EphA2 and Src regulate equatorial cell morphogenesis during lens development

Catherine Cheng1, Moham M. Ansari2, Jonathan A. Cooper2 and Xiaohua Gong1,*

SUMMARY
High refractive index and transparency of the eye lens require uniformly shaped and precisely aligned lens fiber cells. During lens development, equatorial epithelial cells undergo cell-to-cell alignment to form meridional rows of hexagonal cells. The mechanism that controls this morphogenesis from randomly packed cuboidal epithelial cells to highly organized hexagonal fiber cells remains unknown. In EphA2−/− mouse lenses, equatorial epithelial cells fail to form precisely aligned meridional rows; moreover, the lens fulcrum, where the apical tips of elongating epithelial cells constrict to form an anchor point before fiber cell differentiation and elongation at the equator, is disrupted. Phosphorylated Src-Y424 and cortactin-Y466, actin and EphA2 cluster at the vertices of wild-type hexagonal epithelial cells in organized meridional rows. However, phosphorylated Src and phosphorylated cortactin are not detected in disorganized EphA2−/− cells with altered F-actin distribution. E-cadherin junctions, which are normally located at the basal-lateral ends of equatorial epithelial cells and are diminished in newly differentiating fiber cells, become widely distributed in the apical, lateral and basal sides of epithelial cells and persist in differentiating fiber cells in EphA2−/− lenses. Src−/- equatorial epithelial cells also fail to form precisely aligned meridional rows and lens fulcrum. These results indicate that EphA2/Src signaling is essential for the formation of the lens fulcrum. EphA2 also regulates Src/cortactin/F-actin complexes at the vertices of hexagonal equatorial cells for cell-to-cell alignment. This mechanistic information explains how EphA2 mutations lead to disorganized lens cells that subsequently contribute to altered refractive index and cataracts in humans and mice.

KEY WORDS: Eph, Ephrin, Lens, Cataracts

INTRODUCTION
Cataracts, defined as any lens opacity, remain the leading cause of blindness (Asbell et al., 2005). The lens is an avascular, transparent, highly refractive and biconvex structure that focuses light images onto the retina. The lens is composed of a monolayer of epithelial cells that cover the anterior hemisphere of bulk elongated fibers and is wrapped by a basement membrane called the lens capsule. The mechanisms that regulate lens growth, homeostasis and lifelong transparency are still not well understood. During early development, the posterior cells of the lens vesicle elongate to form primary fibers while the anterior cells differentiate into epithelial cells. Throughout life, lens epithelial cells continually differentiate into secondary fiber cells at the lens equator (Platigorsky, 1981).

High refractive index and lifelong transparency of the lens rely on the precise alignment of lens fiber cells that are overlaid on previous generations of fiber cells in a concentric manner. Cell-to-cell organization starts when equatorial epithelial cells undergo morphogenesis to form aligned meridional rows of hexagonal cells just before fiber cell differentiation and elongation at the lens equator (Bassnett et al., 1999).

One of the key events during lens development is the hexagonal shape and packing geometry of lens fiber cells. Lens fiber cells, which are hexagonal in cross-section, are packed tightly to eliminate intercellular space, establish a high refractive index and minimize light scattering (Kuszak, 1995). The structural organization of contractile and adhesive elements (N-cadherin/actin/myosin) may be required for cell packing and appropriate migration and alignment of elongating fiber cells (Bassnett et al., 1999). N-cadherin/actin complexes form a hexagonal lattice in fiber cell basal ends with the contractile proteins myosin and caldesmon, which are localized at the center of the fiber cell (Bassnett et al., 1999). F-actin bundles of one cell are aligned with those in the next. Paxillin, myosin light chain kinase (MLCK) and focal adhesion kinase (FAK) are also detected in fiber cell basal membrane complexes. Tropomodulin 1 is also required for maintaining the hexagonal geometry of interior lens fiber cells in mice (Nowak et al., 2009). However, it is not known which mechanisms control the initiation of lens equatorial cell-to-cell alignment when randomly packed pre-equatorial epithelial cells become organized into the meridional rows of hexagonal cells at the lens equator.

