

Research Article

Effect of α B-Crystallin on Protein Aggregation in *Drosophila*

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Disorganisation and aggregation of proteins containing expanded polyglutamine (polyQ) repeats, or ectopic expression of α -synuclein, underlie neurodegenerative diseases including Alzheimer's, Parkinson, Huntington, Creutzfeldt diseases. Small heat-shock proteins, such as α B-crystallin, act as chaperones to prevent protein aggregation and play a key role in the prevention of such protein disorganisation diseases. In this study, we have explored the potential for chaperone activity of α B-crystallin to suppress the formation of protein aggregates. We tested the ability of α B-crystallin to suppress the aggregation of a polyQ protein and α -synuclein in *Drosophila*. We found that α B-crystallin suppresses both the compound eye degeneration induced by polyQ and the α -synuclein-induced rough eye phenotype. Furthermore, by using histochemical staining we have determined that α B-crystallin inhibits the aggregation of polyQ *in vivo*. These data provide a clue for the development of therapeutics for neurodegenerative diseases.

1. Introduction

Expansion of polyglutamine (polyQ) domains causes inherited neurodegenerative diseases, such as Huntingtons, disease, various types of spinocerebellar ataxia, and spinobulbar muscular atrophy (SBMA) [1, 2]. The formation of intranuclear inclusions of polyQ-containing proteins in the brain is one of the pathological features of these diseases. Inhibition of the aggregation of the expanded polyQ-containing proteins may therefore provide treatment options for polyQ diseases [3]; however, the relationship between polyQ aggregation and cellular toxicity is not well understood. It was demonstrated in earlier studies in human cells that targeted expression of proteins with an expanded polyQ repeat led to nuclear inclusion formation followed by late-onset cell degeneration. Similar observations have been made in *Drosophila* suggesting that the cellular mechanisms underlying human glutamine-repeat diseases are conserved in invertebrates [4]. From a therapeutic view point, *Drosophila* provides a unique system in which to search for novel genetic

factors that suppress cellular toxicity. Previous studies have indicated that QBP1 (polyQ binding peptide 1) binds to the expanded polyQ stretch and inhibits aggregate formation of the expanded polyQ protein *in vitro* [5, 6]. Furthermore, *Ter* 94, the *Drosophila* homolog of the myeloid leukemia factor 1, and heat-shock-transcription-factor-1 (HSF1-) activating compounds are also reported to have a dominant role in suppressing polyQ aggregation [7–9].

Alpha-crystallins (α -crystallins) are major protein components of the vertebrate eye lens. The α -crystallins and especially α B-crystallin are also found outside the lens, and show a wide tissue distribution [10]. The α B-crystallin protein is found to be overexpressed in many neurological diseases, and mutations in α A- or α B-crystallin can cause cataracts and myopathy [11]. The α B-crystallin protein is a member of the small heat shock protein family, and it has a chaperone-like function [12–15]. Therefore, α B-crystallin can bind to the unfolded proteins and inhibit nonspecific aggregation [10]. Consistent with this it has been shown to prevent the fibrillar aggregation of proteins implicated in

human diseases and limit the onset of misfolding diseases [16–18]. Furthermore, accumulation of α B-crystallin is an important mechanism for the prevention of cellular damage associated with heat shock and oxidative stress conditions in the trabecular meshwork [19, 20].

α -synuclein is a presynaptic protein whose major role in neurodegeneration was recognized in Parkinson disease where a mutation in the α -synuclein gene was associated with autosomal dominant inheritance of the disease [21]. It is reported that α B-crystallin interacts with α -synuclein to protect against amyloid aggregation and limit the onset of misfolding diseases *in vitro* [16].

In the present study, we investigate the ability of the molecular chaperone, α B-crystallin, to inhibit the aggregation of protein induced by ectopic expression of polyQ and α -synuclein in fly model. Our results indicate that α B-crystallin suppresses the neurodegenerative effects induced by overexpression of polyQ as well as α -synuclein.

