

## REVIEW

# Optic cup morphogenesis across species and related inborn human eye defects

Marcos J. Cardozo<sup>1,2</sup>, Elena Sánchez-Bustamante<sup>1,2</sup> and Paola Bovolenta<sup>1,2,\*</sup>

## ABSTRACT

The vertebrate eye is shaped as a cup, a conformation that optimizes vision and is acquired early in development through a process known as optic cup morphogenesis. Imaging living, transparent teleost embryos and mammalian stem cell-derived organoids has provided insights into the rearrangements that eye progenitors undergo to adopt such a shape. Molecular and pharmacological interference with these rearrangements has further identified the underlying molecular machineries and the physical forces involved in this morphogenetic process. In this Review, we summarize the resulting scenarios and proposed models that include common and species-specific events. We further discuss how these studies and those in environmentally adapted blind species may shed light on human inborn eye malformations that result from failures in optic cup morphogenesis, including microphthalmia, anophthalmia and coloboma.

**KEY WORDS:** Neural retina, Retina pigment epithelium, Organoids, Zebrafish, Human, Mammals, Anophthalmia, Microphthalmia, Gene regulatory networks, Cavefish

## Introduction

Sight is a fundamental physical sense that allows almost all animal species to reconstruct the surrounding world and interact with it. However, different species require different visual abilities adapted for their lifestyle, behaviour and ecological niche. For example, being diurnal or nocturnal, terrestrial or marine, or being a predator or prey, imposes very different visual needs. These needs have been behind the evolutionary pressures that culminate in the emergence of different visual system configurations (Baden, 2020; Vallergera, 1994), with the eye as one of the most striking examples of variability through a multibranching evolution (Gehring, 2014; Goldsmith, 1990; Lamb et al., 2007; Martínez-Morales and Locascio, 2016).

The eye is the primary visual organ and receives light information through two types of cells: photoreceptors and pigmented cells. This basic unit is thought to constitute the ancestral prototypic eye, from which all existing eyes have arisen (i.e. eye spots, ocellus, compound eyes, eye cups or camera eyes; see Table 1; Arendt, 2003; Gehring, 2014; Goldsmith, 1990; Martínez-Morales and Locascio, 2016). The ‘camera’ eye is perhaps the most sophisticated

among the existing light-capturing structures. Its evolutionary origin implies the acquisition of a refractive and transparent lens through which the light is focused and projected into the retina: the neural structure that processes and transmits light information to the brain (Baden, 2020; Vallergera, 1994). Camera eyes are found in all vertebrates and in some invertebrates, such as spiders, cnidarians and cephalopods (Lamb et al., 2007). Despite different adaptations, all camera eyes have a hemispheric or ‘cup’ shape, which provides a better visual resolution than any other existing eye designs (Lamb et al., 2007; Martínez-Morales and Locascio, 2016).

Past studies have established that, in all vertebrates, the acquisition of this cup shape starts with specification of the eye field (see Glossary, Box 1) in the mid anterior neural plate, followed by the bilateral protrusion of this region into two optic vesicles (OVs), which thereafter fold, forming the optic cups (OCs) (Fig. 1A) (Martínez-Morales et al., 2017). This folding leads to the generation of a transient groove along the ventral pole of the eye rudiment called the optic or choroid fissure (OF; see Glossary, Box 1), which extends along the optic stalk (Fig. 1A; see Glossary, Box 1), the structure that connects the OC to the adjacent neural tube, thereafter forming the optic nerve (see Glossary, Box 1). The OF enables the ingression of cells from periocular mesenchyme (POM) (see Glossary, Box 1) that generate the retinal vasculature and the egression of retinal axons. Thereafter, the borders of the OF fuse forming the optic nerve (Gestri et al., 2018; Morcillo et al., 2006).

The correct execution of all these morphogenetic events depends on the reiterative use of a conserved core set of regulatory molecules, including transcription factors and morphogenetic signalling pathways that collectively form specific gene regulatory networks (GRNs). These GRNs are evolutionarily conserved, although the specific interaction between some of their components may vary to fulfil species-specific visual perception needs (Beccari et al., 2013; Casares and Almudi, 2016; Chen and Desplan, 2020; Hoshino et al., 2017; Martínez-Morales, 2016). In all, these observations suggest that vertebrate eye morphogenesis should occur following very similar principles and mechanics across phylogeny. Many developmental neurobiologists interested in eye development have thus turned to transparent embryos, such as those of the zebrafish or medaka, to study how the vertebrate eye acquires its shape *in vivo* (Heermann et al., 2015; Kwan et al., 2012; Moreno-Mármol et al., 2021; Nicolas-Perez et al., 2016; Sidhaye and Norden, 2017). The successful development of OC organoids derived from fish, mouse or human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) have further boosted the studies on the physical forces behind eye morphogenesis, providing additional insights and indicating species-specific features (e.g. Eiraku et al., 2011; Nakano et al., 2012; O’Hara-Wright and Gonzalez-Cordero, 2020; Zilova et al., 2021).

In this Review, we summarize the data obtained from the above studies and analyse the common principles by which vertebrates

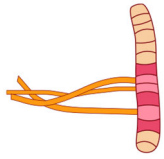
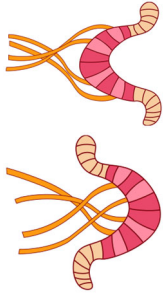
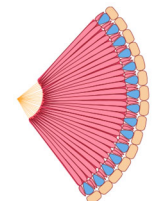
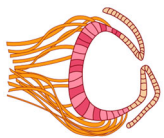
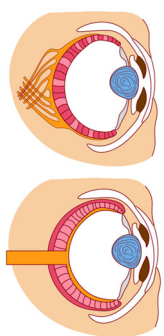
<sup>1</sup>Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, c/ Nicolás Cabrera 1, Cantoblanco, Madrid 28049, Spain. <sup>2</sup>Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), c/ Nicolás Cabrera 1, Cantoblanco, Madrid 28049, Spain.

\*Author for correspondence (pbovolenta@cbm.csic.es)

 P.B., 0000-0002-1870-751X

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**Table 1. Different types of eye structures across species**

Eye type	Description	Prototypic species	Schematic representation*
Eye spot	Spots of a few light-sensitive cells located in the epidermis of some invertebrate species	Ribbon worm, ascidians, amphioxii and starfishes	
Pigment cup eye ocellus	Composed of a cluster of photoreceptors that form a pigmented cup without an optical system. The cup works as a pinhole and filters the light that reaches the photoreceptors, providing poor resolution.	Planaria, honeybees, arthropods and ragworms	
Compound eye	Composed of several ommatidia, which are independent photoreception units. Each unit is composed of a lens, cornea and photoreceptors that distinguish colour and brightness.	Arthropods, insects and crustaceans	
Simple optic cup	Photoreceptor cells form a pigmented cup with a water-filled cavity. A sophisticated pinhole filters the light that reaches the photoreceptors.	Nautilus, squid, spider and Mollusca	
Camera type/ complex eye	An organ composed of a lens, iris, vitreous cavity, photoreceptor cells and pigment cells, as well as additional neuronal cell types. Light travels through the lens to the retina, where visual information is transduced and transmitted along the optic nerve to the brain.	Octopus and vertebrates	

\*Epithelium (nude); iris (brown); lens (light blue); nerve fibres (orange); pigmented cells (red); photoreceptor cells (pink).

generate overall similar cup-shaped eyes, but also indicate relevant differences. Indeed, even among very similar vertebrate eyes, there is variability in organ size, time of development (Fig. 1B) and/or

regenerative capacities. But how has eye morphogenesis adapted to fulfil this variability? What do animal models tell us about human eye development? Can studies on eye morphogenesis help us to

**Box 1. Glossary**

**Eye or retinal field.** The region of the anterior neural plate that comprises the precursors of the neural components of the eye.

