Pleiotropic consequences of misexpression of the developmentally active and stress-inducible non-coding *hsr*ω gene in *Drosophila*

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The non-coding *hsr*ω gene of *Drosophila melanogaster* is expressed in nearly all cell types and developmental stages. However, in the absence of conventional mutant alleles of this gene, its developmental functions remain largely unknown. In the present study, we used a variety of GAL4 drivers to overexpress or ablate this gene's transcripts in specific tissues and examined the developmental consequences thereof. Our results show that a balanced expression of these non-coding transcripts is critical for survival and normal development in all the tissue types tested, since any change in cellular levels of these transcripts in a given cell type generally has detrimental effects, with extreme cases resulting in organismal lethality, although in a few cases the misexpression of these transcripts also suppresses the mutant phenotype due to other genetic conditions. Evidence is also presented for existence of a new spliced variant of the hsrω-n nuclear transcript. Following the RNAi-mediated down-regulation of hsrω transcripts, the omega speckles disappear so that the nucleoplasmic hnRNPs get diffusely distributed, while up-regulation of these transcripts results in greater sequestration of these proteins into omega speckle clusters; either of these conditions would affect activities of the hnRNPs and other hsrω-RNA interacting proteins, which is likely to have cascading consequences. The present findings, together with our earlier observations on effects of altered levels of the hsrω transcripts on induced apoptosis and expanded polyQ-mediated neurodegeneration, further confirm that ncRNA species like the hsrω, far from being evolutionary hangovers, provide critical information for important functions in normal cells.

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1. Introduction

Non-coding RNAs, often regarded as non-functional 'genetic junk', make up the major part of the genome output in higher organisms (Mattick *et al*. 2010). Recent results increasingly suggest that the diverse non-coding transcripts discharge a wide range of functions in the cell through their ability to interact with a diverse repertoire of proteins. A major focus of studies in our laboratory is on one such developmentally active, stress-inducible non-coding gene in *Drosophila*. The *93D* gene, so named because of its location at the 93D4 band on the right arm of chromosome 3 (*www.flybase.org*) is also designated as *heat shock RNA omega* or *hsr*ω (Bendena *et al.* 1989) since it produces multiple heat-inducible transcripts with no identifiable protein product (Lakhotia and Mukherjee 1982; Garbe and Pardue 1986; Hovemann *et al.* 1986; Fini *et al.* 1989). The near ubiquitous presence of hsrω transcripts in *Drosophila* cells (Bendena *et al.* 1991; Mutsuddi and Lakhotia 1995; Lakhotia *et al.* 2001) and the high degree of embryonic lethality associated with *hsr*ω nullisomics

Keywords. EP; hnRNPs; Hrb87F; *hsr*ω; lamin C; non-coding RNA; omega speckles; RNAi

Abbreviations used: AP or A/P, anterior-posterior; DIG, digoxigenin; EP, enhancer promoter; FRISH, fluorescent RNA:RNA *in situ* hybridization; HP1, heterochromatin protein 1; IGC, inter-chromatin granule cluster; ncRNA, non-coding RNA; ORF, open reading frame; RISC, RNA-induced silencing complex; RNAi, RNA interference

(Mohler and Pardue 1984; Lakhotia and Ray 1996) suggest an essential requirement for this gene in vital housekeeping functions during normal development and differentiation of the fly (reviewed in Lakhotia and Sharma 1996; Lakhotia 2003). Since this gene forms one of the largest and transcriptionally most active puffs after a temperature shock (Mukherjee and Lakhotia 1979) and is uniquely inducible by a variety of amides (Lakhotia and Mukherjee 1980, 1984; Tapadia and Lakhotia 1997), these transcripts seem to have a role in management of cellular stress as well (Lakhotia 2003; Jolly and Lakhotia 2006; Lakhotia *et al* 2011).

The *hsr*ω gene is reported to produce a set of three transcripts (Ryseck *et al.* 1985; Garbe *et al.* 1986; Hovemann *et al.* 1986). The largest nucleus-limited hsrω-n transcript is >10 kb and corresponds to the entire transcription unit composed of the 5′ proximal unique region encompassing two exons and an intron followed by a distal stretch of tandem repeats extending for 5 to ~15 kb at the 3′ end. The 1.9-kb-long second nuclear transcript, hsrω-pre-c, is a precursor to the cytoplasmic hsrω-c RNA and results from termination at the first polyadenylation site after the second exon. The ~700 bp intron in hsrω-pre-c is spliced out at the site of transcription (Lakhotia and Sharma 1995) to generate the 1.2 kb cytoplasmic hsrω-c transcript, which carries a short translatable ORF in exon 1, potentially coding for a 24- to 27-amino-acid polypeptide in different species (Garbe and Pardue 1986; Garbe *et al.* 1986; Hovemann *et al.* 1986; Fini *et al.* 1989).

