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Complement component C3aR constitutes a novel regulator for chick eye morphogenesis

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Abstract

Complement components have been implicated in a wide variety of functions including neurogenesis, proliferation, cell migration, differentiation, cancer, and more recently early development and regeneration. Following our initial observations indicating that C3a/C3aR signaling induces chick retina regeneration, we analyzed its role in chick eye morphogenesis. During eye development, the optic vesicle (OV) invaginates to generate a bilayer optic cup (OC) that gives rise to the retinal pigmented epithelium (RPE) and neural retina. We show by immunofluorescence staining that C3 and the receptor for C3a (the cleaved and active form of C3), C3aR, are present in chick embryos during eye morphogenesis in the OV and OC. Interestingly, C3aR is mainly localized in the nuclear compartment at the OC stage. Loss of function studies at the OV stage using morpholinos or a blocking antibody targeting the C3aR (anti-C3aR Ab), causes eye defects such as microphthalmia and defects in the ventral portion of the eye that result in

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Disclosure

The authors declare that they have no competing interests.

Authors' contributions

E.G.E and A.L.M - designed and performed experiments, analyzed data, and co-wrote the paper; J.B, T.H, C.G, Z.M and Z.H discussed and performed experiments and edited manuscript; E.R- discussed experiments, generated reagents and edited manuscript; A.T- generated complement reagents and edited manuscript; J.D.L - discussed experiments, generated reagents and edited manuscript; P.A.T- discussed experiments, analyzed data and edited manuscript; K.D.R.T- conceived idea, supervised the work, designed experiments, analyzed the data, and co-wrote the paper.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2017.05.019>.

coloboma. Such defects were not observed when C3aR was disrupted at the OC stage. Histological analysis demonstrated that microphthalmic eyes were unable to generate a normal optic stalk or a closed OC. The dorsal/ventral patterning defects were accompanied by an expansion of the ventral markers Pax2, *cVax* and retinoic acid synthesizing enzyme *raldh-3 (aldh1a3)* domains, an absence of the dorsal expression of Tbx5 and *raldh-1 (aldh1a1)* and a re-specification of the ventral RPE to neuroepithelium. In addition, the eyes showed overall decreased expression of Gli1 and a change in distribution of nuclear β -catenin, suggesting that Shh and Wnt pathways have been affected. Finally, we observed prominent cell death along with a decrease in proliferating cells, indicating that both processes contribute to the microphthalmic phenotype. Together our results show that C3aR is necessary for the proper morphogenesis of the OC. This is the first report implicating C3aR in eye development, revealing an unsuspected hitherto regulator for proper chick eye morphogenesis.

Keywords

Complement; Eye development; Microphthalmia; Coloboma; C3aR; C3; Optic vesicle

1. Introduction

The complement system is phylogenetically conserved among different organisms and constitutes one of the first lines of defense in innate immunity. It mediates the immune response against pathogens, by directly tagging them for clearance, and by triggering the expression of pro-inflammatory molecules and the activation and recruitment of immune cells at the site of infection. Furthermore, it is involved in the maintenance of tissue homeostasis and adaptive immunity (Clarke and Tenner, 2014; Nonaka and Kimura, 2006; Ricklin et al., 2010). Complement components are predominantly synthesized in the liver and circulate in the blood stream until their activation (Ricklin et al., 2010). The activation results in the cleavage of C3 and C5 generating two small polypeptides, C3a and C5a respectively, that, depending on the context, function as anaphylatoxins and potent chemotactic mediators that recruit immune and non-immune responsive cells (Coulthard and Woodruff, 2015; Ricklin et al., 2010). In addition to their immunological role, complement components have been implicated in multiple functions including angiogenesis (Bora et al., 2007; Nozaki et al., 2006), regulation of neurogenesis, neuroinflammation and neuroprotection (Fonseca et al., 2013; Mukherjee et al., 2008; Rahpeymai et al., 2006; Shinjyo et al., 2009; Stokowska et al., 2017; Veerhuis et al., 2011), stem cell homing and migration (Honczarenko et al., 2005; Ratajczak et al., 2004; Reza et al., 2003; Schraufstatter et al., 2009), neural crest cell migration (Broders-Bondon et al., 2016; Carmona-Fontaine et al., 2011), synapse remodeling (Chu et al., 2010; Stevens et al., 2007), cell proliferation and survival (Benard et al., 2004; O'Barr et al., 2001), cartilage and bone development (Andrades et al., 1996; Huber-Lang et al., 2013; Ignatius et al., 2011; Kalbasi Anaraki et al., 2014; Sakiyama et al., 1994; Sato et al., 1993, 1991), retinopathies (Ding et al., 2015; Hoh Kam et al., 2013; Sweigard et al., 2014; Yanai et al., 2012; Yu et al., 2012), cancer (Sayegh et al., 2014), as well as tissue regeneration (Del Rio-Tsonis et al., 1998; Haynes et al., 2013; Kimura et al., 2003; Leslie and Mayor, 2013; Mastellos et al., 2013; Rutkowski et al., 2010; Todd and Fischer, 2015). C3a and C5a activate the G-coupled receptors C3aR

and C5aR, respectively (Langkabel et al., 1999). Interestingly, the mRNA and protein for C3aR and/or C5aR are found in the central nervous system (CNS) including the cerebellum, medulla, basal ganglia, thalamus, hippocampus, cerebral cortex and spinal cord as well as in adult human and mouse retina (Ames et al., 1996; Davoust et al., 1999; Veerhuis et al., 2011; Vogt et al., 2006; Yu et al., 2012). These receptors are important for differentiation and migration of neurons, glial progenitors and neural crest cells (Carmona-Fontaine et al., 2011; Nataf et al., 2001, 1999; Shinjyo et al., 2009; Stokowska et al., 2017; Veerhuis et al., 2011). Furthermore, the absence of C3a or its receptor C3aR, inhibits adult neurogenesis in ischemic mice (Rahpeymai et al., 2006). Both C3aR and C5aR, are transiently expressed in developing rat cerebellum but they are almost absent in the adult cerebellum suggesting they may have an important role during brain development (Benard et al., 2004, 2008; Rahpeymai et al., 2006). Additionally, several complement components including C3 and C3aR transcripts have been found as early as gastrula stage and in the developing eye in *Xenopus laevis* (McLin et al., 2008). In mouse embryos, C3 and C5aR have been detected throughout neural tube development and even in the developing optic vesicles. Interestingly, C5aR has also been found in the neuroepithelium of early human embryos, implying functions in early embryogenesis and eye development (Denny et al., 2013).

We demonstrated that C3a/C3aR signaling plays an important role in the activation of inflammatory factors and retinal stem/progenitor genes needed to induce embryonic chick retina regeneration (Haynes et al., 2013). Interestingly, in post-hatch chickens, C3aR and C3 have been associated with microglia and their ability to modulate repair in damage-induced retinas (Fischer et al., 2014). Recently, upregulation of C3aR mRNA was associated with Hh-stimulated Müller Glia dedifferentiation in newly hatched chickens (Todd and Fischer, 2015). Here we expand our knowledge of complement components C3/C3aR during eye morphogenesis using the chick embryo as a model.

The vertebrate eye is an intricate organ containing tissues derived from different origins. The surface ectoderm gives rise to the lens and cornea. The neural ectoderm generates the retinal pigmented epithelium (RPE), the retina, and part of the iris and ciliary body. Neural crest and mesodermal cells on the other hand, make periocular skeletal and connective tissues, the stroma of the iris and cornea, the muscles of the ciliary body, the sclera and ocular blood vessels (Graw, 2010; Heavner and Pevny, 2012; Johnston et al., 1979; Sinn and Wittbrodt, 2013). Soon after the neural tube closes, the eye primordium (eye field) contains cells involved in eye formation that express a set of eye field transcription factors (EFTF) including *ET*, *Rx1*, *Pax6*, *Six3*, *Lhx2*, *tll* and *Optx2* (also known as *Six6*) that are highly conserved among different vertebrates (Graw, 2010; Zuber et al., 2003). The first morphological event observed in the developing eye (in mouse at embryonic day (E) 8.5–9.0 or in the chick embryo at stages 9–12) is the formation of the optic vesicle (OV), which is a result of the evagination of the diencephalon's posterior region. Later in development, and under the influence of the lens placode, the distal region of the OV invaginates to generate a bilayered optic cup (OC) that eventually gives rise to the presumptive RPE and presumptive neural retina (NR), while the most proximal region of the OV generates the optic stalk (OS) and the optic fissure (OF) (Fuhrmann, 2010; Graw, 2010; Heavner and Pevny, 2012). Different signaling pathways and factors contribute to the establishment of the dorsal-ventral boundaries for the proper formation of the OC including

fibroblast growth factor (FGF), sonic hedgehog (Shh), wntless-type MMTV integration site family (Wnt) and bone morphogenetic protein (BMP) signaling cascades as well as paired box (Pax) transcription factors such as *Pax2* and *Pax6* (Atkinson-Leadbeater et al., 2014; Hagglund et al., 2013; Kobayashi et al., 2010; Schwarz et al., 2000). In addition, transcription factors such as *Vax1* and *Vax2* (homologous to *cVax* in chick) are activated by *Shh* and cooperate with *Pax2* by directly repressing *Pax6* in the OS (Mui et al., 2002, 2005). Also, the reciprocal repression of *Pax6/Pax2* plays an important role in the dorsal-ventral specification of the OC (Schwarz et al., 2000). Finally, the transcription factors *Vsx2* (also known as *Chx10*) and *Mitf* are necessary to establish the boundaries between the NR and RPE. Although, different transcription factors and pathways have been identified to play a role during the dorsal-ventral patterning of the OC, it is possible that other pathways/factors remain to be elucidated.