2. Material and Methods

2.1. Fly Strains. Fly strains were maintained at 25°C on standard food. The transgenic fly line carrying GMR-GAL4 on X chromosome was described earlier [22] as was the transgenic fly line carrying *gmr-Q92* [4, 7]. The UAS-LacZ flies used in this study were obtained from Bloomington *Drosophila* stock center. Wild-type flies: W; UAS- α *syn*; +, was obtained from Feany and Bender [23].

2.2. Plasmid Construction and Establishment of Transgenic Flies. To construct the plasmid pUAS- α B-crystallin-HA, the full coding sequence of human α B-crystallin cDNA (accession number S45630) corresponding to amino acids (aa) 1 to aa175 was amplified by PCR with primers carrying *Bgl*II site and *Xho*I site (α B-crystallin-*Bgl*II: 5'-*gca gat ctg aca tcg cca tcc acc acc*, α B-crystallin-*Xho*I: 5'-*cgc tcg agc tat ttc ttg ggg gct gc*). The PCR products were digested with *Bgl*II and *Xho*I and inserted into the plasmid pUAST-HA (Lablife).

The P element-mediated germ line transformation with pUAS- α B-crystallin-HA was carried out as describe earlier [24], and F1 transformants were selected on the basis of white-eye color rescue [25]. The established transgenic fly lines and their chromosome linkages are listed in Table 1.

2.3. Western Immunoblot Analysis. Proteins were extracted from eye imaginal discs of third-instar larvae, applied to a SDS-polyacrylamide gel containing 12% acrylamide and transferred to polyvinylidene difluoride membranes (Bio-Rad) in transfer buffer (50 mM borate-NaOH, pH 9.0, and 20% ethanol) by using iBlot system (Invitrogen). The blotted membranes were incubated with monoclonal antibodies to HA (HA124, Invitrogen) at 1:400 dilution or anti- α tubulin IgG (Sigma) at 1:2,000 for 16 h at 4°C. The bound antibodies were detected with peroxidase-conjugated goat anti-mouse IgG and the ECL system (GE healthcare) according to the manufacturer's recommendations. The images were analyzed with a Lumivision ProHSII image analyzer (Aisin Seiki, Aichi, Japan).

TABLE 1: Transgenic fly lines established in this study and their chromosome linkages.

P-element plasmid	Strains	Chromosome linkages
	27	II
	8	II
UAS- α B-crystallin-HA	3	III
	11	III
	12	III

2.4. Scanning Electron Microscopy. Adult flies were anesthetized, mounted on stages, and observed under a VE-7800 scanning electron microscope (Keyence Inc.) in the high vacuum mode. In every experiment, the eye phenotype of at least five adult flies of each line was simultaneously examined by scanning electron microscopy, and these experiments were repeated 3 times. In the experiments, no significant variation in eye phenotype among the five individuals was observed.

2.5. Immunohistochemistry. Third instar larvae were dissected in PBS, and tissues were fixed in 3% paraformaldehyde/PBS for 30 min at 25°C. After washing with PBS/0.3% TritonX-100 (PBS-T), the samples were blocked with PBS-T containing 10% normal goat serum for 20 min at 25°C and incubated with an anti-HA (Invitrogen), anti-FLAG monoclonal antibody (Sigma), or anti- β -galactosidase monoclonal antibody (Developmental Studies Hybridoma Bank, DSHB) at a 1:200 dilution at 4°C for 16 h. After extensive washing with PBS-T, the tissues were incubated with an anti-mouse IgG conjugated with Alexa 594 or Alexa 488 (Molecular Probes) at a 1:400 dilution each at 37°C for 1 h. After extensive washing with PBS-T and PBS, samples were mounted in Fluor Guard Antifade Reagent (Bio-Rad) for microscopic observation under a fluorescence BX-40 microscope (Olympus, Tokyo, Japan) equipped with a cooled CCD camera (ORCA-ER; Hamamatsu Photonics K. K., Shizuoka, Japan).