**Lens ectoderm.** Ectoderm derived from the pre-placodal region and abutting the optic vesicle.

**Lens placode.** A thickening of the lens ectoderm that serves as the precursor to the lens.

**Neural retina.** The light-sensitive tissue of eye, composed of different types of neurons, including photoreceptors and retinal ganglion cells, organized in interconnected layers and responsible of transmitting light information to the brain.

**Optic disc.** Also known as the blind spot of the retina, this is the region where the optic fibres converge to become part of the optic nerve and represents the interface between the optic stalk and the neural retina.

**Optic fissure.** The groove along the ventral region of the optic vesicle that enfolds the axons of the retinal ganglion cells, leaving the eye and the mesenchymal cells that ingress to form the hyaloid artery. This structure is also named the choroid fissure.

**Optic nerve.** The structure derived from the optic stalk and mostly composed of the axons of the retinal ganglion cell layer carrying visual information to the brain.

**Optic stalk.** A derivative of the ventral optic vesicle that connects the vesicle with the neural tube.

**Periocular mesenchyme.** Mesenchymal cells surrounding the developing eye and contributing, among others, to the development of the anterior ocular segments.

**Retinal pigmented epithelium.** The epithelial layer composed of pigmented cells that surrounds the neural retina and supports photoreceptor function.

understand how human congenital eye malformations arise? Here, we try to address these questions, focusing on one crucial event in eye formation: the folding of the OV into an OC. We refer the reader to previous comprehensive reviews for information about the main events that precede (Cavodeassi, 2018; Giger and Houart, 2018; Sinn and Wittbrodt, 2013) or follow (Chow and Lang, 2001; Miesfeld and Brown, 2019) this crucial eye developmental step, or about the GRNs that govern them (Beccari et al., 2013; Casares and Almudi, 2016; Chen and Desplan, 2020; Martinez-Morales, 2016).

**Folding of the optic vesicle into an optic cup in fast-developing species**

In all vertebrate species, the folding of the OV into a cup entails two crucial and concomitant events. First, neuroepithelial precursors acquire two different fates, neural retina (NR; see Glossary, Box 1) and retinal pigment epithelium (RPE) (see Glossary, Box 1), driven by signalling molecules, such as Sonic hedgehog (Shh), fibroblast growth factors (Fgfs) or Wntless-related (Wnt) proteins (Cardozo et al., 2020), which trigger differential transcriptional states in the GRNs controlling cell identity acquisition (Beccari et al., 2013; Buono et al., 2021; Fuhrmann, 2010; Yamada et al., 2021). Second, individual neuroepithelial cells change their shape, giving rise to elongated NR cells, squamous or cuboidal cells of the RPE, and the wedge-shaped cells at the NR-RPE connecting hinges (Figs 1A and 2A).

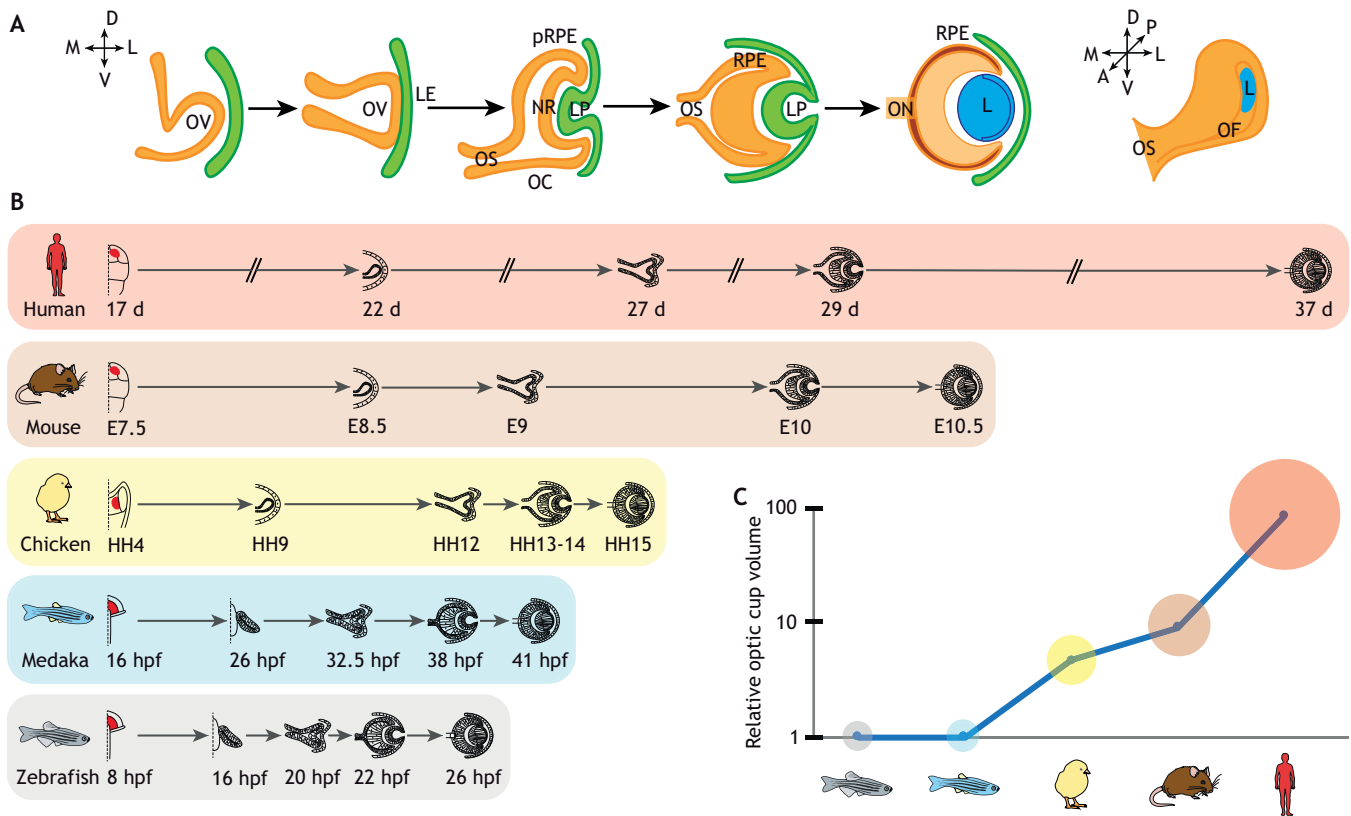
These events are likely common to all vertebrates, but the morphological appearance of the vesicles and the speed of their morphogenesis are parameters that distinguish many teleosts from amniotes. For example, in zebrafish and medaka, the OV is an elongated and flat bilayer composed of a pseudostratified neuroepithelium (Fig. 2A) that folds into a cup in just a few hours (Fig. 1B). The existence of several mutant lines, the easy embryological manipulations and the transparent nature of their

embryos make the zebrafish and medaka ideal models to follow how the eye forms in real time and define the underlying mechanics (Casey et al., 2021).

