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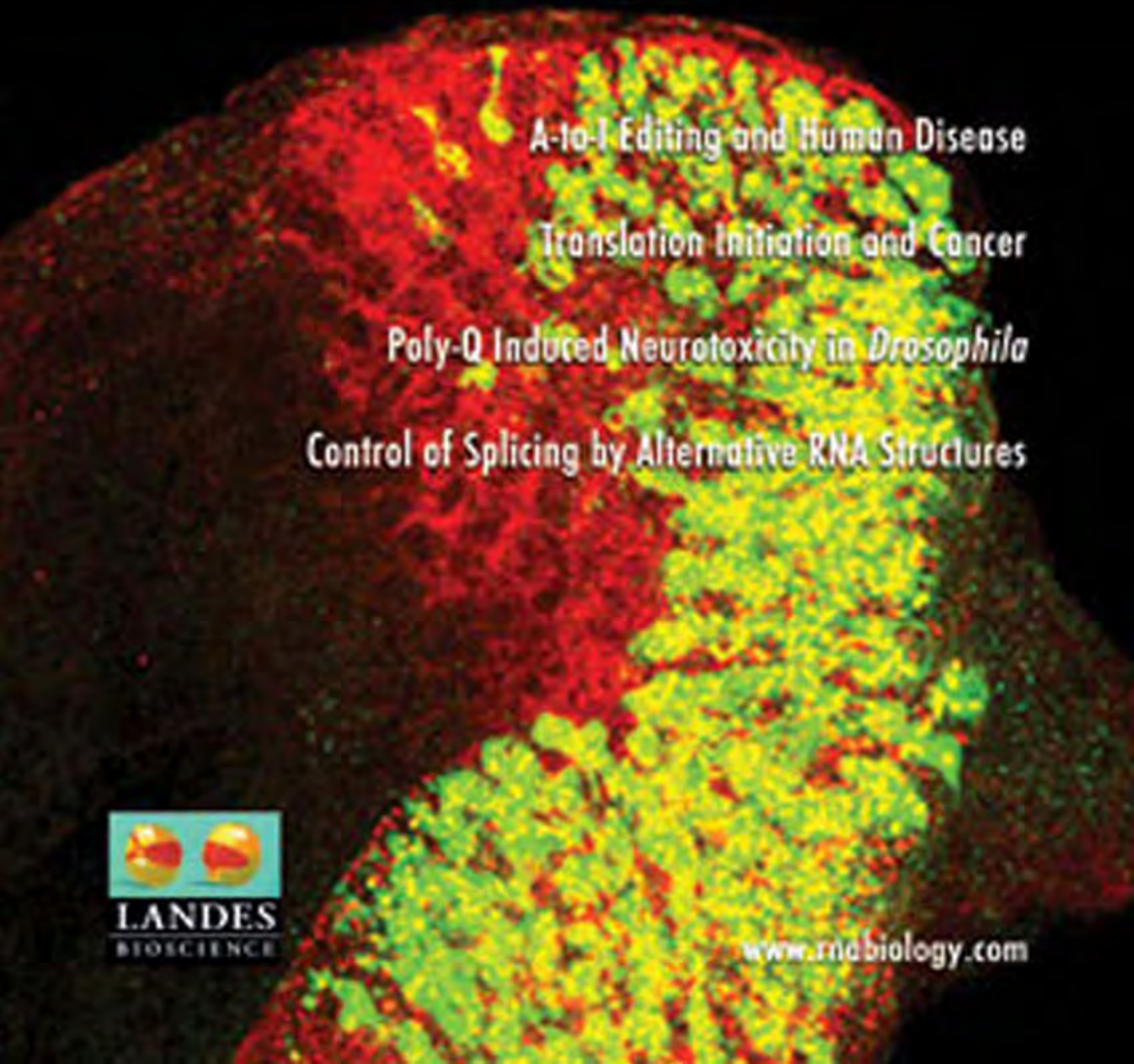

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Research Paper

Altered Expression of the Noncoding *hsrω* Gene Enhances poly-Q-Induced Neurotoxicity in *Drosophila*

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KEY WORDS

omega speckles, hnRNP, Hrb87F, Hsp70, Huntingtin, Spinocerebellar ataxia

ABBREVIATIONS

93D	hsrω gene/93D heat shock locus
DIG	digoxigenin
FRISH	fluorescence RNA-RNA in -situ hybridization
HA	haemagglutinin
HnRNP	heterogeneous nuclear ribonucleoproteins
PBS	phosphate buffered saline
poly-Q	polyglutamine
PSS	Poels' Salt solution

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ABSTRACT

In an earlier report two *P*-transposon insertion alleles of the noncoding *hsrω* gene, *hsrω*^{05241} and *P292* were shown to enhance neurodegeneration caused by expression of ataxin-1 protein with expanded poly-Q in a *Drosophila* model. In present study, we examined the possible relation between *hsrω* gene expression and toxicity due to poly-Q pathogenesis. The *Drosophila hsrω* gene produces several noncoding transcripts in almost all cell types, of which the >10 kb long hsrω-n transcript organizes heterogeneous RNA binding (hnRNPs) and related proteins as nucleoplasmic omega speckles. We show that *P* insertion alleles of the *hsrω* gene, which cause its overexpression, dominantly enhance neurodegeneration in fly eyes expressing either expanded poly-Q (127Q) or mutant huntingtin protein. Null allele of *Hrb87F* gene, encoding hnRNPA1, and a novel gene's mutant allele (*l(3)pl10*^{R}), which affects the omega speckles, also dominantly enhance 127Q-induced neurodegeneration. The hsrω-n transcripts or the hnRNPs do not colocalize with the poly-Q nuclear inclusion bodies, neither in *hsrω* wild type, nor in *hsrω* mutant background. However, the levels of poly-Q and Hsp70 were significantly higher in *hsrω* mutant eye discs. Sequestration of hnRNPs and other related RNA-binding proteins by overexpression of *hsrω* transcripts in *hsrω*^{05241} or in *l(3)pl10*^{R} background or the reduced levels of Hrb87F protein seem to affect nuclear RNA metabolism, thus enhancing the toxicity due to poly-Q expansion.

