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Morphogenesis of the lens placode

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ABSTRACT For over 100 years, the vertebrate eye has been an important model system to understand cell induction, cell shape change, and morphogenesis during development. In the past, most of the studies examined histological changes to detect the presence of induction mechanisms, but the advancement of molecular biology techniques has made exploring the genetic mechanisms behind lens development possible. Despite the particular emphasis given to the induction of the lens placode, there are still many aspects of the cell biology of lens morphogenesis to be explored. Here, we will revisit the classical detailed description of early lens morphological changes, correlating it with the cell biology mechanisms and with the molecules and signaling pathways identified up to now in chick and mouse embryos. A detailed description of lens development stages helps better understand the timeline of the events involved in early lens morphogenesis. We then point to some key questions that are still open.

KEY WORDS: *eye development, chick embryo, mouse embryo, Pax6, apical constriction, thickening*

Introduction

Morphogenesis and induction are central concepts in Developmental Biology that are intrinsically connected. These concepts were first established in landmark experiments in the lens of the eye. These classical experiments occurred before the use of molecular markers, and a common feature was the interpretation of phenotype through careful analysis of changes in histology and cell biology during morphogenesis. More recently, the focus has shifted to pathways and signaling required for lens induction, and phenotypical description has migrated towards gene expression (reviewed in Cvekl and Zhang, 2017). However, considering that gene regulation and cell behavior changes are complementary aspects of the same phenomena, we believe that detailed analysis of histological changes in lens formation remains a valuable tool for understanding the timeline of genetic and cell biology events in lens development. Therefore, here we review classical reports on cell biology changes in lens placode development and use this morphological framework to contextualize signaling events.

In this review, we explore the detailed descriptions of early lens morphological changes in experimentally generated phenotypes to dissect the temporal sequence of the regulatory mechanisms thus far identified in early lens development. In other words, we will present what is currently known about underlying cellular mechanisms in the context of a morphological timeline. This approach has two aims: it correlates tissue shape changes to molecular events. In addition, mechanistic approaches generally focus on a single phase amongst the range of morphological changes that occur during placodal development. Although current understanding of each phase is quite detailed, the unanswered questions lie in the transition between phases. As such, by presenting the molecular events divided in phases, we hope to reveal these gaps and contribute towards future research in linking these distinct compartments of information.

Staging of morphological changes in the lens placode

To integrate the data gathered from both chick and mouse embryos, we adopt here staging criteria based solely on placodal morphology (Fig. 1). Thus, we follow the phase division first proposed by Schook to describe the histological evolution of the lens placode in the chick embryo (Schook P, 1980), which was subdivided into four phases, from the initial contact of the surface ectoderm with the underlying optic vesicle (Phase 0) through establishment of lens vesicle (Phase 4). We did not address in this review zebrafish

Abbreviations used in this paper: BMP, bone morphogenic protein; FGF, fibroblast growth factor; FN, fibronectin; GEF , guanine nucleotide exchange factor; N-cadherin, cadherin-2 or neural cadherin; NCC, neural crest cell; Pax6, paired box protein 6; PPR, preplacodal region; Rho, ras homolog family member of GTPases; R-Smad, receptor-regulated SMADs; Sox2, sex determining region Y-Box 2; TGF-β, transforming growth factor beta.

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eye development, as this species morphogenesis is significantly different from chick and mouse embryos, and has been thoroughly addressed in other reviews (Greiling and Clark, 2009). Below we briefly describe the morphological characteristics of each phase.

Phase 0 (up to HH10 in chick embryo and E9 in the mouse)

Prior to placode formation, the surface ectoderm is comprised of cuboidal cells containing centrally located spherical nuclei (Byers and Porter, 1964). Then, as the basal region of surface ectoderm contacts the underlying optic vesicle, a large number of long microtubules form parallel to the apical-basal axis, the nuclei move towards the basal pole and become oval (Byers and Potter, 1964), and the cells in this region acquire vacuoles (Mckeehan, 1951). During this phase, the contact area between optic vesicle and surface ectoderm expands to its maximal extension (Zwaan and Hendrix, 1973). The basal laminae of the lens placode and optic vesicle, enriched in laminin, fibronectin and collagen IV (Hilfer and Randolph, 1993), remain distinct but closely juxtaposed (Cohen, 1961).

In the mouse embryo, actin-containing filopodia appear throughout the basal surface of the future placode, connecting this region to the underlying optic vesicle (Chauhan *et al.*, 2009; McAvoy, 1980). These filopodia are surrounded by basal lamina, mostly generated by the basal surface of the future lens placode. The filopodia persist throughout phases 1–3 in the mouse. In the chick, scanning electron microscopy images reveal fibrillary structures bridging the optic vesicle and the surrounding margins of lens placode at phase 0, but it is unclear if these structures are collagen fibrils (Yang and Hilfer, 1982).

Phase 1 (HH12-13 in the chick embryo E 9.5 in the mouse)

As the contact between the retinal disc of the optic vesicle and the overlying surface ectoderm continues to increase, the ectoderm cells grow along the basal–apical axis, forming a disc of pseudostratified tissue, the lens placode (Pei and Rhodin, 1970). At this phase, the placode in the chick embryo is 20 μm thick in the central region, with 2 rows of misaligned oval nuclei, tapering down at the edges to the height of the cuboidal surface epithelium (7 μm), surrounding the placodal region (Schook P, 1980). In this setting, proliferation occurs asynchronously, concomitant with interkinetic nuclear migration, positioning mitotic figures at the apical region (Zwaan *et al.*, 1969). Cells undergoing M phase are rounded and accumulate in the apical face whereas S-phase cells are elongated, extending along the entire apical-basal axis (Zwaan and Hendrix, 1973). Only a small portion of