We show here that C3/C3aR proteins are present in neurogenic tissues in chick embryos at stages 10/11. Later in development during OC formation, both proteins were detected in the lens vesicle, neuroepithelium (NE) and ciliary margin (CM). Using reverse transcriptase-polymerase chain reaction (RT-PCR), we confirm the presence of the transcripts for *C3aR* and *C3* at the OV stage. Knockdown (KD) experiments targeting C3aR in the OV and OC by morpholino electroporation, or using a blocking antibody against the receptor (anti-C3aR Ab) at OV stages, exhibited phenotypes such as microphthalmia and defects in the ventral OS and OF resulting in coloboma. Such defects were not observed when blocking C3aR once the OC was established, suggesting that C3aR is critical at OV stage for the proper morphogenesis of the eye. Immunohistochemical analysis of the disrupted eye revealed that *Pax2/Pax6* expression domains were altered, showing a dorsal expansion of *Pax2*, when compared with the confined domain of this transcription factor in the control embryos. The transcription factor *Tbx5*, and the retinoic acid synthesizing enzyme aldehyde dehydrogenase 1 *raldh-1* (also known as *aldh1a1*) that are normally restricted to the dorsal region of the OC were absent in microphthalmic eyes, while ventral markers *cVax* and *raldh-6* (also known as *aldh1a3* or *raldh3*) were expanded dorsally. Nuclear Gli1, an indicator of active Shh signaling, was downregulated in the proximal region of the eye including the NE and RPE. Nuclear β -catenin, a mediator of canonical Wnt signaling pathway, was evenly expressed in the treated eyes in contrast to the restricted presence of active β -catenin in the control eyes, suggesting that blocking C3aR affects Wnt/ β -catenin and Shh pathways. The analysis of cell proliferation and apoptosis, showed a significant decrease in the number of cells entering the cell cycle 3 days post-treatment (dpt), while a higher number of apoptotic cells was detected 2 and 3 dpt when compared with control eyes. Taken together, our results provide evidence that complement component C3aR is necessary for cell survival and cell proliferation, and that blocking C3aR affects the dorsal-ventral pattern of the chick eye, ultimately leading to microphthalmia and coloboma.

2. Materials and methods

2.1. Chick embryos

Fertilized Specific Pathogen-Free (SPF) chicken eggs were purchased from Charles River (Wilmington, MA, USA) and were incubated at 38 °C and 60% humidity in a rotating

incubator. Embryos were staged according to Hamburger and Hamilton (1951) and were collected at the times indicated in the figure legends.

2.2. RT-PCR

Total RNA was extracted from stage 10/11 whole embryos using NucleoSpin RNA II isolation Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's protocol. Approximately 300 ng of total RNA were used for cDNA synthesis using ImProm-II Reverse Transcription System (Promega, Madison, WI) and random-primer hexamers according to the manufacturer's instructions. All PCR reactions were performed using PlatinumTaq DNA polymerase (Invitrogen, Grand Island, NY) and the primers and conditions previously reported for *C3*, *C3aR* and *GAPDH* (Haynes et al., 2013). All the RT-PCR reactions were run from at least three independent biological samples.

2.3. Morpholino electroporation and tissue immunofluorescence quantification

Two different antisense morpholino (Mo) oligonucleotides tagged at the 3'-end with carboxyfluorescein (GeneTools LLC, Philomath, OR, USA) were used to block the initiation of translation (C3aRMo1, 5'-TCTGTAGTTTATTACACCTACCCCA-3) or the splice junction of exon1 intron 1 junction (ENSGALT00000021594) (C3aRMo2, 5'-AGTGGAG ACATTCCCAGTGAAGCAA-3') of the C3aR mRNA. As a control, we used a standard control Mo (5'-CCTCTTACCTCAGTTACAATTTATA-3') (GeneTools LLC, Philomath, OR, USA). Each Mo was re-suspended in Ringer's solution to a 1 mM concentration according to the manufacturer's instructions and an aliquot of 15 µl was heated at 65 °C for 10 min and cooled to room temperature prior to electroporations. Approximately 0.3 µl (~25 pulses) was injected in the neural tube of stage 9, 10/11 or in the OC of stage 15 embryos using a Pico-injector system PLI-100 (Harvard Apparatus, Holliston, MA, USA) and beveled glass needles 50 mm long, 20 µm tip diameter (FHC, Bowdoinham, ME, USA). The electroporation at stages 9–11 was performed using two platinum-iridium (Pt/Ir) electrodes (FHC, Cat. No. 152 UEPMEEVENNND, Bowdoinham, ME, USA) and an ECM 830 electroporator (BTX, Bent, Holliston, MA, USA, *in ovo* gene Model 512) as previously described (Luz-Madrigal et al., 2015; Vergara and Canto-Soler, 2012). To perform stage 15 electroporations, the vitelline membrane covering the right eye was carefully removed using fine forceps and 150 µl of Hanks' Balanced Salt Solution (HBSS) was added above the eye. Each Mo was injected as previously described and two Pt/Ir electrodes were used for electroporation. The anode (+) was placed in the ventro-temporal section of the eye and the cathode (-) was placed perpendicular to the head of the embryo. The electroporation conditions were the same used for stage 10/11 electroporations (Luz-Madrigal et al., 2015). The eggs were sealed with transparent tape, returned to the incubator, and collected at the times indicated in the figure legends. The presence of each Mo in the OV and OC was analyzed 30 min after electroporation using a SteREO Discovery V12 microscope equipped with a PentaFluar S/HBO fluorescence illuminator (Carl Zeiss, Göttingen, Germany) and the images were captured using Zeiss AxioCam MRc5 with AxioPlan 2 and Zeiss AxioVision 4.7.1 extended focus module (AxioVision, Göttingen, Germany). The efficiency of Momediated KD for C3aR was evaluated 24 h after electroporation by immunofluorescence staining and Western blot. Tissue immunofluorescence quantification was performed using ImageJ software (v1.50f,

NIH, <https://imagej.nih.gov/ij/>) as previously reported (Canto-Soler and Adler, 2006; McCloy et al., 2014). Briefly, in each section, the electroporated area of the OC positive for the fluorescein-tagged Mo, was selected using the freeform drawing tool and set as reference to measure the mean fluorescence associated with C3aR immunofluorescence staining. Then, the option “Measure” from the analyze menu was selected and the results were used to calculate the corrected total cell fluorescence (CTCF) applying the formula: $CTCF = \text{Integrated Density} - (\text{Area of selected cell} \times \text{Mean fluorescence of background readings})$. The values are expressed as a percentage of CTCF of the C3aR Mo1 or 2 relative to control Mo from at least five different biological replicates in each experimental group. An unpaired Student's *t*-test was applied for mean comparison using SigmaPlot 8.0 Software. ****P* < 0.001. For the analysis of the chick embryo phenotypes, images were captured using an Olympus BX51-P microscope equipped with a Moticam 3000 camera and Motic Advanced 3.2 software (Motic group, Xiamen, China).

2.4. In situ hybridization

Plasmids containing the sequences of *raldh-1*, *raldh-3* and *cVax* were linearized and transcribed at 37 °C overnight in the presence of DIG RNA labeling mix (Roche, Indianapolis, IN, USA). Eyes collected for in situ hybridization were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded in OCT freezing medium (Sakura Finetek, Torrance, CA, USA). The eyes were sectioned (12 µm) on a Microm HM 505N Cryostat Microtome (MICROM International GmbH, Walldorf, Germany) and rinsed in PBS containing active DEPC for 10 min before hybridizing with the labeled probes as previously described (Belecky-Adams and Adler, 2001).

2.5. Western blotting

Stage 10/11 embryos were electroporated as described above (see Morpholino injection and electroporation section) and the OCs were collected 24 h later and analyzed for the presence of the fluorescently tagged Mo using a stereoscopic zoom microscope SMZ1500 (Nikon, Tokyo, Japan) equipped with a Mercury/Arc lamp. OCs (12–15 per biological sample) were dissected and homogenized immediately in icecold RIPA buffer containing the protease inhibitors Na-orthovanadate, PMSF and protease cocktail (Santa Cruz Biotechnologies, Santa Cruz, CA, USA). At least three independent biological samples were collected for each treatment. The presence of C3aR was detected by standard Western blotting using a 7.5% polyacrylamide gel under reducing conditions, followed by transfer to a PVDF membrane using a semi-dry transfer system. After the membranes were blocked in 4% non-fat milk in 10 mM sodium phosphate, pH 7.4, with 150 mM NaCl and 0.05% Tween 20, they were incubated with 1 µg/ml of the anti-chicken C3aR primary antibody in blocking solution overnight at 4 °C, followed by an anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, 1:5000 dilution in blocking solution) for 1 h at room temperature. Detection was performed using chemiluminescence (Immobilon Western detection reagents; Millipore). Membrane was then stripped using “Restore Western Blot Stripping Buffer” (Thermo Scientific) and developed using anti-actin antibody clone AC-15 (Sigma, St. Louis, MO, USA). Densitometry was measured with ImageLab software (BioRad) and the ratio of C3aR/actin was determined. An unpaired Student's *t*-test was performed and **P* = 0.01.

