Functions of crystallins in and out of lens: *Roles in elongated and post-mitotic cells*

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Abstract

The vertebrate lens evolved to collect light and focus it onto the retina. In development, the lens grows through massive elongation of epithelial cells possibly recapitulating the evolutionary origins of the lens. The refractive index of the lens is largely dependent on high concentrations of soluble proteins called crystallins. All vertebrate lenses share a common set of crystallins from two superfamilies (although other lineage specific crystallins exist). The α-crystallins are small heat shock proteins while the β- and γ-crystallins belong to a superfamily that contains structural proteins of uncertain function. The crystallins are expressed at very high levels in lens but are also found at lower levels in other cells, particularly in retina and brain. All these proteins have plausible connections to maintenance of cytoplasmic order and chaperoning of the complex molecular machines involved in the architecture and function of cells, particularly elongated and post-mitotic cells. They may represent a suite of proteins that help maintain homeostasis in such cells that are at risk from stress or from the accumulated insults of aging.

Keywords

crystallins; chaperone; lens; retina; epithelial cell; stress

1: Introduction. *Evolution of Lens and Crystallins: an elongation connection?*

The vertebrate lens is an avascular cellular structure composed mainly of highly elongated, terminally differentiated fiber cells, most of which lack all organelles (Bassnett et al., 2011; Kuszak et al., 2004). In many species it survives and functions for decades, maintaining transparency and a gradient of refractive index (RI) to produce clear focused images on the light sensitive cells of the retina. Optical adaptation for a particular habitat is generally

achieved by the shape of the gradient in ways that reduce spherical aberration (Kroger et al., 1994) or optimize chromatic aberration (Gustafsson et al., 2008; Kroger and Fernald, 1994). The RI of the lens is largely due to high concentrations of soluble proteins generically known as crystallins (Bloemendal et al., 2004; Slingsby et al., 2013; Wistow, 2012). The lens and its contents evolved at some early point in the history of vertebrates, but long after the evolution of light sensitive cells, the photoreceptors, that perform the key step in vision of transducing photons into neural impulses.

Although fossils are lacking, there are many examples of existing simple eyes, particularly in invertebrates, that have photosensitive patches or organized retinas with no lens (Ayala, 2007; Land and Fernald, 1992; Land and Nilsson, 2002; Shimeld et al., 2005). From the ontogeny of the eye and from the parallel example of existing simple eyes a simple, staged process can be imagined that led to the evolution of the modern lens in discrete steps that all produced a useful, evolutionary selected structure. Indeed, modelling has suggested that such an evolutionary process could be relatively rapid (Nilsson and Pelger, 1994). A layer of cells overlying the retinal anlagen gives rise to a lens in both vertebrates and cephalopods (Harris, 1997). Elongation of these cells, which is an early step in the formation of lens placode in mammals and continues throughout development (Fig 1), gives rise to a structure capable of gathering light. In a remarkable parallel example in vertebrates the parietal or median eye found in reptiles and amphibians acquires a "lens" of a single layer of elongated transparent cells (Fig 1) by a completely different mechanism from that in the familiar lateral eyes (Eakin, 1973; McDevitt, 1972), emphasizing again the connection between cell elongation and lens formation. (In mammals, the ancestral parietal eye is represented only by light sensitive structures in the pineal gland).

Elongation of cells to produce a convex surface is a common feature of cellular lenses, but this alone would not produce an effective lens. The lens needs to be transparent, but also needs an increased refractive index (RI) to diffract and concentrate incoming light. It seems likely that during evolution this was achieved by upregulation of expression of some proteins that were already expressed in the ancestral epithelium and which could maintain elevated cytoplasmic concentrations while remaining transparent. These proteins may have had roles in maintaining the organization of the architecture and homeostasis of the elongating cells, particularly in regard to the enormously extended networks of cytoskeleton and membrane-bound junctional complexes that characterize the fiber cells of the vertebrate lens. In the vertebrate lens the fiber cells are extremely elongated, with the bulk of the plasma membrane derived from the adhesive sides that are enriched in proteins that link to the cytoskeleton. Highly prominent members of the lens membrane proteome are connexins, neural cadherin and lens specialized forms of aquaporin and claudin (Bassnett et al., 2009). In other cells, plasma membrane adhesion molecules link to the cytoskeleton and to organelles via giant linker molecules such as plectins, plakins and nesprins that contain multiple spectrin and plakin repeats and coiled-coils (Wilhelmsen et al., 2005). The α-, β-, and γ-crystallins which are represented in all vertebrate lineages may have evolved from proteins that support this kind of filament system and this may shed light on the functions of these proteins in lens and non-lens cells.

Particularly for image formation in more sophisticated eyes, the lens evolved mechanisms not only for creating increased RI but for regulating RI along the optical axis of the lens from the outer to the innermost layers (Pierscionek and Regini, 2012; Slingsby et al., 2013). This was achieved, at least in part, by increasing the diversity of crystallins through gene duplication and specialization and through regulated expression of crystallins in different layers of the lens to produce and fine-tune a gradient of RI to optimize optical performance.

Later in the evolution of vertebrates, particularly those whose ancestors emerged from the water to live on land, the optics of the lens adapted to requirements of the environment through changes in gene expression; further duplications of crystallin genes; deletion of crystallin genes; and even by *de novo* recruitment (through increased gene expression) of other proteins already expressed in the lens, often metabolic enzymes, as highly expressed crystallins (Wistow, 1993; Wistow and Piatigorsky, 1988).

Although crystallins are predominantly expressed in the lens, they necessarily arose from ancestors which had functions in non-lens cells. The nature of those functions may relate to the maintenance mechanisms of the lens and may also explain the expression of many crystallins outside the lens, particularly in other eye tissues.

The functional roles of the α-crystallins are clearly related to their membership in the chaperone-like small heat-shock proteins family (Clark et al., 2012). sHSPs are considered to bind exposed polypeptides, such as those in stressed, unfolding proteins, thereby blocking or retarding formation of aggregates, including fibrils, that can have toxic effects on the cell. One of the two α-crystallins found in most vertebrates, αB, is stress-inducible, widely expressed in other cells and is particularly associated with protein deposition diseases in neurological and other tissues.

The roles of the βγ-crystallins are not as clear. These proteins belong to a superfamily that includes members in microbes and sponges, yet appear to have been lost in most other metazoan phyla except chordates (Slingsby et al., 2013). An ancestral form of the βγ-crystallins is found in the calcium-enriched otolith, one of the paired pigmented sister cells that function in guidance in the larval stage of tunicates, a urochordate (Shimeld et al., 2005). Recent genome sequence for the phylum Ctenophora (comb jellies, whose few species, characterized by their glass-clear transparency, are common ocean surface swimmers around the world) (Ryan et al., 2013) also predicts the presence of proteins with βγ-crystallin domains. The tunicate and ctenophoran proteins and several microbial βγ-crystallin superfamily members have conserved calcium-binding sites that are lost in the lens crystallins (Kappe et al., 2010; Shimeld et al., 2005). Some of these proteins are involved in specialized calcium-dependent cellular roles associated with formation or maintenance of cellular structures such as spore coats or cysts (Wistow, 1990; Wistow et al., 1985).

Some members of the superfamily in vertebrates also appear to associate with components of cellular architecture such as the cytoskeleton, this is at least implied by the plasma membrane localization of Ep37/EDSP in the embryonic newt *Cynops*, and the rounded shape of melanoma cells which lack the protein AIM1 (Liu et al., 2008; Ray et al., 1996;

Ray et al., 1997; Takabatake et al., 1992; Wistow et al., 1995). This might indicate another chaperone-like role, distinct from that of the sHSPs, in preventing formation of aggregates through "shepherding" other protein complexes (Fan et al., 2012).

The association of microbial superfamily βγ-crystallin members (and some sHsps) with structures such as spore coats also hints at a possible role in maintaining a low water-content, an attribute that could certainly fit some βγ-crystallins for a role in the high protein concentration lens (Chen et al., 2013; Zhao et al., 2013). However this role would seem to be dependent on the presence of high enough concentrations of βγ-crystallins to be able to influence overall water content. For the lower expression of these proteins seen outside the lens, this may not seem likely, however the shepherding role could still be effective at lower than lens concentrations if the crystallins are specifically targeted, perhaps to cytoskeletal filaments. In this scenario, their role in retina, brain and elsewhere could be in preventing tangling and aggregation of filaments, such as in neuronal cell processes thereby acting to maintain transport functions. In retinal cells that lie along the light path to the photoreceptors this could also help to keep the cell cytoplasm transparent by inhibiting formation of light scattering centers. This is a function that might be particularly important in very elongated cells, such as lens fiber cells, or in cell extensions such as axons.

There is another feature that may connect cells that are major sites of crystallin expression. Lens fiber cells, many neuronal cell types and retinal pigment epithelial cells are either post-mitotic or have low rates of turnover and replacement (Anderson et al., 1981; Frederikse et al., 2012). Very long lived cells may suffer stress from the gradual accumulation of deposits, such as amyloid-like fibrils, that are resistant to clearance by normal housekeeping mechanisms (Chiti and Dobson, 2009). Proteins such as α- and βγ-crystallins, which appear to have roles in retarding aggregation or perhaps in reducing tangling or clumping of complex macromolecules, could have evolved to delay some of the effects of aging in cells.

