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## $\beta$ A3/A1-CRYSTALLIN: MORE THAN A LENS PROTEIN

J. Samuel Zigler Jr. and Debasish Sinha

Glia Research Laboratory, Wilmer Eye Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland, USA

### Abstract

Crystallins, the highly abundant proteins of the ocular lens, are essential determinants of the transparency and refractivity required for lens function. Initially thought to be lens-specific and to have evolved as lens proteins, it is now clear that crystallins were recruited to the lens from proteins that existed before lenses evolved. Crystallins are expressed outside of the lens and most have been shown to have cellular functions distinct from their roles as structural elements in the lens. For one major crystallin group, the  $\beta/\gamma$ -crystallin superfamily, no such functions have yet been established. We have explored possible functions for the polypeptides ( $\beta$ A3- and  $\beta$ A1-crystallins) encoded by *Cryba1*, one of the 6  $\beta$ -crystallin genes, using a spontaneous rat mutant and genetically engineered mouse models.  $\beta$ A3- and  $\beta$ A1-crystallins are expressed in astrocytes and retinal pigment epithelial (RPE) cells. In both cell types, these proteins appear to be required for the proper acidification of the lysosomes. In RPE cells, elevated pH in the lysosomes is shown to impair the critical processes of phagocytosis and autophagy, leading to accumulation of undigested cargo in (auto) phagolysosomes. We postulate that this accumulation may cause pathological changes in the cells resembling some of those characteristic of age-related macular degeneration (AMD). Our studies also suggest an important regulatory function of  $\beta$ A3/A1-crystallin in astrocytes. We provide evidence that the cellular function of  $\beta$ A3/A1-crystallin involves its interaction with V-ATPase, the proton pump responsible for acidification of the endolysosomal system.

### Keywords

$\beta$ A3/A1-crystallin; lysosome; astrocyte; retinal pigment epithelial cell; animal model; signaling

## 1. WHAT IS A CRYSTALLIN?

Crystallins are the very abundant, soluble proteins that are the major components of the ocular lens, the most protein-rich tissue in the body. The term crystallin has been traced as

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J. Samuel Zigler, Jr., Assistant Professor, The Johns Hopkins University School of Medicine, The Wilmer Eye Institute, 400 North Broadway, Smith Building Room M037, Baltimore, MD 21231, szigler1@jhmi.edu

Debasish Sinha, Associate Professor, The Johns Hopkins University School of Medicine, The Wilmer Eye Institute, 400 North Broadway, Smith Building Room M035, Baltimore, MD 21231, Debasish@jhmi.edu

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far back as 1830, when Berzelius (cited by Björk, 1964a) first studied the proteins of the lens. He found the bovine lens to be 36% protein and considered this protein to be a single, lens-specific entity. He called it 'crystallin', because it was the major constituent of the 'crystalline lens'. Subsequently, it became apparent that more than one soluble protein was present in the lens; Mörner (1894) was able to separate 3 different soluble proteins, which he called  $\alpha$ -crystallin,  $\beta$ -crystallin, and albumin. Mörner's albumin fraction was later named  $\gamma$ -crystallin by Burky and Woods (1927). These 3 protein fractions were separated first on the basis of their differing net charge and later their differences in molecular mass. Each of these crystallins was initially thought to be a unique protein; however, by the latter half of the 20<sup>th</sup> century it became clear from the work of various laboratories that all 3 crystallin fractions were highly heterogeneous (Björk, 1964b, Spector et al., 1971, Zigler and Sidbury, 1973). The  $\alpha$ -,  $\beta$ -, and  $\gamma$ -crystallins were found to constitute the full complement of crystallins in most vertebrate lenses and were considered to be unrelated to each other. In 1980, Driessen et al. produced the first primary sequence of a  $\beta$ -crystallin, the polypeptide now called  $\beta$ B2, and found it to be homologous to previously determined  $\gamma$ -crystallin sequences. As a result, the  $\beta$ - and  $\gamma$ -crystallins were combined into a single protein superfamily, the  $\beta/\gamma$ -crystallins, which together with the unrelated  $\alpha$ -crystallins constitute the ubiquitous crystallins of the vertebrate lens. Collectively, proteins from these two groups represent over 90% of the total soluble protein in most vertebrate lenses. The relative amounts of the individual crystallins vary widely among vertebrate species; further, there are variations within any species as a function of age. As one example, using a species relevant to much of the data discussed below, Lampi et al. (2002) have analyzed the crystallin composition of the lenses of 16 day old rats. The  $\beta$ -crystallins constituted about 25% of the total, with  $\gamma$ -crystallins being about 60% and  $\alpha$ -crystallins the remaining 15%.  $\beta$ A1-crystallin and  $\beta$ A3-crystallin, in aggregate, constitute between 25% and 30% of the  $\beta$ -crystallins or about 6% of the total crystallin complement.

Until about 25 years ago, it was generally accepted that the crystallins were evolutionarily highly conserved proteins, uniquely adapted to their role as structural elements in the lens, and that they were not present in any tissues except the lens. Several discoveries changed this concept. The first clue came in 1982 when Ingolia and Craig showed that the  $\alpha$ -crystallins shared sequence homology with small heat shock proteins. Horwitz (1992) eventually was able to demonstrate that  $\alpha$ -crystallin, like other members of the small heat shock protein family, has a chaperone-like activity that prevents the uncontrolled aggregation of partially unfolded proteins. At about the same time, it was discovered that in some vertebrate lineages, proteins not related to the  $\alpha$ - or  $\beta/\gamma$ -crystallins were expressed at very high abundance in the lens (Hendriks et al., 1988; Huang et al., 1987). At least a dozen of these so-called taxon-specific crystallins have now been described, and shown to be identical to, or closely related to, cellular proteins such as lactate dehydrogenase,  $\alpha$ -enolase, and glyceraldehyde-3-phosphate dehydrogenase (see Table 1 and literature cited therein). As a general (and very subjective) rule, in order to be considered a crystallin, a protein must be present in the lens of one or more species at a level of at least 5% of total soluble protein (de Jong et al, 1994). Since vertebrate lenses typically have soluble protein levels in the 300–450 mg/ml range this represents a substantial amount of bulk protein. With the discovery of the taxon-specific crystallins and as more sensitive analytical techniques revealed the

presence of  $\alpha$ - and  $\beta/\gamma$ -crystallins in tissues other than the lens, a new paradigm with respect to crystallins has evolved. As numerous authors have noted (Bloemendal et al., 2004, Piatigorsky, 2007), to function as a lens crystallin, a protein must (1) be highly soluble, so that it can be incorporated into the lens at high enough abundance to contribute to the required refractivity, while not being prone to aggregation; (2) be extremely stable, because the lack of protein turnover in mature lens fiber cells means crystallins are extraordinarily long-lived (Wannemaker and Spector, 1968); and (3) be capable of supra-molecular interactions with other crystallins in order to form a stable protein matrix with the high degree of short range order necessary for transparency (Delaye and Tardieu, 1983). These attributes have not changed, but what has changed is the original idea that crystallins are proteins unique to the lens which evolved specifically to meet these criteria. Under the new paradigm, it is understood that all crystallins originated in other cell types, pre-dating the evolution of the lens, and that they had a variety of functions, before they were recruited to the lens to function as 'crystallins'. While some crystallins retain their original function in the lens, others do not. Crystallins are multi-functional proteins, giving rise to the concept of gene sharing (Piatigorsky, 2007). They are a diverse set of proteins that are called "crystallins" only because of their high abundance in the lens; while all crystallins must, at minimum, meet the 3 criteria listed above, the term crystallin does not imply any structural or evolutionary relationship among them.

## 2. $\beta/\gamma$ -CRYSTALLINS: STRUCTURE AND EVOLUTION

The structures of the  $\beta/\gamma$ -crystallins have been extensively studied (see reviews by Bloemendal et al., 2004, Wistow, 1995). Suffice it to say here that the  $\gamma$ -crystallins are monomeric proteins with molecular masses of about 20 kDa, whereas the  $\beta$ -crystallins are a heterogeneous mixture of dimers and higher oligomers with native molecular masses ranging from about 50 kDa to 200 kDa. Our goal in this review is to explore what is known about the role(s) of these proteins outside the lens, focusing on  $\beta A3/A1$ -crystallin. We will provide background information on the  $\beta$ -crystallins, but for the most part will not discuss the  $\gamma$ -crystallin branch of the superfamily.

There are six  $\beta$ -crystallin genes scattered on several chromosomes, coding for homologous polypeptides with molecular masses of ~22–28 kDa (Table 2). Three of the genes code for polypeptides with slightly lower isoelectric points known as the  $\beta A$ (acidic)-crystallins, while the other 3 encode the  $\beta B$ (basic)-crystallins. The  $\beta$ -crystallin polypeptides, as with all members of the superfamily, have 2 domains, each domain being composed of 2 'Greek key' motifs, common structural elements of proteins composed of 4 adjacent anti-parallel beta strands (Blundell et al., 1981). Slingsby et al. (2013) have described how the Greek key motif is the basic building block underlying the generation of the various monomeric and oligomeric  $\beta/\gamma$ -crystallins, and how the multiple forms contribute to the transparency and the refractive index gradient of the lens.  $\beta$ -crystallins, unlike the  $\gamma$ -crystallins, also have N-terminal extensions (acidic and basic polypeptides) and C-terminal extensions (basic polypeptides only). While the role of the terminal extensions is not well understood, they may be involved in the normal association into dimeric and oligomeric forms, or may protect against aggregation to larger forms during lens maturation and aging (Bloemendal et al., 2004; Dolinska et al., 2009). The core domains (excluding the extensions) of the various

$\beta$ -crystallin polypeptides are approximately 50% identical in primary sequence. With respect to the  $\beta$ A3- and  $\beta$ A1-crystallin polypeptides, several laboratories have done extensive work on their structures, association properties, and stability using recombinant proteins (Bateman et al., 2003; Dolinska et al., 2009; Takata et al., 2008). The structures of the genes for the  $\beta$ - and  $\gamma$ -crystallins are different. The two motifs comprising each domain in the  $\beta$ -crystallins are encoded by separate exons, while in the  $\gamma$ -crystallins each domain is encoded by a single exon (Lubsen et al., 1988).

As indicated above, all crystallins are believed to have been recruited from pre-existing genes when the lens evolved in early vertebrates (Piatigorsky, 2007). In the case of the  $\beta/\gamma$ -crystallin superfamily, not only are the functions of the protein precursors unknown, but the ancestral genes and the evolutionary path leading to current  $\beta/\gamma$ -crystallins remain unclear. It has been known for over 25 years that proteins distantly related to  $\beta/\gamma$ -crystallins exist in microbes, including Protein S in the soil bacterium *Myxococcus xanthus* (Wistow et al., 1985) and spherulin 3a in the slime mold *Physarum polycephalum* (Wistow, 1990). It has been postulated that these proteins are involved in resistance to stress, since both become abundant during formation of spores or cysts in response to adverse environmental conditions. These proteins and others found in various microbes (see Piatigorsky, 2007) have weak homology to vertebrate  $\beta/\gamma$ -crystallins and no known functions, although they are all composed of Greek key motifs. Of greater interest in terms of the evolutionary origin of lens  $\beta/\gamma$ -crystallins is a family member present in the urochordate, *Ciona intestinalis* (sea squirt). Urochordates are invertebrates, but are in the Phylum Chordata, as are vertebrates. Shimeld et al. (2005) reported that the *Ciona*  $\beta/\gamma$ -crystallin gene is expressed in a cell-specific manner in sensory organs of *Ciona* and that its promoter is capable of directing expression to the visual system in transgenic *Xenopus* tadpoles. Their data support the idea that a common ancestor of urochordates and vertebrates contained a single domain  $\beta/\gamma$ -precursor gene from which both the *Ciona* and vertebrate  $\beta/\gamma$ -crystallins have evolved. Interestingly, the invertebrate members of the superfamily are calcium binding proteins (Srivastava et al., 2014), while the vertebrate lens  $\beta/\gamma$ -crystallins and a few other members of the superfamily that are expressed in vertebrates have lost this trait or had it greatly attenuated. It seems likely that through a series of gene duplications and fusions in the early vertebrates, the  $\beta$ -crystallins were formed and that the  $\gamma$ -crystallins subsequently arose from a  $\beta$ -crystallin by loss of the intron separating the two motifs comprising the  $\beta$ -crystallin domain (Wistow, 1995).

### 3. EXPRESSION OF $\beta$ -CRYSTALLINS OUTSIDE OF THE LENS

As noted above, mammalian crystallins were, until the 1990s, generally considered lens-specific proteins. With respect to the  $\beta/\gamma$ -crystallins, there had been suggestions as early as 1968 (Clayton et al.) that they were expressed at low levels in non-lens tissues of the chick embryo. Head et al. (1995) were the first to report clear evidence for expression of  $\beta$ -crystallin outside the lens in mammals, finding  $\beta$ B2-crystallin in both murine and feline neural retina and retinal pigmented epithelium (RPE). A number of other laboratories have reported the presence of  $\beta/\gamma$ -crystallins in the retina (Dirks et al., 1998, Magabo et al., 2000), including several that have found increased levels of these proteins following damage to the retina by intense light exposure, ocular hypertension, or retinal tears (Organisiak et al., 2006;

Vazquez-Chona et al., 2004). Piri et al. (2007) found  $\beta$ -crystallins in retina to be predominantly in retinal ganglion cells and their expression to be affected by ocular hypertension. Crystallins, including  $\beta$ A3-crystallin, have been reported to be present in human drusen (Crabb et al., 2002). Reviews of crystallin gene expression in the retina exist (Xi et al., 2003; Andley, 2007). Expression of  $\beta/\gamma$ -crystallins outside of the eye appears to be quite limited. Magabo et al., (2000) demonstrated the presence of  $\beta$ B2-crystallin protein in both rat brain and testis, but found no detectable gene expression in a variety of other tissues tested. Recent studies from our laboratory using *in situ* hybridization and immunohistochemistry have analyzed the expression of  $\beta$ A3/A1-crystallin in the rat eye from embryonic stages to adulthood (Parthasarathy, et al., 2011). Besides robust expression in the lens, expression was also detected in the retinal astrocytes as well as in RPE, and in some retinal ganglion cells. Outside of the eye, we have detected expression only in astrocytes from the brain (Sinha, et al., 2008).

The presence of  $\beta/\gamma$ -crystallins outside of the lens strongly suggests that these proteins have functions other than their refractive function as a lens protein. This is also consistent with the concept that crystallins have all been recruited to the lens from among pre-existing proteins and with the fact that non-lens functions are now known for  $\alpha$ -crystallin and for most, if not all, taxon-specific crystallins (Piatigorsky, 2007).

With respect to  $\beta$ A3/A1-crystallin, while no specific extra-lenticular function has been demonstrated, considerable evidence that such functions do exist has been presented, and several putative functions have been proposed. The first such function, put forth by Srivastava and Srivastava (1999), was that  $\beta$ A3/A1-crystallin has a proteolytic activity. The activity was detected *in vitro* and only under very specific, and seemingly non-physiological, conditions. This finding has not been independently reproduced, and its physiological significance remains to be established.