2.6. Immunohistochemistry

Embryos were fixed overnight in 4% paraformaldehyde (PFA) (Sigma, St. Louis, MO, USA) prepared in 0.1 M phosphate buffer (PB) (0.1 M PB is 0.077 M $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ and 0.022 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, pH=7.2), washed with PBS (NaCl 1.4 M), equilibrated with 30% sucrose in PB buffer, and snap frozen in optimal cutting temperature medium (OCT) (Tissue-Tek, Sakura Finetek, Torrance, CA, USA). Frozen tissues were sectioned at 12 μm thickness on a Microm HM 505N Cryostat Microtome (MICROM International GmbH, Walldorf, Germany) and the sections were washed twice with PBS, permeabilized with 1% saponin (Sigma, St. Louis, MO, USA) in PBS (w/v) for 5 min (15 min for β -catenin PY-489 Ab) and incubated with blocking solution (10% normal goat or normal donkey serum, 0.3% Triton-X100 in PBS) for 30 min at 37 °C in a humidifier chamber. Primary antibodies (Abs) were diluted in blocking solution at the concentrations indicated in the Abs section and incubated overnight at 4 °C. Sections were washed with PBST (0.3% Triton-X100 in PBS) and incubated with a secondary antibody for 2 h, washed with PBST, incubated 5 min with DAPI 30 nM (Invitrogen, USA Grand Island, NY) for nuclear counterstaining and cover-slipped with Vectashield (Vector Laboratories, Burlingame, CA). Confocal images (size 1024 \times 1024) were collected sequentially on a Zeiss 710 Laser Scanning Confocal System (Carl Zeiss, Göttingen, Germany) using a Plan-Apochromat 20x/0.8 M27 or EC Plan-Neofluar 40x/0.75 M27 objective lenses, processed with ZEN 2009 Light Edition (Carl Zeiss, Göttingen, Germany) and CorelDRAW X3 (Corel Corporation, Ottawa, ON, Canada). For negative controls, the primary antibody was omitted and only the secondary antibody was used. Blocking peptide or pre-immune IgG was used as a negative control to show non-specific binding of C3aR or C3 Abs as previously described (Haynes et al., 2013). Results were confirmed using at least three different biological replicates.

2.7. Whole-mount immunohistochemistry

After an overnight fixation in 4% PFA, embryos were washed with 1% PBST (1% Triton-X100 in PBS) and incubated for 2 h in blocking solution (10% goat serum, 0.2% sodium azide in 1% PBST). C3aR or C3 Abs (1:100 in blocking solution) were added and incubated at 4 °C for three days following several washes with 10% goat serum in 1% PBST and 1% PBST only. A solution of secondary antibody (1:100 in 10% goat serum in 1% PBST) was added to the embryo and incubated at 4 °C for 2 days followed by several washes with 1% PBST and PBS. Embryos were analyzed using a SteREO Discovery V12 microscope (Carl Zeiss, Göttingen, Germany) and the images were captured using Zeiss AxioCam MRc5 with AxioPlan 2 and Zeiss AxioVison 4.7.1 extended focus module (AxioVision, Göttingen, Germany). A blocking peptide or pre-immune IgG was used as a negative control to show non-specific binding of C3aR or C3 Abs as previously described (Haynes et al., 2013). The results represent at least three different biological replicates.

2.8. Antibodies

Antibodies against Pax6 (1:10), N-cadherin (6B3, 1:10), β -catenin (PY-489, 1:20) and Laminin (3H11, 1:10) were obtained from Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA). Anti-Pax2 Ab (1:100) was obtained from Covance (Princeton, NJ, USA). Anti-Tbx5 Ab (1:100) and Anti-Gli1 (H-300, 1:50) were purchased from Santa Cruz

Biotechnologies (Santa Cruz, CA, USA). Anti-Mitf Ab (1:500) was obtained from Abcam (Cambridge, MA, USA). Anti-Chx-10 (Vsx2, 1:250) Ab was purchased from ExAlpha (Shirley, MA, USA). Fluorescein/Oregon Green Ab (1:100) was purchased from Molecular Probes (Grand Island, NY, USA). The production of Abs against C3 (targets amino acids 741–761) and C3aR as well as the blocking peptides are described in Haynes et al. (2013). Preimmune IgG was isolated from the corresponding rabbit serum using Protein A affinity purification. All secondary Abs were purchased from Molecular Probes (Grand Island NY, USA) and used at 1:100 dilutions.

2.9. Cell Quantification

Quantification of Gli1 and β -catenin positive cells, in control and treated eyes, was determined by counting three different areas from three different eyes ($1000 \mu\text{m}^2$) of the distal and most proximal regions of NE or RPE using confocal images (1024×1024 taken at $20\times$). The percentage among DAPI positive cells was then calculated and the average number was determined. Student *t*-test was used to establish statistical significance between experimental and control groups. Error bars represent SEM. *** $P < 0.001$. Non-significant (NS) = $P > 0.05$.

2.10. Histology

Embryos used for histology were fixed in Bouin's fixative (Ricca Chem. Comp., Arlington, TX) overnight at room temperature and transferred to 70% ethanol, embedded in paraffin, sectioned at $12 \mu\text{m}$ using a Rotary microtome HM 355S (MICROM International GmbH, Walldorf, Germany), stained with hematoxylin and eosin (H & E) and photographed using an Olympus BX51-P microscope with Magnafire S99800 Camera (Olympus, Tokyo, Japan). Images were analyzed using MagnaFire imaging system (Olympus, Tokyo, Japan).

2.11. C3aR Blocking experiments

Anti-C3aR Ab utilized in the blocking experiments was previously reported and targets the amino acids 257–274 (DIRLLESESDLPHTSLPV) present in the second extracellular loop of C3aR (Haynes et al., 2013) and it was produced using the methods established previously (Carmona-Fontaine et al., 2011). Approximately $0.3 \mu\text{l}$ of anti-C3aR Ab solution ($2.6 \mu\text{g}/\mu\text{l}$) or rabbit IgG (H-270) (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) at the same concentration was injected in the forebrain of stage 10/11 embryos using a Pico-injector system PLI-100 (Harvard Apparatus, Holliston, MA, USA) and beveled glass needles 50 mm long, $20 \mu\text{m}$ tip diameter (FHC, Bowdoinham, ME, USA). Embryos were collected at the times indicated in the figure legends.

2.12. Cell proliferation

Proliferation studies were performed at the times indicated in the figure legends using 5'-ethynyl-2'-deoxyuridine (EdU). A total of $30 \mu\text{l}$ of 5 mM EdU solution (Invitrogen, Grand Island, NY) was added directly on top of the eyes and incubated for 1 h, then the embryos were harvested in 24-well plates containing PBS, fixed in 4% PFA/0.1 M PB overnight at 4°C , processed as indicated in the immunohistochemistry section, and then sectioned at $12 \mu\text{m}$ thickness. Slides were washed $2 \times 5 \text{ min}$ in PBS, $1 \times 5 \text{ min}$

in 1% saponin/PBS, 2 × 5 min in PBS. EdU detection was performed using Click-iT[®] EdU Alexa Fluor[®] 488 (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. One hundred microliters of Click-iT EdU reaction cocktail were added in each slide and incubated 30 min at room temperature (RT) protected from the light, incubated 5 min with DAPI at 30 nM (Invitrogen, USA Grand Island, NY) and cover-slipped with Vectashield (Vector Laboratories, Burlingame, CA). Quantification was done by counting the number of EdU positive cells from at least three sections using confocal images (1024 × 1024 taken at 20×) from three different eyes. The percentage was then calculated and the average number was determined. Student *t*-test was used to determine statistical significance between experimental and control groups. Error bars represent SEM. ****P* < 0.001. Non-significant (NS) = *P* > 0.05.

2.13. TUNEL

TUNEL was performed by using the in situ cell death-detection kit TMR-red (Roche Applied Sciences, Indianapolis, IN, USA). Quantification was done by counting the number of TUNEL positive cells from at least three sections using confocal images (1024 × 1024 taken at 20×) from three different eyes and the average number was calculated. Student *t*-test was used to determine statistical significance between experimental and control groups. Error bars represent SEM. ***P* < 0.01; ****P* < 0.001. Non-significant (NS) = *P* > 0.05.

3. Results

3.1. Expression patterns of C3 and C3aR during eye morphogenesis

We have previously shown that complement components C3/C3aR are expressed in embryonic day 4 (E4) chick eyes and that they play an important role in retina regeneration (Haynes et al., 2013). We demonstrated that blocking the C3a/C3aR signaling pathway using a C3aR specific antibody (anti-C3aR Ab) was sufficient to inhibit C3a-induced retina regeneration (Haynes et al., 2013). To investigate the possible role(s) of complement component C3aR in eye development, we decided to first examine its expression pattern using the chick embryo as a model. Wholemount immunofluorescence performed at the OV stage (stages 10/11) using a previously reported C3aR antibody that targets amino acids 257–274 present in the second extracellular loop (Carmona-Fontaine et al., 2011; Haynes et al., 2013), revealed that C3aR is present in neurogenic tissues including the prosencephalon, mesencephalon, OV, neural tube, and also in the somites (Fig. 1a). In addition, transverse sections of the embryo demonstrated that C3aR is present throughout the distal to ventral OV and in the presumptive lens ectoderm (Fig. 1b). Moreover, C3aR is located predominantly in the membrane as was demonstrated by co-localization with the cell adhesion molecule N-Cadherin (Fig. 1k-m). Later, at stage 14 (E2), C3aR is present in the presumptive RPE, NE and in the lens vesicle (Fig. 1c). At stage 18 (E2.8) and 24 (E4), during the transition from early to well-formed OC, C3aR is detectable in the RPE, NE, CM and in the lens (Fig. 1d and e). Interestingly, at these stages the receptor was detected almost exclusively in the nuclear compartment co-localizing with DAPI (Fig. 1n-p). Consistent with the immunofluorescence staining, Reverse Transcriptase-PCR (RT-PCR) analysis revealed the presence of *C3aR* mRNA in chick embryos at stages 10/11 (Fig. 1q). In addition to C3aR, we also investigated the presence of the complement component C3 during eye