INTRODUCTION

Expansion of CAG trinucleotide repeat sequences is associated with nine neurodegenerative disorders in humans[1] like Huntington's disease (HD),[2] and the various Spinocerebellar Ataxias.[3] All these neurodegenerative diseases result from expanded CAG repeats, encoding a stretch of poly-glutamines or poly-Qs in the translated region of the respective gene. The expanded poly-Q stretch confers toxicity to the protein. These CAG repeat expansion diseases share common features like adult onset, progressive neurodegeneration, generational anticipation and a remarkable threshold-expansion length, which suggests that they may share a common pathogenetic mechanism.[4-7] These diseases are also characterized by vulnerability of only a subset of neurons to the diseased protein despite its widespread expression in brain and other tissues.[8] A pathological hallmark of the poly-Q expansion disorders is the presence of intracellular protein aggregates in nuclei of affected neurons, cultured cells and also in animal models.[4,5,9,10] These inclusion bodies contain misfolded proteins, molecular chaperones, 26S proteasome, ubiquitin conjugating enzyme and several transcription factors.[11-13]

Several neurodegenerative disorders have been modeled in *Drosophila*[14-20] to permit unbiased genetic screens for enhancers and suppressors of neurodegeneration. Such studies have helped in identifying new genes and pathways involved in pathogenesis.[16] Changes in expression of heat shock proteins and components of the ubiquitin/proteasome pathway were found to modulate poly-Q toxicity,[21-24] suggesting that misfolding, impaired degradation, and abnormal aggregation of proteins may be key determinants of the disease. On the other hand perturbation in transcriptional regulation was also found to be one of the primary factors responsible for pathogenesis.[25-27] Mutant huntingtin aggregates sequester transcription factors like CBP (CREB binding protein) and redistribute it away from its usual location and thus repress transcription from CBP/p300 activated promoters in cultured cells.[11,26] In the SCA-1 mouse model, specific neuronal genes were downregulated at a very early stage of pathogenesis, before any known behavioral or pathological changes occurred.[28] Thus, transcriptional mis-regulation and protein folding and turnover seem to be the main cellular processes affected in poly-Q pathogenesis.[29]

In an earlier genetic screen[16] two mutant alleles of the noncoding *hsrω* gene, *hsrω*^{05241} and *P292*, were identified as enhancers of SCA-1 induced neurotoxicity. The *hsrω* gene in *Drosophila melanogaster* produces several noncoding transcripts in a developmentally regulated manner in nearly all cell types and also is also induced by a variety of cellular stresses.[30,31] The >10 kb nucleus limited hsrω-n transcript forms nucleoplasmic omega speckles which provide a dynamic sink for heterogeneous nuclear RNA binding proteins, hnRNPs and other related proteins that are not engaged in RNA processing.[31-33] In unstressed *Drosophila* cells a variety of hnRNPs (Hrb87F, Hrb57A, Hrp40, S5, etc.) localize in two nuclear compartments: the active hnRNPs remain associated with sites of RNA processing on chromatin and the inactive ones are stored, in association with the hsrω transcripts, in the nucleoplasmic omega speckles.[33] The omega speckles are distinct from the interchromatin granule clusters (IGCs), which contain SR proteins but not hnRNPs.[32,33]

In the present study, we examined the role of noncoding *hsrω* gene in poly-Q expansion disease pathogenesis. We show that the *hsrω*^{05241} is a recessive gain of function allele, rather than a loss of function allele as suggested by Fernandez-Funez et al.[16] Furthermore, besides *hsrω*^{05241}, other overexpressing alleles of *hsrω*, viz., *EP3037* and *EP93D*, also enhance the neurodegeneration due to expression of expanded poly-Q (127Q) or of expanded huntingtin protein in developing eye cells of *Drosophila*. A null allele of *Hrb87F* (homologue of vertebrate hnRNPA1) and a mutant allele of a novel gene, *l(3)pl10*^{R}, which affects omega speckles, also dominantly enhance the neurodegeneration. Unlike many other known genetic modifiers of poly-Q-induced neurodegeneration, e.g., transcriptional cofactors, molecular chaperones, ubiquitin/proteasomes, *Drosophila* myeloid leukemia factor and *Drosophila* Apaf-1 related killer (Dark), which directly associate with nuclear inclusion bodies,[9,21-24,26,27,34-38] neither the hsrω-n nor the hnRNPs colocalize with the poly-Q inclusion bodies. It appears that altered organization of omega speckles and the consequent altered distribution of hnRNPs and related proteins perturbs nuclear RNA processing activities. This adds further to the cellular dysfunctioning in poly-Q expressing neural cells and thus aggravates the toxicity.

MATERIALS AND METHODS

***Drosophila* fly stocks.** *Drosophila melanogaster* cultures (wild type and mutants) were reared at 22° ± 1°C on standard food containing agar, maize powder, yeast and sugar. For cytological preparations, staged larvae were grown in Petri plates on food supplemented with additional yeast for healthy growth. *Oregon R*$^{+}$ was used as a wild type strain. The *UAS-127Q* and *UAS-20Q* transgenic lines were obtained from Dr. Parsa Kazemi-Esfarjani,[39] *UAS-Q93* and *UAS-Q20* transgenic lines from Prof. Leslie Thompson[27] and the *Df(3R)Hrb87F* mutant flies were obtained from Prof. S. Haynes.[40] Among the *P*-insertion alleles of *hsrω* gene, *hsrω*^{05241} [41] and *EP3037* were obtained from *Drosophila* stock centers, while *EP93D* insertion was generated in our laboratory (Mallik M, Lakhotia SC, unpublished). The *EP3037* and *EP93D* chromosomes carry the EP transposon,[42] which allows enhanced expression of the *hsrω* gene in response to Gal4 transcription factor. The *l(3)pl10*^{R} mutation was generated in our laboratory (Rajendra TK, unpublished) and later mapped to the 94E13-94F1 cytogenetic region.[43] *GMR-GAL4*, obtained from *Drosophila* stock centre, was used as a driver strain to express the desired gene under the UAS promoter in cells behind the morphogenetic furrow in 3rd instar larval eye discs.[44]

Pseudopupil analysis. Arrangement of rhabdomeres in the ommatidia of the compound eye was visualized using the pseudopupil technique.[45] Adult flies were decapitated, eyes were dipped in a drop of immersion oil on a microscopic slide and directly examined in a Nikon E800 microscope using 60X oil plan-Apo (1.4 NA) objective and images were recorded with a Nikon DXM 1200 digital camera.

Antibodies, clones and RNA probes. Mouse monoclonal P11 antibody was used to immunolocalize the Hrb87F protein[46] (dilution 1:10). Heat inducible form of Hsp70 protein in *D. melanogaster* was detected by the 7Fb rat monoclonal antibody,[47] (dilution 1:200). The Hsp60 was detected with a rabbit polyclonal antibody (SPA-805, Stressgen, Canada, dilution 1:100) and haemagglutinin (HA)-tagged polyglutamine protein with a rabbit polyclonal antibody to haemagglutinin (Y-11, Santa Cruz, dilution 1:20). Axonal connections in eye imaginal discs of third instar larvae were detected by mab22C10, a mouse monoclonal antibody specific for the neurons (Developmental Studies Hybridoma Bank, Iowa, dilution 1:100,). Appropriate secondary antibodies conjugated either with Alexa Fluor 488 (Molecular Probes, dilution 1:200) or Cy3 (Sigma, 1:100) were used for fluorescent detection of the primary antibody localization.