Recent studies, including our work, have reported that EphA2 or ephrin A5 mutations cause cataracts with variable severity or incomplete penetrance in humans and mice (Cheng and Gong, 2011; Cooper et al., 2008; Jun et al., 2009; Kaul et al., 2010; Masoodi et al., 2012; Park et al., 2012; Shi et al., 2012; Shiels et al., 2008; Sundaresan et al., 2012; Tan et al., 2011; Zhang et al., 2009). Bidirectional signals mediated by membrane-anchored ephrins and Eph receptor tyrosine kinases play important roles in a broad range of cell-cell recognition events, including axon pathfinding, early segmentation and organ morphogenesis, by modulating cell repulsive or adhesive signals through multiple downstream proteins, such as Ras/Rho, MAP kinase, Akt or FAK, that are crucial for intracellular signal transduction and cytoskeletal dynamics (Arvanitis and Davy, 2008; Himanen et al., 2007; Kullander and Klein, 2002). Eph receptor kinases mediate forward signaling in one cell while ephrin ligands transmit reverse signaling in the adjacent cell (Davy et al., 1999; Holland et al., 1996). The Eph family of receptor tyrosine kinases includes 16 members, divided into EphA...
Epha2 causing an increased stress response as reflected by elevated Hsp27 junctions (Cheng and Gong, 2011; Jun et al., 2009) as well as lens fiber cells associated with altered N-cadherin adhesion (Shiels et al., 2008; Sundaresan et al., 2012; Tan et al., 2011). In addition, non-synonymous SNPs of Zhang et al., 2009) and recessive (Kaul et al., 2010) cataracts. In significant changes in refractive index (Shi et al., 2012).

Epha2 signaling is essential for lens transparency. Mutations in EPHA2 cause human congenital dominant (Park et al., 2012; Zhang et al., 2009) and recessive (Kaul et al., 2010) cataracts. In addition, non-synonymous SNPs of EPHA2 have been linked to age-related cataracta in humans (Masoodi et al., 2012; Shiels et al., 2008; Sundaresan et al., 2012; Tan et al., 2011). In mice, the loss of EphA2 disrupts the structure and organization of lens fiber cells associated with altered N-cadherin adhesion junctions (Cheng and Gong, 2011; Jun et al., 2009) as well as causing an increased stress response as reflected by elevated Hsp27 (Kapl et al., 2004). Eph-ephrin bidirectional signals have emerged as a major cell-cell contact-dependent communication to coordinate not only developmental processes but also the normal physiology and homeostasis of mature organs. Altered Eph-ephrin function contributes to a variety of diseases (Pasquale, 2008).

Eph-ephrin signaling is essential for lens transparency. Mutations in EPHA2 cause human congenital dominant (Park et al., 2012; Zhang et al., 2009) and recessive (Kaul et al., 2010) cataracts. In addition, non-synonymous SNPs of EPHA2 have been linked to age-related cataracta in humans (Masoodi et al., 2012; Shiels et al., 2008; Sundaresan et al., 2012; Tan et al., 2011). In mice, the loss of EphA2 disrupts the structure and organization of lens fiber cells associated with altered N-cadherin adhesion junctions (Cheng and Gong, 2011; Jun et al., 2009) as well as causing an increased stress response as reflected by elevated Hsp27 (Kapl et al., 2004). Eph-ephrin bidirectional signals have emerged as a major cell-cell contact-dependent communication to coordinate not only developmental processes but also the normal physiology and homeostasis of mature organs. Altered Eph-ephrin function contributes to a variety of diseases (Pasquale, 2008).

In this study, we demonstrate that EphA2 is required for the formation of lens fulcrum and organized meridional rows of hexagonal, differentiating, lens equatorial epithelial cells. The loss of EphA2 results in the absence of the lens fulcrum, disorganized meridional rows, altered equatorial epithelial cell shape and, ultimately, disrupted alignment of lens fiber cells. EphA2 is essential for the activation and phosphorylation of Src and cortactin, which in turn recruit F-actin to the vertices of the equatorial hexagonal cells. This Eph/Src signaling cascade controls lens equatorial cell morphogenesis during development. A lack of EphA2 abolishes Src activation and other downstream regulation to disrupt the lens fulcrum and the alignment of newly differentiating hexagonal cells at the lens equator, which subsequently contributes to the disruption of lens refractive index and transparency.

