Author's response to reviews

Title: AlphaA-Crystallin R49Cneo Mutation Influences the Architecture of Lens Fiber Cell Membranes and Causes Posterior and Nuclear Cataracts in Mice

Authors:

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Author's response to reviews: see over
March 30, 2009

Dr. Rikki Graham, Ph.D.
Senior Assistant Editor
BMC-series journals

Cc: John Kerr
Assistant Editor
BMC Ophthalmology

Re: Manuscript “αA-Crystallin R49Cneo Mutation Influences the Architecture of Lens Fiber Cell Membranes and Causes Posterior and Nuclear Cataracts in Mice”
By U.P. Andley

Dear Dr. Graham,

Thank you very much for your e-mail of February 14, 2009 and the Reviewers’ comments on my manuscript. I thank the reviewers for their thorough analysis of this paper and constructive criticisms. All issues that were brought up were taken into consideration in the revised manuscript and are addressed point-by-point below. Thank you also for allowing extra time to respond to these comments.

I want to re-iterate that I am the only author. Everyone who helped me either worked for the Departmental Core facilities or was a laboratory technician. They did not write the manuscript and do not meet the eligibility requirements to be coauthors. These individuals have been listed in the acknowledgments section.

Thank you for reconsidering this revised manuscript for consideration of publication in BMC Ophthalmology.

Sincerely,

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Please note: In the following text, Reviewers’ comments are denoted by quotation marks.
Reviewer 1: Dr. Jon Martin Collinson

Major revisions:

1) “The author postulates that the reduced severity of the phenotype is due to suppression of transcription and/or processing of the mutant allele by the neo cassette. This makes sense and is quite likely, but no evidence for the hypothesis is presented here in respect of αA crystallin protein levels, comparing WT with R49Cneo with R49C. This evidence should be presented.”

Response: I do not have an antibody that specifically recognizes the mutant protein but not wild type αA-crystallin. I attempted mass spectrometry, but this method does not differentiate between amounts of wild type αA-crystallin and mutant αA-crystallin since the ionization rate of the wild type and mutant peptide could be quite different. Data obtained by gel permeation chromatography suggests that the amount of αA-crystallin expressed decreases (data not shown). Immunoblot analysis shown in Figure 3 supports this conclusion. I have modified the relevant statement in the Abstract and Discussion sections of the revised manuscript.

2) “I am not sure how the insolubility assay is performed as no details are given here, but there should probably be some sort of loading control in figure 3. Also the author should perhaps explain why introduction of the neo cassette would reduce the solubility of the protein, if it is in an intron and should be spliced out. It is possible that the neo cassette acts at the level of mRNA processing and creates a protein that retains the intron, but there is no evidence presented for this either - and this would be simple to assay with Western blot.”

Response: I want to clarify that the insolubility of the protein is not due to the introduction of the neo cassette alone, but is due to expression of αA-R49C mutant protein. Details of insolubility assay are added to Materials and Methods and Figure 3 legend of the revised manuscript. The Figure is a western blot with an antibody to α-crystallin, with overloaded protein so that we could still detect the protein in the homozygous extracts.

Minor Revisions:

1) “Spelling of solubility in Fig 3 legend.”

Response: This has been corrected in the revised manuscript.

2) “Describe genetic background of Cre-EIIA mice. Is it possible that breeding R49Cneo with these leads to mice on a mixed genetic background that affects the severity of the cataracts independently of the change in the R49C allele. A control would have been to report any cataracts in WTneo x CreEIIA mice.”
Response: Cre-EIIA mice were on a C57BL/6 background. I have added this to the Methods section of the revised manuscript.

The severity of cataracts is unlikely to be dependent on the mixed genetic backgrounds, because in each case I had wild-type littermates as controls. I found the phenotype to be stable over a number of generations. However, because I cannot completely rule out the possibility that a mixed genetic background affects cataracts, I have added this statement to the Discussion section.

Reviewer 2: Jochen Graw

Please note: Numerals are added to each point of the critique for ease of evaluation.

1. “The main problem of the actual manuscript is the impression that a new transgenic line is described. This impression is formed by sentences in the Abstract like "we have produced a new mouse cataract model ..." (lines 4-7), or by the last paragraph in the Introduction. The reader assumes that a new transgenic mouse line has been described in the actual manuscript according to previously reported transgenic lines. Only if one is looking in detail for the references 44 and 55, it becomes obvious that these papers deal with the same mutants. Particularly, the Introduction does not mention the previous findings as starting point for the experiments reported in the new manuscript.”

Response: I have changed the Abstract and Introduction to clarify that I am reporting the previously described mutation R49C in αA-crystallin, except that these mice still express the neo cassette.

2. “Moreover, the Material-and-Method section of the new manuscript gives a very detailed repetition of the entire procedure generating the knock-in mice. However, it has been already described in the same detailed manner in the previous paper by Xi et al., 2008; it includes the names of the lines produced and tested (KI3 and KI4) and the primer sequences for genotyping. To avoid misunderstanding, the entire paragraph ("generation of knock-in mice") has to be skipped in a revised version”.