2: Structure of α-Crystallins

In common with most other small heat shock proteins (sHsps), lens α-crystallin is a highly polydisperse assembly. There are no methods for preparing α-crystallin in homogeneous form, and most biophysical techniques indicate an assembly size of around 800 kDa with newly synthesized lens α-crystallin being composed of an approximately 3:1 ratio of αA/αB chains (Horwitz, 2009). Nanoelectrospray mass spectrometry of unfolded/refolded or recombinant αB-crystallin indicates an ensemble of interconverting oligomeric forms smaller than lens alpha-crystallin, ranging from 10 to 40 subunits, centering around 28 subunits, yet including odd and even numbered oligomers (Aquilina et al., 2003). There is no precedent for a soluble, globular assembly with the characteristics of α-crystallin, as most globular protein assemblies have a discrete size in which protomers are arranged around one or more axes of rotational symmetry that form one of the four point groups: cyclic, dihedral, cubic (tetrahedral *23* or octahedral *432*), icosahedral. This structural and conformational dynamism is considered central to the role of α-crystallin both as a lens protein and as a small heat shock protein chaperone.

The sequences of sHsps contain a central, conserved "α-crystallin domain" (ACD), flanked by a long variable N-terminal extension and a shorter better conserved C-terminal extension (Hilton et al., 2013). Because most sHsps are not amenable to crystallography, the compromise in understanding their quaternary structures at atomic resolution has been to solve 3D structures of any monodisperse assembly that can be identified from across the Kingdoms of Life, and look for common structural principles. The first crystal structure of a sHsp assembly came from an archaeal thermophile (MjHsp16.5) and showed how a 24-mer could be built by the assembly of symmetric dimers (pdb 1shs). The structure showed how the fold of the ACD dimerized by strand-exchange and assembled into an octahedral shape with point group *432* symmetry (Kim et al., 1998). Higher assembly in this example was driven only by a C-terminal β-strand extension, which differs from most polydisperse sHsps in which the N-terminal extension also plays a role (McHaourab et al., 2009). Crystal structures of complete sHsp assemblies from yeast and wheat show how, by relaxing the symmetry of the dimer, different orientations of the C-extension created by conformational flexibility of the linker between domain and extension, allow assemblies of (lower) dihedral symmetry, namely a 16-mer with *422* symmetry (pdb 3w1z, (Hanazono et al., 2013), and a 12-mer with *32* symmetry (pdb 1gme, (van Montfort et al., 2001)). The key assembly contact in all three of these sHsps is a sequence motif I-X-I/V in the C-terminal extension in one chain filling pockets in the ACD of another chain. The role of the flexible linker in sHsp assembly was reinforced when modification of this region in MjHsp16.5 (pdb 4eld) by insertion of a segment of human Hsp27 caused a switch in oligomer size from 24-mer to 48-mer, whilst maintaining assembly interactions as well as the point group symmetry (McHaourab et al., 2012). In the eukaryotic structures, relaxed dimer symmetry permits regions of some N-terminal extensions to be resolved as α-helical in conformation and located towards the interior of the assemblies (pdb 1gme; 3w1z).

Sequence analyses of sHsps indicated that the higher assembly components, the I-X-I/V motif and ACD pockets, were conserved, whereas the dimer interface regions diverged, making modelling unreliable. The lack of a monodisperse vertebrate sHsp assembly meant that only smaller assemblies, formed from truncated α-crystallin chains, could be imaged by crystallography. The human αB ACD, stripped of both extensions, formed a different kind of dimer (Bagneris et al., 2009) consistent with the sequence distinction between metazoan and non-metazoan sHsp sequences. Vertebrate α-crystallin ACD dimer structures have an extended anti-parallel β-sheet dimer interface that is conserved in constructs that included C-terminal extensions, although a register shift is permissible (Laganowsky et al., 2010; Laganowsky and Eisenberg, 2010). The docking of the I-X-I/V sequence motif into partner ACD pockets is conserved, though a change in strand direction is allowed, consistent with the palindromic sequence of the motif region. An sHsp structure (pdb 2bol) from tapeworm that has two consecutive ACDs but lacks a C-terminal sequence motif, showed that I-X-P sequences from other regions of the sequence could dock instead into ACD pockets (Stamler et al., 2005). These high resolution snapshots of subassembly species are consistent with solid state NMR spectroscopy of full-length reassociated αB assemblies (Jehle et al., 2010), indicating a conserved role for the I-X-I/V sequence motif in alpha-crystallin assembly. What was missing was the structure of the N-terminal region, although solid state NMR indicated it was likely flexible. The X-ray snapshots showed a deep groove at the anti-

parallel dimer interface, into which we have speculated the N-terminal extensions might bind in a dynamic way (Clark et al., 2011; Slingsby et al., 2013). The idea is that under stress conditions, sHsp sequence extensions compete with unstructured regions of substrate proteins for binding to pockets and groove, in other words the dynamic assembly mechanism provides the basis of chaperone function.

This picture of an interaction between an N-terminal extension and a dimer groove is not in agreement with all the biophysical findings. The shape of the extended beta sheet across the anti-parallel interface is flat in the X-ray crystal structures whereas in the solid state NMR coordinates (pdb 2klr) it is twisted in a way that blocks the groove (Clark et al., 2011; Jehle et al., 2010). Statistical analysis of single particle images taken by electron microscopy can be used to reconstruct 3D electron density maps of large assemblies so long as different views of the object and their symmetric relationships can be distinguished. Early cryoEM studies indicated α-crystallins were too polydisperse to reconstruct with any symmetry, and hence the map showed few features other than a hollow shell (Haley et al., 2000). More recently there have been claims of a more homogeneous αB sample allowing the reconstruction of density maps from negative stain images consistent with a model of a 24-mer with *23* symmetry (Peschek et al., 2009). The structure of the αB ACD dimer coordinates (pdb 2klr) were fitted to this EM map as a trimer of dimers around the 3-fold axes in an arrangement similar to that seen around the 3-fold axes of the MjHsp16.5 and wheat X-ray structures (Jehle et al., 2010; Jehle et al., 2011) and to the negative stain EM structure of the *Mycobacterium tuberculosis* Acr1 12-mer with *23* symmetry (pdb 2byu) (Kennaway et al., 2005). A larger cryoEM dataset subsequently did reveal size polydispersity, but with a significant population of particles that could be classified as 24-mers with *23* symmetry (Braun et al., 2011). A new model was fitted to the map in which a trimer of dimers was modelled into a large anti-parallel beta barrel with the 3-fold axis passing through the middle of the barrel and through density modelled as the self-associating N-terminal extensions (pdb 2ygd) (Braun et al., 2011). When the three N-terminal serines known to be phosphorylated *in vivo* are mutated to three glutamates, several techniques indicated a redistribution towards smaller oligomers, with cryoEM reconstructions showing maintenance of the *23* (tetrahedral) 24-mer model along with 3D reconstructions of 12-mers and 6-mers that now comprise a larger proportion of the sample (Peschek et al., 2013). Other authors have argued that the level of size polydispersity of the αB-crystallin, as measured by gas phase IM-nanoMS is better modeled by an assembly mechanism based on interconverting asymmetric polyhedral models (Baldwin et al., 2011).

The 3D structure of the N-terminal domain of αB-crystallin is unknown which makes the unambiguous fitting of ACD dimer coordinates into EM maps difficult, especially when samples are polydisperse. The two EM based models have N-terminal extensions in completely different locations (Delbecq and Klevit, 2013), however they are each consistent with I-X-I/V motifs fitting in to ACD pockets. Both solid-state and solution NMR spectroscopy support significant occupation of the ACD pockets with I-X-I/V motifs (Delbecq et al., 2012; Jehle et al., 2010), although other NMR methods indicate that the motif is highly flexible at ambient temperatures and hovers over rather than binds into the pockets (Baldwin et al., 2012). A study using a combination of nanoMS and IMS to measure

the impact of neighboring residues of the I-X-I sequence on the strength of its binding with an ACD pocket shows that in the context of a full assembly, increasing the binding of an interdimer contact (motif into pocket) destabilizes the extended anti-parallel beta sheet interface within a dimer (Hilton et al., 2013). These authors argue for a major role of the C-terminal region in regulating quaternary dynamics and hence access to substrate binding sites (see also Benesch review). This idea also fits with the notion that the extended anti-parallel interface groove, which crystallography has shown is blocked in the R120G human disease mutant, forms a regulated peptide binding site (Clark et al., 2011).

An unexpected view on the function of αB comes from studies showing that the peptide sequence DRFSVNLDVKHFSPEELKVK (residues 73–92) from human αB-crystallin can prevent protein aggregation *in vitro*, leading to its designation as a "minichaperone" (Bhattacharyya et al., 2006). Remarkably, following uptake by different mechanisms, exogenous administration of this peptide was found to protect fetal retinal pigment epithelial (RPE) cells from oxidative stress-induced apoptosis (Sreekumar et al., 2013). In the 3D structure this peptide comprises the B3 strand of one sheet of the alpha-crystallin domain and the edge B4 strand of the other sheet along with the arching loop that connects these strands. The seven hydrophobic side chains are buried between the sheets of this strand, although the B3-strand hydrophobic side chains have some solvent exposure depending on the presence of a B2 strand (Clark et al., 2011). The B3 strand occupies a strategic position as it forms the sides of the shared groove of the α-crystallin domain dimer, and Val 91 lines one of the B4/B8 pockets, both candidate substrate-binding regions. However, the peptide on its own would be expected to be dominated by its hydrophobic character and, assuming that αB-crystallin remains largely folded in its biologically active form, it is difficult to reconcile how the peptide can mimic the true function of αB. This suggests that the effect of the "minichaperone" may be through a different mechanism from the native sHsp.