Another potential function for  $\beta/\gamma$ -crystallins relates to stimulation of axonal outgrowth from retinal ganglion cells. This idea originated in studies that demonstrated that injuring the lens was beneficial to optic nerve regeneration following axotomy. Two groups reported this remarkable effect of lens injury (Leon et al., 2000; Fischer et al., 2000; 2008). While the Benowitz laboratory focused on macrophage-derived factors produced in response to lens rupture, the Thanos group has produced evidence both *in vivo* and *in vitro* that  $\beta/\gamma$ -crystallins released by the injured lens stimulate axon regeneration. In 2007, they demonstrated expression of  $\beta$ B2-crystallin in cultured retinal ganglion cells, particularly in filopodia and growth cones, and reported that  $\beta$ -crystallins are secreted into the culture medium.  $\beta$ B2-crystallin, which is extremely stable and highly soluble, was shown to promote neurite outgrowth both in cultures of retinal ganglion cells and also in primary hippocampal neurons (Liedtke et al., 2007). Subsequently, Fischer et al., (2008) generalized these effects to the entire  $\beta/\gamma$ -crystallin superfamily, showing that purified  $\beta$ - or  $\gamma$ -crystallin fractions, but not  $\alpha$ -crystallin or bovine serum albumin, could mimic the effects of lens injury on axon regeneration (Figure 1). They also showed that addition of exogenous  $\beta/\gamma$ -crystallins (1 mg injected intravitreally in the rat eye) activated retinal glial cells, stimulating astrocytes to secrete ciliary neurotrophic factor (CNTF) and up-regulating its downstream signaling pathway (JAK/STAT3). These studies, which have been recently reviewed by

Thanos et al., (2012), suggest that  $\beta/\gamma$ -crystallins are able to alter the activity of signaling pathways by a mechanism, or mechanisms, yet to be identified.

#### 4. $\beta$ A3/A1-CRYSTALLIN AND LEAKY RIBOSOMAL SCANNING

$\beta$ A3/A1-crystallin is unique amongst all crystallins. A single *Cryba1* mRNA uses alternative translation by leaky ribosomal scanning to produce two closely related polypeptides,  $\beta$ A3 and  $\beta$ A1, from two different translation start sites (Werten et al., 1999). The two polypeptides are identical except that the longer one ( $\beta$ A3-crystallin) has 17 additional amino acid residues at its amino terminus. Leaky scanning occurs when the small ribosome subunit scanning over the mRNA molecule skips the first start codon (if the recognition site is poor) and subsequently initiates translation from a downstream alternate start codon. Translation start site selection is a highly regulated process and is critical for differential gene regulation in eukaryotes (Sonnenberg and Hinnebusch, 2009).

#### 5. THE NUC1 RAT: A SPONTANEOUS MUTATION IN $\beta$ A3/A1-CRYSTALLIN

Further insight into the specific functions of the  $\beta$ A3- and  $\beta$ A1-crystallin polypeptides has been provided by our studies on a spontaneous mutant (Nuc1) in the Sprague-Dawley rat (Hose et al., 2005; Sinha et al., 2005; Zhang et al., 2005; Gehlbach et al., 2006; Sinha et al., 2008; Parthasarathy et al., 2011; Zhang et al., 2011). The Nuc1 mutation is a 27 base pair insertion in exon 6 of the *Cryba1* gene that results in loss of an absolutely conserved glycine residue (G178 in  $\beta$ A3) and its replacement by 10 new amino acid residues (Sinha et al., 2008; Figure 2). The mutant protein is synthesized, but based on molecular modeling and analysis of proteins from mutant lenses, does not form normal associations with other  $\beta$ -crystallin polypeptides. The mutant polypeptides appear to aggregate and are found entirely in the void fraction following gel exclusion chromatography, indicating a molecular mass greatly exceeding that of normal  $\beta$ -crystallin oligomers (Sinha et al., 2008; Figure 3).

Initial interest in the Nuc1 rats resulted from the obvious dense nuclear cataracts (Sinha et al., 2005). Subsequent study demonstrated that animals with the nuclear cataract phenotype were heterozygotes for the Nuc1 mutation, and that homozygotes had severe microphthalmia and lenses that ruptured at the posterior capsule before birth (Hose et al., 2005; Sinha et al., 2005; Figure 4). Since numerous crystallin mutations that cause cataracts had been described, both in human patients and in animals, the cataracts in Nuc1 were of limited interest. However, in contrast to these other mutations, which seemed to affect only the lens, Nuc1 had other ocular manifestations. These conditions, which were obvious only in the homozygotes, included, in addition to microphthalmia, an increase in retinal thickness associated with decreased apoptosis, and failure of the hyaloid vasculature to regress normally, a condition known as persistent fetal vasculature, or PFV (Goldberg, 1997; Sinha et al., 2005; Zhang et al., 2005; Gehlbach et al., 2006; Parthasarathy et al., 2008 and Zhang et al., 2011; Figures 5 and 6). Electroretinographic (ERG) deficits were observed in the Nuc1 homozygous animals (Figure 7) as well as abnormalities in the development of retinal neurons, astrocytes and the retinal vasculature (Gehlbach et al., 2006). The evidence, taken together, was consistent with the idea that mutation of  $\beta$ A3/A1-crystallin impaired the ability of the retina to successfully complete the cellular remodeling necessary to become



mature and fully functional. Our data suggest that the effects in the Nuc1 homozygous retina result from total loss of the original or “non-crystallin” function of  $\beta$ A3/A1-crystallin. It is possible that this “non-crystallin” function of  $\beta$ A3/A1-crystallin is also necessary in the lens, since the orientation and organization of differentiating immature lens fibers is abnormal in Nuc1 homozygotes, but not in heterozygotes (Sinha et al., 2005).

To establish which cell types in the retina express  $\beta$ A3/A1-crystallin, laser capture microdissection was performed on wild type (WT) rat retina; *Cryba1* message was detected only in the ganglion cell layer and in the RPE (Sinha et al., 2008). Immunohistochemistry with antibodies specific to  $\beta$ A3/A1-crystallin confirmed its presence in RPE cells, and confocal microscopy showing colocalization of staining in the ganglion cell layer with Glial Fibrillary Acidic Protein (GFAP) demonstrated its presence in astrocytes (Sinha et al., 2008; Figure 8). Subsequent studies utilizing *in situ* hybridization and immunohistochemistry demonstrated expression of  $\beta$ A3/A1-crystallin in some ganglion cells as well (Parthasarathy et al., 2011). While no studies have been conducted on the possible role of  $\beta$ A3/A1-crystallin in ganglion cells, some progress has been made toward understanding its function(s) in RPE cells and in ocular astrocytes.

## 6. $\beta$ A3/A1-CRYSTALLIN IN GLIA OR GLIA-LIKE CELLS OF THE RETINA

### 6.1 Astrocytes

Astrocytes, glial cells unique to the central nervous system (CNS), were first visualized in 1913 by Ramon y Cajal using a gold sublimate stain (Kimelberg, 2004). Astrocytes have usually been viewed as having a passive role in the CNS; only in the past decade has emerging research suggested an active role in brain function and information processing (Parpura and Haydon, 2009; Allen and Barres, 2009). The role of astrocytes in the retina, however, remains to be determined.

Astrocytes are one of the two types of macroglial cells found in mammalian retinas. Unlike Muller cells, which span the entire thickness of the retina and are present in all mammals, astrocytes are mainly confined to the retina’s inner surface (Ling et al., 1989; Watanabe and Raff, 1988) and are closely associated with retinal blood vessels. Avascular retinas contain no astrocytes (Stone and Dreher, 1987; Schnitzer, 1988). Retinas that are diffusely vascularized contain diffusely distributed astrocytes (Stone and Dreher, 1987; Schnitzer, 1988), and those that are vascularized in a restricted region contain astrocytes only in that region (Stone and Dreher, 1987; Schnitzer, 1988).

Retinal astrocytes are immigrant cells in the retina, arising from the neuroepithelial cells of the optic stalk, the primordium of the optic nerve (Small et al, 1987). During retinal histogenesis, they migrate from the optic nerve into the inner retina where they become closely associated with retinal blood vessels (Stone and Dreher, 1987; Watanabe and Raff, 1988; Dorrell and Friedlander, 2006). Astrocytes first appear in the developing rat optic nerve at E (embryonic day) 16 and increase in number until 6 weeks after birth (Miller et al., 1985). They form a corona of processes around the optic nerve head at E18, cover approximately 35% of the retina at birth, and reach the periphery of the retina by P (postnatal day) 8 (Ling et al., 1989).

Our studies to date indicate that  $\beta$ A3/A1-crystallin has an important regulatory function in astrocytes (Sinha et al., 2008; Zhang et al., 2011; Ma et al., 2011; Valapala et al., 2013). There were definite morphological and functional abnormalities in the Nuc1 astrocytes as compared to WT astrocytes. In addition, comparison of retinal flat mounts from WT and Nuc1 animals demonstrated defective formation in the mutants of the normally highly organized honeycomb-like astrocyte network (Sinha et al., 2008; Figure 9, A–D). This network serves as a template for the proper formation of the retinal vasculature in rodents. The network of vessels in the Nuc1 retina was much less dense than in the normal animals, and there was leakage from some vessels in the Nuc1 retina, consistent with a failure in the process of retinal remodeling (Gehlbach et al., 2006; Sinha et al., 2008; Figure 9, E–I and Figure 10). Retinal astrocytes with the Nuc1 mutation were shown to migrate faster than WT astrocytes in vitro. In Nuc1 homozygous animals the mutant astrocytes migrated abnormally out of the retina, ensheathing the hyaloid artery where they likely were a primary cause of the PFV observed in these animals (Zhang et al., 2011; Figure 11).

Further, in loss- and gain-of-function mouse models for  $\beta$ A3/A1-crystallin, as well as in the Nuc1 mutant rat, we showed that this protein is required to form the organized astrocyte template that is essential for the remodeling of retinal vessels (Sinha et al., 2008; Sinha et al., 2012). Remodeling is a complex developmental process in which programmed cell death (PCD) plays a major role. PCD of astrocytes in the rodent retina peaks between P0 and P5 and declines by P15, coinciding with the time of astrocyte template for mation (Chan-Ling et al., 2009). We have shown that  $\beta$ A3/A1-crystallin regulates anoikis (Ma et al., 2011), a type of apoptotic (programmed) cell death, which is initiated by the loss of cell anchorage (Gilmore, 2005; Chiarugi and Giannoni, 2008). PCD is carefully coordinated to control the number and organization of cells in tissues and organs. Our data indicate that loss of  $\beta$ A3/A1-crystallin induces IGF-II and increases cell survival by regulating the PI3K/AKT/mTOR and ERK pathways, thereby protecting astrocytes from anoikis-mediated cell death (Ma et al., 2011).

To better understand which signaling mechanism  $\beta$ A3/A1-crystallin might regulate during the formation of the astrocyte template, we studied the Notch signaling pathway in optic nerve astrocytes. We chose Notch because it is the most significantly enriched signaling pathway in developing tissues and is known to regulate pattern formation in other systems (Collier et al., 1996; Lai, 2004). Moreover, Notch signaling has an important role during differentiation and maturation of brain astrocytes (Givogri et al., 2006; Carlen et al., 2009). We found that optic nerve astrocytes express the receptor Notch 1, as well as the ligand, Jagged1 (Valapala et al., 2013; Figure 12). This suggests that in astrocytes, Notch is involved in juxtacrine signaling, where Jagged1 from one astrocyte activates the Notch receptor on a neighboring astrocyte. This juxtacrine signaling may be critical to the proper organization of astrocytes in the developing retina. Binding of the ligand activates two sets of proteolytic cleavages in the Notch receptor. The second set of proteolytic cleavages, by the  $\gamma$ -secretase multiprotein complex (including presenilin, nicastrin, APH1 and PEN2), results in the release of the Notch intracellular domain (NICD), which translocates to the nucleus to activate Notch target genes (Spasic and Annaert, 2008; Kopan and Ilagan, 2009). Surprisingly, Nuc1 optic nerve astrocytes were found to express Notch1 transcript at twice the concentration of the normal cells (Valapala et al., 2013). However, when a specific



antibody for NICD was used, the Nuc1 cells were shown to have less NICD than the control cells, a finding confirmed by immunofluorescence and confocal microscopy. Expression of the Notch target genes *Hey1* and *Hes1* was also reduced by 65–75% in the Nuc1 cells. When studies were undertaken to determine the mechanism whereby Notch signaling is decreased in the Nuc1 cells, it was found that the proteolytic processing of Notch was decreased in the Nuc1 cells, leading to decreased generation of NICD. When NICD was generated exogenously in the Nuc1 cells, it was translocated efficiently to the nucleus and activated transcription of target genes normally (Valapala et al., 2013).

Notch processing occurs in the lysosome, and the activity of  $\gamma$ -secretase, an acid protease, is dependent upon proper lysosomal acidification (Vaccari et al., 2010). The acidic luminal environment of endosomes, lysosomes and secretory vesicles is established by the vacuolar-type proton ATPase (V-ATPase), which pumps protons into the lumen (Yan et al., 2009; Sun-Wada et al., 2003; Vaccari et al., 2010). Interestingly, in *Drosophila*, various mutants that show impaired acidification due to alteration of V-ATPase activity affect Notch signaling (Yan et al., 2009; Vaccari et al., 2010). The lysosomes in the retinal astrocytes of Nuc1 animals had a significantly higher pH than did those in the control cells and the activity of V-ATPase was reduced (Valapala et al., 2013; Figure 13).

To completely eliminate any possible effects secondary to damage to the lens or other ocular structures affected in the Nuc1 model, astrocytes isolated from the  $\beta$ A3/A1-crystallin floxed mice were transfected with a *Cre*-recombinase adenoviral construct to ablate *Cryba1* expression (Valapala et al., 2013). Data from studies on these cells confirmed the results with the Nuc1 astrocytes: lysosomal pH was elevated, V-ATPase activity was decreased, NICD levels decreased, and expression of Notch target genes also decreased relative to cells transfected with empty vector. All of these abnormalities could be reversed, both in the floxed astrocytes transfected with *Cre* and in primary cultures of Nuc1 astrocytes, by over-expression of  $\beta$ A3/A1-crystallin (Valapala et al., 2013). Thus,  $\beta$ A3/A1-crystallin has a fundamental effect on Notch signaling in astrocytes (Figure 14), and the improper template formation and patterning by retinal astrocytes in the Nuc1 animals may be the result of defective Notch signaling.

Previous studies have shown a direct link between Notch signaling and STAT3 (Signal transducers and activators of transcription 3) activation to be important for astroglial differentiation and regulation of GFAP expression (Nagao et al., 2007; Chenn, 2009). Notch signaling induces astrocyte differentiation in gliogenic cells by STAT3-mediated activation of the GFAP promoter (Ge et al., 2002). Thus, Notch and its downstream signaling mediators are involved in the regulation of GFAP, and this pathway is also important for the regulation of astrocyte-derived VEGF (Vascular Endothelial Growth Factor). This prompted us to determine if STAT3 activation might also be affected by  $\beta$ A3/A1-crystallin in astrocytes, as we have shown for Notch (Valapala et al., 2013). Our data suggests that in astrocytes, STAT3 and  $\beta$ A3/A1-crystallin are co-regulated (Valapala et al., unpublished observations). This could lead to a positive feedback loop in astrocytes, with  $\beta$ A3/A1-crystallin participating in the phosphorylation of STAT3 in the cytosol and, in turn, STAT3 regulating the transcription of *Cryba1* in the nucleus. This modulation of the Notch/STAT3 signaling axis by  $\beta$ A3/A1-crystallin appears to be involved in regulating GFAP, as well as

secretion of VEGF. Loss of *Cryba1* in astrocytes reduces gene promoter activity for GFAP and decreases GFAP protein expression (Gehlbach et al., 2006; Sinha et al., 2008; Valapala et al; 2013). We postulate that  $\beta$ A3/A1-crystallin is a local mediator in astrocytes, regulating the Notch/STAT3 signaling axis and stimulating the secretion of VEGF required for remodeling of retinal vessels.

## 6.2 Retinal Pigment Epithelium

The retinal pigmented epithelium (RPE) is a single layer of pigmented cells situated between the neurosensory retina and the choroid, which is the vascular layer at the back of the eye (Strauss, 2005). The RPE serves many crucial physiological roles that are vital for the normal functioning of the retina (Kevany and Palczewski, 2010). One such function is critical to the well-being of the light sensitive cells of the retina, called the photoreceptors (Kevany and Palczewski, 2010). Photoreceptors continually grow new outer segments (OS), where the photosensitive proteins are deployed to absorb incident light. As the OS are renewed from the base, the terminal portions become damaged and are released into the space between the photoreceptors and RPE. The RPE cells must engulf this material, digest it by a process called phagocytosis, and recycle the constituent molecules back to the retina so the photoreceptors can produce new OS (Kevany and Palczewski, 2010). In a related process known as autophagy, damaged proteins or organelles within the cell are collected, degraded, and removed (Feng et al., 2014). Both phagocytosis and autophagy depend upon the activity of lysosomal hydrolases for final degradation and are essential for keeping the photoreceptors healthy through the constant recycling of OS (Kaarniranta et al., 2013). We recently provided novel evidence that  $\beta$ A3/A1-crystallin, acting through V-ATPase/mTORC1 signaling, is essential for normal lysosome-mediated clearance in the RPE (Valapala et al., 2014).