Cell-autonomous basal constriction of NR progenitors is one of the pillars of vertebrate OV folding (Fig. 2A) based on the discovery of the *ojoplano* (*opo*; ‘flat eye’ in Spanish) medaka mutant, in which the OV remains unfolded (Martinez-Morales et al., 2009). The *opo* gene encodes a transmembrane protein that localizes at the basal end-feet of the NR precursors, controlling the endocytosis of focal adhesion components and their interaction with the actomyosin cytoskeleton (Bogdanović et al., 2012; Martinez-Morales et al., 2009). Pulsatile contraction of the actomyosin cytoskeleton shrinks the basal end-feet of NR cells (Nicolas-Perez et al., 2016; Sidhaye and Norden, 2017), generating a tension that favours OV inward bending, as also confirmed by local interference with myosin II activity (Moreno-Mármol et al., 2021). These events are strongly dependent on basal end-feet laminin and its interaction with the abutting extracellular matrix (ECM) that seems to strengthen contractile forces (Nicolas-Perez et al., 2016). Indeed, genetic inactivation or knock-down of laminin components (*lamc1* and *lama1*) impairs basal contractility and the transmission of mechanical tension, ultimately preventing OV folding (Bryan et al., 2016; Nicolas-Perez et al., 2016).

As the OV bends inwards, the lateral-NR layer keeps growing through the continuous incorporation of neuroepithelial cells from the medial OV layer through a collective migration process (Sidhaye and Norden, 2017; Soans et al., 2022) known as ‘rim involution’ or ‘epithelial flow’ (Heermann et al., 2015; Kwan et al., 2012; Picker et al., 2009; Sidhaye and Norden, 2017). This event is part of broader cellular rearrangements characterized by ‘pinwheel’-like movements that begin with OV evagination, displacing NR and RPE precursors into more posterior positions, and promoting the incorporation of new cells from the neural tube (Kwan et al., 2012). Rim involution takes place mostly at the ventral hinge (Fig. 2A) through the dynamic extrusion of cryptic basal lamellipodia that extend in the direction of migration, attaching to the ECM (Heermann et al., 2015; Kwan et al., 2012; Sidhaye and Norden, 2017; Soans et al., 2022), thereby enabling cellular translocation (Heermann et al., 2015; Sidhaye and Norden, 2017). Notably, the ECM surrounding the OV, in part deposited by periocular neural crest cells (Bryan et al., 2020), undergoes topological changes that are associated with different cellular dynamics, thereby influencing the efficiency of directed collective rim cell migration (Soans et al., 2022). Besides the important role of the surrounding ECM, bone morphogenetic protein (BMP) signalling seems to facilitate this involution (Heermann et al., 2015), which, if disrupted, either by manipulating BMP signalling or by interfering with lamellipodia formation, prevents the acquisition of a proper cup shape. In the absence of cellular translocation, progenitor cells accumulate at the ventral inner layer but they nevertheless acquire NR fate (Heermann et al., 2015; Sidhaye and Norden, 2017), suggesting a fate pre-commitment. Notably, the last cells that translocate into the ventral inner layer and the few that translocate dorsally retain stem cell properties and eventually generate the peripheral rim of the retina or ciliary margins (CMs) (Heermann et al., 2015), which allows the continuous growth of the eye in both amphibians and fish.

Rim involution depletes cells from the inner OV layer, in principle unbalancing the size of the layers. This disequilibrium is minimized by the concomitant spreading of the small patch of remaining outer layer cells, positioned in the dorso-medial OV (Kwan et al., 2012; Li et al., 2000; Moreno-Mármol et al., 2021). This patch of neuroepithelial progenitors is committed to a RPE

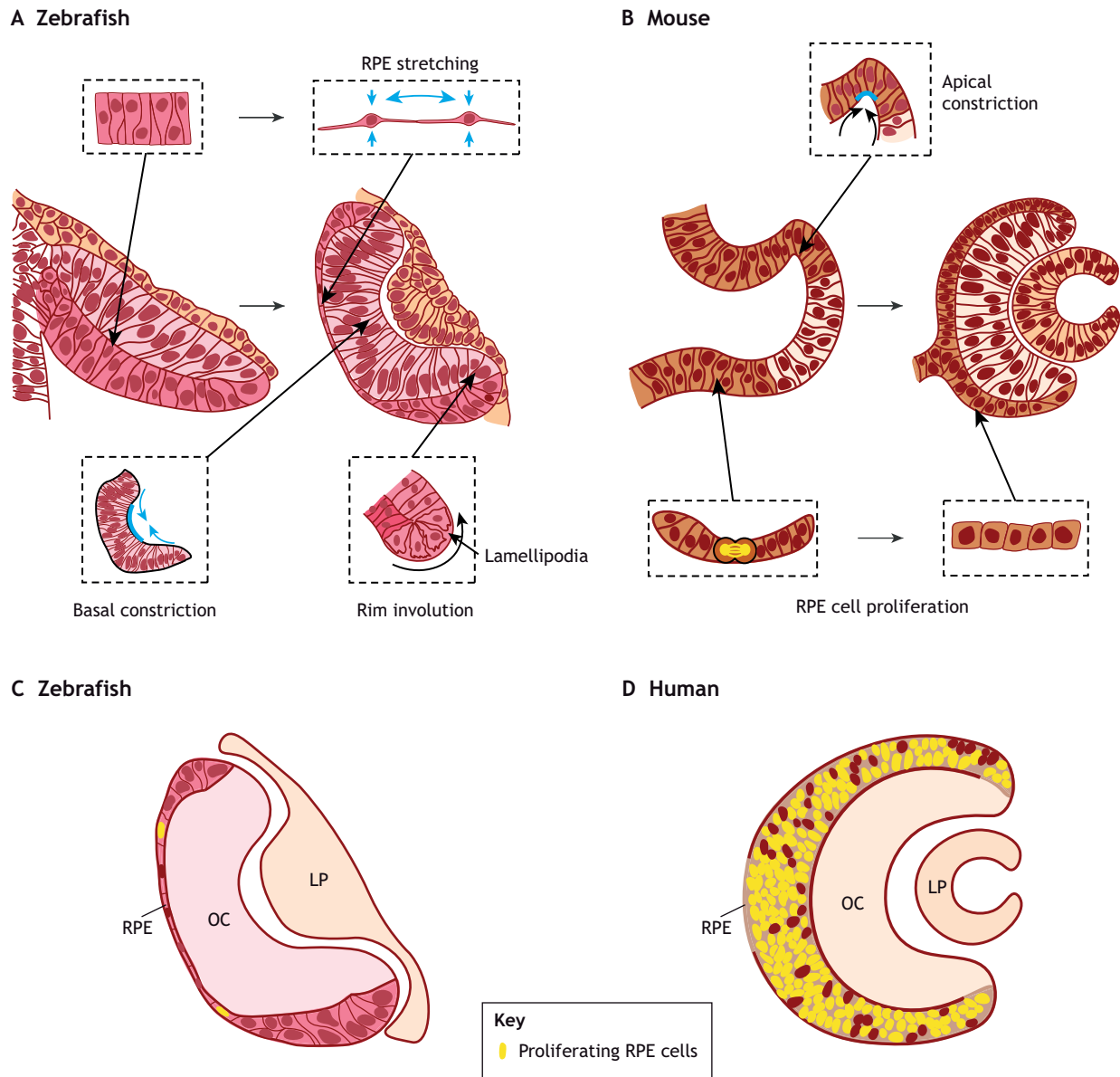


**Fig. 1. Schematic representation of vertebrate eye morphogenesis according to a species-specific schedule.** (A) Steps of eye morphogenesis from the initial optic vesicle (OV) to a fully formed optic cup (OC). Lens tissue is depicted in green; neural derivatives in orange. The fully formed lens (L) is represented in blue. LE, lens ectoderm; LP, lens placode; NR, neural retina; OC, optic cup; OF, optic fissure; ON, optic nerve; OS, optic stalk; pRPE, presumptive retinal pigment epithelium; RPE, retinal pigment epithelium. (B) Timelines of eye development in different vertebrate species. (C) Graph representing the optic cup volume relative to that of zebrafish in the different depicted species: (from the right) human, mouse, chicken, medaka and zebrafish.