morphogenesis. We have previously shown that E4 chick eyes express the complement components *C3*, *Factor D* and *Factor B* that are important for the assembly of the alternative pathway of C3 convertase (C3bBb) that cleaves C3 into C3a and C3b fragments. In addition, the presence of a 68-kDa fragment corresponding to iC3b was found in the whole chick eye, CM, NE and RPE indicating the local activation of chicken C3. Although, C3a is generated during C3 activation, it is difficult to detect by western blotting because of the low abundance under physiological conditions (Haynes et al., 2013). Using an antibody specific for the alpha-chain of chicken C3 (targets amino acids 741–761) (Haynes et al., 2013), we observed similar patterns of expression to those obtained for the C3aR in the whole embryo at the OV stage (Fig. 1f). We also detected the presence of C3 throughout the distal to ventral OV, the presumptive lens ectoderm, presumptive RPE, lens vesicle (Fig. 1g and h) and in the RPE, NE, CM and lens at stage 18 (E2.8) and 24 (E4) respectively (Fig. 1i and j). Similar to *C3aR* mRNA, the transcript for *C3* was detected in the embryo at stages 10/11 (Fig. 1q). Negative controls for the C3 and C3aR immunofluorescence in which the pre-immune IgG control was used or the corresponding blocking peptide was added along with the tested antibody are shown in Supplementary Fig. S1. All together these results demonstrate that complement components C3 and C3aR are present during chick eye morphogenesis.

3.2. C3aR is required for proper eye morphogenesis

To elucidate the role of C3aR during the eye morphogenesis, we knocked down C3aR levels using anti-sense morpholinos (Mos) that block the translation initiation site (C3aR Mo1) or the splicing of exon1-intron1 junction of the C3aR (C3aR Mo2). Each C3aR Mo or control morpholino (control Mo) was injected in the neural tube of stage 10/11 embryos and the OV was electroporated (see Section 2). The KD efficiency was analyzed 24 h after electroporation by immunohistochemistry and Western blot. In both cases, we observed a significant reduction of C3aR protein in the OCs of C3aR-Mo-electroporated embryos compared with those electroporated with control Mo (Supplementary Fig. S2). C3aR morphants collected 3 days after electroporation, once the OC has been completely established, displayed consistently unilateral defects, due to the electroporation of one side of the embryo, microphthalmia being the most frequent (Mo1: 34.8%; Mo2: 39.6%; Fig. 2j and Supplementary Table S1) in which the eyes were more than 50% smaller compared with the controls (Fig. 2a and c). Another common defect consisted of an expanded gap in the ventral portion of the eye compared with the control morphants (Mo1: 30.4%; Mo2: 18.9%; Fig. 2a, e, j and Supplementary Table S1). We also observed in a few cases more severe morphological defects such as lack of lens or retina, anophthalmia, smaller lens and folded retina (Supplementary Table S1 and Supplementary Fig. S3). Electroporation with control Mo did not show any defects and the eyes were similar to the mock electroporated or non-electroporated embryos (Control Mo, 100%, n=36, Fig. 2a, j and Supplementary Table S1). Although, we observed similar defects using two different C3aR-Mos, we verified the morphological defects generated by each Mo, using a well characterized C3aR blocking antibody (anti-C3aR Ab) that targets the second extracellular loop of C3aR and that has been previously used to block the C3aR pathway (Carmona-Fontaine et al., 2011; Haynes et al., 2013). Anti-C3aR Ab injections in the OV at stage 10/11 generated similar defects to those observed with the C3aRMo electroporation consisting of microphthalmia (40%, Fig. 2d, j

and Supplementary Table S1), defects in the ventral portion of the eye (30%, Fig. 2f, j and Supplementary Table S1) and very few severe defects such as no lens or retina, folded retina and anophthalmia (Supplementary Fig. S3 and Table S1). Embryos injected with control IgG did not show any defects and the eyes were normal (100%, n=30, Fig. 2b and j).

The use of C3aR blocking antibody in addition to C3aR-Mos provides strong evidence that the phenotypes observed are specific and are not the result of “off target effects”. These results suggest that blocking C3aR at stages 10/11 interferes with the proper morphogenesis of the eye. To narrow the critical time when C3aR has an impact on eye morphogenesis, we performed electroporations of C3aR-Mo at stage 9 (early OV stage) and stage 15 (when the OC is well established). Similar to the results observed at stage 10/11, when knocking down C3aR at stage 9 we obtained microphthalmic eyes (37%, n=17) and other defects including ventral defects, no lens and anophthalmia (Supplementary Table S2). In contrast, the KD of C3aR at the OC stage had no impact on eye size and the eyes were normal (n=22; Supplementary Fig. S4). Together these results suggest that C3aR signaling is critical at the OV stages for the proper morphogenesis of the eye.

We further investigated the most striking phenotypes including microphthalmic eyes and eyes with ventral defects (coloboma-like eyes) by histology. Frequently, the microphthalmic eyes were unable to form a closed OC (Fig. 2h) even though the NE and RPE (most of the cases dorsal RPE) have been specified (see Fig. 5d for analysis of RPE specification). A small lens was formed; however, it was often displaced (compare control eye, Fig. 2g, with treated eye, Fig. 2h). Similar results were obtained when Pax6 was blocked or Pax2 was overexpressed in early stage 10 chick embryos (Canto-Soler and Adler, 2006; Sehgal et al., 2008). We also observed some embryos in which the eyes with ventral defects also had smaller lenses (Fig. 2i). In most cases the OS was abnormally formed (Fig. 2h and i).

3.3. Interfering with the C3aR pathway generates prominent apoptosis and affects proliferation

To determine if cell death could contribute to the microphthalmic phenotype, we performed TUNEL analysis after OC formation. This analysis showed a high rate of apoptosis in dorsal and ventral structures of eyes at two and three days after treatment (Fig. 3a, c-e and j, $P < 0.001$). Control eyes did not show any significant apoptosis (Fig. 3f, h and j). To determine if defects in proliferation were also contributing to the microphthalmic phenotype, we performed an analysis of EdU incorporation after OC formation. We observed a similar percentage of cells in S phase (EdU+ cells) in microphthalmic and control eyes at two days after treatment (Fig. 3b, g and k), however, at three days after treatment, the number of EdU+ cells was significantly decreased in the microphthalmic eyes compared to control eyes (Fig. 3c-e, i and k, $P < 0.01$). Taking together these results suggest that apoptosis associated with decreased proliferation during OC formation could be the major contributor to the microphthalmic phenotype observed after interfering the C3aR pathway.

3.4. C3aR is required for proper ventral-dorsal specification of the eye

To know if the dorsal-ventral/proximal-distal domains of the eye were also disrupted, we performed immunohistochemistry to analyze the Pax6 (dorsal/distal mostly) and

Pax2 (ventral/proximal) domains in control, microphthalmic and coloboma-like eyes, and determined that the Pax2 domain had expanded (Fig. 4a-f). In addition, Tbx5, a transcription factor that is restricted to the dorsal region of the OC, was absent in the microphthalmic eyes compared to control eyes (Fig. 4g and h). To further characterize gene expression, we performed in situ hybridization using two members of the aldehyde dehydrogenase family, *raldh-1* (also known as *aldh1a1*) and *raldh-6* (also known as *raldh-3* or *aldh1a3*) (Mey et al., 2001; Peters and Cepko, 2002; Suzuki et al., 2000). *Raldh-1*, a gene expressed only in the dorsal portion of the developing retina, was absent in the microphthalmic eyes (Fig. 4i and j). In contrast, *raldh-6* and *cVax* that are normally confined to the ventral area of the developing retina, were not only present in this area but have been expanded towards the dorsal area of the OC recapitulating the pattern of Pax2 in the microphthalmic eyes (Fig. 4k-n). Another interesting characteristic of these eyes was the re-specification of ventral RPE (evaluated by the RPE specific marker Mitf) to NE (Fig. 5d and e) that was accompanied by the expression of Vsx2, a transcription factor that regulates eye organogenesis and is key in the specification of retinal progenitor cell identity (Zou and Levine, 2012) (Fig. 5d and f). In zebrafish, changes in Shh activity generate perturbations in *Pax2* and *Pax6* expression, and as a consequence, changes in eye development (Macdonald et al., 1995). In addition, Shh pathway has been associated with dorsal-ventral patterning of eye structures and ocular tissue specification (Furimsky and Wallace, 2006; Zhang and Yang, 2001). To gain insight into Shh activity, we decided to evaluate the presence of Gli1, a direct target of the Shh pathway and an indicator of Shh activated cells. Gli1 protein was evident in the NE, RPE, lens and the OS of control eyes and showed a clear nuclear pattern (Fig. 6a-c). Although the structure of the eye has been disrupted in the microphthalmic eyes, we observed a significant decrease in the number of Gli1+ cells in the distal NE and proximal NE and RPE (Fig. 6d-g; $P < 0.001$) compared with the control eyes. Interestingly, we observed a gradient of nuclear Gli1 protein increasing towards the distal region of the RPE, where the number of Gli1+ cells was similar to the control eyes (Fig. 6d and g). It could be possible that the reduced number of Gli1+ cells is associated with the prominent apoptosis observed in the ventral-proximal side of the microphthalmic eyes (Fig. 3c, d'). To analyze further whether disturbing C3aR signaling affects the pathways associated with cell specification and eye morphogenesis, we decided to investigate β -catenin, a canonical Wnt pathway effector which is important for RPE commitment and OC formation (Hagglund et al., 2013; Steinfeld et al., 2013; Westenskow et al., 2009). We have previously used an antibody that specifically recognizes the transcriptionally active form of β -catenin (PY-489- β -catenin) (Rhee et al., 2007; Zhu et al., 2014). Using the same antibody, we observed that in the control eyes, active β -catenin was predominantly present in the CM, proximal RPE, and some positive cells in the proximal region of the NE (Fig. 6h-j). However, in contrast to the restricted presence of active β -catenin in the control eyes, β -catenin was ubiquitously present in microphthalmic eyes being more abundant in the proximal NE and distal RPE compared with the control eyes (Fig. 6k-o, $P < 0.001$).

