Expression of Cataract-linked γ-crystallin Variants in Zebrafish Reveals a Proteostasis Network that Senses Protein Stability

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ABSTRACT

The refractivity and transparency of the ocular lens is dependent on the stability and solubility of the crystallins in the fiber cells. A number of mutations of lens crystallins have been associated with dominant cataracts in humans and mice. Of particular interest were γB- and γD-crystallin mutants linked to dominant cataracts in mouse models. While thermodynamically destabilized and aggregation-prone, these mutants were found to have weak affinity to the resident chaperone α-crystallin in vitro. To better understand the mechanism of the cataract phenotype, we transgenically expressed different γD-crystallin mutants in the zebrafish lens, and observed a range of lens defects that arise primarily from the aggregation of the mutant proteins. Unlike mouse models, a strong correlation was observed between the severity and penetrance of the phenotype and the level of destabilization of the mutant. We interpret this result to reflect the presence of a proteostasis network which can “sense” protein stability. In the more destabilized mutants, the capacity of this network is overwhelmed leading to the observed increase in phenotypic penetrance. Overexpression of αA-crystallin had no significant effects on the penetrance of lens defects suggesting that its chaperone capacity is not limiting. While consistent with the prevailing hypothesis that a chaperone network is required for lens transparency, our results suggest that αA-crystallin may not be efficient to inhibit aggregation of lens γ-crystallin. Our work further implicates additional inputs/factors are involved in this underlying proteostasis network and demonstrates the utility of zebrafish as a platform to delineate mechanisms of cataract.

Lens crystallins are water soluble proteins that play a central role in conferring the optical properties of the ocular lens. Lens development entails terminal differentiation of epithelial cells into fiber cells that have negligible protein synthesis and turnover activities (1,2). Mammalian crystallins consist of three classes of proteins, α-, β- and γ-crystallins which together constitute 90% of the total weight of lens fiber cells (3). The glass-like, short-range order packing of the crystallins in fiber cells is critical to the transparency and refractivity of the lens (4,5). Protein-protein interactions between crystallins are consistent with a uniform protein distribution at dimensions comparable to the wavelength of visible light (6). From this perspective, the vertebrate lens is a thermodynamic wonder of protein evolution. The unusual stability of the crystallins along with their carefully tuned interactions was evolved to avert, for a long period...
of the human lifespan, the unavoidable thermodynamic fates of aggregation and precipitation expected for highly concentrated protein solutions.

The α-crystallins are molecular chaperones that belong to the small heat-shock proteins (sHSP) superfamily (7-11). They can bind thermodynamically destabilized proteins and sequester aggregation-prone proteins whereas β- and γ-crystallins are considered structural proteins with undefined function in the development of the lens. In his seminal work, Horwitz hypothesized that once lens proteins cross an energetic threshold of destabilization, they become substrates for α-crystallin which sequesters them away inhibiting their aggregation, thereby delaying light scattering (12-13). Much effort has been dedicated to identifying age-related modifications of the crystallins and understanding the consequent effects on their stability and propensity to aggregate (14-19). The unfolding of destabilized lens proteins following the exhaustion of α-crystallin binding capacity has been hypothesized to lead to the onset of age-related cataract although changes in the surface properties of molecules leading to loss of solubility is another important mechanism of cataracts (20).

Horwitz’s hypothesis stimulated an expansive but successful effort to understand the mechanism of α-crystallin chaperone activity in vitro. α-crystallins bind thermodynamically destabilized proteins as well as sequester aggregation-prone proteins in vitro (10,12,13, 21-25). Binding can involve conformational changes to expose otherwise unavailable binding sites. The oligomer plasticity of α-crystallin is critical to the binding capacity and affinity (26-29).

Despite the successful outline of its function, a divide emerged between the mechanistic principles of α-crystallin chaperone activity established in vitro and the role of this activity in lens aging and cataract. While reliance on model substrates is justifiable in the context of mechanistic studies, its consequence is to overlook the potential specificity of α-crystallin interactions with β- and γ-crystallins. A particularly disconcerting example of this divide is the failure of current models to explain the weak chaperone-based interactions between α-crystallin and destabilized mutants of γ-crystallin which cause dominant cataract in mouse models. For example, γB-crystallin mutant I4F and γD-crystallin mutant V76D were identified in mouse lines with dominant cataracts (30,31). In detailed analyses, Mishra et al. (2012) (32) and Moreau & King (2012) (33) demonstrated that these γ-crystallin mutants have reduced thermodynamic stability. Moreover, they undergo a slow process of aggregation that is relevant in long-lived cells such as lens fiber cells. However, neither α-crystallin subunits can bind these mutants with high affinity or preempts their aggregation in vitro. Together, the two studies reveal a class of mutations that can escape detection by the α-crystallins, a finding seemingly consistent with their ability to induce cataracts in mice (30,31).

