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Expression of βA3/A1-crystallin in the developing and adult rat eye

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Abstract

Crystallins are very abundant structural proteins of the lens and are also expressed in other tissues. We have previously reported a spontaneous mutation in the rat βA3/A1-crystallin gene, termed Nuc1, which has a novel, complex, ocular phenotype. The current study was undertaken to compare the expression pattern of this gene during eye development in wild type and Nuc1 rats by in situ hybridization (ISH) and immunohistochemistry (IHC). βA3/A1-crystallin expression was first detected in the eyes of both wild type and Nuc1 rats at embryonic (E) day 12.5 in the posterior portion of the lens vesicle, and remained limited to the lens fibers throughout fetal life. After birth, βA3/A1-crystallin expression was also detected in the neural retina (specifically in the astrocytes and ganglion cells) and in the retinal pigmented epithelium (RPE). This suggested that βA3/A1-crystallin is not only a structural protein of the lens, but has cellular function(s) in other ocular tissues. In summary, expression of βA3/A1-crystallin is controlled differentially in various eye tissues with lens being the site of greatest expression. Similar staining patterns, detected by ISH and IHC, in wild type and Nuc1 animals suggest that functional differences in the protein, rather than changes in mRNA/protein level of expression, likely account for developmental abnormalities in Nuc1.

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Keywords

Astrocytes; βA3/A1crystallin; Eye development; Ganglion cells; Lens; Retina; In situ hybridization

Introduction

Crystallins are highly abundant, water soluble, structural proteins of the ocular lens that contribute to transparency and refractive power (Delaye and Tardieu 1983). In all mammalian lenses, members of three major families of crystallins, α, β, and γ, are expressed (Piatigorsky 1989). The α-crystallins represent a distinct group, while the β- and γ-crystallins are evolutionarily and structurally related members of a single superfamily, the β/γ-crystallins (Lubsen et al. 1988). Once believed to be lens-specific, many, if not all, crystallins are now known to be present in other tissues (Clayton et al. 1986; Andley 2007). While the α-crystallins are now accepted to be functional small heat shock proteins in numerous tissues, the extra-lenticular functions of the β/γ- crystallins are not clear, although evidence is emerging that many members of the superfamily are calcium binding proteins (Aravind et al. 2009). Further, the β/γ-crystallins are structurally related to microbial stress proteins (Jaenicke and Slingsby 2001), and recently a member of this superfamily has been shown to complex with a trefoil factor to form a secreted frog skin toxin (Liu et al. 2008). A few studies describe possible functions of the β- and γ-crystallins outside the lens, including roles for βB2-crystallin in fertility (Duprey et al. 2007) and the promotion of retinal ganglion cell axon growth (Liedtke et al. 2007), while βA3 crystallin may have intrinsic proteinase activity (Srivastava et al. 2008).

βA3/A1-crystallin is unique among crystallins in that two polypeptides are translated from a single mRNA by utilizing alternate start sites (Peterson and Piatigorsky 1986). In previous studies, we have described a naturally occurring mutation in the βA3/A1-crystallin gene of the Sprague–Dawley rat, termed Nuc1 (Sinha et al. 2005; Sinha et al. 2008). Nuc1 homozygous animals exhibit several developmental eye abnormalities, including lens rupture prior to birth, abnormal vascular patterning in the retina, decreased apoptosis, and persistent fetal vasculature of the eye, while the heterozygous animals are characterized by congenital nuclear cataract (Sinha et al. 2005; Zhang et al. 2005; Gehlbach et al. 2006). The mutation causing the Nuc1 phenotype is a 27 base pair insertion in exon 6 of the βA3/A1-crystallin gene on rat chromosome 10 (Sinha et al. 2008). The 27 base pair insertion is composed of near-perfect tandem repeats of a 7 base pair sequence, TGACTAT. This in-frame insertion results in the loss of a conserved glycine residue in exon 6 and its replacement with 10 new amino acids, resulting in a protein 1083 daltons higher in molecular mass. Theoretically, it also results in an altered protein structure with a protruding C-terminal loop, affecting protein/protein interactions and consequentially, protein function (s).

Our hypothesis, based on our published (Sinha et al. 2005; Sinha et al. 2008; Gehlbach et al. 2006) and unpublished studies, is that βA3/A1-crystallin has a pivotal role in the remodeling of several ocular tissues during development. Here, we show that the expression of βA3/A1-crystallin in the rat lens is detected at a slightly earlier embryonic stage than previously demonstrated (Aarts et al. 1989; Graw et al. 1999; Yancey et al. 1988). We also provide a detailed characterization of both the spatial and the temporal expression patterns of βA3/A1-crystallin in non-lens tissues of the eye to inform future studies into the functional and mechanistic role(s) of βA3/A1-crystallin during eye development.

Materials and methods

Animals

Wild type Sprague–Dawley rats and rats homozygous for the Nuc1 mutation (cryba1^{nuc1}) were housed at Spring Valley Laboratories, Woodbine, MD. All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy Press) and were approved by the Animal Care and Use Committee of Johns Hopkins University School of Medicine. Experiments were performed on eyes of wild type and Nuc1 animals from ages E12.5 to 2 months. Timed pregnancies were identified by the presence of the vaginal plug and the day of appearance of the plug was designated as E0.5. Whole embryos (E12.5), heads (E14.5, E17.5, E19.5) and eyes (P1, P14 and P60), were harvested as required for further experimentation. For studies on lens proteins, whole lenses were dissected from wild type rats by the posterior approach; lens material was isolated from Nuc1 rats by bisecting frozen eyes and manually removing the remaining white lens tissue from the ruptured lenses, as previously described (Sinha et al. 2008).