Digoxigenein (dig)-labeled antisense riboprobe, generated from the pDRM30 clone, which specifically detects the hsrω-n RNA,[30] was used for hybridization to cellular RNA.

Partial squash preparations of larval eye imaginal discs for RNA:RNA in situ hybridization and/or immunostaining. Partial squash preparations of eye portions of the eye imaginal discs from wild type, *hsrω*^{05241}/*hsrω*^{05241}, *UAS-127Q/GMR-Gal4;* +/+ and *UAS-127Q/GMR-Gal4*; *hsrω*^{05241}/*hsrω*^{05241} were prepared essentially following the procedure of Bendena et al.[48] These were processed for Flourescence RNA-RNA in situ hybridization (FRISH) and/or immunostaining as described earlier.[33]

Whole organ immuno-fluorescence staining. Eye imaginal discs from late 3rd instar larvae of desired genotypes were processed for immuno-fluorescence staining as described previously.[33] In each case, the tissues were mounted in antifadent and observed under fluorescence and/or confocal microscope.

Microscopy. The FRISH and/or immuno-fluorescentlly stained preparations were observed either under a Nikon E800 fluorescence microscope using appropriate filter combinations or in a Biorad Radiance 2000 multiphoton laser scanning confocal microscope. The different objectives used were 10X (0.3NA, Plan Fluor), 20X (0.5NA, Plan Fluor) or 60X oil (1.4NA, Plan Apo). The images on the Nikon E800 fluorescence microscope were recorded with a Nikon Digital Camera DXM 1200.

For recoding images of adult eyes, flies were etherized, viewed and photographed at a magnification of 5X and 6X digital zoom using a Sony Digital Camera (DSC-75) fitted onto a Zeiss Stemi SV6 stereo-binocular-microscope.

All the images were assembled using Adobe Photoshop 7.0.

RESULTS

***hsrω*^{05241} is a recessive gain of function allele of *hsrω* gene and affects hnRNP distribution in nuclei.** The *hsrω*^{05241} allele harbors a *P*-element in the -130 bp region of *hsrω* promoter and was earlier reported to be associated with altered expression of *hsrω* in cyst cells of adult testis and consequent recessive male sterility.[41] Though an earlier study[49] using chromogenic detection of hsrω transcripts in situ failed to detect any effect of the mutation in various somatic tissues, our present fluorescence RNA-RNA in situ hybridization (FRISH) studies reveal that compared to the 6–8 fine omega speckles (Fig. 1A–C) in wild type larval eye disc cells, those from *hsrω*^{05241} homozygous larvae show 1-2 large clusters or aggregates of hsrω-n transcripts (Fig. 1D–F). Thus, *hsrω*^{05241} seems to be a gain-of- function allele rather than a loss-of-function allele as presumed by Fernandez-Funez et al.[16]

A variety of hnRNPs, which are not chromatin bound, are known to colocalize with the nuclear transcripts of *hsrω* to form fine nucleoplasmic omega speckles.[32,33] A combined FRISH using hsrω-n specific riboprobe and immunostaining with the P11 antibody which recognizes the *Drosophila* Hrb87F protein,[46] showed a fine speckled distribution of Hrb87F in wild type cells (Fig. 2A–C) whereas in *hsrω*^{05241} homozygous mutant cells, it was present as large aggregates along with hsrω-n RNA (Fig. 2D–F).

***hsrω*^{05241}, *EP* alleles of *hsrω* and a null allele of *Hrb87F* affect organization of rhabdomeres in eyes.** The *EP93D* allele carries a *P*-transposon

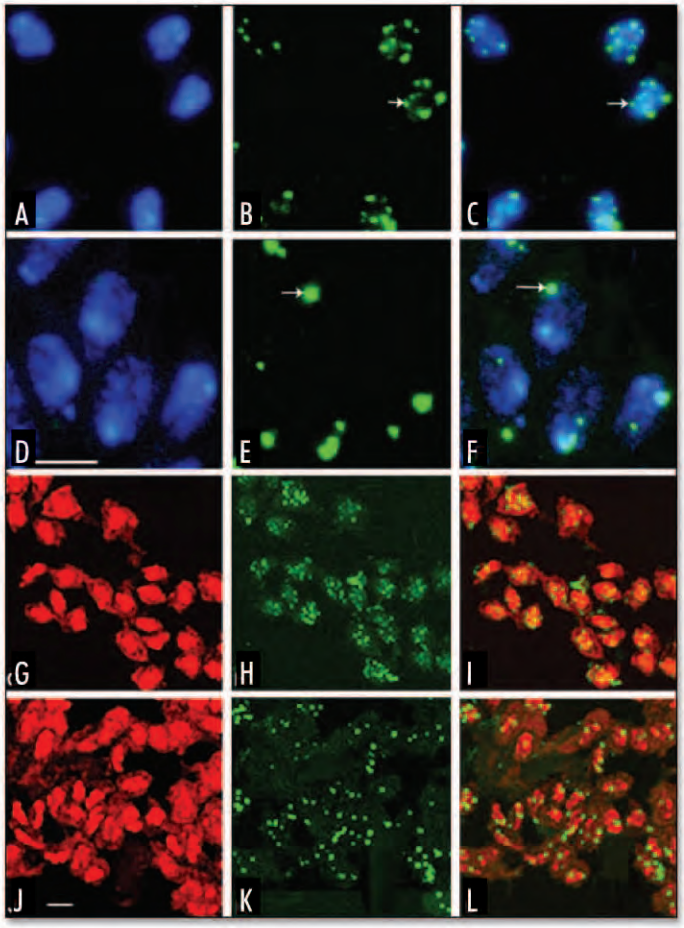

Figure 1. (A-F) Localization of hsrω-n transcripts (green) in eye disc cells of wild type (A-C) and *hsrω*^{05241} homozygous (D-F) larvae by fluorescence RNA-RNA in situ hybridization. Chromatin (A, D) is stained with DAPI (blue). Merged images are shown in C and F. The hsrω-n transcripts (arrows) in wild type eye disc cells are distributed as several small speckles in each nucleus (B and C), but in *hsrω*^{05241} mutants they are present as 2-3 larger clusters in each nucleus (E and F). G-L. hsrω-n transcripts (green, H, K) in brain cells of wild type (G-I) and *l(3)pl10*^{R} homozygous (J-L) late 3rd instar larvae; chromatin (red, G, J) is stained with propidium iodide. I and L show merged images. Unlike the multiple omega speckles in wild type, the *l(3)pl10*^{R} cells show only one or two large cluster/s of hsrω-n transcripts. Scale bars, for A-F in D and for G-L in J, represent 5μm.