In RPE cells of normal rats, it was demonstrated by immunoelectron microscopy that  $\beta$ A3/A1-crystallin specifically localizes to lysosomes (Zigler et al., 2011; Figure 15). In contrast, in rats homozygous for the Nuc1 mutation, which do express the larger mutant forms of  $\beta$ A3- and  $\beta$ A1-crystallins, immunoreactivity was randomly distributed within the cytoplasm of RPE cells, with no indication of lysosomal localization. Ultrastructural analysis revealed abnormalities in the RPE of young Nuc1 mutant animals, including disorganization of basal infoldings and an increased number of vacuoles. By one year of age, lipofuscin-like aggregates and large vacuoles containing undigested cellular organelles and debris as well as photoreceptor outer segment discs were abundant in the RPE of the mutant animals (Figures 16 and 17). Decreased activity of the lysosomal hydrolase, Cathepsin D, and a decrease in the concentration of the active form of microtubule-associated protein 1A/1B light chain 3 (LC3-II, an indicator of autophagy activity) were observed in the mutant RPE. These findings are consistent with the observed decrease in the lysosome-mediated degradation of cargo contained in phagosomes and autophagosomes (Figure 18). This suggests that  $\beta$ A3/A1-crystallin has an important function in the lysosomes of the RPE cell. Two factors must be considered when comparing this putative function with the structural role of  $\beta$ A3/A1-crystallin in the lens. First, the concentration of the protein in the lens is extremely high compared to its concentration in RPE. Siezen et al (1986) and later investigators have clearly demonstrated that *in vitro*, protein concentration markedly

affects the state of oligomerization of  $\beta$ -crystallin. Secondly, the function in the lysosome must occur at the very acidic (~pH 4.5) environment of this organelle, as compared to the neutral pH in the lens. The increased acidity could be expected to cause conformational change in the protein. Thus, it is likely that not only the function, but also the structure, of  $\beta$ A3/A1-crystallin is different in RPE as compared to lens.

To definitively demonstrate that  $\beta$ A3/A1-crystallin has cellular functions outside the lens that are distinct from its refractive function in the lens, a genetic approach was adopted. We generated a conditional knockout mouse in which *Cryba1* was deleted specifically from RPE using the Cre-loxP technology. *Cryba1* floxed mice (Valapala et al., 2013) were mated with Best1-cre mice (Iacovelli et al., 2011) that express Cre recombinase specifically in RPE to produce *Cryba1* cKO mice (Valapala et al., 2014). Live cell imaging of cultured primary RPE cells showed accumulation of (auto)phagosomes in the cells isolated from cKO animals as compared to those isolated from control animals. Over-expression of  $\beta$ A3/A1-crystallin in the cKO cells alleviated this accumulation, while SiRNA knockdown of *Cryba1* expression in control cells mimicked the effect seen in cKO cells. Sub-cellular fractionation of normal RPE cells revealed that  $\beta$ A3/A1-crystallin is a lysosomal luminal protein (Figure 19, A-B). Immunoprecipitation studies suggest that it binds to the  $V_0$ -ATPase  $\alpha 1$  subunit (Figure 20); if this result is confirmed,  $\beta$ A3/A1-crystallin would be the first binding partner identified for the  $V_0$  domain ( $\alpha 1$  subunit) of V-ATPase in a mammalian system. The  $\alpha 1$  subunit is known to mediate phagosome-lysosome fusion in the brain (Peri and Nusslein-Volhard, 2008; Valapala et al., 2014). Since acidification of the endolysosomal compartments is essential for lysosomal clearance of (auto)phagosome cargo, the endolysosomal pH was determined in RPE cells from *Cryba1* floxed and cKO mice. Consistent with the findings in astrocytes, the pH was higher in the knockout cells (Figure 21), and the activity of V-ATPase, the proton pump responsible for the acidification, was significantly decreased (Figure 22). Again, over-expression of  $\beta$ A3/A1-crystallin in the cKO cells rescued both the pH and the V-ATPase activity to the normal range (Valapala et al., 2014; Figures 21 and 22).

Recently, V-ATPase has been shown to be an important component of the mTOR (mechanistic target of rapamycin) signaling pathway (Bar-Peled and Sabatini, 2014). mTOR is an atypical serine/threonine kinase that has been conserved throughout evolution. It interacts with many proteins to form at least two distinct multiprotein complexes called mTORC1 and mTORC2 (Laplante and Sabatini, 2013). On the basis of physical interactions with rapamycin, mTORC1 and mTORC2 have been respectively characterized as rapamycin-sensitive and rapamycin-insensitive complexes (Laplante and Sabatini, 2013). The mTOR-containing complexes also have different upstream inputs and downstream outputs (Zoncu et al., 2011). mTORC1 integrates multiple signals either to promote cellular growth when growth factors, nutrients and energy are available, or to induce catabolic processes during stress (Zoncu et al., 2011). The downstream effects of active mTORC1 include translation of mRNA, suppression of autophagy, ribosome biogenesis and activation of transcription leading to mitochondrial metabolism (Zoncu et al., 2011). mTORC2 promotes cellular survival, in part through regulating cytoskeletal dynamics, ion transport, and growth (Zoncu et al., 2011). V-ATPase has been shown to play a direct role in amino acid signaling to mTORC1 (mTOR complex 1) and to be a component of the mTOR

pathway in lysosomes (Zoncu et al., 2011). Previous studies have shown the existence of bidirectional regulatory loops between mTORC1 and V-ATPase (Pena-Llopis et al., 2011), and it is known that luminal pH is involved in mTORC1 regulation and has important implications for its ability to control autophagy (Korolchuk et al., 2011). Following induction of autophagy *in vivo* by nutrient starvation, there was a significant increase in the level of p-mTOR (active mTOR) in the cKO cells relative to control cells. Further, there was a significant decrease in p-AKT in the knock-out cells, although total AKT and mTOR levels were similar in the cKO and control cells. When cultured cKO astrocytes were transfected with a vector construct over-expressing  $\beta$ A3/A1 crystallin, both of these effects were reversed. Downregulation of *Cryba1* in control cell cultures decreased the level of p-AKT and increased p-mTOR, mimicking the findings in the cKO cells *in vivo* (Valapala et al., 2014; Figure 23). Therefore, crosstalk between the V-ATPase and mTORC1 appears to be necessary for regulating autophagy, and *Cryba1* is required for proper functioning of this signaling axis (Valapala et al., 2014). Based on our studies to date, we speculate that  $\beta$ A3/A1-crystallin may regulate mTORC1 activity in RPE cells by modulating V-ATPase in the lysosomal lumen (Figure 24).

Finally, to determine whether conditional knockout of  $\beta$ A3/A1-crystallin in the RPE had an effect on retinal function, electroretinography was performed on cKO and control mice (Figure 25). At 2 months of age, only the photopic b-wave amplitudes were decreased in the cKO animals, whereas by 7 months of age the a-wave amplitudes were also decreased. This indicates that the cone function is affected first, but that both rods and cones are ultimately affected by loss of *Cryba1* expression in RPE (Valapala et al., 2014).

## 7. $\beta$ A3/A1-CRYSTALLIN IN OCULAR HEALTH AND DISEASE

There is abundant evidence, both from human mutations and from experimental animal studies, that  $\beta/\gamma$ -crystallins play a critical role in the maintenance of lens transparency. Numerous mutations in these proteins cause cataracts (see reviews by Hejtmancik, 2008 and Graw, 2009) with a variety of phenotypes. Almost all of these cataracts are dominant traits, so the human patients studied will be heterozygotes for the mutation; thus abnormal phenotypes resulting from loss or modification of “non-crystallin” functions of the protein in these patients would not be present if such a trait were recessive. In the Philly mouse, which has a deletion mutation in the  $\beta$ B2-crystallin gene, cataract is a dominant phenotype, but sub-fertility, presumably associated with the fact that the protein was detected in both sperm and ovary, was reported only in mice homozygous for the mutation (DuPrey et al, 2007). A single report in the human genetic literature of an individual presumed to be homozygous for a  $\beta$ B2-crystallin mutation is suggestive (Litt et al, 1997). While heterozygotes for this mutation developed cataracts requiring extraction in adulthood, the homozygous individual required cataract surgery by age 5 and lost all visual perception by adolescence. Thus, while the function or functions of  $\beta/\gamma$ -crystallins outside of the lens remain unclear, evidence is accumulating that such functions do exist and that in certain cell types they may be important.

In the Nuc1 rat, which has a mutation in the  $\beta$ A3/A1-crystallin gene, heterozygotes have congenital cataracts (Sinha et al., 2005), whereas non-lens ocular effects are apparent only in

the homozygotes. One such abnormality that we have studied is the persistence of the fetal vasculature (PFV) (Zhang et al., 2005; Zhang et al., 2011). During early development of the mammalian eye, there is a transient network of blood vessels arising from the area of the optic nerve, extending through the vitreous, and surrounding the developing lens. Called the fetal vasculature, these blood vessels nourish the lens and retina before formation of the retinal vasculature. When vessels begin to appear in the retina, the fetal vasculature normally degenerates and disappears completely, prior to birth in humans and within the first few weeks of post-natal development in rodents, to provide an optically clear path between the cornea and the retina. One of the more common congenital, developmental disorders of the eye, persistent fetal vasculature, results from the complete or partial failure of this vascular regression (Goldberg, 1997). PFV is a disease that leads to blindness or serious loss of vision, with few treatment options at present, in otherwise normal children. The Nuc1 animal model provides unique opportunities to investigate molecular mechanisms underlying PFV disease, thereby helping us to better understand the disease etiology and potentially to develop therapies to prevent or ameliorate the condition. Our findings suggest that abnormalities in Nuc1 astrocytes cause them to migrate faster and to ensheath the hyaloid vasculature, thereby inhibiting its regression. Further, the Nuc1 mutant rats, with an aberrant astrocyte template and defective retinal remodeling, show an age-dependent increase in the expression of colony stimulating factor-1 receptor (CSF-1R) and infiltration of microglia, suggesting the activation of an immune response (unpublished observations). Microaneurysm formation, intravascular deposits, vessel leakage and blockage of vessels, characteristic clinical features of diabetic retinopathy, have been observed in Nuc1 rats (Gehlbach et al., 2006). The Nuc1 model may provide mechanistic insights as to how abnormalities in astrocytes contribute to both neuronal cell death and vascular changes in diabetic retinopathy.

As noted above, we have reported evidence suggesting that  $\beta$ A3/A1-crystallin interacts directly with the  $V_0$ -ATPase a1 subunit (Valapala et al., 2014). *In vivo*, V-ATPase activity is regulated by several mechanisms, including reversible dissociation of the  $V_1$  and  $V_0$  domains (Forgac, 2007). We speculate that binding of  $\beta$ A3/A1-crystallin to  $V_0$ -ATPase a1 regulates H<sup>+</sup>-pumping activity by assembly/disassembly of the holoenzyme. Interestingly, presenilin 1, a protein that is often mutated in Alzheimer's disease, is required for the  $V_0$  a1 subunit of V-ATPase to be translocated to lysosomes (Lee et al., 2010). In neurodegenerative diseases of the brain, such as Alzheimer's and Parkinson's, a number of studies suggest that defective lysosomal clearance is involved in the disease pathogenesis (Bergamini et al., 2004; Keller, 2004; Shintani and Klionsky, 2004). It should also be noted that in Alzheimer's disease, optic nerve degeneration and loss of retinal ganglion cells have been reported (Hinton et al., 1986). Further knowledge of the binding complex between V-ATPase and  $\beta$ A3/A1-crystallin in the RPE could identify a specific role of  $\beta$ A3/A1-crystallin in the acidification of the endolysosomal system, thereby revealing new therapeutic targets for retinal degenerative diseases that result from abnormalities in lysosomal function.

It is also likely that the accumulation of undegraded intracellular material disturbs RPE homeostasis and triggers a parainflammatory response in an attempt to restore normal RPE

function. Tissue homeostasis is monitored by resident tissue macrophages that normally remain in a basal homeostatic state. When cellular homeostasis is disrupted, a state of para-inflammation will be initiated (Medzhitov, 2008). However, when tissue malfunction is excessive, the support provided by resident macrophages may be insufficient, requiring recruitment of additional macrophages and thereby, induction of chronic inflammation. We believe that prolonged impairment of lysosomal-mediated clearance in RPE, as seen in our *Cryba1* cKO model, can convert para-inflammation to chronic inflammation (Valapala et al., 2014b). Indeed, dysregulated para-inflammation, leading to a chronic inflammatory state, has been proposed to play an important role in other diseases, including type 2 diabetes, cardiovascular disease and age-related diseases of the retina (Medzhitov, 2008; Xu et al., 2009).

Aging is the main risk factor for the development of various diseases, including age-related macular degeneration (AMD); therefore, understanding the mechanisms regulating aging may lead to new avenues of treatment that could delay the development of such diseases. The mTOR pathway regulates many major cellular processes and is implicated in an increasing number of pathological conditions, including cancer, obesity, type 2 diabetes and neurodegeneration (Efeyan et al., 2012). In an elegant study (Zoncu et al., 2011), V-ATPase was shown to be a component of the mTOR pathway in lysosomes. Previous studies have shown the existence of bidirectional regulatory loops between mTOR and V-ATPase (Pena-Llopis et al., 2011). This crosstalk between V-ATPase and mTOR is necessary for regulating cellular processes, including autophagy, and  $\beta$ A3/A1-crystallin may be an essential component of this signaling axis (Valapala et al., 2014). A multicenter study (Interventions Testing Program) conducted by the National Institute of Aging, NIH, reported that inhibition of mTOR with rapamycin expands maximal and median life spans in mice (Harrison et al., 2009). The study suggested that mTORC1 inhibition may be effective in treating age-related diseases even when the treatment is initiated in middle-aged humans. However, the mechanism by which mTORC1 inhibition increases longevity in mammals is unresolved. It should also be noted that strong inhibition of mTORC1 would have considerable adverse effects, particularly if treatment were required long term for chronic conditions. A better understanding of the functions of the m-TOR interacting proteins might permit the development of more specific modulators of the mTOR complexes that perturb their function only toward specific effectors. Since defective lysosomal homeostasis in RPE has been suggested to be involved in the pathogenesis of AMD disease (Bird, 2010; Chen et al., 2011; Tseng et al., 2013), one could speculate that  $\beta$ A3/A1-crystallin might be one such effector that could be targeted in an effort to restore or maintain normal lysosomal function.