Construction of recombinant vectors and generation of sense and anti-sense probes

Full-length cDNA sequences of wild type and Nuc1 βA3/A1-crystallin gene were cloned into the TOPO vector containing dual promoters SP6 and T7 (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Recombinant transformants were identified by blue/white screening and colony PCR for the inserts. Orientation of the cDNA was determined by vector sequencing at the Johns Hopkins University DNA core facility. The resulting construct was used to generate Digoxigenin (DIG)-labeled sense or anti-sense probes by in vitro transcription. Briefly, linearized DNA template obtained from either BamH1 or NdeI digestion (1 μg/μl) of the recombinant vector, was incubated with RNA polymerase (SP6 or T7), DIG labeling mix, and RNAse inhibitor along with the transcription buffer for 2 h at 37°C. The DIG-Labeled RNA probes were purified from the mix, aliquoted and stored at –20°C until use.

Tissue fixing, embedding and in situ-Hybridization

Whole embryos, heads or eyes were fixed in ice-cold 4% paraformaldehyde (PFA) as follows: E12.5: 30 min to 1 h; E14.5: 1–3 h; E17.5-P0: 4–8 h and post-natal stages: 4–6 h. After fixation, they were washed briefly in Phosphate Buffered Saline (PBS) and equilibrated overnight at 4°C in 20% sucrose. For ISH and fluorescent immunohistochemistry (fIHC), tissues were embedded in OCT compound and transverse sections (heads) or sagittal sections (eyes) of 12–16 lm thickness were cut using a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany) and stored at –80°C until use. For non-fluorescent immunohistochemistry (nfIHC), tissues were fixed as described above, embedded in paraffin and cut at 12 μm thickness. Nonradioactive ISH was performed according to Yang et al. (Yang et al. 2007) with 0.2–0.3 μg labeled probes/slide. Briefly, sections were post fixed with 4% PFA, permeabilized with Proteinase K (20 μg/ml, Invitrogen) for 1 min, and acetylated with acetic anhydride in 0.1 M triethanolamine prior to incubation with the riboprobes for 18 h at 55°C. Following the hybridization, sections underwent high stringency washes in 50% Formamide solution and were digested with RNAse A (20 μg/ml, Invitrogen) in buffer containing 0.5 M NaCl, 10 mM Tris–HCl (pH 7.5) and 5 mM EDTA (pH 8.0) for 30 min to remove the unbound probe. An alkaline phosphatase conjugated-anti-digoxigenin antibody (1:5,000: Roche Biosciences, Nutley, NJ) was used to detect the RNA probe by incubation at 4°C for 16 h. Color reactions were developed for desired times with BCIP/NBT (Roche) as substrate.

For hematoxylin and eosin (H&E) staining, eyes were enucleated and fixed initially in 2.5% glutaraldehyde for 72 h, followed by 10% buffered formalin. The eyes were embedded in methyl-methacrylate, sectioned and stained as previously described (Sinha et al. 2005).

Immunohistochemistry

Immunofluorescence studies on frozen sections was performed according to Sinha et al. (Sinha et al. 2008). Briefly, sections were incubated with PBS containing 5% goat or donkey serum for 30 min, prior to overnight incubation with primary antibody at 4°C. Following the incubation, sections were washed in PBS, incubated with secondary antibody for 1 h at room temperature, washed, drained and mounted using DAKO Paramount (DAKO Corporation, Carpinteria, CA). The primary antibodies used in this study included a rabbit polyclonal peptide antibody raised against a sequence common to both the βA3 and βA1-crystallin proteins (1:1,000), rabbit polyclonal antibodies to GFAP (glial fibrillary acidic protein) (DAKO, Carpinteria, CA; 1:1,000), and goat polyclonal antibodies to Brn3b (Brain-specific homeobox 3b) (Santa Cruz Biotechnology, Santa Cruz, CA; 1:500). Goat anti-rabbit IgG labeled with Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:300), Donkey anti-rabbit IgG labeled with Alexa Fluor 555 (Invitrogen; 1:200) and Donkey anti-goat IgG labeled with Alexa Fluor 488 (Invitrogen; 1:200) were used as a secondary antibodies along with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen; 1:1000) to stain nuclei when required. nfIHC on paraffin sections was performed using ImmPRESS™ Peroxidase Kit (Vector Laboratories Inc., Burlingame, CA) according to manufacturer's instructions and counterstained with hematoxylin by standard protocols.

Western blot

Astrocytes were cultured from the brains of 2-day-old wild type and Nuc1 rats as previously described (Sinha et al. 2008). The cells were rinsed in PBS, and homogenized in M-PER (Thermo Fisher Scientific, Rockford, IL) with 1% 0.5 M EDTA and 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO). Samples were placed on an end over end mixer at 4°C for 30 min followed by centrifugation at 13,0009*g* for 15 min. Protein quantification was performed using the Quick Start Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA). Approximately 25 μg of protein from the supernatant was mixed with 2× LDS sample buffer (Invitrogen, Carlsbad, CA) and then heated in a boiling water bath for 3 min. Each sample was loaded onto a 4–12% Bis–Tris Nu-PAGE gel and run with MES Buffer (Invitrogen, Carlsbad, CA). The gels were stained with Colloidal Coomassie brilliant blue. For western blotting, proteins were transferred to nitrocellulose membranes (Invitrogen, Carlsbad, CA) for 90 min and then blocked with 3% BSA in TTBS (Tris buffered saline, 0.1% Tween-20) overnight at 4°C. Blots were incubated with polyclonal antibody to βA3/A1-crystallin at 1:8,000 dilution for 1 h at room temperature followed by 4 washes of 10 min each. Blots were incubated with HRP-conjugated secondary antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 1 h at room temperature at a dilution of 1:20,000 followed by 4 washes of 10 min each. ECL western blotting detection reagents (GE Healthcare, Piscataway, NJ) were used for detection with varying exposure times.

Lens tissue was homogenized in 1× LDS buffer and centrifuged to remove insoluble material. Electrophoresis and western blotting was performed as for astrocyte lysates except that detection was with a lower sensitivity colori-metric reagent (4-CN, Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Microscopy

Bright-field and fluorescent micrographs were captured using a Leica DM5500B microscope and Leica DFC310FX camera (Leica Microsystems). LAS-AF (Leica) software

was used to merge single monochromatic fluorescent micrographs when required and Adobe Photoshop CS3 software (Adobe System Inc., San Jose, CA) was used to assemble the multiple panels.