In this paper, we explore the in vivo mechanisms by which the two thermodynamically destabilized γ-crystallin mutants can lead to lens defects (30,31). To better define the role of α-crystallin in lens development and dissect its chaperone activity in vivo, we have initiated studies using zebrafish as a model system. The zebrafish lens affords many advantages and simplifications relative to mouse models that include the cost and speed of generation of transgenic and knockout lines and the size of embryo clutches, a factor that is critical for the studies reported here. In a previous study, we demonstrated that αA-crystallin plays a critical role in the development of the embryonic zebrafish lens (34). Partial rescue by the transgenic expression of rat αA-crystallin reveals a conserved function for this protein in lens development. Here we challenge the proteostasis network of the zebrafish lens with targeted and controlled expression of the γD-crystallin mutants. Not unexpectedly, we find that embryos expressing destabilized and aggregation-prone mutants show lens defects. In contrast to mouse models, we observed a range of penetrance and severity determined by the underlying stability of the mutants. The mechanism of lens defects appears to be primarily linked to the tendency of the mutants to aggregate while other typical cellular dysfunctions are not prominently detected. We interpret these findings as revealing the presence of a chaperone network (35) capable of “sensing” thermodynamic stability and aggregation-propensity of proteins. Consistent with in vitro studies on destabilized γD-crystallin mutants (32,33), as well as on other modified crystallin
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proteins (36,37) which demonstrated that αA-crystallin does not interact strongly with these aggregation-prone mutant proteins, the buffering capacity of αA-crystallin in the lens is not limiting suggesting the contribution of other chaperones to lens proteostasis.

RESULTS

Transgenic expression of human γD-crystallin variants induce embryonic lens defects in zebrafish—To assess the in vivo interactions between cataract-linked γD-crystallin variants and lens chaperones in the context of the native environment of the lens fiber cells, we generated transgenic zebrafish lines that express three destabilizing human γD-crystallin (Hsa.CRYGD) variants 14F, V76D, and 14F/V76D (32). Using transgenesis protocols described previously (34), the γD-crystallin mutants were expressed specifically in the lens under the control of the zebrafish cryaa promoter. We opted for this promoter to keep the overall level of the mutants low compared to native γ-crystallins since aggregation is strongly concentration-dependent. In addition, all transgenic lines expressed myl7 promoter-driven Cerulean fluorescent protein to provide a convenient selection marker for transgenic animals (Fig. 1Ab).

Expression of the γD-crystallin variants led to apparent abnormalities in the embryonic lens starting at 3dpf (Fig. 1B), while the overall morphology of the embryos were completely unaffected (Fig. 1Aa). The nature of the lens defects were similar to those previously described for αA-crystallin knockout lines (34). The phenotypic features appeared as either round, shiny crystal-like droplets spread across the lens that were classified as “minor” defects (Fig. 2Af, arrows; also shown in Fig. S1). The latter class of lens defects led to increased light reflectance compared to the WT siblings in all three transgenic lines expressing γD-crystallin variants (Fig. 2Aa-c, 2B; V76D not shown). Transverse and sagittal sections of γD 14F/V76D transgene (Fig. 2Cb, Cd) retinæ at 4 dpf stained with toluidine blue showed normal retinal laminar architecture when compared to the wild-type (Fig. 2Ca, Cc), and further confirmed that the observed defects were localized in the lens, showing multiple darkly-stained speckles or inclusion bodies (Fig. 2Cb, Cd, arrowheads) that resembled to cytosolic protein aggregates (31).

We screened a large number of embryos for each mutant scoring them based on the severity of the phenotype. A strong correlation emerged between the penetrance of lens defects and the reduction in the thermodynamic stability of γD-crystallin mutant proteins (Fig. 1B). In the embryos carrying γD -crystallin 14F/V76D transgene, which is the most unstable among the three mutants, the majority of embryos (~80%) showed abnormalities in lens morphology, and more than half of these were categorized as major lens defects (Fig. 2Af). In the lines expressing the less destabilizing γ D-crystallin mutants, 14F and V76D, we observed lower percentages of embryos exhibiting lens defects (14F, ~30%; V76D, ~62%), but nonetheless more frequent and severe when compared to WT embryos.