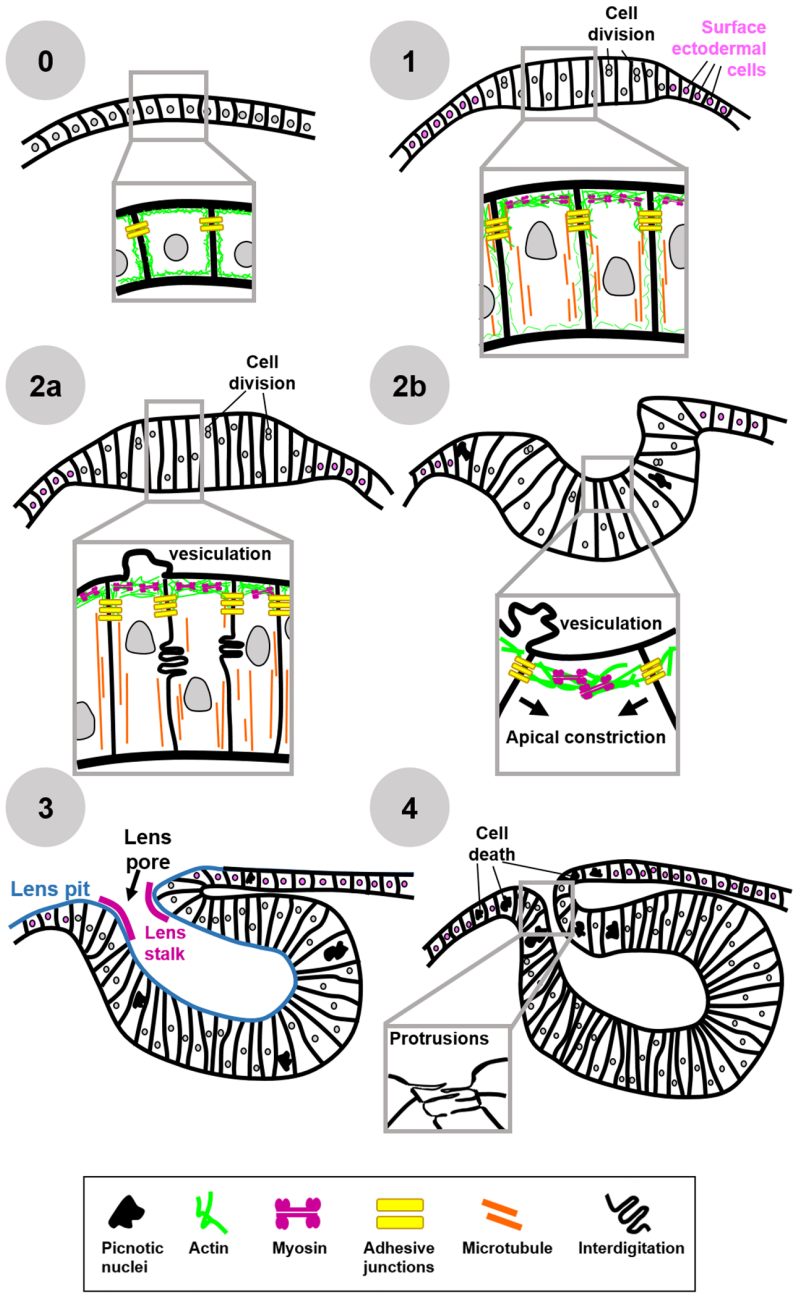

Fig. 1. Key events in lens placode development morphogenetic processes. *The morphogenetic events involved in lens placode formation and invagination are represented in 6 important steps.* **(Phase 0)** *Pre-placodal ectoderm is composed of cuboidal cells with spherical nuclei in the center. Actin is diffuse, distributed at the cell faces.* **(Phase 1)** *Early thickening of lens placode. The placodal cells increase in height forming a pseudostratified tissue with 2 rows of misaligned oval nuclei. Microtubules are organized parallel to apico-basal axis and an actin-myosin network begins to accumulate at the apical surface. Also there is an increase of intercellular junctions.* **(Phase 2a)** *Late thickening of lens placode. The height and cell density of lens placode increases. The pseudostratified tissue has now 3-4 rows of nuclei. The apical surface becomes irregular due to the multiple vesicles.* **(Phase 2b)** *Lens placode begins to invaginate. Placodal height does not increase and the apical constriction begins, forming the early lens pit. Cell death begins to appear in the surface ectoderm and placodal cells.* **(Phase 3)** *Lens placode invagination. The apical surface maintain an irregular configuration due to the vesiculation. The lens stalk is formed and defines the ridges of the lens placode.* **(Phase 4)** *End of lens placode invagination. While the invagination progresses and approaches the end, cell death at the interface between surface ectoderm and lens placode increases. The cells at the lens placode ridges emit protrusions that bridge the lens stalk.*

the cells are in M phase.

In addition to the apical-basal microtubule network, there is a gradual enrichment of actin-myosin network at the apical surface of the elongated cells (Borges *et al*., 2011; Byers and Porter, 1964) together with an increase in intercellular junctions in the subapical zone of cells in the centermost region of the placode. The extracellular matrix between the pre-placodal ectoderm and the optic vesicle becomes enriched in laminin and collagen IV, deposited as a dense fibrous array that associates the basal surface of the lens placode with the optic vesicle (Johnston *et al*., 1979; Svoboda *et al*., 1987). This attachment site is especially distinctive at the center of the retinal disc and of the lens vesicle. At this site, the basement membranes of the retinal disc and the lens placode fuse into a single lamina (Hilfer and Randolph, 1993).

Phase 2 (HH14+ in the chick embryo, E 9.75 in the mouse)

During this phase, the lens placode further increases its height (36 μm and four rows of nuclei in the chick embryo), with cells arranged in a fan-shape around the central indentation (see below). This shape is attained through increase in cell density (Zwaan and Hendrix, 1973), without a corresponding expansion of the contact area between optic vesicle and the placode, which remains constant since phase 0. In the apical surface, a small indentation appears, deepest at the dorsal side. The appearance of the indentation marks the start of the invagination of the lens placode, during which there is a reduction of the ratio between apical and basal surface areas of the placodal cells. At the indentation, the apical surface becomes increasingly irregular, due to the appearance of multiple intracellular vacuoles and small microvilli. The vacuoles range from 0.5–1.5 μm in diameter (Schook, 1980b). At the subapical region, the actin and myosin fibers now form a well-defined network (Borges *et al*., 2011; Plageman *et al*., 2010). In the mouse embryo, the number of actin-myosin filopodia increases at the basal surface. In the chick embryo, fibrillary connections containing collagen IV bridge the basal surfaces of the retinal disc and the lens placode (Hilfer and Randolph, 1993). There is progressive accumulation of glycosaminoglycans in the extracellular matrix between the lens placode and the optic vesicle as the invagination proceeds (Hendrix and Zwaan, 1975; Huang *et al*., 2011).

At the end of phase 2 (defined here as phase 2b), the indentation in the center of the lens placode deepens into the lens pit, without its surface opening (also known as the lens pore) changing its width.