Results and discussion

βA3/A1-crystallin expression in the developing embryonic rat lens

Expression analysis of the βA3/A1-crystallin gene in the wild type rat by ISH showed that the mRNA was expressed as early as embryonic stage (E) 12.5 in the posterior region of the lens vesicle (Fig. 1A, panels a–d). By E14.5, mRNA expression was robust throughout the lens fiber cells, and this pattern was seen also at E17.5 and E19.5 (Fig. 1A, panels b–d). Analysis of the protein expression by IHC showed that although mRNA was expressed at E12.5 in the lens vesicle, protein expression was detected only from E14.5 (Fig. 1A, panels e, f) and was also confined to lens fibers.

Previously, Aarts et al. reported that mRNA expression of βA3/A1-crystallin in the rat lens was detectable around E18 by Northern blotting, while another study in mice reported that by RT–PCR βA3/A1-crystallin mRNA expression was evident around E14.5 (Aarts et al. 1989; Graw et al. 1999). The present study indicates that the βA3/A1-crystallin gene is expressed earlier in lens development than reported in these studies and is similar in timing (E12.5) to that of γ-crystallin in embryonic rat lens (Van Leen et al. 1987). The discrepancy likely arises from the ability of in situ hybridization to detect mRNA in a subset of cells in a tissue, unlike the previous Northern blotting/RT–PCR studies. In fact, another study, which employed ISH, has reported βA3/A1-crystallin expression at essentially the same time (E13) in the rat lens (Yancey et al. 1988). It is important to recognize that determination of the time of inception of expression is inherently imprecise, since different technologies and specific methods of application result in different sensitivities of detection, and because the time of conception is not known exactly.

The Nuc1 embryonic eye showed no differences in terms of either morphology, or βA3/A1-crystallin gene expression, from wild type during stages E12.5 (Fig. 1B, panels a, d). At E17.5, βA3/A1-crystallin mRNA, as well as protein expression, was seen throughout the lens fibers, as in the wild type (Fig. 1B, panels b, e), though the lens appeared smaller. We have previously reported that the Nuc1 homozygous lens shows several abnormalities by E20, including rupture of the posterior capsule, poor migration of the lens fibers along the capsule, and retention of nuclei in the mature lens fiber cells that normally have undergone denucleation (Sinha et al. 2005). While all crystallins have a structural role in the lens that is critical to the refractive property and transparency of the organ, our data suggest that βA3/A1-crystallin also has a distinct cellular function in the lens fibers during differentiation that affects denucleation and migration.

βA3/A1-crystallin expression in postnatal rat lens development

After birth, the mRNA expression in the wild type rat lens, similar to the embryonic stages, appeared to be present in all fiber cells at P1 and P14 (Fig. 2A, panels a–d). However, by 2 months of age (P60), expression was confined to the outer cortical region of the secondary fiber cell layer and was absent in the lens nucleus (Fig. 2A, panels e, f). This is consistent with the fact that the mature lens fibers comprising the inner regions of the lens have lost their cell nuclei and the ability to synthesize mRNA. The transcripts appear to be more abundant in the equatorial region than in the anterior and posterior areas. The equatorial area stains first (data not shown), and appears slightly darker post development (Fig. 2A, panels a–f), similar to the pattern reported for the α-crystallins in mice (Robinson and Overbeek 1996). Although this study focuses on development and does not extend beyond the indicated time points, the data are in agreement with our previous study (Sinha et al. 2008)

and the study by Aarts et al. (1989), who showed that total βA3/A1-crystallin mRNA in the rat lens declines after 2 months and becomes negligible by 8 months of age. Presumably, the expression becomes further confined to the rim of the lens as more and more lens cells are denucleated (Fig. 2A, P60). This pattern is also seen for the α-crystallins in mouse lens, although it occurs much earlier, by P9 (Robinson and Overbeek 1996). No staining was seen when wild type sense RNA control was used (Fig. 2A, panels g, h) and this was true for all other stages tested (data not shown).

Analysis of expression in the wild type postnatal lens epithelial cells showed a pattern similar to embryonic stages; that is, neither βA3/A1-crystallin mRNA or protein was detected (Fig. 2A, panels b, d, f and data not shown, respectively). The expression in the equatorial region of the lens is likely to be in the presumptive lens fiber cells undergoing differentiation and not in the epithelial cells per se, as determined by Yancey et al. (1988). In their study, they found that βA3/A1-crystallin gene expression in neonatal rat lens was induced during differentiation of epithelial cells to fiber cells in the zone of elongation and that the anterior epithelial cells were devoid of this crystallin transcript during the embryonic and neonatal stages tested, similar to our results. In another study, a quantitative PCR analysis of α, β and γ-crystallin expression in 3 day-old rat lens epithelial cells also showed that βA3/A1-crystallin mRNA was poorly expressed at this stage, consistent with our data (Wang et al. 2004).

The Nuc1 lens showed morphological differences in structure as well as in βA3/A1-crystallin expression in the postnatal stages. At P1, lens rupture at the posterior pole of the lens is clearly seen (Fig. 2B, panel a, c), although the mRNA expression in the lens was similar to that of the wild type, with expression throughout the lens fiber cells and none in the epithelium (Fig. 2B, panel b). At P14, the damage to the Nuc1 lens is extensive with diminishing βA3/A1-crystallin mRNA expression (Fig. 2B, panels d, e). By the adult stages, the integrity of the lens is further destroyed, as reflected by the minimal βA3/A1-crystallin mRNA expression (Fig. 2B, panels g, h). Similar to that seen in wildtype tissues, use of Nuc1 sense mRNA control showed no staining (Fig. 2B, panel i) suggesting that there is no alteration in transcriptional control. It is likely that the decrease in mRNA seen in Nuc1 postnatal lens results from the structural and morphological damage to the lens, rather than to changes in transcription capabilities arising directly from the Nuc1 mutation. Unlike the mRNA, however, the βA3/A1-crystallin remains very abundant in the lens tissue of the Nuc1 rat after birth (Fig. 2C lanes 2 and 4), suggesting that the mutant βA3/A1-crystallin remains stable.