fate. RPE progenitors first expand in the anteroposterior direction through a limited number of cell divisions and then, in virtual absence of cell proliferation, they stretch (Cechmanek and McFarlane, 2017; Kwan et al., 2012; Li et al., 2000; Moreno-Mármol et al., 2021) by strongly reducing their apico-basal axis while undergoing around an eightfold increase in their apical cell surface area (Moreno-Mármol et al., 2021). This transition from a pseudostratified to a squamous epithelium (Fig. 2A) occurs as RPE progenitors become fully specified with the onset of GRNs that make RPE cells rapidly molecularly diverge from their progenitors (Buono et al., 2021). Consistent with their squamous appearance, RPE cells begin to express keratins, connexins and desmosomal proteins that, in a speculative view, may confer a particular mechanical strength and a ‘syncytial-like’ behaviour to this tissue (Moreno-Mármol et al., 2021). These molecular changes, together with an important cytoskeletal reorganization, sustain RPE cell stretching (Moreno-Mármol et al., 2018; Moreno-Mármol et al., 2021). Indeed, inhibition of myosin II in a few RPE cells, or interference with microtubule depolymerization in RPE progenitors, prevents both cell flattening and OV folding, whereas similar focal perturbations in the NR impact on OV convexity but not in RPE flattening, suggesting a cell-autonomous control. Thus, cell-autonomous RPE stretching is a rather powerful mechanical force for teleost OC formation given that compromised stretching in only a few cells is sufficient to decrease OV folding (Moreno-Mármol et al., 2021). In summary, morphogenesis of the teleost OC depends on the tension generated by the coordinated shape changes that the NR and the RPE undergo, with the additional

contribution of cell involution from the inner to outer layer of the OV (Fig. 2A).

An unresolved issue is whether and how these morphogenetic forces are coordinated and sensed. Part of the coordination may rely on inner-outer layer interactions, mediated by molecules such as semaphorins and their plexin receptors, as abrogation of their expression results in incomplete ventral OC formation (Cechmanek et al., 2021). It is tempting to speculate that mechano-sensor and -transducer proteins, such as Yes1-associated transcriptional regulator 1 (Yap1), may instead be involved in sensing RPE tension, a function that Yap1 exerts in many different tissues (Totaro et al., 2018). This possibility is supported by the observation that, in zebrafish *yap1* mutants, the RPE develops with a patchy appearance (Miesfeld et al., 2015), suggestive of failed cellular interaction. Furthermore, heterozygous loss-of-function mutations in *YAP1* have been found in individuals affected by coloboma (DeYoung et al., 2022; Williamson and FitzPatrick, 2014), a defect in which the OF does not seal up (discussed in more detail later). Nevertheless, it should be noted that Yap1 also acts as a co-factor for the Tead family of transcription factors (TFs), which are downstream effectors of Hippo signalling and are among the first TFs to be recruited during RPE specification (Buono et al., 2021). Accordingly, RPE cells are completely absent in *yap-taz* zebrafish double mutants (*taz* being a *yap* paralog), indicating that the Tead/Taz/Yap1 complex is involved in RPE specification (Miesfeld et al., 2015). This is also supported by the observation that, in *Yap1* mouse mutants, the RPE acquires NR characteristics (Kim et al., 2016). Furthermore, different studies have demonstrated that Yap1



**Fig. 2. Comparison between teleost and mammalian optic cup morphogenesis.** (A,B) Schematic representations of the zebrafish (A) and mouse (B) transition from optic vesicle to optic cup (OC). The dashed outlines indicate the shape changes that each cell type undergoes as it acquires the identity of neural retina (NR), retinal pigmented epithelium (RPE) and hinge cells. (C,D) Schematic representations of a section through the zebrafish (C) and human (D) OC, illustrating the differences observed in RPE cell proliferation (green nuclei). LP, lens placode.

participates in the regulation of RPE, NR and CM proliferation at later stages of mouse eye development (Moon et al., 2018; Sun et al., 2020). It might, therefore, be difficult to distinguish the potential mechano-sensing activities of Yap1 in the RPE from these other functions.

**Folding of the optic vesicle in species with a longer embryonic developmental time**

In contrast to teleosts, the transition of the amniote OV into an OC takes place in the order of days, with a considerable variability according to the gestational period of the species (Fig. 1B), so that human OV folding is much slower than that of the zebrafish (Fig. 1B). The morphology of the OV is also different, with a balloon shape constituted by a pseudostratified epithelium everted from the neural tube (Fig. 2B). Its dorsal region differentiates as RPE, the intermediate region as NR, whereas the optic stalk

originates from the most ventral region (Box 2) (Martinez-Morales et al., 2017). The NR/RPE hinge regions become apparent as the NR bends inwards (Figs 1A and 2B).

Initial descriptions on how this ‘balloon’ folds came mostly from the analysis of static images from chick, mouse and even human embryos (Coulombre, 1969; Hilfer, 1983; O’Rahilly and Müller, 2010). These studies supported the ‘induction’ hypothesis, proposed by Hans Spemann, according to which the interaction between the OV neuroepithelium and the overlying surface ectoderm was required to form the OC. The nearing of the OV to the ectoderm was thought to induce ectoderm thickening to form the lens placode (see Glossary, Box 1), which, in turn, triggered OV folding (Chow and Lang, 2001). This idea was further supported by the observation of cytoplasmic extensions connecting the two tissues in different species, including humans (Chauhan et al., 2009; Mann, 1928; McAvoy, 1980), and by a number of genetic studies.

For example, specific inactivation of *Sox2* and *Pax6* (two TFs belonging to the eye GRNs; Beccari et al., 2013) in the mouse lens ectoderm (see Glossary, Box 1) not only prevents lens placode formation but also OV folding (Smith et al., 2009), supporting a role for the lens ectoderm in OC morphogenesis.

This long-lasting belief started to be questioned with the generation of the first OC organoids derived from mouse ESCs (Eiraku et al., 2011). This seminal study showed that mouse ESCs (mESCs) can acquire eye field identity and then develop into an OC through an intrinsic self-organizing program, independently of the presence of a lens ectoderm/placode (Eiraku et al., 2011). This self-organization was subsequently reproduced using human ESCs (Lowe et al., 2016; Mellough et al., 2015; Nakano et al., 2012) and iPSCs (Gabriel et al., 2021; Meyer et al., 2011), and it is perhaps further supported by the histological analysis of the eye of individuals with congenital primary aphakia, a rare disorder in which the lens is missing but the OC still forms (Manschot, 1963; Valleix et al., 2006). Nevertheless, the low and variable frequency with which properly folded OCs form, especially when originating from human ESC or iPSCs (Capowski et al., 2019; Nakano et al., 2012), improves in culture conditions in which additional forebrain, primordial lens- and cornea-like structures also develop (Gabriel et al., 2021). There is also little understanding of the cell type identities associated with OV organoids that support their integrity (Decembrini et al., 2014) or of the composition of the surrounding ECM, which may have an important role in the successful generation of OCs. In fact, the ECM seems to promote early NR invagination in chick embryos (Oltean et al., 2016). In all, the proposed tissue interactions between the OV and the lens ectoderm/placode (Chow and Lang, 2001; Graw, 2003) may still have, at least, the role of optimizing OV folding into a proper cup and/or influencing OV elongation. According to computational models, this elongation seems to be needed to optimize OV invagination (Hosseini and Taber, 2018).