The hsrω-n transcripts are confined to the nucleus and are present, apart from at the site of transcription, as small granules or speckles distributed in the nucleoplasm in close vicinity of the chromatin (Lakhotia *et al.* 1999; Prasanth *et al.* 2000). In unstressed cells, several RNA-binding proteins, including those that belong to the heterogeneous nuclear RNA-binding protein (hnRNP) family, are also bound to the hsrω-n RNA speckles (Lakhotia *et al.* 1999; Prasanth *et al.* 2000). These speckles, which are distinct from the well-known interchromatin granule clusters or IGCs, (Spector 1993), have been named 'omega speckles' (Prasanth *et al.* 2000). The hsrω-n transcripts are essential for organizing the omega speckles since in hsrω nullosomic cells or in those with RNAi-driven ablation of the hsrω-n transcripts, the hnRNPs and other omega-speckle-associated proteins remain diffused in the nucleoplasm (Prasanth *et al.* 2000; Jolly and Lakhotia 2006; Mallik and Lakhotia 2009a, b).

Stress conditions lead to a dramatic reorganization of the omega speckles; following a 40 min heat treatment at 37°C, the minute nucleoplasmic omega speckles form large aggregates that finally get localized to the 93D chromosomal site itself (Lakhotia *et al.* 1999; Prasanth *et al.* 2000). The omega speckles are believed to function as storage sites for the unengaged hnRNPs and related proteins, and depending upon the cellular needs, these are dynamically released from or sequestered in these structures (Lakhotia *et al.* 1999; Prasanth *et al.* 2000; Jolly and Lakhotia 2006). The dynamic trafficking of hnRNPs and related proteins between 'active' and 'inactive' cellular compartments is regulated by the hsrω transcripts to provide an efficient and well-orchestrated mechanism for coordinating nuclear RNA processing activities to ensure maintenance of homeostasis in normal as well as adverse cellular conditions (Jolly and Lakhotia 2006).

A genetic approach to understanding the functions of the non-coding *hsr*ω gene has not been possible because of the high tolerance of base sequence changes in this gene (Mohler and Pardue 1984). Therefore, we developed *EP* (Rorth 1996) and *RNAi* transgenic lines for bringing about conditional and directed changes (Brand and Perrimon 1993) in cellular levels of these transcripts to better understand functions of this gene in normal and stressed cells. In earlier studies using these transgenic lines, we showed that *GAL4*-mediated down-regulation of hsrω transcript levels inhibits induced apoptosis as well as the neurodegeneration following expression of transgenes with expanded polyQ stretches (Mallik and Lakhotia 2009a, b, 2010). With a view to know if this gene has developmental functions as well, we have now examined effects of conditional overexpression or ablation of the hsrω transcripts in different tissues during normal development. We show that alterations in cellular levels of these non-coding transcripts in defined target tissues affects their normal development and differentiation, with global alterations resulting in organismic lethality. We believe that the critical global role of the hsrω-n transcripts in spatial and temporal regulation of nuclear RNA processing activities, through modulation of availability and/or activity of the diverse hnRNPs and other associated regulatory proteins, is responsible for the pleiotropic phenotypic effects following this gene's conditional misexpression.

2. Materials and methods

2.1 *Drosophila stocks*

All fly stocks were reared on standard agar-cornmeal medium at 24±1°C. For cytological preparations, staged larvae were grown in Petri dishes on food supplemented with additional yeast for healthy growth. *Oregon-R*$^{+}$ was used as the wild type. The transgenic lines used for targeted down-regulation (*UAS-hsr*ω*-RNAi*^{3}, referred to in the following as *hsr*ω*-RNAi*) or overexpression (*EP93D* and *EP3037*) of hsrω transcripts have been described previously (Mallik and Lakhotia 2009a). The different *GAL4* drivers {*dpp-GAL4/CyO* (Staehling-Hampton *et al.* 1994), *P{GawB}elav*^{C155} *UAS-mCD8::GFP* (Lin and Goodman 1994), *ptc-GAL4* (Hinz *et al.* 1994), *P{GawB}ap*^{md544}*/CyO* (Calleja *et al.* 1996), *P{GawB}l(2)C805*^{C805}*/CyO* (Hrdlicka

et al. 2002), *P{w[+mW.hs] = GawB}c825* (Manseau *et al.* 1997; Hrdlicka *et al.* 2002), *GMR-GAL4* (Hay *et al.* 1994) and *P{Act5C-GAL4}25F01/CyO* (Ekengren *et al.* 2001)} used for targeted expression of the *EP* or the *RNAi* transgenes were obtained from the Bloomington Stock Center. The homozygous viable *UAS-lamC/UAS-lamC* stock (Gurudatta *et al.* 2010) was obtained from Dr V Parnaik.