3. Results and Discussion

3.1. Ectopic Expression of α B-Crystallin Results in Abnormal Eye Morphology. We first established lines of transgenic flies carrying the *uas-human- α B-crystallin*. In order to confirm that human- α B-crystallin is functional in *Drosophila*, we used the *gmr-gal4* driver strain to provide ectopic expression of α B-crystallin-HA from the morphogenetic furrow to the posterior end of the *Drosophila* eye imaginal disc [26]. By inspection with the scanning electron microscope, the compound eyes of adult flies carrying the *gmr-gal4* alone showed normal eye morphology (Figures 1(a) and 1(b)), while those of flies carrying both the *gmr-gal4* and the *uas- α B-crystallin* exhibited a rough-eye phenotype (Figures 1(d) and 1(e)).

To further confirm the expression of α B-crystallin carrying an HA tag in its C-terminal end in eye imaginal discs, we

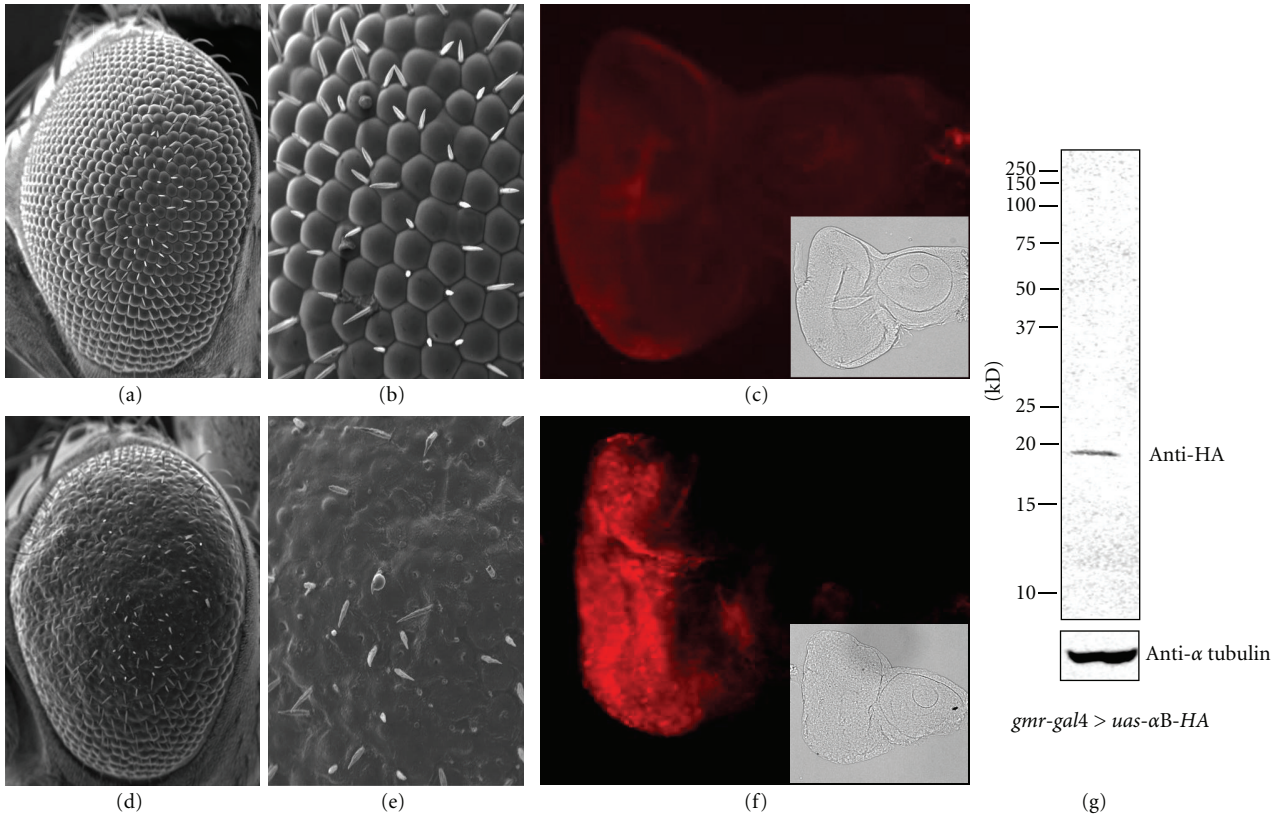


FIGURE 1: Scanning electron micrographs of adult compound eyes. (a, b) *gmr-gal4; +; +*. (d, e) *gmr-gal4; uas- αB -crystallin-HA; +; +*. (c, f) Immunostaining of the eye imaginal discs with anti-HA antibody. The eye disc from *gmr-gal4; +; +* flies (c) and *gmr-gal4; uas- αB -crystallin-HA; +; +* flies (f). The insets indicate Nomarski images. (g) The anti-HA antibody specifically recognized αB -crystallin-HA in the Western Blot of the eye imaginal discs of *gmr-gal4; uas- αB -crystallin-HA; +; +* flies.

carried out immunostaining of eye imaginal discs from third instar larvae using anti-HA antibody. Only *gmr-gal4; uas- αB -crystallin* flies showed ectopic αB -crystallin-HA signals from the morphogenetic furrow to the posterior end of eye discs (Figure 1(f)). In contrast, the *gmr-gal4* line that was used as the negative control showed no signal (Figure 1(c)). The expression of αB -crystallin-HA was further confirmed by the Western immunoblot analysis of eye imaginal disc extracts with anti-HA antibody, detecting a single αB -crystallin-HA band at around 20 kDa (Figure 1(g)).