**MATERIALS AND METHODS**

**Mice**

Mouse care and breeding were performed according to approved protocols (UC Berkeley Animal Care and Use Committee and Fred Hutchinson Cancer Research Center Institutional Animal Care and Use Committee) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. EphA2−/− mice were acquired from The Jackson Laboratory (strain B6; 006028). The EphA2−/− mice were genotyped according to the method provided by The Jackson Laboratory using a standard PCR method (Gong et al., 1997). Src−/− mice were generated as previously described (Soriano et al., 1999). Src+/− mice were intercrossed to generate Src−/− mice. Knockout mice were genotyped using three primers: Exon 2, 5′-AGCA-ACAAAGAACGAGCCCGGAGC-3′; Exon 2.3, 5′-GTTGACGCGTGCCC-GAGGAGTGGAA-3′; and 9603, 5′-TCAAGCCGACTAGCGCTTCCT-CCAC-3′. PCR was performed with the following program: 93°C for 3 minutes; then 40 cycles of 93°C for 35 seconds and 49°C for 40 seconds; and finally 65°C for 2 minutes. This PCR produced a 200 bp band from the Src WT allele and a 410 bp band from the Src knockout allele.

**Imaging of GFP-positive live lenses**

GFP-positive (GFP+) transgenic WT mice, in which GFP expression is under the chicken β-actin promoter (Okabe et al., 1997), were mated with EphA2−/− mice to generate GFP−/− knockout mice, which were screened as previously described (Cheng and Gong, 2011). Mutant and WT mice with one copy of the GFP transgene were used for image analysis. Fresh intact GFP lenses from postnatal day (P) 21 mice were dissected in DMEM without Phenol Red immediately before imaging. Images of lens epithelial and fiber cells with a mosaic GFP expression pattern were collected using a Zeiss LSM700 confocal microscope. Lenses were maintained in DMEM on the stage of the confocal microscope. z-stack images of the lens equator were collected with 1 μm z-steps. ZEN 2010 software (Zeiss) was used to analyze equatorial epithelial and fiber cells and create three-dimensional reconstructions.

**Immunohistochemistry**

Frozen lens sections from P14 mice were processed and collected as previously described (Gong et al., 1997) for immunostaining. Lens capsule flat-mounts from P21 mice were prepared using a previously described protocol (Cheng and Gong, 2011; Sugiyama et al., 2010). Anti-EphA2 (R&D Systems), anti-β-actin (Sigma-Aldrich), anti-E-cadherin (Invitrogen), anti-cortactin (Millipore), anti-cortactin-pY466 (Invitrogen) and anti-Src-pY416 (equivalent residue is Y424 for mouse; Cell Signaling) primary antibodies, appropriate fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories) and phalloidin-Rhodamine (Invitrogen) were used. Samples were mounted with DAPI VectorShield mounting medium (Vector Laboratories). Confocal and z-stack images were collected using a Zeiss LSM700 confocal microscope. Staining was repeated at least three times, and representative results are shown.

**Wheat germ agglutinin staining**

Rhodamine-conjugated wheat germ agglutinin (WGA; Vector Laboratories) was used to stain P21 whole fixed lenses for confocal imaging. WGA was previously shown to stain the plasma membranes of lens epithelial and fiber cells (Bond et al., 1996). Enucleated eyeballs with a small posterior opening were fixed in fresh 4% paraformaldehyde for 30 minutes on ice. Eyeballs were then briefly washed twice with cold 1× PBS and stored overnight in 1× PBS at room temperature before processing. Lenses were carefully dissected from fixed eyeballs and placed in blocking solution (3% BSA, 1× PBS at room temperature before processing. Lenses were carefully dissected from fixed eyeballs and placed in blocking solution (3% BSA, 1× normal goat serum, 0.3% Triton X-100) for 15 minutes at room temperature. Lenses were then placed in DAPI VectorShield mounting medium for 30 minutes at room temperature. After washing twice with 1× PBS, lenses were finally placed in a 1:10 dilution of WGA (in 1× PBS) for 30 minutes at room temperature. Lenses were washed again in 1× PBS twice before imaging on a Zeiss LSM700 confocal microscope as described above.

