AKAP2 anchors PKA with aquaporin-0 to support ocular lens transparency

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A decline in ocular lens transparency known as cataract afflicts 90% of individuals by the age 70. Chronic deterioration of lens tissue occurs as a pathophysiological consequence of defective water and nutrient circulation through channel and transporter proteins. A key component is the aquaporin-0 (AQP0) water channel whose permeability is tightly regulated in healthy lenses. Using a variety of cellular and biochemical approaches we have discovered that products of the A-kinase anchoring protein 2 gene (AKAP2/AKAP-KL) form a stable complex with AQP0 to sequester protein kinase A (PKA) with the channel. This permits PKA phosphorylation of serine 235 within a calmodulin (CaM)-binding domain of AQP0. The additional negative charge introduced by phosphoserine 235 perturbs electrostatic interactions between AQP0 and CaM to favour water influx through the channel. In isolated mouse lenses, displacement of PKA from the AKAP2–AQP0 channel complex promotes cortical cataracts as characterized by severe opacities and cellular damage. Thus, anchored PKA modulation of AQP0 is a homeostatic mechanism that must be physically intact to preserve lens transparency.

INTRODUCTION

Approximately 20 million people worldwide suffer from severely reduced vision caused by clouding of the ocular lens known as cataract (Brian & Taylor, 2001). Understanding the environmental and genetic risk factors for cataract is therefore a major global health challenge, which is being aided by basic research of lens physiology. The ocular lens is adapted for optimal focusing of light on the retina. It lacks a conventional vascular system, and its mature fibre cells contain no organelles, which enables minimal light scattering. To accommodate this unusual physiology, the lens has developed an internal circulation system based upon an array of membrane channel and transporter proteins (Mathias et al, 2007). Foremost amongst these is the water channel aquaporin-0 (AQP0), also known as major intrinsic polypeptide (MIP), which constitutes more than 60% of the total membrane protein content of fibre cells (Bloemendal et al, 1972). Consistent with its critical role in the lens, mutation or malfunction of AQP0 causes a loss of lens transparency known as cataract (Berry et al, 2000; Francis et al, 2000; Gu et al, 2007).

Aquaporins form channels for water permeation across biological membranes by virtue of 6-transmembrane helices that assemble a hydrophilic water-conducting pore (Gonen & Walz, 2006). AQP0 serves as a prototype to understand how water channels are regulated at the molecular level (Gonen & Walz, 2006). Three mechanisms reduce AQP0 water permeability. Water channels close when AQP0 tetramers from opposing cells adhere to one another to form adhesive junctions (Gonen et al, 2004). Upon elevation of pH, the de-protonation of His40 and His66 at either end of the pore reduces water flow (Gonen et al, 2004; Nemeth-Cahalan et al, 2004). Binding of
Ca\textsuperscript{2+}/CaM to a C-terminal cytoplasmic region between residues 225 and 263 of AQP0 decreases water permeability (Varadaraj et al, 2005). Conversely, phosphorylation of AQP0 (Ball et al, 2004) within this regulatory region opens the water pore (Reichow & Gonen, 2008; Rose et al, 2008). However, the intracellular signals and effector molecules that sustain this pore (Reichow & Gonen, 2008; Rose et al, 2008) within this regulatory region opens the water channel for water transport. Conversely, phosphorylation of AQP0 residues 225 and 263 of AQP0 decreases water permeability of cortical lens cataracts upon incubation of lenses with a PKA activator (Varadaraj et al, 2005). AKAP2 is the principal A-kinase anchoring protein in the lens (Carr et al, 1992; Kinderman et al, 2006). We reasoned that such an anchoring protein might be a component of the AKAP2 complex, so in this study we have explored the role of PKA and AKAPs in regulating AQP0. Biochemical and immunohistochemical experiments show that A-kinase anchoring protein 2 (AKAP2) interacts directly with the C-terminal domain of AQP0 on membranes of outer cortical lens fibre cells. AKAP2 thus anchors PKA in proximity to AQP0, enabling phosphorylation of the water channel at Ser235. The importance of AKAP2 and PKA to AQP0 regulation is underlined by the development of the water channel at Ser235. AKAP2 anchors PKA at cell membranes in the lens outer cortex.

