Hsp60D - A novel modifier of polyglutamine-mediated neuro degeneration in Drosophila

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ABSTRACT

Background: Several dominant neurodegenerative disorders result from alleles carrying expanded stretch of polyglutamine (polyQ) tracts in the encoded proteins, which become toxic and form insoluble cytoplasmic and/or nuclear aggregates or "inclusion bodies" in the affected neuronal cells. **Purpose:** Present study identified Hsp60D, a member of the Drosophila Hsp60 family, as a novel modifier of neurodegeneration in fly models of polyQ disorders, viz., Spinocerebellar Ataxia type 3 (SCA3, caused by mutated MJDtr-Q78 allele) or the 127Q model. **Methods:** Immunostaining of third instar larval eye discs was carried out and the eye discs of 50-hours old pupae of different genotypes were stained with Phalloidin-Rhodamine to examine the organization of actin filaments in ommatidial units. The nail polish imprints of adult eye surfaces were examined and the assay was performed using a glass Y-maze. **Results:** We showed that the reduction in the cellular levels of Hsp60D protein through directed RNAi in the polyQ expressing developing eye cells not only improved external eye morphology, retinal structure and vision, but also reduced the number of inclusion bodies and the associated expression of Hsp70. **Conclusion:** Our results suggest that Hsp60D may be required for folding of polypeptides with polyQ stretches so that in its absence, due to targeted RNAi, the expanded polyQ polypeptides fail to fold in a manner that can produce the toxic inclusion bodies.

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Introduction

Several dominantly inherited neurodegenerative diseases like Huntington's disease (HD), spinobulbar muscular atrophy (SBMA or Kennedy's disease), dentatorubropallidoluysian atrophy (DRPLA), and a variety of spinocerebellar ataxias (SCAs) are primarily due to dynamic mutations resulting in expansion of CAG trinucleotide repeats that encode polyglutamines (polyQ) in the respective proteins leading to dysfunction of specific neurons and late onset neurodegeneration.[1,2] Studies on these pathogenic proteins reveal that the enlarged polyQ domain alters protein conformation, which in turn affects a range of pathways, including protein folding,[3-5] protein degradation,[6,7] and cell death. Proteins with expanded polyQ repeats form characteristic entangles which are seen as nuclear and/or cytoplasmic inclusion bodies (IBs) in the affected neurons.[8,9] Various components of normal cellular machinery, such as ubiquitin, chaperones and other endogenous proteins containing short nonpathogenic polyQ tracts, get entangled within these IBs, resulting in global cellular mis-regulation and toxicity.[10-12]

Several neurodegenerative diseases have been modeled and extensively studied in Drosophila melanogaster.[13-15] In the present study, we used the SCA3 (also known as MJDtr-Q78) and 127Q models and studied the role of Hsp60D in neurodegeneration. Spinocerebellar Ataxia-3 (SCA3) or Machado-Joseph Disease (MJD), caused by expansion of polyQ in the Ataxin-3 protein, is a dominantly inherited ataxia characterized by degeneration of the cerebellum, brain stem and basal ganglia in humans.[16-18] The affected human gene is ATXN3 where the number of glutamine repeats in exon 10 increases beyond the normal range of 14 to 40 in affected individuals.[19] Expression of truncated form of mutated Ataxin-3 with 78 polyQ (MJDtr-Q78) repeats induces progressive neurodegeneration in fly eyes and the central nervous system, typically mimicking the mammalian pathogenesis with formation of ubiquitinated nuclear IBs that are also positive for Hsp70 and other proteins.[20-22]

In addition to various disease models, transgenic lines in which only pure polyQ repeats are expressed in a tissue dependent manner have also been developed to study the correlation between length of repeat and severity of disease.[23-26] The 127Q model carries a stretch of 127 repeats of CAG triplets joined in frame with the HA-tag under the UAS promoter . Expression of this transgene in fly eyes produces severe loss of pigmentation and neuronal integrity while a wider expression in the central and peripheral nervous system results in organismal lethality at various stages of development.[25]

Heat shock proteins (Hsps) are molecular chaperones that mediate correct folding, assembly, and degradation of misfolded proteins.[27] Hsp27, Hsp40, Hsp70, and Hsp100 are known to modify the polyQ pathogenesis in various model systems.[20,28-30] Ectopic expression of Hsp70 or Hsp40 in truncated Ataxin-3 expressing flies suppresses neurodegeneration by improving the solubility of the toxic Ataxin-3 protein.[7,20,29] Chaperone proteins can modulate polyQ diseases through multiple pathways, e.g., i) by preventing formation of polyQ aggregates, ii) by channeling the mutant polyQ protein to the ubiquitin-proteasome pathway (UPP) for degradation[31,32] and iii) by modulating cell death pathways,[33] they can prevent apoptotic cell death and thus the neurodegeneration.

The Hsp60 family proteins are a major group of conserved chaperones with organellar as well as cytosolic forms and have diverse roles.[34] Their role in polyQ pathogenesis has not been studied much. Drosophila melanogaster has four genes for Hsp60 family proteins, designated as Hsp60A, Hsp60B, Hsp60C and Hsp60D, respectively.[34] While Hsp60A appears to be ubiquitously distributed mitochondrial chaperonin,[35] Hsp60B has spermatogenesis-specific functions.[36,37] On the other hand, Hsp60C has significant roles in tracheal morphogenesis, spermatogenesis and oogenesis.[38,39] Hsp60D is essential for progression of induced apoptosis since in its absence, DIAP1 (Drosophila inhibitor of apoptosis protein 1) does not appear to dissociate from inactive or active caspases.[40]

In order to investigate if Hsp60D affects polyQ pathogenesis, we used GAL4-inducible Hsp60D-RNAi transgene[40] in the 127Q and SCA3 fly models. These transgenes were expressed ectopically in eye discs or pan-neuronally, using the well established UAS-GAL4 system.[41] Interestingly, we find that, contrary to the earlier noted suppression of polyQ pathogenesis following over-expression of the molecular chaperones, it is the down regulation of Hsp60D that suppresses expanded polyQ mediated neurodegeneration.