**Quantification of immunostaining signal intensity**

Confocal images of EphA2, β-actin, E-cadherin, cortactin, cortactin-pY466 and Src-pY424 staining in WT hexagonal equatorial epithelial cells were analyzed to compare the signal intensity at cell vertices versus the broad/short sides of the cells. Three separate staining samples for each antibody were evaluated. Each image was first exported in grayscale and then cropped to the same size. A heat map for each image was generated in ImageJ (NIH) using the HeatMap Histogram plug-in. Heat maps were pseudocolored between purple (0) and red (255) for signal intensity. A circular area (1.6 μm in diameter or 2.01 μm² in area) was marked at each vertex and along each side of a cell. Mean intensities at the vertices and on the broad and short sides of three individual cells were collected from each image. A total of nine cells were analyzed for each antibody, and mean intensities and standard deviation were calculated and plotted in Excel (Microsoft). Student’s t-test was used to determine significance (P<0.001).
RESULTS
EphA2 plays an important role in the formation of meridional rows at the lens equator
To elucidate the role of EphA2 in the lens, we first examined lens cell morphology in live GFP+ wild-type (WT) and Epha2−/− lenses using a laser confocal microscope. In the WT lens, equatorial epithelial cells with typical mosaic GFP expression became hexagonal and organized into meridional rows (Fig. 1A, arrowheads), and newly differentiating fiber cells were straight, uniform in width and aligned precisely (Fig. 1C). By contrast, the majority of Epha2−/− equatorial epithelial cells were not hexagonal and failed to form organized meridional rows (Fig. 1B, arrows). In addition, underlying fiber cells in the Epha2−/− lens were wavy, variable in width and misaligned (Fig. 1D). Three-dimensional reconstruction of z-stacks through the lens equator demonstrated, in both anterior-posterior and transverse views of lens bow regions, that normal elongated fiber cells were precisely overlaid in WT lenses (Fig. 1E,G). The lens fulcrum (Sugiyama et al., 2009) or modiolus (Zampighi et al., 2000) is a distinct region at the lens equator where the apical tips of elongating epithelial cells constrict to form an anchor point before fiber cell elongation in the WT lens (Fig. 1E, arrowhead). Epha2−/− lenses had an abnormal lens fulcrum (Fig. 1F, arrowheads) and irregularly shaped and disorganized fiber cells (Fig. 1H). Thus, a loss of EphA2 resulted in a disruption of the lens fulcrum (also see Figs 5-7) and a failure of equatorial epithelial cells to fully convert into hexagonal cells to form organized meridional rows.

EphA2 proteins accumulate at the vertices of hexagonal epithelial cells
To investigate the molecular basis for how EphA2 signaling controls epithelial cell shape and alignment at the lens equator, we examined EphA2 protein localization in these cells. Double immunostaining of the flat-mounted lens epithelium (attached to the lens capsule) revealed that both EphA2 and β-actin were enriched and colocalized at the vertices of the equatorial hexagonal epithelial cells that formed organized meridional rows in the WT sample (Fig. 2, arrowheads). By contrast, β-actin clusters were no longer restricted to the cell vertices and appeared at other points along the cell membrane in Epha2−/− equatorial epithelial cells (Fig. 2, arrows). E-cadherin was detected at cell-cell boundaries of WT hexagonal epithelial cells, but were not enriched at the cell vertices (Fig. 3A). However, we were unable to detect any obvious change in E-cadherin protein distribution in these equatorial epithelial cells between WT and Epha2−/− flat-mounted samples (Fig. 3A). In order to understand the mechanism for the alteration of β-actin clusters in the Epha2−/− lens, we characterized the localization of cortactin, a filamentous actin (F-actin)-binding protein. Cortactin specifically accumulated at the vertices of the hexagonal WT epithelial cells (Fig. 3B, arrowheads). Moreover, cortactin partially colocalized with EphA2 at cell vertices. Interestingly, similar to the change in β-actin staining, cortactin proteins accumulated in abnormal clusters on the membranes of Epha2−/− epithelial cells (Fig. 3B, arrows). Thus, cortactin is likely to play an important role in the regulation of F-actin needed to maintain equatorial epithelial cell shape and alignment.