Response: I have reduced the Materials and Methods section describing the generation of the knock-in mice to a brief description of the process.

3. “Also some of the figures given in the new manuscript are similar to those of the previous papers: Fig. 1 is more or less identical to Fig. 1a of the paper by Xi et al., 2008, and the lens phenotypes given in Fig 2a-f give the same information for 2 months and 4 months as previously reported for 5 and 16 weeks (Xi et al., 2008, Fig. 3a-f). However, the already published figures are slit lamp pictures with densitograms giving much more information than the pictures in the new manuscript.”
Response: I thank the reviewer. Figure 2A was mistakenly the same as in the previous manuscript. I have replaced this with a new figure 2A. I do not agree that Figures 2 b-h have been previously published. However, I have replaced them with Figures 2B-D in the revised manuscript. These are clearly different from the previously published manuscript. I was careful to ascertain that the lenses described in this manuscript are different from those in the published manuscript; they are from a different line of mice carrying the neo cassette, and experiments were performed in a different time frame. They do not overlap with figures published previously. I want to emphasize that the point here is to show that the cataract is stable even when the neo cassette is still expressed. Data for the newborn mouse lens without the neo cassette has been retained as Figure 2E and F of the revised manuscript.

4. “Figure 3 of the new manuscript gives just an acrylamide gel for water-soluble lens extracts (there is no corresponding method given - just a reference to Immuno-blots, but it seems to be a Coomassie-staining). The immunoreactivity, which is summarized on the right side of this figure, was already reported in much more detail in both previous papers (Xi et al., 2008; Andley et al., 2008 - references 44 and 55). As a minor point in this context, the legend of the figure is not helpful for understanding (amount of protein? age and number of animals/lenses? single or pooled lens extract? etc); in the right part, the number of extracts tested is missing as well as error bars (either for SD or SEM).”

Response: This figure is from mice still expressing the neo cassette. It is different from the figure reported previously. The corresponding method is given in the Methods section of the revised manuscript. Other details have been added to the legend of the revised manuscript.

5. “Moreover, the immunofluorescence analysis of the R49C transgenic mice using a MIP antibody (Fig 8 of the new manuscript) was described also previously in more detail in the paper by Xi et al., 2008 (Fig. 9).”

Response: I do not agree. The MIP immunofluorescence staining is very different in the current manuscript than in Figure 9 of the published manuscript. Please note the position of vacuoles or swollen cells in the MIP figure of the current manuscript. For the homozygous mice, the neo-deleted mouse lens is much smaller, and there is greater disruption of the fiber cell morphology in the previously published work than in the MIP figure of the revised manuscript. In the revised manuscript, the membrane changes are seen mainly in a band of fibers in the inner cortical region, surrounded by normal fiber cells on both sides (Figure 8).

6. “The new aspects of the manuscript are in the figures 4-7 and 9 giving a detailed morphological analysis including electron microscopy; also Fig. 2g, h gives new information for the mutant line lacking the neo-cassette and giving rise to a hypomorphic allele.”

Response: I appreciate the Reviewer’s positive and constructive comment.
7. “If Dr. Andley wants to submit a revised version, she should make clear in the abstract and more detailed in the Introduction that the new manuscript continues the characterization of already existing transgenic lines. In the Introduction, the previous findings should be shortly summerized and the aim of the new study should be emphasized. The result section should deal with new data only.”

Response: I agree with the Reviewer, and have made the necessary changes.

8. “If the results of the transgenic mice without the neo-cassette will be demonstrated, the promoter of the Cre gene should be mentioned to understand in which tissues the neo-cassette will be lost. Moreover, instead of speculations about the reduced number of transcripts in some of the transgenic lines (p8, para 1 and 2), one can make the corresponding experiment and determine their relative concentration using real-time PCR.”

Response: I mentioned that I used Cre-EIIa mice, and have emphasized the promoter used in the revised manuscript.

Minor points:

9. The genetic symbol Cryaa (encoding aA-crystallin; in italics) should be used throughout.

Response: I have made this change.

10. The revised version should be written with 1.5 line spacing; the Introduction and Discussion should be shortened to 1.5 and 2 pages, resp.

Response: I have reduced the length of the Introduction and Discussion sections and line spacing has been adjusted to 2.0, as required by the Journal guidelines.

11. Finally, it is surprising that an experimental paper is written by one author only.

Response: Everyone who helped me either worked for the Departmental Core facilities or was a laboratory technician. They did not write the manuscript and do not meet the eligibility requirements to be coauthors. These individuals have been listed in the acknowledgments section.

Reviewer 3: John West

Major Compulsory Revisions

(1) “A clear statement should be included at the end of the Introduction to explain that the aim of the study was to characterise the lens abnormalities in
WT/R49Cneo and R49Cneo/R49Cneo mice and compare them to previous reports of WT/R49C and R49C/R49C lenses (refs 44 and 55). Currently this only becomes really clear once the reader reaches the Discussion.”