Methods

Fly stocks and crosses

Fly cultures were maintained at 23±1^{0}C on standard food containing agar, maize powder, yeast and sugar. Oregon R$^+$ was used as the wild type strain. Various transgenic lines carrying UAS-MJDtr-Q78, UAS-MJDtr-Q27,[22] UAS-127Q, UAS-20Q,[25] UAS-DTS5-11,[42] UAS-DIAP1-RNAi,[42] UAS-Uba2-116E,[44] GMR-p35,[45] UAS-DIAP1,[46] UAS-Pros26.1 UAS-Prosβ2 (BL# 6787) transgenes or mutant alleles of thread, viz., th^5 or th^{SL48} were obtained from different labs. Of the three different lines available for UAS-MJDtr-Q78 transgene, which express different levels of the SCA3 protein,[43] we used the strong allele of UAS-MJDtr-Q78. GMR-GAL4 and elav-GAL4 stocks were obtained from the Bloomington stock center. The Hsp60D-RNAi^3 line, generated in our laboratory and described earlier,[40] was used in the present study and is referred to in the text as Hsp60D-RNAi. Most of the genetic interactions were carried out with single copy of Hsp60D-RNAi transgene unless mentioned otherwise. Appropriate crosses were carried out to generate progeny of the desired genotypes.

Immunostaining of third instar larval eye discs and Phalloidin staining of pupal eyes

For immunostaining, the eye discs were dissected from late 3rd instar larvae of the desired genotypes and fixed in freshly prepared 4% para-formaldehyde in PBS for 20 min and processed as described earlier.[49] For detection of the protein/s of interest, the desired primary antibody/antibodies [anti-HA (Y-11, Santa Cruz, 1:40); anti-Hsp70 (7Fb from Dr. S. Lindquist, 1:100)] was/were added singly or in the desired combination to eye discs at the indicated dilution/s. For the detection of primary antibody, secondary antibodies, anti-rabbit Cy3 (1:200) and anti-rat AF488 (1:200), were added for 1 hr at 37^{0}C. The discs were washed in PBST, 4 times at 15 min interval, at room temperature. In order to visualize nuclei, the discs were counterstained with DAPI (4^1,6-diamidino-2-phenylindole dihydrochloride, 1μg/ml) for 10 minutes and washed twice in PBST for 10 min each. Finally, the tissues were mounted in DABCO (an antifadent).

Eye discs of 50-hours old pupae of different genotypes mentioned in the text were stained with Phalloidin-Rhodamine to examine the organization of actin filaments in ommatidial units. White pupae of desired genotypes were collected in fresh food vials and aged for 50 h. The aged pupae were dissected and the eye discs associated with brain complex were taken out and fixed in 4% para-formaldehyde for 60 min, washed thrice in PBST (with 0.3% TritonX-100) and placed in the antibody blocking solution[40] for 60 min. 30 μl of Phalloidin-Rhodamine (1:100, Sigma) was added to the tissues followed by overnight incubation at 4^{0}C. Counterstaining with DAPI and washing steps were performed as mentioned above.

Confocal imaging was carried out with a LSM510 Meta Zeiss confocal microscope using appropriate dichroics and filters. 10-15 discs were examined in each case. All images were assembled using the Adobe Photoshop software.

Western Blotting

Protein samples were prepared from 15 heads of one-day-old flies of different genotypes. The heads were homogenized in the protein sample buffer (100 mM Tris, pH 6.8, 1M DTT, 10% SDS, 100 mM PMSF, pH 6.8, 1% bromophenol blue and 1% glycerol) and boiled for 10 min followed by quick chilling and centrifugation at 5000 rpm for 10 min at 4^{0}C. Protein samples were electrophoresed in denatured condition in vertical SDS-poly-acrylamide gel having 4% stacking and 12% resolving gel using a discontinuous buffer system. After electrophoretic separation of proteins, proteins in the stacking and resolving parts of the gel were transferred onto the PVDF membrane (Millipore, USA) using standard protocol. For detection of polyQ proteins, anti-HA primary antibody raised in rabbit (Y-11, Santa Cruz, 1:400) and anti-rabbit HRP secondary antibody (Bangalore Genei, India, 1:1500) were used. The same blot was stripped of the antibodies using 100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris pH 6.7 and processed again for immunodetection of tubulin, as loading control. The antibodies used for tubulin detection were anti-tubulin primary antibody raised in mouse (Developmental Studies Hybridoma Bank, Iowa, 1:400) and antimouse HRP (Bangalore Genei, India 1:500). The antibody binding was detected using Super Signal West Dura kit according to the manufacturer's instructions (Pierce, USA).

Nail polish imprints

The nail polish imprints of adult eye surfaces were prepared as described earlier.[50] These were examined under DIC optics in a Nikon Ellipse 800 microscope.

Phototaxis assay

The assay was performed using a glass Y-maze.[51] Briefly, flies of different genotypes were independently introduced into the stem of a Y-maze, one arm of which was illuminated from an

outside light source. The other arm and the stem of the Y were wrapped with a black paper and thus were completely dark inside. Flies were introduced into the maze through the stem and allowed to run into the Y-maze phototactically for one minute. The flies with vision positively moved towards light (i.e. in the illuminated chamber) while those moving randomly were functionally blind. Flies in both the chambers were etherized and counted separately. In each experiment, the number of flies moving to the different arms was converted into % value and the mean % value (±SD) was calculated for three experiments.

Results

Down-regulation of Hsp60D suppresses SCA3 or 127Q mediated neurodegenerative eye phenotypes

Expression of a truncated form of Ataxin-3 (MJDtr-Q78) under control of the GMR-GAL4 driver resulted in, as already reported,[22] massive degeneration of retinal cells giving rise to rough and de-pigmented eyes in freshly eclosed flies (n = 306, Fig. 1A, D). It was noticed that over-expression of Hsp60D did not affect any of the polyQ eye phenotypes significantly (data not shown). However co-expression of a single copy of Hsp60D-RNAi transgene in the MJDtr-Q78 eye discs rescued the eye degeneration since the eyes showed normal pigmentation and more or less regularly organized ommatidial arrays (n = 364, Fig. 1B, E), nearly comparable to those in wild type eyes.