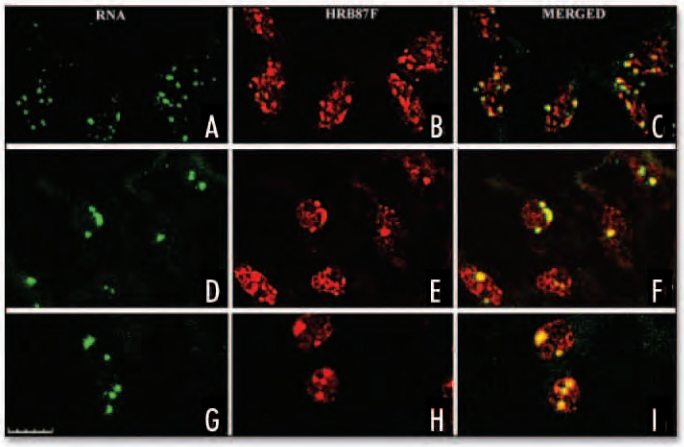

Figure 2. Confocal images of lightly squashed eye disc cells of wild type (A-C), *hsrω*^{05241}/*hsrω*^{05241} (D-F) and UAS-127Q/GMR-Gal4; *hsrω*^{05241}/*hsrω*^{05241} larvae (G-I) showing distribution of hsrω-n RNA (green A, D and G) and Hrb87F protein (red, B, E and H). The characteristic patterns of omega speckles in wild type or the *hsrω*^{05241} cells are not altered in cells expressing expanded poly-Q. C, F and I show merged images. Scale bar represents 5μm.

(*EP* transposon) at the same site as in *hsrω*^{05241} allele, from which it was generated by *P*-transposon swapping (Mallik M, Lakhotia SC, unpublished). Like the *hsrω*^{05241} flies, the *EP93D* homozygous males are also sterile and as in the *hsrω*^{05241} homozygous larval eye discs, the omega speckles in *EP93D* homozygous eye disc cells also appeared clustered (not shown).

Eyes of *hsrω*^{05241} homozygous flies appear normal externally. However, pseudopupil analysis of adult eyes revealed significant disorganization of rhabdomeres in majority of the ommatidial units (see Fig. 3A, B, D and E). Eyes of *EP93D* and *EP3037* homozygous flies also showed disorganization of ommatidial units; however, the disorganization in these cases is milder (not shown).

Df(3R)Hrb87F homozygotes do not produce any Hrb87F protein (hnRNPA1 homolog) but are viable without any apparent phenotype.[40] However, eyes of *Df(3R)Hrb87F* homozygous flies showed slight roughening and pseudopupil analysis revealed loss of photoreceptor neurons (Fig. 3C and F). *Df(3R)Hrb87F*/+ heterozygotes did not show any detectable abnormalities in eyes (not shown).

Gain of function alleles of *hsrω* enhance poly-Q-induced cellular toxicity. We used two different *Drosophila* models to examine poly-Q induced (*UAS 127Q* and *UAS Htt-ex1p-93Q*) toxicity developed by Kazemi-Esfarjani and Benzer[39] and Steffan et al.[27] respectively. Transgenic flies expressing a stretch of 20 glutamine residues under a UAS promoter served as controls in each case. These transgenes were expressed in differentiating eye-disc using the glass multimerized receptor (GMR) driver.[44,50] Effects of expression of the different poly-Q transgenes on eye Emorphogenesis in *hsrω* wild type or *hsrω* mutant backgrounds was examined.

As reported earlier,[27,39] expression of the unexpanded poly-Q repeat, 20Q, did not result in any eye phenotype, neither in *hsrω* wild type (Fig. 4A), nor in *hsrω* mutant background (data not shown). Gal4 driven expression of expanded poly-Q (*UAS-127Q* or *UAS-Htt-ex1p-93Q*) in developing eye discs resulted in loss of pigmentation and disruption of the regular ordered arrays of ommatidia in adult eyes without affecting the eye size (Figs. 4B and I). Presence of *hsrω* gain of function alleles in *127Q* or *Htt-ex1p-93Q* expressing eyes resulted in an enhancement in the severity of degeneration of eyes. Eyes of flies expressing 127Q in *hsrω*^{05241}/+ background were significantly reduced in size (Figs. 4C and J). In some cases, necrotic lesions resulting in parts or whole of the eye turning black were also seen. GMR-Gal4 driven expression of a single copy of *EP* alleles (*EP93D* or *EP3037*) of *hsrω* in combination with *127Q* resulted in a severe enhancement of poly-Q toxicity. Eyes of almost all *GMR-Gal4/UAS-127Q; EP93D/+* or *GMR-Gal4/UAS-127Q; EP3037/+* flies showed extensive black necrotic lesions (Fig. 4D and E). Such lesions were rarely seen in the absence of a mutant allele of the *hsrω* gene (Fig. 4B) and even when seen, the necrotic patches in *GMR-Gal4/UAS-127Q; hsrω*$^{+}$/*hsrω*$^{+}$ flies were much smaller.

A null allele of *Hrb87F* dominantly enhances poly-Q toxicity. The nuclear transcript of *hsrω*, hsrω-n organizes the heterogeneous RNA binding proteins (hnRNPs) not actively engaged in RNA processing, into nucleoplasmic omega speckles.[33] Since the hsrω-n transcripts associate with hnRNPs in omega speckles, the effect of *Df(3R)Hrb87F*, a null allele of the hnRNPA1-encoding gene *Hrb87F*[40] on poly-Q toxicity was examined. Eyes of *GMR-Gal4/UAS-127Q; Df(3R)Hrb87F/+* flies (with only one functional copy of *Hrb87F*) showed dark necrotic patches covering almost the entire eye (Fig. 4F), the likes of which were never seen in *GMR-Gal4/UAS-127Q; +/+* flies (Fig. 4B). Thus, the *Df(3R)Hrb87F* allele also acts as a dominant enhancer of poly-Q toxicity.

***l(3)pl10*^{R} is also a dominant enhancer of poly-Q-induced neurodegeneration.** *l(3)pl10*^{R}, a mutant allele of an as yet unidentified gene in *Drosophila*, located in the 9413-94F1 cytogenetic region,[43] affects the distribution of omega speckles and causes prolonged larval life and pupal lethality when homozygous. In all *l(3)pl10*^{R} homozygous late larval cells, the

omega transcripts localize to one or a few large clusters (Fig. 1G–L), mostly at the *hsrω* locus itself (larval brain cells from wild type and $l(3)pl10^{R}$ homozygous larvae are shown in the Figure1G–L since the eye discs in $l(3)pl10^{R}$ homozygous larvae are too small to be distinctly identified) and this is associated with prolonged larval life and pupal lethality. The hnRNPs also remain clustered with the hsrω-n RNA in these cells.[43] Since this recessive late larval/early pupal lethal mutation affected intranuclear distribution of omega speckles and hnRNPs, we examined if this mutant allele affected poly-Q induced neurodegeneration.