Independently of the aforementioned open questions, live imaging of *in vitro* OC generation has shown that neuroepithelial cells undergo a stepwise and domain-specific (NR versus hinge versus RPE) transformation of their morphology (Fig. 2), with tissue-autonomous capacity to generate and interpret mechanical forces (Eiraku et al., 2011, 2012; Okuda et al., 2018). Careful analysis of these changes, combined with computational modelling, has generated the so called ‘relaxation-expansion’ model of *in vitro* mammalian OC formation (Eiraku et al., 2011, 2012; Okuda et al., 2018). According to the model, the OV folds in different phases that involve changes in individual cell morphology (e.g. apical or basal surface contraction, cell flattening, etc.), in physical properties (stiffening and softening) and in adhesion (to the surrounding cells and ECM), together with cell division and positional rearrangements.

ESCs or iPSCs, cultured in media formulated for generating OCs, initially form spherical vesicles, some of which begin to acquire NR and RPE characteristics. Two main changes drive the folding of the sphere – the cell-autonomous convex invagination of the NR and the constriction of hinge cells along their apico-basal length (Fig. 2B) (Okuda et al., 2018) – both of which require cytoskeletal rearrangements. While acquiring their fate, NR cells redistribute their actomyosin content with increased accumulation at the basolateral surfaces and a decrease at the apical side. According to several studies of epithelial sheet bending (Heisenberg and Bellaïche, 2013) and pharmacological interferences with actomyosin function (Eiraku et al., 2011; Lowe et al., 2016; Okuda et al., 2018), this actomyosin reorganization induces a differential basal constriction and apical relaxation in neuroepithelial cells, so that their end-feet are, respectively, stiffer

and softer, promoting an autonomous inward bending of the tissue (Okuda et al., 2018). This bending is likely strengthened by a more prominent intracellular adhesion at the basal side and the interaction with the ECM (Boucherie et al., 2013; Lowe et al., 2016), as observed in zebrafish and chick embryos (Nicolas-Perez et al., 2016; Oltean et al., 2016). The inward bending of the NR layer, in turn, imposes a particular strain on hinge cells. In organoids, these cells assume a rather sharp wedge shape (Fig. 2B), owing to lateral constriction along the apico-basal axis mediated by strain-induced calcium transients (Okuda et al., 2018). This constriction mechanism further facilitates OV folding and likely explains part of the differences between the shape of NR and hinge cells. However, a substantial redistribution of actomyosin with an inverted apico-basal distribution must also occur, given that, in hinge cells, the apical end-feet are quite narrow (Fig. 2B). Whether these changes are the result of the sole NR-derived strain or whether the RPE also exerts mechanical forces on hinge cells, as proposed in fishes (Heermann et al., 2015; Moreno-Mármol et al., 2021), is unclear. However, in ESC-derived organoids, the generation of abutting NR and RPE is crucial for the differentiation of the CM (Kuwahara et al., 2015). Furthermore, in mouse mutants in which the RPE is not specified but acquires a NR identity (Häggglund et al., 2013; Martínez-Morales et al., 2001; Tang et al., 2010), wedge-shaped hinge cells are virtually absent and OV folding is strongly compromised (Martínez-Morales et al., 2001).

Additional studies support the relevance of RPE and CM identity acquisition for amniote OV folding. In mouse organoids, failed RPE specification prevents the initial steps of OV invagination (Eiraku et al., 2011), although, once the OC is formed, the RPE seems to function as a shell, with high mechanical rigidity and a strong actomyosin activation that, if broken, does not impact on the convex NR shape (Eiraku et al., 2011). OC malformations have also been reported after genetic manipulations of components of the Wnt and Fgf signalling pathways that interfere with the acquisition of RPE and CM identity and/or OV growth (Balasubramanian et al., 2021; Bankhead et al., 2015; Carpenter et al., 2015; Fuhrmann et al., 2022). For example, failed secretion of lens-derived Wnt ligands perturbs proliferation of the peripheral region of the eye cup, with a more evident effect on cells of the RPE (Carpenter et al., 2015). This defect is associated with a poorly folded, ‘saucer’ shaped OC (Balasubramanian et al., 2021; Carpenter et al., 2015). Similarly, preventing post-translational modifications, and thus secretion, of Wnt ligands in the eye field diminishes OV growth and impairs its folding (Fuhrmann et al., 2022). In other words, RPE specification (Balasubramanian et al., 2021; Häggglund et al., 2013; Martínez-Morales et al., 2001; Tang et al., 2010;) and growth (Carpenter et al., 2015; Fuhrmann et al., 2022) are prerequisites for OC generation *in vivo*.

More likely, the NR and RPE prospective layers need to maintain an adequate proportion to support OV folding: if one of the two layers is shorter or larger than the other, the vesicle does not invaginate (Balasubramanian et al., 2021; Carpenter et al., 2015; Moreno-Mármol et al., 2021; Okuda et al., 2018), perhaps because the generated tension is unbalanced.

### Differences in OC morphogenesis among vertebrate species

The need for a balanced proportion of NR and RPE holds true for both fast-developing teleosts and slow-developing amniotes. However, the mechanism by which these species achieve the right proportion of layers represents one of their salient morphogenetic differences. In teleosts, cell proliferation is dispensable for OC formation (Kwan et al., 2012) and its inhibition has no effect on RPE expansion during OV folding (Cechmanek and McFarlane,

2017). Rather, the RPE seems to cease mitotic division and differentiates simultaneously with OV folding (Buono et al., 2021; Moreno-Mármol et al., 2021). Proliferation also minimally contributes to the growth of the prospective zebrafish NR, which increases its surface through rim involution, well in line with the observation that in zebrafish the outer OV layer loses cells (from 587 to 432), while the inner layer gains them, in numbers that cannot be explained only by cell proliferation (Li et al., 2000). Thus, cell stretching and cell flow are the solutions adopted by fast-developing species to maintain an appropriate equilibrium between the two OV layers (Moreno-Mármol et al., 2021), which, in both teleosts and amniotes, seems to be a prerequisite for OV folding (Carpenter et al., 2015; Moreno-Mármol et al., 2021; Okuda et al., 2018). Indeed, the 6 h duration of this process is far less than the estimated 10 h needed for a single cell cycle in the OV neuroepithelium (Li et al., 2000).

In species with slower development, rim involution has not so far been described, and perhaps rightly so, as the OV has the time to grow by cell division. This also explains the different RPE development in amniotes, in which cells do not undergo a marked stretching and keep dividing during OV folding, with a remarkable inverse correlation between proliferation rate and apico-basal length among species (Moreno-Mármol et al., 2021). In humans, the OC RPE layer is composed of a highly mitotic pseudostratified epithelium that barely differs from the NR layer (Moreno-Mármol et al., 2021), in striking contrast to the zebrafish OC morphology (Fig. 2C,D). Thus, in humans and in mammals more broadly, the RPE acquires a final cuboidal, not squamous, appearance at a slower pace, with a step-wise differentiation process, as recently supported by RNA-seq analysis of human OC (Hu et al., 2019).