GMR-GAL4 driven expression of 127Q-transgene, which has only the expanded glutamine tract without any disease protein context, induced more severe eye degeneration than following expression of truncated Ataxin-3, and resulted in, as already reported,[25] glazed, de-pigmented and collapsed eyes (n = 312, Fig. 1G, J). In this case also, the eye degeneration was suppressed following co-expression of a single copy of Hsp60D-RNAi resulting in significant improvement of external eye morphology and pigmentation (n = 283, Fig. 1H, K), although to a lesser extent than found in the case of truncated Ataxin-3 expressing flies (compare Fig. 1B, E with 1H, K). Over-expression of Hsp60D did not significantly affect the 127Q induced eye phenotype (not shown). In presence of two copies of Hsp60D-RNAi transgenes, while the recovery in case of MJDtr-Q78 expressing eyes (n = 306 Fig. 1C, F) was nearly same as that with single copy of Hsp60D-RNAi, recovery of the 127Q induced eye damage was much better (n = 285, Fig. 1I, L).

Internal eye degeneration in polyQ flies is also reduced by Hsp60D-RNAi

We compared the integrity of photoreceptor cells in MJDtr-Q78 and 127Q expressing mid pupal stage (50 hour old pupae) retinas by staining the cellular actin cytoskeleton with Phalloidin-Rhodamine and nuclei with DAPI. In the MJDtr-Q78 expressing eye discs the integrity of photorecepter cells was poor when compared with wild type pupal retina. Small vacuoles were present in MJDtr-Q78 expressing retinas and the characteristic arrangement of the seven photoreceptors and the actin filaments within them was also altered (Fig. 2, compare A with B). The 127Q expressing pupal eyes showed greater disorganization and in many cases a complete loss of photoreceptor cells was also seen (Fig. 2, compare A with E).

Integrity of photoreceptor cells in the ommatidial units improved in a dosage dependent manner when one or two copies of Hsp60D-RNAi was/were co-expressed with MJDtr-Q78 (Fig. 2C and D, respectively) or 127Q (Fig. 2F and G, respectively). Compared to the partially restored organization of the photoreceptor cells in each ommatidial unit in pupal eye discs in which only one copy of Hsp60D-RNAi (Fig. 2C and F) was co-expressed, discs expressing two copies of the Hsp60D-RNAi transgene showed nearly normal organization of photoreceptor cells in most of the ommatidial units (Fig. 2D and 2G).

Accumulation of toxic polyQ aggregates and Hsp70 is reduced by Hsp60D-RNAi

Presence of nuclear inclusions and stress-inducible Hsp70 are characteristic features of polyQ neurodegenerative disorders.[7,9,20] In order to understand if the rescue of degenerated eye phenotypes by Hsp60D-RNAi is associated with any change in the polyQ inclusion bodies, third instar larval eye discs expressing GMR-GAL4 driven expanded polyQ transgene (either MJDtr-Q78 or 127Q) alone or in conjunction with one or two copies of Hsp60D-RNAi were co-immunostained for the polyQ and stress-inducible Hsp70 proteins (Fig. 3). Consistent with the previous findings, a large number of nuclear inclusions were observed in the posterior differentiating cells of MJDtr-Q78 (Fig. 3A) or 127Q (Fig. 3D) expressing eye discs. In addition, a cytoplasmic distribution was also seen in all cells of MJDtr-Q78 and in the anterior rows of cells of 127Q expressing eye discs (Fig. 3A, D). In agreement with greater degeneration seen in adult eyes following 127Q expression compared to those expressing mutant SCA3 (Fig. 1 and 2), the levels of polyQ as well as Hsp70 were generally higher in 127Q expressing discs. Co-expression of a single copy of Hsp60D-RNAi slightly reduced the number of nuclear inclusions in MJDtr-Q78 or 127Q-expressing eye discs, although the cytoplasmic protein were much less affected in any of the genotypes (Fig 3. compare 3B with A and E with D). Interestingly, co-expression of two copies of Hsp60D-RNAi transgene resulted in substantial reduction of inclusion bodies in the MJDtr-Q78 (Fig. 3C) as well as 127Q (Fig. 3F) expressing discs. The cytoplasmic levels of MJDtr-Q78 or 127Q were also somewhat reduced in eye discs co-expressing two copies of Hsp60D-RNAi (compare Fig. 3A, with C and D with F).

To confirm the above results, total MJDtr-Q78 protein in adult fly heads was assessed by Western blotting. In agreement with earlier report,[52] most of the polyQ proteins were trapped in the stacking gel because of large entangles. It is clear from Fig. 4 that flies expressing MJDtr-Q78 (lane1) alone had a greater amount of the aggregated protein trapped in the stacking gel, whereas in Hsp60D-RNAi co-expressing eyes, the SDS-insoluble fraction was considerably reduced in a dosage dependent manner (Fig. 4, lanes 2, 3).

Immunostaining with the stress-inducible Hsp70 specific 7Fb antibody[53] revealed that eye discs expressing only the MJDtr-Q78 or the 127Q transgene showed high levels of Hsp70 (Fig. 3, A and

D, respectively). A marked reduction in stress-inducible Hsp70 was observed when a single copy of Hsp60D-RNAi was simultaneously expressed (compare Fig. 3, A with B and D with E). Interestingly, co-expression of two copies of Hsp60D-RNAi resulted in a complete absence of Hsp70 protein, in parallel with that of the polyQ inclusion bodies (Fig. 3C and F). These observations suggest that depletion of cellular Hsp60D not only reduces the accumulation of toxic protein in inclusion bodies but also the cellular stress.