Eyes of flies expressing expanded Poly-Q and carrying a single copy of $l(3)pl10^{R}$ allele (*GMR-Gal4/UAS-127Q*; $l(3)pl10^{R}$/+) showed black necrotic patches that covered almost the entire eye and even eye size was reduced (Fig. 4K). The aggravation of neurodegeneration by single copy of the $l(3)pl10^{R}$ mutant allele shows the $l(3)pl10^{R}$ allele to be a dominant enhancer of Poly-Q toxicity.

Expression of UAS-127Q transgene leads to disorganized axonal connections from ommatidial units to brain. In order to examine the organization of individual ommatidial cells soon after -the onset of 127Q expression, eye discs of larvae expressing *GMR-Gal4* driven 127Q in $hsr\omega^{+}$/$hsr\omega^{+}$ or $hsr\omega^{05241}$/$hsr\omega^{05241}$ background were stained with the mAb22C10 antibody. As controls, eye discs from wild type, $hsr\omega^{05241}$ homozygotes and *UAS-20Q/GMR-Gal4* larvae were stained with mAb22C10 antibody. The ommatidia and axonal projections in $hsr\omega^{05241}$ homozygotes and 20Q expressing larvae were organized similar to those in wild type (Fig. 5A–C). *127Q* expression in $hsr\omega^{+}$ eye discs leads to disarrayed axonal projections in the optic stalk (Fig. 5D). Expression of *127Q* in $hsr\omega^{05241}$ mutant, on the other hand, caused a much more severe disorganization of the ommatidial units and their projections since the individual ommatidial cellular integrity was lost and axonal connections to the optic stalk appeared highly irregular (Fig. 5E).

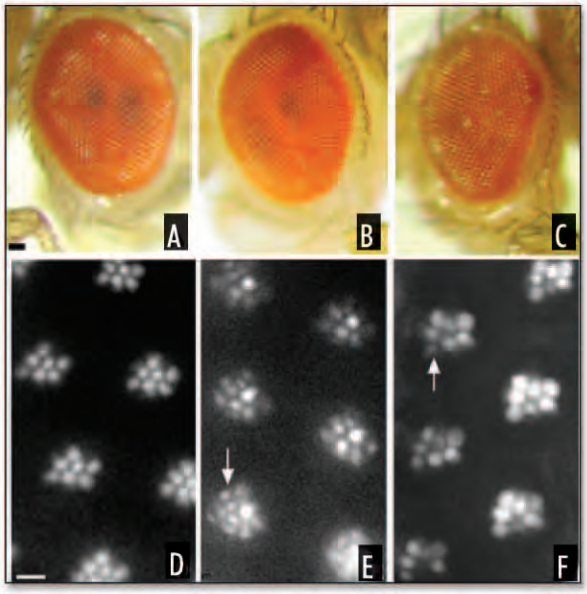

Figure 3. Eyes of wild type (A), $hsr\omega^{05241}$ (B) and *Df(3R)Hrb87F* (C) adult flies. Eye of $hsr\omega^{05241}$ (B) is almost comparable to that of wild type (A), whereas mild roughness is evident in eyes of *Df(3R)Hrb87F* flies (C). Pseudopupil images of ommatidia in wild type, $hsr\omega^{05241}$ and *Df(3R)-Hrb87F* (D–F). In wild type eyes (D), each ommatidium has seven visible photoreceptors, whereas $hsr\omega^{05241}$ mutant eye (E) shows extra/fragmented photoreceptors (arrow). *Hrb87F* (F) null mutant eye displays frequent loss of photoreceptor neurons (arrow) in ommatidial units. Scale bar represents 10 μm.

Expression of expanded poly-Q does not affect omega speckle organization, nor do the poly-Q inclusion bodies with omega speckles and hnRNP speckles. To examine whether expression of expanded poly-Q protein causes alterations in organization of omega speckles, fluorescence RNA-RNA in situ hybridization using hsrω-n riboprobe and immunostaining with the P11 antibody was carried out in lightly squashed preparations of eye discs from late 3rd instar larvae expressing *127Q* under *GMR-Gal4* driver in $hsr\omega^{+}$ or $hsr\omega^{05241}$ mutant background. It was seen that in either cases, poly-Q expression did not alter the organization of omega speckles. In the $hsr\omega^{+}$ and poly-Q expressing cells, the hsrω-n transcripts and the Hrb87F localized in 6–8 small omega speckles (not shown) while in the $hsr\omega^{05241}$ mutant background, the aggregates of omega speckles were similar to those in the $hsr\omega^{05241}$ mutant cells not expressing the expanded poly-Q protein (Fig. 2D–I).

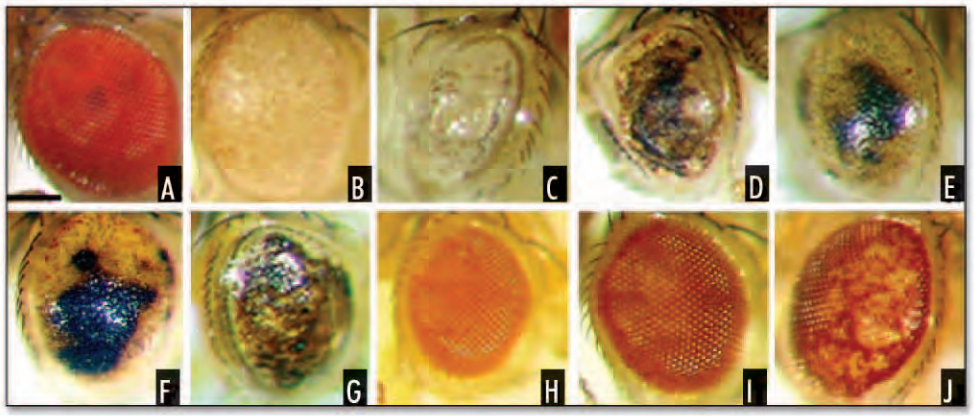

Figure 4. Dominant enhancement of poly-Q induced neurodegeneration by mutant alleles of non-coding *hsrω* and *Hrb87F* genes. Light microscopic images of eyes of *GMR-Gal4/UAS-20Q;* $hsr\omega^{+}$/$hsr\omega^{+}$ (A), *GMR-Gal4/UAS-127Q;* $hsr\omega^{+}$/$hsr\omega^{+}$ (B), *GMR-Gal4/UAS-127Q;* $hsr\omega^{05241}$/$hsr\omega^{+}$ (C), *GMR-Gal4/127Q; EP93D/*$hsr\omega^{+}$ (D), *GMR-Gal4/127Q; EP3037/*$hsr\omega^{+}$ (E), *GMR-Gal4/127Q; Df(3R)Hrb87F/*+ (F), *GMR-Gal4/127Q;* $l(3)pl10^{R}$/+ (G),*GMR-Gal4/UAS-htt exon-1- 20Q;* $hsr\omega^{+}$/$hsr\omega^{+}$ (H), *GMR-Gal4/UAS-htt-exon1-93Q;* $hsr\omega^{+}$/$hsr\omega^{+}$ (I) GMR-GAL4/UAS-htt exon-1-93Q; $hsr\omega^{05241}$/$hsr\omega^{05241}$ (J) flies. Scale bar represents 50 μm.