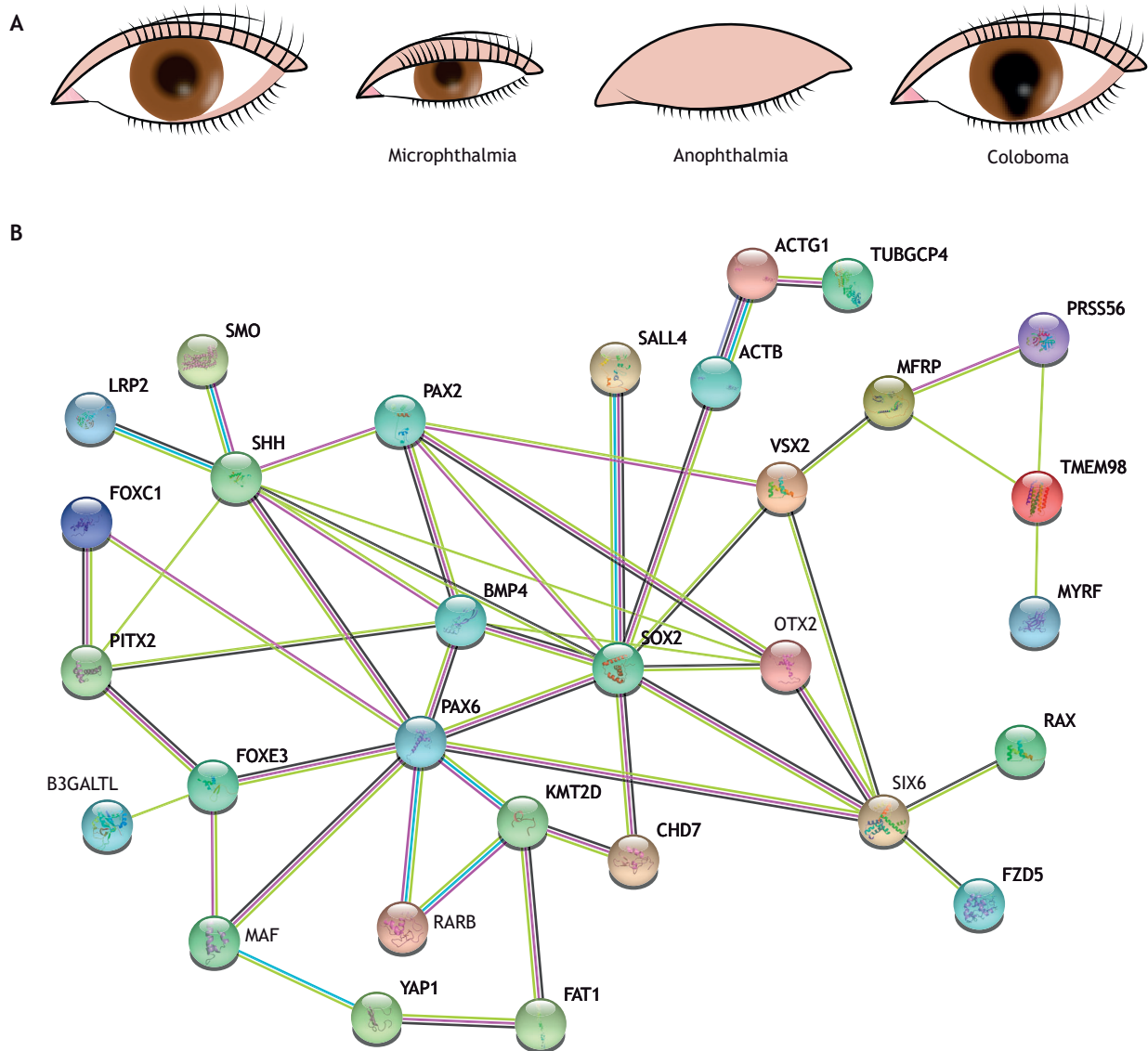
Other differences in OV morphogenesis between species relate to the species-specific organ size and intrinsic developmental clocks. This is evident, for example, when comparing the development of eye organoids from mESCs and human ESCs (hESCs). OC derived from human cells requires a longer generation time, as occurs during embryonic development (Fig. 1B). This internal clock is also present in teleosts, as also shown by the isochronic transplantation of zebrafish blastomeres into a medaka host, where they develop into a NR according to their faster intrinsic dynamics (Fuhrmann et al., 2020). Similarly, hESC-derived OC are consistently larger (about twofold) and the NR layer is twice as thick as that obtained from mouse cells, reflecting the relative differences in the respective embryos at equivalent stages (Nakano et al., 2012). The human NR layer seems also to retain an intrinsic tendency to become apically convex, if isolated from the rest of the organoid after initial invagination, a behaviour not observed with mouse cells (Eiraku et al., 2011; Nakano et al., 2012). This difference perhaps depends on a distinct composition of the basal membrane or is favoured by a more apical localization of the nuclei in the human cells that forces apical expansion (Nakano et al., 2012). This observation is intriguing, especially considering that the large majority of human iPSC eye organoids develop as retinal spheres with an ectopic RPE patch (O'Hara-Wright and Gonzalez-Cordero, 2020). Although culture conditions might be, in part, responsible for this difference, it will be interesting to determine what the underlying molecular differences between human iPSC- and ESC-derived OC organoids are, as this may provide additional insights on eye morphogenesis.

Evolutionary developmental comparisons of the morphogenetic events that shape the eye primordium have just begun and with the large variability of existing camera eyes in the animal kingdom (Koenig and Gross, 2020), we envisage that additional variations will be discovered, especially when analysing non-conventional

model species. In amniotes, apoptosis has a relevant role in refining eye morphogenesis, with massive apoptotic death in the developing lens, OF fusion and differentiating retina (Frade et al., 1997; Trousse et al., 2001). However, its potential participation in OV folding has not been specifically addressed, although mutations in genes involved in apoptosis are among the genetic causes of congenital eye defects [e.g. holocytochrome C synthase (HCCS); Indrieri et al., 2013]. The potential relevance of apoptosis in morphogenesis is further supported by the evidence that, during their extrusion from the neural tube epithelium, apoptotic cells generate forces that contribute to the acquisition of the neural tube conformation (Roellig et al., 2022). Similarly, this mechanism could be particularly important to shape the tubular eyes present in some deep-sea fishes (de Busserolles et al., 2020) or other environmental adaptations of the camera eye. These, in turn, might provide hints for a better understanding of congenital human eye malformations.