Although the structural studies provide conflicting models of vertebrate sHsps, they underscore the dynamic nature of the system. The sHsps may have been selected for a role in the eye lens because they are large and polydisperse, and hence unlikely to crystallize or phase separate in the concentrated cytoplasm of the lens. We have outlined potential protein binding sites in the ACD that also function in (quasi)-assembly. We have suggested that the ability to form extended lattices between assemblies through their I-X-I/V motifs might be the basis of the water-insoluble alpha-crystallin which is characteristic of high refractive index regions of mature lenses without compromising transparency (Slingsby et al., 2013). Or they may have been recruited because of their role as stress proteins, with their main function in the lens being to bind other disordered proteins that cannot survive for a lifetime without the energy-hungry machines that provide quality control.

3: The roles of α-crystallins outside the lens

αB-crystallin is expressed in many tissues, notably in the retina, muscle and brain where it is often a biomarker of pathology (Andley, 2007). The two genes (*CRYAA, CRYAB*) that encode the lens α-crystallin A and B polypeptide chains, belong to the small heat shock protein (sHsp) family, whose ten members (HSPB1-B10) have distinctive tissue distributions (Carra et al., 2013; Kappe et al., 2003). Current understanding is that heat

shock proteins play roles in cellular proteostasis where they support various stress mechanisms (Balch et al., 2008; Tyedmers et al., 2010; Vembar and Brodsky, 2008) with a particular importance in retinal degeneration (Athanasiou et al., 2013). In the cytosol the Heat Shock Response (HSR), under the control of a heat shock transcription factor (HSF), leads to upregulation of several classes of chaperone proteins including some sHsps and ATP-driven chaperones including Hsp70s for protein binding and refolding, while the Ubiquitin-Proteosomal system (UPS) ensures terminally misfolded proteins are tagged and trafficked to proteasomes for degradation (Basha et al., 2012). Quality control within the ER maintains the integrity of membrane and secreted proteins using several chaperones, predominantly Hsp70 family member HSPA5, also known as ER lumenal Ca(2+)-binding glucose-regulated protein 78 (GRP78), or BiP, which has an ER localization signal sequence. If the ER becomes overloaded, the Unfolded Protein Response (UPR) surveillance system is activated and sends defective proteins back to the cytosol for degradation (Walter and Ron, 2011). Larger protein aggregates can be trafficked to lysosomes for catabolism and recycling. If these mechanisms are overwhelmed by protein aggregation, the cells die by apoptosis or necrosis. As the exogenous expression of sHsps is strongly cytoprotective in many cell types they have become attractive as potential neuroprotective agents (Arrigo, 2013; Stetler et al., 2010).

However, the exact role of sHsps in protein homeostasis is unclear. Some members, including αB and αA, can prevent denaturation of model stressed proteins *in vitro* (Horwitz et al., 1998). Mutations in sHsp family members, particularly those that are upregulated by HSF, may lead to self-aggregation that can overwhelm the degradation pathways directly (Clark et al., 2012), or indirectly by a loss of other (largely unknown) functions in cellular homeostasis pathways. The activity of small heat shock proteins is considered to be networked to other chaperones, which use the energy of ATP to drive conformational changes needed for unfolding/refolding of damaged proteins in readiness for recycling or destruction (Buchberger et al., 2010). For those sHsps that behave as ATP-independent chaperones, it is possible that cytoprotection stems entirely from an ability to bind non-native proteins, so keeping them in a soluble state.

This ancestral ability of sHsps to bind non-native, probably unstructured, polypeptide chains may have been exploited for other cytoplasmic roles, such as an adaptor/scaffold/tether, for incorporation into other pathways involved in proteostasis, autophagy or cell death. An example is binding of the small heat shock protein HSPB8 to Bag3. Structural biology provides clear evidence that the Bag domain acts as a regulator of Hsp70 (HSPA8) ATPase activity by acting as a nucleotide exchange factor, and that the interactive surface residues are conserved in the Bag domain of human Bag3 (Sondermann et al., 2001). Outside the Bag domain, I-P-V sequences in Bag3 can bind HSPB8, most likely by insertion into B4/B8 pockets of the alpha-crystallin domain (Fuchs et al., 2010), in the same way that these sequence motifs have been observed to facilitate assembly of several crystallized sHsps (Slingsby et al., 2013). This interaction provides a link between a sHsp and a motor chaperone through a single adaptor/tether.

There are many studies reporting binding of αB to several different components of the apoptosis pathway (Acunzo et al., 2012). Interestingly, cytoprotection activity from

ischemic brain injury in mice by overexpression of Hsp27 (HspB1) was chased back from cytochrome c towards the upstream activated kinase ASK-1, thus blocking cell death signaling (Stetler et al., 2008). The presence of classic phosphorylation sites in N-terminal extensions of the α-crystallins and other sHsps has long been held to denote a role in signaling following cytoskeletal stress (Launay et al., 2006). In the case of heat-induced necrotic cell death, a study in *C elegans* showed that preconditioning with heat resulted in localization of Hsp16.1 to the medial Golgi where it maintained calcium-homeostasis through interaction with a transmembrane cation transporter, ATPase PMR-1 (Kourtis et al., 2012). Hsp16.1 may stabilize the PMR-1 pump to allow clearance of calcium from the cytoplasm under stress. Human PMR-1 contains an I-P-V motif in the N-terminal cytoplasmic region, suggesting it too may be a target for sHsp binding. Recent evidence has suggested that αB does support the folding of multispan transmembrane proteins and be involved in their transport from the cytosol to the lumen of the ER (D'Agostino et al., 2013).

In order to unravel the role of sHsps in cell homeostasis it is instructive to look at the impact of knockouts. αA(HSPB4) is massively expressed in the eye lens, whereas αB(HSPB5) in addition to high expression in the lens is highly expressed in striated muscle, and is also found in many other cell types, particularly the neuroglia. This tissue distribution is broadly in keeping with current understanding of gene regulatory regions in the two α-crystallin encoding genes, and with the phenotypes of the homozygous mouse knockouts of *cryaa* (which caused early cataract) and *cryab* (which caused muscular degeneration without cataract), although the latter also knocked out a second muscle sHsps, HspB2 (Brady et al., 1997; Brady et al., 2001). An essential role for αB in human skeletal muscle was indicated when severe infantile myofibrillar myopathy was found to be caused by either a homozygous frameshift in exon 1 of *CRYAB* or a homozygous frameshift in exon 3 within the ACD, in different families (Forrest et al., 2011; Selcen, 2011). It was suggested that "the normal process of translocation of αB-crystallin to the Z-disk following muscle contraction is disrupted with consequent failure of the normal cellular mechanism for repair and maintenance of the functioning contractile apparatus" (Forrest et al., 2011). Myofibrillar myopathies are associated with disintegration of the Z-disks (specialization of muscle sarcomere for attachment of plus ends of actin filaments), with most myopathy mutations mapping to Z-disc components: desmin, αB-crystallin, myotilin, ZASP, filamin C and Bag3 (Selcen, 2011).

Some αB particles can be seen by electron microscopy to interact directly with desmin filaments (Houck et al., 2011). There is conflicting evidence concerning direct interaction between small heat shock proteins and with actin or tubulin filaments, leaving their role in cytoskeletal dynamics elusive (Mymrikov et al., 2011). In a recent phylogenomic analysis of muscle components, of the 28 components of Z-discs, four are unique to vertebrates (synaptopodin-2; telethonin; myozenin-1; small ankyrin-1, isoform 5) (Steinmetz et al., 2012). One possibility is that αB, which arose with the vertebrates, has co-evolved a cytoskeletal function with one of these Z-components in vertebrate striated muscle. Alternatively, high levels of cytoskeletal and membrane junction components containing I-X-I/V sequence motifs may require the support of substantial levels of αB.

A more subtle retinal vasculature phenotype has been described in *cryab* knockout mice in which retinal vessel density in the inner plexiform layer was reduced (Kase et al., 2010). Ocular angiogenesis is regulated by secretion of VEGF-A with increased ocular neovascularisation correlating with increased levels of VEGF-A. This has led to the idea that the level of VEGF-A protein expression is post-translationally regulated by αB, and that lowering levels of αB might be a useful therapy for many stress-induced ocular disorders (Kerr and Byzova, 2010; Liu et al., 2013). The finding that αB-crystallin and VEGF-A are colocalized in the endoplasmic reticulum in stressed RPE cells (Kase et al., 2010), most likely represents αB doing its cytosolic job under stress: binding the cytoplasmic regions of membrane proteins ready for trafficking through the ER-Golgi. VEGF-A contains a signal peptide sequence followed by a PDGF domain that has a cystine knot consisting of three intertwined disulfide bridges, and will likely need to be supported by the redox chaperones in the ER for their correct assembly. If endoplasmic-reticulum-associated protein degradation (ERAD) is activated and oxidized VEGF-A is sent back to the reducing cytosol, it could bind cytosolic αB in a manner similar to the *in vitro* insulin chaperone assay for the α-crystallins, possibly to regions suggested by protein array experiments (Ghosh et al., 2007b). Retinal proteome analysis in a mouse model of oxygen-induced retinopathy showed that knockdown of ORP-150 (an Hsp70 family member with an ER localization signal peptide) decreased the level of secreted VEGF in RPE and decreased neovascularization (Kim et al., 2012). Overall it seems that when cells are subjected to high levels of stress, several arms of the stress response are activated to prolong the life of cells and that this may include a role for αB that remains to be clarified.