Hsp60D-RNAi improves vision in polyQ expressing flies

To confirm that suppression of polyQ toxicity by Hsp60D-RNAi is associated with improved vision, phototaxis assay was performed with one and four day old flies of different genotypes (Fig. 5 A, B). Flies with degenerated retina following expression of MJDtr-Q78 or 127Q moved randomly in light/dark chambers of the Y-maze (Fig. 5A, B).[54,55] Consistent with the above noted morphological rescue of eye morphology and photoreceptor organization in flies co-expressing the polyQ and Hsp60D-RNAi transgenes, we observed that such flies also showed improved phototaxis, indicative of restored vision. Co-expression of one or two copies of Hsp60D-RNAi transgene significantly restored the vision in one day old MJDtr-Q78 expressing flies, since majority of them moved to the lighted chamber (Fig. 5A). The effectiveness of the rescue in 127Q co-expressing flies was less compared to that in the MJDtr-Q78 expressing flies. One day old flies co-expressing 127Q and one copy of the Hsp60D-RNAi transgenes did not show any significant phototaxis; but the flies co-expressing two copies of Hsp60D-RNAi showed positive

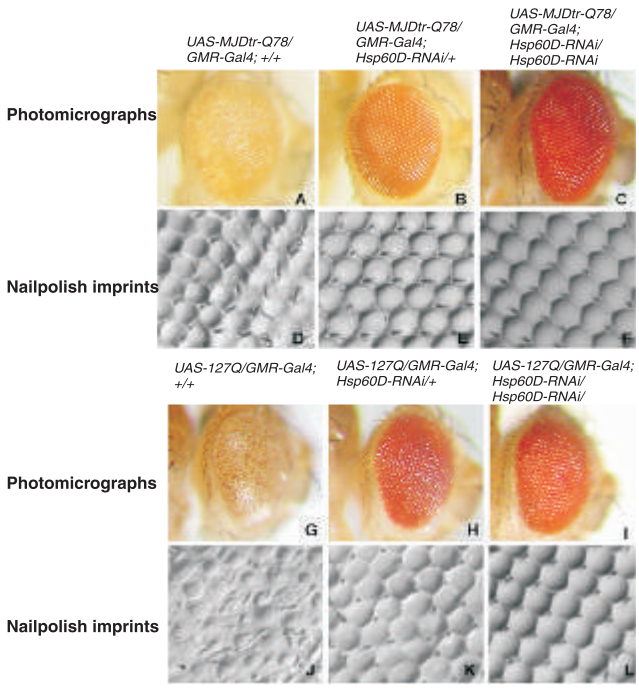

Fig. 1 : External eye morphology and ommatidial arrays in MJDtr-Q78 (A-F) or 127Q (G-L) models are restored by ablation of Hsp60D in a dosage dependent manner as seen in photomicrographs (A-C and G-I) and nail polish imprints (D-F and J-L) of adult eyes expressing transgenes in various genetic combinations (noted above each column of panels).

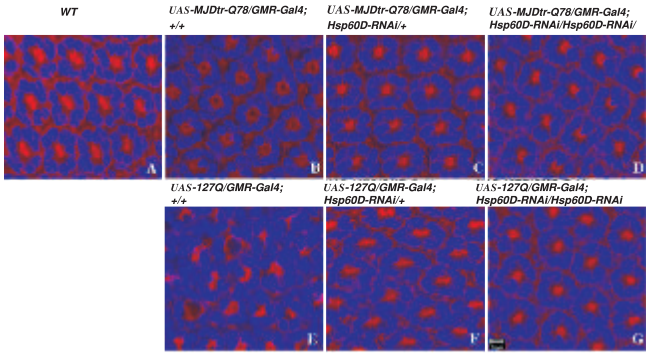

Fig. 2 : Hsp60D-RNAi restores photoreceptor organization in MJDtr-Q78 or 127Q expressing pupal eyes in a dosage-dependent manner. Confocal projections of eye discs form 50 hours old wild type (WT, A), UAS-MJDtr-Q78/GMR-GAL4; +/+ (B), UAS-MJDtr-Q78/GMR-GAL4; Hsp60D-RNAi/+ (C), UAS-MJDtr-Q78/GMR-GAL4; Hsp60D-RNAi/ Hsp60D-RNAi (D) 127Q /GMR-GAL4; +/+ (E), 127Q /GMR-GAL4; Hsp60D-RNAi/+ (F) and 127Q/GMR-GAL4; Hsp60D-RNAi/ Hsp60D-RNAi (G) pupae stained with Phalloidin-Rhodamine (red) and counterstained with DAPI (blue) to show nuclei. Scale bar represents 5μm and is common for all images).

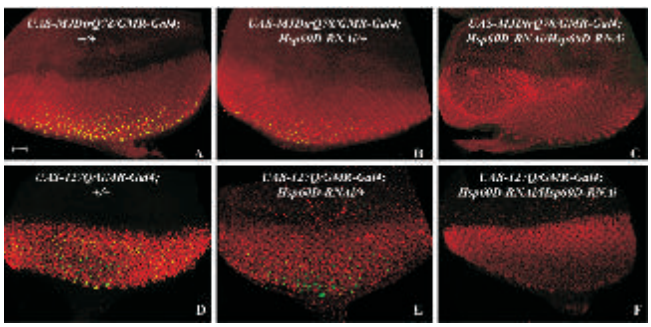

Fig. 3 : PolyQ inclusion bodies (red) and Hsp70 (green) are reduced following down-regulation of Hsp60D in a dosage dependent manner as seen in confocal projections of eye discs of different genotypes (noted on top left corner in each panel) co-immunostained for polyQ protein (red) and the stress-inducible Hsp70 (green). Scale bar in A is common for all the panels and represents 20 μm.