βA3/A1-crystallin expression in the retina of the developing rat eye

Expression of βA3/A1-crystallin mRNA in the wild type retina is seen first in the ganglion cell layer (GCL) at P1 (Fig. 3A, left panel-arrow). This is the first study, to our knowledge, that has examined expression of this gene in the neonatal rat retina. By P14, βA3/A1-crystallin mRNA expression was seen in both the GCL and weakly, in the inner nuclear layer (Fig. 3A, middle panel), while by P60 only the GCL was positive in the neural retina (Fig. 3A, right panel-arrow). A similar pattern was seen in Nuc1 rats suggesting that the mutant mRNA is not undergoing nonsense mediated decay (data not shown). The result at P14 is similar to that seen by Piri et al. (Piri et al. 2007), where an analysis of βA3/A1-crystallin expression by ISH showed βA3/A1-crystallin expression in the GCL and INL. In our previous studies, laser capture microdissection of the retinal layers followed by QRT–PCR analysis showed that at P24, expression was predominantly in the GCL with a weak signal from the INL, while by 4 months it was confined to the GCL (Sinha et al. 2008). Protein analysis indicated that the strongest immunoreactivity in the retina, was in the GCL at both 15 days and 30 days (Fig. 3B, C). As seen in Fig. 3B, there also seems to be weaker staining in the inner plexiform layer (IPL). In one other study, a comprehensive analysis of

various crystallins in the retina showed that β-crystallins are expressed in all layers of the retina (Xi et al. 2003). However, the antibody used in this study reacted with all 7 β-crystallins, not just βA3 or βA1-crystallin, making comparison to our study difficult.

The identity of the cell type transcribing βA3/A1-crystallin in the GCL was determined by staining for astrocytes and retinal ganglion cells using GFAP and Brn3b antibodies, respectively. As seen in Fig. 3B, there is clear co-localization of βA3/A1-crystallin (red) and GFAP (green), indicating presence of the crystallin in astrocytes, as we have previously reported (Sinha et al. 2008). To determine whether βA3/A1-crystallin is also expressed by retinal ganglion cells, sections were double-labeled for βA3/A1-crystallin and Brn3b (Fig. 3C). Brn3b is a member of the POU4f/Brn3 family of transcription factors exclusively expressed by retinal ganglion cells (Badea and Nathans 2010). Brn3b is present only in the cell nuclei (Fig. 3C-center panel). In the merged image (Fig. 3C, right panel) it is evident that for at least some retinal ganglion cells, the cytoplasm surrounding the cell nucleus stains for βA3/A1-crystallin. Piri et al., reported that βA3/A1-crystallin was synthesized in the retinal ganglion cells in 2-week-old rat retina (Piri et al. 2007). Here we show that βA3/A1-crystallin protein is produced in the GCL of the neural retina by both astrocytes and ganglion cells, thereby expanding our previous studies. As seen in Fig. 3B, there is again positive staining for βA3/A1-crystallin in the IPL at 2 months, possibly arising from the dendritic arbors extending into the IPL from ganglion cells. Note also that by 2 months of age the presence of βA3/A1-crystallin in the RPE is apparent. Protein production in the astrocytes was further confirmed by western blot analysis for βA3/A1-crystallin protein from primary astrocyte cultures. Both the wild type and Nuc1 astrocytes synthesize the crystallin (Fig. 4). Note that the βA3/A1-crystallins in the Nuc1 sample migrate slightly slower than those in wild type, consistent with the fact that the mutant proteins are approximately 1.1 kDa larger in mass because of the Nuc1 insertion mutation (Sinha et al. 2008). Although the Nuc1 astrocytes clearly have increased amounts of βA3/A1-crystallin, it is not possible to know from present data whether this reflects greater synthesis, slower turnover, or increased stability of the mutant proteins.

Expression of βA3/A1-crystallin in RPE, to our knowledge, has not been demonstrated to date, and is addressed in a separate study. mRNA analysis of this layer, using laser capture microdissection followed by Real time RT-PCR, indicated that the βA3/A1-crystallin mRNA is indeed present in RPE (Zigler et al., in press). As seen in Fig. 3C, there is clear labeling of βA3/A1-crystallin protein in RPE at 2 months of age in wild type rat retina, while no such labeling is evident at P15 (Fig. 3B).

The expression of βA3/A1-crystallin in the neural retina, together with results from our studies on the Nuc1 spontaneous mutant rat, imply a cellular function for this protein in the retina. We have suggested previously that impaired apoptosis may be the key factor in the Nuc1 phenotype. In the Nuc1 animals, histological and morphometric analysis of the retina showed that all retinal layers contain more cells and are thicker than normal by P3 (Gehlbach et al. 2006). Also, neuronal and vascular development was impaired in these animals in the postnatal stages, although the neuroblastic layers at E16/17 appeared to be normal. Subsequent studies showed that the normal honeycomb template formation of the retinal astrocytes was lost in the Nuc1 animals, which contributed to the impaired neuronal and vascular developmental pattern (Sinha et al. 2008). Astrocytes, the only immigrant cell type in the neural retina, form a corona of processes around the optic nerve head by E18, cover approximately 35% of the retina at birth, and reach the periphery of the retina at P8 (Miller et al. 1985; Ling et al. 1989). Moreover, the ganglion cells are the first cell type to differentiate during neurogenesis of the retina. A near complete loss of retinal ganglion cells leads to a drastic reduction in all other retinal cell types (Moshiri et al. 2008). It has also been postulated that interactions between developing astrocytes and retinal ganglion cells