Phase 3 (HH15-16 in the chick embryo, E 10.5 in the mouse)

During this phase, there are no further changes in the overall thickness of the lens placode. As the lens pit deepens, it constricts, forming a duct that becomes the lens stalk. Vesiculation at the apical surface of the invaginating placode increases in intensity proportional to the depth of invagination. Surface blebs, composed of lumps of cytoplasm and empty membranous spheres appear in the apical surface of lens pit cells, becoming especially pronounced at the bottom of the lens vesicle. In contrast, the apical surface of the ectoderm cells that surround the lens remain smooth (Bancroft and Bellairs, 1977; Schook, 1980a; Wrenn and Wessells, 1969). The localized nature of this blebbing, formed by membrane bulging above the adhesive junctions, strongly suggests that it plays an important, although as yet unidentified, role in invagination. Interaction between the apical region of neighboring cells is maintained by adhesive junctions and membrane interdigitations (Schook, 1980a; Wrenn and Wessells, 1969).

At the start of phase 3, the dorsal border of the lens pore – at the junction with the neighboring ectoderm – displays dying cells with picnotic nuclei. As the pit deepens, the field of dying cells expands, forming a ring around the lens pore (García-Porrero *et al*., 1979).

From this stage onwards, the chick lens vesicle is no longer closely associated to the optic tissue, now evolved to an optic cup. Accordingly, the extracellular matrix is organized into separate, thin basal membranes surrounding the lens (known as lens capsule) and the optic cup, with a wider intervening zone with globular and filamentous components in electron micrographs (Hendrix and Zwaan, 1975). The remaining attachment sites are in two locations: the margin of the optic cup with the outermost radius of the lens vesicle and the center of the retina with proximal center of the lens vesicle (Yang and Hilfer, 1982). In the mouse embryo, the number of basal filopodia connecting the placode to the optic vesicle decreases at this phase (Plageman *et al*., 2011).

Phase 4 (HH17 in the chick embryo, E 11.5 in the mouse)

Invagination of the lens placode is complete at this phase. The apical surface of the cells that line the lens pit gradually stop producing large membranous bulges (Schook, 1980a). Now, the lens stalk is progressively obliterated so that the lens placode separates from the surface ectoderm and becomes the lens vesicle. The marginal border of the lens pore starts to fuse. During this step, the boundary between surface ectoderm and lens vesicle accumulates dying cells interspersed with phagocytic cells. In the pore itself, cellular protrusions bridge the narrow lumen by projecting towards the opposing rim and interdigitate with neighboring protrusions (Schook, 1980c). The point of contact between protrusions is electron-dense, probably indicating the establishment of new junctions. As the lumen becomes filled with projections, obliteration of the stalk is complete and the lens vesicle becomes distinct from the surface ectoderm. The surface ectoderm that covers the lens vesicle becomes similar to the surrounding surface ectoderm, presenting polygonal cells with distinct borders.

Although this review will not broach the development of periocular tissues, it must be emphasized that, from phase 0 throughout the end of phase 4, there are no intervening mesenchyme/crest cells between the lens placode/vesicle and the optic cup. Cephalic neural crest cells migrate bilaterally from the neural tube (stage HH10 in chick embryos) and infiltrate the head mesenchyme to surround the optic vesicle. Initially, these cells come in contact with the head ectoderm posterior to the pre-lens placode field (Sullivan *et al*., 2004). There, they will later contribute to the establishment of a diversity of periocular tissues (Johnston *et al*., 1979; Noden, 1975). However, the gap between the lens and optic cup is noticeably devoid of mesenchymal tissue throughout the developmental stages described above.

Molecular changes of the lens placode in the context of morphological phases

In the previous section, we established a timeline of morphological changes. Here, we will utilize this timeline as a framework

to dwell deeper into the cellular and molecular mechanisms that drive each phase.

Phase 0: induction, maintenance and restriction of pre-placodal lens fate

In vertebrates, all the cranial placodes arise from the anterior portion of the neural plate border, located between the neural plate and the non-neural ectoderm (reviewed in Grocott *et al.*, 2012). At early neurula stages, the subdomains are not yet molecularly or spatially segregated, occupying a common preplacodal region (PPR), which subdivides into the olfactory, trigeminal, otic, epibranchial and lens placodal regions as development progresses (Fig. 2). Although PPR cells have the potential to form any sensory placode at neurula stages, their initial default competence is to develop into the lens, as indicated by the fact that, when cultivated *in vitro*, preplacodal ectoderm cells express lens placode specific markers (Bailey *et al.*, 2006).

Lens fate can be followed by the expression pattern of the transcription factor Pax6. Pax6 expression occurs very early in the head ectoderm. Chick explants isolated from the PPR before neurulation express Pax6 (Bailey *et al.*, 2006). Later, during eye development, Pax6 expression becomes restricted to the optic vesicle and lens placode (Antosova *et al.*, 2016; Ashery-Padan *et al.*, 2000; Plageman *et al.*, 2010; Walther and Gruss, 1991). Pax6 expression is sufficient and necessary for eye development (Walther and Gruss, 1991): ectopic expression of Pax6 induces ectopic lens in amphibians (Altmann *et al.*, 1997), and inhibition of Pax6 expression in the pre-placodal ectoderm arrests lens development at phase 0 (Antosova *et al.*, 2016; Ashery-Padan *et al.*, 2000; Huang *et al.*, 2011; Plageman *et al.*, 2010). Sox2, an additional marker co-expressed with Pax6 in the pre-placodal lens, is only required for lens placode progression into phase 2 (see next section). In the PPR, expression of Sox2 and Pax6 occur through independent genetic pathways (Smith *et al.*, 2009). Nonetheless, later in development, Sox2 and Pax6 control the same effectors, although in different moments. For instance, expression of N-cadherin depends on Sox2 but not Pax6 during phase 0-1, but, during phase 2 through 4, N-cadherin expression depends on Pax6 (Smith *et al.,* 2009).These results suggest a stage-specific regulation of Sox2 and Pax6 activity that is required for lens placode development.