AKAP2 anchors PKA at cell membranes in the lens outer cortex
The architecture of the mammalian lens is illustrated in Fig 2A. AQP0 is concentrated on the membranes of tightly packed hexagonal fibre cells, where it sustains fluid microcirculation in the absence of a conventional vascular system (Mathias et al, 2007). Immunoblotting of sheep lens fractions demonstrated that AKAP2 is present in the lens cortex (Fig 2B, top panel, lane 3) but is not detected in the lens core (Fig 2B, top panel; lane 2). In contrast, AQP0 is detected in both core and cortical fractions (Fig 2B, bottom panel; lanes 2 and 3). Subcellular fractionation revealed that AKAP2 and AQP0 partition with cortical membranes (Fig 2B, bottom lane). In addition, 1 M NaCl released AKAP2 into the supernatant fraction (Fig 2C, top panel, lane 4), implying that AKAP2 is peripherally attached to cortical membranes via ionic interactions, whereas, AQP0 is an integral membrane protein. Analysis by RII overlay gave consistent results (Fig S1 of Supporting Information).

Immunohistochemical analyses of fixed mouse lenses extend these findings. In axial sections, AKAP2 is detected in the epithelium and outer cortical fibre cells (Fig 2D and F). Its signal is strongest at sites proximal to the equator, as delineated by an inwardly migrating band of nuclei (Fig 2E and F). Equatorial lens sections imaged at higher magnification revealed that AKAP2 predominates at the plasma membrane in the outer cortex (Fig 2G and J), as indicated by a striated pattern that overlaps with the membrane marker wheat germ agglutinin (WGA; Fig 2H and J). Furthermore, the AKAP2 signal diminishes ~100 μm from the lens surface at the transition zone between the cortex and the core, as demarked by the bow region where lens fibre differentiation begins (Fig 2G and I). At 100× magnification of lens equatorial sections a punctate staining pattern for AKAP2 is discernible (Fig 2K and M) that overlaps with fibre cell membranes (Fig 2L and M). Related studies show that the RII and C subunits of PKA are also present at the same location (Fig S2 of Supporting Information).

AKAP2 binds directly to the C-terminal tail of AQP0
AKAPs often sequester their anchored enzymes with preferred substrates (Smith et al, 2010). Accordingly, AQP0 was enriched in anti-AKAP2 immune complexes (Fig 3A bottom panel, lane 3). Immunofluorescence staining demonstrated a co-distribution of both proteins at the membranes of outer cortical lens fibre cells (Fig 3B–D and Fig S3 of Supporting Information). To identify amino acid determinants that participate in this protein–protein interaction, a series of immobilized AQP0 peptides (20-mers in 2 amino acid increments) that encompass the cytoplasmic tail of the channel were incubated with purified recombinant AKAP2 (Fig 3E and F). Equivalent amounts of each immobilized peptide were confirmed by detection with
ultraviolet light (Fig 3F). AKAP2 bound to peptides 3–8 as determined by immunoblotting (Fig 3F). In separate studies, peptide 7, which corresponds to residues 237–256 of AQP0, was used to release native AKAP2 from association with the water channel in solubilized lens homogenates (Fig 3G, lower panel, lane 3), suggesting that a core sequence within this segment of the AQP0 regulatory domain is both necessary and sufficient for AKAP2 binding. A control peptide of identical amino acid composition but scrambled sequence did not prevent co-purification of the AKAP2–AQP0 complex (Fig 3G, lower panel, lane 4). Systematic alanine scanning of the AQP0 237–256 sequence (LKGTRPSESNGQPEVTGEPV) revealed that replacement of Arg241 with alanine eliminated AKAP2 binding, and substitution of the surrounding residues Lys238, Thr240, Glu244 or Asn246 reduced AKAP2 association with the immobilized peptide (Fig 3H). Collectively these biochemical and SPOT array experiments indicate that AKAP2 binds directly to the C-terminus of AQP0, in a manner that involves binding determinants between residues 238 and 246 of the channel.
Figure 2. Lens distribution of AKAP2.