Overall, these results show that RPE specification (evaluated by Mitf presence) and dorsal-ventral patterning (Tbx5, *raldh-1*, *raldh-6* and *cVax* expression), as well as Gli1 and active β -catenin expression patterns are affected when interfering with the C3aR signaling during chick eye morphogenesis.

It has been shown that ectopic neural retina and coloboma occur when Pax2 is overexpressed in chick eyes (Sehgal et al., 2008). In our study, some of the eyes showed signs of coloboma (wide ventral gaps) at 3 dpt (Fig. 2e and f). To further characterize this phenotype, we blocked C3aR on stage 10/11 chick embryos and analyzed the eyes 8 days later. At this time, we were able to clearly determine the lack of closure of the OF (compare Fig. 7a and d). Immunohistochemistry for laminin, which delineates the basement membrane of the RPE as well as of the vitreous surface of the retina, distinctly showed that when C3aR signaling is blocked, the proximal region of the retina is not continuous, as it is in the control eye (Fig. 7b, c, e and f), but resembles the pattern found in stage 24 (E4) eyes, where the OF is still open (Fig. 7g-i). The degradation of the basement membrane enclosing each opposing face of the RPE-retina is essential for normal closure of the OF (Hero, 1989, 1990; Hero et al., 1991; Torres et al., 1996), therefore our results indicate a lack of closure of the OF and confirm the coloboma phenotype observed anatomically when looking at the whole eye.

4. Discussion

The presence of C3aR in the adult mouse and human retina has been reported (Vogt et al., 2006; Yu et al., 2012), however its role in eye development has not been explored. Even though complement components including C3 and C3aR have been shown to be expressed during embryogenesis from gastrula to organ bud stage in *Xenopus laevis*, no detailed expression studies on eye structures were performed (McLin et al., 2008). Therefore, this is the first report describing complement components C3/C3aR protein expression during early eye development, and certainly it is the first study that demonstrates that C3aR is necessary for proper eye morphogenesis. The anaphylatoxin receptors C3aR and C5aR are members of the rhodopsin family of G-proteincoupled receptors (GPCR) (Norgauer et al., 1993; Rollins et al., 1991). Although the human C3aR (hC3aR) and C5aR receptors share some identity between them (37% in the nucleotide sequence), the C3aR has interesting characteristics including an uncommon second extracellular loop (more than 160 amino-acid residues) between the TM4 and TM5 domains (Ames et al., 1996; Tornetta et al., 1997). Importantly, this loop is thought to be involved in the interaction with the ligand C3a and the receptor function (Chao et al., 1999; Gao et al., 2003). In addition, the human C3aR contains a presumptive nuclear localization (NLS) signal FRKKAR at amino acid 442 (FRAKAR in chick at amino acid 418, NP_001025940) that is also conserved between mouse, rat, and chimpanzee (Supplementary Fig. S5). This NLS motif is also present in other 17 GPCRs including the receptors for angiotensin, bradykinin, and apelin (Lee et al., 2004). Furthermore, apelin receptor is localized in the nucleus of neurons present in the cerebellum and hypothalamus. Mutations on the apelin NLS motif impair its nuclear localization (Lee et al., 2004). In a recent study, the GPCR coagulation factor II receptor-like 1 (F2r11), which is important for new blood vessel formation during retinal development, was shown to translocate from the plasma membrane to the cell nucleus in retinal ganglion cells (Joyal et al., 2014). Although C3aR contains this NLS motif, its functional significance remains to be determined. Interestingly, we found that C3aR protein had a dynamic distribution during eye morphogenesis, shuffling between the membrane and the nucleus as eye development progressed. This change of C3aR cellular localization during the transition from OV (membrane) to OC (nuclear) could be associated with some of its functions regulating

dorsal-ventral eye development and the closure of the OF. Consistent with these results, hC3aR has been shown to translocate to the nuclear compartment after stimulation with C3a in human mesenchymal stem cells (MSC) (Schraufstatter et al., 2009). Moreover, in the same study, the C3a-dependent stimulation of C3aR generated sustained and robust ERK1/2 and Akt phosphorylation that could lead to activation of transcription, cell proliferation, and differentiation (Schraufstatter et al., 2009). It is possible that the shift in cellular localization of C3aR from membrane to the nucleus could coincide with the specification of the OC, however, detailed studies should be performed to unravel this interesting observation.

The role of C3aR during mammalian eye morphogenesis has not been evaluated, even though C3aR knockout (KO) mice have been generated. Analysis of KO adult mouse eyes show that the retina has continuing loss of neurons including photoreceptors and interneurons. These mice have progressive loss of sight and abnormal RPE function. However, no analysis of choroid fissure closure was reported as these studies centered on the role of complement components in retina maintenance and macular degeneration (Yu et al., 2012).

The proper establishment of the dorsal-ventral/proximal-distal domains in the vertebrate eye is critical for the accurate closure of the OF. These domains are regulated by several transcription factors including Pax2, Pax6, Vax 1/2, AP-2 alpha and Tbx5 along with several signaling pathways such as Shh, BMP, Wnt, retinoic acid and FGF. Disruption of any of these during OV to OC formation results either in microphthalmia, ventral domain expansion, defective OS, retina duplications and/or coloboma (Adler and Belecky-Adams, 2002; Alldredge and Fuhrmann, 2016; Atkinson-Leadbetter et al., 2014; Bassett et al., 2010; Fujimura et al., 2009; Hagglund et al., 2013; Kobayashi et al., 2010; Koshiba-Takeuchi et al., 2000; Marsh-Armstrong et al., 1994; Molotkov et al., 2006; Morcillo et al., 2006; Mui et al., 2002, 2005; Schulte et al., 1999; Schwarz et al., 2000; Torres et al., 1996; Westenskow et al., 2009; Zhang and Yang, 2001; Zhao et al., 2010; Zhou et al., 2008). We considered defects on pathways involved in the maintenance of dorsal neuroretinal patterning of the eye such as the Wnt pathway to possibly explain the phenotype obtained by blocking/knocking down C3aR during OV formation. We observed loss of Tbx5 and *raldh-1* as well as expansion of the ventral Pax-2 and *cVax* domains similar to what was reported in the Lhx2- β -catenin KO mice, where the canonical Wnt pathway was disrupted before the formation of the OC. In addition, Lhx2- β -catenin KO mice also displayed a NE in place of the RPE (double NE) in the dorsal side (Hagglund et al., 2013). We found RPE to NE transformation but only in the ventral side. This ventral restriction could be due to the timing when we interfered with C3aR. Interestingly, when β -catenin is knocked out in mice, specifically in the RPE after the OC is formed, the RPE is not maintained and a NE forms in the dorsal as well as the ventral side (Fujimura et al., 2009). We have also reported that RPE can transdifferentiate to neural retina in chicks after retina removal when the Wnt pathway is inhibited (Zhu et al., 2014). However, we note that unlike the Lhx2- β -catenin KO mice, in which the OVs did not form an OC, we observed a rudimentary OC formation in the majority of microphthalmic eyes. Moreover, nuclear β -catenin (an indicator of active Wnt-canonical pathway) showed a more ubiquitous expression throughout the NE and RPE in contrast to the differential expression in a correctly formed OC of the control eyes. Our results resemble more the phenotype obtained when Axin2, an antagonist of Wnt/ β -catenin,

is disrupted leading to developmental eye defects such as microphthalmia, lens defects and coloboma including defects in the closure of the OF and ventral OC (Alldredge and Fuhrmann, 2016). Moreover, constitutive activation of Wnt/ β -catenin signaling in lens fiber cells results in abnormal and delayed fiber cell differentiation resulting in cataracts and microphthalmia (Antosova et al., 2013). Finally, we have previously observed that nuclear β -catenin⁺ and p27⁺ (Cyclin-dependent kinase inhibitor 1B) cells of the RPE do not proliferate suggesting that nuclear β -catenin prevents those cells from entering the cell cycle (Zhu et al., 2014). We observed a significant reduction of cell proliferation at 3 dpt, this reduction could be associated with the prominent amount of active β -catenin detected. Collectively, our results suggest that C3aR is regulated by or is regulating the canonical Wnt pathway.

In addition, we observed that our results also closely resemble phenotypes obtained when Shh is overexpressed (Adler and Belecky-Adams, 2002; Morcillo et al., 2006; Zhang and Yang, 2001; Zhao et al., 2010), where the ventral-proximal domain characterized by Pax2 is expanded and the Pax6 dorsal-distal domain is reduced (Ekker et al., 1995; Macdonald et al., 1995; Nasrallah and Golden, 2001; Perron et al., 2003; Zhang and Yang, 2001). In addition, the eyes were also microphthalmic and some displayed coloboma (Adler and Belecky-Adams, 2002; Nasrallah and Golden, 2001; Zhang and Yang, 2001). Moreover, we observed cell death in the ventral retina of the disrupted C3aR embryos similar to what has been reported for Shh overexpression in chick eyes (Zhang and Yang, 2001). However, in our results, Gli1 an indicator of active Shh signaling, was downregulated in the NE and proximal RPE in the microphthalmic eyes. This reduction coincides with the prominent apoptosis observed in the same region. Interestingly, blocking Shh at Stage 10 (optic vesicle) in the chick also results in cell death and generates eyes with reduced size and disrupted ventral patterning (Zhang and Yang, 2001), suggesting that the cells in the ventral-proximal part of the eye that underwent cell death are those with disrupted C3aR and inhibited Shh/Gli1 signaling. In line with this, it has been shown that loss of Shh generates apoptosis mediated by p53 in the retina (Prykhozij, 2010). Clearly, more work is needed to unravel the connection between C3aR, Wnt/ β -catenin and Shh pathways.