may be important for the generation of normal retinal architecture (Burne and Raff 1997; Wechsler-Reya and Barres 1997). The retinal abnormalities observed in the Nuc1 spontaneous mutant rat occur concomitantly with the time of expression of the βA3/A1-crystallin gene in the astrocytes and ganglion cells, suggesting that βA3/A1-crystallin plays a crucial role in the remodeling of the retina during development. Apoptosis is critical to this remodeling; our studies indicate that impairment in the programmed cell death process, rather than increased proliferation of progenitor cells, is the major cause of the abnormal thickness of the retina in the Nuc1 rat (Sinha et al. 2008; Gehlbach et al. 2006). In addition, as the pattern of expression of βA3/A1-crystallin is similar in both wild type and Nuc1 retina, it is likely, as stated earlier, that functional abnormalities resulting from the mutated protein are the primary cause of the developmental changes in Nuc1 rat eyes.

Our current data suggest that βA3/A1-crystallin has a critical role in the developing lens and retina. Experiments under way in our laboratory to eliminate or modulate the expression of βA3/A1-crystallin specifically in astrocytes, lens or RPE, using genetically engineered mice, may help us to understand the functions of βA3/A1-crystallin in these different cell types. The expression pattern and the transcriptional control of βA3/A1-crystallin in the lens and the retina, as reported here are the bases for such future investigations.

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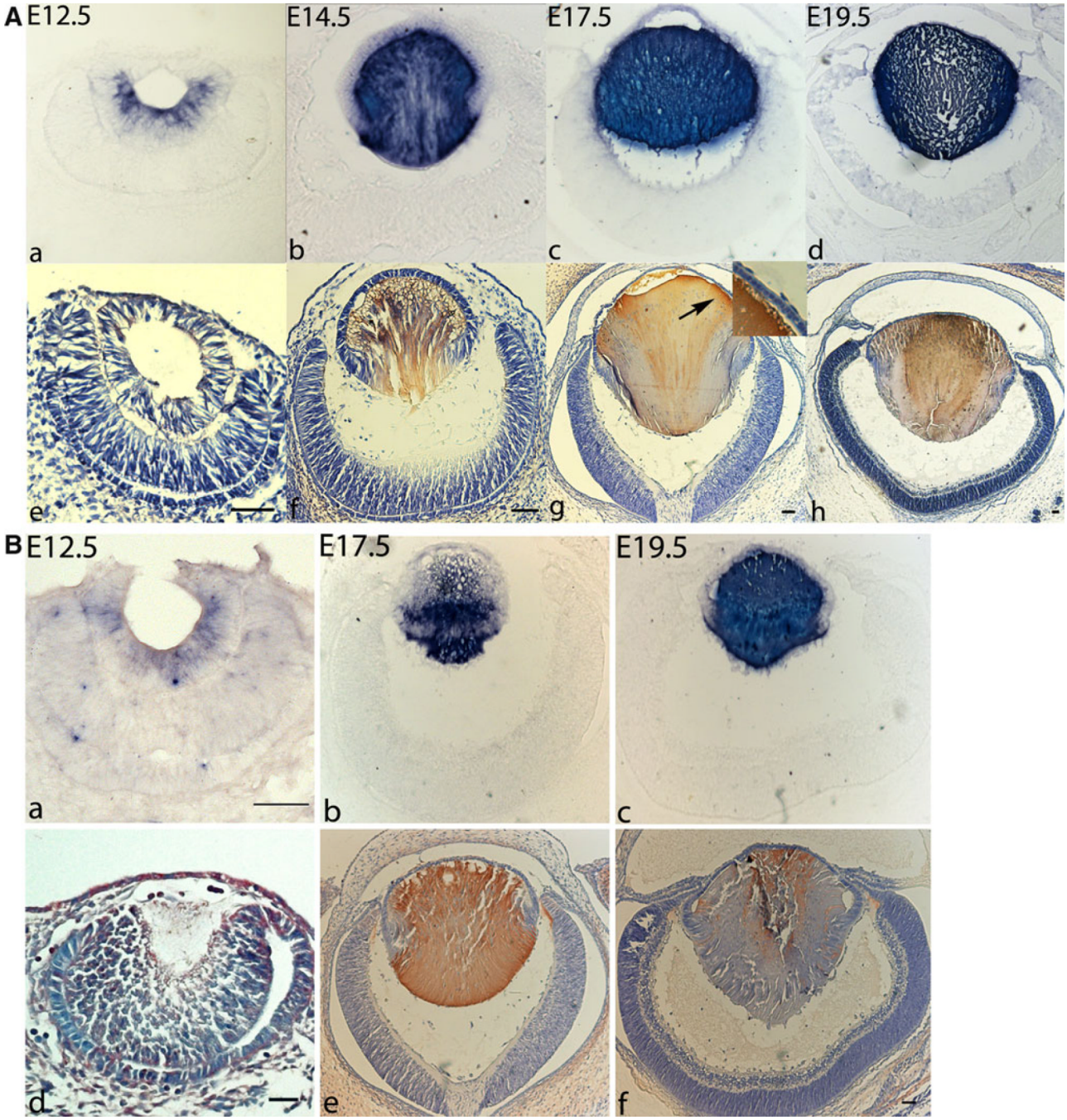
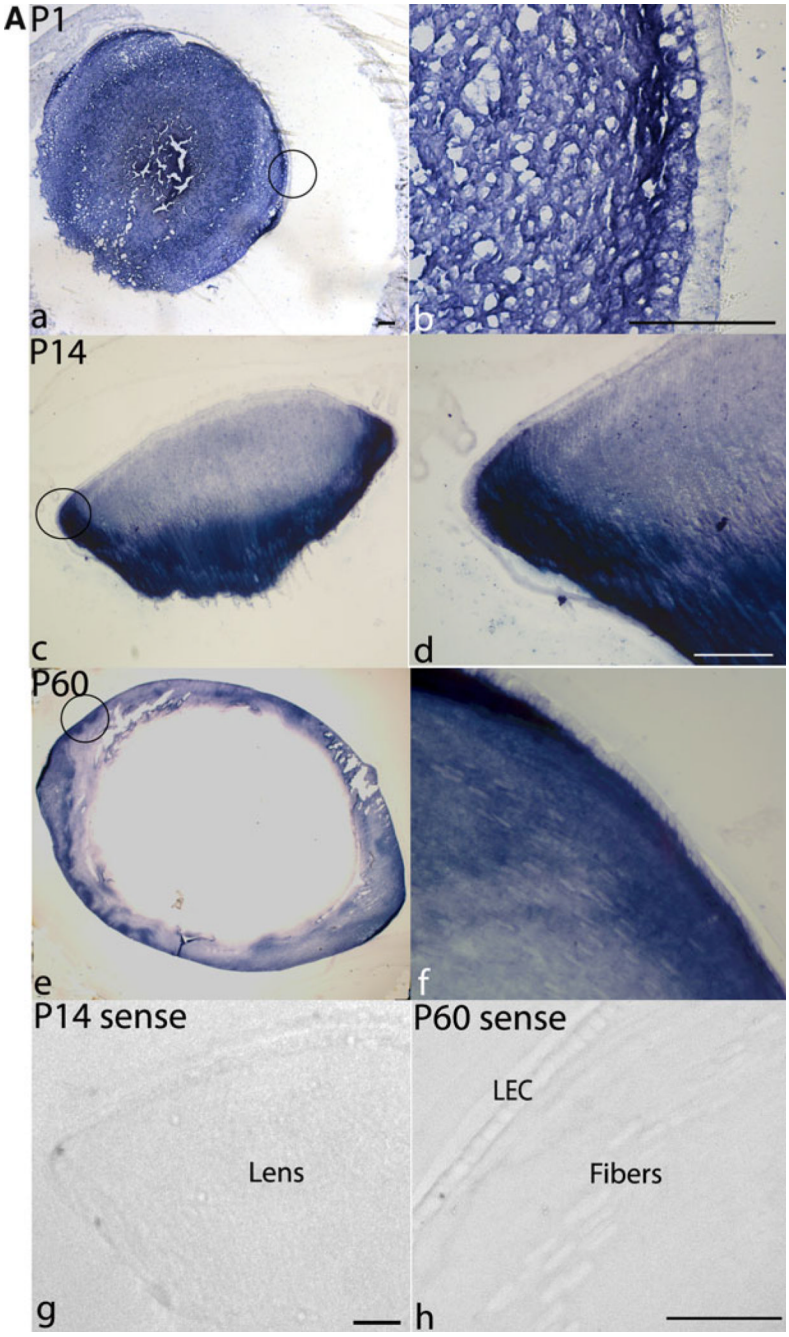