As in human cells,[51] the expanded poly-Q protein forms aggregates in the cytoplasm, peri-nuclear region or within the nucleus in eye cells of 127Q expressing larvae. Combined in situ hybridization with hsrω-n riboprobe and immunostaining with anti-HA antibody in eye disc cells expressing 127Q in $hsr\omega^{+}$ or $hsr\omega^{05241}$ mutant background revealed that the nuclear inclusion bodies did not colocalize with the hsrω-n RNA (Fig. 6A–C). In a few $hsr\omega^{+}$ cells, omega speckles and the nuclear poly-Q aggregates were seen to be adjacent but not overlapping. In eye disc cells of $hsr\omega^{05241}$ homozygous larvae expressing 127Q, the nuclear inclusion body/bodies and the aggregates of omega speckles also did not colocalize (data not shown).

Double immunostaining with anti-HA (to localize nuclear inclusions bodies formed by the 127Q protein) and the Hrb87F antibodies in eye disc cells of larvae expressing expanded poly-Q also showed that the poly-Q inclusion bodies and the speckled hnRNPs did not colocalize (Fig. 6D–F).

***$hsr\omega^{05241}$* mutation enhances the level of GMR-Gal4 driven 127Q protein in eye disc cells.** Eye discs of late third instar larvae expressing either 20Q (*GMR-Gal4/UAS-20Q;* $hsr\omega^{+}$/hsrω^{+}) or 127Q in $hsr\omega^{+}$ (*GMR-Gal4/UAS-127Q;* $hsr\omega^{+}$/$hsr\omega^{+}$) or $hsr\omega^{05241}$ mutant background (*GMR-Gal4/UAS-127Q;* $hsr\omega^{05241}$/$hsr\omega^{05241}$) were immunostained for poly-Q protein to see if the $hsr\omega^{05241}$ mutation affected the expression of expanded poly-Q. As reported earlier by Kazemi and Benzer,[39] there was no staining in eye

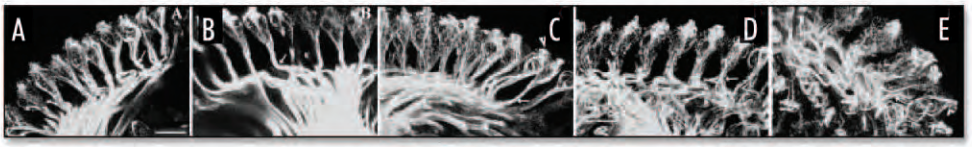

Figure 5. Confocal images of late third instar larval eye discs immuno-stained with the mAb22C10 antibody to show axonal projections from developing rhabdomeres in *hsrω*$^{+}$/*hsrω*$^{+}$ (A), *hsrω*^{05241}/*hsrω*^{05241} (B), *GMR-Gal4/UAS-20Q; hsrω*$^{+}$/*hsrω*$^{+}$ (C), *GMR-Gal4/UAS-127Q; hsrω*$^{+}$/*hsrω*$^{+}$ (D) and *GMR-Gal4/UAS-127Q; hsrω*^{05241}/*hsrω*^{05241} larval eye discs (E). Scale bar represents 10 µm.

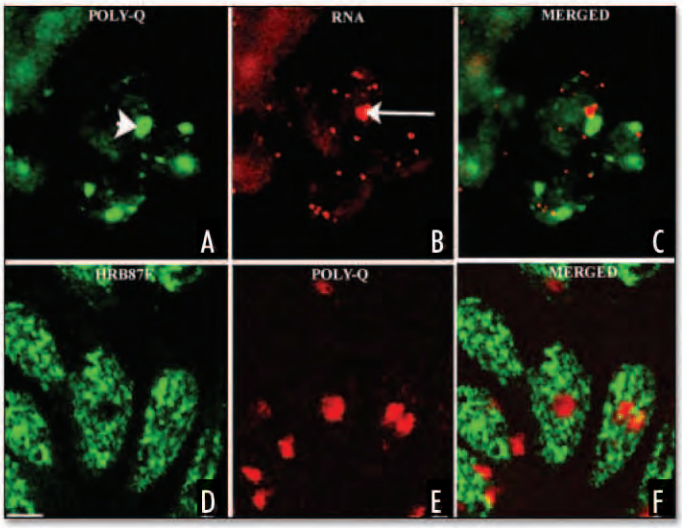

Figure 6. The nuclear speckles containing hsrω-n transcript and Hrb87F protein do not colocalize with poly-Q protein aggregates. (A–C) Fluorescence microscopic localization of inclusion bodies (Green, arrowhead in A) and hsrω-n RNA (red, B) in eye disc cells expressing 127Q (arrow in B points to an omega speckle adjacent to the inclusion body). (D–F) Confocal images show Hrb87F hnRNP speckles (green, D) and the nuclear inclusion bodies (red, E) of -poly-Q protein. Merged images are shown in (C and F). Scale bar represents 10 µm.

discs expressing 20Q, but in 127Q expressing hsrω^{+} larvae, immunostaining was seen as diffuse cytoplasmic staining as well as in form of cytoplasmic and nuclear aggregates (Fig. 7A–C). Interestingly, eye discs expressing 127Q protein in homozygous *hsrω*^{05241} mutant background showed an enhanced cytoplasmic expression of poly-Q protein in rows of cells closer to the advancing morphogenetic furrow (Fig. 7D–F). Furthermore, the cytoplasmic and nuclear poly-Q aggregates formed by the expanded poly-Q protein were also more frequent in the posterior differentiating cells of eye discs from *UAS-127Q/GMR-Gal4; hsrω*^{05241}/*hsrω*^{05241} larvae (Fig. 7D–F) compared to *UAS-127Q/GMR-Gal4*; *hsrω*$^{+}$/*hsrω*$^{+}$ larvae (Fig. 7A–C).

Expression of Hsp70 and Hsp60 in cells expressing expanded poly-Q. Presence of abnormally folded poly-Q proteins in the form of cytoplasmic and nuclear aggregates is known to induce a stress response.[21] Eye antennal discs of *GMR-Gal4/UAS-127Q*; *hsrω*$^{+}$/*hsrω*$^{+}$ and *GMR-Gal4/UAS-127Q; hsrω*^{05241}/*hsrω*^{05241} third instar larvae were immunostained with the 7Fb antibody that specifically identifies the heat-inducible Hsp70.[47] As reported earlier with the fly model of SCA-3,[21] the 127Q expressing cells in the present study also showed significant levels of Hsp70 in cytoplasm as well as in the nuclear inclusion bodies of 127Q expressing larvae (Fig. 8A–C). Interestingly, the levels of Hsp70 in cytoplasm as well as in the nucleus in eye discs expressing 127Q in *hsrω*^{05241} mutant background, was significantly increased compared to that in *hsrω*$^{+}$ background (Fig. 8D–F). In the absence of expanded poly-Q expression, eye discs of *hsrω*$^{+}$/*hsrω*$^{+}$ or *hsrω*^{05241}/*hsrω*^{05241} larvae grown at 24°C did not show any Hsp70 (not shown).