The difference of severity and penetrance between the γD-crystallin transgenic lines could be due to differential expression level of each line given the random nature of the genomic insertion events, collectively termed position effects (38-40). That is, the higher level of expression of γD-crystallin transgenes, the more penetrant lens phenotype will be observed. To exclude position effects as the origin of the variable phenotype, we performed quantitative RT-PCR to compare the expression level between γD-crystallin V76D and I4F transgenes, as well as between γ D-crystallin V76D embryos showing major and minor defects (Fig. S2). Even though the V76D transgenic line displayed lens phenotypes in higher percentage of embryos relative to the I4F transgene (Fig. 1B), there was actually a slightly lower expression for the V76D transgene (Fig. S2a). Moreover, no significant difference was detected in the transgene expression between embryos exhibiting major and minor lens defects for the same mutant (Fig. S2b). Thus, we conclude that the level of transgenic expression of γD-crystallin mutants does not dictate the severity or penetrance of the lens phenotype; rather the correlation with the stability of the mutants demonstrates that the variability is a function of the mutation sites/types. We interpret this finding to manifest the presence of an intrinsic mechanism(s) in the lens that can buffer these aggregation-prone mutants. Titration of an intrinsic mechanism(s) in the lens that can buffer these aggregation-prone mutants. Titration of an intrinsic mechanism(s) in the lens that can buffer these aggregation-prone mutants. Titration of an intrinsic mechanism(s) in the lens that can buffer these aggregation-prone mutants. Titration of an intrinsic mechanism(s) in the lens that can buffer these aggregation-prone mutants.
for the observed dependence on the stability of the mutants.

**Destabilized γD-crystallin proteins form aggregates in the lens**—Previous studies inferred that congenital mutations of γ-crystallins identified in humans and mouse tend to form aggregates (31, 41-43), which then result in opacification and cataract formation. However, this conclusion is drawn based on sparse biochemical evidence rather than on in vivo data.

To directly test the hypothesis that the observed lens phenotypes arise from the propensity of the destabilized γD-crystallins to form protein aggregates, we tagged the γ D-crystallin mutants with red fluorescent protein in order to visualize their association state (see Experimental procedures for details). The Gal4/UAS targeted gene expression system (44) was utilized to generate lens fiber mosaics by injecting embryos derived from a lens-specific transgenic driver line, Tg[cryaa:Gal4VP16] (45), with UAS responder constructs expressing fluorescently tagged human γ -crystallin (Hsa.CRYGD-mCherry) wild-type (as control) and three variants, I4F, V76D and I4F/V76D double mutant (Fig. 3A). When the fluorescence of tagged γD-crystallins was measured at 4dpf, we frequently observed distinct red punctates in lenses expressing γD-crystallin I4F/V76D mutant (Fig. 3Bd-f), whereas the fluorescence was diffuse with rare punctates in the lens expressing wild-type γD-crystallin (Fig. 3Ba-c). Importantly, the highest frequency of the fluorescent punctate formation was observed in the lenses of embryos expressing the most destabilized γD-crystallin variant (I4F/V76D double mutant; Fig. 3C) compared to γD-crystallin I4F or V76D. This correlation between the occurrence of fluorescent punctates in the lens and the free energy of unfolding of γD-crystallin mutants suggests that reduction of γ D-crystallin stability does enhance the likelihood of aggregate formation in native cellular environments, i.e. in lens fiber cells.

Supporting this conclusion, the fluorescent punctates were found to co-localize with the lens defects in parallel DIC and fluorescence microscopy (Fig. 3Bf, arrow). Thus the lens phenotypes associated with expression of the γ-crystallin mutants are primarily due to formation of protein aggregates consistent with the histological analysis as described above (Fig. 2C).

**Quantitative proteomics reveals formation of water insoluble aggregates in adult zebrafish lens**—In addition to the embryonic lens defects, we set out to examine the long-term consequence of the expression of destabilized human γD-crystallin variants in adult transgenic fishes. For this purpose, we utilized the iTRAQ quantitative proteomics strategy (46,47) to analyze the relative changes of global protein abundance in both water-soluble (WSF) and -insoluble (WIF) fractions for the lens of 3-month and 15-month old adult fish. The detailed analyses will be described elsewhere. Directly relevant to this study is the finding that in the 15-month adult lens samples, there was a significant increase of human γD-crystallin peptides (enzymatically digested for iTRAQ label) in the WIF observed in the fish lens expressing the mutant γD-crystallin I4F/V76D compared to I4F (Fig. 4 and Fig. S3). Together with the fluorescence detection of aggregation described above, these results suggest that the aggregates by the more destabilized mutants of γD-crystallin do not remain soluble, and preferentially reside in the insoluble fraction, thus providing a molecular basis for the increase in light scattering by the lens.

**Exogenous expression of rat αA-crystallin showed no protective effects on lens defects induced by γD-crystallin I4F/V76D**—In vitro biochemical studies showed that cataract-linked γD-crystallin mutants could evade quality control by α-crystallins (32,33), consistent with their ability to induce cataracts in mouse models (30,31). We set to probe the interactions between γD-crystallin and α-crystallins in vivo using our transgenic zebrafish model. The goal was to test if the α-crystallin binding capacity is limiting, being titrated out by the mutant γ-crystallins. By crossing the fish line carrying γD-crystallin I4F/V76D transgene with a transgenic line expressing rat α.A-crystallin in the lens under the control of the same promoter (34) and subsequently using PCR genotyping to separate embryos with single and double transgenes, we found no suppression for the penetrance of lens defects in double transgenic embryos (γD-crystallin I4F/V76D; αA WT) compared to embryos with γD-crystallin I4F/V76D transgene alone (Fig. 5A).