BMP signals are important for specification and maintenance of lens fate (Furuta and Hogan, 1998; Huang *et al.*, 2015; Jidigam *et al.*, 2015; Pandit *et al.*, 2011; Sjödal *et al.*, 2007). In the mouse, BMP4 is expressed in both the pre-placodal ectoderm and in the optic vesicle (phase 0), whereas, in the chick, it is restricted to just the pre-placodal lens ectoderm and not detected in the optic vesicle (Furuta and Hogan, 1998; Huang *et al.*, 2015; Jidigam *et al.*, 2015). Suppression of BMP signaling in the lens PPR directs its cells to a nasal fate; conversely, increase in BMP signaling in nasal placodes converts them to a lens fate (Sjodal *et al.*, 2007). However, BMP signaling is not required for the maintenance of Pax6 expression: knockout of BMP or its receptors, Alk3 or Alk2 in mice still express Pax6; however, they lack Sox2 expression in the presumptive placodal region. The Pax6-positive domain in these mice does not progress to Phase 2 (Furuta and Hogan, 1998; Huang *et al.*, 2015; Rajagopal *et al.*, 2009; Wawersik *et al.*, 2005). Thus, BMP is required for preserving phase 0 lens fate in the preplacodal lens, whereas Pax6, which is not induced by BMP, is necessary for progression into the following phases. The current model for early induction of Pax6 expression in the PPR places it downstream of the somatostatin-nociceptin pathway activated by the head mesoderm (Hintze *et al.*, 2017; Lleras-Forero *et al.*, 2013) (Fig. 2).

The canonical BMP pathway depends on phosphorylation of R-Smads and their translocation from the cytoplasm to the nucleus after interaction with Smad4 (Wang *et al.*, 2014). However, although phosphorylated Smads are present in both the lens placode and in the optic vesicle from phase 0 to phase 4, knockout mice for Smad1, 5 and 4 showed no change in lens placode or lens vesicle formation (Huang *et al.*, 2011; Jidigam *et al.*, 2015; Rajagopal *et al.*, 2009). This suggests that, in the context of the lens placode, BMP signal follows as yet undefined, non-canonical pathways.

Since the PPR has a strong preference to form lens placode, the development of the lens can also be viewed as a selective

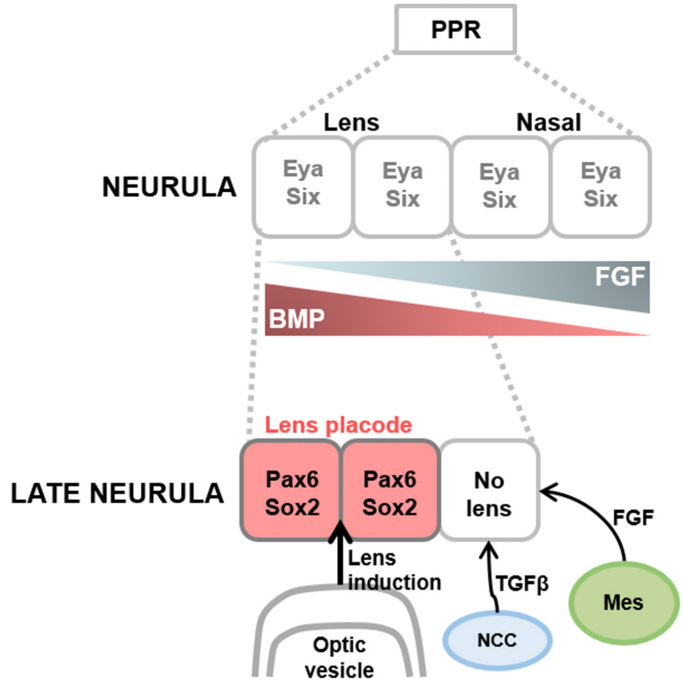

Fig. 2. Pre-placodal region and lens field induction on the surface ectoderm. *At neurula stages, the pre-placodal region (PPR) is defined at the surface ectoderm. The PPR express placode precursor's specific genes (Six1-4 and Eya1-2) and, initially, has a strong preference to form lens placode. Later, the balance between FGF and BMP signal defines the specific placodes fields in the PPR. The maintenance of FGF induces olfactory fate, while BMP signal maintains lens placode fate. At late neurula stages, the optic vesicle contacts the overlying PPR and reinforces lens formation. Thus, lens fate is suppressed everywhere except at ectoderm overlying the optic vesicle. FGF is secreted by the anterior neural ridge at the anterior portion of the PPR and the head mesoderm (Mes, green circle), thus restricting the lens placode fate by repressing lens characteristics. At late neurula stages, BMP is also required for definition of lens placode fate. The optic vesicle approaches the pre-placodal ectoderm, and induces lens placode-specific gene expression, like Sox2 and Pax6. After the approximation of optic vesicle to the ectoderm, the restriction of lens character continues with the migration of neural crest cells (NCC, blue circle). Neural crest cells secrete TGFβ, which inhibit Pax6 activity through activation of Smads.*

maintenance of the lens fate in the ectoderm overlying the optic vesicle, with lens specification suppressed everywhere but at the ectoderm over the optic vesicle, as development proceeds. Thus, FGF secreted by the anterior neural ridge at the anterior portion of the PPR changes its fate, promoting the expression of olfactory placode markers and repressing lens specification (Bailey *et al*., 2006; Sjodal *et al*., 2007). FGF is also produced by the mesoderm underlying the otic placode field, suppressing lens placode fate in posterior regions of the head (Bailey *et al*., 2006; Sjödal *et al*., 2007)(Fig. 2).

Despite its essential role in defining the otic and olfactory placodes in the (Pax6-positive) early head ectoderm, FGF is insufficient to suppress the expression of lens markers in long-term cultures (Bailey *et al*., 2006). Indeed, neural crest cells have also been implicated in the suppression of lens fate: ablation of cranial neural crest cells before evagination of optic vesicle results in ectopic expression of lens placode genes (Sox2 and Pax6) and lens development in extraocular ectoderm (Bailey *et al*., 2006) (Fig. 2). However, as previously mentioned, neural crest cells do not reach the pre-lens placodal ectoderm overlying to the optic vesicle, despite being present throughout the head mesenchyme (Sullivan *et al*., 2004). Together, these data suggest that the neural crest cells negatively regulate Pax6 expression, restricting the lens placode to the contact region above the optic vesicle. Migrating neural crest cells secrete TGF-β, which in turn activates Smad3 and induces the expression of Wnt2b in non-lens ectoderm. The TGF-β and Wnt pathways cooperate to suppress Pax6 expression (Fig. 3). Activated Smad3 inhibits Pax6 function whereas β-catenin, an effector of Wnt signaling represses its transcription (Grocott *et al*., 2011). In support of this, modulation of beta-catenin levels is necessary and sufficient to change Pax6 expression. Loss of β-catenin in surface ectoderm results in ectopic formation of lens structures (Kreslova *et al*., 2007; Smith *et al*., 2005) and, conversely, β-catenin gain of function in the pre-lens placodal ectoderm decreases Pax6 expression, inhibiting lens placode formation (Smith *et al*., 2005).