Several functions have been ascribed to α-crystallin in the retina, including roles involved in cytoprotection, cell survival, inflammation, and autophagy (Kannan et al., 2012), although they may all stem from roles in homeostasis and the stress response. A large number of potential interaction partners have been described (Kannan et al., 2012), although there are few examples of direct interactions. All these observations suggest that αB (and perhaps also αA) has a broad range of housekeeping duties helping to organize and protect complex cytoplasmic and membrane bound proteins to maintain homeostasis in many cell types. In the lens, this contributes to preserving the order and stability of the hugely elongated fiber cells, with extensive junctional and cytoskeletal complexes and a protein dense cytoplasm.

4: Structure of β- and γ-crystallins

The two domains

β- and γ-crystallin gene families have evolved through the duplication and diversification of proteins based on a common 3D architecture (Slingsby et al., 2013; Wistow, 2012). These modular proteins have been examined in detail as they provide insights into the evolution of protein assemblies (Figure 2). Their recent rapid expansion in vertebrates by gene duplication has resulted in an extensive family of two-domain proteins, oligomeric β-crystallins and monomeric γ-crystallins, that constitute around 50% of the cytoplasmic lens proteins in mammals. Each β- and γ-crystallin chain folds into four Greek key motifs that are characterized by an unusual folded β-hairpin. Each domain is formed from two Greek key motifs that intercalate to form a β-sandwich with each pair of motifs arranged about an

approximate dyad. The consecutive domains on each chain are connected by a short linker. The second motif in each lens βγ-crystallin domain is more conserved than the first. These conserved motifs have characteristic tyrosine and tryptophan corner residues buried in the core of the domain that underpins the β-sandwich fold, while a set of conserved hydrophobic and polar residues face outwards and are essential for higher domain assembly.

A version of the presumed ancestral single domain of the vertebrate proteins can be seen in urochordate *Ciona intestinalis* ciona-crystallin (pdb 2bv2) which binds two calcium atoms. Very similar calcium-binding is seen in the 3D structures of several bacterial, archaeal, and mold βγ-crystallin-like domain structures (Slingsby et al., 2013). The binding sites can be detected in the sequences by the presence of the fingerprint **D/N**-X-X-**S** on each Greek key motif of a βγ-domain, with each calcium site requiring one conserved side chain from two motifs. Confidence in assigning one or two calcium binding sites in cephalochordate (amphioxus) βγ-domain sequences (Kappe et al., 2010) is bolstered by the single calcium binding site observed in the crystal structure of a βγ-domain protein from the sponge *Geodia cydonium* (pdb 4iau, (Vergara et al., 2013)). The recent genomic sequence determination for the Ctenophore (comb jelly) *Mnemiopsis leidyi,* a representative of another nonbilaterian metazoan lineage (Ryan et al., 2013), predicts the presence of several βγ-domain proteins, apparently from intron-less genes, some of which also contain the calcium binding site fingerprint. Conservation of calcium binding sites across so many lineages strengthens the idea that these nonbilaterian, archaeal and possibly bacterial βγ-domains are ancestrally related and that they are therefore likely related to the chordate βγ-crystallins too. However, this means that βγ-crystallin-like domains were frequently lost in many animal lineages (such as arthropods) and that the ancestral calcium-binding sites were themselves lost from lens crystallins for reasons unknown.

In terms of domain structure, the urochordate *Ciona* calcium-bound single domain (pdb 2bv2) which lacks the full set of domain pairing residues, does not domain pair in the crystal lattice and is a monomer in solution. While the monomeric structure could be ancestral in this lineage, it is also possible that the simple ocellus in *Ciona* represents a secondary loss of a more complex eye, and that in the same way ciona-crystallin may have lost its full set of domain-pairing hydrophobic residues. Indeed, although the *Geodia* sponge sequence comprises two βγ-crystallin-domains, the 3D structure shows that domain duplication is on a different evolutionary pathway from the chordates as the domain pairing interface is unrelated (Vergara et al., 2013). The ancestral relationship between lens crystallins and a βγ-domain (D3) from mammalian brain CRYBG3 (a relative of AIM1(Wistow, 2012)) is observed in the conserved domain pairing hydrophobic residues in the second Greek key motif (pdb 4fd9); the isolated domain forms a low stability monomer in solution but the domains pair like βγ-crystallins within the crystal lattice (Rajanikanth et al., 2012). Given the strong similarity in gene structure between β-crystallins and the AIM1 family (Ray et al., 1997), this was perhaps to be expected.

Missense mutations or post-translational modifications that alter highly conserved residues of the βγ-crystallin domain are likely to perturb the fold with an impact that will depend on the overall thermodynamic and kinetic stability of the protein. One of the most conserved residues in the βγ-crystallin Greek key motifs is a glycine which allows a sharp turn in the

characteristic folded β-hairpin. A cataract-causing mutation, G18V, is located in the first folded hairpin in human γS-crystallin (Ma et al., 2009; Sun et al., 2005); the branched hydrophobic residue cannot take up the twisted backbone conformation available to glycine. The NMR solution structure of mutant γS-crystallin G18V under conditions where it is monomeric (pdb 2m3u) (Kingsley et al., 2013) shows the mutation causes local changes to the backbone and side chains compared with wildtype (pdb 2m3t). At neutral pH, under solution conditions where the mutant forms multimers that interact strongly with αB-crystallin, solution NMR has shown αB-crystallin binding to the N-terminal domain of γS-crystallin G18V (Kingsley et al., 2013).

The corner tryptophan in each of the lens βγ-crystallin domains, buried between β-sheets, is highly fluorescent in the absence of a second tryptophan contributed by a 3_{10} helix, which acts as a quencher in each domain (Chen et al., 2009). These four tryptophans are conserved in all human lens βγ-crystallin domains except for the quenching tryptophan in the second domain of βB2 and βA2, while in fish γM-crystallins, the pair can be absent from one or both domains (Chen et al., 2013; Zhao et al., 2013). The *Ciona* calcium-bound domain has both corner and quenching tryptophans, suggesting that the pair may have an ancestral function, while in βγ-domains within AIM1 and CRYBG3, the quenching tryptophan is only moderately conserved, and it is absent in the sponge domains. As γ-crystallins have evolved into very stable proteins, particularly in mammals, the fold has become very tolerant of mutations, even in the core. For example, in human γD-crystallin a point mutation (W42R) to the corner tryptophan in the first domain causes nuclear congenital cataract, yet the solved structure (pdb 4gr7) shows only minor changes (Ji et al., 2013). However, the protein was less soluble, less stable, and more susceptible to tryptic digestion.

γ-crystallins

In γ-crystallins the inter-domain linker is bent and domain pairing results in monomer formation (Figure 2). In solution, all γ-crystallins exhibit low frictional ratios and partial specific volumes compared with other proteins (Chen et al., 2013). A survey of the amino acid composition of all proteins found that γ-crystallins have unusually high contents of residues with higher refractive index increments which may make a significant contribution to increased refractive power of the lens (Zhao et al., 2011). Some of these residues may also have important roles in protein interactions and the role of γ-crystallins in the low hydration environment of the lens.

A key sequence difference that correlates with presence in the higher refractive index lens core region is the Arg/Lys ratio. γS-crystallin, the single most abundant γ-crystallin in the human lens (Lampi et al., 1997; Wistow et al., 2002), is the most conserved family member and it is the major γ-crystallin of the more hydrated, cortical (outer) region of the lens. In human γS-crystallin sequence the Arg/Lys ratio is 13/10, while in orthologs in the zebrafish, *Danio rerio,* it is 16/8 (γS1) and 15/8 (γS2). In contrast, the major human γ-crystallins of the denser lens core, γC- and γD-crystallins, have Arg/Lys ratios of 20/3 and 21/1, respectively, while in *Danio rerio* γM2b- and γM7-crystallins the ratios are 21/3 and 20/2 respectively. The extremely high RI in the core of fish lenses may also benefit from γM-crystallins having very large numbers of methionines on the surface (Chen et al., 2013; Mahler et al., 2013;

Zhao et al., 2013). The presence of these residues is associated with extremes in the hydrodynamic properties of compactness and low frictional ratio which are common to all γ-crystallins but particularly prominent in γM-crystallins of fish lenses.