Together with the fluorescence data, these genetic results support the observations that these
destabilized γD-crystallins cause lens abnormalities in zebrafish and we presume cataracts in mouse models due to their propensity to form protein aggregates in the lens and thus scatter light (see above section). The lack of an effect of αA-crystallin overexpression is consistent with lack of significant interactions with γD-crystallin mutants as previously reported in vitro (32). Thus, the pool of available αA-crystallin binding sites is not limiting and its titration by the mutant is not at the origin of aggregation and formation of lens defects.

Reduced dosage of α-crystallin exacerbated the lens defects induced by γD-crystallin I4F expression—To further assess the role of α-crystallin in modulating the phenotype, we expressed the γ-crystallin mutants in zebrafish strains with reduced chaperone capacity. We hypothesized that expression of destabilized γD-crystallin variants in embryos with inheritably compromised chaperone network would accentuate the severity and penetrance of the lens defects. The γD-crystallin I4F transgene that elicited abnormalities in the zebrafish lens with moderate penetrance (around 30%; Fig. 1B) provides an ideal platform to study genetic synergism or antagonism for modifying genes/factors. In light of our previous finding showing that deficiency in αA-crystallin resulted in compromised lens development in zebrafish (34), we tested our hypothesis by expressing γD-crystallin I4F transgene in the cryaa heterozygous background (cryaa+/−).

Specifically, crossing the γD-crystallin I4F transgenic line with the cryaa homozygous mutant (cryaa−/−) and the wild-type fish, we observed a significant increase (about two-fold) in the number of embryos exhibiting major lens defects induced by γD-crystallin I4F at 4dpf when one copy of endogenous cryaa gene was deleted (Fig. 5B; comparing I4F; cryaa−/− to IF4). In addition, when compared to the non-transgenic siblings (cryaa+/−), there was also a marked increase in the percentage of embryos showing major lens defects at 4dpf in the cryaa−/− embryos that carry the γD-crystallin I4F transgene (Fig. 3; comparing [I4F; cryaa−/−] to [cryaa−/−] only). These results indicated that compromising level of αA-crystallin exacerbated the lens defects induced by γD-crystallin I4F expression in zebrafish lens. This observed additive effect does not necessarily imply a direct physical interaction between the two crystallin proteins. Rather it may reflect that a single copy loss of endogenous cryaa gene essentially creates a “sensitized” background allowing two weak perturbations to synergistically converge on the downstream chaperone network. Furthermore, it implicates a requirement for maintaining a proper “gene dosage” of α A-crystallin to keep the underlying chaperone networks operating properly.

Expression of γD-crystallin variants do not induce apoptosis or disrupt the denucleation process in the lens—Cell death has been implicated in cataract phenotypes, including radiation-induced cataracts and diabetic retinopathy-associated cataracts (48-51) particularly with regard to age-related cataract formation (52). Staining with TUNEL (Fig. 6G,H) and acridine orange (Fig. S4) to detect apoptotic cell death, we found no evidence of elevated apoptosis in the embryos carrying γD-crystallin I4F/V76D transgene, as well as I4F and V76D variants (not shown), when compared to WT. Therefore, cell death by apoptosis did not seem to play a major role in the lens defects observed in the γD-crystallin transgenes (Fig. 2A, C). Similar phenotypes (i.e. the lack of cell death) were also observed in loss-of-function analyses by morpholino knockdowns of crybgx, cryaa, and crybb1c in zebrafish (53).

Failure of lens fiber cell denucleation has been associated with various congenital cataracts (54-57). Utilizing nuclear staining (DAPI; Fig. 6D,E) and a transgenic line expressing nuclear localized GFP (Fig. 6A,B), we found no evidence of any noticeable impairment or delay in the lens fiber cell denucleation process, while clearly observed an incomplete denucleation in cloche mutants (Fig. 6C,F), consistent with published results (58). Thus, the lens defects induced by mutant γD-crystallin expression were unlikely a result of the disruption of the lens fiber cell denucleation process.

DISCUSSION
The finding that expression of destabilized mutant γD-crystallins induces defects in the embryonic zebrafish lens is consistent with previous studies in mouse models, which associated these mutants with cataract formation (30,31). However, the penetrance and severity of
the phenotype was heterogeneous in zebrafish revealing a critical difference between the two model systems. The phenotypic variability was not only dependent on the degree of destabilization, but was also observed within the embryo population expressing the same mutant. Hence, there are embryos expressing the V76D mutant that have undisturbed lenses while others show severe lens defects.