Overall, these data suggest a two-step model for lens fate restriction. Prior to neural crest migration, activation of FGF initiates restriction of the Pax6-positive field in the head ectoderm. Later, the arrival of neural crest cells to the area subjacent to the periplacodal region activates the TGF-β pathway, which acts (both directly and indirectly) to limit the lens field to the crest-free region overlying the optic vesicle (Grocott *et al*., 2011).

Phases 1 and 2: increase in cell height

During apical-basal growth of the placode (also known as elongation or thickening) microtubules parallel to the apical-basal axis appear (Byers and Porter, 1964). Also, the number of lens cells increases exponentially, while the placodal area – the contact between the placode and the optic vesicle – and individual cell volume remain constant (Mckeehan, 1951). As a result, cell density increases. Each of these cytological changes have been proposed to be the driving force of placodal elongation (Hendrix *et al*., 1993).

The role of microtubules in cell elongation was dismissed by experiments centered on colcemid-induced microtubule depolymerization (Beebe *et al*., 1979; Pearce and Zwaan, 1970). Treatment of phase 2 chick lens placodes with the drug did not affect maintenance of its pseudostratified structure nor their invagination. However, the authors did not expose placodes during initiation of elongation (phase 1). Thus, the contribution of the microtubule arrays to the establishment of this process remains unclear.

The cell crowding hypothesis for placodal elongation proposes that, as individual cell volume and placodal area remain unchanged while cell number increases (Zwaan and Hendrix, 1973; Zwaan and Pearce, 1971), the resulting tissue volume increase will squeeze the cells, forcing them to increase in height at the expense of their cross-sectional area. This phenomenon depends heavily on maintenance of placodal area, which has been theoretically attributed to increased adhesion of placodal cells to the underlying extracellular matrix. Indeed, the tightness of adhesion – verified by resistance to mechanical separation (Mckeehan, 1951) – correlates with changes in histological staining of the extracellular matrix. PAS-staining becomes more intense and glucosamine-containing macromolecules accumulate in the space between the lens and the underlying optic vesicle (Hendrix and Zwaan, 1975). Moreover, experimental changes to the extracellular matrix affect apical-basal growth: global deletion of fibronectin-1 expands the placodal field and decreases placodal height, without altering expression of the lens markers Pax6 and Sox2 (Huang *et al*., 2011). Interestingly, lens-specific deletion of fibronectin-1 does not alter placodal elongation or invagination, suggesting that the primary source of fibronectin is not the placode itself or that expression of fibronectin-1 in the placode occurs prior to the establishment of the knockout-phenotype (Huang *et al*., 2011) (Fig. 3). The production of fibronectin-1 (as well as extracellular matrix components Versican and Tenascin-C) in the placodal basal lamina requires Pax6 (Huang *et al*., 2011): lens-specific removal of Pax6 expanded the contact area between optic vesicle and placode, reduced the distance between the two and inhibited placodal columnar growth. In this scenario, ectodermal spreading limitation, which leads to cell crowding, depends on Pax6-induced changes in the extracellular matrix that increase its adhesivity. Thus, Pax6 is also a key link between apical-basal growth and changes in the extracellular matrix.

These same parameters, placodal area, placodal volume and cell density, have been proposed as the main mathematical variables that determine changes in placodal curvature during elongation and invagination (Hendrix *et al*., 1993). For their mathematical analysis, these authors subdivided placodal elongation into two phases: firstly, there is an increase in cell number with expansion of placodal area; this is followed by continued division with restriction of placodal area. The timing of these phases coincides with the conversion from phase 0-1 to phase1-2. Histologically, these two phases differ in cell height and number of rows of nuclei: two in phase 1 and four in phase 2.

TABLE 1

ROLE OF INDIVIDUAL GENOMIC COMPONENTS FOR PAX6 IN THE TIMELINE OF LENS MORPHOGENESIS

Mutants	Phase 1	Phase 2	Reference
$Pax6^{+/-}$	yes	no	Huang et al., 2011
$Pax6^{-/-}$	no	no	Huang et al., 2011
$Pax6^{\Delta SIMO/Sey}$	yes	no	Antosova et al., 2016
$Pax6^{\Delta EE/\Delta EE}$	yes (delayed)	yes (delayed)	Dimanling, 2001
$Pax6^{\Delta EE;\Delta SIMO/\Delta EE;\Delta SIMO}$	n/a	no	Antosova et al., 2016

Pax6 heterozygotes present placodes with intermediate thickness whereas homozygote knockouts do not develop a placode, demonstrating a dose-dependent effect on placodal growth. Removal of one copy of the SIMO genomic element upstream of Pax6 halts progression into phase 2, whereas removal of both copies of EE genomic element delays but does not suppress neither phase 1 or 2, resulting in the formation of smaller lens vesicles. Placodes that do not progress to phase 2 do not form lens vesicles.

Using the phase criteria proposed here to re-evaluate lens phenotypes reported in published studies allows further refinement of the contribution of genes to the establishment of the placode. For instance, deletion of different genomic elements that control Pax6 expression result in arrest at phase 0 or phase 1 (Table 1). Together, these data suggest that application of more stringent criteria to classify phenotypes histologically could contribute to a more detailed understanding of the elongation process.

Phases 2 and 3: invagination

Lens invagination is the result of the sum of several cell biology events, some generated by placodal cells and some resulting from the action of non-placodal cells. Among the former are included the shape-changing forces generated internally by placodal cells, such as reduction of apical area, mitosis and membrane blebbing, whereas the role of basal filopodia in tethering the placode to the optic cup is an example of events external to the placodal cells.

Apical constriction

The apical constriction (reduction of apical area) found in different instances of morphogenesis requires a common machinery of apical actin, myosin and adhesive junctions. In it, attachment of F-actin to the cytoplasmic side of adhesive junctions firmly anchors the cytoskeletal network to cell membrane, and non-muscle type II myosin generates contractile force (upon activation through phosphorylation by the kinase ROCK, a downstream effector of Rho GTPase). As a result, the circumference of the actin network decreases, consequently reducing the perimeter of the attached apical membrane. The establishment of these components in the apical subdomain and the trigger for the constriction vary in different cell types (reviewed in Martin and Goldstein, 2014). In the lens, apical enrichment of actin and myosin II occurs during phases 1 and 2 and requires the activity of the Rho GTPase pathway (Borges *et al.*, 2011; Plageman *et al.*, 2010). Consistent with this, RhoA is preferentially localized to the cell apex (Borges *et al.*, 2011; Jidigam *et al.*, 2015). Genetic or pharmacological interference with either Rho or its effector ROCK disrupts apical contraction and, consequently, lens morphogenesis (Borges *et al.*, 2011; Plageman *et al.*, 2011). In contrast, loss of Rac1 phosphorylates Myosin II and enhances apical constriction, suggesting a balance between these two GTPase pathways (Chauhan *et al.*, 2011).