In evolutionary terms, the ancestral γ-crystallin would most likely have resembled fish γS-crystallin more than the specialized core γ-crystallins. γS-crystallin domains have the same paired fluorescing and quenching tryptophan arrangement as ciona-crystallin, whereas the urochordate sequence has no arginines and no methionines. Thus the adaptation to a role for a high refractive index medium in the vertebrate lens appears to correlate with substitution of arginine for lysine along with increased content of methionine. Arginine is distinctive in having some aromatic character with the positive charge delocalized around the three terminal nitrogen atoms making it versatile in its ability to form fluctuating ion pairs (as glimpsed in the disorder in the very high resolution human γD-crystallin crystal structure, pdb 1hk0, (Basak et al., 2003), or interactions with other aromatics side chains; put another way it promotes protein-protein interactions, rather than interacting with high levels of solvent water. In an analogous way, the relatively polarizable electrons of the sulfur atom in the methionine side chain may also contribute to interactions with aromatic side chains (Valley et al., 2012).

Being monomeric, the entire surface of γ-crystallins is accessible to solvent. Their surfaces have extensive networks of polar interactions, particularly rich in arginine-containing ion pairs, while in fish, γM-crystallins are also rich in surface methionines. These features produce surfaces with a low potential for tight binding of surface water while allowing complex interactions with both water and other proteins. This may allow γ-crystallins to be highly soluble while binding relatively little water and perhaps to interact with and stabilize other proteins without tight binding and aggregation.

Lens formation requires a protein in solution to be concentrated almost to the solid state. Several human congenital cataracts are associated with catastrophic loss of protein solubility caused by mutations of arginines (Clark et al., 2012; Wistow, 2012) which could be due to perturbation of the low macroscopic dipole moment of γ-crystallins (Purkiss et al., 2007). The lens core γ-crystallins have been characterized as having attractive interactions that enable concentrated solutions to exist close to fluid-fluid phase separation (Bloemendal et al., 2004; Wang et al., 2010), an attribute likely correlated with their function as dense packing proteins. The balance of forces that enable a protein with a low overall charge yet high solubility to exist close to a critical point whilst maintaining short-range order is likely delicate. The optical perfection of eye lenses is testimony of the power of natural selection to find the right "surface solution" for protein packing density appropriate to a given position along the optical refractive index gradient.

β-crystallins

The two classes of β-crystallins, the basic (βB1, βB2 and βB3) and acidic (βA3/1, βA2 and βA4), all have N-terminal sequence extensions, compared to γ-crystallins, while the basic ones also have C-terminal extensions. βB2-crystallin dimerizes by domain swapping (pdb 2bb2; 1ytq) whereby the domain pairing of two domains in γ-crystallins is recapitulated except that the "paired domain" interaction is intermolecular and the connecting peptide

extended (Figure 2) (Bax et al., 1990). By contrast, in homo-oligomeric, N-terminally truncated human βB1-crystallin [pdb 1oki] domain pairing is intrachain as in the γ-crystallins, with the monomer-monomer (dimer) interface recapitulating the domain swapped dimer-dimer interface found in the crystal lattices of bovine and human βB2-crystallin (Figure 2) (Van Montfort et al., 2003). Homo-oligomers of βB3 and βA4-crystallins (solved by high throughput structural genomics consortia) show intrachain domain pairing like that in γ-crystallin and truncated βB1-crystallin (Figure 2). In the human βB3-crystallin structure [pdb 3qk3] the calculated biological assembly is a trimer, although exploration of the crystal lattice also reveals a dimer contact similar to that in βB1-crystallin (Figure 2 (Downard et al., 2011). In human βA4-crystallin [3lwk] the calculated biological assembly has the same dimer arrangement as βB1-crystallin (Figure 2). Only rudimentary stumps of the sequence extensions can be seen in these β-crystallin crystal structures. βB2-crystallin is generally the predominant β-crystallin in the lens and forms dimers and hetero-oligomers with all the β-crystallin chains, but there are no 3D structures of the heterodimers or higher-order oligomers. The domain swapped dimeric βB2-crystallin is thermodynamically the least stable of all crystallins, is involved in dynamic subunit exchange with other β-crystallins *in vitro* (Bateman et al., 2003; Dolinska et al., 2009; MacDonald et al., 2005) and can act as a "chaperone" to stabilize/co-assemble other β-crystallins *in cellulo* (Marin-Vinader et al., 2006). βB2- and βA3-crystallin homodimers undergo subunit exchange to form a mixture of homo- and heterodimers (Hejtmancik et al., 1997; Takata et al., 2009). If βA3 homodimers associate like βA4 (Figure 2), then βB2- and βA3-crystallin homodimers would have their own distinctive mode of domain pairing, so heterodimerization would involve linker remodeling by one of the chains. Homodimers of mouse βB1-crystallin and human βA3-crystallin form a dimer-tetramer equilibrium (Chan et al., 2008). When most of the βB1 N-terminal extension was removed such that it resembled an *in vivo* processed chain, heterotetramer formation was enhanced, whereas the βA3-crystallin N-terminal extension was dispensable (Dolinska et al., 2009). The truncated βB1 construct resembles that used to solve the crystal structure of βB1-crystallin (pdb 1oki). Again, assuming the βA3-crystallin dimer resembles the structure of βA4, there are two possible scenarios for higher assembly. If, on mixing, the dimers underwent subunit-exchange and domain swapping to form heterodimers, they could then form a heterodimer-heterodimer using the released interface, to form a heterotetramer like the βB2-crystallin lattice tetramer (Figure 2). Or, if they both remain domain paired as in the homodimers, a new interface could be used to form the higher assembly. In this case the assembly could be accomplished in a similar manner to interface 1 in the lattice of truncated βB1-crystallin whereby the stumps of the sequence extensions of one dimer patch a domain surface on another dimer (see Figure 4a in reference (Van Montfort et al., 2003).

When post-translational modifications such as deamidation, or cataract causing point mutations, occur on the assembly interfaces, they have an impact on quaternary structure. For example, with age in human lens, significant levels of chemical modification occur to a pair of glutamines conserved in β-crystallins that are close to the hydrophobic patch involved in domain pairing in nearly all vertebrate βγ-crystallins (Lampi et al., 2001). The geometry of the paired domain arrangement places each glutamine close to the approximate dyad where it lies alongside its partner glutamine from a second domain (Figure 3). If one of

these glutamines becomes deamidated in βB2-crystallin, where the paired domains come from different chains, one negative charge will be added to each of the paired domains at the dimer interface. If a single glutamine is deamidated in βB1-crystallin, then one negative charge is added to each of the intramolecular paired domain interfaces. Congenital cataract with microcornea is caused by S129R mutation in βB1-crystallin which occurs at the dimer interface, close to the 2-fold axis (Figure 3) (Wang et al., 2013). The altered side chain, in this case the replacement of small neutral serine for bulky positively charged arginine, results in two close positive charges at the dimer interface, in line with the experimental finding that the homodimer shifted to monomer.

The evolution of this complex mixture of dynamic interacting β-crystallins has involved the addition of sequence extensions to a conserved "domain-pair". To what extent these extensions play roles in oligomer assembly, or, by acting as spacers between assemblies, contribute to setting up the RI gradient, is unclear. In terms of the structural biology, what is lacking is the definition at the atomic level of the role of the intact extensions in hetero-oligomer assembly. A strong case can be made for oligomer polydispersity in lens α- and β-crystallins being advantageous for transparency (Slingsby et al., 2013). To what extent the expansion of the β-crystallin family in vertebrates has benefited other vertebrate-specific functions is less clear. Evolution may have selected for properties specifically in the lens that were nevertheless useful in non-lens cell types. Furthermore, as the gene expression patterns and native structures of β-crystallins evolved co-dependently for lens, this may have favored expression of the whole suite of proteins, rather than individual members, outside the lens when needed.

5: Non-lens Expression of β- and γ-crystallins

Although crystallins were originally thought to be specific to the lens, it gradually became clear that they could also be found at much lower levels in other tissues. This was first observed for δ-crystallin of the chicken lens which was immunochemically detected in retina (Thomson et al., 1978; Thomset et al., 1981). Later it was discovered that many "taxon-specific" crystallins were identical to metabolic enzymes which retained their ancestral roles in many cell types while serving as recruited crystallins (Wistow and Piatigorsky, 1987; Wistow et al., 1987; Wistow and Piatigorsky, 1988). It was also discovered that the α-crystallins were related to sHSPs (Ingolia and Craig, 1982) and eventually it was shown that αB is indeed a functional sHSP that is stress inducible in many tissues (Bhat and Nagineni, 1989; Clark et al., 2012). However, while the non-lens expression of αB-crystallin is conceptually straightforward - it is a *bona fide* sHSP with stress response roles in several tissues - the expression of the lens-specialized αA outside the lens is not so easy to rationalize from a biological point of view. The same is true for the β- and γ-crystallins. Like αA, they have most often been detected in other parts of the eye, particularly the retina.

The evolutionary history of lens places it in the ontological context of the rest of the eye (Grainger, 1992; Piatigorsky, 1981). As proteins were recruited to serve as crystallins, they were probably selected from among those that were already part of the repertoire of the cells of eye cup and overlying tissues. The genes for these proteins would only have needed some limited modification in their promoter sequences to achieve higher level expression; no

major chromatin remodeling or wholesale promoter engineering would have been needed. As such, it is to be expected that the crystallins (or their ancestors) had useful roles in non-lens tissues of the eye. Such roles could still exist in the modern vertebrate eye. However, since the shared ontology of different eye tissues is reflected in overlap in transcriptional mechanisms for genes in different parts of the eye (cell types of related developmental lineages are likely to have many transcription factors in common), expression of crystallins outside the lens, particularly in the retina and brain, could also be the result of transcriptional "leakiness".