3.2. Expression of αB -Crystallin Suppresses the Compound Eye Degeneration Induced by PolyQ. To examine the effect of αB -crystallin protein on polyQ-induced neurodegeneration in *Drosophila*, we crossed *gmr-Q92* fly lines expressing a Q92 peptide in the eye discs under the direct control of the *gmr* promoter [5, 7], and *gmr-gal4 > uas- αB -crystallin-HA* (*uas- αB -HA*) flies in which αB -crystallin-HA expression is driven by *gmr-gal4*.

Expression of the FLAG-tagged 92-glutamine peptides induced compound eye degeneration such as rough eye and loss of red eye pigment (loss-of-pigment phenotype) as described (Figures 2(a) and 2(d)) [5, 7] and the eye phenotype, especially the loss-of-pigment phenotype, was

effectively rescued by coexpression of αB -crystallin-HA (Figures 2(c) and 2(f)). In contrast, expression of the control lacZ protein did not affect the polyQ-induced phenotype (Figures 2(b) and 2(e)).

3.3. Expression of αB -Crystallin Suppresses PolyQ Aggregate Formation in the Eye Imaginal Discs. In order to investigate the ability of αB -crystallin to inhibit polyQ aggregation *in vivo*, we examined polyQ aggregate formation in the eye imaginal discs of third instar larvae in the *gmr-Q92* fly lines with and without expression the αB -crystallin-HA. PolyQ forms inclusion bodies in the region posterior to the morphogenetic furrow in eye discs of the *gmr-Q92* flies and many of these aggregate formations could be detected by immunostaining with anti-FLAG antibody (Figures 3(A1) and 3(C1)).

Expression of αB -crystallin-HA completely suppressed formation of the polyQ aggregates in the line exhibiting a mild eye phenotype (*gmr-Q92*) (Figure 3(B1)) and dramatically suppressed it in the line exhibiting a severe eye phenotype (*2xgmr-Q92*) (Figure 3(D1)), while expression of control lacZ protein exerted no suppressing effect (Figures 3(A1) and 3(C1)). These results suggest correlation between formation of the polyQ aggregates and neurodegeneration.

