

Improved Activities of CREB Binding Protein, Heterogeneous Nuclear Ribonucleoproteins and Proteasome Following Downregulation of Noncoding *hsw* Transcripts Help Suppress Poly(Q) Pathogenesis in Fly Models

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ABSTRACT

Following earlier reports on modulation of poly(Q) toxicity in *Drosophila* by the developmentally active and stress-inducible noncoding *hsw* gene, we investigated possible mediators of this modulation. RNAi-mediated downregulation of the large nuclear *hsw-n* transcript, which organizes the nucleoplasmic omega speckles, suppressed the enhancement of poly(Q) toxicity brought about by reduced availability of the heterogeneous nuclear ribonucleoprotein (hnRNP) Hrb87F and of the transcriptional regulator, cAMP response element binding (CREB) binding protein (CBP). Levels of CBP RNA and protein were reciprocally affected by *hsw* transcript levels in eye disc cells. Our data suggest that CBP and hnRNPs like Hrb57A and Hrb87F physically interact with each other. In addition, downregulation of *hsw* transcripts partially rescued eye damage following compromised proteasome activity, while overexpression of *hsw* and/or poly(Q) proteins disrupted the proteasomal activity. Rescue of poly(Q) toxicity by *hsw*-RNAi required normal proteasomal function. We suggest that *hsw*-RNAi suppresses poly(Q) toxicity by elevating cellular levels of CBP, by enhancing proteasome-mediated clearance of the pathogenic poly(Q) aggregates, and by inhibiting induced apoptosis. The direct and indirect interactions of the *hsw* transcripts with a variety of regulatory proteins like hnRNPs, CBP, proteasome, *Drosophila* inhibitor of apoptosis protein 1 (DIAP1), etc., reinforce the view that the noncoding *hsw* RNA functions as a “hub” in cellular networks to maintain homeostasis by coordinating the functional availability of crucial cellular regulatory proteins.

A number of human neurological disorders like Huntington's disease (HD), spinal and bulbar muscular atrophy (SBMA), dentatorubropallidolusian atrophy (DRPLA), and several spinocerebellar ataxias (SCAs) are caused by expansion of polyglutamine stretches beyond the characteristic threshold range in different proteins (GUSELLA and MACDONALD 2000; EVERETT and WOOD 2004; GATCHEL and ZOGHBI 2005). The expanded polyglutamine domain confers novel toxicity to these proteins in neuronal cells such that these diseases are characterized by selective vulnerability of neurons despite a widespread expression of the diseased protein in brain and other tissues. A hallmark feature of most of these diseases is the formation of insoluble intracellular aggregates or inclusion bodies (IBs) in the affected human neurons as well as in cell culture and animal models due to abnormal folding of the mutant poly(Q) proteins (DAVIES *et al.* 1997; KLEMENT *et al.* 1998; CHEN *et al.* 2001, 2002). It is debated if the IBs are causal to or a consequence of disease pathogenesis or represent a

cellular protective mechanism (DiFIGLIA *et al.* 1997; SAUDOU *et al.* 1998; WARRICK *et al.* 1998; YANG *et al.* 2002; YOO *et al.* 2003; ARRASATE *et al.* 2004). Nevertheless, our earlier results (MALLIK and LAKHOTIA 2009a) utilizing several fly models of poly(Q) disorders suggested a strong correlation between poly(Q) aggregation and neurodegeneration since an almost complete or significant inhibition of IB formation was seen following downregulation of the *hsw-n* transcripts, which in turn was associated with reduction in Hsp70 levels and suppression of poly(Q)-induced eye degeneration.

Proteins with expanded poly(Q) tracts interact with and sequester many transcriptional regulators containing short poly(Q) repeats or proline-rich regions like cAMP response element binding (CREB) binding protein (CBP), TBP-associated factor 4 (TAFII130), Sp1 transcription factor (SP1), p53, etc., in the IBs and thus compromise their normal regulatory functions (NUCIFORA *et al.* 2001; DUNAH *et al.* 2002; LI *et al.* 2002; SCHAFFAR *et al.* 2004; HELMLINGER *et al.* 2006). IBs also sequester molecular chaperones and components of the ubiquitin proteasome pathway (UPP), suggesting that misfolding, impaired degradation, and abnormal aggregation of proteins also contribute to the pathogenesis (WARRICK

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et al. 1999; CHAN and BONINI 2000; CUMMINGS *et al.* 2001).

The *hsw* gene produces independently regulated multiple noncoding transcripts (LAKHOTIA 2003). Their overexpression aggravates (SENGUPTA and LAKHOTIA 2006) and RNAi-based downregulation ameliorates (MALLIK and LAKHOTIA 2009a) the poly(Q) toxicity. The large nucleus limited *hsw-n* transcript organizes the nucleoplasmic omega speckles and is known to associate with a variety of proteins (LAKHOTIA *et al.* 1999; PRASANTH *et al.* 2000; JOLLY and LAKHOTIA 2006). We recently showed (MALLIK and LAKHOTIA 2009a) that *GMR-GAL4*-driven coexpression of the *hsw*-RNAi transgene and the pathogenic poly(Q) proteins does not affect the poly(Q) transgene transcription but prevents accumulation of the poly(Q) aggregates and consequently the associated pathogenesis (MALLIK and LAKHOTIA 2009a). Thus the *hsw*-RNAi-dependent recovery from poly(Q) pathogenesis must be effective after the mutant poly(Q) proteins are synthesized in the target cells. It was also seen that *hsw*-RNAi does not suppress the pathological consequences of overexpression of wild-type or mutant tau protein and thus appears to be specific for poly(Q)-induced neurodegeneration (MALLIK and LAKHOTIA 2009a).

With a view to understand how depletion of these transcripts suppresses poly(Q) aggregation and thus the neurotoxicity, we investigated interaction(s) of the *hsw* transcripts with heterogeneous nuclear ribonucleoproteins (hnRNPs), CBP, and components of the UPP, which are some of the reported modifiers of poly(Q) pathogenesis (BONINI and FORTINI 2003; NELSON *et al.* 2005; SHAO and DIAMOND 2007; BRANCO *et al.* 2008). We show for the first time that in developing eye disc cells *hsw* transcript levels reciprocally affect cellular levels of CBP. Our results also suggest that CBP and hnRNPs like Hrb57A and Hrb87F physically interact in eye disc cells. Various hnRNPs and some other RNA-binding/processing proteins are known to be sequestered by the *hsw-n* transcripts as part of cellular regulation (LAKHOTIA *et al.* 1999; PRASANTH *et al.* 2000; JOLLY and LAKHOTIA 2006) and downregulation of *hsw* transcripts abolishes the omega speckles (MALLIK and LAKHOTIA 2009a). Therefore, we believe that *hsw*-RNAi augments the availability of hnRNPs, etc., in functional compartments. The enhanced availability of various hnRNPs and CBP following depletion of the *hsw* transcripts thus seems to contribute to suppression of poly(Q) toxicity by not only reducing the formation of IBs but also compensating their functional depletion in poly(Q)-expressing cells. The present results also confirm our earlier suggestion (MALLIK and LAKHOTIA 2009a) that the residual toxicity of the poly(Q) proteins is taken care of by the proteasomal clearance machinery since the almost complete suppression of the poly(Q) toxicity by *hsw*-RNAi required functional proteasomal activity in eye disc cells.

We earlier reported (MALLIK and LAKHOTIA 2009b) that RNAi-mediated depletion of the *hsw* transcripts also suppresses induced apoptosis in *Drosophila* through stabilization of *Drosophila* inhibitor of apoptosis protein 1 (DIAP1) and through suppression of c-Jun N-terminal kinase (JNK) signaling. Since poly(Q)-induced neurodegeneration also involves enhanced apoptosis and hyperactivation of JNK signaling (EVERT *et al.* 2000; BERKE *et al.* 2004; MORFINI *et al.* 2006; SCAPPINI *et al.* 2007), these appear to be additional components through which depletion of *hsw* transcripts can suppress the neurotoxicity. It is remarkable that the noncoding *hsw* transcripts affect several components implicated in the poly(Q) pathogenesis.

MATERIALS AND METHODS

Fly stocks: All flies were raised on standard agar–cornmeal medium at $24^{\circ} \pm 1^{\circ}$, and fly crosses were carried out at the same temperature unless otherwise stated. Oregon R⁺ was used as wild type. The *UAS-127Q*, *UAS-MJDr-Q78(S)*, *UAS-httex1p Q93*, *UAS-hsw-RNAi²*, *UAS-hsw-RNAi³*, *EP93D*, and *EP3037* transgenic lines have been described previously (MALLIK and LAKHOTIA 2009a). The *GMR-GAL4* (HAY *et al.* 1994), *P{Act5C-GAL4}25F01/CyO* (EKENGREN *et al.* 2001), and *UAS-Pros26'.B; UAS-Prosβ 2'* (BELOTE and FORTIER 2002) stocks were obtained from the Bloomington Stock Center. *UAS-CBP FL-AD* (acetylase dead version, F2161A), *UAS-CBP RNAi*, *UAS-CBPΔNzk*, *UAS-CBPΔQ*, and *UAS-CBPΔBHQ*, all homozygous viable stocks, were provided by J. P. Kumar (KUMAR *et al.* 2004; ANDERSON *et al.* 2005). The *UAS-DTS5-11* (SCHWEISGUTH 1994), *P{Act5C-GAL4}25F01/CyO* (EKENGREN *et al.* 2001), and *UAS-Pros26'.B; UAS-Prosβ 2'* (BELOTE and FORTIER 2002) stocks were obtained from the Bloomington Stock Center. *UAS-CBP FL-AD* (acetylase dead version, F2161A), *UAS-CBP RNAi*, *UAS-CBPΔNzk*, *UAS-CBPΔQ*, and *UAS-CBPΔBHQ*, all homozygous viable stocks, were provided by J. P. Kumar (KUMAR *et al.* 2004; ANDERSON *et al.* 2005). The *UAS-DTS5-11* (SCHWEISGUTH 1994) transgenic line, obtained from N. M. Bonini, produces a GAL4-inducible dominant-negative form of the β-subunit of proteasome. *UAS-Ub^{G76V}-GFP* (DANTUMA *et al.* 2000) was obtained from J. Paul Taylor and was used as a GFP reporter for proteasome function. The *P{w⁺ tsr⁺}/P{w⁺ tsr⁺}*, *ry Df(3R)Hrb87F/ry Df(3R)Hrb87F* (ZU *et al.* 1996) stock was provided by S. Haynes. The *Df(3R)Hrb87F* deletion removes the Hrb87F coding region together with ~0.6 kb of the flanking testis-specific *Tsr* gene, resulting in recessive male sterility of *Df(3R)Hrb87F* homozygotes, although the *Df(3R)Hrb87F/+* heterozygotes are normally fertile; to compensate for this, the stock carries a transgenic copy of *Tsr* on chromosome 2 (ZU *et al.* 1996).

Appropriate crosses were performed following standard protocols to obtain progenies of desired genotypes.

Examination of eye structure: For recording images of external morphology of adult eyes, flies of the desired genotype were etherized and their eyes photographed using a Sony Digital Camera (DSC-75) attached to a Zeiss Stemi SV6 stereobinocular microscope. The surface architecture of adult eyes was examined by the nail polish imprint method (ARYA and LAKHOTIA 2006).

The arrangement of photoreceptor rhabdomeres in adult eyes was examined by pseudopupil analysis using a 60× planapo oil immersion objective of a Nikon E800 microscope (SENGUPTA and LAKHOTIA 2006).

Pupal lethality assay: The numbers of larvae of different genotypes that pupated in a given cross were counted and the fate of these pupae was monitored. Numbers of pupae that died before or after differentiation and of flies that emerged were counted separately.

RNA:RNA *in situ* hybridization: A 445-bp PCR-amplified fragment corresponding to the *Drosophila nevine* (CBP) transcript

was cloned into the pGEM^R-T Easy (Promega, Madison, WI) vector in appropriate orientation for subsequent manipulations. A digoxigenin-labeled CBP-specific antisense riboprobe was generated from this clone and used for hybridization to cellular RNA. RISH was carried out essentially as described earlier (PRASANTH *et al.* 2000).

Whole organ immunostaining and confocal microscopy:

For antibody staining, eye discs from wandering third instar larvae of the desired genotypes were dissected, fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min, and processed for immunofluorescence staining as described earlier (PRASANTH *et al.* 2000). The following primary antibodies were used: (1) 1:30 dilution of a rabbit polyclonal antibody to hemagglutinin (HA) (sc-805, Santa Cruz) for HA-tagged 127Q and MJDtr-Q78(S) polyglutamine proteins, (2) 1:50 dilution of a polyclonal goat huntingtin antibody (sc-8767, Santa Cruz) for the human huntingtin protein, (3) 1:10 dilution of the P11 mouse monoclonal antibody (SAUMWEBER *et al.* 1980) for Hrb87F hnRNP, (4) 1:10 dilution of the Q18 mouse monoclonal antibody (SAUMWEBER *et al.* 1980) for Hrb57A hnRNP, and (5) 1:800 dilution of an affinity-purified guinea-pig polyclonal CBP antibody (LILJA *et al.* 2003).