Among the β- and γ-crystallins, some genes are highly conserved with orthologs throughout the vertebrates. These include all the β-crystallins (βA1, 2, 4; βB1, 2, 3) and two γ-crystallins (γS, N) (Wistow et al., 2005). Their ancient origins make these candidates for proteins with ancient, conserved, non-lens functions. Even so, some of them are apparently dispensable even in the lens and have been lost or suppressed in some species (Wistow, 2012), suggesting that they lack an important conserved, non-lens role. For example, in primates *CRYGN* appears to be a pseudogene (Wistow et al., 2005) while *CRYBA2* is expressed in lens but produces very little protein (Lapko et al., 2003; Wistow et al., 2002), probably through a block in translation. Many γ-crystallins have also been lost during evolution. The large class of γM-crystallins in fish seems to have been completely eliminated in terrestrial species. Their replacements in mammals, γA-F-crystallins, also seem to be dispensable, with some members eliminated in primates and even in the guinea pig (Simpanya et al., 2008). In birds, no genes for either the γM or γA-F classes exist. Genes coding for γS and γN are present in bird genomes but their level of expression is low and it is not clear that they produce functional proteins.

Non-lens ocular expression of βγ-crystallins

The first reports of crystallins, including β-crystallins, in the retina came from work on chicken embryos (Clayton et al., 1985; Thomson et al., 1978; Thomson et al., 1981). This work particularly associated crystallin expression with the phenomenon of transdifferentiation of chick neural retina or retinal pigment epithelium (RPE) in culture into "lentoid" bodies (Clayton et al., 1986), underscoring the close developmental linkage of lens with other tissues of the eye cup. The first description of a β-crystallin in mammalian retina was for βB2-crystallin in mouse and cat neural and pigmented retinas and in cat iris (Head et al., 1995). The first detection of non-lens expression of a γ-crystallin was for γS-crystallin which was detected in retina and cornea in bovine eye (Jaworski and Wistow, 1996) and in mouse retina (Sinha et al., 1998). Later γA-F-crystallins were detected in mouse retina (Jones et al., 1999).

All six γ-crystallins, along with αA, αB and γS-crystallins were detected in rat retina by mass spectroscopy and they were found to be upregulated upon exposure to intense light, suggesting a protective role (Sakaguchi et al., 2003). Later work provided evidence that distribution of αB and γ-crystallins in photoreceptors was under circadian regulation (Organisciak et al., 2011). α- and β-crystallins were also found to be upregulated in the degenerating retina of the *rd1* mouse (Cavusoglu et al., 2003). All mouse crystallins were detected in retina using RTPCR (Xi et al., 2003). Microarray analysis of rat retina injured by

mechanical tearing showed upregulation of αA, αB, βA1/3, βA4, βB2, βB3, γA, γC, γE (Vazquez-Chona et al., 2004). This study also found upregulation of highly lens-specific components of differentiated lens fiber cells, including BFSP1 (an intermediate filament protein) and Lim2 (a claudin) in retina, raising the question of possible contamination with RNA from lens itself. Similarly, another study using RNAseq described upregulation of crystallins and several fiber cell specific genes in a mouse model of diabetes (Kandpal et al., 2012). Again the possibility of contamination or leakage of RNA from lens, whether by damage during dissection or through effects of the disease model needs to be considered.

In addition to expression in the neural retina, αB, βA1/3, βA4, βB2 and γS-crystallins were identified by mass spectroscopy in drusen, deposits basal to the RPE that may be associated with age-related macular degeneration (AMD), in humans, while βB2 and γS-crystallins were similarly detected in monkey drusen (Crabb et al., 2002; Umeda et al., 2005). In a mouse model for RPE damage induced by dietary D-galactose, after 20 weeks exposure upregulation of expression of γS, βA1/3, βA4, βB2 and αA in RPE was detected (Tian et al., 2005), again suggesting a stress-related or protective role. Like lens fiber cells and many neuronal cell types, RPE cell types have little or no mitotic activity (Anderson et al., 1981; Dunn et al., 1996). Unlike lens cells, they are highly metabolically active and mediate transport between the vasculature and the photoreceptors. As RPE cells age, pigmented deposits accumulate while basal deposition of proteins and lipids is associated with AMD (Curcio et al., 2011; Gehrs et al., 2006; Wyatt et al., 2013). Perhaps elevation of expression of crystallins is part of a late stage stress-response to preserve RPE, or at least its function as a component of the blood-retina barrier, as long as possible.

Recently, an examination of crystallin expression patterns in retina taken from large scale microarray analyses of multiple strains of laboratory mice showed extremely large variation in expression levels from strain to strain (Templeton et al., 2013). Further studies showed poor correlation of RNA and protein levels and also found large variation between eyes in the same animal (Templeton et al., 2013). Such variation calls into question the importance of non-lens expression. At the same time, variable degrees of contamination of retina samples by lens material during dissection could also contribute to higher levels of transcript in some samples. The authors pointed out that the crystallin genes, along with some others with similar expression patterns, share promoter elements, particularly Maf response elements, raising the issue of "leaky" expression in retina of genes that have been optimized for very high expression in lens.

With regard to non-lens expression of γ-crystallins, particularly in mouse retina, an interesting early experiment used the promoter of the mouse *Cryge* gene, encoding γE (then called γ2), to direct expression of diphtheria toxin in a transgenic mouse (Breitman et al., 1989). Lens fiber cells were eliminated as expected, however, although iris was absent, the sensory retina, although convoluted, was apparently intact in the microphthalmic transgenic eye. While this might argue against expression of the gene in the retina, it is also possible that the construct used lacked sequences required for retina expression or that only a limited number of cells were targeted. This might suggest a quite discrete expression pattern in particular cell subtypes.

Proposed functional ocular roles outside the lens

It seems clear that there is at least some level of expression of βγ-crystallins in various parts of the retina. But do the genes have functional roles in the retina? In the Nuc1 rat there is a mutation in βA1/3 which is associated with defects in normal development and regression of intraocular blood vessels (Zhang et al., 2005). β-crystallins and γ-crystallins were detected in astrocytes surrounding intraocular vessels in the normal developing rat eye and crystallins were found to be induced by hypoxia in cultured human astrocytes (Zhang et al., 2005). The authors initially suggested that βA1/3 was specific to astrocytes in the retina and showed that there were defects in intermediate filaments in Nuc1 astrocytes (Sinha et al., 2008). Subsequently, expression of βA1/3 was detected in astrocytes, ganglion cells and RPE (Parthasarathy et al., 2011). Later it was shown that βA1/3 was localized to lysosomes in the RPE and that phagocytosis of outer segments was defective in the Nuc1 rat (Zigler et al., 2011). Nuc1 astrocytes were also shown to be defective for "anoikis", apoptosis related to defective cell-matrix contacts, and this was associated with problems in Golgi trafficking (Ma et al., 2011). Overexpression of the mutant allele under the control of the GFAP promoter in a transgenic mouse produced defective astrocytes and was associated with differences in retinal vasculature (Sinha et al., 2012). Astrocytes from Nuc1 eye were also shown to be defective in endolysosomal acidification and this was linked to defects in processing of Notch and inhibition of Notch target genes (Valapala et al., 2013).

The authors of the Nuc1 studies propose several essential roles for βA1/3 in astrocyte development and function, in filaments, lysosomes, endosomes, Golgi and in "life death decisions". However, it seems at least plausible that in several of these phenomena the phenotype might be due to a gain-of-function of the misfolded mutant βA1/3 interfering with subcellular processes such as trafficking. Nevertheless, several cataractogenic mutations of βA1/3 are known in both human (Bateman et al., 2000; Kannabiran et al., 1998; Yang et al., 2011; Yang et al., 2012) and mouse (Graw et al., 1999). These involve exon skipping or loss of key residues that would certainly produce a misfolded, non-functional protein in astrocytes. However no ocular vascular phenotype has been noted for these mutations. This suggests that there may be a rather specific effect in Nuc1 rather than a general loss-of-function or gain-of-function effect for βA1/3.

In the lens, it seems that β-crystallins exist predominantly as hetero-oligomers (Slingsby et al., 2013). This raises the possibility that individual members of the family may not have specific roles as homo-oligomers. In lens and in other tissues, the deleterious effects of misfolded β-crystallins may be amplified by their missing or defective interaction with other, normal β-crystallins, perhaps recruiting them into misfolded, potentially toxic aggregates.

Another series of papers has focused on a proposed role for βγ-crystallins in the function of retinal ganglion cells (RGC) and optic nerve (Piri et al., 2013). It was observed that damage to the lens was associated with neuroprotective and regenerative effects on ganglion cells in a model of traumatic optic nerve damage in rats (Heiduschka et al., 2004). Later it was shown that βB2 was present in filopodial protrusions and axons of RGC and in the supernatant of retina primary cultures (Liedtke et al., 2007). Overexpression of βB2 in RGCs promoted axonogenesis which was blocked by antibody to βB2 and conditioned medium

containing βB2 also promoted axon growth. Green fluorescent protein tagged βB2 fusion protein was shown to be taken up by cultured cells (Liedtke et al., 2007). The role of βB2 was expanded to RPE derived cells by showing that βB2 or neuronal progenitor cells (NPC) expressing βB2 could protect ARPE-19 cells in culture from UV damage and that the βB2 expressing NPCs injected into the vitreous could protect RPE in a rat model of optic nerve trauma (Bohm et al., 2013). An autocrine role for βB2 was proposed. In contrast, others also observed regenerative effects on RGCs associated with lens damage but have attributed this to macrophage activation rather than a direct role by crystallins (Leon et al., 2000; Yin et al., 2003).