A. Schematic of lens cellular architecture. Hexagonal fibre cells are visible in equatorial sections; migrating nuclei at the equator are depicted in axial sections. Epithelial cells are continually generated at the anterior surface and migrate into the outer cortex of the lens at the equator, where they differentiate to fibre cells. Fibre cells develop an elongated hexagonal shape that facilitates packing of neighbouring cells into a lattice. As new fibre cells are added to the cortex, older fibre cells become buried into the core of the lens, where they are terminally differentiated—with losing their nuclei and other organelles.

B. Sheep lens homogenate (lane 1) was separated into core (lane 2) and cortex (lane 3). The cortex was subfractionated into membrane (lane 4) and supernatant (lane 5).

C. Cortical membranes were incubated with LLB containing either 0 M NaCl (lanes 1 and 2) or 1 M NaCl (lanes 3 and 4) before subfractionation into membrane (lanes 1 and 3) and supernatant (lanes 2 and 4) fractions. (B and C) Immunoblotting against AKAP2 (upper panel) and AQP0 (lower panel) is shown for each fraction.

D–F. 10× magnification imaging of axial sections of mouse lens stained with anti-AKAP2 antibody (D, red in F) and the nuclear marker DRAQ5 (E, blue in F). Three separate images were aligned to cover the lens from the anterior pole to the equator.

G–J. Immunofluorescence (40×) images of equatorial lens sections are shown following staining with anti-AKAP2 antibody (G, red in J), membrane marker WGA (H, green in J) and DRAQ5 (I, blue in J).

K–M. High-magnification images of equatorial sections of outer lens cortex are shown following staining with anti-AKAP2 antibody (K, red in M), WGA (L, green in M) and DRAQ5 (blue in L).
PKA prevents AQP0–CaM association through Ser235 phosphorylation

Our mapping studies suggest that AKAP2 could position PKA in a way that favours phosphorylation of AQP0. In keeping with this postulate, the C-subunit of PKA efficiently phosphorylated purified AQP0 in a PKI-sensitive manner (Fig 4A top panel, lanes 2 and 3). MS/MS sequencing of Lys-C-digested AQP0 identified a conserved PKA phosphorylation site at Ser235 in a peptide
Figure 4. Structural consequence of PKA phosphorylation in AQP0.

A. Autoradiograph (top panel) showing $^{32}$P incorporation into purified AQP0 (anti-AQP0 IB, lower panel) following incubation with $^{32}$P-$\gamma$ATP $+$ $/$ $-$ PKA C subunit and $+$ $/$ $-$ PKI.

B. Product ion scan of SVSERpSILK peptide. Neutral loss of $\text{H}_3\text{PO}_4$ is strong. A series of $\gamma-\text{H}_3\text{PO}_4$ and $\beta-\text{H}_3\text{PO}_4$ ions were observed enabling determination of the phosphorylation site location at Ser235.

C. Normalized intensity of SVSERpSILK peptide with retention time during LC following incubation of AQP0 with PKA (red), without PKA (black) and with both PKA $+$ PKI (blue).

D. Autoradiograph (right column) following PKA phosphorylation of arrayed AQP0 233–252 peptides with $^{32}$P-$\gamma$ATP. Equal spotting efficiency is demonstrated by UV illuminescence. Each serine and threonine residue was mutated to alanine as indicated.

E. Structural representation using the coordinates of AQP0 (Gonen et al, 2004). Inset: location of the PKA phosphorylation site, AKAP2 binding domain (green) and helical segment (yellow) in the C-terminal domain of AQP0 (PDB ID 2B6P; Gonen et al, 2004).

F. Native gel electrophoresis of CaM alone (lane 1) or in the presence of unphosphorylated (lane 2) or Ser235-phosphorylated (lane 3) AQP0 223–242 peptide.

G. Electrostatic surface representation of the AQP0 C-terminal helix situated between residues 227 and 239 in its unphosphorylated (left) and Ser235-phosphorylated (right) states.