Appropriate secondary antibodies conjugated either with Cy3 (1:200, Sigma-Aldrich, India) or Alexa Fluor 488 (1:200; Molecular Probes, Eugene, OR) were used to detect the given primary antibody. Chromatin was counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (1 µg/ml). Confocal imaging was carried out on a Zeiss LSM 510 Meta laser scanning confocal microscope at appropriate settings, using a plan-apo 63× oil immersion objective.

Reverse transcription-PCR: Total RNA was purified from eye discs of third instar larvae or heads of adult flies of the desired genotype, using TRIzol reagent following the manufacturer's recommended protocol (Sigma-Aldrich, India). cDNA was synthesized for semiquantitative PCR as previously described (MALLIK and LAKHOTIA 2009a). One-tenth volume of the reaction mixture was subjected to PCR using the following primers: *hsrw*-n forward 5'-GGCAGACATACGTACAC GTGGCAGCAT-3', *hsrw*-n reverse 5'-TTGCGCTCACAGGAGTCAA-3'; *CBP*-forward 5'-GGCCGATCACTTAGACGAAC-3', *CBP*-reverse 5'-CTGGAGTAGTGTGCTGAGTT-3'; *127Q*-forward 5'-CAGCGTCTGATAAGTGAATTCG-3', *127Q*-reverse 5'-TACGTAGACTAGTCTGTTGCTG-3'; and *GFP*-forward 5'-CATGAAGCAGCAGCACTT-3', *GFP*-reverse 5'-TGTTCTGCTGGT AGTGGT-3'. Amplicons obtained following reverse transcription (RT)-PCR for GPDH [primer pair described earlier (MALLIK and LAKHOTIA 2009a)] were used as loading controls. The PCR products were electrophoresed on a 2% agarose gel with appropriate molecular weight markers. Gel pictures were recorded using the G:BOX bioimaging system (Syngene).

Western blotting: The Hrb87F protein levels in +/+ and *w; P{w⁺ tsr⁺}/+*; *Df(3R)Hrb87F/+* were compared by homogenizing 30 pairs of eye imaginal discs from third instar larvae of each genotype in sample buffer as described earlier (PRASANTH *et al.* 2000). The lysates were electrophoretically separated on a 12% SDS-polyacrylamide gel (with 5% stacking gel) and transferred to an immobilon-P membrane (Millipore, Bedford, MA). The primary antibodies used were 1:200 dilution of mouse β-tubulin antibody (E7, Developmental Studies Hybridoma Bank) and 1:100 dilution of Hrb87F antibody. Immunoblots were developed with 1:1000 dilution of goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody (Bangalore Genei, India) using the SuperSignal West Pico chemiluminescence reagent (Pierce, Rockford, IL). Blots were reprobed, as required, after stripping the earlier bound antibodies as described previously (MALLIK and LAKHOTIA 2009a).

To compare CBP levels in different genetic backgrounds, extracts from 50 pairs of eye-antennal discs of the following

genotypes were lysed in sample buffer and homogenized as above: (1) *w; GMR-GAL4/GMR-GAL4; +/+*; (2) *w; GMR-GAL4/GMR-GAL4; hsrw-RNAi³/hsrw-RNAi³*; (3) *w; GMR-GAL4/GMR-GAL4; EP93D/EP93D*; (4) *w; GMR-GAL4/UAS-MJDtr-Q78(S)*; and (5) *w; GMR-GAL4/UAS-MJDtr-Q78(S); hsrw-RNAi³/hsrw-RNAi³* (PRASANTH *et al.* 2000). The lysates were slot blotted on PVDF membrane (Millipore), using a Millipore-S slot blot apparatus. They were probed with CBP (1:8000) and β-tubulin (1:200) antibodies. Blots were developed using HRP-conjugated secondary antibodies [1:5000 dilution of goat anti-guinea pig, (KPL) or 1:1000 dilution of goat anti-mouse (Bangalore Genei)] and SuperSignal West Pico chemiluminescence reagent (Pierce).

For relative quantitation of the levels of the desired protein in Western blots, the ratio of band densities of the target and the reference (β-tubulin) proteins for each sample on the blot was calculated. Means (±SD) of ratios from three independent blots for experimental and control samples were compared.

Immunoprecipitation: CBP complexes were immunoprecipitated with the affinity-purified CBP antibody from extracts of 50 pairs of wild-type and *GMR-GAL4*-driven *hsrw-RNAi²*-expressing third instar eye imaginal discs. Approximately 1 µg of affinity-purified CBP antibody was used for each immunoprecipitation as described earlier (PRASANTH *et al.* 2000). Recovered proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and probed sequentially with the Hrb87F and Hrb57A antibodies at a dilution of 1:100 each.

All images were assembled using Adobe Photoshop 7.0.

RESULTS

As reported earlier (MALLIK and LAKHOTIA 2009a,b), we established several independent *hsrw-RNAi* transgenic lines. The *hsrw-RNAi³* line, in which the *UAS-hsrw-RNAi* transgene is inserted on chromosome 3, was used for most of the experiments reported in this study. Another line, *hsrw-RNAi²*, carrying the *UAS-hsrw-RNAi* transgene on chromosome 2 and being more effective in reducing *hsrw* transcript levels than the *hsrw-RNAi³* line (MALLIK and LAKHOTIA 2009b), was used in certain cases as noted later. The *hsrw-RNAi³* line is referred to in the following as *hsrw-RNAi*. For ectopic overexpression of the *hsrw* gene, two EP lines, *EP93D* and *EP3037*, with the EP transposon (RORTH 1996) inserted at -130- and -144-bp positions, respectively, in the *hsrw* gene promoter (MALLIK and LAKHOTIA 2009a), were used. Targeted expression of the desired UAS transgenes in developing eye discs/eyes was achieved by using the *GMR-GAL4* driver (HAY *et al.* 1994). In certain cases, the *Act5C-GAL4* driver (EKENGREN *et al.* 2001) was used for ubiquitous expression of the desired UAS transgene(s).

Enhancement of poly(Q) toxicity by reduced levels of Hrb87F is ameliorated by *hsrw-RNAi*: The Hrb87F protein, a Drosophila homolog of the mammalian hnRNP A2/B1 (HAYNES *et al.* 1991), is associated with the omega speckles (PRASANTH *et al.* 2000). As already reported (SENGUPTA and LAKHOTIA 2006), a null allele, *Df(3R)Hrb87F* (ZU *et al.* 1996), dominantly enhances the 127Q-induced toxicity leading to formation of extensive black lesions on the eye surface (compare Figure 1B and 1G, *N* = 392, with 1A and 1F, *N* = 608). In the present study, we found that it also results in dominant

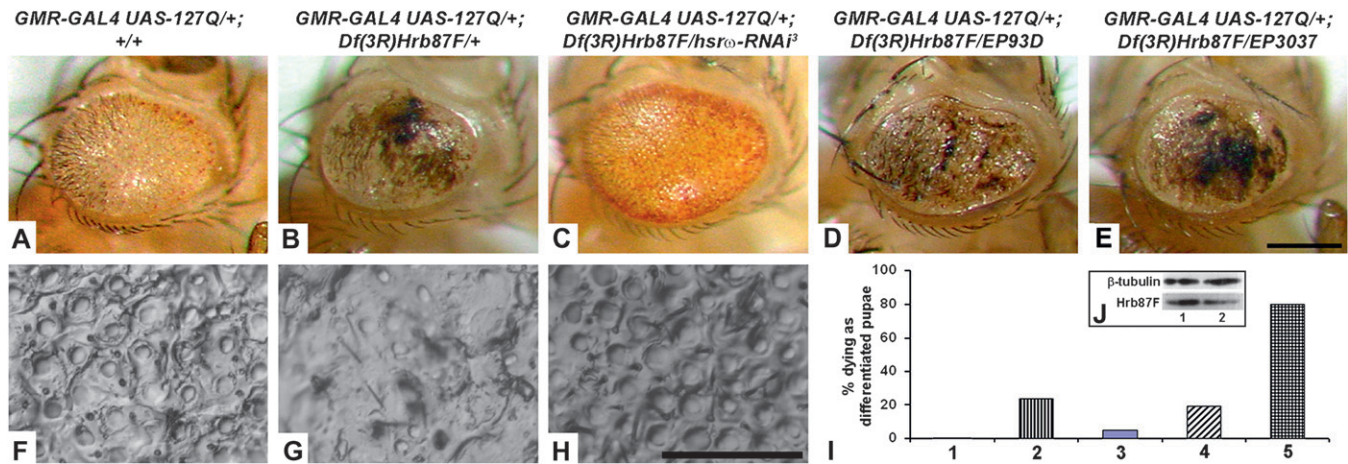


FIGURE 1.—Enhancement of 127Q-mediated eye degeneration by depletion of the Hrb87F hnRNP is modulated by levels of *hsrω-n* RNA. (A–E) Photomicrographs of eyes of adults whose genotypes are indicated at the top; (F–H) nail polish imprints of eyes of genotypes as in A–C, respectively. Bars for A–E in E and F–H in H: 20 μ m. (I) Histograms of frequencies (%) of different genotypes that die as differentiated pupae: bar 1, *GMR-GAL4 UAS-127Q/+; +/+* ($N = 608$); bar 2, *GMR-GAL4 UAS-127Q/+; Df(3R)Hrb87F/+* ($N = 514$); bar 3, *GMR-GAL4 UAS-127Q/+; Df(3R)Hrb87F/hsrω-RNAi²* ($N = 343$); bar 4, *GMR-GAL4 UAS-127Q/+; Df(3R)Hrb87F/EP93D* ($N = 466$); bar 5, *GMR-GAL4 UAS-127Q/+; Df(3R)Hrb87F/EP3037* ($N = 166$); the frequencies of dying pupae in genotypes 2, 3, 4 and 5 are significantly different ($P < 0.05$, χ^2 -test) from that in 1. (J) A Western blot to show the reduced level of Hrb87F protein (bottom panel) in *Df(3R)Hrb87F/+* (lane 2) larval eye discs compared to that in wild type (lane 1). The top panel shows β -tubulin levels as a loading control.

lethality since 24% of progeny carrying one copy of this deletion in a *GMR-GAL4:127Q* background died as differentiated pupae (Figure 1I, bar 2; $N = 514$). Downregulation of *hsrω-n* RNA level with one copy of *hsrω-RNAi* not only improved the external eye morphology relative to those expressing *GMR-GAL4*-driven 127Q in a *Df(3R)Hrb87F/+* background (Figure 1, C and H, $N = 326$) but also substantially reduced the mortality such that only 4.9% differentiated pupae died (Figure 1I, bar 3; $N = 343$). That the *Df(3R)Hrb87F/+* heterozygotes have reduced levels of the Hrb87F protein was confirmed by semiquantitative Western blotting. Compared to wild-type larval eye discs, the Hrb87F level in *Df(3R)Hrb87F/+* heterozygotes was found to be $\sim 20\%$ less [mean of ratio of Hrb87F level in *Df(3R)Hrb87F/+* and wild-type eye discs = 0.797 ± 0.07 ; $N = 3$, Figure 1J].

An increase in the level of *hsrω* transcripts by co-expression of *EP93D* or *EP3037* in the *Df(3R)Hrb87F/+* and 127Q background significantly enhanced the appearance of black lesions on the eye surface (Figure 1, D, $N = 378$, and E, $N = 33$, respectively) and resulted in death of a greater number (80.1%, $N = 166$) of *w/w; GMR-GAL4 UAS-127Q/+; Df(3R)Hrb87F/EP3037* (Figure 1I, bar 5) pupae. Intriguingly, however, expression of the *EP93D* allele with 127Q in the Hrb87F depleted background ($N = 466$) did not increase the frequency of dying pupae (Figure 1I, compare bar 4 with bar 2). The reason for this difference in the survival of *w/w; GMR-GAL4 UAS-127Q/+; Df(3R)Hrb87F/EP3037* and *w/w; GMR-GAL4 UAS-127Q/+; Df(3R)Hrb87F/EP93D* genotypes is not clear but it may be related to different locations of the two *EP* insertions in the *hsrω* gene promoter

that may affect its developmental expression in some critical cells/tissues. This needs further examination.

Genetic interactions between CBP, *hsrω* transcripts, and poly(Q) proteins: As noted in the Introduction, the sequestration of CBP into poly(Q) aggregates, and the consequent reduction in soluble CBP levels, results in transcriptional dysregulation as part of the poly(Q) pathogenesis (NUCIFORA *et al.* 2001; JIANG *et al.* 2003; TAYLOR *et al.* 2003). It is also reported that overexpression of CBP reduces the poly(Q) damage (TAYLOR *et al.* 2003; ROUAUX *et al.* 2004). With a view to examine if *hsrω-RNAi*-mediated suppression of the poly(Q) damage involved CBP, we examined genetic interactions between CBP, *hsrω* transcripts, and poly(Q) proteins using the *UAS-CBP FL-AD* transgene, which carries a dominant-negative mutation in the acetyltransferase domain of CBP (LUDLAM *et al.* 2002), or the *UAS-CBP RNAi* transgene (KUMAR *et al.* 2004), using the eye-specific *GMR-GAL4* driver.