Fig. 1.

Developmental expression of βA3/A1-crystallin in the embryonic eye. **A** *Panels a–d* show the results of in situ hybridization (ISH) with the wild type full-length antisense RNA probe. *Panels e–h* show non-fluorescent immunohistochemistry (nfIHC) using a peptide antibody raised against βA3/A1-crystallin. βA3/A1-crystallin mRNA is expressed as early as E12.5 in the posterior cells of the lens vesicle and subsequently in the primary and secondary lens fibers, but not in other cells. Protein expression, also limited to lens fibers, is evident at E14.5. *Inset* in *g* is magnification of the region indicated by the *arrow*, shown to demonstrate lack of expression in the lens epithelium. *Bars* represent 50 μm and apply to the

corresponding in situ sections as well. **B** *Panels a–f* are Nuc1 embryonic eye sections showing results of ISH (*a–c*) with Nuc1 full-length antisense RNA probe and nfIHC (*d–f*). The Nuc1 lens during embryonic development shows a similar expression pattern as wild type lenses. *Bar* represents 50 μm. *Panels b, c, e, f* are of the same magnification. All sections are of 12 μm thickness. Sections for ISH were cryo-sectioned while those for nfIHC were embedded in paraffin



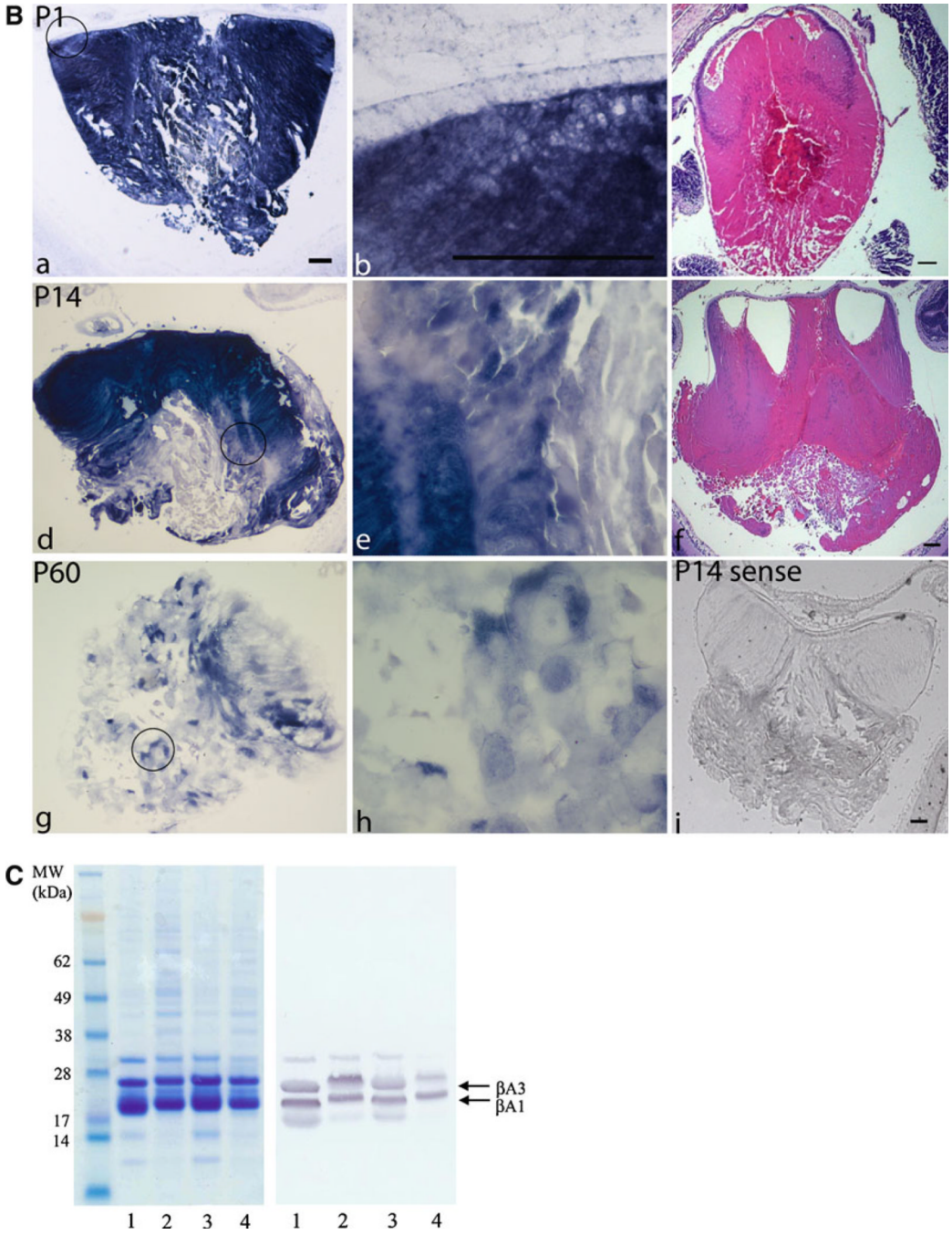


Fig. 2.

mRNA synthesis of βA3/A1-crystallin in postnatal stages of the wild type (**A**) and Nuc1 (**B**) lenses. The βA3/A1-crystallin protein staining pattern in postnatal lens (not shown) is similar to the embryonic stages with strong expression in the fiber cells, and none in the epithelium. In **A** *panels b, d, f* show higher magnifications of the indicated *circled areas* in *a, c, e* respectively. *Panels a, c, e* are of same magnification. *Panels g, h* show ISH with wild-type sense mRNA at P14 and P60 respectively. In *panel h*, the locations of the lens epithelial cells (*LEC*) and fibers are indicated. *Bars* represent 100 μm. In **B** *panels b, e, h* show higher magnifications of the indicated *circled areas* in *a, d, g* respectively. *Panels a, d, g* are of same magnification. *Bars* represent 100 μm. *Panels c, f* show representative H&E stained Nuc1

sections at ages P1 and P14 respectively. The differences seen in morphology in ISH and H&E sections are a result of different embedding and sectioning procedures (methyl methacrylate vs. cryo-sections) rather than time differences or variations between experiments. *Panel i* shows ISH at P14 with Nuc1 sense RNA control. **C** The presence of βA3/A1-crystallin in the lenses of wild type and Nuc1 rats at 1 and 4 months of age. Lens protein extracts were loaded on the gel as follows: *Lanes 1* wild type, 1 month; *Lanes 2* Nuc1, 1 month; *Lanes 3* wild type, 4 months; *Lanes 4* Nuc1, 4 months. The *left panel* shows Coomassie blue staining patterns. The *right panel* shows western blot results for duplicate loadings of the same samples run on the same gel. After transfer to nitrocellulose, the blot was probed with antibody against βA3/A1-crystallin. Note that consistent with the larger size of the mutant proteins (about 1,100 Da), the bands for Nuc1 migrate slower than the wild type. *Arrows* denote the positions of the wild type proteins