## 8. FUTURE DIRECTIONS

In loss and gain-of-function mouse models for  $\beta$ A3/A1-crystallin and in the Nuc1 mutant rat, we show that this protein is needed to establish the specific mechanisms and signaling molecules involved in crucial physiological processes. It must be recognized that these mechanisms could be different in the different cell types where the protein is expressed. Complicating the efforts to get at this most important question are several other issues which remain unresolved and which could have major impact on our understanding of these functions. First, two distinct proteins ( $\beta$ A3- and  $\beta$ A1-crystallin) are produced from the



*Cryba1* gene by leaky ribosomal scanning. Are they equivalent and inter-changeable in terms of their role as crystallins in the lens and with respect to their putative functions in other cell types? Alternatively, does each protein have an independent function in some or all of these situations? Secondly, do  $\beta$ A3- and  $\beta$ A1-crystallins exert their functions independently or only when associated with other  $\beta$ -crystallin polypeptides. The dogma from studies on the lens is that the  $\beta$ -crystallins exist only as dimers or higher oligomers of the seven polypeptides constituting the family. This conclusion is based on *in vitro* studies of lens homogenates and association studies using dilute solutions of isolated or recombinant proteins, which may not reflect the situation in the intact lens with its extremely high protein concentration. Recent studies have cast doubt on the long established concept of  $\alpha$ -crystallin structure in the lens (Gangalum et al., 2012), and the same concerns regarding extrapolation from *in vitro* to *in vivo* status could apply in this situation as well. With respect to the situation in other cell types, it is not even clear at this point what complement of  $\beta$ -crystallins, aside from  $\beta$ A3/A1-crystallin, is expressed in RPE cells, astrocytes, or ganglion cells. In summary, a growing body of evidence supports the hypothesis that  $\beta/\gamma$  crystallins, and in particular  $\beta$ A3/A1-crystallin, have specific cellular functions distinct from their role in the lens as structural elements contributing to refractivity and transparency. In the case of  $\beta$ A3/A1-crystallin it appears that these “non-crystallin” functions are mediated via signaling pathways. It should be noted that it is highly likely that the  $\beta$ A3/A1-crystallin in lysosomes, where the “non-crystallin” function occurs, is different not only in function, but also in structure from the protein in the lens. Studies on recombinant proteins *in vitro* have shown that at low concentration,  $\beta$ A3/A1-crystallin exists as a mixture of monomers and dimers rather than the dimers and higher oligomers believed to be present in the lens (Sergeev et al., 2000). The very acidic conditions within lysosomes could also be expected to affect the conformation of the polypeptides.

It is likely that  $\beta$ A3/A1-crystallin regulates multiple cellular processes in astrocytes and RPE by modulating specific signaling pathways mediated by V-ATPase. Our *in silico* docking and co-immunoprecipitation results suggest that  $\beta$ A3/A1-crystallin and  $V_0$ -ATPase  $\alpha 1$  subunit may interact, with formation of a complex. Future Nuclear Magnetic Resonance (NMR) studies may establish that such binding actually occurs and may also further our understanding of the structure-function relationship of this binding. Should the binding of  $\beta$ A3/A1-crystallin and  $V_0$ -ATPase  $\alpha 1$  be confirmed by NMR, it could be speculated that the putative lysosomal sorting signal present on the surface of  $\beta$ A3/A1-crystallin (Zigler et al., 2011) might be important in the trafficking of V-ATPase to lysosomes in astrocytes and RPE cells, since previous studies reported that the  $V_0$ -ATPase  $\alpha 1$  subunit is important in organelle targeting (Lee et al., 2010). We have also demonstrated that  $\beta$ A3/A1-crystallin participates in the mTOR signaling pathway in the lysosomes of RPE cells (Valapala et al., 2014). To understand how  $\beta$ A3/A1-crystallin affects lysosomal function, it will be necessary to elucidate its interaction with mTORC1. V-ATPase, previously shown to be a component of the mTORC1 pathway in lysosomes (Zoncu et al., 2011), may be the source of this interaction. Other studies have shown the existence of bi-directional regulatory loops between mTOR and V-ATPase (Pena-Llopis, 2011). This crosstalk is necessary for regulating cellular processes and  $\beta$ A3/A1-crystallin may be an essential component of this signaling axis.

To better understand the “crystallin” function of  $\beta$ A3/A1-crystallin, we will determine how its mutation, or deletion, affects the expression, oligomerization, and interactions of the other  $\beta$ -crystallins in the lens. Further, we will determine whether the loss of these two major polypeptide constituents from the highly ordered lens protein matrix affects the expression levels and distribution of the other major lens proteins, the  $\alpha$ - and  $\gamma$ -crystallins, and investigate how changes in the complement of lens crystallins affects lens growth and transparency. Moreover,  $\beta$ A3/A1-crystallin may have other cellular functions in lens, apart from its structural role. Based on our studies (Sinha et al., 2005), we will investigate whether it is involved in the orientation of elongating lens fiber cells and the subsequent loss of organelles from the terminally differentiated fibers.

Finally, it remains to be determined whether mutations in  $\beta$ A3/A1-crystallin can contribute to, or cause, human ocular disease aside from cataract. Multiple mutations in *Cryba1* are known to cause autosomal dominant cataracts of various descriptions (Hejtmancik, 2008; Graw, 2009). These are undoubtedly due to disruption of the “crystallin” function of the proteins, leading to protein aggregation and light scattering in the lens. Based on animal studies, effects of  $\beta$ A3/A1-crystallin mutations, other than cataract, are apparent only in homozygotes; to date, such homozygous individuals have not been identified among human patients. Since  $\beta$ A3/A1-crystallin has been shown to be expressed in the human retina and RPE (unpublished observations), the animal studies suggest that some mutations would negatively affect these tissues, perhaps producing retinal degenerative changes.

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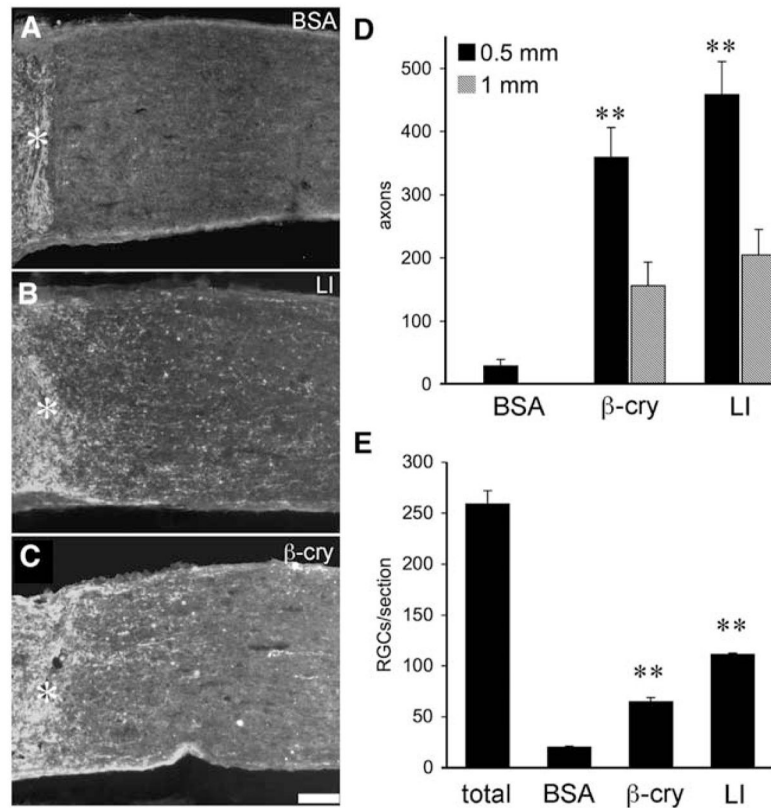


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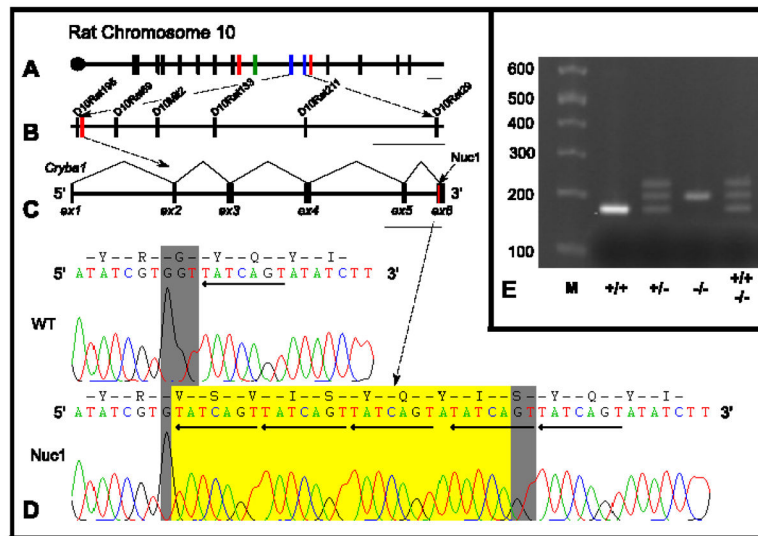


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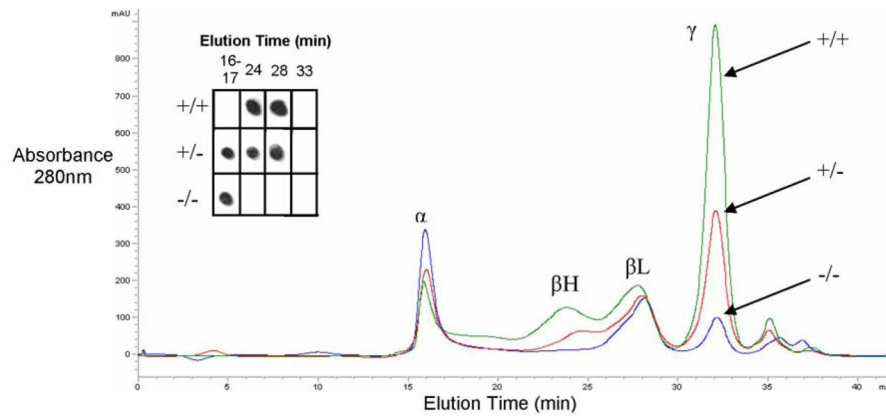


**Figure 1.** Effects of crystallins on axonal regeneration in the crushed optic nerve and survival of axotomized RGCs. (A–C) Longitudinal sections through the optic nerve showing GAP-43-positive axons distal to the injury site (asterisk) 2 weeks after optic nerve crush (ONC) and (A) two intravitreal injections of a solution containing BSA (1 mg) (B) lens injury or (C) two injections of  $\beta$ -crystallins (1 mg). Scale bar = 100  $\mu$ m. (D) Quantitation of regeneration (number of axons growing 500  $\mu$ m and 1000  $\mu$ m beyond the injury site per optic nerve) 2 weeks after surgery. Scale bar: 100  $\mu$ m. (E) Quantitation of surviving RGCs ( $\beta$ III-tubulin-positive RGCs per retinal cross-section) 2 weeks after ONC. \*\* $p < 0.001$  compared with BSA-treated controls. (reproduced with permission from Fischer et al, 2008)



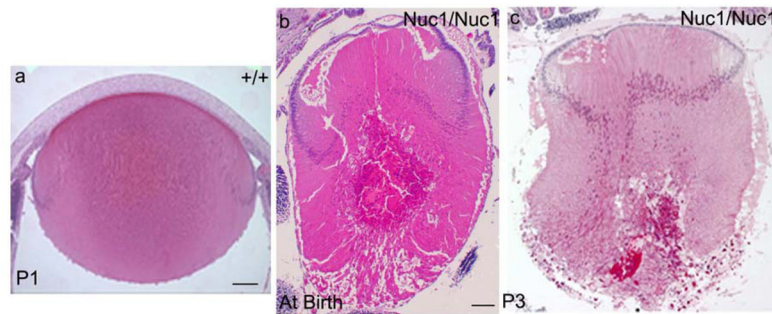
**Figure 2.**

The positional cloning of the  $\beta$ A3/A1-crystallin gene to rat chromosome 10. Markers used are designated by bisecting lines and are drawn to scale by physical distance. (A) shows the initial Nuc1 interval bounded by red lines (D10Rat125 and D10Rat98), D10Rat32 (green line) segregated perfectly with the disease and gave a LOD score of 6.02. The final Nuc1 interval is bounded by blue lines (D10Rat195 and D10Rat29) and this region is magnified in b. Scale bar = 5Mb. (B) shows magnification of final linkage interval and markers, in which position of  $\beta$ A3/A1 gene (*Cryba1*) is marked with a red line. Scale bar = 1 Mb. (C) shows the genomic structure of *Cryba1* with the site of the Nuc1 mutation highlighted in red. Scale bar = 1 Kb. (D) displays the sequence at the site of mutation in +/+ and Nuc1/Nuc1 animals. Chromatograms display the sense strand of genomic amplimers. Grey highlight indicates the highly conserved glycine codon in normal *Cryba1*. Sequence inserted by the Nuc1 mutation is highlighted in yellow and the TGACTAT repeats are marked by reverse arrows. Agarose gel analysis of PCR products from the region of *Cryba1* including the 27 base insertion is shown in E. Expected bands are observed for wildtype and homozygote. The 3<sup>rd</sup> (heavier) band seen in the heterozygote is the result of heteroduplex formation since it is also present in the wildtype plus homozygote heteroduplex control sample. (reproduced from Sinha et al., 2008)



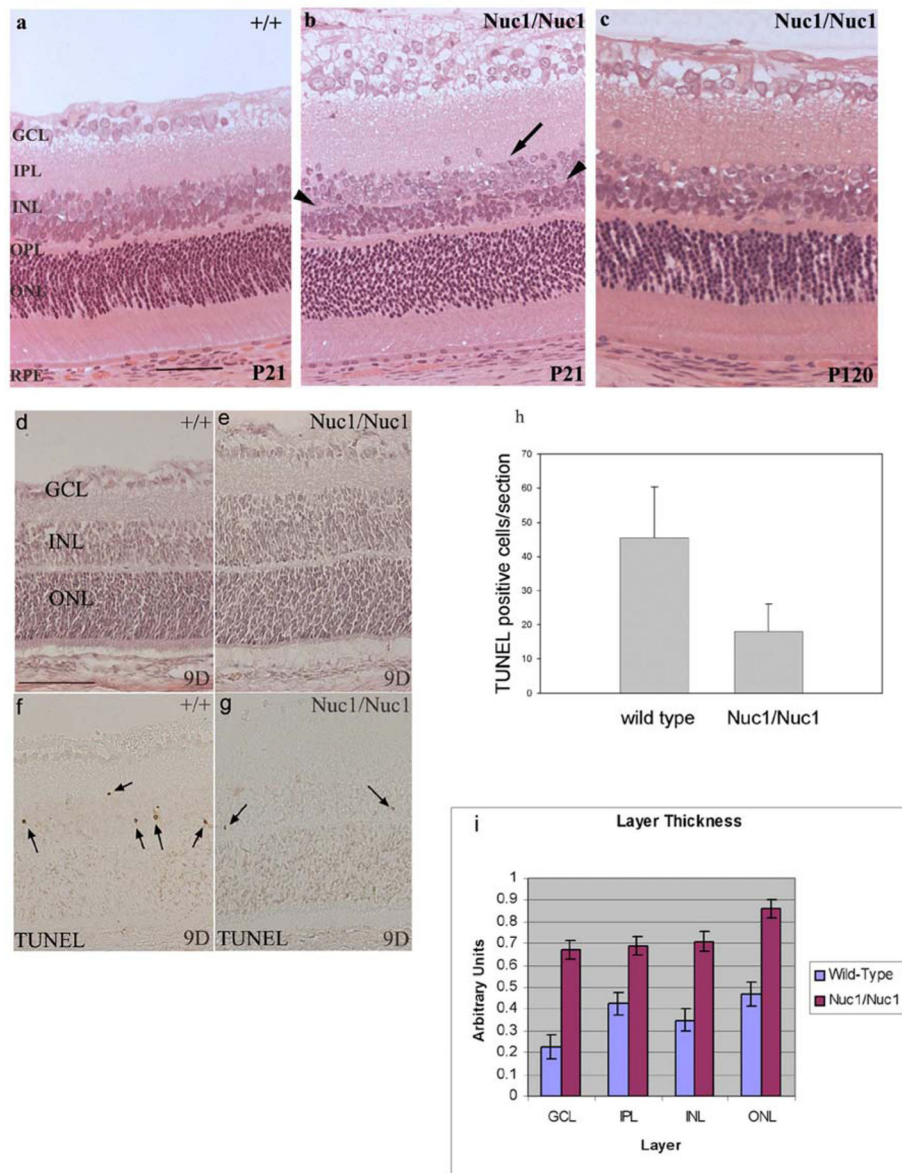
**Figure 3.**

The effects of the  $\beta$ A3/A1-crystallin mutation on the composition of soluble crystallins in the lens. The elution patterns show trends in the distribution of soluble proteins (+/+ = green, +/- = red, -/- = blue). The pattern from normal lens has four major peaks: a void volume peak which contains  $\alpha$ -crystallin plus any heavy molecular weight protein aggregates, two peaks of  $\beta$ -crystallins of different size which have been described in many species ( $\beta_H$  and  $\beta_L$ ), and  $\gamma$ -crystallin which is the dominant peak in the rat lens. In the cataract lenses, the relative proportion of the void fraction increases from about 10% in the wildtype lens to 18% in the heterozygote lens and to 32% in the homozygote.  $\gamma$ -crystallin, which constitutes 47% of the soluble protein in the wildtype lens, is reduced to 37% in the heterozygote and to 16% in the homozygote. As a percentage of the soluble protein,  $\beta$ -crystallin is quite constant in all phenotypes, however there is a dramatic shift to the lower molecular weight species (dimers) in the heterozygote and especially the homozygous lens. The inset shows a dot blot in which an antibody specific for  $\beta$ A3/A1-crystallin (reactive with both normal and mutant forms) is used to localize those polypeptides in the chromatographically separated peaks. In the wildtype lens the protein is present only in the  $\beta$ -crystallin peaks as expected (fractions 24 and 28). In the heterozygote lens, reactivity is present in those fractions, but also in the void peak. In the homozygote, reactivity is essentially limited to the void peak. This indicates that the mutant protein aggregates and does not participate in the formation of stable oligomers as does normal  $\beta$ A3/A1-crystallin. (reproduced from Sinha et al., 2008)