Eyes of *GMR-GAL4/UAS-CBP FL-AD* flies ($N = 589$) were moderately rough with significant reduction in the number of interommatidial bristles (Figure 2, A and D). Coexpression of one copy of the *hsrω-RNAi* transgene in this background ($N = 523$) resulted in substantial improvement in eye morphology and ommatidial arrays (Figure 2, B and E). On the other hand, enhancement of *hsrω* transcript levels through expression of *EP3037* (Figure 2, C and F) or *EP93D* (not shown) exaggerated the eye damage ($N = 499$ and $N = 466$, respectively).

Expression of the *CBP FL-AD* transgene in 127Q or *MJDtr-Q78(S)* background resulted in varying degrees of lethality at the pupae stage (see below). The surviving

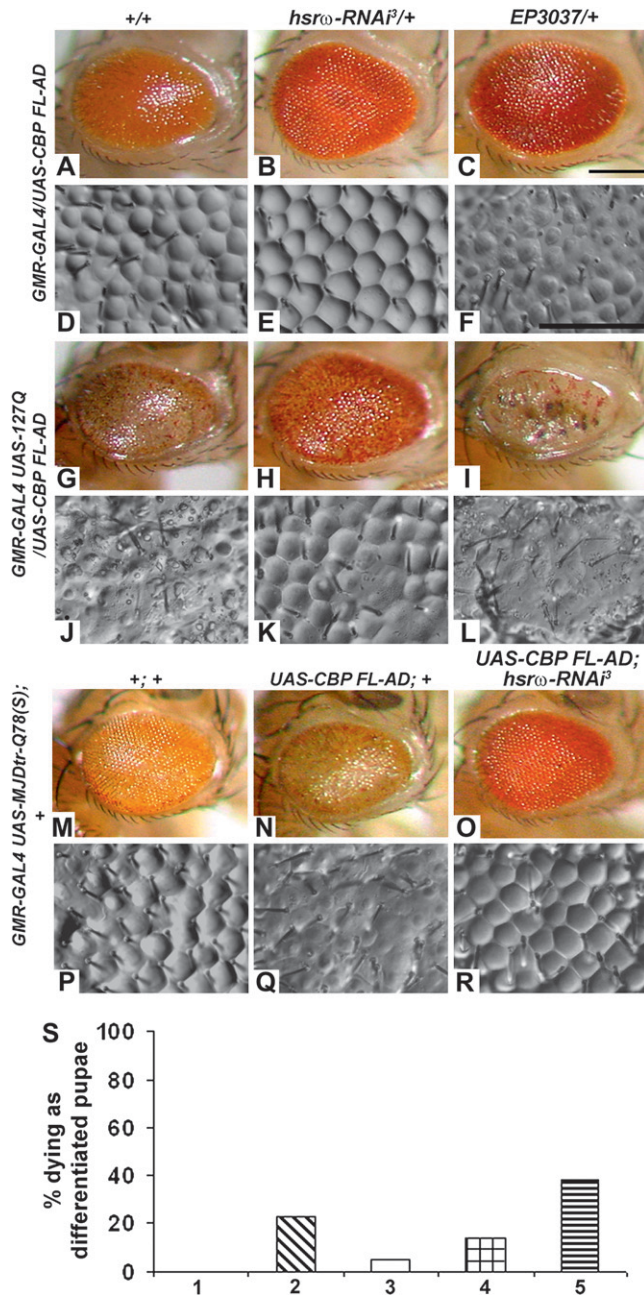


FIGURE 2.—Depletion of *hsrw*-n transcripts overcomes the deleterious effects of mutant forms of CBP in absence as well as presence of pathogenic poly(Q) expression. Genotypes common to rows are indicated on the left while those for columns are indicated at the top. A–C, G–I, and M–O are photographs and D–F, J–L, and P–R are nail polish imprints of adult eyes. Bars for A–C, G–I, and M–O in C and D–F, J–L, and P–R in F: 20 μ m. (S) Histograms of frequency (%) of differentiated pupae of the indicated genotypes that die before emergence: bar 1, *GMR-GAL4 UAS-127Q/+; +/+* ($N = 608$); bar 2, *GMR-GAL4 UAS-127Q/UAS-CBP FL-AD; +/+* ($N = 503$); bar 3, *GMR-GAL4 UAS-127Q/UAS-CBP FL-AD; hsrw-RNAi3/+* ($N = 334$); bar 4, *GMR-GAL4 UAS-127Q/UAS-CBP FL-AD; EP93D/+* ($N = 649$); bar 5, *GMR-GAL4 UAS-127Q/UAS-CBP FL-AD; EP3037/+* ($N = 295$); the frequencies of dying pupae in all the genotypes are significantly different ($P < 0.01$, χ^2 -test) from that in *GMR-GAL4 UAS-127Q/+; +/+*.

flies in either case showed reduction in eye size [compare Figure 2G ($N = 382$) with Figure 1A ($N = 608$) and Figure 2N ($N = 335$) with 2M ($N = 835$)] and a greater disruption in ommatidial arrays (compare Figure 2J with Figure 1F and Figure 2Q with 2P). Coexpression of one copy of the *hsrw-RNAi* transgene improved the eye morphology and size (Figure 2, H and K, $N = 316$, O and R, $N = 481$). On the other hand, elevation of *hsrw* transcript levels in *GMR-GAL4 UAS-127Q/UAS-CBP FL-AD; EP3037/+* (Figure 2, I and L, $N = 182$) or *GMR-GAL4 UAS-127Q/UAS-CBP FL-AD; EP93D/+* ($N = 558$, not shown) flies resulted in greater reduction in the eye size along with near complete loss of ommatidial arrays and bristles and appearance of black lesions on the eye surface.

Expression of the dominant-negative CBP in concert with *127Q* under *GMR-GAL4* control caused 23% ($N = 503$) lethality at the differentiated pupae stage (Figure 2S, bar 2). Introduction of one copy of the *hsrw-RNAi* transgene in this background reduced the differentiated pupal death to only 5.4% (Figure 2S, bar 3; $N = 334$). On the other hand, coexpression of *EP3037* enhanced differentiated pupal lethality to $\sim 38\%$ (Figure 2S, bar 5; $N = 295$). However, *GMR-GAL4*-driven coexpression of dominant-negative *CBP*, *127Q*, and *EP93D* resulted in only 14% pupal death (Figure 2S, bar 4; $N = 649$). As noted above (Figure 1I), this difference between the two *EP* alleles may be related to the different locations of the two *EP* transposons in the *hsrw* promoter.

Expression of the *CBP FL-AD* transgene in the *MJDTtr-Q78(S)* background also resulted in 15% pupal death ($N = 394$), which was significantly ($P < 0.01$, χ^2 -test) reduced to 3% ($N = 495$) following coexpression of a single copy of the *hsrw-RNAi* transgene.

To further examine interactions between CBP and the noncoding *hsrw* transcripts, we downregulated CBP through RNA interference, using a *UAS-CBP RNAi* transgene (KUMAR *et al.* 2004). *GMR-GAL4*-driven expression of *UAS-CBP RNAi* resulted in depigmented and somewhat smooth eyes with individual facets being less distinct, especially in the posterior region (Figure 3, A and D; $N = 487$). Depletion of *hsrw*-n transcripts in the *CBP RNAi* background ($N = 513$) brought about significant improvement in pigmentation and eye morphology, including in the posterior part (Figure 3, B and E). Augmentation of *hsrw* transcript levels using *EP3037* (Figure 3, C and F; $N = 433$) or *EP93D* (not shown; $N = 495$) in a CBP depleted background enhanced eye degeneration with the loss of ommatidial integrity extending to most of the eye.

Downregulating CBP levels in the *127Q* or *MJDTtr-Q78(S)* background caused high incidence of late pupal lethality (see below) and dramatically enhanced poly(Q)-mediated eye degeneration in the few surviving flies (Figure 3, G and J, $N = 22$, and M and P, $N = 2$, respectively). Expression of the *hsrw-RNAi* transgene

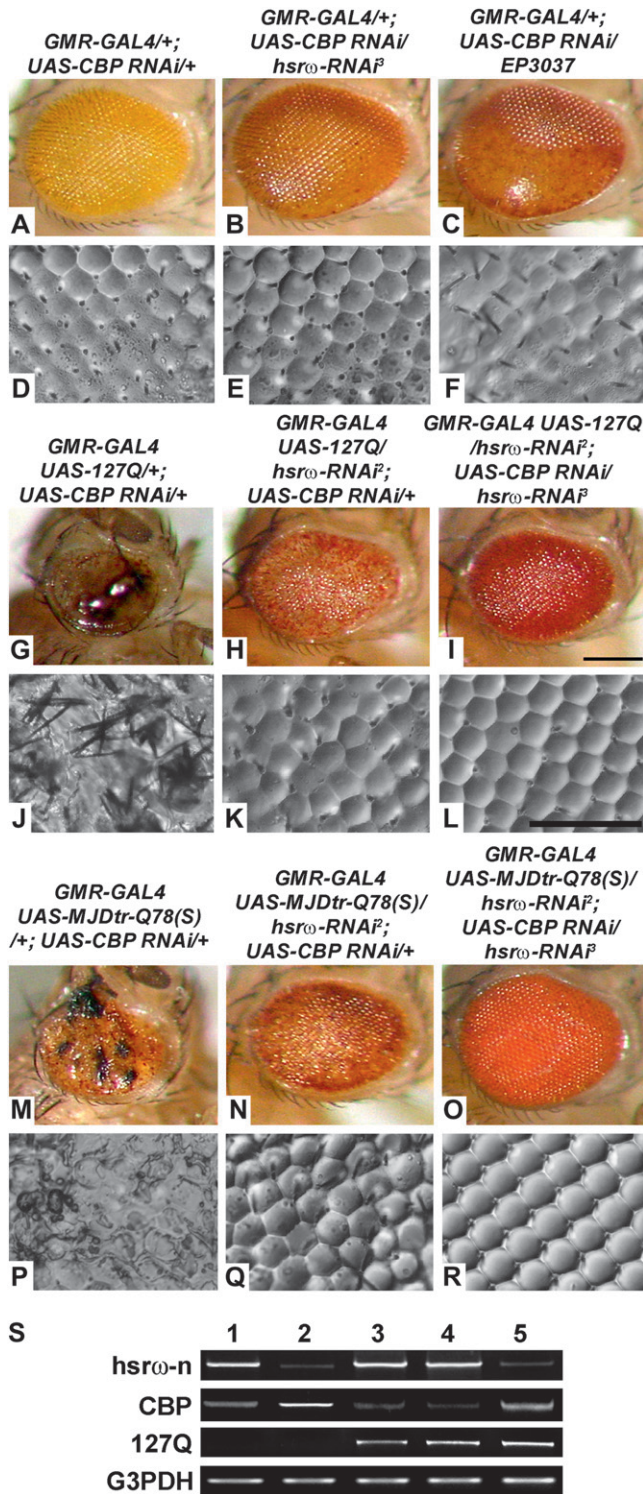


FIGURE 3.—*hsrω-RNAi* suppresses deleterious effects of CBP RNAi in absence as well as presence of pathogenic poly(Q) expression. A–C, G–I, and M–O are photomicrographs and D–F, J–L, and P–R are nail polish imprints of adult eyes of the genotypes indicated above the columns. Bars for A–C, G–I, and M–O in I and D–F, J–L, and P–R in L: 20 μ m. (S) Semiquantitative RT–PCR amplicons for *hsrω-n*, *CBP*, and *127Q* transcripts in total RNA from heads of *GMR-GAL4/+; UAS-CBP RNAi/+* (lane 1), *GMR-GAL4/+; UAS-CBP RNAi/ hsrω-RNAi²* (lane 2), *GMR-GAL4 UAS-127Q/+; +/+* (lane 3), *GMR-GAL4 UAS-127Q/+; UAS-CBP RNAi/+* (lane 4), and *GMR-GAL4 UAS-127Q/ hsrω-RNAi²; UAS-CBP RNAi/+* (lane 5),

not only robustly suppressed the enhanced eye damage following depletion of CBP but also substantially reversed the *127Q* or *MJDtr-Q78(S)*-induced eye degeneration in a dose-dependent manner since the rescue with one copy each of *hsrω-RNAi²* and *hsrω-RNAi³* (Figure 3, I and L, $N = 137$, and O and R, $N = 576$) was better than that seen with one copy of *hsrω-RNAi²* (Figure 3, H and K, $N = 166$, and N and Q, $N = 174$). Eyes of adult flies coexpressing one copy each of the *hsrω-RNAi²* and *hsrω-RNAi³* transgenes with *127Q* or *MJDtr-Q78(S)* appeared near normal.