### Understanding inborn eye malformations through vertebrate environmental adaptations

Microphthalmia, anophthalmia and coloboma (MAC) is a spectrum of rare conditions (the estimated prevalence is 2-14/100,000 births), in which the eye is either reduced in size (microphthalmia) or virtually absent (anophthalmia). Coloboma instead derives from the poor growth of any of the tissues that compose the anterior segment of the eye and/or the retina/optic stalk or from alterations in the molecular components that enable OF fusion (Fig. 1A and 3A) (Plaisancié et al., 2019; Williamson and FitzPatrick, 2014). MAC can exist in isolation or, more frequently, as part of complex syndromes. These malformations are thought to arise from abnormal eye morphogenesis and more than half of the cases already have a molecular diagnosis (Slavotinek, 2019; Williamson and FitzPatrick, 2014). However, the precise defective morphogenetic events responsible for these malformations are still unclear (Plaisancié et al., 2019). *De novo* or inherited heterozygous mutations in the human *SOX2* or *OTX2* loci are the most frequently identified genetic causes of MAC, with a phenotypic severity that can vary even among family members (Plaisancié et al., 2019). *SOX2* and *OTX2* have a crucial role in the specification of the entire and anterior neural plate, respectively (Acampora et al., 2001; Pevny and Nicolis, 2010), but they have been shown to co-regulate the expression of RAX (Danno et al., 2008), a TF that is required for the initial evagination of the OV (Mathers et al., 1997; Rembold et al., 2006). Indeed, their haploinsufficiency seems to affect mostly the eye, perhaps because the specification of the retinal, telencephalic and hypothalamic precursors seems to require different activity levels of these TFs (Beccari et al., 2012; Bernard et al., 2014; Martinez-Morales et al., 2001). Other and less frequent mutations, continuously updated in a MAC gene database (<https://panelapp.genomicsengland.co.uk/panels/509/>), have been found in genes belonging to GRNs that specify eye tissues, including TFs and members of signalling pathways (Table 2). Other mutations are instead in effector genes involved in cell adhesion, cytoskeletal rearrangements and mechano-sensing, or ECM components (Table 2), all molecular classes that have been implicated in OC morphogenesis. Notably, enrichment analysis of possible protein-protein interactions using the STRING software (<https://string-db.org/>) shows that many of the proteins encoded by MAC-responsible genes are potentially functionally connected, with *SOX2* acting as principal node (Fig. 3B). Recent studies have also shown that *Sox2* can recruit distant elements that are necessary for the expression of many forebrain genes, including those involved in eye specification (Bertolini et al., 2019; D'Aurizio et al., 2022). Furthermore, several



**Fig. 3. Inborn eye malformations and possible interactions among causative defective proteins.** (A) Schematic representation of the eye in individuals affected by microphthalmia, anophthalmia or ventral coloboma. (B) STRING analysis of proteins encoded by genes responsible for microphthalmia, anophthalmia and coloboma (MAC). Many proteins are potentially functionally connected, with SOX2 acting as the principal node.

of these identified putative SOX2-regulated enhancers are linked to MAC-responsible genes, including some of the above-mentioned loci or other factors either associated with related eye defects (e.g. nuclear factor erythroid 2-related factor 2 (NR2F); Bertacchi et al., 2019) or proposed as MAC candidates, such as myeloid ecotropic viral integration site 1 (MEIS1) (Marcos et al., 2015), cell adhesion-associated, oncogene regulated (CDON) (Cardozo et al., 2014; Reis et al., 2020; Zhang et al., 2009) and ventral anterior homeobox 1 (VAX1) (Slavotinek et al., 2012).

Taking these observations together, it is tempting to speculate that the insufficient level of SOX2 or OTX2 expression levels in individuals with MAC, particularly in those with anophthalmia and microphthalmia, may arise by a reduced recruitment of progenitors into the eye field and/or their poor specification as eye tissue. Eye field defects might be followed by an imbalance between the two OV layers, thereby destabilizing OC morphogenesis and its related cellular reorganizations. These initial destabilizations might be

further reinforced by potentially lower levels of expression of a wide variety of SOX2- (Bertolini et al., 2019) and OTX2- (Sakai et al., 2017) regulated genes, many of which are similarly involved in morphogenetic events. Mutations in genes functionally linked to SOX2 and/or OTX2, or to their downstream effectors or to variations in regulatory elements, to which these TFs might bind, could have similar effects in destabilizing OV/OC morphogenesis and growth, eventually leading to its regression. However, no studies so far have directly addressed these possibilities. Indeed, the large number of available mouse mutants with MAC-resembling phenotypes have not yet been analysed with a specific focus on OC formation. Furthermore, most of the studies taking advantage of human retinal organoids have been directed towards solving disorders that cause blindness, such as retinitis pigmentosa or age-related macular degeneration (Gagliardi et al., 2019). Therefore, a detailed analysis of how MAC arises awaits further investigation.



**Table 2. List of genes associated with microphthalmia, anophthalmia and coloboma (MAC) inborn eye defects**

Category	Gene (protein)	Role in OC morphogenesis	References
Transcription factors	<i>RAX</i>	Eye field specification and OV evagination	Brachet et al. (2019)
	<i>PAX6</i>	Multiple steps of eye development	Schedl et al. (1996)
	<i>PAX2</i>	Specification of the OS, OF and optic disc (see Glossary, Box 1)	Sanyanusin et al. (1995)
	<i>SIX6</i>	Multiple steps of eye development	Aldahmesh et al. (2013); Gallardo et al. (2004)
	<i>VSX2</i>	Specification of ventral retina OS, OF and OD	Bar-Yosef et al. (2004)
	<i>TFAP2A</i>	Retinoic acid-responsive TF involved in eye morphogenesis	Gestri et al. (2009)
	<i>MITF</i>	Implicated in RPE specification	George et al. (2016)
	<i>SALL1</i>	OD development but still poorly characterized	Kohlhase et al. (1999)
	<i>SALL4</i>	Still poorly characterized function in eye and ON development	Ullah et al. (2017)
	Signalling cascade components	<i>SHH</i>	Proximo-distal patterning of the optic primordium
<i>BMP7</i>		Eye specification and OD development	Wyatt et al. (2010)
<i>CHRD1</i>		Development of the anterior part of the eye	Webb et al. (2012)
<i>LRP2</i>		Receptor involved in Shh signalling and involved in eye development	Kantarci et al. (2007)
<i>LRP5</i>		Development of the eye vasculature	Ergun et al. (2017)
<i>PORCN</i>		Early morphogenesis of the eye	Williamson and FitzPatrick (2014)
<i>SMO</i>		Shh transducer protein	Twigg et al. (2016)
<i>MFRP</i>		Transmembrane glycoprotein involved in RPE development and ocular size determination	Zenteno et al. (2009)
<i>GDF6</i>		Regulation of eye size	Asai-Coakwell et al. (2007)
Cell adhesion molecules		<i>CLDN19</i>	Transmembrane protein involved in tight junctions in the RPE
	<i>FAT1</i>	Involved in OF closure	Lahrouchi et al. (2019)
	<i>GJA1</i> (connexin 43)	Expressed in the RPE and involved in lens development	Vitiello et al. (2005)
	<i>GJA8</i> (connexin 50)	Involved in lens formation and eye growth	Ceroni et al. (2019)
	<i>TENM3</i> (tenurin 3)	Involved in early eye morphogenesis (tenurin 3)	Chassaing et al. (2016)
	<i>TMEM216</i>	Tetraspan transmembrane protein involved in photoreceptor development	Valente et al. (2010)
Cytoskeletal rearrangements and mechano-sensing proteins	<i>ACTB</i> (actin $\beta$ )	A filamentous cytoskeletal protein implicated in nearly all cellular processes	Procaccio et al. (2006)
	<i>ACTG1</i> (actin $\gamma$ 1)	An essential cytoskeletal protein implicated in nearly all cellular processes	Rivière et al. (2012)
	<i>TUBGCP4</i> (tubulin $\gamma$ complex-associated protein 4)	Involved in eye development	Scheidecker et al. (2015)
	<i>RAB18</i>	Lysosomal trafficking	Bem et al. (2011)
	<i>YAP1</i>	Involved in RPE specification and control of cell proliferation	Williamson et al. (2014)
	ECM components	<i>COL18A1</i> (collagen type XVIII)	Involved in development of the anterior and posterior pole of the eye
<i>COL4A1</i> (collagen type IV alpha 1 chain)		Ubiquitously expressed in basement membranes, including in the cornea, lens and retina	Deml et al. (2014)
<i>FBN1</i> (fibrillin)			Kainulainen et al. (1994)
<i>FREM1</i>		Poorly defined role in eye development	Slavotinek et al. (2011)
<i>FREM2</i>		Development of eyelids and eye growth	Jadeja et al. (2005)
<i>FRAS1</i>		Development of eyelids and eye growth	McGregor et al. (2003)

Consult the MAC gene database (<https://panelapp.genomicsengland.co.uk/panels/509/>) for actualizations.