Response: In accordance with the Reviewer’s suggestion, I have added clear statements in the Abstract and Introduction sections of the revised manuscript. I have also added a statement of aims of the current study at the end of the Introduction section.

(2) “The results are mostly presented as a series of illustrations without indicating how representative they are. The number of mice of each genotype that were examined at each age should be stated with an indication of how variable the results are.”

Response: I have added the number of samples tested and indicated how representative the illustrations are in the Results section.

(3) “The methods used to determine the percentage water insoluble alphaA-crystallin protein (Fig 3) should be included in the Materials and Methods. The two panels of Fig 3 should be labelled ‘A’ and ‘B’ and should be described more fully in the legend. In the first panel of Fig 3, the 3 lanes differ in genotype. The age and cataract severity should be given in the legend. The second panel shows estimates of percentage of insoluble protein for lens with cataracts severities 1, 2 and 3. The histogram should show error bars and the legend should explain how many samples there were per severity class and what genotypes and ages they were.”

Response: I have added this information to the revised manuscript.

(4) “Fig 7C legend should explain what the cell numbers mean (e.g. cell numbers per section or field of view or lens?) Should genotypes WT/R49C and R49C/R49C be WT/R49Cneo and R49Cneo/R49Cneo in 7C?”

Response: Cell numbers are given per section. I have added this to the legend of Figure 7C of the revised manuscript. I have corrected the genotypes in accordance with the Reviewer’s suggestion.

(5) “P8: In the Discussion it seems to be assumed that R49Cneo causes reduced expression of alphaA-crystallin (e.g. ‘These lower levels of R49C alphaA-crystallin evidently have given us a novel way to examine the effect of lower expression levels of the mutant R49C alphaA-crystallin protein on lens opacity and histology.’). Is there any direct evidence for this or it is speculation?”

Response: I have clarified that these statements are based on studies published in the literature.

(6) “Fig 4 should show all three genotypes at both 3 days and 3 months (so a 3
day homozygous R49Cneo/ R49Cneo and a 3month heterozygous WT/
R49Cneo should be included.”

Response: The Reviewer asked later in this critique that I combine some of the Figures. Instead, I have simplified Figure 4. It now shows age-matched 3 month old wild type and R49Cneo/ R49Cneo homozygous lenses.

Minor Essential Revisions
(such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)
(7) “Figure legends: It would better if the figure legends were used to explain fully what the figure shows and other comments moved to the text of the results section. For example, in Fig 3 legend the sentence ‘The increased severity of cataract after deletion of the neo cassette is putatively caused by an increase in R49C-alpha-A crystallin expression.’ would be better in the main text of the Results. Similarly some of the commentary in Figs 6 and 7 would be better in the text.”

Response: I agree with the Reviewer, and have moved these statements to the Results section.

(8) “Page 5/last paragraph: The Methods section should not include results such as ‘Changes appear by 3 weeks in heterozygous and homozygous lenses’ etc. This type of information should be in the Results.”

Response: I have made this change.

(9) “Fig 1: The legend should clarify whether the asterisk above exon 1 indicates the mutation and should indicate that the numbered blue rectangles are exons, the filled triangles are loxP sites and ‘X’ is the Xhol site. It might also be worth modifying the diagram slightly because the lines illustrating recombination between the construct and the wild type allele could be misinterpreted as unequal crossing over. On the right, one line goes from intron 1 to intron 3 whereas the other one goes from intron 1 to intron 2.”

Response: I have modified the diagram in Figure 1 in accordance with the reviewer’s suggestion.

(10) “The age of the mice shown in Figs 2B and 2C should be given in the legend.”

Response: I have added the age of mice to the legends of Figure 2B and 2C.

Discretionary Revisions
(which are recommendations for improvement but which the author can choose to ignore)
(11) “The title is a bit misleading because the manuscript is about the R49Cneo
mutation not R49C.”

Response: I have added the word neo to the title of the manuscript.

(12) “p3/para 1/line 12: Consider whether ‘lens membranes’ would be better as ‘lens cell membranes’ (e.g. to avoid confusion with capsule)?”

Response: I have made this change on page 3.

(13) “P5/para2/3: ‘Cre-recombinase sites’ should be changed to ‘loxP sites’ which is more accurate and consistent with the Results (line 4).”

Response: I have made this change.

(14) “p5/2/5: The mouse strain name should probably be ‘129/SvJ’ rather than ‘129SvJ’.”

Response: I have made this correction.

(15) “p5/2/12 and 13: The mouse strain name should be given in full (‘C57BL/6’ rather than ‘C57’ or ‘C57BL6’).”

Response: I have made these changes.

(16) “It may be better to combine some of the figures.”

Response: I have simplified Figure 4, but prefer not to combine other figures for ease of reading.