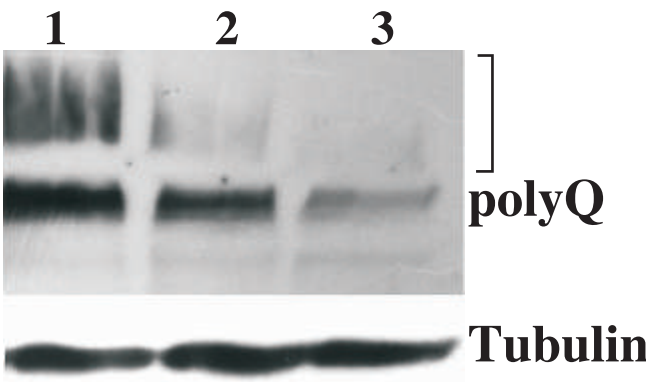

Fig. 4 : Knockdown of Hsp60D reduces aggregation of polyQ protein as revealed by western blotting of total proteins from adult fly heads (15 heads in each lane). Lane 1 = GMR-GAL4/MJDtr-Q78 showing high levels of polyQ protein in the stacking gel (indicated by the "]" mark on right), Lane 2 = GMR-GAL4/MJDtr-Q78; Hsp60D-RNAi/+ with reduced level of insoluble polyQ protein and Lane 3 = GMR-GAL4/MJDtr-Q78; Hsp60D-RNAi/Hsp60D-RNAi where the presence of Hsp60D-RNAi in double copy significantly lowered the polyQ protein accumulation. Tubulin levels shown in lower panel were used as loading control.

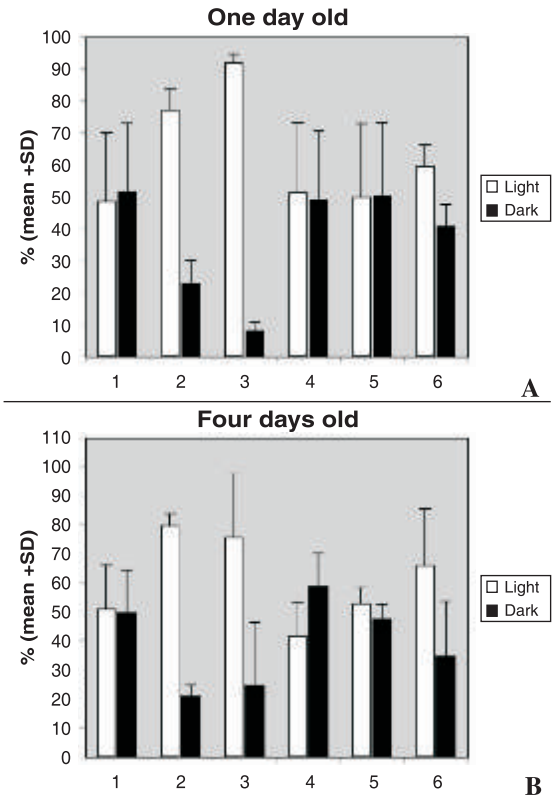

Fig. 5 : Phototaxis in 1 day (A) and 4 days old (B) flies of different genotypes as indicated following (1) UAS-MJDtr-Q78/GMR-GAL4; +/+, (2) UAS-MJDtr-Q78/GMR-GAL4; Hsp60D-RNAi/+, (3) UAS-MJDtr-Q78/GMR-GAL4; Hsp60D-RNAi/ Hsp60D-RNAi, (4) 127Q /GMR-GAL4; +/+, (5) 127Q /GMR-GAL4; Hsp60D-RNAi/+ ,(6) 127Q/GMR-GAL4; Hsp60D-RNAi/ Hsp60D-RNAi. Positively phototactic flies (white bars) preferentially move to the lighted chamber of the Y-tube.

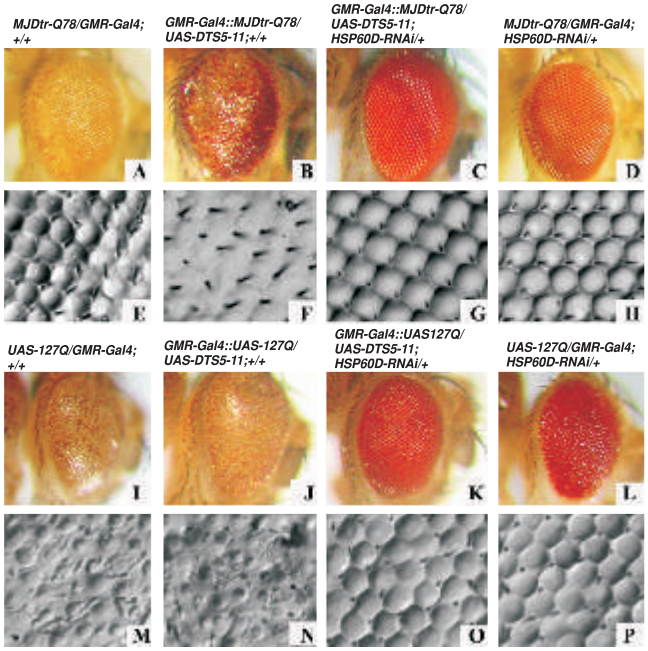

Fig. 6 : Compromising the proteasomal activity does not affect suppression of polyQ toxicity by Hsp60D-RNAi as seen in the photomicrographs (A-D and I-P) and nail polish imprints (E-H and M-P) of adult eyes of various genotypes noted above the columns.