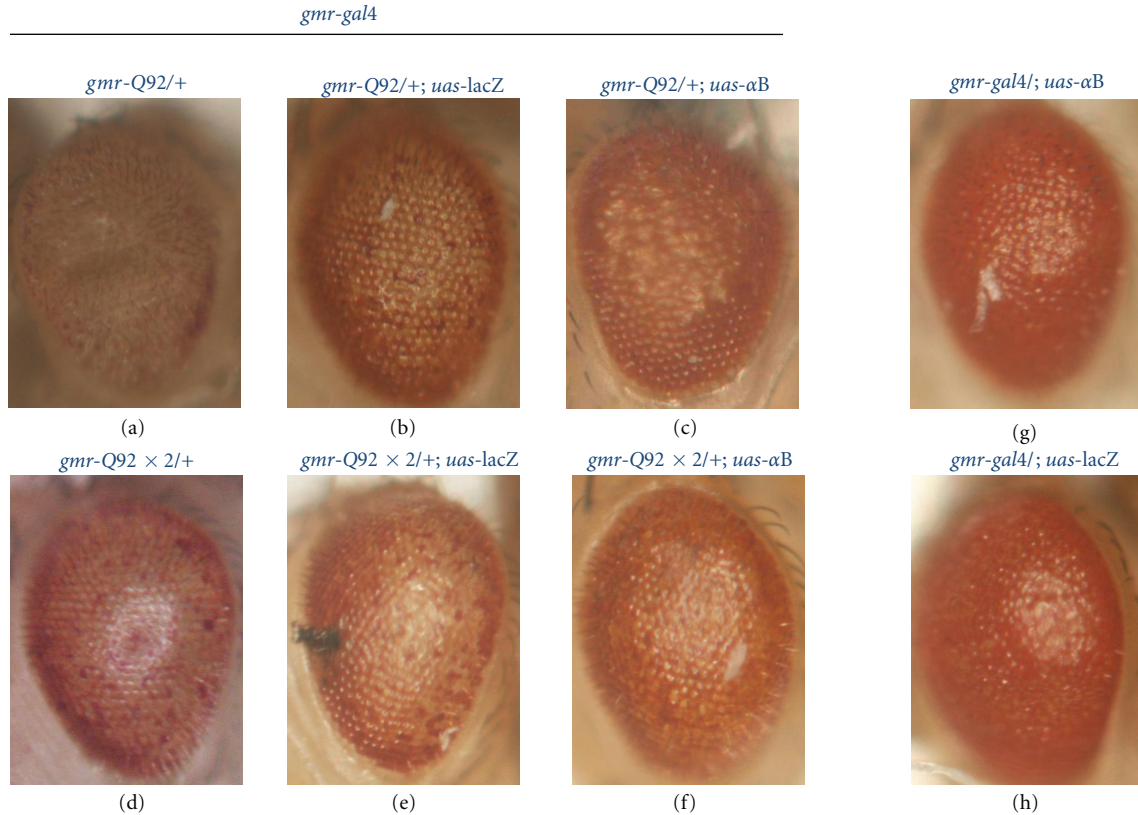


FIGURE 2: Suppression of compound eye degeneration by coexpression of α B-crystallin in the Q92-expressing flies. Light microscopic images of the adult compound eyes of the polyQ lines expressing only one Q92 peptide (a) or 2xQ92 peptide (d) under control of the *gmr* promoter. (c, f) Transgenic flies coexpressing Q92 peptides and α B-crystallin (*gmr-gal4; gmr-Q92/+; uas- α B-crystallin-HA*). (b, e) Transgenic flies coexpressing Q92 peptides and control LacZ protein (*gmr-gal4; gmr-Q92/+; uas-LacZ*). (g) Transgenic flies expressing α B-crystallin alone (*gmr-gal4; uas- α B-crystallin-HA*). (h) Transgenic flies expressing lacZ alone (*gmr-gal4; uas-LacZ*). Magnification x 90.