GMR-GAL4-driven expression of *127Q* or *MJDtr-Q78(S)* in the *CBP RNAi* background caused near complete lethality (>97%) at the pharate stage (Table 1). Introduction of a single copy of the *hsrω-RNAi³* transgene did not improve adult emergence since the pharate stage lethality remained at 97.2 and 86% in *127Q* and *MJDtr-Q78(S)* backgrounds, respectively (Table 1). However, expression of a single copy of the *hsrω-RNAi²* transgene improved their emergence to adult stage [28% survivors in *127Q* and 86% in *MJDtr-Q78(S)*, Table 1]. When both the *hsrω-RNAi²* and the *hsrω-RNAi³* transgenes were coexpressed with *127Q* or *MJDtr-Q78(S)* in the *CBP RNAi* background, 32 and 95% pupae, respectively, emerged as adult flies (Table 1). Following coexpression of either *EP93D* or *EP3037* with *127Q* and *CBP RNAi*, lethality at the differentiated pupae stage remained 100% (Table 1).

Since in several of the above genotypes, only one copy of the *GMR-GAL4* driver is available for activating the multiple UAS-regulated transgenes, it remains possible that the different UAS promoters may not be adequately stimulated to transcribe the downstream gene. We confirmed that the multiple UAS transgenes in different genotypes were indeed activated as expected in response to the *GMR-GAL4* driver through RT–PCR (see Figure 3S) with total RNA from heads of *GMR-GAL4/+; UAS-CBP RNAi/+* (lane 1), *GMR-GAL4/+; UAS-CBP RNAi/ hsrω-RNAi²* (lane 2), *GMR-GAL4 UAS-127Q/+; +/+* (lane 3), *GMR-GAL4 UAS-127Q/+; UAS-CBP RNAi/+* (lane 4), and *GMR-GAL4 UAS-127Q/ hsrω-RNAi²; UAS-CBP RNAi/+* (lane 5) adult flies using appropriate primers (see MATERIALS AND METHODS) for *hsrω-n*, *CBP*, and *127Q* transcripts. Relative abundance of RT–PCR amplicons in the different genotypes in Figure 3S reflects the expected expression levels of the corresponding transcripts: (i) the *hsrω-n* transcript levels were reduced in genotypes carrying the *hsrω-RNAi* transgene (lanes 2 and 5); (ii) *CBP* transcript levels following expression of the *CBP-RNAi* transgene varied in relation to the presence or absence of the *hsrω-RNAi* and/or *127Q* transgenes (lanes 1–5); and (iii) the *127Q* transcripts were equally abun-

and *GMR-GAL4 UAS-127Q/ hsrω-RNAi²; UAS-CBP RNAi/+* (lane 5) adult flies show that multiplicity of UAS target sequences in different genotypes does not limit the *GMR-GAL4* activity. *G3PDH* was coamplified as a loading control.

TABLE 1
Downregulation of hsrw-n RNA suppresses CBP-RNAi-mediated pupal lethality of 127Q- or MJDtr-Q78(S)-expressing individuals

Genotype	No. of pupae	% dead differentiated pupae
127Q model		
<i>GMR-GAL4 UAS-127Q/+; UAS-CBP RNAi/+</i>	933	97.0
<i>GMR-GAL4 UAS-127Q/+; UAS-CBP RNAi/hsrw-RNAi³</i>	457	97.2
<i>GMR-GAL4 UAS-127Q/hsrw-RNAi²; UAS-CBP RNAi/+</i>	584	71.6
<i>GMR-GAL4 UAS-127Q/hsrw-RNAi²; UAS-CBP RNAi/hsrw-RNAi³</i>	432	68.3
<i>GMR-GAL4 UAS-127Q/+; UAS-CBP RNAi/EP93D</i>	461	99.6
<i>GMR-GAL4 UAS-127Q/+; UAS-CBP RNAi/EP3037</i>	157	100.0
MJDtr-Q78(S) or SCA3 model		
<i>GMR-GAL4 UAS-MDtr-Q78(S)/+; UAS-CBP RNAi/+</i>	627	99.7
<i>GMR-GAL4 UAS-MDtr-Q78(S)/+; UAS-CBP RNAi/hsrw-RNAi³</i>	608	86.3
<i>GMR-GAL4 UAS-MDtr-Q78(S)/hsrw-RNAi²; UAS-CBP RNAi³/+</i>	201	13.4
<i>GMR-GAL4 UAS-MDtr-Q78(S)/hsrw-RNAi²; UAS-CBP RNAi/hsrw-RNAi³</i>	606	5.0

dant in all three genotypes in which the 127Q transgene was present irrespective of the presence of other transgenes (lanes 3–5 in Figure 3S). In agreement with results presented later, expression of the *hsrw-RNAi* transgene is associated with an increase in CBP transcript levels (lanes 2 and 5 in the CBP row in Figure 3S). These results, together with our earlier report (MALLIK and LAKHOTIA 2009a), thus clearly establish that under our experimental conditions, the GAL4 produced by one copy of the *GMR-GAL4* transgene was adequate to activate the multiple UAS targets present in a given genotype.

With a view to further examine genetic interaction between CBP and *hsrw* transcripts, we used the *Act5C-GAL4* driver to ubiquitously express *UAS-CBP RNAi* in absence (*Act5C-GAL4/+; UAS-CBP RNAi/+*) or presence of the *hsrw-RNAi* (*Act5C-GAL4/hsrw-RNAi²; UAS-CBP RNAi/hsrw-RNAi³*) transgenes. Results presented in Table 2 reveal that global expression of *CBP RNAi* caused some degree of embryonic and larval lethality (Table 2, column 2), which was enhanced in presence of the *hsrw-RNAi* transgenes (Table 2, column 4). It may be noted that ubiquitous expression of the *hsrw-RNAi* transgene by itself also caused significant embryonic and larval lethality (Table 2, column 3) and, therefore, ubiquitous coexpression of *CBP RNAi* and *hsrw-RNAi* is expected to result in greater early lethality. The high frequency of unfertilized eggs in all these crosses reflects some perturbations in gametogenesis because of *Act5C-GAL4*-driven *CBP RNAi* or *hsrw-RNAi* (our unpublished observations). The high frequency of embryonic and larval lethality in the cross in column 3 of Table 2 is also contributed by death of *CyO* or *Act5C-GAL4* homozygous progeny.

All of the surviving progeny larvae in the *Act5C-GAL4/CyO; +/+ X +/+; UAS-CBP RNAi/UAS-CBP RNAi* cross

pupated but nearly 50% of them died as early (majority) or differentiated (a few) pupae (rows 7 and 8 in column 2, Table 2). The *Act5C-GAL4/+; UAS-CBP RNAi/+* genotype was completely absent in eclosing flies while the *CyO/+; UAS-CBP RNAi/+* flies emerged in the expected (~50%) proportion. Significantly, *Act5C-GAL4*-driven coexpression of one copy of *CBP-RNAi* with one copy each of the *hsrw-RNAi²* and *hsrw-RNAi³* transgenes dramatically reduced pupal lethality to just ~9% so that 29% of the pupae eclosed as phenotypically normal, fertile *Act5C-GAL4/hsrw-RNAi²; UAS-CBP RNAi/hsrw-RNAi³* (row 11 in column 4, Table 2) flies. It may be noted that *Act5C-GAL4*-driven expression of two copies of the *hsrw-RNAi³* transgene by itself resulted in death of ~8% pupae (rows 7 and 8 in column 3, Table 2). These results, therefore, indicate that *hsrw-RNAi* can substantially overcome the lethal consequences of global depletion of CBP.

Cellular levels of hsrw transcripts reciprocally affect CBP mRNA and protein levels in eye discs: To understand the basis of the above noted genetic interaction between CBP and *hsrw*, we examined if altered levels of the *hsrw* transcripts affected CBP gene expression. We estimated CBP transcript levels in *Act5C-GAL4/CyO; +/+* and *Act5C-GAL4*-driven *hsrw-RNAi*-expressing larval tissues (Figure 4, A–F). RNA:RNA *in situ* hybridization performed with a CBP-specific riboprobe demonstrated that *Act5C-GAL4*-mediated depletion of *hsrw-n* RNA dramatically increased the level of CBP transcripts in third instar larval eye imaginal discs (Figure 4D) as well as salivary glands (Figure 4, E and F) when compared with only GAL4-expressing eye discs (Figure 4A) or salivary glands (Figure 4, B and C), respectively.

We also estimated CBP transcript levels by semiquantitative RT-PCR with RNA isolated from eye discs of third instar larvae expressing *GMR-GAL4*-driven *EP93D*

TABLE 2
hsr ω -RNAi rescues *Act5C-GAL4*-driven *UAS-CBP* RNAi-mediated pupal lethality

Crosses	<i>Act5C-GAL4/CyO</i> ; +/+ \times +/+; <i>UAS-CBP</i> RNAi/ <i>UAS-CBP</i> RNAi	<i>Act5C-GAL4/CyO</i> ; <i>hsrω-RNAi</i> ³ / <i>hsrω-RNAi</i> ³ \times <i>Act5C-GAL4</i> / <i>CyO</i> ; <i>hsrω-RNAi</i> ³ / <i>hsrω-RNAi</i> ³	<i>Act5C-GAL4</i> / <i>CyO</i> ; <i>hsrω-RNAi</i> ³ / <i>hsrω-RNAi</i> ³ \times <i>hsrω-RNAi</i> ³ / <i>UAS-CBP</i> RNAi/ <i>UAS-CBP</i> RNAi
1. Total no. of eggs examined	1009	1088	1953
2. No. (%) of unfertilized eggs	147 (14.6%)	177 (16.3%)	680 (34.8%)
3. No. (%) of fertilized eggs	862 (85.4%)	911 (83.7%)	1273 (65.2%)
4. No. (%) of dead embryos ^a	42 (4.9%)	246 (27.0%)	78 (6.1%)
5. No. (%) of dead first and second instar larvae ^a	116 (13.4%)	344 (37.8%)	294 (23.1%)
6. No. (%) of pupae ^a	704 (81.7%)	321 (35.2%)	901 (70.8%)
7. No. (%) of dead undifferentiated pupae ^b	354 (50.3%)	5 (1.6%)	72 (8.1%)
8. No. (%) of dead differentiated pupae ^b	13 (1.8%)	20 (6.2%)	8 (0.9%)
9. No. (%) of <i>Act5C-GAL4</i> -driven <i>CBP</i> RNAi-expressing flies that eclosed ^b	0	NA	NA
10. No. (%) of Curly winged flies (expressing <i>Act5C-GAL4</i> -driven <i>hsrω-RNAi</i>) ^b	NA	296 (92.2%)	NA
11. No. (%) of <i>Act5C-GAL4</i> -driven <i>CBP</i> RNAi and <i>hsrω-RNAi</i> -expressing flies that eclosed ^b	NA	NA	260 (28.8%)
12. No. (%) of Curly winged flies (without <i>Act5C-GAL4</i> driver) ^b	337 (47.9%)	NA	561 (62.3%)

^aThe percentage values in rows 4–6 were calculated with respect to the total number of developing eggs (row 3).

^bThe percentage values in rows 7–11 were calculated with respect to the total number of pupae (row 6); NA, not applicable since these genotypes are not produced in the given crosses.

or *hsr ω -RNAi*. As shown in Figure 4G, compared to the CBP levels in *GMR-GAL4/GMR-GAL4*; +/+ eye imaginal discs (lane 1), a distinct increase (~42%) in transcript level was seen in *hsr ω -RNAi*-expressing discs (Figure 4G, lane 3). On the other hand, the level of CBP transcripts was detectably reduced (to ~90%) in *GMR-GAL4/GMR-GAL4*; *EP93D/EP93D* eye discs (Figure 4G, lane 2). The earlier noted (Figure 3S) results of RT-PCR of total RNA from adult heads of different genotypes also showed that even in the presence of CBP-RNAi, *hsr ω -RNAi* significantly increased the CBP transcript levels (Figure 3S, lane 2). These results further showed that, as reported earlier (TAYLOR *et al.* 2003), expression of 127Q resulted in decreased levels of CBP transcripts (Figure 3S, lane 3), which were further reduced by coexpressing the CBP-RNAi transgene; significantly, *hsr ω -RNAi* in this case also enhanced the CBP transcript levels (Figure 3S, lane 5).

To check if the enhanced level of CBP transcripts in *hsr ω -RNAi*-expressing cells was accompanied by increase in the protein also, we compared levels of CBP in Western slot blots of total proteins from *GMR-GAL4/GMR-GAL4*; +/+, *GMR-GAL4/GMR-GAL4*; *hsr ω -RNAi*³/*hsr ω -RNAi*³, and *GMR-GAL4/GMR-GAL4*; *EP93D/EP93D* eye discs (slots 1, 2, and 3, respectively, in Figure 4H). Analysis of three independent blots showed that compared to wild type, the CBP level in *hsr ω -RNAi*-expressing discs was, on average, enhanced by 17.6% (mean ratio of CBP level in *GMR-GAL4/GMR-GAL4*; *hsr ω -RNAi*³/*hsr ω -*

*RNAi*³ and *GMR-GAL4/GMR-GAL4*; +/+ eye discs = 1.176 \pm 0.04); on the other hand in *GMR-GAL4/GMR-GAL4*; *EP93D/EP93D* discs it was reduced, on an average, by 30% (mean = 0.698 \pm 0.04; Figure 4H).