**Figure 4.** Structural disorganization of developing Nuc1 lens and identification of macrophage/macrophage-like cells. Histological analysis of lenses from wild-type (a) and homozygous (Nuc1/Nuc1) mutants (b and c). Representative hematoxylin and eosin-stained sections show protrusion at the posterior pole of the lens leading to rupture of the capsule with expulsion of lens material very early in post-natal development in Nuc1/Nuc1 (b and c). Lens abnormality in structure is clear in Nuc1/Nuc1 (b) at birth when compared to wild type (a) at post-natal day 1. The morphological damage to the lens becomes more severe as post-natal development progresses (c, post-natal day 3) compared to b (at birth). (Reproduced from Hose et al., 2005)

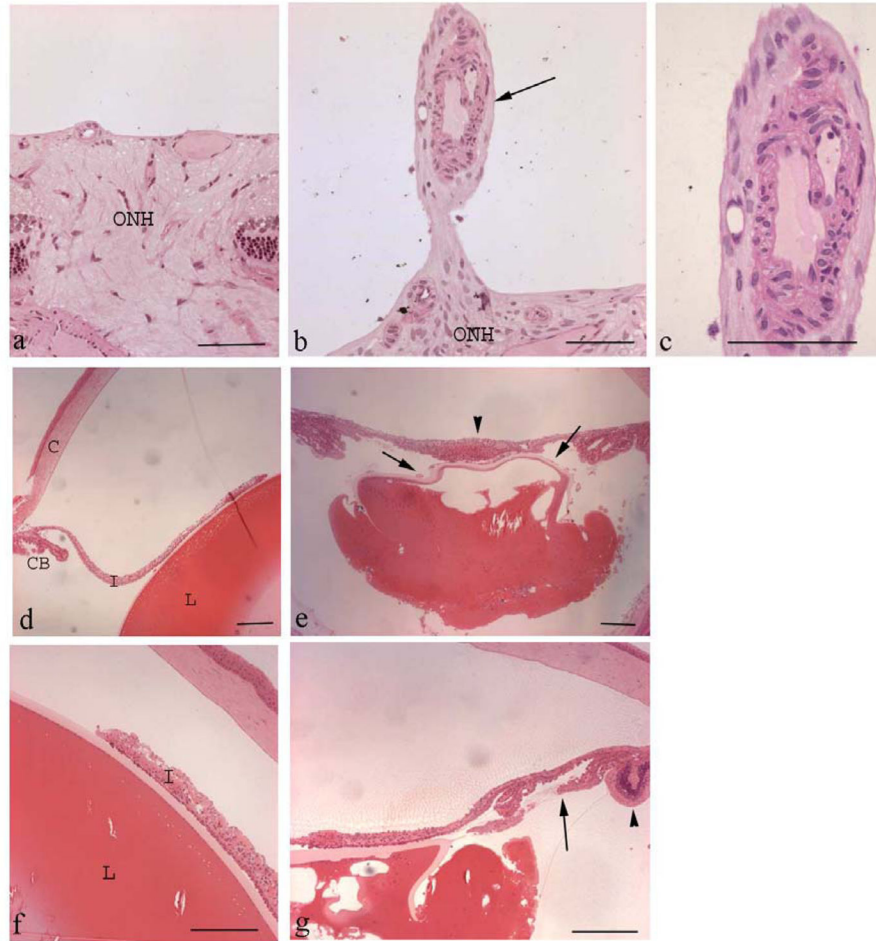




**Figure 5.**

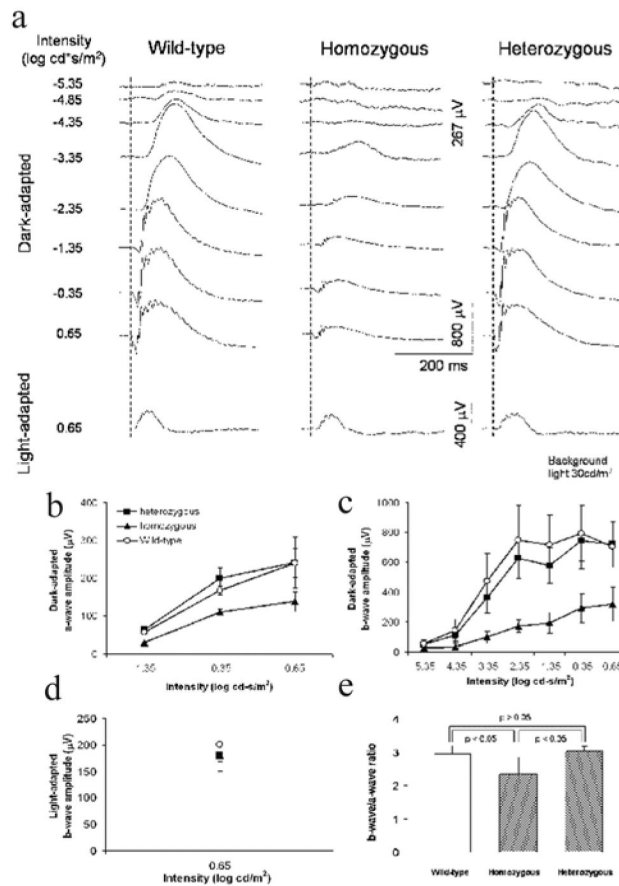
Aberrant retinal morphology in Nuc1/Nuc1 mutation. a) Wild-type retinal tissue showing normal structure at post-natal day 21. b) In contrast, Nuc1/Nuc1 has a much thicker retina at the same age. More cells are present in Nuc1 homozygotes (b) compared to wild-type littermates (a). An additional nuclear layer (arrow in b) is present in Nuc1 homozygous retina. This layer is greatly diminished by 4 months after birth (c). The transient fiber layer of Chievitz (b, arrowheads) persists in Nuc1 retinas and only disappears around 4 months of age, indicative of delayed development. Photomicrographs in this figure are from the posterior region of the retina. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner retina (c) nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Bar=50 $\mu$ m. TUNEL labeling and H&E staining of postnatal day 9 wild type and Nuc1/Nuc1 rat retinas. The thickness of Nuc1/Nuc1 retina (e) was significantly increased

compared to the wild type retina (d). TUNEL labeling showed more positive cells (arrows) in the wild type (f) than in the Nuc1/Nuc1 retina (g). Semi quantitative analysis also indicated reduced number of apoptotic cells in Nuc1/Nuc1 homozygotes compared to wild-type littermates (n=7; h). Also, every layer of Nuc1 retina was thicker than wild-type (i) (n=7; i). Bar=100 $\mu$ m. (reproduced from Sinha et al., 2005)



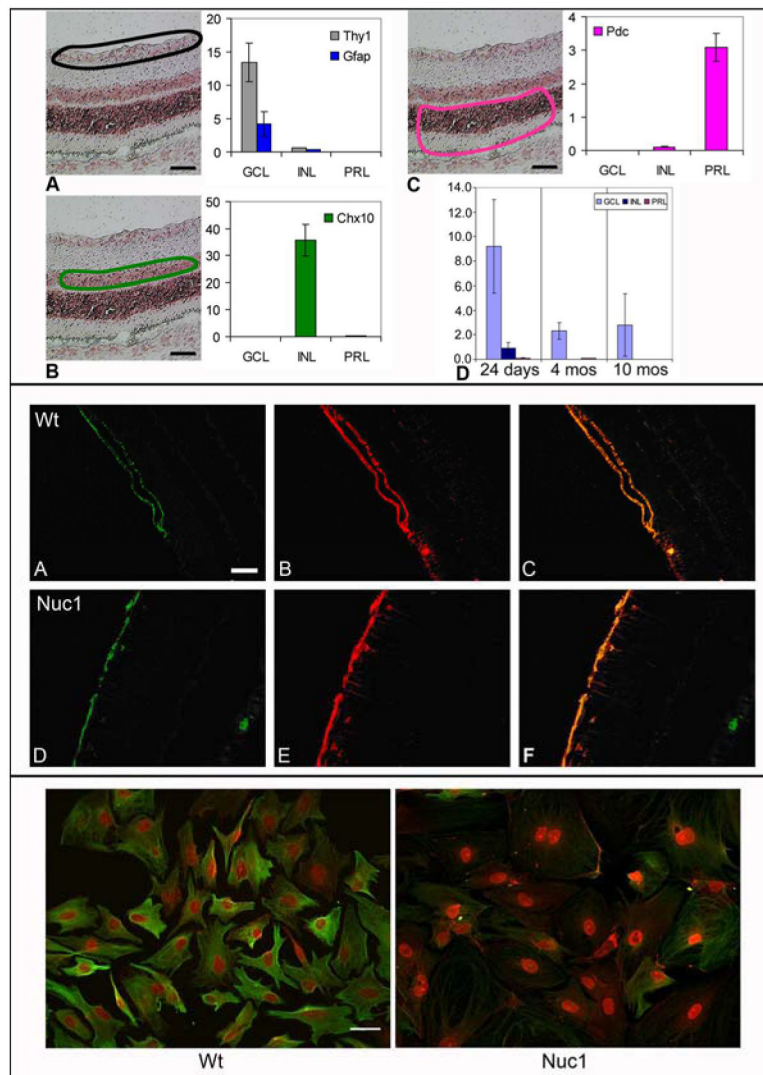
**Figure 6.**

Defective regression of embryonic vasculature in Nuc1 mutant rat. In wild type animals (a), the hyaloid artery had completely regressed by 5 weeks of age, showing a normal optic nerve head (ONH). In 5 week old Nuc1/Nuc1 rats (b,c), the hyaloid artery and adjacent tissue were still present on the surface of the optic nerve head projecting into the vitreous (arrow). Please note in (c), the thick vessel wall of the artery at higher magnification showing cellular morphology of the retained vessel. Representative H & E stained sections from 25-day-old animals (d) show normal lens (L), iris (I), ciliary body (CB) and cornea (C). In (e) the pupillary membrane is still evident in the Nuc1 homozygous animals (arrows) whereas it has fully regressed in the wild type eye (d). Iris hyperplasia was also noted in the Nuc1/Nuc1 eyes (arrowheads). The lens shows abnormal shape and disorganization of structure. The normal eye structure at 120 days in wild-type eyes is shown in (f). In Nuc1 homozygote (g), the ciliary process (arrow) is dragged centrally towards the disrupted lens, resulting in traction, which causes peripheral retinal dragging and folding (arrowhead). Scale bar= 50 $\mu$ m. (reproduced from Zhang et al., 2005)



**Figure 7.**

Wave-form morphology shows diminished a-wave and b-wave amplitudes in homozygous but not heterozygous animals, as compared with wild-type at 10 weeks. Light-adapted wave forms are preserved in all animals (a). ERG amplitude vs. flash intensity (V-log I) curves for the dark adapted a-wave (b), dark-adapted b-wave (c) and light adapted b-wave (d) in each group of rats: wildtype (open circles,  $n=6$ ), Nuc1 heterozygotes (filled squares,  $n=6$ ) and Nuc1 homozygotes (filled triangles,  $n=5$ ) are presented. Error bars indicate standard deviation. The dark-adapted b-wave amplitudes in the Nuc1 homozygous rats were significantly smaller than the wildtype and the Nuc1 heterozygous rats ( $P<0.01$  at all intensities). The dark adapted a-wave amplitudes of the Nuc1 homozygous rats were smaller at the highest flash intensities relative to the wildtype ( $P<0.05$ ) and the Nuc1 heterozygous ( $P<0.05$ ) rats. The light-adapted b-wave amplitudes of the Nuc1 homozygous rats were comparable to the wildtype and the Nuc1 heterozygous rats ( $P>0.05$ ). (e) Indicates the dark-adapted ERG b-wave/a-wave ratio of the homozygous Nuc1 rats was smaller than that of the control ( $P<0.05$ ) and the heterozygous ( $P<0.05$ ) rats. (reproduced from Gehlbach et al., 2006)

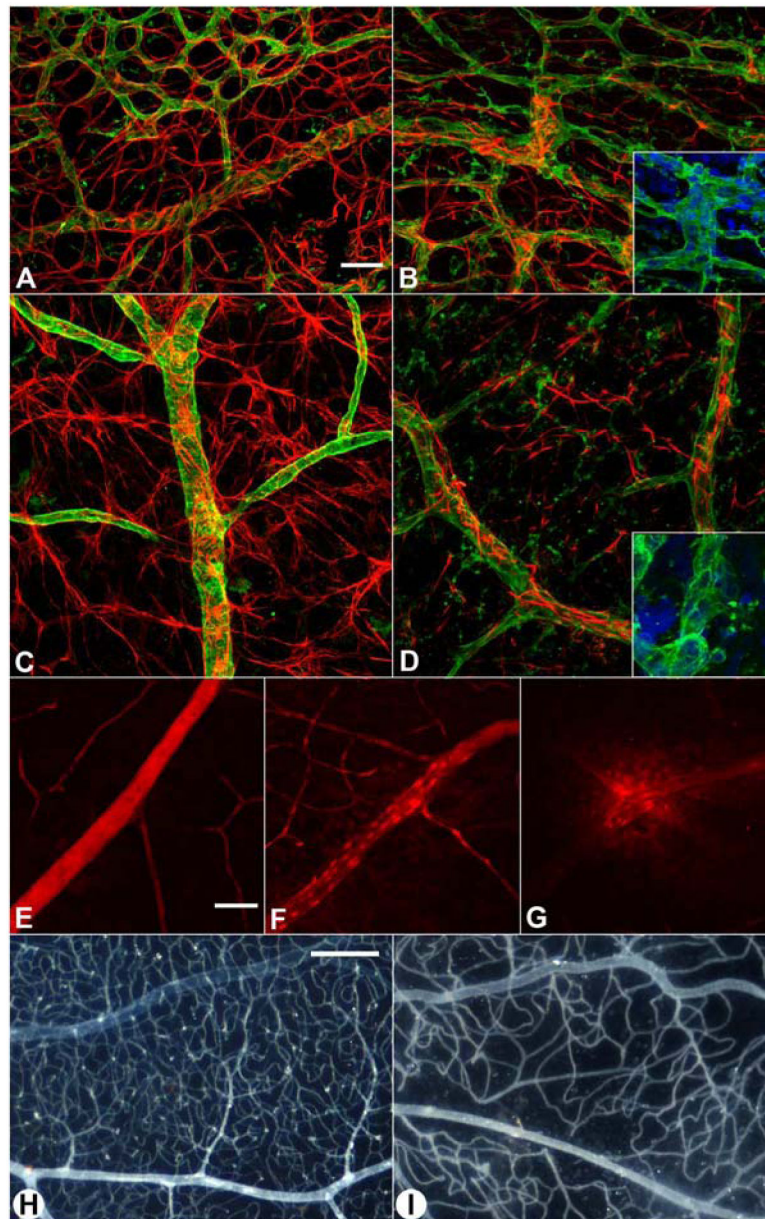


**Figure 8.**

Localization of  $\beta$ A3/A1-crystallin in the retina. Top-panel: Laser-capture microdissection studies. (A), (B) and (C) respectively show the location of samples taken from the ganglion cell layer (GCL), outlined in black, the inner nuclear layer (INL) outlined in green and the photoreceptor layer (PRL) outlined in pink. Scale bar = 50 $\mu$ m. For each region quantitative RT-PCR demonstrates the purity of the dissected tissue by measuring specific markers for each cell type. In (D), quantitative RT-PCR shows the relative expression of  $\beta$ A3/A1-crystallin in the 3 retinal regions of wildtype Sprague-Dawley rat at 24 days, 4 months and 10 months of age. Data shown as mean  $\pm$  SD. Middle-panel: Confocal microscopy showing GFAP positive staining at the internal limiting membrane of 4 week old wildtype (A) and Nuc1 (D) retinas. (B) and (E) show  $\beta$ A3/A1-crystallin staining on the same section, while (C) and (F) are merged images showing co-localization of GFAP and  $\beta$ A3/A1-crystallin in the wildtype and Nuc1 retinas, respectively. The fluorescence is enhanced because of the reduced amount of GFAP present in the mutant cells. Scale bar = 50 $\mu$ m Bottom-panel: Wildtype and Nuc1 astrocytes cultured from brain of neonatal rats. Note