H. Electrostatic surface of the CaM binding cleft.

I. Location of the PKA recognition motif for Ser235 in WT and R233K mutant (Gu et al, 2007) AQP0.

J. Phosphorylation of WT (lanes 1 and 2) and R233K mutant (lanes 3 and 4) AQP0 (223–242) peptides with $^{32}$P-ATP was attempted in solution with PKA in the presence (lanes 2 and 4) and absence (lanes 1 and 3) of PKI.
corresponding to residues 229–238 of the channel (Fig 4B and Fig S4 of Supporting Information). Quantitative analysis of the SVSRLpSILK phosphopeptide revealed that Ser235 phosphorylation was increased fivefold in the presence of PKA over untreated AQP0 (Fig 4C). Incubation with PKI diminished this enhancement threefold (Fig 4C). MS detected no other phosphorylation sites in the SVSRLpSILK peptide, and follow-up peptide array experiments confirmed that substitution of Ser235 with alanine prevented PKA phosphorylation of AQP0 233→252 peptide (Fig 4D). Furthermore, sequential elimination of other serine or threonine residues within this region had no qualitative effect on phosphate incorporation (Fig 4D).

Inspection of the AQP0 crystal structure (Gonen et al, 2005) reveals that Ser235 is located at the centre of the CaM binding site on AQP0, in a helical segment within ~20 Å of AKAP2 binding determinants (Fig 4E). Band-shift assays using native gel electrophoresis indicate that CaM is capable of binding to AQP0 223→242 (Fig 4F, lane 2), but that Ser235 phosphorylation prevents this interaction (Fig 4F, lane 3). Surface charge analysis reveals complementarities between the charges presented by the AQP0 peptide (Fig 4G) and the reciprocal binding cleft of CaM (Fig 4H). Phosphorylation at Ser235 introduces negative charge at the centre of the AQP0 CaM-binding interface (Fig 4G). Therefore, it is likely that repulsion between like negative electrostatic charges is the basis of decreased CaM binding following PKA phosphorylation at AQP0 residue Ser235. This provides a mechanistic explanation as to why phosphorylation of this region favours the displacement of CaM from AQP0 to open the water pore (Kalman et al, 2008; Reichow & Gonen, 2008; Rose et al, 2008). Independent experiments showed that PKA efficiently phosphorylated an AQP0 peptide bearing Ser235 in solution (Fig 4I and J). Basophilic protein kinases such as PKA recognize arginine or lysine side chains at the phosphorylation site on A-Kinase anchoring proteins. How-

Disruption of A-Kinase anchoring generates outer cortical cataract

Lens transparency is preserved by the constant flow of water carrying it ions, second messengers and metabolites (Mathias et al, 2007). It has been proposed that circular diffusion patterns are driven by an ionic current that is directed inward at the poles and outward at the equator of the lens and supported by water influx through AQP0 (Donaldson et al, 2010). An alternate model is that water passage through AQP0 gap junctions is sufficient to provide the necessary microcirculation in the lens (Beebe & Truscott, 2010). Irrespective of which fluid circulation model prevails, the phosphorylation of this channel is vital for regulating the rate of water influx. To assess the physiological importance of AKAP–PKA anchoring and AQP0 phosphorylation in maintaining lens transparency, we utilized a cell-permeable disruptor peptide of PKA anchoring (Ht31; Vijayaraghavan et al, 1997), to release the kinase from association with AQP0 in vivo. Mouse lenses were incubated in artificial aqueous humour (AAH) in the presence or absence of 10 μM Ht31 (Fig 5A and B). Following 72 h incubation in AAH alone, lens transparency was maintained throughout the lens (Fig 5A). Addition of Ht31 over the same time period led to cataract development in the lens cortex but not the lens core (Fig 5B). Changes in lens opacity were quantified by densitometry (Fig 5C) confirming that displacement of PKA increased development of cortical lens cataracts (p < 0.001, n = 8). The development of cortical cataracts following disruption of PKA–AKAP interactions is consistent with the cumulative data presented in Figs 1–4.