Cellular levels of *hsr ω* transcripts reciprocally affect CBP levels in poly(Q)-expressing eye discs also: In view of the above results, we examined if downregulation of *hsr ω -n* transcripts affected the cellular distribution and levels of CBP in poly(Q)-expressing eye discs also. Poly(Q) aggregation and levels of CBP were assayed by co-immunostaining eye discs from late third instar larvae expressing *127Q*, *htt ϵ x1p Q93*, or *MJDtr-Q78(S)* alone or in combination with two copies of *hsr ω -RNAi* (Figure 5). As described earlier (MALLIK and LAKHOTIA 2009a), the poly(Q) protein and the IBs were distributed in characteristic patterns in the different disease models (Figure 5, A–G). Likewise, the patterns of CBP distribution also varied in the three models (Figure 5, H–N). In *127Q*- (Figure 5H, *N* = 10) or *MJDtr-Q78(S)*- (Figure 5M, *N* = 8), but not in *htt ϵ x1p Q93* (Figure 5K, *N* = 7) -expressing eye disc cells, the diffuse CBP staining on chromatin appeared to be less than that in discs not expressing the mutant poly(Q) proteins (Figure 6B). On the other hand, the *127Q*- or *htt ϵ x1p Q93*-expressing discs showed many cytoplasmic and some nuclear granules of CBP (Figure 5, H and K, respectively), but in those expressing *MJDtr-Q78(S)*, such granules were very few (Figure 5M). Most of the nuclear and cytoplasmic granules of CBP in the *127Q* discs did not colocalize

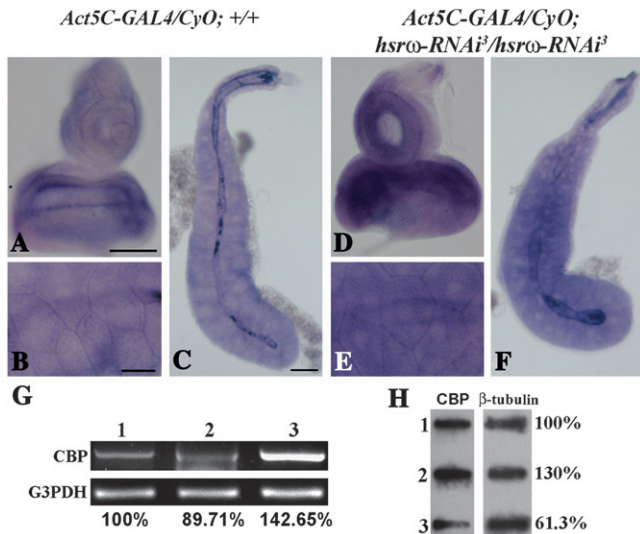


FIGURE 4.—Depletion of *hsr-w* RNA upregulates CBP expression. Light microscope images (A–F) show localization of CBP RNA in *Act5C-GAL4/CyO; +/+* (A–C) and *Act5C-GAL4*-driven *hsr-w-RNAi*-expressing (D–F) third instar larval eye imaginal discs (A and D) and salivary glands (B, C and E, F); higher magnification images of the basal regions of salivary glands shown in C and F are shown in B and E, respectively. Bars for A and D in A and B and E in B, 50 μ m; and for C and F in C, 100 μ m. (G) Levels of CBP transcripts following a semiquantitative RT–PCR with total RNA from larval eye discs of different genotypes using CBP-specific primers (lane 1, *GMR-GAL4/GMR-GAL4; +/+*; lane 2, *GMR-GAL4/GMR-GAL4; EP93D/EP93D*; lane 3, *GMR-GAL4/GMR-GAL4; hsr-w-RNAi³/hsr-w-RNAi³*). The bottom panel shows coamplified G3PDH as a loading control. (H) A Western slot blot showing the amount of CBP (left) in extracts from *GMR-GAL4/GMR-GAL4; +/+* (slot 1), *GMR-GAL4/GMR-GAL4; hsr-w-RNAi³/hsr-w-RNAi³* (slot 2), and *GMR-GAL4/GMR-GAL4; EP93D/EP93D* (slot 3) eye imaginal discs. The right panel, showing amounts of β -tubulin in each of the extracts, was used as a loading control; values on the right show CBP levels in each sample relative to that in wild type (slot 1), which was taken as 100%.

with the poly(Q) IBs (Figure 5, H and O) but in the *httex1p Q93*-expressing discs, the CBP aggregates (Figure 5K) almost always colocalized (Figure 5R) with the huntingtin IBs (Figure 5D). Coexpression of two copies of *hsr-w-RNAi* with the expanded poly(Q) transgenes not only reduced poly(Q) IBs and CBP aggregates (Figure 5, B, C, E, and G and I, J, L, and N) but also substantially increased the levels of diffuse CBP staining on chromatin (Figure 5, I and J, $N = 11$; L, $N = 11$; and N, $N = 6$). As reported earlier (MALLIK and LAKHOTIA 2009a), *hsr-w-RNAi* in *127Q*-expressing eye discs results in either partial or complete rescue of neurodegeneration. Accordingly, some eye discs coexpressing *127Q* and *hsr-w-RNAi* ($N = 11$) showed reduced levels of poly(Q) aggregates (Figure 5B, 54.5% eye discs) while others showed little or no poly(Q) staining (Figure 5C, 45.5% discs). Very significantly, the levels of CBP in these discs were, respectively, marginally (Figure 5, I and P; $N = 6$, 54.5% eye discs) or significantly (Figure 5, J and Q; $N =$

5, 45.5% discs) elevated. Consistent with the above immunofluorescence results, analysis of three independent Western slot blots demonstrated that third instar larval eye imaginal discs coexpressing *MJDtr-Q78(S)* and *hsr-w-RNAi* (inset in Figure 5U, slot 2) showed, on an average, $\sim 24\%$ increase (mean ratio = 1.244 ± 0.06) in the CBP level compared with eye discs expressing *MJDtr-Q78(S)* alone (inset in Figure 5U, slot 1).

CBP and hnRNPs show physical association in eye disc cells: With a view to understand the basis of the above noted interactions between *hsr-w* transcripts and CBP, we immunostained eye discs of various genotypes to examine the distribution of CBP and the hnRNPs like Hrb57A or Hrb87F that are normally associated with the *hsr-w* transcripts in omega speckles (PRASANTH *et al.* 2000). Nuclei of the photoreceptor cells of late third instar wild-type (Figure 6, A–C, $N = 15$; J–L, $N = 8$) as well as *GMR-GAL4/GMR-GAL4; +/+* (Figure 6, A'–C', $N = 7$) larval eye discs showed CBP and both the hnRNPs to be distributed in diffuse as well as speckled/granular patterns; the cytoplasm in these cells also showed distinct granules of CBP and the hnRNPs. Interestingly, in the peripodial cells of eye discs, CBP and Hrb87F, but not Hrb57A, were much more abundant than in the disc proper cells but were restricted mostly to the nucleus in diffuse and granular domains (not shown). The Hrb57A-containing cytoplasmic granules were distinctly larger than those stained by the Hrb87F antibody (compare Figure 6A and 6J). The diffuse nuclear CBP staining overlapped with that for Hrb87F (Figure 6C) and Hrb57A (Figure 6L). On the other hand, the CBP granules, especially those in the photoreceptor cell cytoplasm, were often adjacent to or partially or fully overlapped with those of Hrb87F (Figure 6C) and Hrb57A (Figure 6L).

As seen in Figure 6, A'–C', the distribution of Hrb87F and CBP in photoreceptor cells in *GMR-GAL4* homozygous eye discs was more or less comparable to that in wild type (Figure 6, A–C). However, *GMR-GAL4*-driven expression of the *hsr-w-RNAi* transgene resulted in significant redistribution of both hnRNPs and CBP (Figure 6, D–F, $N = 14$; M–O, $N = 12$). Nuclear granules of the hnRNPs (Figure 6, D and M) and CBP (Figure 6, E, F, N, and O) were nearly absent while cytoplasmic granules of the hnRNPs, especially those of Hrb57A (Figure 6M) and of CBP, were more abundant. It is interesting to note that the cytoplasmic granules of Hrb57A and CBP also showed a greater overlap following expression of *hsr-w-RNAi* (Figure 6O). As noted above (Figure 5), the overall staining for CBP was enhanced in eye discs with reduced *hsr-w* transcripts (Figure 6, E and N). In contrast, *GMR-GAL4*-driven expression of *EP93D* in larval eye discs resulted in enhanced nuclear accumulation of the Hrb87F protein and an overall reduced staining for CBP with the nuclear as well as cytoplasmic granules also being much less prominent (Figure 6, G–I; $N = 9$). In these discs, the

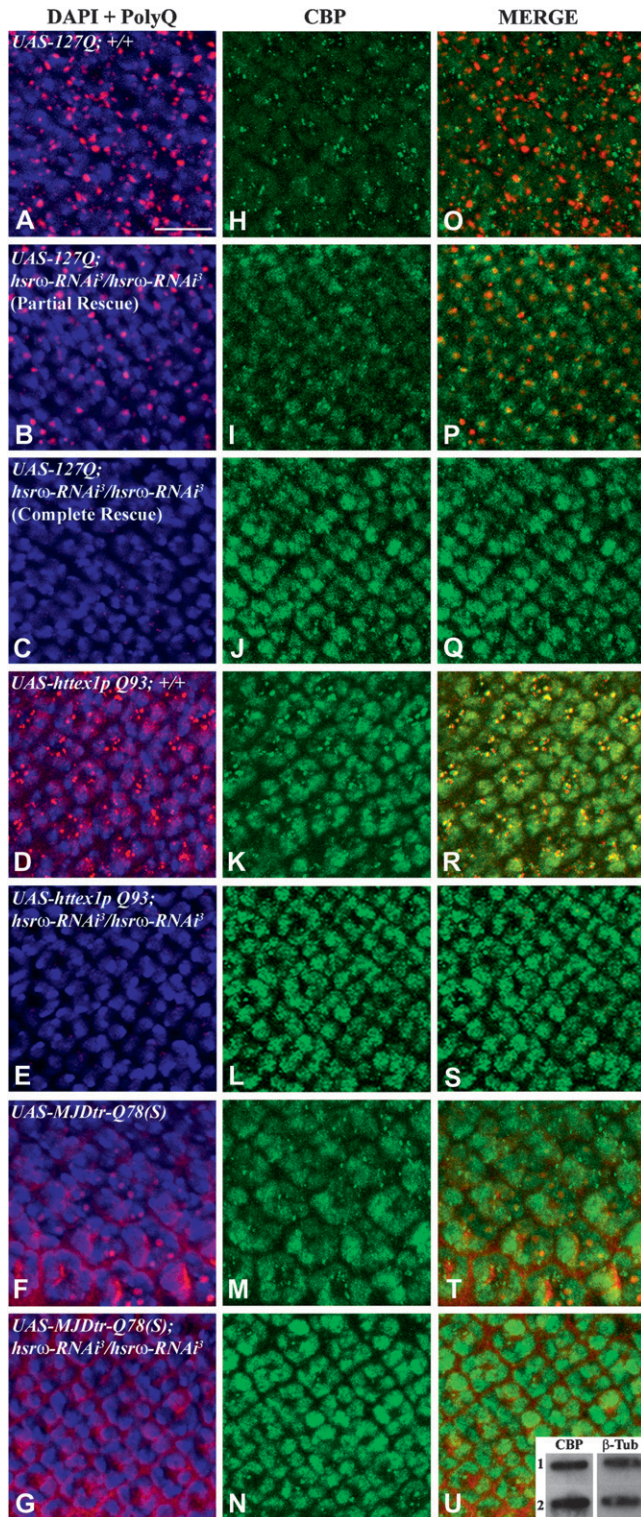


FIGURE 5.—Depletion of *hsrw* transcripts elevates CBP levels and inhibits aggregation of poly(Q) proteins. Confocal projections of some optical sections of eye imaginal discs, excluding the peripodial membrane, co-immunostained for poly(Q) protein (red, A–G) and CBP (green, H–N); genotype for each row is indicated in the first column; all of them also carry one copy of *GMR-GAL4*. In each case, chromatin is counterstained with DAPI (blue, A–G). Merged images of poly(Q) and DAPI-stained nuclei are shown in A–G and of poly(Q) and CBP in O–U. The morphogenetic furrow in

ommatidial arrays were less organized as revealed by DAPI staining of photoreceptor nuclei (Figure 6G).

The apparent association between CBP and the hnRNPs was confirmed by immunoprecipitation followed by Western blotting. As shown in Figure 6P, immunoprecipitation of crude extracts of eye imaginal discs from wild-type (lane 1) and *GMR-GAL4/GMR-GAL4; hsrw-RNAi³/hsrw-RNAi³* (lanes 2 and 4) third instar larvae with the CBP antibody pulled down Hrb87F (lanes 1 and 2) as well as Hrb57A (lane 4).

***hsrw*-RNAi-mediated rescue of eye degeneration due to dominant-negative CBP mutants requires the CBP transactivation domain:** To identify the domain(s) through which the *hsrw* transcripts interact with CBP, we overexpressed a series of CBP loss-of-function mutants (KUMAR *et al.* 2004; ANDERSON *et al.* 2005) in conjunction with single copies of *hsrw-RNAi* or *EP93D* or *EP3037* in the developing eye and looked for modulation of the retinal phenotypes associated with the expression of the CBP variants alone.