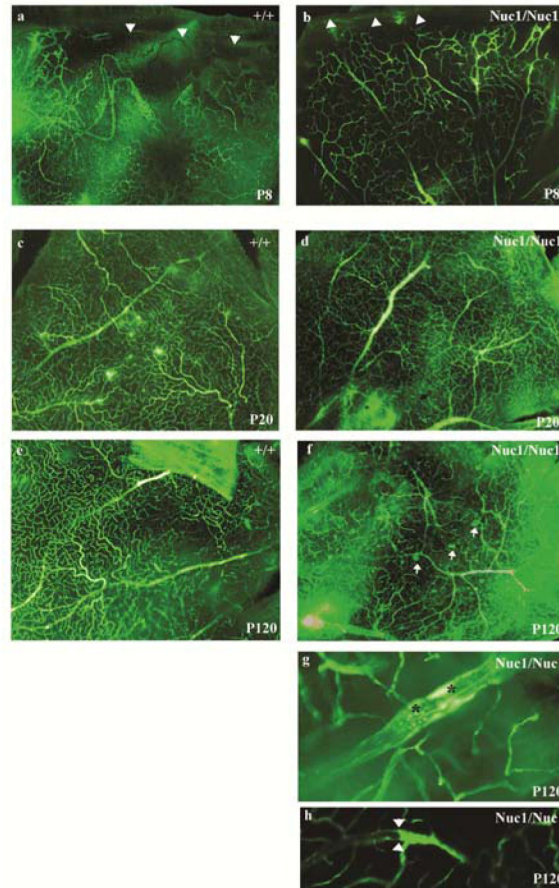
$\beta$ A3/A1-crystallin (red) is expressed strongly in the cell nuclei of both normal and mutant astrocytes but also in the cytoplasm. The astrocytes from Nuc1 homozygous rats show striking changes in cell morphology; they are larger in size with disrupted processes and reduced expression of GFAP (green). Scale bar = 40 $\mu$ m (reproduced from Sinha et al., 2008)





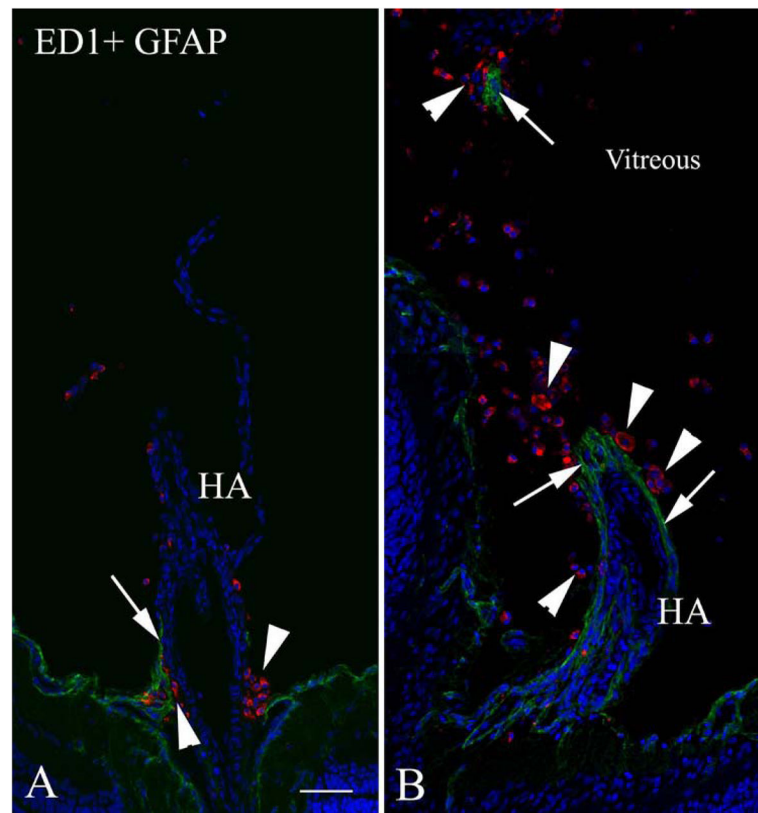
**Figure 9.** Retinal blood vessel abnormalities in Nuc1 homozygous rats. GFAP (red) and GSA lectin blood vessel staining (green) in wildtype (A, C) and Nuc1 retinas (B, D) at postnatal day 6 (A, B) and 10 weeks of age (C, D). In the wildtype (A), there is a dense capillary plexus (top) and a capillary-free zone around arteries. The capillary-free zones are missing in Nuc1 (B) and the capillary plexus pattern is abnormal. By 10 weeks, the wildtype retina is remodeled and the astrocyte processes are extensive and enwrapping the blood vessels (C). In Nuc1, the number of astrocytes and their processes are greatly reduced (D) suggesting fewer interactions between astrocytes and endothelial cells. In the insets of B and D, it is apparent that the Nuc1 blood vessels have too many endothelial cells suggesting that remodeling during maturation of the retinal vasculature has not occurred. Evans blue

injections at 4 weeks of age demonstrate that the wildtype retinal vasculature does not leak (E) but the Nuc1 retinal blood vessels do leak (F and G at higher magnification). Using the ADPase flat-embedding technique, the normal vascular pattern is apparent in the 10 month old wildtype (H) while the Nuc1 vascular pattern is sparse, appears to be lacking a deep capillary plexus and has an abnormal pattern (I). Scale bars = 20 $\mu$ m (A–G) and 0.25mm (H–I). (reproduced from Sinha et al., 2008)

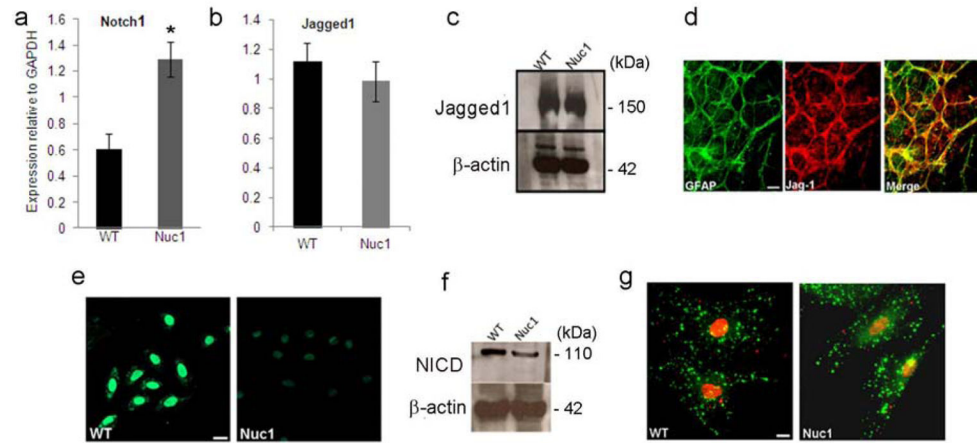


**Figure 10.**

Fluorescence microscopy of flatmounts from FITC–dextran perfused wildtype and Nuc1 homozygous retinas show that vascularization of the Nuc1 homozygous retina reaches the ora serrata in advance of wildtype (a and b, arrowheads). However, by P20 the vascular patterning of both Nuc1 homozygous and wildtype appears to be similar with more large caliber vessels present in the Nuc1 homozygotes (c and d). By P120, the Nuc1 homozygote shows clear difference in the morphology and patterning of the vasculature compared with wildtype (e and f). We find evidence of microaneurysm formation in Nuc1 homozygotes (f, small arrows) and intravascular deposits (g, asterisks). Confocal microscopy (h) shows blockage of blood flow inside some vessels (arrowheads). (reproduced from Gehlbach et al., 2006)



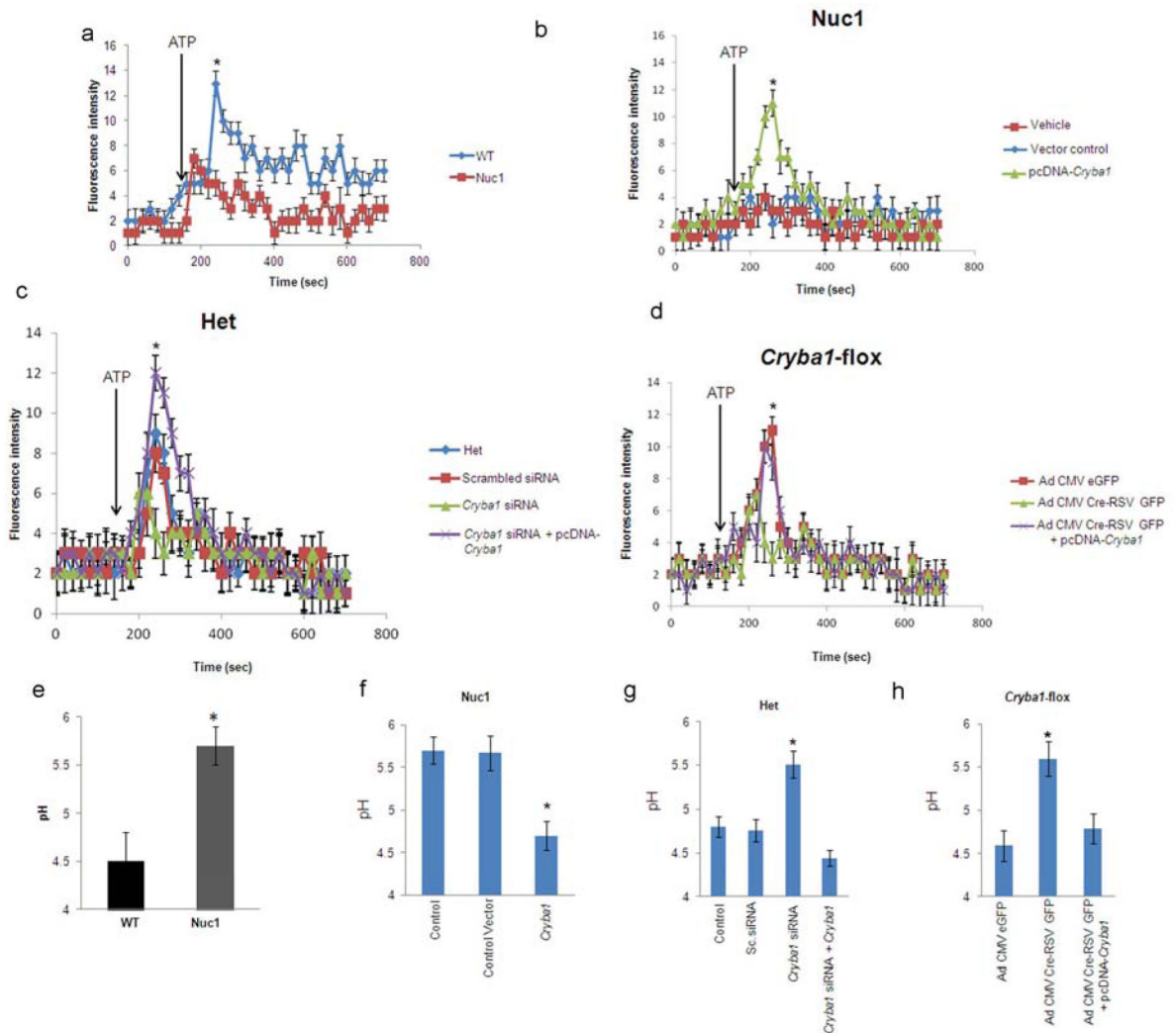
**Figure 11.** Double labeling with the macrophage marker ED1 (red) and GFAP (green) in 20-day-old wild type and Nuc1 rat eyes. A. In the wild type rat, some GFAP-positive cells (arrow) are seen at the base of the regressing hyaloid artery (HA). Clusters of ED1-positive cells (arrowheads) are also observed at the base of the involuting hyaloid artery. B. In the Nuc1 rat, the persistent hyaloid artery is surrounded by a layer of GFAP-positive astrocytes (arrows), with abundant ED1-positive cells (arrowheads) in the vicinity of the hyaloid artery. A fragment of GFAP-positive cells (arrow) is also observed in the vitreous, presumably from a tangential section of the hyaloid artery, surrounded by ED1-positive cells (arrowhead). Scale bar=50  $\mu$ m, n=5 wild type, n=5 Nuc1 homozygote. (reproduced from Zhang et al., 2011)



**Figure 12.**

Levels of Notch1 and Jagged1 in immunopanned, postnatal day 2 WT and Nuc1 optic nerve astrocytes. a, b. Quantitative reverse transcriptase (qRT) PCR using Taqman expression probes shows that Notch1 was elevated by  $>2$  fold in Nuc1 astrocytes (\*P=0.027), relative to WT astrocytes. WT and Nuc1 astrocytes had similar levels of Jagged1. c. Western analysis for Jagged1 shows similar protein levels in WT and Nuc1 astrocytes. d. Retinal flat mounts from a 3-day old WT rat showing significant co-localization (yellow) of Jagged1 (red) with GFAP (green) positive astrocytes. e, f. Immunostaining and western analysis for active Notch (NICD) using anti-V1744 antibody shows that NICD was decreased by 76% in Nuc1 astrocytes compared to WT astrocytes (\*P=0.0031). g. Expression and distribution of NICD and Jagged1 as revealed by immunostaining with anti-V1744 active Notch (red) and anti-Jagged1 antibodies (green). Decreased nuclear staining for NICD was evident in Nuc1 cells, but Jagged1 staining was similar in WT and Nuc1 astrocytes. Scale bar = 20 $\mu$ m. In all panels, graphs show mean values and error bars represent s.d. from a triplicate experiment representative of at least three independent experiments. Statistical analysis was performed by a two-tailed Student's *t*-test: \*P < 0.05. (reproduced from Valapala et al., 2013)



