) and Erk1/2 (c) in total cell lysates. Quantification of the changes in pAkt (b) and pErk1/2 (c) are presented on the graphs. Scale bar – 200 μm.

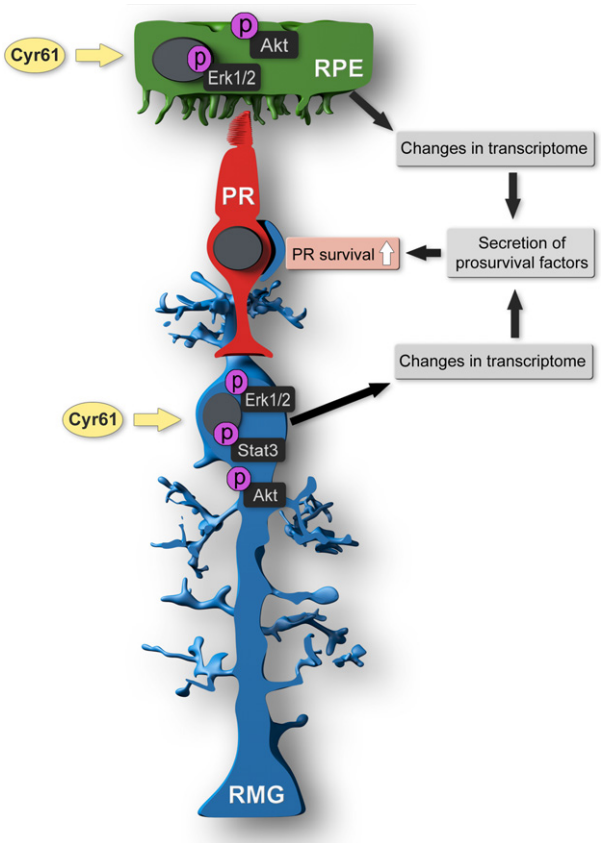

Fig. 6 Model of Cyr61 neuroprotection. Cyr61 binds to yet unknown receptors on retinal Müller glial cells (RMG) and retinal pigment epithelium (RPE) cells causing an increase in phosphorylation of Akt, Stat3 and Erk1/2. Stat3 and Erk1/2 are known to translocate to the nucleus after activation, inducing transcriptional changes. As a result RMG and RPE cells may release increased levels of neuroprotective agents that in a direct way stimulate PR cells leading to their prolonged survival.

potential therapeutic application in models for peripheral ischaemic disease. Nevertheless, Cyr61 provides better perfusion making it a preferred option for therapy development (Fataccioli *et al.* 2002; Rayssac *et al.* 2009). Moreover, over-expression of Cyr61 was already successfully applied as a therapeutic regime in an animal model of retinopathy of prematurity (Hasan *et al.* 2011).

In conclusion, our study shows, that Cyr61 protects PR in an indirect way, most likely through stimulation of RMG and RPE cells, which in turn most likely secrete a variety of neuroprotective factors supporting PR survival (Fig. 6). A similarly complex signalling pattern was described for Cyr61 activity in the context of cutaneous wound healing (Chen *et al.* 2001). The results of this study indicate that application of Cyr61 to the degenerating retina is neuroprotective and opens the possibility of future *in vivo* characterizations of Cyr61 action within the retina.

Acknowledgements and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Viability of primary porcine PR.

Figure S2. Purity of primary porcine PR.

Figure S3. Cyr61 does not stimulate primary PR.

Figure S4. PR cell size in short-term and long-term retinal explants.

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