Activation of cortactin and Src is EphA2 dependent
The function of cortactin is regulated by phosphorylation mediated by Src kinases (Knöll and Drescher, 2004). The activation of Src kinase is regulated by its upstream kinases, including EphA2 (Baldwin et al., 2006; Faoro et al., 2010; Parri et al., 2007). Thus, we examined the phosphorylated forms of cortactin and Src. Immunostaining revealed that cortactin phosphorylated at Y466 (cortactin-pY466) was enriched at the vertices of hexagonal WT
Epha2 to the irregular shape of E-cadherin, at cell vertices (supplementary material Fig. S1). Owing epithelial cells confirmed the enrichment of these proteins, except immunostaining signal distribution in WT hexagonal equatorial actin, E-cadherin, cortactin, cortactin-pY466 and Src-pY424 particles) in flat-mounted WT or antibodies alone did not produce any intracellular aggregates (or epithelial cells. However, cortactin-pY466 was not only absent from cell-cell boundaries, but also formed intracellular aggregates (or particles) in Epha2−/− equatorial epithelial cells (Fig. 4A). In addition, Src phosphorylated at Y424 (Src-pY424), which was also enriched at the vertices of WT hexagonal equatorial epithelial cells, was missing from cell-cell boundaries and formed intracellular aggregates (or particles) in Epha2−/− equatorial epithelial cells (Fig. 4B). Immunostaining with secondary antibodies alone did not produce any intracellular aggregates (or particles) in flat-mounted WT or Epha2−/− equatorial epithelial cells (data not shown).

Quantitative analysis of fluorescence intensities of EphA2, β-actin, E-cadherin, cortactin, cortactin-pY466 and Src-pY424 immunostaining signal distribution in WT hexagonal epithelial cells confirmed the enrichment of these proteins, except E-cadherin, at cell vertices (supplementary material Fig. S1). Owing to the irregular shape of Epha2−/− equatorial epithelial cells, it was difficult to accurately determine the location of cell vertices and to perform similar quantitative analysis on mutant samples. Thus, EphA2 probably regulates cell-to-cell alignment by spatially controlling the activation of Src and cortactin as well as F-actin recruitment to the vertices of lens equatorial epithelial cells. Other kinases probably mediate the activation of mislocalized intracellular Src and cortactin in Epha2−/− cells.

Immunostaining of frozen lens sections confirmed that EphA2, cortactin-pY466 and Src-pY424 proteins were restricted to the apical and basal sides of equatorial epithelial cells in WT lens sections (Fig. 5). However, cortactin-pY466 and Src-pY424 were almost absent from Epha2−/− lens sections (Fig. 5). Src-pY424 was also absent at the lens fulcrum in the Epha2−/− lens. EphA2 and Src-pY424, but not cortactin-pY466, were also localized and enriched at the lens fulcrum (Fig. 5, arrowheads). Sporadically distributed intracellular aggregates of cortactin-pY466 and Src-pY424, as observed in flat-mounted samples (Fig. 4), were not seen in lens sections (Fig. 5). If we look at transverse sections of the data collected in Fig. 4 (a similar view to frozen lens sections), it would be difficult to discern the sporadically distributed intracellular aggregates in Epha2−/− cells. In addition, flat-mount staining of the lens epithelial cells requires different fixation and sample processing than frozen lens sections.