Interfering with the Rho and Rac1 pathways does not interfere with lens fate: their effects are restricted to morphogenetic phenotypes (Borges *et al.*, 2011; Jidigam *et al.*, 2015; Plageman *et al.*, 2011). Likewise, inhibition of BMP signaling with Noggin in phase 1 placodes does not change lens fate, but disrupts apical localization of RhoA, and of actin (Jidigam *et al.*, 2015). The elements linking the BMP and RhoA pathways are still unknown (Fig. 3). In contrast, the apical localization of Shroom3 – a cytoskeletal adaptor protein also required to maintain the apical actin-myosin network – depends on lens fate, in that Pax6 is necessary and sufficient for its transcription (Plageman *et al.*, 2010), whereas the apical placement of Shroom3 depends on RhoA activity (Plageman *et al.*, 2011). Global disruption of Shroom3 expression generates discontinuities in the apical actin-myosin network of phase 2 lens placodes (Plageman *et al.*, 2010). Thus, the current model for instructing lens apical constriction is comprised of three components with a co-dependent relationship: RhoA, Shroom3 and ROCK. RhoA, as a GTPase, depends on guanine nucleotide exchange factors (GEFs) to be activated, and depletion of the GEF Trio inhibits both endogenous and Shroom3-induced apical constriction (Plageman *et al.*, 2011). Activated RhoA recruits actin, myosin II and Shroom3 to the apical domain; Shroom3 then interacts with both actin and ROCK (Mohan *et al.*, 2012). In vitro, ROCK binds simultaneously to Shroom and RhoA, through different domains. Thus, Shroom could act as a scaffolding protein that brings to-

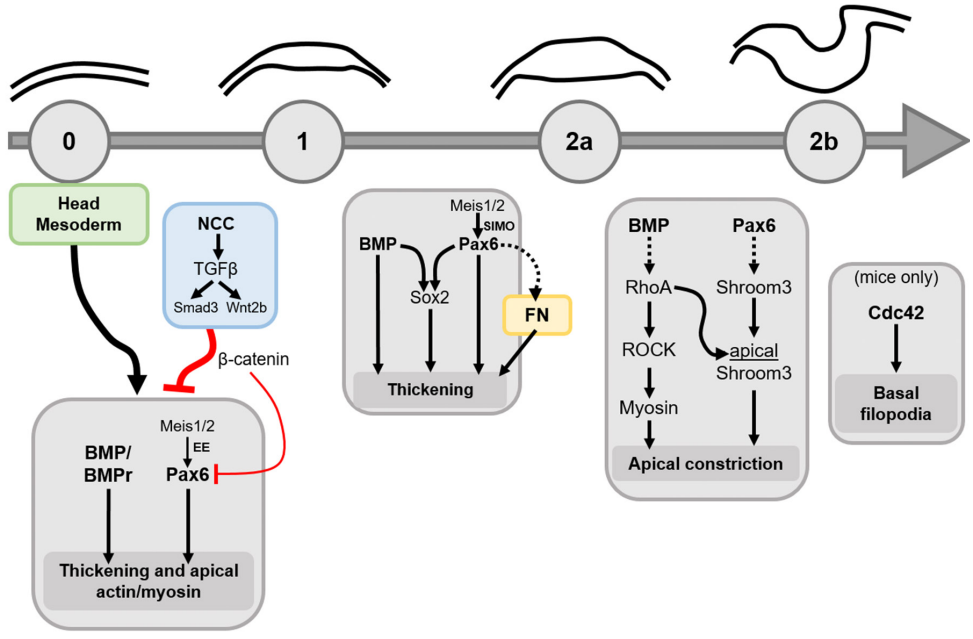

Fig. 3. Main pathways involved in the early stages of the lens placode development. *The cephalic mesoderm (green box) in the anterior region is important to induce the lens fate and Pax6 expression on the surface ectoderm. BMP and Pax6 signaling are crucial for the placode thickening and organization of the cytoskeleton in the transition from phase 0 to phase 1. At this stage, Pax6 expression depends on Meis1/2 and the genomic element EE. Neural crest cells (NCC, blue box) secrete TGFβ that inhibits lens placode formation and Pax6 expression in other regions of the ectoderm. BMP, Pax6 and Sox2 are necessary for the transition from phase 1 to phase 2a. Pax6 regulates the composition of the extracellular matrix (yellow box) between the placode and the optic vesicle, where the presence of fibronectin (FN) is crucial for thickening, but not for the organization of the cytoskeleton. At phase 1, the expression of Sox2 depends on BMP and Pax6. Pax6 expression is modulated by Meis1/2 via SIMO. Between phases 2a and 2b, apical constriction depends on RhoA that concentrates Shroom3 and activates myosin motors in the apical region of the placodal cells. BMP and Pax6 are also required for the apical displacement of RhoA and expression of Shroom3 respectively. In mouse embryos, the progression of placode invagination depends on Cdc42 activity for the emission of filopodia that anchor the placode in the optic vesicle. Solid black arrow, activation; black dotted arrow, indirect activation; red line, inhibition.*

gether activated RhoA and ROCK in the apical domain (Mohan *et al.*, 2013). This event would trigger the signaling cascade that culminates in phosphorylation of myosin and resulting shrinkage of the apical surface area. It is currently unclear what stimuli activate the GEF Trio that is at the top of this pathway.

Although most signaling events that prevent placodal elongation also interfere with apical concentration of actin-myosin network, global knockout of fibronectin-1 in the extracellular matrix suppresses lens elongation but not apical localization of actin. This suggests that lens elongation and apical segregation of the cytoskeleton occur through distinct pathways (Huang *et al.*, 2011).