Immunofluorescence staining of lens sections revealed that the tight hexagonal packing of fibre cells was preserved in control lenses, as shown by membrane staining at low (Fig 5D) and high magnification (Fig 5E and G). RII remained associated with the cell membrane (Fig 5F and G), suggesting that AKAP2-mediated PKA anchoring was preserved under control conditions. In contrast, fibre cell packing in the outer cortex appeared disordered following incubation with Ht31 for 72 h as detected by WGA staining of membranes (Fig 5H). At higher magnification, the catastrophic effects of displacing PKA were more apparent (Fig S1–K). Many fibre cells are enlarged in a manner that is consistent with intracellular swelling. Moreover, extracellular gaps developed that are indicative of extracellular swelling and tissue architecture breakdown. Additionally, membrane aggregates are present from ruptured cells. Importantly, RII was no longer concentrated at the periphery of fibre cells close to membranes upon treatment with Ht31 (Fig 5I and K). Collectively, the data in Fig 5 argue that the pathological effects on fibre cell swelling and cataract formation may be a consequence of PKA holoenzyme release from AKAP2 in the equatorial cortex of the lens. Accordingly, fluid microcirculation would be seriously interrupted with deleterious effects on lens morphology.

DISCUSSION

We show that AKAP2 is a water-channel-associated anchoring protein that restricts PKA within the immediate vicinity of AQP0 in the equatorial lens cortex. This could likely direct phosphorylation within a regulatory region of AQP0 to modulate water permeability. These findings ascribe the first directly demonstrated function for AKAP2 anchoring proteins. However, a broader implication is that we may have uncovered an unanticipated mechanism for anchored PKA. Our model proposes that anchored PKA may participate in the phosphorylation dependent activation of AQP0 to preserve fluid circulation within the lens. This postulate is substantiated by evidence presented in Fig 5 showing that perturbation of PKA anchoring evokes a pathophysiological state that accentuates the development of cortical cataracts. The involvement of this kinase can now be inferred from pathological mutations in the AQP0 gene (Francis et al, 2000; Gu et al, 2007; Lin et al, 2007). Of
particular interest is an autosomal dominant cataractogenic mutation that encodes an arginine to lysine substitution at position 233 of AQP0. This corresponds to the P-2 position of a PKA phosphorylation site at Ser235 (Zetterqvist & Ragnarsson, 1982). Individuals carrying this mutation exhibit fibre cell swelling, severe light scattering and the early onset of cataracts (Lin et al., 2007). At first glance it may seem surprising that a conservative arginine to lysine mutation reduces phosphorylation of Ser235 to this extent. However, PKA phosphorylation of an AQP0 peptide (residues 223–242) harbouring this R233K mutation was reduced by 5.8-fold (n = 3, p < 0.001) when compared to the WT sequence (Fig 4H and I). On the basis of these observations we can postulate that mutations that prevent the phosphorylation of Ser 235 favour CaM binding to sustain the closed conformation of the water channel. Hence, we propose that the phosphorylation state of Ser235 has profound effects on AQP0 water permeability.

Two CaM molecules bind per AQP0 tetramer in a cooperative and calcium dependent manner to reduce water conductance (Reichow & Gonen, 2008). Calmodulin (CaM) binds a helical segment located between residues 223 and 242 of each AQP0 protomer. On the basis of our biochemical and cellular data we propose that PKA phosphorylation of Ser235 compromises the electrostatic AQP0–CaM interface to favour the active open state of the water pore. Presumably, the added charge and bulk of a phosphate group at position 235 as depicted in Fig 4G perturbs CaM binding to the regulatory domain of the channel. This allows us to put forward an intriguing new mechanism, whereby, cAMP/PKA phosphorylation must override inhibitory Ca2+/CaM action to permit water conductance through AQP0. One implication of this concept is that bidirectional control of Ser235 phosphorylation would require that the AQP0–AKAP2–PKA complex both has access to cAMP (Alvarez et al., 2003; Goldberg et al., 2004), which can diffuse through gap junctions (Alvarez et al., 2003; Goldberg et al., 2004), and is targeted in proximity to phosphatases. By analogy to other AKAP signalling complexes (Dodge et al., 2001; Dodge-Kafka et al., 2005; Klauck et al., 1996; Tasken et al., 2001), signal termination enzymes for second messenger action may also be binding partners for AKAP2. Although we have not yet investigated the components, complexity or the entire spectrum of AKAP2 binding partners in the lens, it seems reasonable that phosphodiesterases or phosphatases could be recruited to the AKAP2–AQP0 network. In sum, our study charts how a single anchored PKA phosphorylation event that subtly changes the conformation of a key lens membrane protein can be magnified leading to