Another pathway that regulates eye morphogenesis is the BMP pathway. When noggin is overexpressed in developing chick eyes to inhibit BMP signaling, the ventral RPE seems to transdifferentiate towards OS fate as it is Pax2⁺/Pax6⁻. Even though we observe that the ventral portion of the eye has no Mitf⁺ RPE, it mostly expresses Vsx2 and not Pax2, and therefore differs from the observations obtained when the BMP pathway is blocked (Adler and Belecky-Adams, 2002).

C3aR KD studies during early chick eye development reveal microphthalmic eyes as well as eyes displaying colobomas. One of the mechanisms by which C3aR may be regulating eye morphogenesis is by controlling survival of cells in the dorsal/ventral region, and possibly their migration. We observed significant cell death at 2 and 3 days posttreatment with anti-C3aRAb and C3aR Mos. We also observed a decrease in cell proliferation. In support of our results, C3a has been implicated in cell proliferation and survival in liver cells undergoing regeneration (Markiewski et al., 2009, 2004; Min et al., 2016; Strey et al., 2003).

In conclusion, this is the first report describing complement components C3/C3aR expression during early eye development, and certainly it is the first study to unravel the unsuspected role of C3aR in eye morphogenesis. C3aR signaling is necessary for the proper formation of the OC and regulates the closure of the OF. Interfering with C3aR during OC formation affects the proximal-ventral *Pax2/cVax/ raldh-6+* domain as well as the *Tbx5/ raldh-1+* dorsal domain and affects the ventral RPE fate. The evidence gathered so far also suggests that Shh and Wnt/ β -catenin pathways are affected by blocking C3aR, which in turn leads to prominent apoptosis, decrease in cell cycle entry leading to microphthalmic eyes and coloboma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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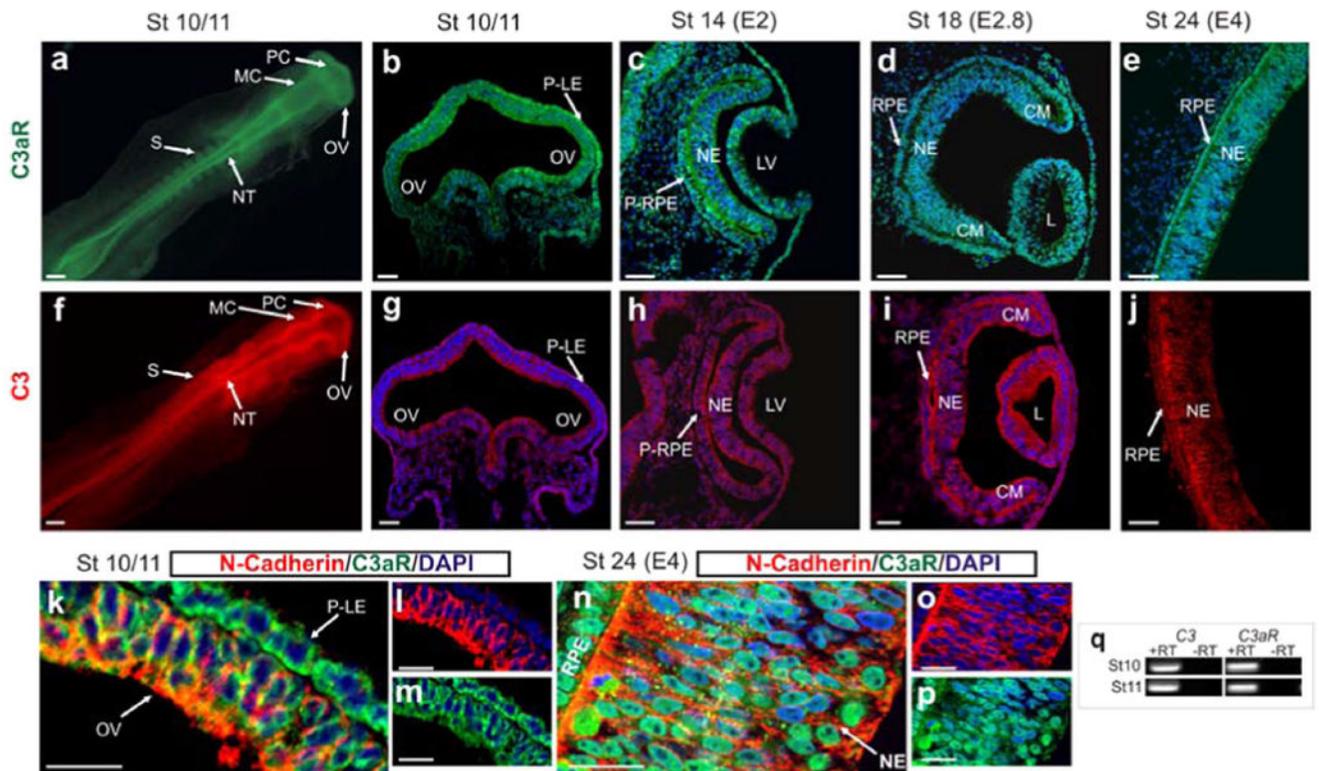


Fig. 1.

C3 and C3aR are present in developing chick eye. (a-j) Immunofluorescence staining showing the presence of C3aR (green) and C3 (red) in (a,f) whole chick embryo and (b-e, g-j) transverse sections of the chick eye at different developmental stages. DAPI was used to counterstain the nuclei. (k-m) Immunofluorescence staining of N-Cadherin (red) and C3aR (green), at the OV stage. (n-p) Representative high resolution confocal image from a transverse section at stage 24 (E4) showing C3aR primarily localized in the nuclear compartment and co-localizing with DAPI. (q) RT-PCR analysis of *C3* and *C3aR* transcripts of whole embryos at stage (St) 10 and 11. Control reactions (-RT) were included in which the reverse transcriptase was omitted. OV=Optic vesicle; PC=Prosencephalon; MC= Mesencephalon; S=Somites; NT=Neural tube; P-LE=Presumptive lens ectoderm; P-RPE= Presumptive RPE; LV=lens vesicle; NE=Neuroepithelium; CM=Ciliary margin; RPE=Retinal Pigmented Epithelium; L=lens; Scale bars in a and f: 200 μ m; in b-e, g-j: 50 μ m and in k-p: 20 μ m.

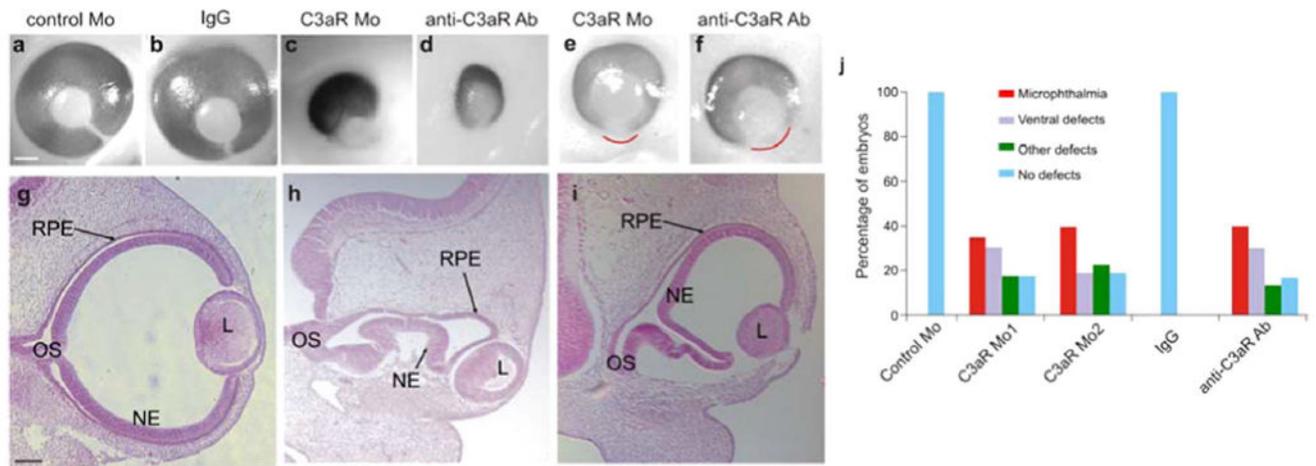


Fig. 2.

Interfering with C3aR signaling causes microphthalmia and defects in the ventral portion of the eye. C3aR Mo1, C3aR Mo2 or control Mo were electroporated at the OV stage (St 10/11) and collected 3 days later. (c, e) Lateral views of representative C3aR morphant eyes showing microphthalmia and ventral defects (red line in e). Embryos treated with control Mo (a) displayed normal development. (d, f) Embryos injected with anti-C3aR antibody (anti-C3aR Ab) and collected 3 days later generated similar microphthalmic eyes and eyes with ventral defects (red line in f) when compared to electroporated C3aR morphants. (b) Embryos injected with control IgG showed normal development. (g-i) Histological sections illustrating control, microphthalmic and ventral defective eyes, respectively. (j) Percentage of embryos with defects when treated with either C3aR Mo1 (that blocks the translation initiation; n=23), Mo2 (blocks the splicing of the exon#1-intron#1 junction; n=53), control Mo (n=36), anti-C3aR Ab (n=30) or control IgG (n=30). RPE=Retinal Pigmented Epithelium; L=lens; NE=Neuroepithelium; OS=Optic Stalk. Scale bars in a: 0.5 mm and applies to b-f; in g: 100 μ m and applies to h and i.