In a continuous epithelial tissue such as the placode, the concomitant apical shrinkage of all its cellular components bends it into a convex curve, increasing mechanical stress on its components. Prior to invagination, the placode cells establish interdigitations, and increase adhesive junctions at their apical subdomain, to withstand the mechanical stress and maintain tissue integrity (Schook P, 1980). Consistent with this, loss of lens beta-catenin from adhesive junctions disrupts the apical actin network after invaginations starts, and the lens does not invaginate further, remaining a shallow cup. At later stages, the cells are completely disorganized and no longer form a coherent epithelium (Smith *et al.*, 2005). However, lens fate is not affected, as the expression of Pax6, Prox1 and β-crystallin remain and apical actin localization in early stages (phase 1) is also unchanged (Smith *et al.*, 2005).

Mitosis

Although increase in cell density seems to be required for placodal elongation (see previous section), the actual number of cells required for invagination seems to be flexible. Reduction of cell number by interference with FGF or BMP signaling after lens induction does not affect invagination (Pan *et al.*, 2006; Rajagopal *et al.*, 2009) and they successfully undergo morphogenesis to form lens vesicles, although the lenses thus formed are smaller than normal. However, if the rate of proliferation and accumulation of placodal cells appears not be crucial for morphogenesis, the duration of each phase of mitosis might be relevant.

The conversion of cell shape from columnar to trapezoidal, characteristic of the early stages of phase 2, has been attributed to apical constriction but also to basal expansion, the increase in area at the basal surface of the placode. Cell cycle in the pseudostratified placode is coupled to interkinetic nuclear migration (reviewed in Spear and Erickson, 2012). In this process, cells undergoing S phase position their nuclei basally, whereas cells at M phase become spherical and accumulate in the apical region of the placode (Mckeehan, 1951; Zwaan and Hendrix, 1973). The translocation of the nucleus in S, G1 and G2 phase cells is followed by local increase in cellular volume, resulting in basal expansion. Since interkinetic nuclear migration in the lens placode occurs at phase 1, basal expansion would occur prior to the onset of apical constriction (phase 2). Indeed, in the otic placode, increase in basal surface occurs prior to apical shrinkage (Alvarez and Navascués, 1990). Although placodal cells divide asynchronously, for the total surface ratio between apical and basal regions to decrease, the majority of the cells should have basally located nuclei and thus be in S phase. This is made possible by the fact that the duration of each placodal cell cycle phase differs, with most of mitosis spent in S phase, with basally locate nuclei (Zwaan *et al.*, 1969). This pattern becomes more pronounced as the placode differentiates. Duration of cell cycle phases changes between phase 0 to phase 1, to show a significant decrease in M phase duration (Zwaan and Hendrix, 1973; Zwaan and Pearce, 1970). During neurulation, the columnar neuroepithelium (neural plate) bends at its medial region while the lateral neuroepithelium remains relatively planar (Smith and Schoenwolf, 1988). Bending at the medial region of the neuroepithelium results from a combination of apical constriction and basal expansion. Overall, medial neuroepithelium cell cycle is longer than in the lateral neuroepithelium. Also, medial neuroepithelium cells display much longer S phase than their lateral counterparts. Thus, as changes in cell cycle length and duration of each cycle phase are associated with nuclear localization (Kosodo *et al.*, 2011), and since nuclear localization generates basal expansion in pseudostratified epithelial tissues, control of cell cycle also contributes to morphogenesis.

The mechanism that regulates progression or retention through cell cycle phases in placodal cells is still unclear. In the neural tube, interkinetic nuclear migration has been proposed to involve both active and passive means. During G2 phase, nuclear migration from basal to apical compartment depends on Tbx2-mediated transport on microtubules (Kosodo *et al.*, 2011; Spear and Erickson, 2012). In contrast, apical-to-basal migration is a passive process due to increased nuclear density at the apical region of the tissue. Arrest of cell cycle in G1 phase, through overexpression of $p18^{Inc4c}$, results in nuclear retention at the basal face. Although the nuclear distribution in cortical neuroepithelium is different from the placode – more nuclei are at the apical layer of the former – it is possible that placodal cells actively manipulate duration of the cell cycle to favor basal retention of the nuclei that will result in basal expansion. An alternative possibility is that the basal location of the nuclei is a response to decreased volume in the apical region, caused by the onset of apical constriction. However, since basal accumulation of the nuclei occurs prior to apical constriction, this second alternative is less likely.

Membrane blebbing

It is possible that the intense membrane blebbling observed during invagination is a mechanism to shed the leftover cell membrane resulting from apical constriction. Indeed, intensity of membrane blebbing correlates well with the degree of invagination (Schook, 1980). At phases 2b and 3, blebbing is more severe at the floor of the lens pit than at its walls. Blebbing also occurs during invagination of the otic placode, the precursor of the inner ear. As in the lens placode, the apical domain in otic placode cells undergoes apical constriction, and presents abundant intercellular junctions and cell interdigitations (Meier, 1978). The commonalities in lens and otic placodes suggest that membrane blebbing could be a general consequence of apical constriction, although it could also be a driving force.

Alternatively, it is possible that membrane blebbing is a quick way of actively shedding cell volume. Thus, the excess volume generated by apical shrinkage that is not used to increase cell height could be shed as membrane blebs. Indeed, inactive forms of Shroom3 or inhibition of BMP signaling result in wider apical area and in shorter placodal cells, thus maintaining a constant cell volume through changes in cell height (Jidigam *et al.*, 2015; Plageman *et al.*, 2010). Inactivation of RhoA also reduces apical shrinkage. If the same principle of cell volume conservation was applied, it would be expected that cell height would decrease. Instead, there

is an increase in cell height, reflecting an increase in cell volume. Inactivation of Rac1 enhances apical shrinkage and generates shorter columnar placodal cells, thus showing that cell volume decreased (Chauhan *et al.*, 2011). This apparent paradox raises the possibility that RhoA and Rac1 pathways not only modulate apical constriction but also control total cell volume. Considering that, in other biological settings, Rho-ROCK are involved in the dynamics of membrane blebbing (reviewed in Fackler and Grosse, 2008), it is possible that, in the placode, RhoA mutants have increased cell volume due to a decrease in membrane blebbing. Similarly, Rac1 mutants might display decreased cell height by shedding total cell volume through a large increase in membrane blebbing.