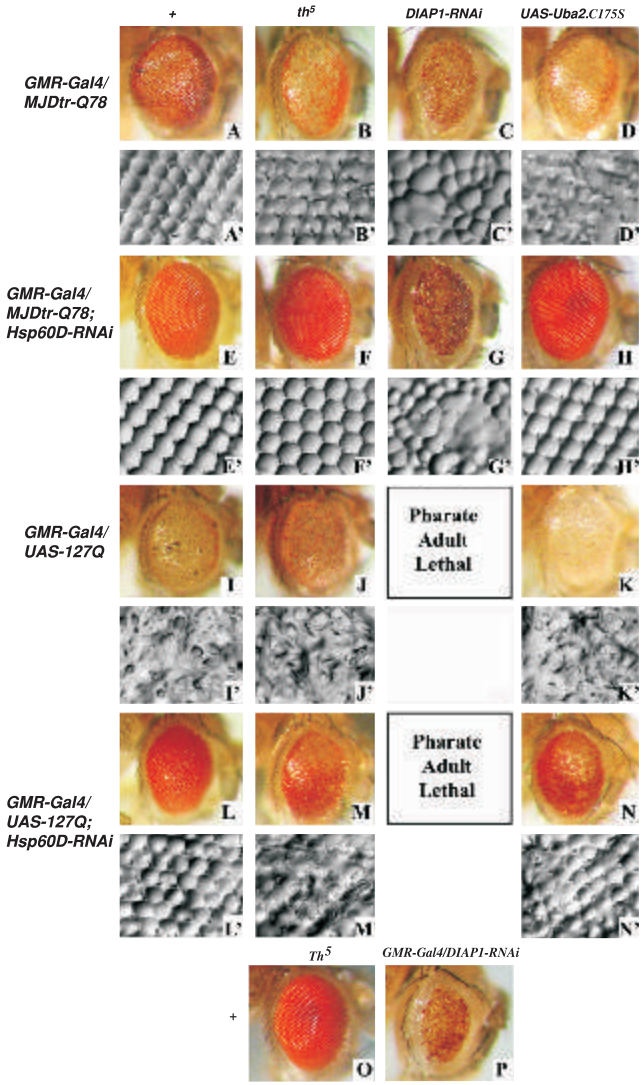

Fig. 7 : Loss of function mutations of DIAP1 or Uba2 partially mitigate the rescue of polyQ toxicity by Hsp60D-RNAi as seen in photomicrographs (A-D, E-H,I-K, L-N and O,P) and nail polish imprints (A'-D', E'-H',I'-K' and L'-N') of eye of different genotypes (indicated by combination of markers indicated for rows and columns).

phototaxis (Fig. 5A). Together, these results thus indicate that Hsp60D-RNAi mediated suppression is less effective in 127Q expressing flies, presumably because this transgene causes more severe damage than the MJDtr-Q78 transgene.

Neurodegeneration progresses with age. Therefore, to check the effectiveness of the Hsp60D-RNAi mediated rescue, we tested vision of the same set of flies after four days. The rescue in case of MJDtr-Q78 was still maintained, as most of the flies with one or two copies of Hsp60D-RNAi still moved into the lighted chamber (Fig. 5B). Likewise, 127Q expressing flies carrying one copy of Hsp60D-RNAi continued to be blind at day four as well while those with two copies of Hsp60D-RNAi remained positively phototactic (Fig. 5B).

Hsp60D-RNAi suppresses organismal lethality caused by pan-neuronal expression of expanded polyQ proteins

To know if the suppression of polyQ toxicity by Hsp60D-RNAi

Table 1. Hsp60D ablation suppresses pupal lethality following pan-neuronal expression of the pathogenic polyQ transgenes

Genotype	Total number of pupae examined	% Early pupal death	% Late pupal death	% Flies emerged	Life span (days)
elav-GAL4; UAS-MJDtr-Q27/+; +/+	151	0.0	0.0	100	>30
elav-GAL4; UAS-MJDtr-Q78(S)/ +; +/+	476	15.8	84.2	0.0	0.0
elav-GAL4;UAS-MJDtr-Q78(S)/+; Hsp60D-RNAi/+	486	3.7	93.4	3.2	6.0
elav-GAL4;UAS-Q20/+; +/+	246	0.0	0.0	100	>30
elav-GAL4;UAS-127Q/+; +/+	199	77.9	11.1	0.0	0.0
elav-GAL4;UAS-127Q/+; Hsp60D-RNAi/+	311	54.3	42.1	0.0	0.0

extends to rest of the nervous system, the expanded polyQ proteins alone or together with Hsp60D-RNAi were expressed in the central and peripheral nervous system using the pan-neuronal elav-GAL4 driver. As known from previous studies,[22] elav-GAL4 driven expression of MJDtr-Q78 or 127Q in all cells of the central and peripheral nervous system resulted in 100% lethality at pupal stage (Table 1). Genotypes expressing UAS-MJDtr-Q27 or UAS-20Q transgenes under the elav-GAL4 driver were used as controls for MJDtr-Q78 and 127Q, respectively. Co-expression of Hsp60D-RNAi with MJDtr-Q78 or 127Q resulted in partial suppression of lethality (Table 1). Compared to the death of 15.8% undifferentiated MJDtr-Q78 expressing pupae, co-expression of Hsp60D-RNAi reduced the early pupal lethality to 3.7% (Table 1). More significantly, in contrast to the complete failure of MJDtr-Q78 expressing pupae to emerge as adults, co-expression of Hsp60D-RNAi caused approximately 3% pupae to successfully come out as adults, although the emerging flies were short-lived with a maximum life span of 6 days. Similarly, co-expression of Hsp60D-RNAi with 127Q resulted in a shift from early to late pupal lethality, although no flies emerged in this case (Table 1).

Rescue by Hsp60D-RNAi is largely independent of requirement of functional proteasome

To examine if the reduction in polyQ toxicity following Hsp60D-RNAi was dependent on functional proteasome, a GAL4-inducible dominant temperature sensitive proteasome mutant transgene (UAS-DTS5-11) was co-expressed with MJDtr-Q78 or 127Q. This dominant negative mutation affects the β6 subunit of proteasome, and thus the mutant flies can survive at 25°C but die at 29°C.[56] Bilen and Bonini[57] reported that expression of UAS-DTS5-11 at 25°C partly compromises the proteasome activity; they further reported that although expression of this dominant-negative form at the permissive temperature does not have any detectable effect on SCA3 mediated neurodegeneration, expression of single copy of this mutant allele reduces the efficacy of several polyQ suppressors that are dependent on proteasome for their action. Contrary to the above report of Bilen and Bonini,[57] we found that the degeneration caused by GMR-GAL4 driven expression of MJDtr-Q78 (n = 175, Fig. 6B, F) or 127Q was enhanced, more so in the case of MJDtr-Q78, by co-expression of DTS5-11 (n= 186, Fig. 6J, N). Interestingly however, co-expression of DTS5-11 did not significantly affect the suppression of the polyQ damage by Hsp60D-RNAi in either SCA3 (n = 137, Fig. 6C, G) or 127Q fly eyes (n = 122, Fig. 6K, Q); since such flies exhibited nearly comparable recovery in eye phenotypes as in those with functional proteasome (Fig. 6D, H and L, P). Conditional expression of UAS-Pros26^{1};UAS-probeta2, dominant negative forms of β2 and β6 subunits of proteasome[47] was also used to confirm the above results. GMR-GAL4 driven expression of this dominant negative proteasomal form at 25°C did not have any external eye phenotype by itself but co-expression with MJDtr-Q78 or 127Q enhanced the polyQ degeneration (n= 83 and 55, respectively). Interestingly, Hsp60D-RNAi could still restore the polyQ eye phenotype even when the UPP activity was compromised by expression of the dominant negative proteasome (n = 58 and 63 for MJDtr-Q78 and 127Q, respectively; figures not shown).