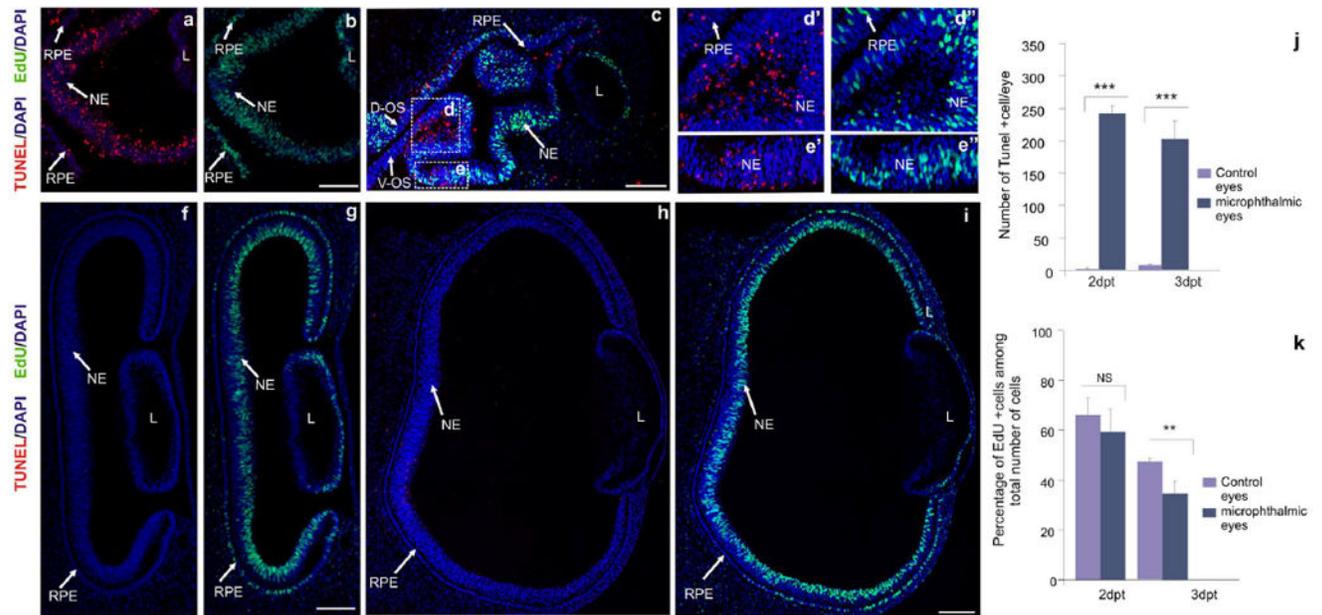
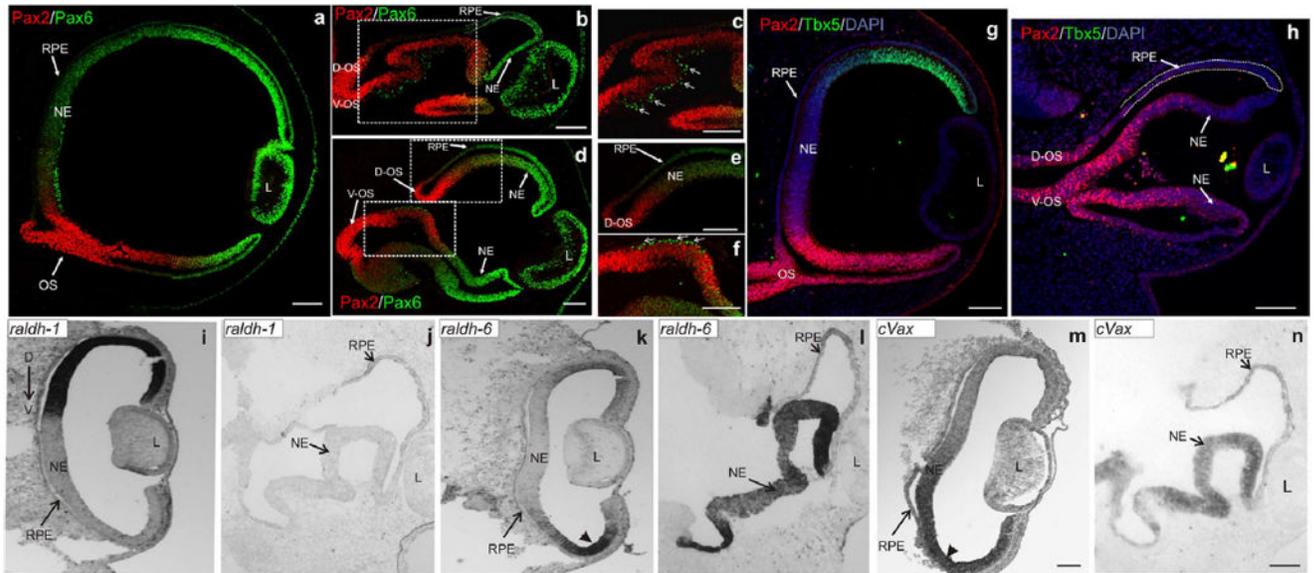


Fig. 3. Disrupting C3aR generates prominent apoptosis as well as decreased cell proliferation in microphthalmic eyes. (a, c-e) TUNEL (red) analysis of eyes collected 2 and 3 days post treatment (dpt), showed microphthalmic eyes with a prominent number of apoptotic cells in the NE and RPE. (d', e') Magnification of boxed areas in c. (b, c-e) EdU (green) was widely incorporated in NE, RPE and L at 2 dpt; in contrast, at 3 dpt there was significant reduction of EdU incorporation compared to the control eyes. (d'', e'') Magnification of boxed areas in c showing EdU positive cells. Control eyes collected at the same time points showed normal development (f, h) low to null levels of cell death and (g, i) prominent EdU incorporation. (j, k) Quantitative analysis of TUNEL and EdU positive cells on microphthalmic and control eyes at 2 and 3 dpt. The Student's *t*-test was used to determine significance. Graphic data are presented as mean \pm SE. ** $P < 0.01$, *** $P < 0.001$; actual values in Table S3. DAPI (blue) was used to counterstain the nuclei. RPE= Retinal Pigmented Epithelium; NE= neuroepithelium; L= lens; D-OS= Dorsal Optic Stalk; VOS= Ventral Optic Stalk; dpt= days post-treatment; NS= non-significant. Scale bar in b,g, and i is 100 μ m and applies to a,f, and h respectively. Scale bar in c is 100 μ m.

**Fig. 4.**

C3aR is required for proper ventralization of the eye. (a-f) Pax2 (red)/Pax6 (green) immunofluorescence staining of eyes electroporated with (a) Control Mo, (b) C3aR Mo1 or (d) exposed with anti-C3aR antibody at St 10/11 and analyzed 3 dpt. Treated eyes showed a dorsal expansion of Pax2 expression when compared with its confined expression in the ventral neuroepithelium (NE) and optic stalk (OS) observed in control eyes. (c, e, f) Magnification of the boxed areas in (b) and (d), where a few Pax2+ cells are localized in the Pax6 domain (arrows in c and f); (g) Immunofluorescence staining against Pax2 (red) and Tbx5 (green) of control (IgG-injected) and (h) microphthalmic eyes (injected with anti-C3aR Ab) lacking Tbx5+ cells and having an expansion of Pax2+ cells in the dorsal domain. DAPI (blue) was used to counterstain the nuclei. (i-n) *In situ* hybridization of control eyes (IgG-injected) and microphthalmic eyes (injected with anti-C3aR Ab) for (i,j) *raldh-1*, expressed only in the most dorsal region of control eyes and absent in treated eyes. (k,l) *raldh-6* and (m,n) *cVax* are expressed in the most ventral region in both the control and microphthalmic eyes but expanded in the dorsal region of treated eyes. RPE= Retinal Pigmented Epithelium; NE=neuroepithelium L= lens; OS= Optic Stalk; D-OS= Dorsal Optic Stalk; V-OS=: Ventral Optic Stalk; Scale bar in a-h is 100 μ m. Scale bar in m: 100 μ m, and applies for i, k; Scale bar in n: 100 μ m and applies for j, l.