Flies expressing the *CBP* Δ NZK variant, lacking the N-terminal half of the protein that includes the domains for binding to CREB and to nuclear hormone receptors (KUMAR *et al.* 2004; ANDERSON *et al.* 2005), exhibited a slight roughening of the external retinal surface and disruption of the ommatidial bristle arrangement because of missing or extra bristles at some places (Figure 7, A and E). Downregulation of *hsrw-n* RNA in this background resulted in near wild-type eye morphology (Figure 7, B and F). On the other hand, enhancement of *hsrw* transcripts through expression of *EP93D* (Figure 7, C and G) or *EP3037* (Figure 7, D and H) enhanced the eye damage (Figure 7, C, D, G, and H). The *EP3037* allele had a slightly greater enhancing effect.

As reported earlier (KUMAR *et al.* 2004), *GMR-GAL4*-driven expression of the *CBP* Δ Q construct that deletes the alanine- and glutamine-rich transactivation domain alone resulted in 100% lethality ($N = 594$) at the early pupae stage before eye differentiation. The eye phenotype following expression of *CBP* Δ BHQ, which removes the BROMO, HAT, and poly(Q) domains (KUMAR *et al.* 2004; ANDERSON *et al.* 2005), was very severe ($N = 1086$). The external surface of the adult eye was flattened and reduced in size (Figure 7, I and M). Interestingly, the early pupal lethality resulting from the expression of *CBP* Δ Q or the eye phenotype due to *CBP* Δ BHQ was not affected by either *hsrw-RNAi* (Figure 7, J and N; $N = 1024$) -mediated down regulation or *EP*-mediated overexpression (*EP93D*, Figure 7, K and O, $N = 877$; or *EP3037*, Figure 7, L and P, $N = 826$) of *hsrw* transcripts.

all cases is toward the top. Bar, 10 μ m. The inset in U is a slot blot showing CBP levels (left pane) from *GMR-GAL4/UAS-MJDr-Q78(S); +/+* (slot 1) and *GMR-GAL4/UAS-MJDr-Q78(S); hsrw-RNAi³/hsrw-RNAi³* (slot 2) -expressing larval eye imaginal discs. The right pane in the inset shows similar levels of β -tubulin in both the slots.

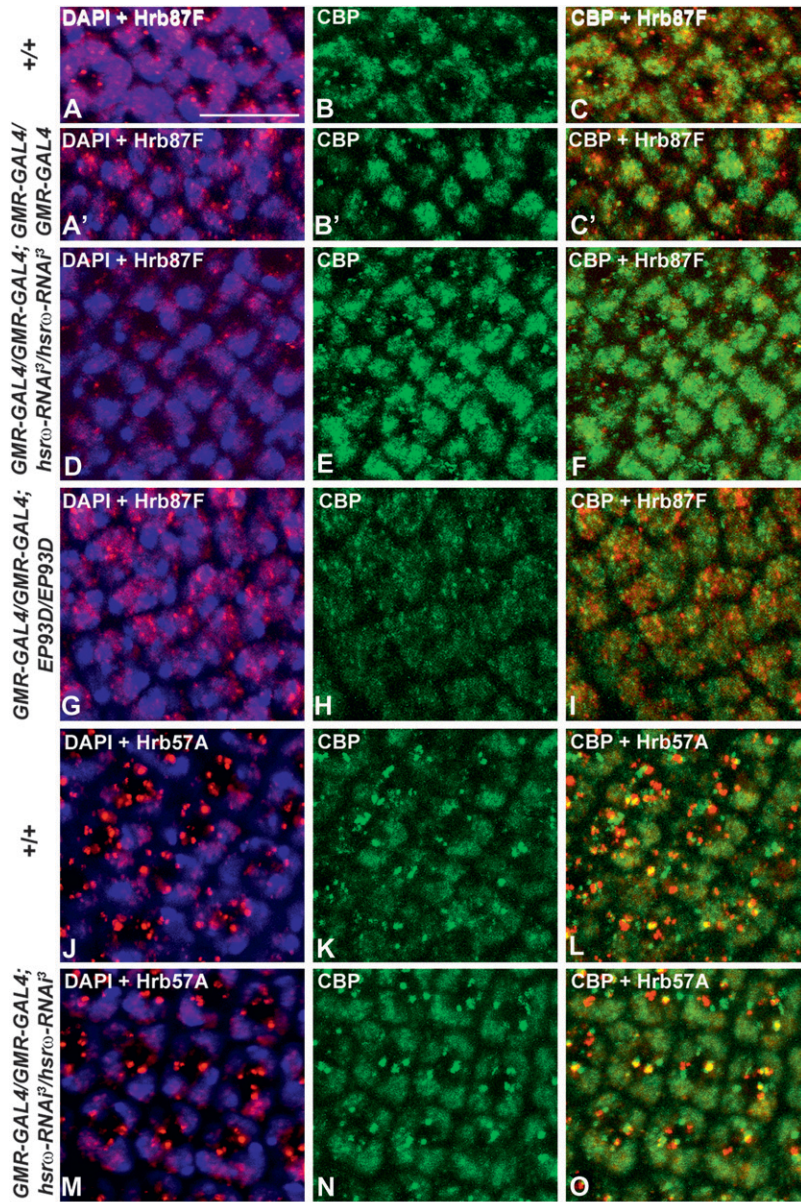
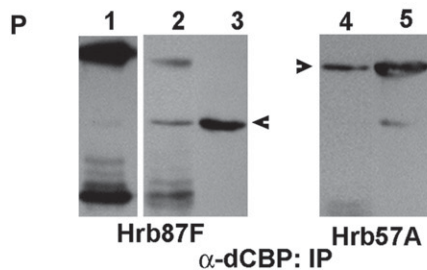


FIGURE 6.—CBP shows association with hnRNPs and its cellular levels are reciprocally modulated by levels of the hsrw transcripts. Shown are projections of confocal images of late third instar larval eye discs (genotypes indicated on the left) co-immunostained with Hrb87F (red, A, A', D, and G) and CBP (green, B, B', E, and H) or with Hrb57A (red, J and M) and CBP (green, K and N) antibodies. Merged images of hnRNPs and DAPI-stained nuclei (blue) are shown in A, A', D, G, J, and M; of CBP and Hrb87F in C, C', F, and I; and of CBP and Hrb57A in L and O. Bar, 10 μ m. (P) CBP antibody co-immunoprecipitated endogenous Hrb87F (arrowhead in lanes 1–3) from wild-type (lane 1) and from *GMR-GAL4*-driven *hsrw-RNAi* (lane 2) third instar larval eye-imaginal discs. Likewise, immunoprecipitation with CBP also pulled down Hrb57A from *GMR-GAL4*/*GMR-GAL4*; *hsrw-RNAi*²/*hsrw-RNAi*² eye discs (lane 4). Lanes 3 and 5 show Hrb87F and Hrb57A proteins, respectively, in the crude extracts used for immunoprecipitation; the filter in lane 3 was re-probed with the Hrb57A antibody to produce the image in lane 5 and, therefore, a residual signal corresponding to Hrb87F is also seen in this lane. Lane 1 was overdeveloped compared to lanes 2–5 to enable visualization of the Hrb87F band in this lane.



Thus, any variations in the cellular hsrw transcript levels failed to modulate phenotypes caused by ectopic expression of CBP loss-of-function mutants that lack the poly(Q) domain.

UPP activity is modulated by hsrw transcripts: In our earlier study (MALLIK and LAKHOTIA 2009a) we raised the possibility that the pathogenic poly(Q) polypeptides that fail to aggregate following hsrw-RNAi are degraded

by the proteasomal machinery of the cell so that the source of toxicity is substantially eliminated. Therefore, we first examined if the proteasome activity is affected by the hsrw transcripts, using two different approaches.

In the first approach, we used the *UAS-Pros26^l.B* and *UAS-Pros β 2^l* transgenes that carry temperature-sensitive missense mutations in the 20S proteasome subunits β 6 and β 2, respectively (BELOTE and FORTIER 2002).

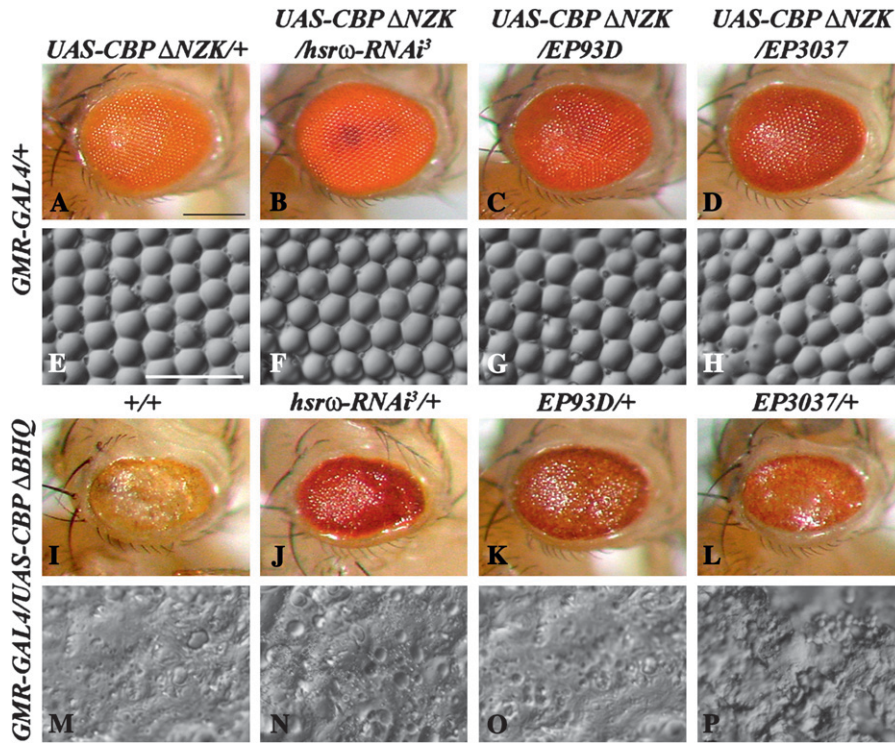


FIGURE 7.—Effect of altered levels of *hsrω* transcripts on eye degeneration following expression of individual CBP variants. In all cases, the CBP variant was expressed from a UAS construct driven by *GMR-GAL4*. Genotypes common to rows are indicated on the left while those for columns are indicated at the top. A–D and I–L are photomicrographs and E–H and M–P are nail polish imprints of adult eyes. Bars for A–D and I–L in A and for E–H and M–P in E: 20 μ m.

Although these temperature-sensitive mutant alleles have maximum effect at 29° (BELOTE and FORTIER 2002), we reared the larvae and flies carrying these transgenes at 25° since expression of the mutant proteasome subunits even at 25° affects eye structure but the additional poly(Q) toxicity due to elevated temperature is avoided.

Externally, eyes of flies coexpressing the two proteasome mutant transgenes under control of *GMR-GAL4* at 25° appeared near normal (Figure 8, A and B; $N = 225$); coexpression of *hsrω-RNAi* along with the proteasome mutant transgenes also did not alter the external eye morphology (Figure 8, D and E; $N = 281$). However, the pseudopupil images of eyes (Figure 8C, $N = 9$) of flies expressing the proteasome mutants exhibited severe retinal damage, which was substantially, although not completely, rescued by coexpression of one copy of *hsrω-RNAi* (Figure 8F, $N = 12$).

In another approach, we used the *UAS-Ub^{G76V}-GFP* transgenic line (DANTUMA *et al.* 2000) to examine if misexpression of *hsrω* transcripts compromises the endogenous UPP activity. The *UAS-Ub^{G76V}-GFP* transgene is a fluorescent reporter of UPP function carrying an uncleavable ubiquitin-conjugated GFP variant under control of the UAS promoter. This modified protein has an extremely short half-life and therefore any dysfunction of the endogenous UPP results in GFP fluorescence in the transgene-expressing cells while in cells with normal proteasome activity, the GFP-tagged ubiquitin is efficiently degraded so that very low GFP fluorescence is seen (DANTUMA *et al.* 2000). When *UAS-Ub^{G76V}-GFP* was coexpressed with a single copy of the dominant-negative

proteasome subunit transgene, *UAS-DTS5-11*, at 29° using the *GMR-GAL4* driver, a robust GFP fluorescence was seen in eye imaginal discs as expected (Figure 8G, $N = 5$); the ommatidial units in these discs were also considerably disorganized as revealed by the DAPI fluorescence of nuclei (Figure 8J). This validated the effectiveness of the GFP-reporter assay in this transgenic line. Eye imaginal discs from flies expressing the *UAS-Ub^{G76V}-GFP* reporter along with one copy of *hsrω-RNAi* at 25° showed a very low GFP fluorescence signal (Figure 8H, $N = 7$) and the DAPI staining (Figure 8K) revealed well organized ommatidial units. On the other hand, *GMR-GAL4*-driven expression of a single copy of *EP3037* at 25° resulted in a strong *Ub^{G76V}-GFP* (Figure 8I, $N = 12$) fluorescence together with disruption in the organization of ommatidial units (Figure 8L).