3.4. Expression of α B-Crystallin Suppresses the Human α -Synuclein Induced Rough-Eye Phenotype. A previous *in vitro* study indicated that α B-crystallin was very effective in suppressing fibrillization of both α -synuclein wild-type and A30P α -synuclein mutant [15]. Therefore, in this study, to evaluate ability of α B-crystallin to suppress the rough-eye phenotype induced by ectopic expression of α -synuclein, we performed coexpression of α B-crystallin and α -synuclein in fly eyes and compared the results with those of control flies coexpressing lacZ and α -synuclein. As noted previously, overexpression of wild-type α -synuclein induced rough eye phenotype (Figures 4(g) and 4(h)). The rough eye phenotype was significantly suppressed by coexpressing α B-crystallin (Figures 4(a) and 4(b)), while no effect on the rough eye was observed by coexpressing control lacZ (Figures 4(c) and 4(d)). These results indicate that α B-crystallin protein effectively suppresses the events induced by overexpression of α -synuclein.

In this study, we investigated a role of α B-crystallin on polyQ aggregation in a *Drosophila* model to gain insight into pathways which could be useful in the treatment of neurodegenerative diseases. We used immunohistochemical methods to show that the GMR-GAL4/UAS- α B-crystallin flies expressed an abundant α B-crystallin protein in the

region from the morphogenetic furrow to the posterior end of eye discs. Consequently, flies exhibit rough-eye phenotype in adults. These results indicate that the human α B-crystallin protein is functional in flies.

Previous studies showed that α B-crystallin can act as a molecular chaperone to prevent fibril formation and aggregation of α -synuclein, β 2-microglobulin, and κ -casein *in vitro* [10, 12, 15–17, 27–29]. Moreover, chaperone activity sites of α B-crystallin were identified in the sequences from residues aa73-aa85 and aa101-aa110 [30–32], suggesting that expression of full length or a part of chaperone sequences of this protein is capable of suppressing polyglutamine-induced protein aggregation. In the present study, we found that the full-length α B-crystallin could inhibit aggregation of the 92-polyQ peptide *in vivo*. The results support the hypothesis that interactive domains in α B-crystallin play a role in prevention of protein aggregation. In contrast, the recent study shows that α B-crystallin does not inhibit or modify the fibrillar aggregation of SpAcQ52 *in vitro* [33]. Because glutamine is a hydrophilic amino acid, the surface hydrophobicity of polyQ may not be changed as aggregation progresses. Furthermore, the polyQ region is thought to be in a condensed disordered state in the native conditions [34]. Therefore, the physiochemical properties of the polyQ tract