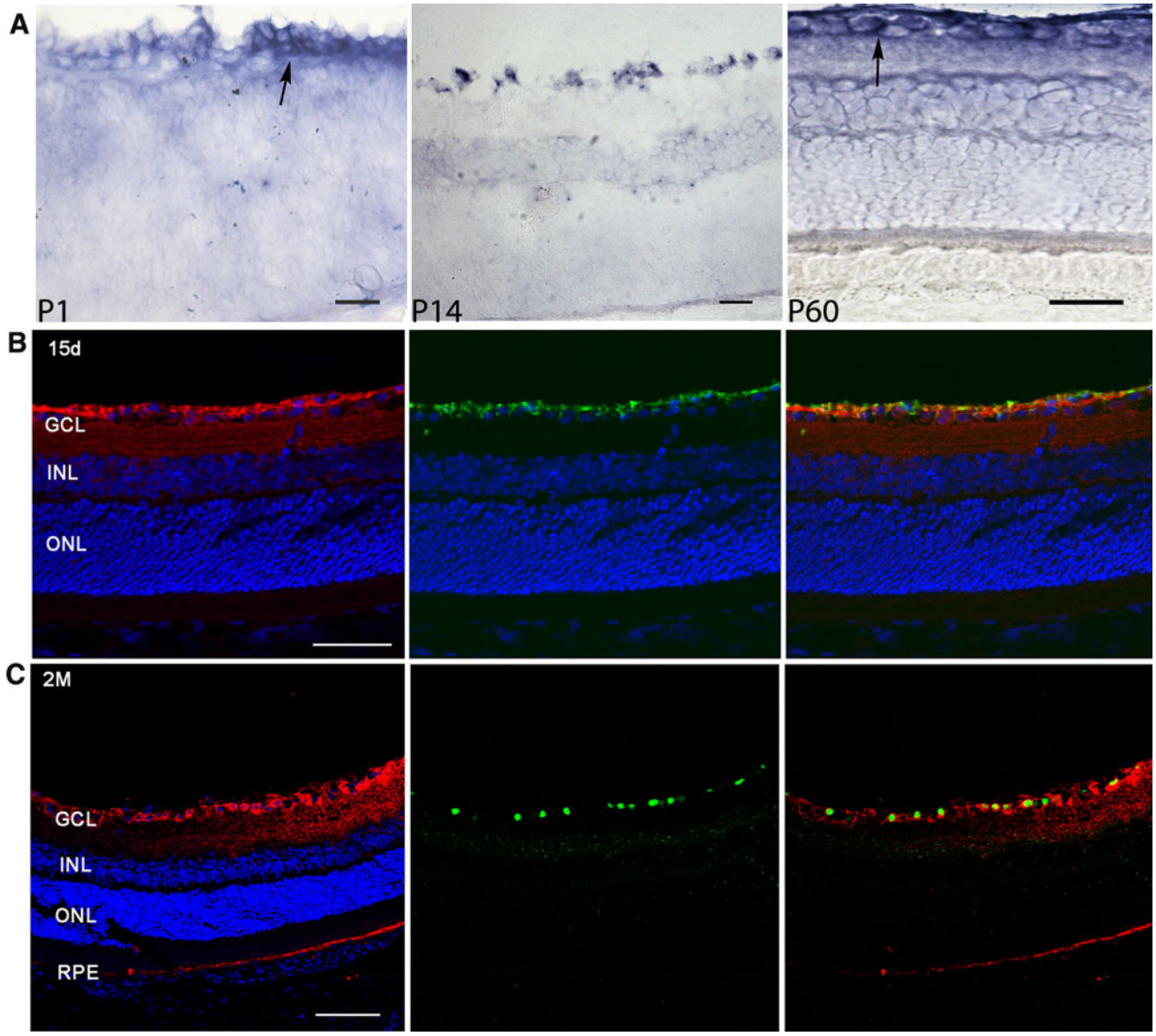


Fig. 3.

Expression of βA3/A1-crystallin in the wild type postnatal retina. **A** ISH of cryo-sectioned eyes with wild type full-length RNA antisense probes at P1, P14 and P60 days. *Arrows* indicate regions of intense staining in the ganglion cell layer during postnatal development. *Bars* represent 25 μm. **B** fIHC on 12 μm thick cryo-sections at postnatal day 15, using a peptide antibody raised against βA3/A1-crystallin (*red*) and GFAP (astrocyte marker, *green*). The *left panel* shows strong βA3/A1-crystallin staining in the ganglion cell layer. The *center panel* shows GFAP-positive astrocytes in the ganglion cell layer. The merged image (*right*) demonstrates significant co-localization along the ganglion cell layer, indicating that GFAP-positive astrocytes are also positive for βA3/A1-crystallin. *Bar* represents 50 μm. **C** fIHC on 12 μm thick cryo-sections at postnatal day 60, using a peptide antibody raised against βA3/A1-crystallin and Brn3b (ganglion cell marker). The *left panel* shows strong βA3/A1-crystallin staining in both the ganglion cell layer and RPE. Specific staining of ganglion cell nuclei by Brn3b is shown in the middle panel. A significant portion of the βA3/A1-crystallin cytoplasmic staining (*red*) surrounds the Brn3b (*green*)-positive ganglion cells as shown in

the merged image (*right*) indicating cytoplasmic staining for βA3/A1-crystallin in retinal ganglion cells. *Bar* represents 50 μm

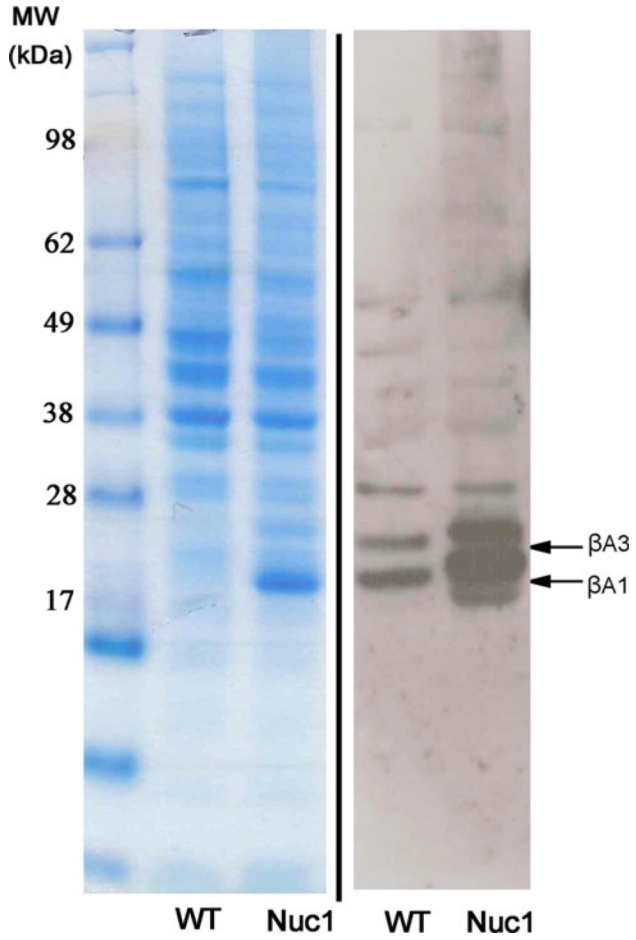


Fig. 4.

Western blotting confirms the presence of βA3/A1-crystallin in astrocytes. *Left panel* shows the Coomassie blue staining pattern and the *right panel* the western blot from parallel lanes of the same gel with identical loading, probed with antiserum against βA3/A1-crystallin. Wild type and Nuc1 astrocyte extracts from P2 primary cultures from brain are shown. Both the βA3 and βA1 polypeptides are detected in all samples. Note that the βA3 and βA1 polypeptides migrate slightly slower in the Nuc1 samples, consistent with the insertion mutation, which increases the molecular mass by about 1,100 Da. *Arrows* denote the positions of the wild type proteins