Appropriate crosses were performed to obtain the desired progeny genotypes for misexpression of the hsrω transcripts through the desired *GAL4* driver.

2.2 *Reverse transcriptase–polymerase chain reaction and southern hybridization*

For determining the levels of hsrω transcripts following *Act5C-GAL4*-driven expression of the *RNAi* or the *EP* transgenic lines, RT-PCR (figure 1) was carried out using total RNA from heads of adult flies of the desired genotype (s) using TRIzol reagent. First-strand cDNA synthesis was carried out as described earlier (Mallik and Lakhotia 2009a). One-tenth volume of the reaction mixture was subjected to PCR using primers that specifically detect the hsrω-n (forward primer LP: 5′-GGCAGACATACGTACA CGTGGCAGCAT-3′ and reverse primer R1: 5′-TTGCGCTCACAGGAGATCAA-3′; *see* figure 2A) and G3PDH (forward primer 5′-CCACTGCCGAGGAGGTCA ACTA-3′ and reverse primer 5′-GCTCAGGGTGAT TGCGTATGCA-3′) transcripts, as described previously (Mallik and Lakhotia 2009a).

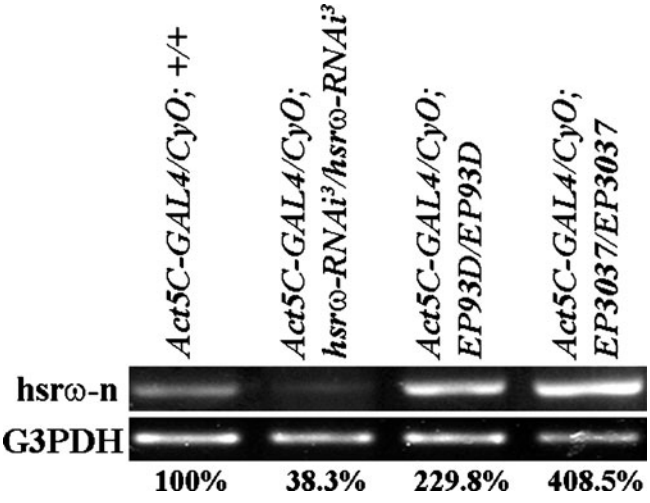

Figure 1. *GAL4*-driven expression of the *hsrω-RNAi* transgene or the *EP* alleles modulates cellular levels of hsrω transcripts. Semi-quantitative RT-PCR, using the LP and R1 primers (see figure **2**A), with total RNA isolated from third instar larvae of different genotypes, noted above each lane, shows that *GAL4*-driven expression of the *hsrω-RNAi* transgene depletes cellular levels of the hsrω-n RNA, while expression of any of the *EP* (*EP93D* or *EP3037*) alleles of the gene enhances hsrω transcript levels. G3PDH amplicons in each sample were used as loading control. The percent value below each lane indicates the relative levels of the hsrω-n RNA in different genotypes.

In another set, RT-PCR reaction was carried out with total RNA isolated from 50 pairs of wild-type third instar larval eye discs using the hsrω-n-specific P1 (5′-GGAAAC AATGAAACCATACGC-3′) and the R1 (as above) primers. The electrophoresed PCR products were blotted onto a positively charged nylon membrane (Roche, Germany) for Southern hybridization using a 1.2-kb-DIG-labelled fragment corresponding to the 93D cDNA from the *pJG10* vector (Bendena *et al.* 1991). After detection of the hybridization signal, the membrane was deprobed in 0.2 N NaOH and 0.1% SDS at 65°C for 40 min with constant shaking and re-hybridized with the 0.6-kb-DIG-labelled *pDRM18* probe corresponding to the intron present in the *hsrω* gene (Bendena *et al.* 1991). Hybridization signals were detected using anti-DIG-alkaline phosphatase (1:10000; Roche, Germany) and dioxetane (CSPD) chemiluminescent substrate according to the manufacturer's instructions (Roche, Germany).

2.3 *Immunoprecipitation*

Total cellular extracts from 50 pairs of wild-type third instar larval eye discs were immunoprecipitated with the mouse monoclonal Hrb87F (P11; Saumweber *et al.* 1980) antibody, as described earlier (Prasanth *et al.* 2000). RNA was extracted from the Hrb87F immunoprecipitate by addition of 300 µl TEL buffer (200 mM Tris-HCl, pH = 7.4, 25 mM EDTA, 100 mM LiCl) containing 1% SDS, followed by treatment with TRIzol reagent as per the manufacturer's protocol (Sigma-Aldrich). The extracted RNA was used for RT-PCR using the above-noted hsrω-n-specific P1 and R1 primers (figure 2A).