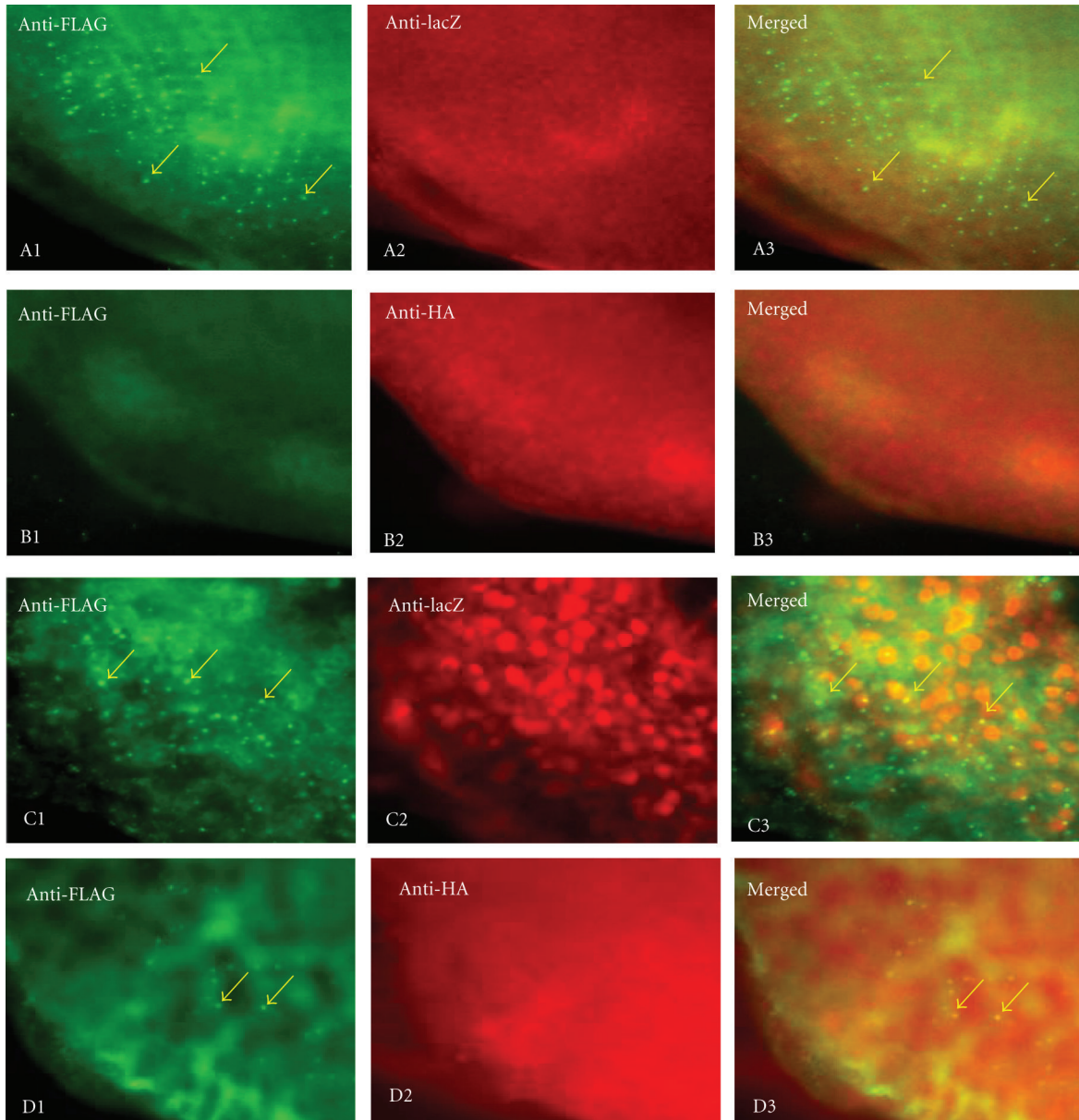


FIGURE 3: Suppression of Q92 aggregate formation by expression of α B-crystallin in the imaginal discs of the Q92 flies. The eye discs were immunostained with anti-FLAG antibody to detect Q92 tagged with FLAG (green) and anti-HA or anti-LacZ antibodies to detect α B-crystallin-HA (red) and lacZ, respectively (red). The arrows indicate examples of Q92 aggregates. (A1–A3) *gmr-gal4; gmr-Q92/+; uas-lacZ*. (B1–B3) *gmr-gal4; gmr-Q92/+; uas- α B-crystallin-HA*. (C1–C3) *gmr-gal4; 2x(gmr-Q92)/+; uas-lacZ*. (D1–D3) *gmr-gal4; 2x(gmr-Q92-FLAG)/+; uas- α B-crystallin-HA*.

may lead it to interact with chaperonin differently *in vitro* compared with *in vivo*. The effectiveness of suppression of polyQ disorders may also depend on the other factors, such as the temperature, concentrations of chemical compounds, or the inhibitor proteins [33, 35, 36]. Here, our data indicate that inhibition of polyQ-aggregation by α B-crystallin in flies carrying one copy of the Q92 gene is complete, but flies carrying two copies of the Q92 gene show only partial suppression, suggesting the importance of optimization to determine the effective level of inhibitors for therapies for polyQ diseases. More interestingly, it is reported that α B-crystallin is a potent *in vitro* inhibitor of the fibrillization

of α -synuclein, a protein involved in neurodegenerative diseases, both wild-type and the two mutant forms (A30P and A53T) [15, 16]. The data we present here suggests that this is achieved by the interaction of α B-crystallin and α -synuclein inhibiting further aggregation.