Given the dependence of the phenotype on the mutants’ thermodynamic stability but not on their level of expression, we propose that the severity and penetrance reflects the presence of a chaperone network that functions to bind, sequester and possibly refold the destabilized, aggregation-prone mutants. The buffering capacity of this network effectively imparts a capability to “sense” the stability of the expressed mutant proteins, which is manifested in the remarkable dependence on the free energy of unfolding. Thus, at the low level of unfolded and aggregation-prone protein population expected for the I4F mutant, the chaperone network is more efficient at sequestering aggregation-prone proteins than for the double mutant I4F/V76D where the free energy of unfolding is significantly reduced and partially and/or globally unfolded states are highly populated even at equilibrium. This mechanistic model is supported by the detection of aggregates of γD-crystallin mutants that localize morphologically with lens defects. Our results not only confirm previous studies in mouse models that these mutants are associated with cataracts (30,31), but also uncover an important role of a chaperone network in buffering the aggregation-prone populations.

The variability of lens phenotypes in the embryo population expressing the same aggregation-prone mutant must reflect genetic or epigenetic differences in the same network that imparts the “sensing” of the γ-crystallin mutants stability. The identity of this network will be a subject of future investigations although we presume it must be associated with molecular chaperones.

A prominent member of the lens chaperone network is the sHSP αA-crystallin. Using our knockout zebrafish line of αA-crystallin (34), we verified that reduction in its expression enhances penetrance of the lens phenotype induced by destabilized γD-crystallin mutants. However, increasing the available pool of αA-crystallin did not affect the expressivity of the phenotype. This result suggests that the aggregation of the γD-crystallin mutants is not depleting the pool of available αA-crystallin.

One the fundamental differences between the zebrafish models described here and mouse models where these mutants were identified (30,31) is the level of expression of the mutant proteins. Whereas in the mouse the γ-crystallin mutants were under the control of their respective native promoters, the use of the αA-crystallin (cryaa) promoter to drive the expression in the zebrafish lens may reduce the overall concentration of these proteins. This may allow the distinction of intrinsic differences in the aggregation propensity of different γD-crystallin mutants. In the mouse it is possible that the level of expression even of the mildly destabilized I4F mutant overwhelms the chaperone network hence obscuring these differences among various γ-crystallin mutants.

Our work establishes a new model to explore the mechanisms of cataract-linked mutants and provide direct evidence for the presence and activity of a chaperone network that maintains balanced proteostasis in the lens. Although consistent with in vitro studies (32,33), the lack of rescue for lens phenotypes by αA-crystallin is somewhat counterintuitive with the abundant expression of this chaperone in mammalian lenses. Further experiments to test the modulation of the phenotypes described here by the other resident chaperone αB-crystallin are under way.

EXPERIMENTAL PROCEDURES

Zebrafish maintenance and breeding—AB wild-type strain zebrafish (Danio rerio) were used. The embryos were obtained by natural spawning and raised at 28.5°C on a 14/10 hour light/dark cycle in 0.3x Danieau water containing 0.003% PTU (w/v) to prevent pigment formation. Embryos were staged according to their ages (dpf; days post-fertilization). The following mutant and transgenic fish lines were used: cryaa<sup>my532</sup>, Tg(cryaa:Rno.Cryaa,myl7:Cerulean)<sup>my531Tg</sup> (34), Tg(cryaa:Gal4vp16)<sup>mw46Tg</sup>, Tg(UAS:GFP)<sup>kca33Tg</sup> (45), clocche<sup>m39</sup> (58). All animal procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee. The same feeding procedures were performed on
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all zebrafish lines used in this study, according to their appropriate ages.

Zebrafish transgenesis—To establish the transgenic zebrafish expressing human (Homo Sapiens) CRYGD gene (Hsa.CRYGD) specifically in the lens, Tg(cryaa:Hsa.CRYGD,myl7:Cerulean) was constructed by inserting Hsa.CRYGD cDNA (32) downstream of zebrafish cryaa promoter (1.2 kb)(59) in the pT2HBLR vector that also contains myl7 promoter-driven Cerulean as the selection marker. Tol2 mediated transgenesis were performed as previously described (34) for all three Hsa.CRYGD (14F, V76D, and 14F/V76D) transgenes. At least two founder lines (F0) for each construct were screened and out-crossed to established stable F1 generations. Each F1 line was propagated and raised into F2 and F3 generations. The lens defects data collected were from F3 or F4 embryos and similar penetrance of lens defects was observed in individual stable lines that express the same γD-crystallin constructs.

Assembly of the UAS expression constructs—All UAS expression constructs were created by MultiSite Gateway (Invtrogen) assembly reactions using protocols established previously (Tol2kit)(60). Specifically, Tol2kit vectors, p5E-UAS and p3E-mCherry were assembled with pME-Hsa.CRYGD (wild-type and three variants: 14F, V76D, and 14F/V76D) and recombined into pDestTol2CG2 vector, which was then used for microinjections.