The proposed role for βB2 in RGCs is quite surprising. The mechanism of secretion of βB2 into medium is unclear since βB2 lacks any sort of secretion signal. Furthermore, βγ-crystallins are intracellular proteins and contain relatively exposed cysteine and methionine residues that are vulnerable to oxidation. In the reducing environment of the cell they are protected but outside the cell oxidation, including disulfide crosslinking would be expected. Aside from their vulnerability to the extracellular environment, βγ-crystallins have no structural similarity to any known signaling proteins and there is no evidence for any receptor that might bind them. However the possibility remains that crystallins could be exported from one cell to another via exosomes, small membrane vesicles that have been proposed to act as transport systems for a number of factors in different cell types (Raposo and Stoorvogel, 2013). Whether such a mechanism could deliver biologically useful amounts of crystallins to a target cell is unclear.

Several experiments on β-crystallins in the RGC model used proteins purified from lens and these could contain low levels of other factors with biological activity. Other experiments used cells expressing βB2 that might also be secreting other factors. A knockout mouse for βB2 has been described (Zhang et al., 2008). Age-related cataract was observed but no retina effects have been reported, although it is quite possible that subtle phenotypes would not have been noticed. Similarly, several cataractogenic mutations of βB2 have been documented in humans (Wistow, 2012) and again no retina phenotype has been reported.

Expression of βγ-crystallins in brain and other tissues

Beyond expression in neural and other tissues of the eye, β-crystallins, most abundantly βB1 (http://mouse.brain-map.org/) and βB2, have also been detected in other tissues, particularly in the brain (Dirks et al., 1998; Head et al., 1991; Head et al., 1995; Magabo et al., 2000). Indeed, examination of the brain in the O377 mouse mutant (Ganguly et al., 2008) of βB2 have revealed reduced hippocampal size while behavioral and neuroanatomical tests revealed defects in sensorimotor gating and changes in neuronal activity in the dentate gyrus (Sun et al., 2013). Some alterations in gene expression, including genes for NMDA receptor subunits were also noted. The O377 mutant has a splice junction defect which leads to insertion of 19 residues in the middle of the C-terminal domain (Ganguly et al., 2008). This would certainly be expected to lead to misfolding and probably aggregation of the mutant protein. Again this raises the possibility of a gain-of-function effect from the misfolded protein in addition to any loss-of-function.

Other proposed functions

Within their shared structural fingerprint of repeated pairs of modified Greek-key motifs that form tightly folded domains, all known β-crystallins and many γ-crystallins share conserved pairs of tryptophan residues (Wistow et al., 2005). These make important contributions to the hydrophobic core of each domain and to domain stability (Mahler et al., 2013). Tryptophan side chains can absorb and emit photons in the UV range. This can make them susceptible to oxidative damage in the lens while transmitted UV could be harmful for the retina. In βγ-crystallins, these problems are obviated by a UV quenching mechanism that is based on an interplay of the Trp residues and the protein backbone that significantly reduces and red shifts the Trp emission spectrum in the folded protein (Chen et al., 2009; Kosinski-Collins et al., 2004). This mechanism can help protect both lens and retina from UV damage and would seem to be a useful role in both tissues.

While UV exposure might not be a major problem for modern aquatic species, it is thought that during the Cambrian period, when the precursor of the vertebrate lens/cornea began to evolve, UV levels were much higher so that a UV quenching mechanism could have been beneficial. Even in modern surface swimmers, such as zebrafish, a UV filter mechanism in the lens could contribute to the function and discrimination ability of the short wave cone pigments in the retina (Bowmaker and Hunt, 2006).

The βγ-crystallin superfamily contains many members expressed in diverse micro-organisms. From the first member noted, Protein S of *Myxococcus xanthus* (Wistow et al., 1985) it became apparent that many of these microbial proteins contain calcium binding sites which can be clearly visualized in structural studies (Bagby et al., 1994; Clout et al., 2001). However, the conserved fingerprint of the calcium binding site is not present in vertebrate βγ-crystallins (Suman et al., 2011) and although it has been suggested that they may retain some affinity for calcium, presumably through a different site, it is not clear that this is biologically significant. No bound calcium has ever been observed in any of the crystal structures for lens βγ-crystallins.

A more surprising idea is that human βA3-crystallin possesses autodegradative serine protease activity (Gupta et al., 2010). While there is evidence for degradation of the purified recombinant βA3 protein under some conditions, βγ-crystallins show no similarity to any known proteases (or indeed any other class of enzyme).

A common organizational role in lens and non-lens?

Is there a broad rationale for the role of βγ-crystallins in retina that might tie together some of these observations? In the lens, βγ-crystallins play a role in maintaining the short range order required for transparency (Bloemendal et al., 2004; Slingsby et al., 2013). They belong to a superfamily that contains members associated with cellular architecture in eukaryotes (Ray et al., 1996; Ray et al., 1997; Takabatake et al., 1992; Wistow et al., 1995) or structures including spores or cysts in microorganisms (Wistow, 1990; Wistow et al., 1985). In mammals there are three genes for large proteins that contain multiple βγ-crystallin superfamily domains; AIM1 (absent in melanoma 1) and its two relatives, AIMIL/CRYBG2 and CRYBG3 (Wistow, 2012). In addition to their β-crystallin-like domains, these proteins

contain a C-terminal trefoil domain. Intriguingly, a non-lens βγ-crystallin relative identified in skin secretions of a frog (*Bombina maxima*) and designated βγ-CAT (Liu et al., 2008), has been shown to dimerize with a trefoil domain protein. Trefoil domains are widespread and may have many different functions, but some are known to be important in organization of actin filaments (Jansen et al., 2011). Recently, examination of a knockout mouse model for γS-crystallin has revealed a role for this highly conserved protein in the organization of actin filaments (Fan et al., 2012) (Figure 1), suggesting that the protein and its relatives can act to stabilize and prevent aggregation of large macromolecular complexes such as cytoskeletal filaments. In the lens this would help prevent the formation of light scattering centers. It could also help maintain normal trafficking networks that rely on such structures. Indeed, clearance of degraded organelles in the maturing lens fiber cells is also defective in the γS KO mouse (Fan et al., 2012). Such a role could be useful in retinal neurons, particularly in axons. These cells, which lie over the photoreceptors in the path of incoming light, need to be transparent and to scatter as little light as possible (indeed some retinal cells act as wave guides to enhance light transmission (Franze et al., 2007)). Perhaps crystallins (including α-crystallins (Ghosh et al., 2007a)) help to avoid tangling and clumping of intracellular components, including cytoskeleton and the large complexes involved in junctions and intracellular transport, in these cells. They might play a similar role in another transparent ocular tissue, the cornea (Jaworski and Wistow, 1996; Ren et al., 2010). Normal function in many neuronal and glial cells also requires efficient transport of many components along the axons and cell processes. Again, crystallins could act to stabilize filaments and tubules to promote and protect neuronal function and this might be particularly important under certain conditions of stress.

A generalized protective role in keeping complex cell components from aggregating and in maintenance of cytoarchitecture could also be important in long-lived, non-mitotic or post-mitotic cells such as lens fibre cells, neurons and RPE. A protective role for sHsps in protein deposition disease is already quite clearly implicated (Clark et al., 2012); perhaps increased levels of βγ-crystallins could add another level of protection in cells subject to such disease?

Acknowledgments

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Summary and Prospects

The crystallins must have arisen from proteins with functions that existed before the evolution of the lens. They were recruited from among the transcriptome/proteome of cells from the same embryonic layers that give rise to brain and retina. Some of those proteins that were able to achieve high concentration, maintain optical clarity and also act to stabilize the structures of highly elongated cells became crystallins. It is possible that they retain their ancient roles in neuronal cells of retina and brain and in other long-lived post-mitotic cells such as retinal pigment epithelium. They may help to maintain functionality in these cells by stabilizing cytoskeleton and other complexes, and preventing tangling, aggregation and deposition.

Animal models should shed more light on functional roles of crystallins, but it should be recognized that in lens and in other cell types multiple crystallins are co-expressed. It may be that they exert their full effects as interacting complexes of crystallins rather than as individual proteins. This may confound interpretation of some single gene deletions in which phenotypes might either be masked by redundancy or exaggerated by gain-of-function defects in larger complexes missing one component. Better models might involve expression or deletion of groups of crystallins, rather than single genes, in specific cells. Indeed, it would be interesting to see whether expression of a suite of crystallins would have protective effects in a range of aging or stressed cells.

Although crystallins are expressed elsewhere, they are at their highest concentration in the lens and so too may be some of their important potential partners, such as proteins of the cytoskeleton and junctional complexes. Proteome analyses that search for interacting partners of crystallins in lens may give important clues to function.