Functional depletion of DIAP1 affects Hsp60D-RNAi mediated suppression of polyQ toxicity

Previously, we identified that down-regulation of Hsp60D through RNAi inhibits induced apoptosis and this suppression requires DIAP1.[40] To check whether the Hsp60D-RNAi mediated suppression of polyQ toxicity also required DIAP1, a loss of function allele of DIAP1 (th^{5}) and the UAS-DIAP1-RNAi transgene were employed to deplete DIAP1 levels. The th^{5}/+ flies showed near normal eye phenotype (Fig. 7-O). On the other hand, a single copy of this mutant allele enhanced the eye degeneration seen in MJDtr-Q78 expressing flies (n = 111, Fig. 7B, B') and more so in those expressing 127Q (n = 87, Fig. 7J, J'). Interestingly, in th^{5}/+ background while the Hsp60D-RNAi mediated suppression of SCA3 was not much affected ((n= 122), Fig. 7 compare E, E' with F, F'), that of the 127Q phenotypes was significantly affected (n = 97, compare Fig. 7L, L' with M, M').

Effect of GMR-GAL4 driven DIAP1-RNAi, which by itself results in a degenerated eye phenotype (Fig. 7P), on rescue of the polyQ damage by Hsp60D-RNAi was also examined. Co-expression of the DIAP1-RNAi transgene in the MJDtr-Q78 expressing flies resulted in enhanced eye degeneration with reduction in eye size (n = 107, Fig. 7C, C'), whereas, with 127Q, it resulted in death at pharate stage; these pharates displayed highly reduced eyes and under-developed heads (n = 133, not shown). Co-expression of Hsp60D-RNAi (one copy or two copies) failed to rescue the eye degeneration in MJDtr-Q78 and DIAP1-RNAi expressing flies (n=78, compare Fig. 7E, E' with G, G'). Likewise, the pharate stage lethality due to co-expression of DIAP1-RNAi and 127Q was also not rescued by Hsp60D-RNAi (n=98). Thus the availability of DIAP1 seems to be essential for rescue of polyQ

phenotype by Hsp60D-RNAi.

Functional depletion of sumoylation pathway does not significantly affect Hsp60D-RNAi mediated suppression of polyQ toxicity

Post-translational modification of proteins by sumoylation affects the polyQ pathogenesis since its inhibition suppresses the Huntington phenotype in Drosophila model[58] whereas a dominant negative form of Uba2 (a SUMO activating enzyme subunit 2) enhances the toxicity in SBMA and MJDtr-Q78 models.[59,60] As reported earlier,[59] expression of dominant negative allele of Uba2 (Uba2.C175S) in MJDtr-Q78 expressing eyes enhanced the degeneration, resulting in more glazed eyes with little pigmentation (n = 72, compare Fig. 7A, A' with D, D'). In 127Q expressing eyes also, co-expression of Uba2.C175S enhanced the phenotype, (n = 69, compare Fig. 7I, I' with K, K'). While the suppressive effect of Hsp60D-RNAi in MJDtr-Q78 expressing eyes did not appear to be affected by functional depletion of Uba2 protein (n = 80, compare Fig. 7H, H' with E, E'), in the case of 127Q, the recovery brought about by Hsp60D-RNAi in presence of the Uba2 mutant allele was slightly less than in functional SUMO background (n = 87, compare Fig. 7N, N' with L, L').

Discussion

The present study identified Drosophila Hsp60D as a novel modifier of polyQ toxicity since the severe neurodegeneration following expression of mutated Ataxin-3 or 127Q proteins is dominantly suppressed by co-expression of Hsp60D-RNAi in developing eyes as well as pan-neuronally. Co-expression of Hsp60D-RNAi transgene recovers, in a dose-dependent manner, not only the external and internal eye structures damaged by polyQ toxicity, but also their functionality as assayed by positive phototropism. The accumulation of toxic polyQ protein aggregates and stress inducible Hsp70 protein in the diseased cells is also reduced in proportion to the extent of depletion of Hsp60D.

Heat shock proteins are well known modifiers of polyQ toxicity.[3] Over-expression of Hsp70 in mouse and Drosophila polyQ models delays the neuronal dysfunction, improves cell survival, and thus suppresses neurodegeneration.[20,59,61,62] Ectopic expression of human Hsp70 in a fly model of SCA3 reduces the retinal toxicity whereas mutation of its co-chaperone Hsp40 enhances the degenerative phenotype.[20] The polyQ toxicity in 127Q expressing eye discs is suppressed by over-expression of heat shock protein Hdj1 and/or Tpr-2, which contains a DNAJ domain.[25] In addition to Hsp40, other cofactors of Hsp70, like CHIP and Bag-1 are also known to modulate polyQ pathogenesis in SCA1, SCA3 and Huntington disease models.[63-66] It is known that many of the chaperone proteins also co-localize with nuclear inclusion bodies in the diseased cells.[64] However, Hsp60 has not been found to be associated with the aggregates.[7] We have also seen (data not presented) that unlike the other molecular chaperones, over-expression of Hsp60D in polyQ expressing cells does not have any enhancing or suppressive effect on neurodegeneration. On the contrary, the results presented here show that, unlike the suppression of polyQ toxicity by over-expression of Hsp70 or other Hsps and their co-factors, it is the reduction in levels of Hsp60D that suppresses the polyQ toxicity.