Quantitative RT-PCR—Total RNAs were isolated from embryos at desired stages (2dpf and 4dpf) by using Trizol Reagent (Invitrogen), followed by DNase treatment (Ambion). The iScript cDNA synthesis kit (Bio-Rad) was used for reverse transcriptions and subsequent qRT-PCR reactions (SsoAdvanced Universal SYBR Green Supermix, Biorad) were performed on Bio-Rad CFX96 Real-Time PCR Detection System according to manufacturer's protocols (the cycling parameters were: 95 °C for 10mins, 40 cycles of 95°C for 15s, and 60°C for 30s.). The PCR products were analyzed by gel electrophoresis to confirm that they were of the expected size. The CFX Manager software provided with the thermocycler (Bio-Rad) was used to determine Ct values. Expression differences between samples were calculated by the −ΔΔCt (Comparative Ct) method (61) and reported without Log2 conversion to fold changes. Three pools of ten embryos from separate clutches of each transgenic line were collected and each sample was analyzed in triplicate. The following primers used qRT-PCR are shown here: 5′-CGAGCTGTCCAACTACCGAG-3′ and 5′-TCGACCTGAGTCAGGAGAA-3′ for Hsa.CRYGD (for all γD transgenes); 5′-CGAGCTGTCTTCCCCATCCA-3′ and 5′-TCACCAACGTAGCTGTCTTCTG-3′ for actb2 (β-actin) as an internal control for calibration of gene expression levels between samples.

Cell death assays—Embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS, dehydrated with 100% MeOH and stored in -20°C. The procedures of TUNEL staining were carried out following the manufacturer's suggested protocol (In Situ Cell Death Detection Kit, TMR red; Sigma-Aldrich #12156792910). Acridine Orange staining was performed by immersing live embryos in 5ug/ml Acridine Orange for 10 min at room temperate, then imaged with a FITC filter immediately after (62).

Histology and Immunohistochemistry—Plastic histological sections for embryonic eyes were performed as described (63), and 1µM sections were stained by Toluidine blue. The DAPI staining was performed on cryosections as described in Uribe and Gross (2007)(64).

Microscopy and image processing—Lenses of live embryos in 0.3x Daneau water with PTU/tricane were analyzed by bright field microscopy (Zeiss Axiovert 200) at 4dpf and classified into three classes on the basis of the severity of lens defects as defined in our previous study (34). Briefly, Class 1 consisted of embryos that did not appear to have visible lens defects (“WT-like”). Class 2, referred as the “minor” lens defect group, showed small and sporadic mostly spherical droplets in the lens. The Class 3, referred as “major” lens defects, was characterized by large and/or numerous droplets covering large areas of the lens. Fluorescence images were taken with Zeiss AxioZoom.V16 microscope. Differential interference contrast (DIC) and reflectance analysis were performed by Zeiss LSM510 inverted confocal microscope with λem=488 on 4dpf embryos which were embedded in 3% methylcellulose. DIC images were taken with a 20x objective lens and used as a reference ROI (region of interest) for reflectance analysis, which
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was subsequently digitally analyzed by the image-processing program Image J.

**iTRAQ quantification of protein changes**—To quantify protein expression changes in transgenic zebrafish lenses, one lens from 15 month old fish (F3 adults) of each type (WT, WT \( \gamma \)D, \( \gamma \)DI4F and \( \gamma \)DI4F/V76D) was homogenized in 50 mL homogenizing buffer (25 mM Tris, 150 mM NaCl, 1 mM DTT, 5 mM EDTA, pH 7.5). The sample was centrifuged at 20,000g for 30 mins and pellets were washed with another 50 mL of homogenizing buffer followed by centrifugation at 20,000g. The supernatant was pooled together as the water soluble fraction (WSF). Protein concentrations for WSF fractions were measured by a Bradford assay (Thermo Scientific, Rockford, IL). The pellets were considered as the water insoluble fraction (WIF). WIF was washed with water twice, suspended in 100 mL of water, and an aliquot was mixed with equal volume of 5% SDS for a BCA assay (Thermo Scientific, Rockford, IL). 100 mg of WSF from each lens was reduced with 50 mM TCEP (Tris-(2-carboxyethyl)phosphine) at 60 oC for 1 hour, alkylated with 200 mM MMTS (methyl methanethiosulfonate) at room temperature for 10 min., and digested with sequencing-grade trypsin overnight. 20ug of each WSF fraction was then labeled with iTRAQ reagents according to the manufacturer’s instructions (AB Sciex, Foster City, CA) (114 for WT, 115 for WT \( \gamma \)D, 116 for \( \gamma \)DI4F and 117 for \( \gamma \)D I4F/V76D). For WIF fractions, the entire reconstituted pellet for each WIF sample was digested with trypsin, and 18ug was labeled with iTRAQ reagents. Reagents were reconstituted in ethanol such that each protein sample was iTRAQ-labeled at a final concentration of 90% ethanol, and labeling was performed for 2 hours.