Basal filopodia

In the absence of Shroom3 activity, lens invagination still occurs in mouse embryos, but the shape of the invaginating placode is distorted. Instead of an oval curvature, a sharp invaginating V is formed. The occurrence of invagination, albeit misshaped, in the absence of apical constriction suggests that extrinsic forces also contribute to this process (Plageman *et al.,* 2010). In the mouse embryo, the space between the lens placode and the optic vesicle is bridged by cytoplasmic filopodia, most derived from lens pit cells (Chauhan *et al.*, 2009). The establishment of these filopodia depends on the Cdc42-IRSp53 pathway (Chauhan *et al.*, 2009) (Fig. 3). Removal of either of these components of the pathway results in intense loss of filopodia, which is accompanied by an increase in inter-epithelial space and a shallower angle of the lens cup. Both of these latter phenotypes support an active role for basal filopodia in lens invagination. Indeed, changing the length of the filopodia and refining the curvature of the invagination requires myosin II contractility. Basal filopodia in the lens placode were described for rat as well, but not for chick embryos (McAvoy, 1980). Instead, the chick interepithelial space is a densely packed matrix containing collagen fibrils (Yang and Hilfer, 1982). Also, the interepithelial space between lens placode and optic vesicle in the chick is significantly narrower (1-3 μm) than rat (6 μm) or mouse (15 μm) (Chauhan *et al.*, 2009; Hunt, 1961; McAvoy, 1980). Thus, it is possible that the tethering of the lens placode to the optic cup in the avian embryo is mediated by strong interactions (Chauhan *et al.*, 2009) with the extracellular matrix.

Phase 4: lens pit closure

At phase 4, invagination of the lens placode is complete and the borders approximate, to fuse and form the lens vesicle. The detachment of the lens placode from the surface ectoderm involves the obliteration of the lens stalk, where significant cell death activity is observed (Garcia-Porrero *et al.*, 1979; Schook, 1980c). Cell death progresses during lens placode closure and the fusion of the surface ectoderm (García-Porrero *et al.*, 1979). The importance of cell death has not yet been explored in lens development, but it has been explored to some extent in neural tube closure. Cell death events concentrate at the dorsal marginal edges of the merging neural tube. Further, apoptosis-deficient embryos display abnormalities in cranial neural tube closure (Yamaguchi *et al.*, 2011). Caspase inhibition in chick embryos also results in neural tube closure defects, suggesting that programmed cell death is crucial for correct closure of this structure (Weil *et al.*, 1997). Considering that lens vesicle detachment shares multiple histological features with neural tube closure, it may also require cell death.

Detachment requires loss of adhesion between the cells at the borders of the lens and their surrounding neighbors at the surface ectoderm. During placode invagination and closure, placodal cells express N-cadherin, while Pan-cadherin is expressed only in the surface ectoderm, suggesting that the segregation of these two tissues is mediated by changes in cadherin subtypes (Smith *et al.*, 2009). Loss of Pax6 alters the cadherin expression pattern, with loss of N-cadherin and expansion of the Pan-cadherin expression domain towards the lens stalk. In this condition, the lens stalks persists and the lens vesicle does not finalize its separation (Smith *et al.*, 2009).The same phenotype is observed with lens-specific loss of N-cadherin. Together, these data indicate that expression of N-cadherin in the lens induced by Pax-6 is crucial for its detachment (Smith *et al.*, 2009). Similarly, during neural tube invagination, the neuroepithelium expresses N-cadherin while surrounding ectodermal cells express E-cadherin (Dady *et al.*, 2012). This configuration is required for neural tube closure and separation from non-neuro ectodermal surface. Again, N-cadherin misexpression results in disorganized neural tube formation (Bronner-Fraser *et al.*, 1992; Hong and Brewster, 2006; Radice *et al.*, 1997).

Another important event observed during phase 4 is intense emission of cell extensions in the lens stalk region towards the lumen of the lens pore (Schook, 1980c). Again, this process is similar to protrusions observed during neural tube closure in chick and mouse embryos (reviewed in Nikolopoulou *et al.*, 2017). In mouse embryos, filopodia and ruffles (lamelipodia) are found during different moments of neurulation (Ray and Niswander, 2016; Rolo *et al.*, 2016). In chick embryos, as neural tube invagination completes, the non-neural ectoderm cells emit filopodia-like protrusions that promote contact between the neural tube ridges and the surface ectoderm (Schoenwolf, 1979). In both models, cellular protrusions participate in neural tube closure (reviewed in Nikolopoulou *et al.*, 2017). In mice, Rac1 (but not Cdc42) is necessary for lamelipodia formation and neural tube closure (Rolo *et al.*, 2016). Likewise, cellular protrusion emission might be important for lens pore closure and lens vesicle formation.

Open questions

By reviewing research on the molecular aspects of early lens placode development, in the framework provided by classic, histology and cell-biology-based phenotypic description of lens formation, a number of key questions still requiring investigation are revealed.

During phase 0, it is clear that BMP signaling is crucial for the induction, maintenance and morphogenesis of the lens placode (Furuta and Hogan, 1998; Huang *et al.*, 2015; Jidigam *et al.*, 2015; Pandit *et al.*, 2011; Sjödal *et al.*, 2007). However, results obtained in Smad-deficient mice suggest that BMP acts through a pathway that is alternative to the canonical (Huang *et al.*, 2011; Jidigam *et al.*, 2015; Rajagopal *et al.*, 2009). The exact identity of this pathway remains to be identified. Later, at phases 1-2, during placode thickening and invagination, the cell cycle is coupled to interkinetic nuclear migration, a characteristic of embryonic pseudostratified epithelia. As a result, the cells are mostly in the S phase and the nuclei concentrate in the basal compartment. It is possible that nuclear localization contributes to the initial inward bend of the placode by promoting basal expansion. Thus, the mechanisms that determine the duration of each cell cycle phase in lens placode, and in other pseudostratified tissues, merit investigation.

During phase 2-3, invagination is driven by apical constriction through recruitment of RhoA-Shroom-myosin, which is also central to apical shrinkage in other tissues. In the lens, the most upstream element of the cascade is the GEF Trio (Plageman *et al.*, 2010; Plageman *et al.*, 2011). However, it is not yet known what upstream factors are involved in its activation. Finally, at phase 4, during lens closure and detachment from the surface ectoderm, cell death and the emission of protrusions abound at the region of detachment. Such events are also observed during the closure and detachment of the neural tube (reviewed in Nikolopoulou *et al.*, 2017). Whether they play an active role in either lens or neural tube morphogenesis remains an open question.

In conclusion, throughout the complex multi-staged process of lens morphogenesis, there is a sequential activation of cell biology processes that are common to other developmental events. Due to its anatomical location, the lens remains an excellent experimental paradigm that is easily accessible to imaging analysis and detailed description of its histological evolution. By tying together gene expression, cell signaling and detailed description of changes in cell biology, results obtained from the study of the lens can contribute to our understanding of morphogenesis in general.

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