As discussed in introduction, various cellular protein degradation pathways like UPP, autophagy are known to degrade toxic, misfolded and non-functional proteins, and, therefore, these pathways have also been implicated in the modulation of polyQ toxicity. Bilen and Bonini[57] identified a number of modifiers of SCA3, some of which suppress the polyQ pathogenesis through the proteasome pathway while others reduce the polyQ toxicity even in absence of functional proteasome. In the present study also it was noticed that abrogation of the proteasomal machinery did not significantly affect suppression of polyQ toxicity by Hsp60D-RNAi suggesting that the proteasome pathway may not have significant role in suppression of polyQ toxicity by Hsp60D-RNAi.

Similar to ubiquitin and chaperone proteins, SUMO (small ubiquitin-like modifier) proteins are associated with the neuronal inclusion bodies and are known to modify polyQ pathogenesis, although the exact role of sumoylation in polyQ pathogenesis is not clearly understood.[67] It was shown that in fly models of truncated androgen receptor protein with expanded polyQ and in SCA3, expression of a mutant form of the SUMO-1 activating enzyme, Uba2, enhanced the toxicity.[59] Over-expression of Hsp70 was ineffective in suppressing neurodegeneration when the functional Uba2 levels were compromised.[59] In the present study, however, expression of mutated SUMO-1 did not substantially mitigate the effectiveness of Hsp60D-RNAi in suppressing the SCA3 toxicity although in the case of 127Q, the suppression was slightly reduced. However, it is possible that the somewhat less effective rescue of 127Q damage by Hsp60D-RNAi in Uba2 mutant background may be due to an additive effect of the compromised Uba2 function and the 127Q damage rather than a direct requirement of SUMO pathway in Hsp60D-RNAi mediated suppression.

Present findings suggests that following Hsp60D-RNAi, the polyQ inclusion bodies were reduced or nearly absent and concurrently, the stress inducible Hsp70 was also correspondingly reduced or absent from the polyQ expressing eye discs suggest that the reduced levels of Hsp60D may suppress polyQ toxicity at an early step, presumably prior to the formation of aggregates of mis-folded polyQ proteins. It is possible that being a chaperone, the Hsp60D participates in folding of mutated polyQ proteins into the toxic conformation. Requirement of Hsp60 homologs in folding of potentially toxic polypeptides into pathological proteins is known. Carrio and Villaverde[68] showed that normal Hsp60/GroEL function is required for formation of aggregates or inclusion bodies containing the misfolding prone but partially soluble VP1LAC hybrid protein since in GroEL/Hsp60 mutant *E. coli* cells, such aggregates or inclusion bodies were not formed. Likewise, Hsp60/GroEL is also required for the conversion of normal prion protein (PrP^{C}) into the prion-disease causing PrP^{Sc} form.[69,70] The significant reduction or absence of polyQ inclusion bodies

(Fig. 3) and insoluble multimeric forms of polyQ proteins (Fig. 4) following Hsp60D-RNAi suggest that in the fly models also, aggregation of the expanded polyQ proteins into pathogenic inclusion form may require Hsp60D so that in its absence, they do not get mis-folded and thus the polyQ aggregates are not formed. In addition, inhibition of caspase activity following Hsp60D-RNAi[40] may also prevent production of toxic subunits of the mutant polyQ proteins since caspase mediated cleavage of expanded polyQ proteins is reported to be a necessary step in polyQ toxicity.[71] Taken together, Hsp60D-RNAi in fly models seems to prevent the source of polyQ toxicity so that Hsp70 is not induced and the subsequent damage to cells is reduced.

Apoptosis has been implicated in several neurodegenerative disorders,[72] although the reported effects of expression of the different anti-apoptotic factors on polyQ toxicity have been varying.[57, 73-76] In our previous study,[40] we found that Hsp60D-RNAi inhibits induced apoptosis. This raises the possibility that Hsp60D-RNAi suppresses polyQ neurodegeneration also by suppression of apoptosis of sensitive neurons.

Effect of DIAP1 mutants on Hsp60D-RNAi mediated suppression of polyQ toxicity is intriguing. It was observed that co-expression of single copy of th[5] (a DIAP1 loss of function allele), although without any apparent effect on the Hsp60D-RNAi mediated suppression of SCA3 damage, partially mitigated the Hsp60D-RNAi mediated suppression in the 127Q model. In agreement with this, a stronger reduction of DIAP1 protein levels by expressing DIAP1-RNAi, inhibited the Hsp60D-RNAi mediated suppression not only in case of 127Q but also of SCA3. These observations suggest that the DIAP1 protein is required for the Hsp60D-RNAi mediated suppression. The inhibition of Hsp60D-RNAi mediated suppression of the polyQ damage by DIAP1-RNAi could be a consequence of additive toxicity due to activation of caspases following DIAP1-RNAi, which can not be suppressed by Hsp60D-RNAi in the absence of DIAP1.[40]

Other recent studies in our laboratory[77-79] demonstrate that, like the Hsp60D-RNAi, depletion of the stress-inducible non-coding hsrω RNA in Drosophila also suppresses induced apoptosis and polyQ-mediated neurodegeneration. It is interesting that down regulation of Hsp60D as well as the hsrω transcripts suppressed the aggregate formation.[40, 77] Notwithstanding these similarities in the end-results of depletion of either the Hsp60D or the non-coding hsrω RNA, there are notable differences in their mechanisms of suppression of induced apoptosis as well as polyQ neurodegeneration. For instance, while polyQ suppression by depletion of hsrω transcripts requires functional proteasomal machinery,[78] the suppression by Hsp60D-RNAi is largely independent of the proteasome function. Further studies are required to know if the pathways through which these two modulators of polyQ toxicity act have any direct interaction.

Results presented here identify Hsp60D as a novel modifier of polyQ damage, although identification of the specific mechanism/s through which reduced levels of Hsp60D suppress the polyQ toxicity requires further studies. Nevertheless, these findings open new avenues for search of effective therapeutic agents in human diseases involving apoptosis and/or toxic proteins.

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