The iTRAQ-labeled samples were mixed, acidified with TFA, and were subsequently desalted by a modified Stage-tip method prior to LC-MS/MS analysis. iTRAQ labeled samples were analyzed using MudPIT analysis with 13 salt pulse steps (0mM, 25mM, 50 mM, 75mM, 100 mM, 150mM, 200 mM, 250mM, 300 mM, 500 mM, 1 M and 2 M ammonium acetate). Peptides were introduced via nano-electrospray into a Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA). The Q Exactive was operated in data-dependent mode acquiring HCD MS/MS scans (R = 17,500) after each MS1 scan (R = 70,000) on the 20 most abundant ions using an MS1 ion target of \( 1 \times 10^6 \) ions and an MS2 target of \( 1 \times 10^5 \) ions. The maximum ion time for MS/MS scans was set to 100ms, the HCD-normalized collision energy was set to 30, dynamic exclusion was set to 30s, and peptide match and isothe separation were enabled. For iTRAQ data analysis, mass spectra were processed using the Spectrum Mill software package (version B.04.00, Agilent Technologies). MS/MS spectra acquired on the same precursor m/z (±0.01m/z) within ± 1 s in retention time were merged. MS/MS spectra of poor quality which failed the quality filter by not having a sequence tag length >1 were excluded from searching. A minimum matched peak intensity requirement was set to 50%. For peptide identification, MS/MS spectra were searched against a Uniprot zebrafish database (Jun 21, 2012) appended with the human CRYGD protein sequence. Additional search parameters included: trypsin enzyme specificity with a maximum of three missed cleavages, ± 20 ppm precursor mass tolerance, ± 20 ppm (HCD) product mass tolerance, and fixed modifications including MMTS alkylation of cysteines and iTRAQ labeling of lysines and peptide N-termini. Oxidation of methionine was allowed as a variable modification. Autovalidation was performed such that peptide assignments to mass spectra were designated as valid following an automated procedure during which score thresholds were optimized separately for each precursor charge state and the maximum target-decoy-based false-discovery rate (FDR) was set to 1.0% (46,65).

**Statistics**—Differences among groups were analyzed by Student’s t-test. Data are shown as means ± standard error (SE). Statistical significance was accepted when p < 0.05.

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FIGURE LEGENDS

FIGURE 1. Transgenic expression of human CRYGD mutants induce lens defects in 4dpf zebrafish embryos. (A) Embryos of lens-specific Hsa.CRYGD transgenes displayed Cerulean marker in the heart. An overlay image of DIC and Cerulean fluorescence (Aa). A white arrow marked the Cerulean marker in the heart (Ab). (B) Comparison of percentage of embryos showing lens defects between WT and three Hsa.CRYGD transgenes, I4F, V76D and I4/F/V76D.

FIGURE 2. The lens defects and reflectance in zebrafish embryos expressing human CRYGD mutants. (A) Compared to WT siblings (non-transgenic carriers), embryos carrying Tg(cryaa:Hsa.CRYGD_I4F) and Tg(cryaa:Hsa.CRYGD_I4F/V76D) at 4dpf exhibited various degrees of lens defects (d-f, arrows), as well as changes in reflectance (a-c). (B) Reflectance analysis of WT lens and Hsa.CRYGD_I4F and Hsa.CRYGD_I4F/V76D lens at 4dpf suggested that the lens of embryos expressing γD-crystallin variants scattered more lights than WT lens (t-test; p<0.001). (C) Histological sections of the lens in Tg(cryaa:Hsa.CRYGD_I4F/V76D) embryos stained by Toluidine blue showed distinct dark inclusion bodies (b,d), compared to WT siblings (a,c). Transverse section (a, b); Sagittal section (c,d).

FIGURE 3. Destabilized γD-crystallin proteins forms aggregation in the lens. (Aa) Schematic of the experiment utilizing the Gal4/UAS targeted gene expression system. Double transgenic line, Tg(cryaa:Gal4); Tg[UAS:GFP], males were outcrossed to AB females. Fertilized zygotes were injected with tol2 mRNA and UAS responder Tol2 constructs expressing fluorescently tagged human γD-crystallin (Hsa.CRYGD-mCherry), including wild-type and three variants, I4F, V76D and I4F/V76D double mutant. Embryos possessing lens fibers positive for mCherry expression were selected and imaged at 4dpf. Examples of green lens (from UAS:GFP; Ab) and mosaic expression of mCherry-tagged γD-crystallin (Ab'). (B) Mosaic expression of mCherry-tagged human γD-crystallins, wild-type (Ba-Bc) and I4F/V76D double mutant (Bd-Bf). Apparent mCherry punctuates/aggregates were observed in the lens expressing Hsa.CRYGD_I4F/V76D-mCherry (white arrow in Be and Bf), but not in those expressing wild-type γD-crystallin construct (mostly shown a diffused and elongated pattern, which is consistent with the shape of lens fiber cells). DIC images (Ba and Bd), florescent images (red channel; Bb and Be) and merged images (Bc and Bf). (C) The frequency of embryos showing mCherry punctuates in the lens (wild-type and three variants, I4F, V76D and I4F/V76D double mutant).