Together, these results show that overexpression of *hsrω* transcripts compromises UPP while their depletion through *hsrω-RNAi* may actually help restore the compromised proteasome functions.

Suppression of poly(Q) protein toxicity by *hsrω-RNAi* requires a functional proteasome: We next expressed the *UAS-Pros26^l.B* and *UAS-Prosβ2^l* transgenes either in *127Q* or in *127Q* and *hsrω-RNAi* backgrounds using the *GMR-GAL4* driver. Expression of *127Q* in the compromised proteasome background enhanced the poly(Q) eye phenotype (compare Figure 9A and 9E, $N = 286$, with 9C and 9G, $N = 293$). Interestingly, unlike the substantial suppression of poly(Q)-induced eye damage by coexpression of one copy of *hsrω-RNAi* in a genetically functional proteasome background (Figure 9, B and F, $N = 244$; also see MALLIK and LAKHOTIA

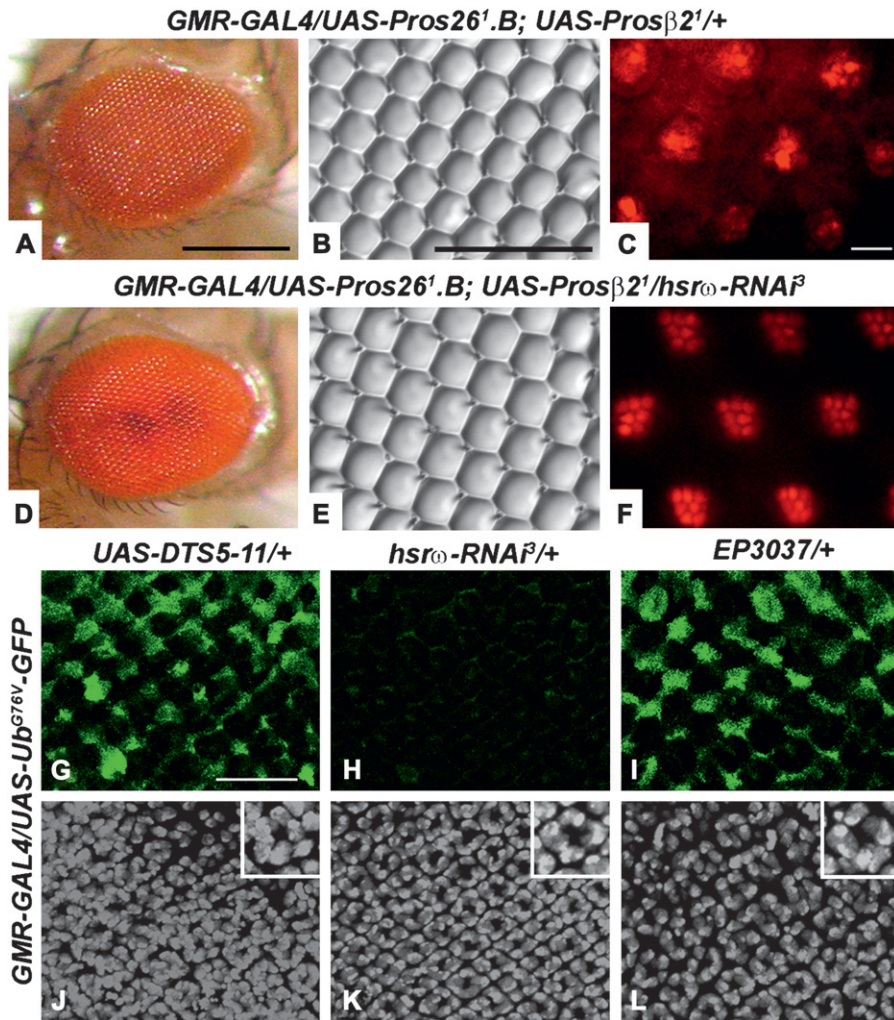


FIGURE 8.—Retinal damage due to impaired proteasomal function is suppressed by *hsr*-RNAi but overexpression of *hsr* transcripts per se compromises the proteasome activity. Genotypes are indicated at the top of each row in A–F. For G–L the genotype for chromosome 2 is indicated on the left while that for chromosome 3 is at the top of each column. A and D are photomicrographs, B and E are nail polish imprints, and C and F are pseudopupil images of adult eyes. G–I are confocal projections of larval eye discs of the indicated genotypes showing the extent of proteasomal dysfunction through GFP expression (green, G–I). J–L show DAPI-stained nuclei in the same sets of confocal projections shown in G–I, respectively; compared to the wild-type-like distribution of nuclei of developing ommatidial units in a typical ring-like arrangement in ordered diagonal arrays in third instar eye imaginal discs from *GMR-GAL4/UAS-Ub^{G76V}-GFP; hsr-RNAi³/+* (K), the nuclei of ommatidial units in eye discs from *GMR-GAL4/UAS-Ub^{G76V}-GFP; UAS-DTS5-11/+* (J) as well as *GMR-GAL4/UAS-Ub^{G76V}-GFP; EP3037/+* (L) appear disorganized. Insets in J–L show higher magnification images of a single ommatidial cluster in the corresponding disc. Bars for A and D in A, for B and E in B, and for G–L in G, 20 μ m; and for C and F in C, 10 μ m.

2009a), suppression of the 127Q damage by *hsr*-RNAi was significantly less when the UPP activity was compromised by expression of the dominant-negative transgenes (Figure 9, D and H; $N = 231$).

We have shown earlier (MALLIK and LAKHOTIA 2009a) that expression of two copies of *hsr*-RNAi with the 127Q transgene resulted in $\sim 50\%$ discs with reduced IBs and 50% with no IBs. In the present study, we examined the effect of one copy of the *hsr*-RNAi transgene in 127Q-expressing eye discs with normal and ectopically compromised UPP activity. As expected, one copy of the *hsr*-RNAi transgene in the absence of any mutant UPP activity also reduced the IBs but fewer ($\sim 30\%$) discs exhibited nearly complete disappearance of the IBs (Table 3). Coexpression of the proteasome mutants in the poly(Q) background increased aggregate formation at the expense of the diffuse poly(Q) staining in rows nearer to the morphogenetic furrow (Table 3; compare Figure 9A' with 9C'). Significantly, discs coexpressing 127Q and *hsr*-RNAi in concert with the proteasome mutants also exhibited enhanced incidence of poly(Q) aggregates than those with normal UPP activity (Table 3; compare Figure 9B' with 9D').

Coexpression of the two transgenes encoding mutant proteasomal subunits with *MJDtr-Q78(S)* under control of the *GMR-GAL4* driver did not further enhance the *MJDtr-Q78(S)* eye degenerative phenotype (compare Figure 9I and 9M with 9K and 9O). However, coexpression of the mutant proteasomal transgenes in the SCA3 fly model also abrogated the ability of *hsr*-RNAi to rescue *MJDtr-Q78(S)*-induced eye damage (compare Figure 9J and 9N with 9L and 9P).

Expression of the Ub^{G76V}-GFP reporter in flies expressing 127Q and *MJDtr-Q78* alone or in conjunction with one copy of the *hsr*-RNAi transgene revealed that as reported earlier (CHAN and BONINI 2000; VENKATRAMAN *et al.* 2004; BENNETT *et al.* 2005), poly(Q) expression by itself disrupted UPP activity, resulting in substantial Ub^{G76V}-GFP reporter fluorescence (Figure 9, Q and S; $N = 8$ in both cases). Interestingly, GFP fluorescence in eye imaginal discs from third instar larvae coexpressing one copy of the *hsr*-RNAi transgene and 127Q (Figure 9R, $N = 8$) or *MJDtr-Q78* (Figure 9T, $N = 12$) was very low, suggesting substantial restoration of normal proteasome function despite expression of the expanded poly(Q) protein. Results of RT-PCR presented in the

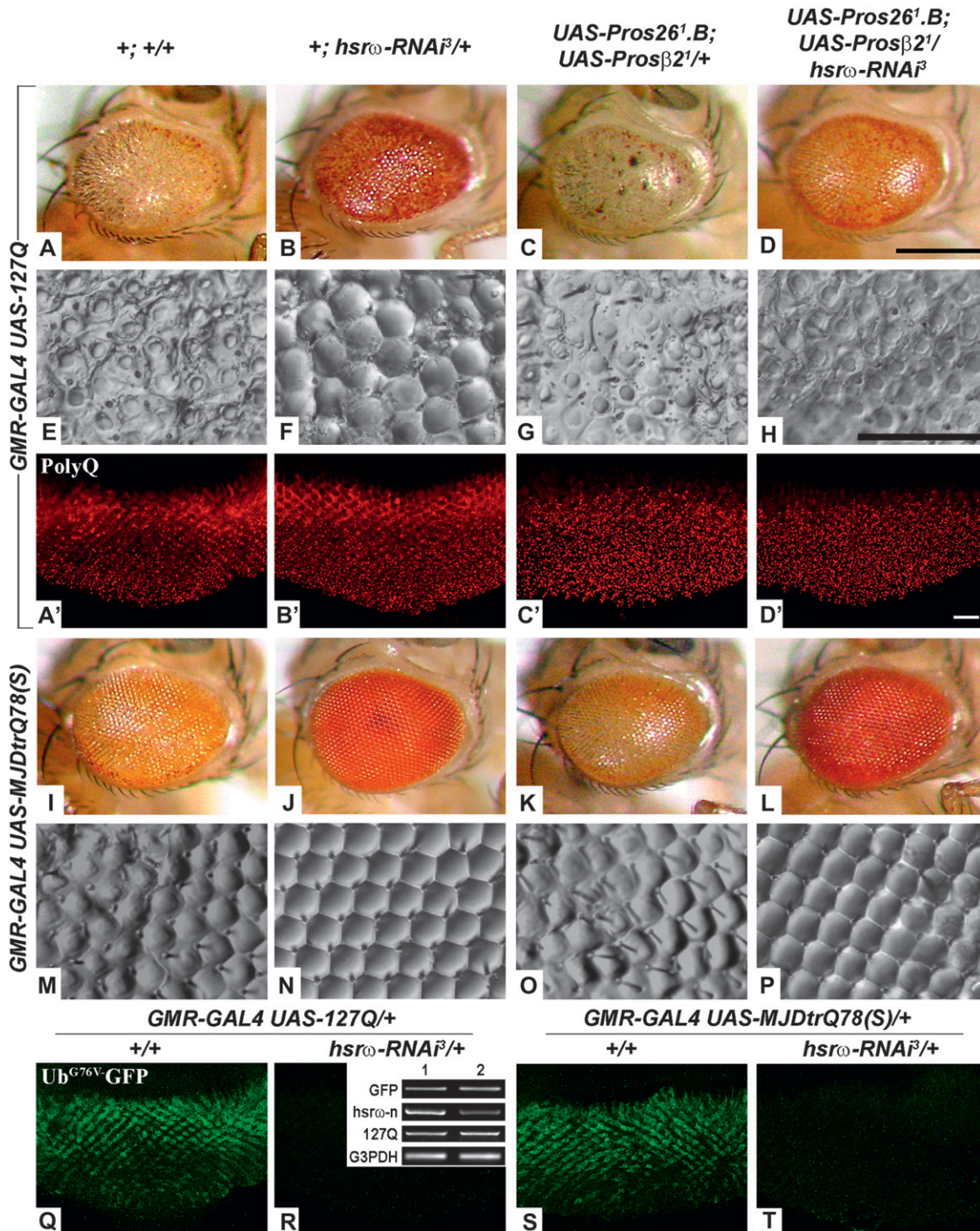


FIGURE 9.—Functional proteasomal activity is required for suppression of poly(Q) damage to eyes and for suppression of formation of IBs in eye discs. A–D and I–L are photomicrographs and E–H and M–P are nail polish imprints of the eye surface. Genotypes common to rows are indicated on the left while those for columns are indicated at the top. (A'–D') Confocal projections of third instar larval eye imaginal discs immunostained for the poly(Q) protein (red, A'–D'). (Q–T) Confocal projections showing Ub^{G76V}-GFP reporter activity in eye imaginal discs from *GMR-GAL4 UAS-127Q/+; +/+* (Q), *GMR-GAL4 UAS-127Q/+; hsr^ω-RNAi^β/+* (R), *GMR-GAL4 UAS-MJDtrQ78(S)/+; +/+* (S), and *GMR-GAL4 UAS-MJDtrQ78(S)/+; hsr^ω-RNAi^β/+* (T) third instar larvae. Bars for A–D and I–L in D, for E–H and M–P in H, and for A'–D' and Q–T in D': 20 μm. Inset in R shows semiquantitative RT-PCR levels of EGFP, hsr^ω-n, and 127Q transcripts in total RNA from heads of *w/w; GMR-GAL4 UAS-127Q/+; +/+* (lane 1) and *w/w; GMR-GAL4 UAS-127Q/+; hsr^ω-RNAi^β/+* (lane 2) adult flies, respectively. G3PDH was coamplified and used as a loading control.