Expression of Hsp60, another major molecular chaperone, was also examined in eye discs of the above genotypes using an anti-Hsp60 antibody, SPA-805 (StressGen, Canada), which is known to recognize *Drosophila* Hsp60.[52] Immunostaining in wild type eye cells (not expressing 127Q) showed presence of constitutive cytoplasmic HSP60 protein (Fig. 8G–I). Hsp60 staining in 127Q expressing eye disc cells was generally comparable to that in wild type eye disc cells (Fig. 8J–L). Expression of 127Q in homozygous *hsrω*^{05241} background also did not significantly affect the Hsp60 level (Fig. 8M–O).

DISCUSSION

Fernandez-Funez et al.[16] reported that two *P*-insertion alleles of the non-coding *hsrω* gene, *hsrω*^{05241} and *P292*, enhance expanded SCA-1 induced toxicity in flies. To understand how a non-coding gene affects poly-Q toxicity, we examined the effects of a number of mutant alleles of the *hsrω* gene and of two other genes that seemingly interact with the hsrω transcripts on neurodegeneration caused by the expression of expanded poly-Q (127Q) or mutant huntingtin (*Htt-ex1p-93Q*) proteins.

Fernandez-Funez et al.[16] (also see Driscoll and Gerstbrein, ref. 19) described *hsrω*^{05241} as a recessive loss of function allele. However, earlier studies in our laboratory[41] and the present results show *hsrω*^{05241} to be a gain of function allele. The distribution of hsrω-n transcripts is altered in homozygous *hsrω*^{05241} eye disc cells, as instead of 6–8-fine omega speckles in wild type cells, these cells show 2–3 larger aggregates. Thus, we believe that the enhancing effect of *hsrω*^{05241} mutation on poly-Q-induced neurodegeneration in fly models is due to the altered expression, rather than absence of expression of *hsrω* gene transcripts. This is further supported by the enhancement observed with the GMR-Gal4 driven overexpression of the two *EP* alleles of *hsrω*.

Our study has also identified two new enhancers, viz., *Df(3R)-Hrb87F* and *l(3)pl10*^{R}, of poly-Q (127Q) induced toxicity. The Hrb87F protein is a homolog of the vertebrate heterogeneous nuclear RNA binding protein A1 (hnRNPA1).[53] Hrb87F and other hnRNPs are known to bind to the hsrω-n transcripts and form omega speckles.[32,33] It seems likely that loss-of-function alleles of other hnRNP-encoding genes would also act as enhancers of poly-Q neurotoxicity. Since the *l(3)pl10*^{R} mutation alters the distribution of omega speckles in nuclei, it appears that the *Hrb87F* and *l(3)pl10*^{R} gene mutations enhance poly-Q induced neurotoxicity via the noncoding nuclear transcripts of the *hsrω* gene.

It is interesting that the *hsrω*^{05241} mutation, and to a lesser extent the *EP93D* and *EP3037* alleles, by themselves cause some degeneration in adult eyes. The hsrω transcripts thus seem to be important for development and differentiation of eye cells. Eyes of *Hrb87F* null flies also showed disorganization of ommatidial units. Even though a complete absence of the Hrb87F hnRNP is well tolerated,[40] it is apparent from present observations that this has subtle effects on specific developmental pathways like ommatidial differentiation.

In keeping with the enhanced neurodegeneration in *GMR-GAL4* driven expanded poly-Q expressing flies due to the *hsrω*^{05241} mutant background, it was seen that the level of poly-Q protein, particularly the nuclear inclusion bodies, was also significantly enhanced in these eye discs. Although we did not directly examine the levels of poly-Q

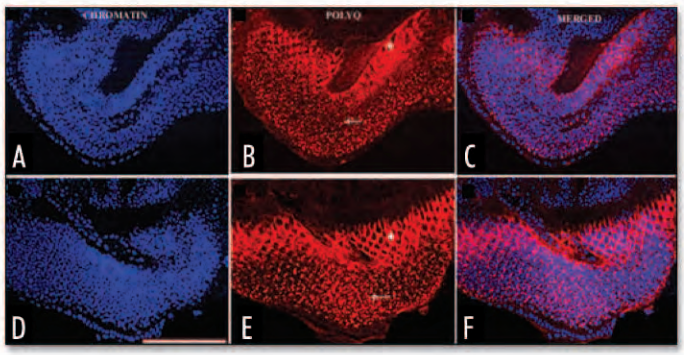

Figure 7. Confocal images of eye discs showing chromatin (blue, A and D) and poly-Q protein (red, B and E) of *GMR-Gal4/UAS-127Q; hsrω*$^{+}$*/hsrω*$^{+}$ (A–C) or *GMR-Gal4/UAS-127Q; hsrω*^{05241}*/hsrω*^{05241} (D-F) larvae. Arrowheads in (B and E) indicate rows of cells immediately behind the morphogenetic furrow while the arrows indicate rows of cells in more posterior regions where ommaidial units are differentiating. Scale bar represents 50 µm.

in the other mutant (e.g., *EP93D, EP3037, Df(3R)*Hrb87F or *l(3)pl10*^{R}) backgrounds, we presume that in these cases also the poly-Q levels are significantly higher than in corresponding wild type backgrounds. We do not know whether increased accumulation of the poly-Q in *hsrω*^{05241} mutant cells was due to its greater synthesis or reduced turnover of poly-Q proteins. Since the hsrω-n transcripts associate with a variety of RNA-binding proteins,[31] it is possible that excess of these transcripts (as in the various *hsrω* alleles used in this study) may sequester some of the transcription factor/s that normally repress the poly-Q transgene transcription or may sequester some protein/s involved in clearance of the poly-Q inclusion bodies. This needs further examination.

It is known from previous studies that the presence of expanded poly-Q proteins in cell induces the expression of heat shock proteins like Hsp70 and other stress proteins.[22,34,54-56] In the present study also it was seen that GMR-Gal4 driven expression of 127Q in eye disc cells was accompanied by the appearance of stress inducible Hsp70, which is specifically recognized by the 7Fb antibody.[47] In this context, it is interesting to note that the organization of omega speckles remained unaltered in 127Q expressing *hsrω*$^{+}$ cells. Heat shock and other kinds of stress are known to bring about specific changes in the organization of omega speckles.[32,33] Apparently, poly-Q induced stress is not perceived by the cell in the same manner as heat stress. The *hsrω* transcripts have not been reported so far to chaperone the misfolded proteins and therefore, presence of poly-Q inclusion bodies may not be expected to stimulate change in *hsrω* expression. It may be further noted that the clustering of omega speckles in *hsrω*^{05241} mutant cells, which is reminiscent of heat shock induced clustering in wild type cells[32,33] is not accompanied by the appearance of Hsp70 in these cells (not shown). Thus, although the *hsrω* gene is a member of the heat shock gene family, clustering of its transcripts or its elevated level of activity need not be associated with induction of the stress-inducible Hsp70 or other HSPs.