Figure 5. Mislocalization of PKA adversely affects lens transparency.
A–B. Mouse lenses were imaged by dark-field light microscopy before and after 72 h incubation in control conditions (A), and in the presence of 10 μM Ht31 (B).
C. Quantification of regional changes in lens opacity following 72 h incubations (n = 8).
D–G. Images of equatorial sections of lens cortex were collected following 72 h incubation in control conditions. A low-magnification image is shown following staining with WGA (D). High magnification images are shown following staining with WGA (E, green in G) and anti-RII antibody (F, red in G).
H–K. Equivalent sections are shown following treatment in the presence of 10 μM Ht31. A low magnification image is shown following WGA staining (H). High magnification images are shown following staining with WGA (I, green in K) and anti-RII antibody (J, red in K).
major pathological effects at the cellular and tissue levels. Interestingly, the water channel AQP0 is only one of several lens proteins that may come under the control of anchored PKA. For example, gap junctions are likewise vital for the circulation system and they are also phosphorylated (Mathias et al., 2010). Conversely, contributing factors to cataract formation in other regions of the lens may include uncontrolled association of crystallins and the formation of high-molecular weight aggregates at the membrane (Mathias et al., 2007). Thus, defects in the PKA–AKAP2–AQP0 axis reported in this article may only represent one of several pathophysiological mechanisms that augment cataract formation.

MATERIALS AND METHODS

Lens fractionation
Lens were dissected from sheep eyes obtained from the Wolverine Packers slaughterhouse (Detroit, MI). Lenses were decapsulated and fibre cells were homogenized in lens lysis buffer (LLB; 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 1 mM AEBSF and 1 μM Pepstatin A). The soft cortical tissue was separated from the dense core tissue using a surgical blade. Fibre cell membranes were fractionated from the soluble lysate by ultra-centrifugation at 50,000 g for 30 min. Protein concentrations of lens extracts were determined by BCA (Pierce).

AKAP identification
RII overlay was performed as described previously (Carr et al., 1992). The D/D domain of PKA RII (residues 1–45) was cloned into pGEX6P1 enabling expression of GST-D/D with a PreScission protease cleavage site between GST and D/D. Both GST and GST-D/D were expressed in Escherichia coli (E coli) BL21 cells and lysed in 25 mM Tris pH 7.5, 500 mM NaCl, 2 mM DTT, 0.5 mM EDTA and 0.1 μl lysozyme prior to purification by affinity to glutathione sepharose (GE Healthcare). Lens lysates were prepared for D/D pulldown experiments by homogenizing sheep lenses in LLB. Lens homogenates were solubilized by supplementing LLB with 1% Triton X-100, followed by sonication for 5 s and centrifugation at 14,000 x g for 30 min. Ten millilitres of the resulting supernatant containing 50 mg/ml protein was incubated for 2 h with 200 μl glutathione sepharose beads loaded with 300 μg of either GST or GST-D/D. After 5 x 1 ml washes with LLB with 1% Triton X-100, the beads were incubated overnight with 2 μg PreScission protease (GE Healthcare) in 100 μl LLB with 1% Triton X-100. Eluted proteins were separated by SDS–PAGE, Coomassie-stained and the region of the gel containing proteins of apparent molecular weight between 100 and 130 kDa were excised. Proteins were in-gel digested with trypsin, and the resulting peptides were sequenced by Linear Trap Quadrupole Fourier Transform MS. Proteins were in-gel digested with trypsin, and the resulting peptides were sequenced by Linear Trap Quadrupole Fourier Transform MS. Proteins were in-gel digested with trypsin, and the resulting peptides were sequenced by Linear Trap Quadrupole Fourier Transform MS. Proteins were in-gel digested with trypsin, and the resulting peptides were sequenced by Linear Trap Quadrupole Fourier Transform MS. Proteins were in-gel digested with trypsin, and the resulting peptides were sequenced by Linear Trap Quadrupole Fourier Transform MS.
**The paper explained**

**PROBLEM:**
Cataract, or clouding of the ocular lens, is the leading cause of blindness worldwide. Deterioration of lens tissue can occur as a consequence of defective water and nutrient circulation through channel and transporter proteins. A key element of the lens circulatory system is the aquaporin-0 (AQP0) water channel. AQP0 permeability is upregulated by phosphorylation at Ser235 but the intracellular signals and effector molecules that sustain this channel activation event had not previously been identified.