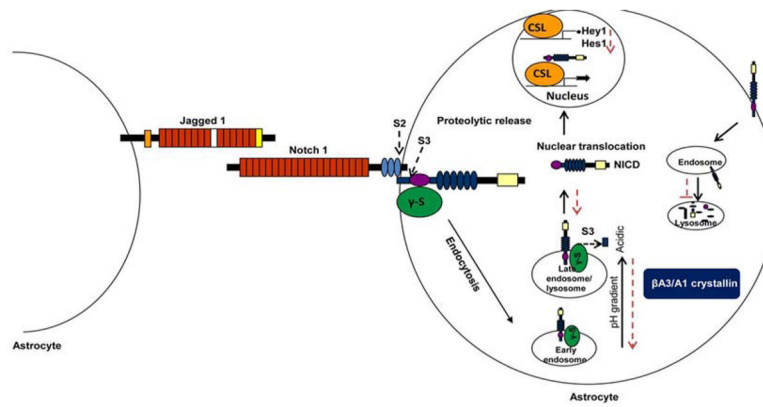


**Figure 13.**

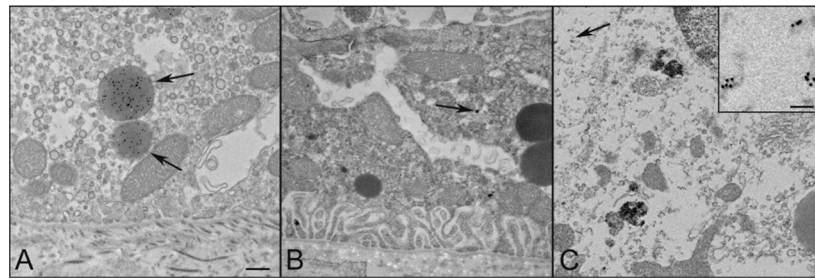
Impaired V-ATPase activity in astrocytes lacking functional  $\beta$ A3/A1-crystallin a. V-ATPase activity was measured by acridine orange fluorescence in WT and Nuc1 astrocytes after intravesicular  $H^+$  uptake was initiated by the addition of Mg-ATP. Increase in acridine orange fluorescence upon addition of ATP was significantly greater in WT astrocytes compared to Nuc1 astrocytes (\* $P=0.017$ ). During the measurement of lysosomal pH, bafilomycin a1 was added to the ATP treated lysosomes for control purposes. b. In Nuc1 astrocytes, over-expression of  $\beta$ A3/A1-crystallin rescued activity to >80% of normal level (\* $P=0.020$ ). Empty vector had no effect. c. V-ATPase activity in heterozygote astrocytes was significantly reduced by *Cryba1* siRNA knockdown (\* $P=0.033$ ), but was rescued to normal levels when  $\beta$ A3/A1-crystallin was subsequently over-expressed in the same astrocytes (\* $P=0.024$ ). d. In astrocytes from mice with floxed *Cryba1*, deletion of *Cryba1* by Cre-recombinase decreased V-ATPase activity relative to control astrocytes (\* $P=0.018$ ). The V-ATPase activity was rescued to normal levels by subsequent over-expression of  $\beta$ A3/A1-crystallin in *Cryba1* knockout mouse astrocytes (\* $P=0.027$ ). e. Measurement of endolysosomal pH in WT and Nuc1 astrocytes was performed using a fluid phase



fluorescent probe, FITC-dextran. 3 hours after incubation of WT and Nuc1 astrocytes with FITC-dextran the fluorescence emission was measured at 520 nm with excitation at 450 nm and 495 nm. The fluorescence excitation ratio at 495 nm and 450 nm ( $I_{495\text{nm}}/I_{450\text{nm}}$ ) was calculated, and endolysosomal pH in WT and Nuc1 astrocytes was measured to be ~4.5 and 5.7 respectively (\*P=0.031). f. The elevated pH in Nuc1 homozygous astrocytes was restored to near normal acidic range by over-expression of  $\beta$ A3/A1-crystallin (\*P=0.026). Empty vector had no effect. g. Further knockdown of  $\beta$ A3/A1-crystallin in Nuc1 heterozygote astrocytes by *Cryba1* siRNA elevated the endolysosomal pH to near Nuc1 homozygous levels (\*P=0.036). Subsequent over-expression of  $\beta$ A3/A1-crystallin decreased pH to the level characteristic of WT astrocytes (\*P=0.021). Scrambled siRNA had no effect. h. pH was elevated to near Nuc1 homozygote level in *Cryba1* knockout mouse astrocytes (\*P=0.029) and was rescued to near normal levels by subsequent over-expression of  $\beta$ A3/A1-crystallin to same cells (\*P=0.024). In all panels, graphs show mean values and error bars represent s.d. from a triplicate experiment representative of at least three independent experiments. Statistical analysis was performed by a two-tailed Student's *t*-test: \*P <0.05. (reproduced from Valapala et al., 2013)

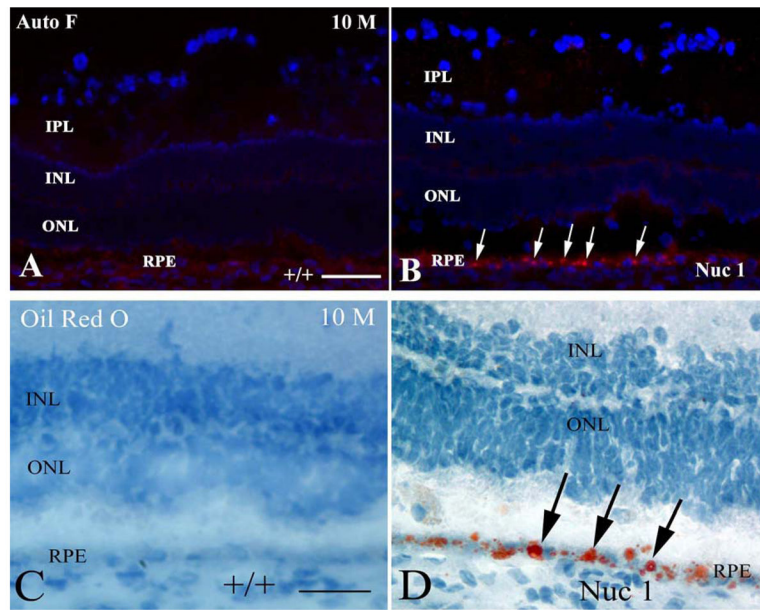


**Figure 14.** Schematic model of Notch signaling and  $\beta$ A3/A1-crystallin. Steps affected by loss of  $\beta$ A3/A1-crystallin shown by red dotted arrows. Impaired endolysosomal acidification inhibits Notch receptor processing and degradation, decreases NICD, ultimately reducing transcription of Notch target genes. (reproduced from Valapala et al., 2013)



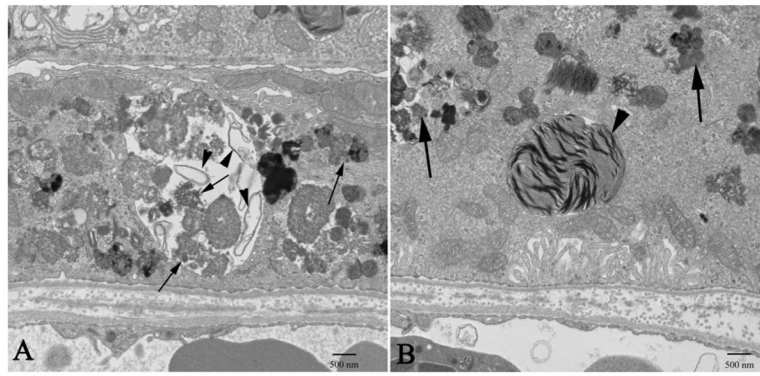
**Figure 15.**

Immuno-electron microscopy to determine the intracellular localization of  $\beta$ A3/A1-crystallin in RPE cells. In Panel A is shown a representative image from a 5.5 month old wild type animal. The arrows indicate lysosomes which contain nearly all the gold particles. In contrast, Panel B is an image from an age-matched Nucl1 animal showing several lysosomes which lack gold particles. In this sample, the gold is randomly distributed within the cytoplasm, the arrow indicates an example. Image C is from a 22 month old Nucl1 animal. Part of a large lysosome is visible at the lower right and does not contain gold particles. As in B, gold particles are distributed throughout the cytoplasm. In this section they are not as apparent because a shorter silver enhancement step was used. To demonstrate the particles the area indicated by the arrow is shown at higher magnification in the insert. Scale bar = 500nm (A–C) and 125nm (C, inset) (reproduced from Zigler et al., 2011)



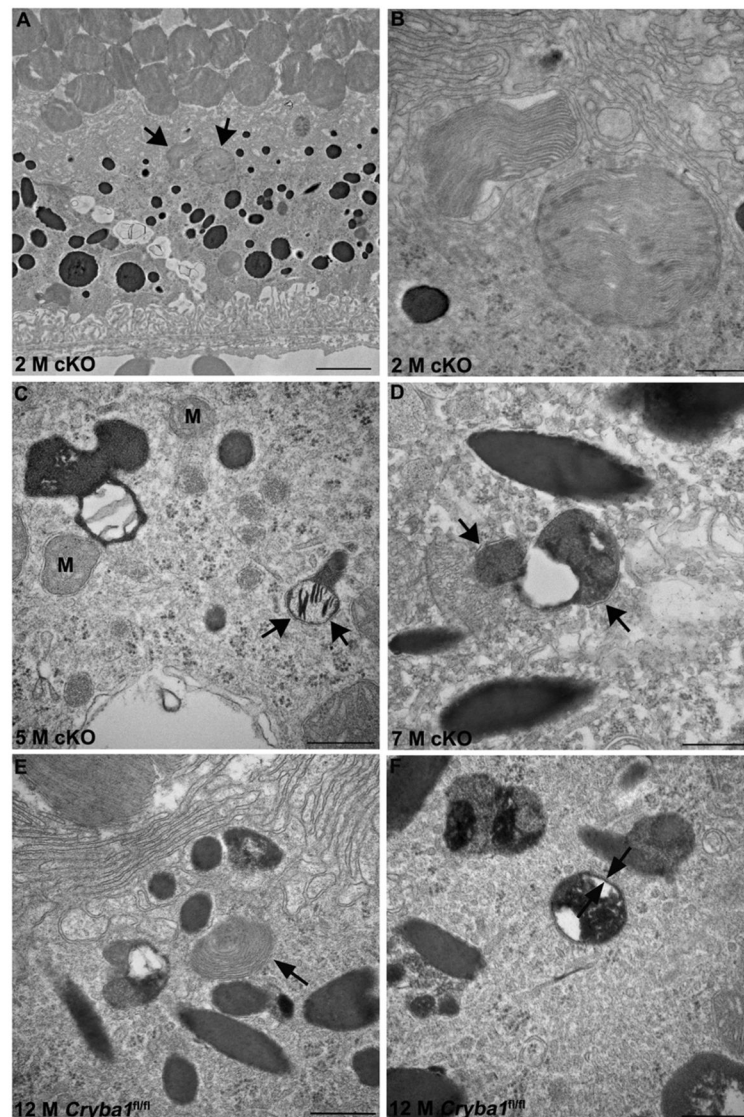
**Figure 16.**

Demonstration of abnormal lipid accumulation in the Nuc1 RPE. At 10M of age, minimal autofluorescence is seen in the RPE cells of the wild type animals (A). However, abundant autofluorescence is seen in the RPE cells of the wild type animals (A). However, abundant autofluorescence (arrows) is observed in the RPE of 10M Nuc1 animals, consistent with accumulation of lipofuscin. (B). Autofluorescence viewed with red filter (560–620nm). Oil red O staining, indicating presence of neutral lipids, shows abundant intense staining in the RPE cells of the 10M old Nuc 1 retinas (arrows, D), but not in the control wild type animals (C). Scale bar = 50  $\mu$ m (reproduced from Zigler et al., 2011)



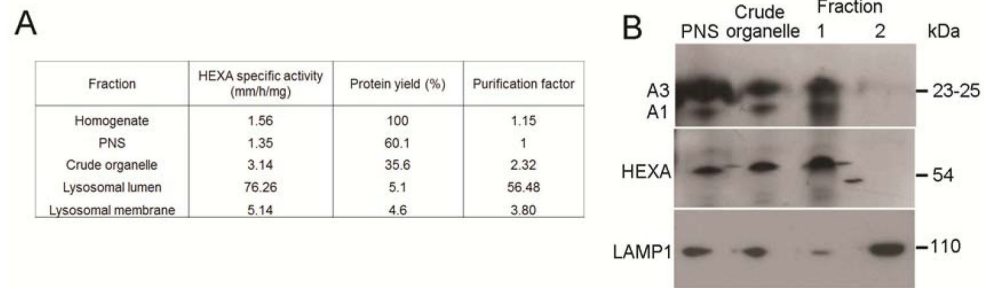
**Figure 17.**

At higher magnification prominent features of RPE cells from 1 year old Nucl1 animals are shown: A. RPE cell showing large aggregates of lipofuscin-like material (arrow). A large vacuole contains many degenerated cellular organelles (arrowheads) intermixing with lipofuscin-like aggregates (arrows). B. Abundant lipofuscin-like aggregates (arrows) and a large phagosome containing undigested outer segment discs (arrowhead) are present in the cytoplasm of an RPE cell. Scale bar = 500 nm. (reproduced from Zigler et al., 2011)



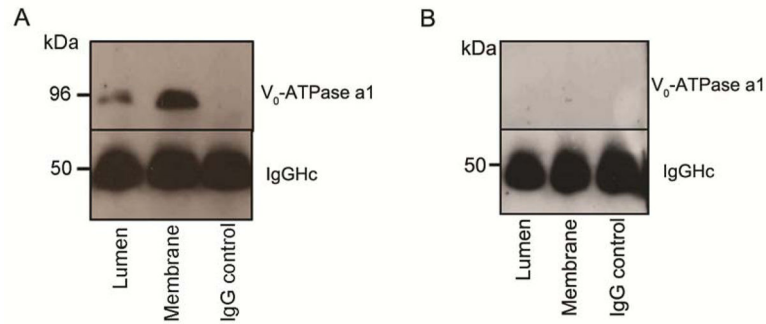
**Figure 18.** Phagosomes and Autophagosomes in conditional knockout of  $\beta$ A3/A1- crystallin in RPE cells. (A) Transmission electron microscopy image from a 2-month old RPE of *cryba1* cKO mouse showing phagosomes containing shed outer segment discs (arrows). Bar, 2  $\mu$ m. (B) TEM showing the same phagosomes (as in A) at higher magnification to demonstrate that they are enclosed by a single membrane. Bar, 500 nm. (C) A 5-month old *Cryba1* cKO mouse RPE shows autophagosomes with double membranes (arrows). M designates mitochondria, which also have double membranes. Bar, 500 nm. (D) TEM images from RPE of a 7-month old *Cryba1* cKO mouse showing an autophagosome with double membrane (arrows). Bar, 500 nm. (E) A 12-month old starved *Cryba1*<sup>fl/fl</sup> mouse RPE shows a phagosome containing shed outer segment discs (arrow). Bar, 500 nm. (F) TEM showing an autophagosome with double membrane (arrows) in a 12-month old *Cryba1*<sup>fl/fl</sup> mouse following starvation. Bar, 500 nm. (reproduced from Valapala et al., 2014)





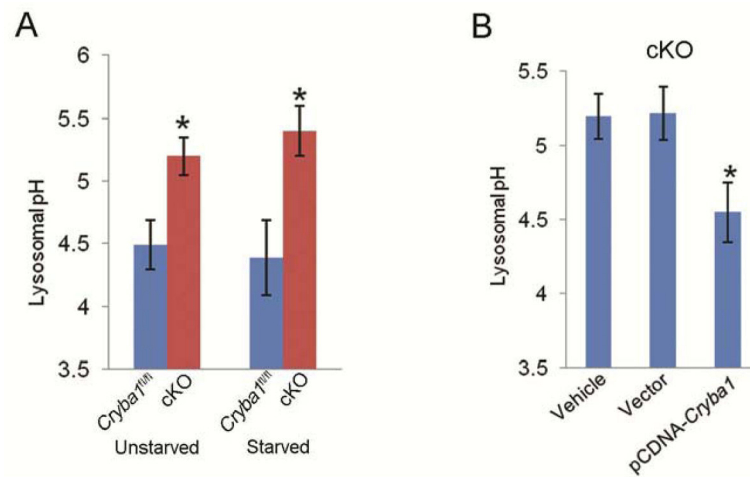
**Figure 19.**

Loss of *Cryba1* affects lysosomal acidification. (A) Subcellular fractionation as assessed by measuring the activity of HEXA, a lysosomal luminal enzyme. The purification factor is calculated from the ratio of the specific activities by normalizing the specific activity of the post-nuclear supernatant (PNS) to 1. (B) Western blotting of lysosomal fractions showed  $\beta$ A3/A1-crystallin to be in the lysosomal lumen fraction (fraction 1), with minimal immunostaining in the lysosomal membrane fraction (fraction 2). LAMP1 and HEXA indicate the purity of the fractions. (reproduced from Valapala et al., 2014)