Src has been shown to play a role in the downregulation of E-cadherin-mediated adhesion junctions for promoting the epithelial-to-mesenchymal transition (EMT) (Walker et al., 2007). Lens epithelial-to-fiber cell differentiation is not a typical EMT process, but shares similar cellular processes, such as cell rearrangement,
migration and elongation. Thus, we further examined the E-cadherin distribution near the lens equator. As expected, in the WT lens section E-cadherin proteins were located at both the apical and basal junctions of equatorial epithelial cells and accumulated at the lens fulcrum (Fig. 6, arrowheads), similar to Src-pY424 and EphA2 (Fig. 5, arrowheads), and disappeared in newly differentiating fiber cells. However, in the EphA2− lens section, E-cadherin accumulated at multiple areas in the apical, basal and lateral sides of equatorial epithelial cells without a normal lens fulcrum (Fig. 6, open arrowheads). In addition, the E-cadherin signal was sustained in newly differentiated EphA2− fiber cells (Fig. 6, arrows). Phalloidin-stained F-actin accumulated strongly at the apical interface and lens fulcrum in the WT lens section (Fig. 6, arrowhead in top middle panel), whereas the EphA2− lens section showed F-actin signals in multiple areas without a typical lens fulcrum (Fig. 6, open arrowheads in the bottom middle panel).

In order to determine the roles of Src in lens fulcrum and meridional row formation during lens equatorial cell morphogenesis, we examined Src− lenses labeled with Rhodamine-conjugated wheat germ agglutinin (WGA) and Src− lenses sections co-stained with phalloidin. The WGA-stained WT lens showed aligned meridional rows (Fig. 7A, left, arrows), whereas both EphA2− and Src− lenses revealed misaligned meridional cells (Fig. 7A, middle and right, arrows). The lens fulcrum was detected as a WGA-stained sharp boundary separating surface epithelial cells and underlying fibers in the WT lens (Fig. 7B, left, arrows). No obvious WGA-stained boundary or lens fulcrum was detected in EphA2− and Src− lenses (Fig. 7B, middle and right, arrows). Compared with a severe disruption of fiber-to-fiber organization in EphA2− lenses, a mild perturbation of the fiber-to-fiber organization occurred in Src− lenses (Fig. 7B, right, arrowheads). Phalloidin staining results confirmed a typical lens fulcrum in the bow region of the WT lens section (Fig. 7C, arrowhead), but no obvious lens fulcrum in EphA2− or Src− lens sections (Fig. 7C, open arrowheads). Thus, both EphA2 and Src play an essential role in the formation of the lens fulcrum and organized meridional rows.

**DISCUSSION**

This work reveals that EphA2 signaling and Src activation are essential parts of the signaling cascade that controls the formation of the lens fulcrum and the organization of meridional rows of hexagonal epithelial cells during lens equatorial cell morphogenesis (Fig. 8). EphA2/ephrin bidirectional signaling phosphorylates downstream molecules, including Src and cortactin, to regulate the dynamics of the actin cytoskeleton. The actin cytoskeleton controls cell shape, cell-to-cell interaction and/or migration, which are needed for the formation of organized meridional rows. Both EphA2− and Src− lenses show disrupted lens fulcruims and misaligned equatorial epithelial cells. Without EphA2, neither Src nor cortactin is phosphorylated (activated) at cell vertices, and altered actin filaments disrupt the formation of organized meridional rows and the hexagonal shape of EphA2− equatorial epithelial cells. Thus, EphA2 and Src are the key signaling molecules that control the morphogenesis and differentiation of lens equatorial cells during development.
The initiation mechanism for EphA2 accumulation at the vertices of equatorial epithelial cells remains unclear. The clustering of EphA is important for its kinase activity to regulate downstream targets (Groves and Kuriyan, 2010; Salaita et al., 2010; Xu et al., 2011). Presumably, clustering of EphA2 (or ephrin ligands) is one of the early or initiating events that trigger downstream signaling through Src and cortactin to recruit actin to the vertices of hexagonal cells in the meridional rows. EphA2 receptors are known to undergo clustering at the cell membrane by self-oligomerization or through interaction with ephrin ligands (Himanen et al., 2010; Salaita et al., 2010). The center of the EphA2 cluster has the greatest tyrosine phosphorylation and tightest cell adhesion to the substrate, which results in reorganization of the actin cytoskeleton. Further studies of ephrin ligands will be needed to elucidate the mechanism for EphA2 clustering in equatorial lens epithelial cells.