Much is known about structural details of individual crystallins, but further insights into their functions probably require molecular analysis of full-length hetero-dimers and higher oligomers. Biophysical tools are emerging whereby spectroscopy can provide residue specific details of interaction between crystallins, such as between alpha-crystallin and destabilized mutant gamma-crystallins. Improved detectors for cryoEM of single particles extend the complementary use of this technique with protein crystallography, which is continually being improved through collaborative efforts such as the CCP4 (http://www.ccp4.ac.uk/). These imaging techniques can clarify the nature of interactions between crystallins and candidate cytoskeletal and junctional complexes, helping to distinguish those components making direct contact from those that are part of a much bigger complex, or merely a constituent of a highly complex, regulated biochemical process, such as cell death. Now that super-resolution fluorescence microscopy is closing the resolution gap between protein 3D structure and subcellular (filament) systems, the simple lens cell may be a window into unknown terrains of cell biology.
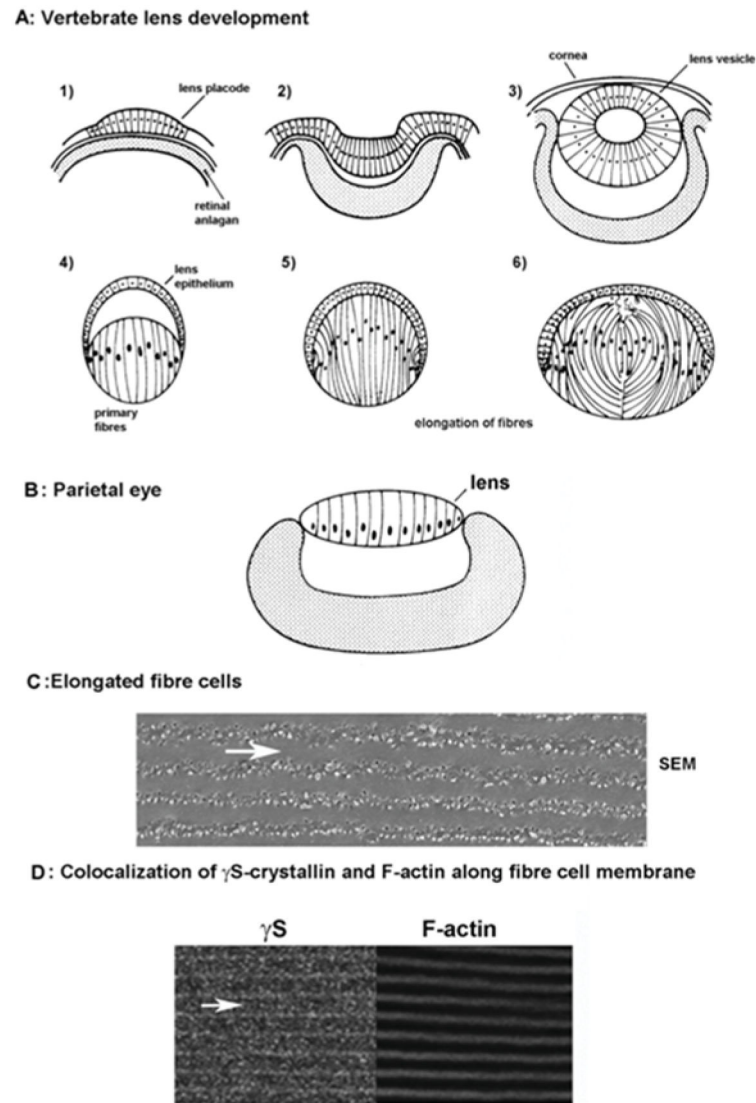
Figure 1. Lens formation is associated with cell elongation
A: Steps in the embryonic development of the vertebrate lens. (Adapted from (Wistow, 1993))

1. Epithelial cells overlying the retinal anlagen elongate to form the lens placode

2. Elongation continues as the optic vesicle invaginates.

3. Formation of the lens vesicle and cornea

4. Within the lens vesicle, primary fiber cells elongate

5. Fibres fill the lens

6. New layers of secondary fibres form throughout life through differentiation and elongation of equatorial epithelial cells.

B: Schematic structure of the lizard parietal eye (adapted from (Eakin, 1973)). A monolayer of elongated cells forms the simple cellular lens.

C: Elongation and intercalation of mature fiber cells. Scanning electron micrograph of a fiber cells from adult mouse lens. Arrow indicates the longitudinal axis of a fiber cell. Extensive cell-cell contacts are formed through intercalation of complex junctions. (For methods, see (Fan et al., 2012))

D: Co-localization of γS-crystallin and F-actin along the plasma membranes of mature mouse fiber cells (adapted from (Fan et al., 2012)).
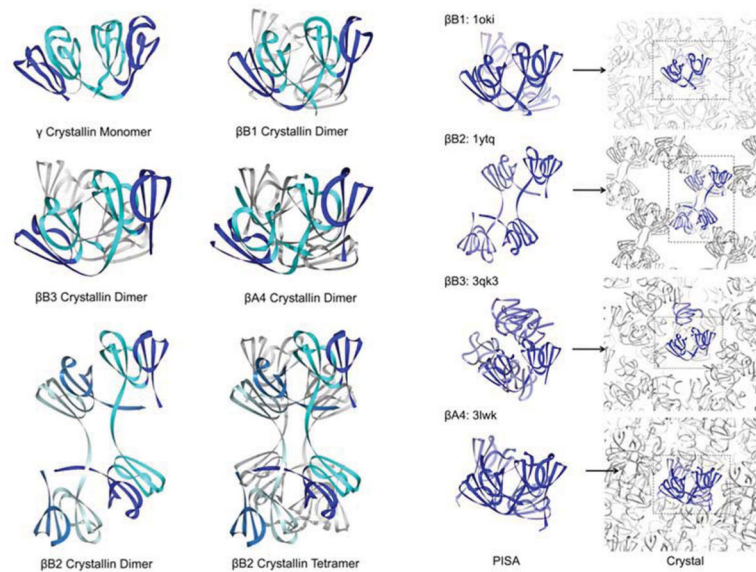
Figure 2. Modular assembly of β- and γ-crystallins from Greek key motifs and a "paired domain" interface

The two columns on the left show structures of the β- and γ-crystallins currently in the PDB Protein Data Bank. The two columns on the right show calculated dimer structures of the β-crystallins, with their PDB identifiers, along with their arrangements within the crystal lattices. A γ-crystallin monomer has four Greek key motifs that form two similar domains organized about a pseudo twofold axis. The first motif of each domain is shown in dark blue and the second motif is in cyan. The cyan motifs provide the conserved tyrosine and tryptophan corners, as well as hydrophobic residues that form the "paired domain" interface as well as some conserved side chains including glutamines. γ-crystallin is monomeric because the "paired domain" interface is intra-chain. The same interface is present in all the resolved β-crystallins. βB2-crystallin is different as the linker is extended so the "paired domain" interface forms between two different chains which creates a domain swapped dimer. In βB1, βB3 and βA4-crystallin, the subunit domains are paired as in γ-crystallins, and so these dimers need an additional interface. This new interface is the same as that observed in the βB2 tetramer formed in the crystal lattice. The third column shows the calculated biological assemblies as calculated by PISA (Krissinel and Henrick, 2007). The fourth column shows the location in the crystal lattice of the conserved dimers highlighted in blue from the surrounding grey crystal lattice. In the case of βB3-crystallin, the calculated assembly unit is a trimer, but the conserved dimer interface is formed in the crystal lattice. This figure is adapted from Figure 5 and SFigure 1 from Slingsby et al (Slingsby et al., 2013).
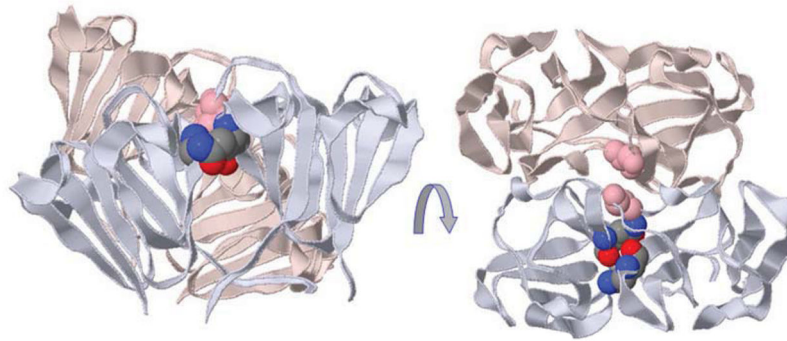
Figure 3. Changes to side chains that perturb assembly interactions

Four domains are shown from βB2 tetramer, with the "paired domains" painted in uniform colors. In a domain swapped dimer, the "paired domains" come from different chains, but are from the same chain in other β-crystallin homodimers and in γ-crystallins. In βB2 tetramer (shown) the four domains at the dimer-dimer interface are also from different chains, whereas in other β-crystallin homodimers, they come from two chains. Two glutamines colored in CPK are appended to the paired N- and C-terminal lavender domains showing their side-by-side location close to the local 2-fold between the domains. The side chain topologically equivalent to Ser129 in βB1 is shown (misty rose) appended to both C-terminal domains showing that a single mutation will clash with itself at this interface in a homo-oligomer.