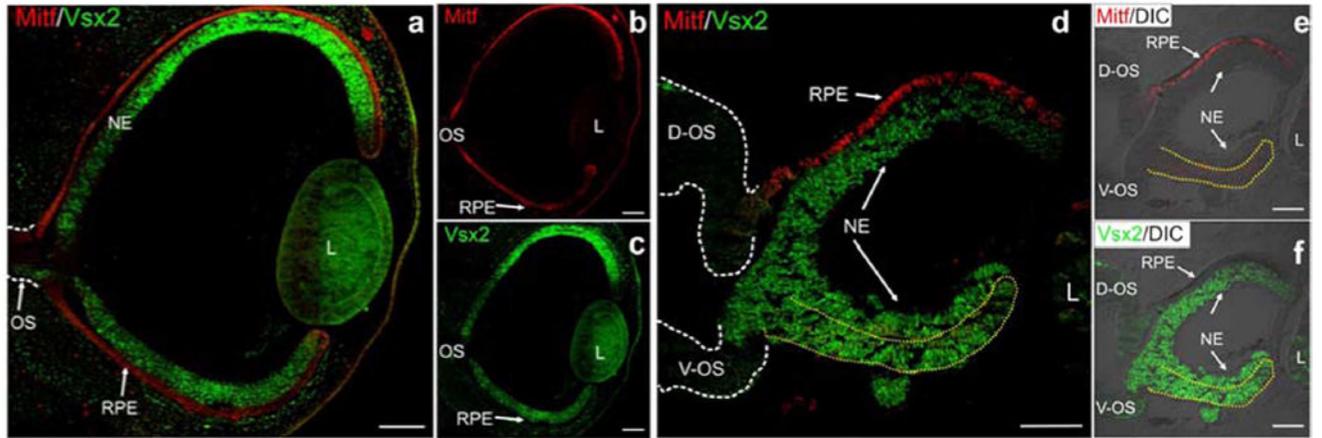


Fig. 5. Blocking C3aR causes re-specification of ventral RPE to neuroepithelium. (a-c) Immunofluorescence staining against the RPE specific marker *Mitf* and retina progenitor marker *Vsx2* in control and (d-f) microphthalmic eyes depicting a lack of ventral RPE and the presence of a *Vsx2*+ bilayered neuroepithelium (yellow dotted line). NE= neuroepithelium; RPE=Retinal Pigmented Epithelium; L= lens; OS= Optic Stalk; D-OS= Dorsal Optic Stalk; V-OS= Ventral Optic Stalk; All scale bars: 100 μ m.

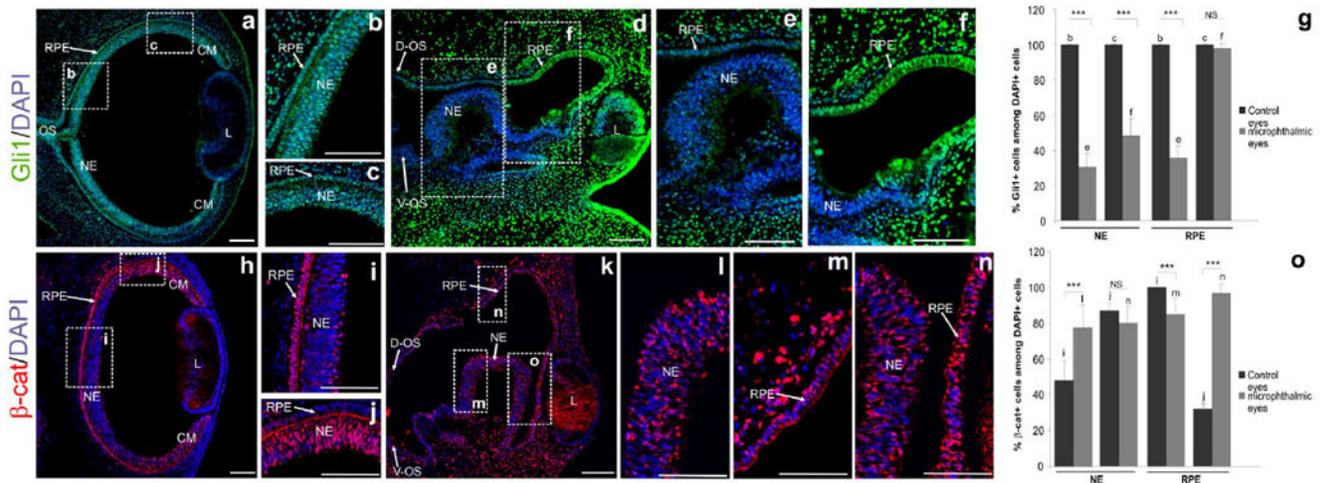


Fig. 6. Blocking C3aR pathway affects Gli1 and nuclear β -catenin protein expression. (a-f) Gli1 immunofluorescence staining of (a) control eyes, showing a nuclear localization in the proximal (b) and distal (c) NE and RPE. (d) Microphthalmic eye, showing a change in distribution and a decrease of Gli1 protein in the NE- (boxed area magnified in e) while keeping a higher protein level in the presumptive dorsal-distal RPE (boxed area magnified in f). (h-n) active β -catenin immunofluorescence staining of (h) control eyes, showing a nuclear localization in the (i) proximal and (j) distal RPE and NE. (k) Microphthalmic eye, showing nuclear β -catenin through the whole eye with specified areas magnified (l-n). (g, o) Quantitative analysis of Gli1 and nuclear β -catenin positive cells among DAPI positive cells in control and microphthalmic eyes. The quantified value of each bar was based on the regions indicated by the letters. The Student's t -test was used to determine significance *** $P < 0.001$. Graphic data are presented as mean \pm SE of pictures taken at 20X (see cell quantification in materials and methods; actual values in Table S3). NE= neuroepithelium; RPE= Retinal Pigmented Epithelium; L= lens; D-OS= Dorsal Optic Stalk; V-OS= Ventral Optic Stalk; N.S. Not significant. Scale bar 100 μ m in all panels.

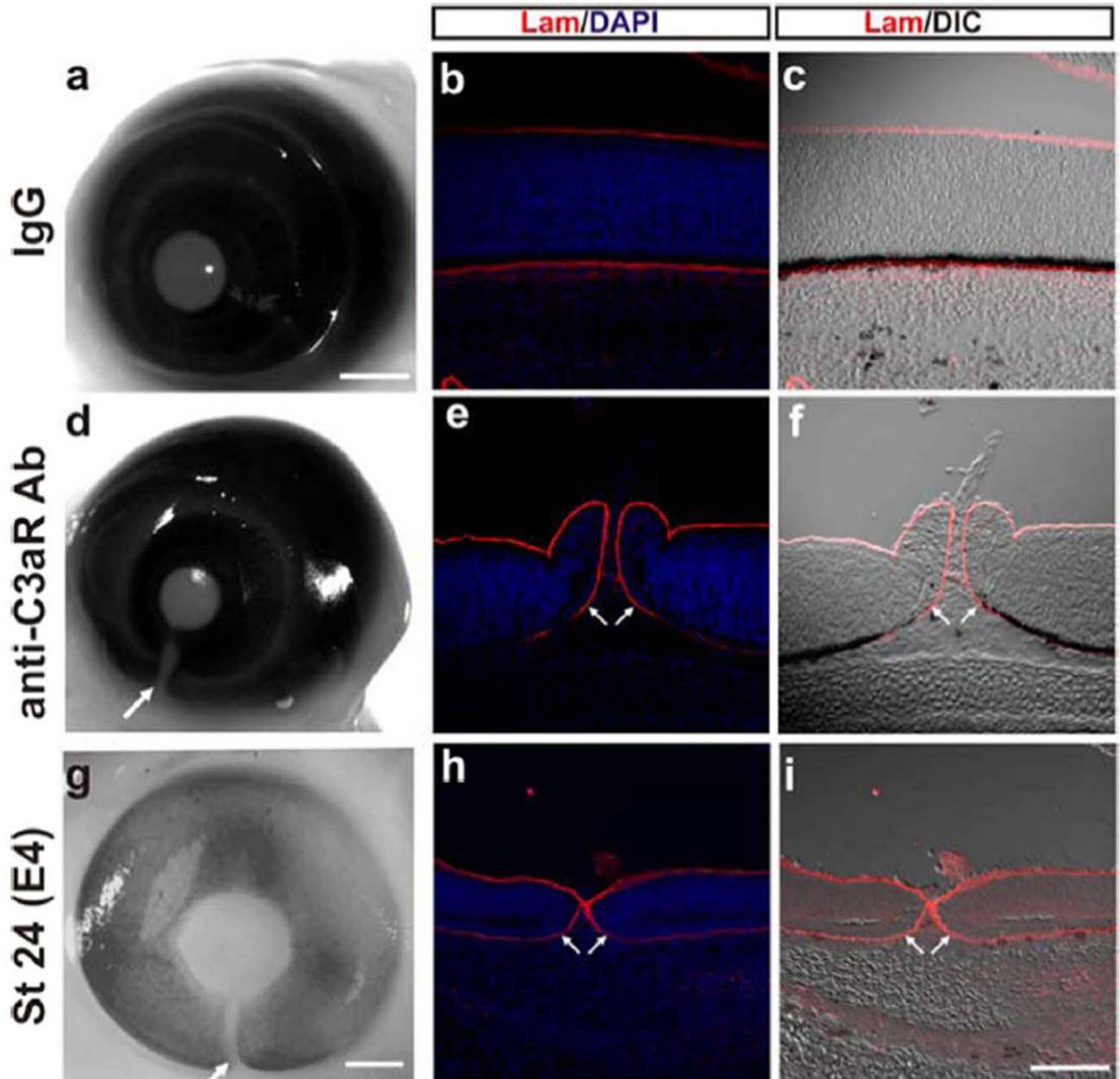


Fig. 7. Blocking C3aR causes coloboma. Lateral view of an eye injected with (a) control IgG or (d) injected with anti-C3aR Ab at stage 10/11 and collected 8 days later. The eye injected with anti-C3aR Ab shows that the optic fissure (OF) is not closed; the arrow indicates the cleft on the ventral side. (g) St 24 (E4) developing eye showing a typical open optic fissure on the ventral side (arrow). Laminin immunofluorescence staining (Lam) of sagittal sections from (b,c) eyes injected with control IgG demonstrating a closed choroid fissure. (e,f) eyes injected with anti-C3aR Ab or (h, i) St 24 control eyes depicting an unclosed choroid fissure

(with arrows). DAPI was used to counterstain the nuclei. Scale bar in a: 1 mm and applies to d; Scale bar in g is 0.5 mm; Scale bar in i is 100 μ m and applies to b, c, e, f and h.

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