It is remarkable that both phosphorylated Src and phosphorylated cortactin are absent from Epha2−/− epithelial cells. Src activation mainly relies on EphA2 signaling at the lens equator. The data from Src−/− lenses confirmed that Src activation is essential for the formation of the lens fulcrum and for organization of the meridional rows, and Src also seems to be partly responsible for fiber cell organization. Src activation plays a key role in lens equatorial cell morphogenesis probably by regulating changes in the actin cytoskeleton. Src is known to participate in cell homeostasis and in a vast range of physiological functions including cell proliferation and survival, cell shape control, regulation of the cytoskeleton, maintenance of normal intercellular contacts, cell-matrix adhesion dynamics, motility and migration (Thomas and Brugge, 1997; Yeatman, 2004). Src mediates major signaling events from receptor tyrosine kinases (RTKs) and from adhesion receptors, including integrins and E-cadherin (Bjorge et al., 2000). RTKs and adhesion receptors can act synergistically to affect cell survival, proliferation, cytoskeleton reorganization and invasion, and these receptors can directly associate with signaling complexes in which Src is used as a common signaling molecule (Abram and Courtneidge, 2000; Huveneers and Danen, 2009; McLean et al., 2005).

Interestingly, this work suggests that Src activation is spatially and temporally controlled by EphA2 to regulate cell-to-cell alignment at the lens equator. It is known that EphA2 can directly phosphorylate Src in other cell types (Parri et al., 2007). It might be important to study other molecules that are involved in the restricted activation of Src and cortactin that establish organized meridional rows prior to fiber cell elongation and differentiation. It has recently been reported that Fyn, another member of the Src family, is utilized to regulate N-cadherin junctions in the chick lens fiber cell (Leonard et al., 2013). Since the disruption of Src−/− lens fiber cells is less severe than that of Epha2−/− lens fiber cells (Fig. 7B), EphA2 might also control other members of Src family, such as Fyn, to regulate...
and Menko, 2004). However, Src has not been reported to be directly associated with human cataracts. Future studies will be necessary to understand the involvement of the Src family of proteins in human lens disorders.

Contactin phosphorylation depends on the presence of EphA2 in equatorial epithelial cells packed into organized meridional rows. Previous work has suggested that contactin phosphorylation promotes cell migration. Contactin associates with the F-actin cytoskeleton through the F-actin-binding tandem contactin repeats and the N-terminal acidic domain that interacts with the actin-related protein (Arp) 2/3 complex for dendritic actin nucleation (Kelley et al., 2010; May, 2001). The C-terminus of contactin interacts with FAK at focal adhesions and may be phosphorylated by the FAK-Src complex (Tomar et al., 2012; Wang et al., 2011). The tyrosine phosphorylation of contactin by the FAK-Src complex reduces its interaction with FAK, most likely to increase its turnover at focal adhesions to promote cell motility in other cell types. A similar mechanism might govern cell movement and restructuring as equatorial epithelial cells undergo cell shape changes to align into meridional rows. However, the changes in contactin could be either the cause or effect of altered organization in EphA2+/− equatorial epithelial cells.