4. Conclusion

The present study has demonstrated an essential role of α B-crystallin in suppressing the neurodegeneration induced by polyQ expansion and ectopic expression of α -synuclein *in vivo* and suggest a possible target for the development of

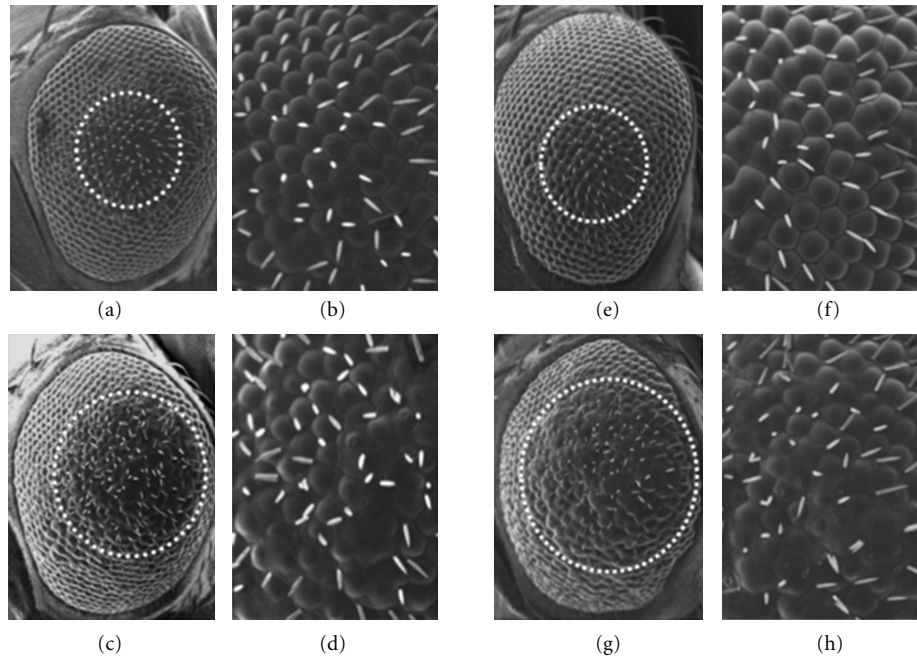


FIGURE 4: Suppression of compound eye degeneration by coexpression of α B-crystallin in the α -synuclein-expressing flies. Light microscope images of the adult compound eyes. (a, b) Transgenic flies coexpressing α -synuclein and α B-crystallin (*gmr-gal4/+; uas- α B-crystallin-HA/+; uas- α -synuclein/+*). (c, d) Transgenic flies coexpressing α -synuclein and control LacZ protein (*gmr-gal4/+; uas-LacZ/+; uas- α -synuclein/+*). (e, f) Control flies (*gmr-gal4/+; +; +*). (g, h) Transgenic flies expressing α -synuclein (*gmr-gal4/+; uas- α -synuclein/+; +*). (a, c, e, g) Magnification x 200. (b, d, f, h) Magnification x 700.

therapeutics for neurodegenerative diseases. Moreover, the assay system to suppress neurodegeneration using transgenic flies described in the present study should be a powerful tool to evaluate the *in vivo* effect of peptides carrying potential chaperone activities, and the α B-crystallin-overexpressing flies established in this study might provide an invaluable standard for the evaluation.

Acknowledgments

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