OC, optic cup; OD, optic disc; OF, optic fissure; ON, optic nerve; OS, optic stalk; OV, optic vesicle; TF, transcription factor; RPE, retinal pigmented epithelium.

Additional hints on how MAC phenotypes may develop come from the study of vertebrate species that are blind as a result of evolutionary adaptations to extreme environments, such as cave fish or terrestrial subterranean animals, such as the mole. Among the many teleosts that have undergone environmental adaptations (Ravi and Venkatesh, 2008), the *Astyanax mexicanus* (the Mexican tetra, or 'blind cave fish') has emerged as an attractive model for studying the development of a colobomatous, microphthalmic eye and its regression. *Astyanax mexicanus* exists as a river-dwelling surface form and a blind cave-dwelling morph. The adult cave morph has no

eyes, although its embryos develop well-formed but small OCs that then regress in a stereotypical manner (Jeffery, 2020). Detailed comparison of the surface and cave early embryos shows that the cavefish begins to neurulate slightly earlier and has a smaller eye field than the corresponding surface morph (Agnès et al., 2022). This reduction has been associated with an earlier and more-expanded expression of Shh and Fgf8 (Pottin et al., 2011; Yamamoto et al., 2004), two morphogens that contribute to anterior neural plate patterning. This smaller eye field has less precise molecular borders with the adjacent telencephalic and hypothalamic

fields, and shows decreased expression levels of *rx3* (an ortholog of the mammalian *Rax* gene), one of the TFs that confers eye identity and enables OV evagination (Rembold et al., 2006). Reduced *rx3* expression, together with DNA hypermethylation of other eye-specific genes (Gore et al., 2018), might explain why the cavefish OV evagination and elongation are somewhat abnormal and proceed for longer than they do in the surface form (Devos et al., 2021). As in the surface form, basal constriction of the cavefish retinal progenitors induces the initial bending of the OV but folding does not progress efficiently so that the OC remains rather flat (Devos et al., 2021), resembling the *ojoplano* phenotype (Martinez-Morales et al., 2009). Abnormal rim involution and RPE flattening, together with lens alterations, are the likely causes of these abnormal shapes that further lead to an evident coloboma (Devos et al., 2021).

In parallel, SOX2 haploinsufficiency may cause poor activation of RAX with consequences similar to those observed in *Astyanax*. In support of this possibility, a recent study points to the compromised activity of a distant enhancer as an explanation for the poor and fuzzy *rx3* expression in the *Astyanax* cave morph (Leclercq et al., 2022 preprint). Furthermore, *Sox2* seems to directly activate *Rax/Rx3* expression in different species (Beccari et al., 2012). Accelerated changes in ocular-specific transcriptional enhancers have been also shown to occur in the genome of different types of moles (Partha et al., 2017), with genes involved in lens and retina development being the most affected. Thus, variants in regulatory elements controlling the expression of genes belonging to eye GRNs may likely explain at least part of the significant number of MAC cases that remain molecularly undiagnosed.

### Conclusions and perspectives

The acquisition of a proper 3D shape is crucial for the correct function of the eye in human and other vertebrate species. Any deviation from the expected eye geometry leads to poor or even absent vision. Therefore, understanding how the eye tissues develop and rearrange to generate a final cup-shaped camera eye may help to find strategies for solving visual defects. There have been many advances in our basic knowledge of how the eye acquires its shape early in development, mostly enabled using transparent vertebrate embryos and the increasing progress in mammalian organoid technology. These are accessible models amenable to genetic, pharmacological, surgical and mechanical manipulations that, together with mathematical and physical models, have identified the motors of OC formation.

Yet there are several unanswered questions that need to be addressed, such as the puzzling interdependence between lens and neural tissue. The lens tissue is no doubt a source of factors that influence OV development and specification, but does it also exert mechanical forces that help OV folding? Related to these questions, a recent study has reported that conditional deletion of *Arl13b*, a gene required for ciliogenesis in the mouse OV, disrupts morphogenesis so that the lens is abnormally surrounded by an inverted OC, in which the RPE layer faces the surface (Fiore et al., 2020). The primary cilium is an important cellular sensing structure and mutations in genes encoding its structural components are among identified causes of MAC (Plaisancié et al., 2019; Williamson and FitzPatrick, 2014). The *Arl13b* mutant phenotype seems to be linked to abnormal sensing of Shh signalling, but how this inverted shape arises is unclear and indicates an important contribution of the primary cilium to eye morphogenesis. When does the cilium form in the progenitors? Is it important only in a subset of OV progenitors? Does it contribute to sense cell tension and how?

Other highly relevant questions relate to the contribution of the surrounding mesenchyme and cephalic neural crest cells to OC formation. Although there is related information, these questions have not been systematically addressed. A recent interesting study has shown that the extensive evagination and invagination movements of the zebrafish eye exert traction forces on the adjacent olfactory placode, influencing its development (Monnot et al., 2022). Does the opposite hold true? Does this influence exist in other species in which the two structures are located further apart? Many of these questions could be addressed by studying early eye morphogenesis in species that are not currently raised in laboratory conditions. In this sense, the use of unconventional animal models such as *Astyanax*, or fish with other environmental adaptations, such as fish with tubular eyes (which have a main and an accessory retina), may help explain different aspects of morphogenesis and their underlying motors.

Very recent technological improvements use mESCs to grow *ex utero* post-gastrulation synthetic whole embryos (sEmbryos) that can reach a development stage comparable with embryonic day (E)8.5 embryos (Amadei et al., 2022; Tarazi et al., 2022). Although this stage has limited usefulness for studying eye-field specification, future advances may enable further development of sEmbryos and thereby facilitate *ex utero* manipulations and imaging of the mammalian eye, furthering our understanding of its morphogenesis. Furthermore, the generation of OC organoids from iPSCs derived from individuals with MAC will allow their 3D development to be followed in real time. For example, generating such models from individuals carrying *SOX2* or *OTX2* mutations will certainly be worthwhile. Furthermore, improving the development of human iPSC-derived OC organoids may in itself represent an important opportunity for learning more about how the eye forms. Indeed, in many cases the RPE develops as a clumped tissue attached to one side of the NR, failing to enwrap it. Investigating why it develops in this way might reveal more about the relationship between NR and RPE in OC formation. Lens, neural and mesenchymal ‘assembloids’ are also expected as future developments that should further clarify how the eye forms.

Many of the questions raised here can be addressed across species, with the possibility of comparing the developmental program of species-specific eye organoids as has been achieved for other organoids, including the possibility of generating inter-species assembloids. All these approaches may ultimately enhance our understanding of human eye morphogenesis and how MAC arises in humans; we predict that the field of eye morphogenesis and related malformations has exciting times ahead.

### Acknowledgements

We thank L. Beccari, E. Serrano-Sanz and J. R. Martinez-Morales for critically reading the manuscript.

### Competing interests

The authors declare no competing or financial interests.

### Funding

Work in our laboratory is supported by the Ministerio de Ciencia e Innovación/ Agencia Estatal de Investigación (PID2019-104186RB-I00 and RED2018-102553-T), the European Regional Development Fund (A way of making Europe), the European Union (NextGenerationEU/PRTR) and ERA-NET Neuron (PCI2022-132938/ NEURON\_NDD-255). Open access funding provided by the Agencia Estatal de Investigación. Deposited in PMC for immediate release.

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