It has been suggested that the Eph-ephrin bidirectional signaling pathway and epithelial cell junction molecules work closely together to regulate epithelial cell integrity or to accomplish cell sorting processes during epithelial cell proliferation or differentiation (Miao and Wang, 2009). The activation of EphA2 changes the expression of E-cadherin and reduces cell-cell or cell-matrix adhesion (Zantek et al., 1999), and the upregulation or downregulation of E-cadherin can also alter the expression of Eph receptors or ephrin ligands (Orsulic and Kemler, 2000). Ephs and ephrins have dual roles in facilitating cell sorting into opposing compartments by shutting down intercompartmental communication through gap junctions (Mellitzer et al., 1999). Our data suggest that EphA2-dependent activation of Src and contactin is likely to provide key signaling that recruits actin filaments to form basal-lateral cell vertices for establishing the hexagonal cell shape and for controlling the precise organization of epithelial cells into meridional rows. However, at the lens fulcrum, EphA2-dependent Src activation initiates the accumulation of E-cadherin and F-actin without the phosphorylation of contactin. Presumably, a different F-actin-binding protein is involved at the lens fulcrum. A loss of EphA2 prevents Src-Y424 activation and cortactin-Y466 phosphorylation, which results in alterations in F-actin and E-cadherin protein distribution, leading to changes in basal-lateral cell shape and cell-cell alignment in the transition from epithelial to fiber cells at the lens equator.

Our results suggest that EphA2 and Src control lens fulcrum formation at the apical ends of equatorial epithelial cells as well as cortactin/F-actin complex localization at the basal-lateral vertices of these hexagonal cells at lens equator. The activation of Src at the apical tips (lens fulcrum) and at the vertices of hexagonal equatorial epithelial cells is solely controlled by EphA2. Src activation is important for the formation of the lens fulcrum and the organization of meridional rows (cell-to-cell alignment) at the lens equator. Src−/− lenses do not have obvious opacities, and no defects are apparent in Src−/−/− mice. Interestingly, in contrast to EphA2−/− lenses that have normal lens weight but severely disrupted fiber cell organization, Src−/− lenses
show a ~15% reduction in lens weight with mild fiber cell alterations (data not shown). The phenotypic variation between EphA2−/− and Src−/− lenses suggests that other RTKs are likely to control Src activation to regulate the proliferation of lens epithelial cells and lens size; other members of the Src family, such as Fyn, may act as downstream targets of EphA2/ephrin signaling in the regulation of lens fiber cell organization by affecting N-cadherin localization (Jun et al., 2009; Leonard et al., 2013). This work is consistent with a recent study revealing disorganized lens cells and altered lens refractive index in EphA2−/− lenses (Shi et al., 2012). Dysfunction of EphA2 due to mutations and other insults is likely to perturb lens equatorial cell morphogenesis, alters the organization and interaction among lens fiber cells, causes abnormal refractive index and leads to both congenital and age-related cataracts in humans and mice (Jun et al., 2009; Kaul et al., 2010; Park et al., 2012; Shiels et al., 2008; Sundaresan et al., 2012; Tan et al., 2011; Zhang et al., 2009).

Acknowledgements
We thank Dr Chun-hong Xia for her critical reading of this manuscript.

Funding
This work was supported by grants from the National Institutes of Health [R01-EY013849 and R01-EY021519 to X.G.; R01-CA41072 and R01-NS080194 to J.A.C.]. Deposited in PMC for release after 12 months.

Competing interests statement
The authors declare no competing financial interests.

Author contributions
C.C. and X.G. designed experiments, interpreted data and wrote the manuscript. C.C. performed experiments. M.M.A. and J.A.C provided and processed Src−/− samples. J.A.C. interpreted data and edited the manuscript.

Supplementary material
Supplementary material available online at http://dev.biologists.org/lookup/suppl?doi:10.1242/dev.100727/-/DC1

References


Fig. 8. Model for EphA2 functions in the organization of hexagonal equatorial epithelial cells into meridional rows. Hexagonal equatorial epithelial cells are packed into organized meridional rows at the lens fulcrum. On the basal-lateral sides of equatorial epithelial cells, EphA2 is likely to interact with an ephrin ligand to cluster at the vertices of the hexagonal cells. EphA2 phosphorylates Src, which consequently activates cortactin to trigger actin to cluster at cell vertices. This signaling cascade and cell adhesion events lead to the organization of equatorial epithelial cells into meridional rows, which is probably a prerequisite for fiber cell organization. On the apical side of equatorial epithelial cells, EphA2 clustering phosphorylates and activates Src to recruit E-cadherin and actin to form the lens fulcrum, which serves as an anchor point for the transition from epithelial cells to fiber cells.