**RESULTS:**
Protein products of the A-kinase anchoring protein 2 gene (AKAP2/AKAP-KL) bind directly to AQP0 to bring protein kinase A (PKA) into proximity with the water channel. This enables PKA phosphorylation of Ser235 within a calmodulin (CaM)-binding domain of AQP0. The additional negative charge introduced by phosphoserine 235 disrupts electrostatic interactions between AQP0 and CaM thus favouring water influx through the channel. In cultured intact lenses isolated from mice, displacement of PKA from the AKAP2–AQP0 channel complex with a peptide disruptor promotes cortical cataracts as characterized by severe opacities and cellular damage.

**IMPACT:**
The major implication of this study is that we have uncovered an unanticipated mechanism for anchored PKA in the preservation of fluid circulation within the lens. This notion is consistent with a previously identified cataractogenic mutation in AQP0 that alters the PKA recognition motif for Ser235 phosphorylation. Thus, anchored PKA modulation of water channels in the lens may be an important homeostatic mechanism that maintains lens transparency.
by normalizing the intensity of the SVSERLpSILK phosphopeptide to that of the total ion chromatogram. Extracted ion chromatograms for this phosphopeptide showed excellent agreement and reproducibility in observed retention times, with peaks from all three runs appearing within 30 s (<1% retention time shift) of one another.

Native gel electrophoresis
6His-CaM was purified by affinity to Ni-NTA resin (Qiagen) and Superdex-75 (GE Healthcare) size exclusion chromatography following pET vector-based expression in E. coli. Native gel electrophoresis was performed with 5 mg/ml CaM in 50 mM Tris pH 8.0, 5 mM CaCl2, 0.5 mM MgCl2, 5 mM glucose, 20 mM sucrose supplemented for 30 min with 1 mg/ml AQP0 223–242 peptides (Anaspec), as appropriate. Protein mobility was imaged by silver stain.

Structural analysis
The coordinates of full-length AQP0 (PDB ID 2B6P; Gonen et al, 2005) and CaM (PDB ID 1CDM; Meador et al, 1993) were analysed. AQP0 residue Ser235 was modelled as phosphoserine to investigate the effect of PKA phosphorylation on the electrostatic surface. Electrostatic surface potentials were calculated and presented using Chimera (Pettersen et al, 2004).

Cataract formation assays
Lenses were dissected from 8- to 10-week-old WT C57BL/6 mice and incubated at 37°C in AAH (125 mM NaCl, 4.5 mM KCl, 10 mM NaHCO3, 2 mM CaCl2, 0.5 mM MgCl2, 5 mM glucose, 20 mM sucrose and 1% penicillin/streptomycin). Lenses that developed cataracts after 6 h in AAH were discarded (~30%). Remaining lenses were incubated with either AAH or AAH supplemented with 10 μM stearated Ht31 (Promega). Lens transparency following 72 h incubations was assessed by dark-field light microscopy. Changes in lens opacity were quantified using ImageJ software (NIH). For cortical opacity, white level intensities (range 1–255) were recorded in 54-pixel boxes evenly spaced around the circumference of each lens at a depth of 100 μm from the surface. The six intensities were averaged and a background intensity, taken from outside of the lens, was subtracted. For core opacity a single measurement was taken at the centre of each lens from which the background intensity was subtracted.

Statistical analysis
All data are presented as the mean ± standard error. Statistical analysis was determined using the unpaired Student t-test. A p-value of less than 0.05 was considered statistically significant. *p < 0.05; **p < 0.01; ***p < 0.001.

Author contributions
MG performed experiments, provided funding, analysed data and wrote the manuscript; SR performed experiments, analysed data and wrote the manuscript; SO performed experiments and analysed data; LL analysed data and wrote the manuscript; JB provided funding and analysed the data; TG provided funding, analysed the data and wrote the manuscript; JS provided funding, analysed data and wrote the manuscript.

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