FIGURE 4. Human CRYGD mutants show different solubility in adult lens. In the 15-month adult lens analyzed by iTRAQ quantitative proteomics, there was a significantly increased amount of human γD-crystallin peptides detected in the WIF in the fish lens expressing the γD-crystallin I4F/V76D compared to I4F, based on the ratio between ΔWIF and ΔWSF. The ΔWIF and ΔWSF from each sample (γD-crystallin I4F/V76D and γD-crystallin I4F) were calculated as fold changes of the expression level relative to the lens expressing γD-crystallin WT.

FIGURE 5. Percentages of the lens defects induced by human CRYGD_I4F expression at 4dpf embryos were not suppressed by exogenous expression of αA-crystallin, but were exacerbated by reducing dosage of endogenous αA-crystallin. (A) Adult Tg(cryaa:Hsa.CRYGD_I4F/V76D) and Tg(cryaa:Rno.Cryaa) transgenic zebrafish lines were crossed. The lens defects of resulting embryos (I4F/V76D) were examined and classified into 3 severity classes as previously mentioned. The proportion of I4F/V76D embryos showing lens defects showed no significant changes with or without the presence of Rno.Cryaa transgene. (B) A significantly higher proportion of Tg(cryaa:Hsa.CRYGD_I4F) embryos showed major lens defects when losing one copy of cryaa (I4F; cryaa+/−; ~39%), compared to those in wild-type background (I4F; ~14%), as well as to the non-transgenic cryaa+/− or cryaba+/− embryos.

FIGURE 6. Denucleation failure or apoptotic cell death was not induced by transgenic expression of human CRYGD mutants. Unlike in cloche mutants, an incomplete denucleation process was clearly
observed; either by nuclear-localized H2B-GFP visualization (C) or by DAPI staining (F), the lens of 
*Tg(cryaa:Hsa.CRYGD_I4F/V76D)* embryos (B,E) showed no signs of denucleation failure and appeared 
similar to WT siblings (A,D). (G,H) The TUNEL staining revealed no significant increase of apoptosis in 
*Tg(cryaa:Hsa.CRYGD_I4F/V7D)* embryos compared to WT siblings.
Figure 1

A

γD-crystallins Tg carrier

non-Tg carrier

B

% of embryos with lens defects

|          | Minor | Major |          | Minor | Major |
|----------|-------|-------|----------|-------|-------|
| WT       | 10    | 80    | I4F      | 20    | 60    |
| V76D     | 50    | 90    | I4F/V76D | 30    | 70    |

n=291, n=408, n=236, n=55

** n=291, *** n=408, *** n=236

Downloaded from http://www.jbc.org/ on July 9, 2020
A

Wild-type (AB) or mutants

Inject γD crystallin-mCherry fusion cDNAs (UAS-driven)

Grow to desired stages; Select embryos with mosaic expression of mCherry in the lens

mCherry-expressing lens

B

Figure 3
Ratio for relative changes between WIF and WSF ($\Delta$WIF/$\Delta$WSF)
Figure 5

A

![Bar graph with percentages of embryos with lens defects for I4F/V76D and I4F/V76D; alphaA WT.]

B

![Bar graph with percentages of embryos with lens defects for I4F, I4F; cryaa +/-, and cryaa +/- controls.]

Major

Minor

n=303

n=291

n=283

% of embryos with lens defects

n=58

n=46

0 20 40 60 80 100

I4F/V76D I4F/V76D; alphaA WT

% of embryos with lens defects

n=303

n=291

n=283

I4F I4F; cryaa +/- cryaa +/-
Figure 6

Table:

|   | WT          | yD I4F/V76D | cloche<sup>−/−</sup> |
|---|-------------|-------------|-----------------------|
| A | ![WT A](image) | ![yD I4F/V76D A](image) | ![cloche<sup>−/−</sup> A](image) |
| B | ![WT B](image) | ![yD I4F/V76D B](image) | ![cloche<sup>−/−</sup> B](image) |
| C | ![WT C](image) | ![yD I4F/V76D C](image) | ![cloche<sup>−/−</sup> C](image) |
| D | ![WT D](image) | ![yD I4F/V76D D](image) | ![cloche<sup>−/−</sup> D](image) |
| E | ![WT E](image) | ![yD I4F/V76D E](image) | ![cloche<sup>−/−</sup> E](image) |
| F | ![WT F](image) | ![yD I4F/V76D F](image) | ![cloche<sup>−/−</sup> F](image) |

Images:

- **Tg[bactin2::H2B-GFP]**
- **3 dpf**

- **DAPI**
- **4 dpf**

- **TUNEL**
- **2 dpf**
Expression of Cataract-linked γ-crystallin Variants in Zebrafish Reveals a Proteostasis Network that Senses Protein Stability
Shu-Yu Wu, Ping Zou, Alexandra W. Fuller, Sanjay Mishra, Zhen Wang, Kevin L. Schey and Hassane S. Mchaourab

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