TABLE 3

Suppression of poly(Q) aggregation by hsr ω -RNAi is inhibited by coexpression of dominant-negative proteasome subunits

Genotype	No. of discs	Relative level of poly(Q) IBs ^a (% discs)			
		+++	++	+	–
<i>GMR-GAL4/UAS-127Q; +/+</i>	17		100		
<i>GMR-GAL4/UAS-127Q; hsrω-RNAi³/+</i>	13			69.2	30.8
<i>GMR-GAL4 UAS-127Q/UAS-Pros26¹.B; UAS-Prosβ 2¹/+</i>	9	100			
<i>GMR-GAL4 UAS-127Q/UAS-Pros26¹; UAS-Prosβ 2¹/hsrω-RNAi³</i>	8	100			

^a The levels are expressed in arbitrary categories with +++ indicating maximum and – indicating few or no inclusion bodies (IBs).

inset in Figure 9R clearly show that the absence of GFP fluorescence following hsr ω -RNAi in any of these genotypes is not due to inhibition of the expression of the *Ub^{G76V}-GFP* transgene by reduced levels of hsr ω transcripts since the *Ub^{G76V}-GFP* as well as the *127Q* transcripts were equally abundant whether the hsr ω transcript levels were normal (lane 1) or lowered by hsr ω -RNAi (lane 2).

Taken together, these observations show that poly(Q) expression disrupts normal UPP function that is restored by hsr ω -RNAi. However, if the cell's proteasomal activity is impaired by expression of dominant-negative subunits, reduced levels of hsr ω transcripts fail to ameliorate the poly(Q) toxicity.

DISCUSSION

Earlier reports from our laboratory showed that while overexpression of the noncoding hsr ω transcripts enhanced (SENGUPTA and LAKHOTIA 2006), reducing the cellular levels of these transcripts through RNAi suppressed the neurodegeneration caused by mutant proteins with expanded poly(Q) stretches (MALLIK and LAKHOTIA 2009a). As shown earlier (MALLIK and LAKHOTIA 2009a), expression of the *hsr ω -RNAi* transgene had no effect on poly(Q) transcription but it diminished/eliminated the source of toxicity by inhibiting formation of the IBs and by enhancing clearance of the mutant poly(Q) proteins. The present study provides useful insights into the possible mechanisms through which these noncoding transcripts modulate cellular toxicity of the mutant poly(Q) proteins.

Studies in a variety of poly(Q) model systems have reported that many essential cellular proteins, *e.g.*, transcription factors like CBP, TBP, etc. (LI and LI 2004; BAE *et al.* 2005), chaperone proteins (CUMMINGS *et al.* 1998; CHAN *et al.* 2000; WAELTER *et al.* 2001), and proteasome subunits (DiFIGLIA *et al.* 1997; CUMMINGS *et al.* 1998; WAELTER *et al.* 2001), etc., are sequestered by the expanded poly(Q) proteins. In agreement with earlier reports (CHAI *et al.* 1999; CHAN *et al.* 2002; JIANG *et al.* 2003; TAYLOR *et al.* 2003; SENGUPTA and LAKHOTIA 2006), the present study shows that the poly(Q) damage

is enhanced by functional depletion of hnRNPs, CBP, or proteasome components because of expression of dominant-negative mutants or RNAi or null mutations. We show that hsr ω -RNAi substantially rescued the poly(Q) toxicity even when additional damage was caused by the presence of mutant alleles of Hrb87F or CBP. On the other hand, compromised proteasome activity affected the rescue of poly(Q) damage by hsr ω -RNAi.

It is significant that while complete absence of Hrb87F does not affect normal development of *Drosophila melanogaster* (ZU *et al.* 1996), ~20% reduction in cellular levels of Hrb87F, as seen in the *Df(3R)Hrb87F/+* eye discs (Figure 1J), resulted in a significant enhancement in the poly(Q) eye phenotype (Figure 1, also see SENGUPTA and LAKHOTIA 2006). As noted earlier, the mutant poly(Q) proteins deplete the functional availability of many essential proteins and thus disrupt cellular homeostasis. Therefore, even a 20% depletion of the otherwise dispensable Hrb87F exaggerates the poly(Q) toxicity. We have shown (MALLIK and LAKHOTIA 2009a) earlier that hsr ω -RNAi results in disappearance of the omega speckles so that the various proteins, including Hrb87F, sequestered in them (LAKHOTIA *et al.* 1999; PRASANTH *et al.* 2000) become available in the soluble cellular pool. The increased availability of such essential proteins in the functional pool following hsr ω -RNAi compensates not only for the genetic deficiency of Hrb87F but also for the functional depletion of this and other proteins by the poly(Q) IBs. This finds support in the fact that targeted overexpression of hnRNP A2/B1 and its *Drosophila* homologs, Hrb87F and Hrb98DE, suppresses CGG repeat-induced neurodegeneration in the FXTAS fly model (SOFOLA *et al.* 2007). It has recently been shown (JI and TULIN 2009) that poly(ADP) ribosylation and deglycosylation of hnRNPs modulate their activity and their binding with the hsr ω transcripts; the authors suggested that only nonribosylated hnRNPs can be sequestered by these transcripts. It is, therefore, likely that the release of hnRNPs from the omega speckles following hsr ω -RNAi provides for a greater pool of the hnRNPs being available for ribosylation and thus activity.

CBP is one of the important regulators of chromatin structure and transcription and its sequestration by the

mutant poly(Q) proteins is believed to be a major cause for neurodegeneration (LI and LI 2004; BAE *et al.* 2005). It is also reported that overexpressing CBP or enhancing its activity suppresses poly(Q) IB formation and neurodegeneration (TAYLOR *et al.* 2003). Our findings that developmental defects in eyes caused by expression of dominant-negative forms of CBP or by its depletion through RNAi are rescued by *hsr* ω -RNAi clearly show that the *hsr* ω transcripts can modulate CBP metabolism in eye disc cells. This possibility is confirmed by our finding that levels of CBP transcripts and that of the CBP protein are elevated following *hsr* ω -RNAi and are lowered by *hsr* ω overexpression.

Our observations that cellular distributions of hnRNPs, like Hrb87F and Hrb57A, partially overlap with that of CBP and that these proteins are co-immunoprecipitated suggest that CBP interacts with Hrb57A and Hrb87F. Downregulation of *hsr* ω -n RNA results in disappearance of the omega speckles and redistribution of the hnRNPs (MALLIK and LAKHOTIA 2009a). In view of the physical association of these proteins, we speculate that the enhanced availability of hnRNPs in the diffuse cellular pool may pull more CBP into the diffuse fraction so that a greater amount of CBP becomes available for activity rather than remaining stored/sequestered. Additionally, caspase-6-mediated cleavage and degradation of CBP followed by a subsequent decrease in histone acetylation, another critical step common to several neuropathologies (ROUAUX *et al.* 2004), may also be inhibited by *hsr* ω -RNAi since our other studies showed that *hsr* ω -RNAi inhibits caspase activity through stabilization of DIAP1 via its interaction with Hrb57A (MALLIK and LAKHOTIA 2009b). The levels of *hsr* ω -n transcripts may also affect CBP mRNA levels through the variety of RNA-processing and transcription factors that directly or indirectly associate with the *hsr* ω transcripts (JOLLY and LAKHOTIA 2006). As reported earlier (TAYLOR *et al.* 2003; SAHA and PAHAN 2006), the net increase in CBP levels, following *hsr* ω -RNAi, would inhibit formation of poly(Q) IBs and restore the histone acetylation homeostasis.

It is remarkable that while *hsr* ω -RNAi suppressed the eye phenotypes resulting from expression of *CBP-FL AD* or *CBP RNAi* or *CBP Δ NZK*, it failed to rescue the lethality or the eye damage following expression of *CBP Δ Q* or *CBP Δ BHQ*, respectively. This indicates that the transactivation domain of CBP is required for the suppressive action of *hsr* ω -n RNAi. It is likely that the hnRNPs like Hrb87F, Hrb57A, etc., interact with CBP through its Q domain so that the hnRNPs released by disappearance of the omega speckles following *hsr* ω -RNAi fail to compensate the damage caused by expression of dominant-negative *CBP Δ Q* or *CBP Δ BHQ*. Further studies are required to understand the mechanism(s) of these interactions.

Our studies also reveal interaction of UPP with *hsr* ω transcripts and an important role of this interaction in

the modulation of poly(Q) toxicity. Restoration of the eye phenotype following targeted disruption of the normal proteasomal activity by *hsr* ω -RNAi indicates that the proteasome activity improves when levels of these non-coding transcripts are reduced. This is also confirmed by the direct demonstration, through the GFP-reporter expression, that the intrinsic UPP is compromised in cells overexpressing *hsr* ω . Our finding that proteasome activity is impaired in 127Q-expressing flies is consistent with earlier reports that poly(Q) toxicity *in vivo* is enhanced by proteasome mutations or by inhibitors of proteasome activity (CHAN *et al.* 2002; VENKATRAMAN *et al.* 2004; BENNETT *et al.* 2005; DIAZ-HERNANDEZ *et al.* 2006; WANG *et al.* 2008). It is significant that *hsr* ω -RNAi ameliorated the proteasomal dysfunction due to poly(Q) expression, since the proteasome-GFP reporter expression was very low in cells coexpressing poly(Q) and *hsr* ω -RNAi (Figure 9). Restoration of proteasome function in mutant poly(Q)-expressing cells is thus an additional pathway through which *hsr* ω -RNAi suppresses the neurodegeneration. This finds further support in the observation that when the endogenous UPP function is intrinsically compromised by expression of dominant-negative mutants, *hsr* ω -RNAi is no longer as effective in suppressing the poly(Q) damage as in cells with normal proteasome function. The mechanism(s) through which the *hsr* ω transcripts regulate UPP pathways remain to be understood.

Many of the poly(Q) proteins involved in CAG repeat expansion disorders contain caspase consensus cleavage sites and caspase-mediated cleavage of the mutant protein appears necessary for pathogenesis (EVERT *et al.* 2000). Inhibition of activity of caspases like caspase-1, caspase-3, or caspase-8 or alteration of the caspase cleavage sites in the mutated protein delays and reduces the expanded poly(Q) protein pathogenicity (GOLDBERG *et al.* 1996; WELLINGTON *et al.* 1998, 2002; ELLERBY *et al.* 1999; ONA *et al.* 1999; SANCHEZ *et al.* 1999; BERKE *et al.* 2004). Our other studies show that in cells in which apoptosis is ectopically induced, *hsr* ω -RNAi stabilizes DIAP1 through enhanced association with Hrb57A (MALLIK and LAKHOTIA 2009b). Elevated levels of DIAP1 inhibit caspase activity and thus apoptosis (HAY 2000; ARYA *et al.* 2007). Further, expression of expanded poly(Q) proteins brings about hyperactivation of JNK (MERIENNE *et al.* 2003; MORFINI *et al.* 2006; SCAPPINI *et al.* 2007), which contributes to neuronal dysfunction and cell death in neurodegenerative disorders. Significantly, *hsr* ω -RNAi suppresses activation of the JNK pathway also (MALLIK and LAKHOTIA 2009b). Inhibition of caspase and JNK activities thus appear to be other paths through which *hsr* ω -RNAi suppresses the poly(Q) toxicity in the fly models.

In summary, we suggest that *hsr* ω -RNAi suppresses poly(Q) toxicity by modulating several components involved in the pathogenesis of these debilitating diseases. First, *hsr* ω -RNAi enhances the availability of

hnRNPs and CBP in functional pools. This in turn would suppress IB formation and restore histone acetylation and transcriptional regulation in cells expressing the mutant poly(Q) proteins (STEFFAN *et al.* 2001; TAYLOR *et al.* 2003; LI and LI 2004; IJIMA-ANDO *et al.* 2005). Second, the proteasomal activity is improved when hsr ω RNA levels are reduced and this helps the cells to get rid of toxic proteins. Third, the release of hnRNPs from omega speckles following hsr ω -RNAi stabilizes DIAP1 (MALLIK and LAKHOTIA 2009b), resulting in inhibition of apoptosis so that neuronal cells, that otherwise would have died, survive. Additionally, in view of the above noted role of JNK in poly(Q) damage, the suppression of JNK activation in eye disc cells following hsr ω -RNAi (MALLIK and LAKHOTIA 2009b) may also contribute to amelioration of the poly(Q) damage. Further, the hsr ω transcripts are known to interact with several other proteins, including Hsp90 (JOLLY and LAKHOTIA 2006), and therefore, it remains possible that other network effects also contribute to the observed suppression of the poly(Q) damage. The observed pleiotropic effects reflect involvement and, therefore, critical importance of the hsr ω noncoding transcripts in cellular homeostasis. Since most of the wild-type poly(Q) proteins, whose mutations result in neurodegeneration, are themselves involved in diverse regulatory processes (CARLSON *et al.* 2009), alterations in the non-coding hsr ω transcript pool can be expected to bring about unpredictable and divergent consequences in cells with genetically compromised regulation. These transcripts apparently function as hubs for coordination of several cellular networks and thus ensure homeostasis. Such multiple networking interactions provide a basis for the context-dependent actions of the same molecule in different cells or in the same cell under different conditions (ARYA *et al.* 2007). The multipronged action of these noncoding transcripts also provides a new paradigm for a therapeutic target for the human poly(Q) disorders.

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