It is intriguing that expression of chaperone like Hsp70 in eye discs was significantly enhanced when 127Q was expressed in *hsrω*^{05241} mutant background. Several earlier studies have shown that overexpression of Hsp70 ameliorates poly-Q induced neurodegeneration in fly models as well as in mammalian systems. However, inspite of significant increase in the levels of Hsp70 in 127Q expressing *hsrω*^{05241} mutant eye disc cells, neurodegeneration was

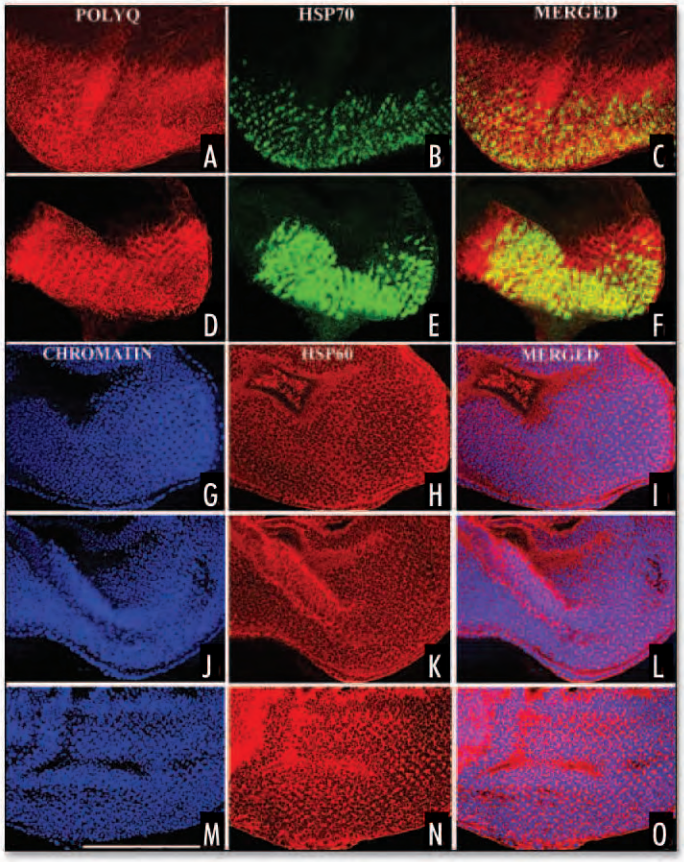

Figure 8. (A–F) Eye discs immunostained for poly-Q (red, A and D) and Hsp70 (green, B and E) from *GMR-Gal4/UAS-127Q; hsrω*$^{+}$*/hsrω*$^{+}$ (A–C) or *GMR-Gal4/UAS-127Q; hsrω*^{05241}*/hsrω*^{05241} (D–F) larvae. (G–O) Eye discs immunostained for Hsp60 (red, H, K and N) from wild type (G–I), *GMR-Gal4/UAS-127Q; hsrω*$^{+}$*/hsrω*$^{+}$ (J–L) or GMR-Gal4/UAS-127Q; *hsrω*^{05241}*/hsrω*^{05241} (M–O) larvae. (G, J and M) show DAPI-stained nuclei while (I, L and O) show merged images. Scale bar represents 50 µm.

enhanced rather than reduced as may be expected from earlier reports of protective effects of the molecular chaperones. It is quite possible that the extent of neurotoxicity following poly-Q expression in *hsrω*^{05241} and similar mutant cells is too high to be efficiently repaired by even the higher levels of Hsp70 proteins.

The axonal projections of differentiating photoreceptor neurons were disorganized in larvae expressing expanded poly-Q protein and this was more pronounced in larvae expressing 127Q in the *hsrω*^{05241} mutant background, which correlates well with the greater degeneration in adult eyes. This is in contrast to the Warrick et al.'s[15] report that disorganization of axonal connections in eye discs expressing truncated form of MJD protein with an expanded poly-Q repeat (MJDtr with 78Q expansion) in a fly model of SCA-3 was not seen till the mid-pupal stage of development. The disorganization of axonal connections is indicative of an early neuropathological event following the onset of expression of 127Q in neuronal cells of the eye. This may be related to the difference in the length of poly-Q stretch (78Q in Warrick et al.'s study versus 127Q in our study). However, our finding of axonal disorganization in eye cells is of relevance in context of Huntington`s disease since in a HD mice model (R6/2 mice expressing 115-150 CAGs under HD gene promoter) and in HD patient brains, axons of striatal projection neurons are seen to degenerate.[57] Thus, an early neuropathological event that is

intrinsically associated with human diseases is also seen in flies expressing expanded poly-Q (127Q).

Our results seem to rule out a direct interaction of the hsrω-n transcripts or hnRNPs with poly-Q nuclear inclusion bodies since none of them colocalize with the poly-Q nuclear inclusion bodies. This is in contrast to earlier observations where modifiers like transcription factors, e.g., TATA binding protein (TBP), CREB binding protein (CBP), p53, eye-absent nuclear receptor corepressor (N-CoR), mSin3A, TAFp130, Sp1,[11,58-60] molecular chaperones, like Hsp70, and cochaperone protein like dHDJ1/Hsp40[21,22] colocalized with nuclear inclusion bodies and thereby modulated the poly-Q toxicity.

It appears that the enhancement of expanded poly-Q neurotoxicity by either the recessive gain of function allele of *hsrω* or by loss function alleles like *Df(3R)Hrb87F* and *l(3)pl10*^{R} is due to altered levels of hnRNPs and other related RNA-binding proteins in these mutant cells. The nucleoplasmic hnRNPs and other related RNPs which at any given time are not productively engaged in the processing of hnRNAs are sequestered for storage by the hsrω-n RNA.[32,33] It is likely that the clustering of the hsrω–n RNA in *hsrω*^{05241} cells or in *l(3)pl10*^{R} cells sequesters or soaks away more hnRNPs and related proteins from active nuclear compartment, resulting in depletion of available hnRNPs for nuclear processing. The *Df(3R)Hrb87F* allele would also affect the level of hnRNPA1 in cells. This depletion of functional hnRNPs and other proteins from active nuclear compartments may compromise the normal functions of hnRNPs in RNA processing and/or transport. This may add further to the cellular dysfunctions resulting from the toxic effects of poly-Q expansion and thus lead to enhancement of the disease phenotype. While the specific mechanism of the effect of *hsrω* gene mutations needs further analysis, it is significant that this noncoding gene has a distinct phenotypic effect.

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