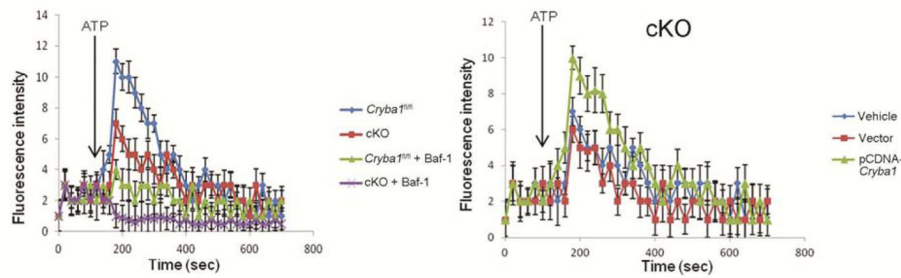
**Figure 20.**

(A) Coimmunoprecipitation of lysosomal lumen and membrane extracts from *Cryba1*<sup>fl/fl</sup> cells with  $\beta$ A3/A1-crystallin antibody and immunoblotting with V<sub>0</sub>-ATPase a1 antibody, demonstrating pull-down of V<sub>0</sub>-ATPase a1 subunit by the crystallin antibody. Non-specific IgG was used as negative control. (B) Coimmunoprecipitation of lysosomal lumen and membrane extracts from *Cryba1* cKO cells with  $\beta$ A3/A1-crystallin antibody and immunoblotting with V<sub>0</sub>-ATPase a1 antibody, demonstrating that the crystallin antibody does not pull-down V<sub>0</sub>-ATPase a1 as there is no detectable band on the gel. Non-specific IgG was used as negative control. (adapted from Valapala et al., 2014)



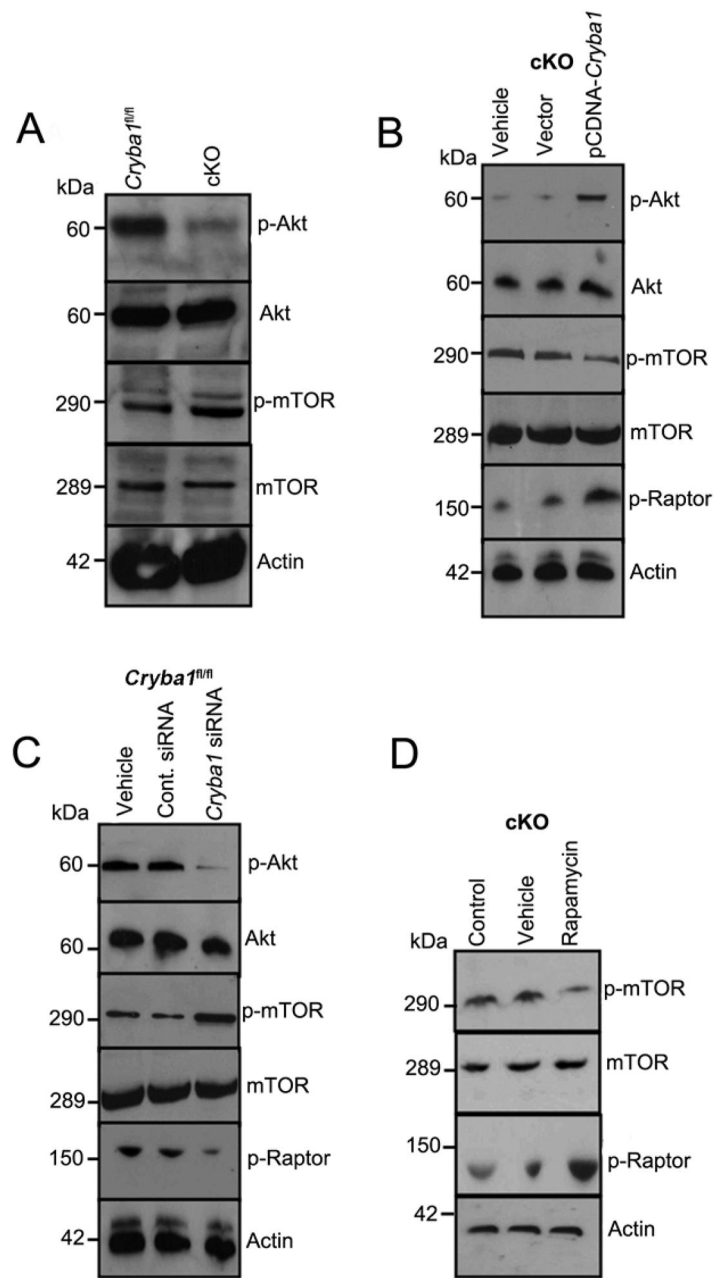
**Figure 21.**

(A) Lysosomal pH in cultured primary RPE cells from *Cryba1<sup>fl/fl</sup>* mice was ~4.5, while in cKO cells it was ~5.2. The lysosomal pH was not significantly changed when cells were starved (St) to induce autophagy. (B) Overexpression of  $\beta$ A3/A1-crystallin in cKO cells reduced lysosomal pH to the normal value of 4.5, while empty vector had no effect. Graphs show mean values and error bars represent s.d. from a triplicate experiment, representative of at least 3 independent experiments. Statistical analysis was performed by a two-tailed Student *t*-test: \* $P < 0.05$ . (reproduced from Valapala et al., 2014)



**Figure 22.**

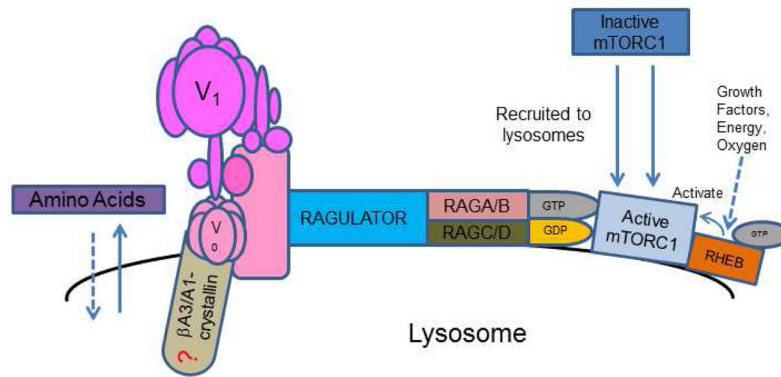
V-ATPase is regulated by  $\beta$ A3/A1-crystallin in RPE cells. Left panel: V-ATPase activity in isolated lysosomes from *Cryba1<sup>fl/fl</sup>* RPE cells is about twice that of cKO cells. Bafilomycin A<sub>1</sub> (Baf-1) reduced activity markedly in both cell types. Right panel: Overexpression of  $\beta$ A3/A1-crystallin in cultured RPE cells from cKO mice restored lysosomal V-ATPase activity to near normal level. The point of addition of ATP (1.4  $\mu$ M final concentration) to activate the enzyme is indicated. Each point represents the average of 3 determinations; error bars represent mean  $\pm$  s.d. (reproduced from Valapala et al., 2014)

**Figure 23.**

*Cryba1* regulates the mTORC1 signaling pathway. (A) Following induction of autophagy *in vivo* by starvation, RPE/choroid was isolated and levels of signaling intermediates determined. In the cKO samples p-Akt was decreased compared to *Cryba1<sup>fl/fl</sup>*, while p-mTOR was increased. Total Akt and total mTOR were not changed. (B) In starved RPE cells cultured from cKO mice, overexpression of  $\beta$ A3/A1-crytallin increased p-Akt and p-Raptor expression, while p-mTOR decreased. (C) Conversely, downregulation of  $\beta$ A3/A1-crytallin expression in *Cryba1<sup>fl/fl</sup>* cells using siRNA, decreased p-Akt, and p-Raptor while p-mTOR was upregulated. (D) Rapamycin, an inhibitor of mTOR, was effective at

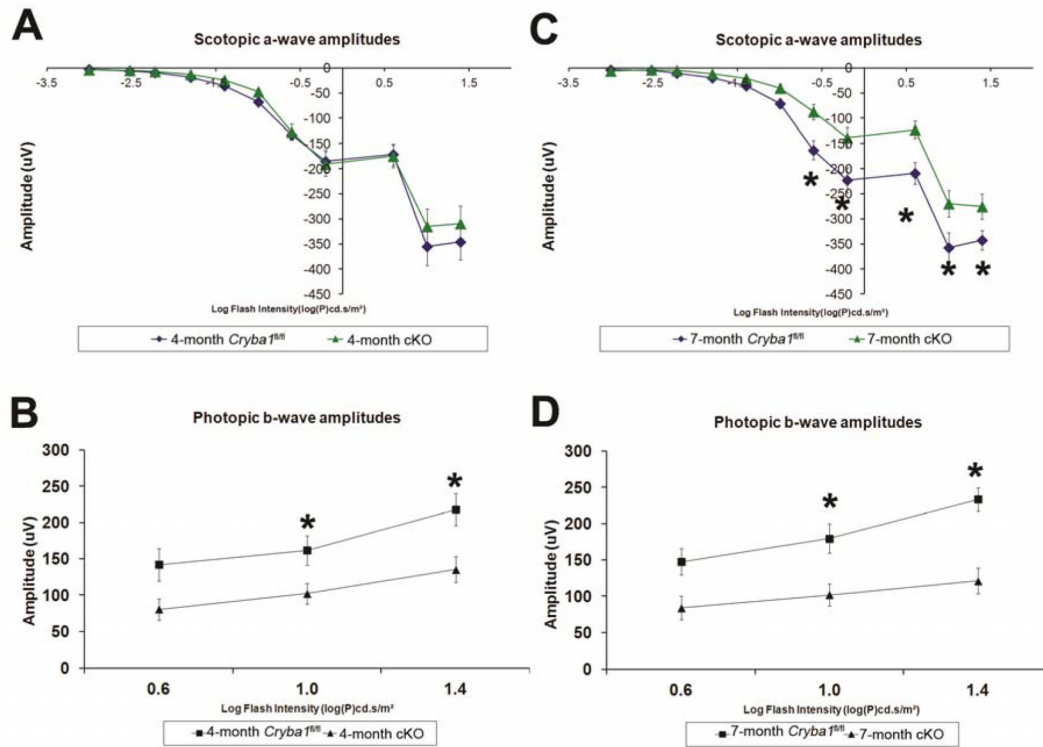
decreasing p-mTOR and increasing p-Raptor levels in the cKO cells. (adapted from Valapala et al., 2014)





**Figure 24.**

A schematic diagram showing the mechanism of mTORC1 activation at the surface of the lysosomes and a possible role for  $\beta$ A3/A1-crystallin in the process. In the “inside-out” model of amino acid sensing postulated by Zoncu et al. (2011), the proton pump, vacuolar H (+)-adenosine triphosphate ATPase (V-ATPase) interacts in an amino acid sensitive manner with pentameric Ragulator, a scaffolding complex that anchors the heterodimeric Rag GTPases to the lysosomes. This leads to the translocation of the inactive mTORC1 to the lysosomal surface. Once mTORC1 is on the surface of the lysosomes, it is activated by Rheb that is also localized to the lysosomal surface. It has been postulated that amino acids are probably translocated to the lysosomal lumen by V-ATPase and the amino acid signaling from the lysosomal lumen plays an important role in the complex process of recruiting mTORC1 to the lysosomal surface and activating it. We have recently shown that  $\beta$ A3/A1-crystallin is localized to the lysosomal lumen of RPE cells and is a binding partner of V-ATPase. We speculate that  $\beta$ A3/A1-crystallin may regulate the activity of mTORC1 by modulating V-ATPase and thereby amino acid signaling from the lysosomal lumen of RPE cells.



**Figure 25.**

Decreased lysosomal clearance leads to loss of cellular homeostasis in RPE of *Cryba1* cKO mice. (A) Scotopic ERGs from eyes of 4-month old *Cryba1* cKO mice showed similar *a*-wave amplitudes as those in *Cryba1*<sup>fl/fl</sup> mice (n = 10 for each group), indicating that rod photoreceptor function was not affected. (B) Photopic ERGs of cKO mice from the same age (4 months) showed smaller *b*-wave amplitudes in comparison to *Cryba1*<sup>fl/fl</sup> mice (n = 10 for each group), indicating reduced cone photoreceptor function (\**P* 0.018 by ANOVA with Dunnett correction for multiple comparisons). (C) Scotopic ERGs from 7-month old cKO mice showed decreased *a*-wave amplitudes when compared to *Cryba1*<sup>fl/fl</sup> mice (n = 10 for each group), demonstrating that rod photoreceptor function was reduced significantly (\**P* 0.0002 by ANOVA with Dunnett correction for multiple comparisons). (D) Photopic ERGs from these 7-months old cKO mice also showed further decrease in *b*-wave amplitudes compared to *Cryba1*<sup>fl/fl</sup> mice (n = 10 for each group), indicating the cone function had been reduced (\**P* 0.0001 by ANOVA with Dunnett correction for multiple comparisons). (reproduced from Valapala et al., 2014)

**Table 1**

## Vertebrate Lens Crystallins

	<b>Distribution</b>	<b>Identical to or (Related to)</b>
$\alpha$	ubiquitous	small heat shock proteins
$\beta, \gamma$	ubiquitous	?
$\lambda$	rabbit	L-gulonate-3-dehydrogenase
$\eta$	elephant shrew	retinaldehyde dehydrogenase
$\zeta$	guinea pig, camelids, Japanese tree frog	NADPH: quinone oxidoreductase
$\mu$	kangaroo	(ornithine cyclodeaminase)
$\nu$	platypus	lactate dehydrogenase A
$\psi$	rhesus monkey	betaine-homocysteine methyltransferase
$\delta 1, \delta 2$	birds, reptiles	argininosuccinate lyase
$\epsilon$	birds, crocodiles	lactate dehydrogenase B4
$\tau$	turtle, lamprey, fish	$\alpha$ -enolase
$\pi$	diurnal geckos	glyceraldehyde 3-phosphate dehydrogenase
$\iota$	diurnal geckos	cellular retinal binding protein type 1
$\rho B$	diurnal geckos	(aldose reductase)
$\rho$	frogs	(hydroxysteroid dehydrogenase)

For references, see Piatigorsky, 2007 and literature cited therein.

**Table 2** $\beta$ -crystallin polypeptides

	<b># Residues</b> <sup>^</sup>	<b>Molecular Mass (Da)</b> <sup>^</sup>	<b>Gene</b>	<b>Extra-lenticular expression</b>
$\beta$ A1-crystallin	198	23,191	<i>Cryba1</i>	retina, brain, RPE
$\beta$ A2-crystallin	196	21,964	<i>Cryba2</i>	testes, retina, brain
$\beta$ A3-crystallin	215	25,150	* <i>Cryba1</i>	retina, brain, RPE
$\beta$ A4-crystallin	195	22,243	<i>Cryba4</i>	retina
$\beta$ B1-crystallin	251	27,892	<i>Crybb1</i>	ciliary body, retina
$\beta$ B2-crystallin	204	23,249	<i>Crybb2</i>	testes, retina, brain, RPE
$\beta$ B3-crystallin	211	24,230	<i>Crybb3</i>	retina

\*  $\beta$ A3- and  $\beta$ A1-crystallins encoded by a single gene (*Cryba1*)

<sup>^</sup> Data for human polypeptides given (other vertebrates are similar)