2.4 *Adult cuticle preparations*

Freshly eclosed male flies of the desired genotypes were boiled in 10% KOH until their eye colour faded. The samples were then dehydrated through a graded alcohol series and left in clove oil for clearing. Finally, the desired parts of the adult cuticle were dissected out and mounted in clove oil.

2.5 *Pupal lethality assay*

The numbers of larvae of different genotypes that pupated in a given cross were counted and the fate of these pupae monitored. Pupae that died without differentiation or after

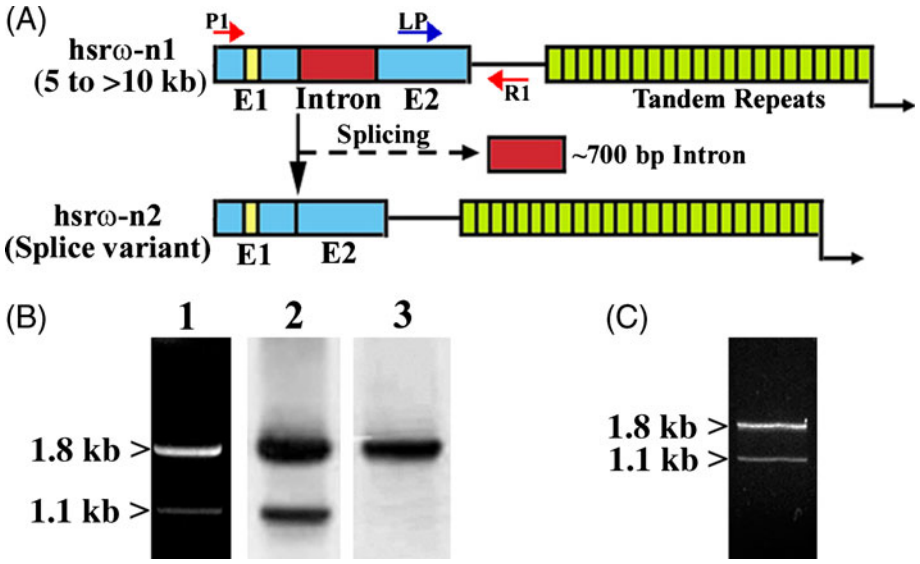

Figure 2. (**A**) A fourth hsrω transcript is generated by splicing of the intron from the hsrω-n RNA **A.** Diagram of the *hsr*ω gene locus showing the two alternate forms of the large nuclear transcripts (hsrω-n1 and hsrω-n2). Locations of the P1, LP and R1 primers (red or blue arrows) used for RT-PCR are also indicated. (**B**) Lane 1 shows ethidium bromide stained PCR amplification products obtained by reverse transcription of total RNA extracted from wild-type larval eye imaginal discs, using the primers P1 and R1. Lanes 2 and 3 show southern hybridization signals, using a blot of the gel in lane 1, following sequential hybridization with DIG-labelled *pJG10* (representing exon1 and exon 2 of the *hsr*ω gene) (lane 2) and then with *pDRM18* (representing the intron of the *hsr*ω gene) (lane 3). (**C**) Ethidium-bromide-stained agarose gel showing the RT-PCR amplicons generated from Hrb87F immunoprecipitated hnRNP complexes from wild-type third instar larval eye imaginal disc homogenates. Sizes of the amplicons are noted on the left margin.

differentiation or those that emerged as flies were counted. In some cases, differentiated pupae were classified as male and female, based on the presence or absence, respectively, of the sex-comb visible through the pupal case.

2.6 *Longevity assay*

For assaying longevity, flies carrying the *elav-GAL4* driver and the desired UAS-responder genes were collected within 8–12 h of eclosion and maintained at 24±1°C. The number of dead flies was counted every day and the survivors transferred to fresh food-vials at 2–3 days interval. At least three replicates were examined for each genotype.

2.7 *Combined fluorescence RNA in situ hybridization and immunostaining*

Malpighian tubules from third instar larvae of the desired genotypes were dissected in Poels' salt solution (PSS; Lakhotia and Tapadia 1998). The tissues were fixed in 4% paraformaldehyde for 1 min, transferred to a drop of 45% acetic acid and lightly squashed under a coverslip on poly-L-lysine coated slides. The coverslips were flipped off with a sharp blade after freezing the slides in liquid nitrogen, and the slides were dehydrated in 95% ethanol. Fluorescence RNA *in situ* hybridization (FRISH), using a DIG-labelled antisense riboprobe generated from the *pDRM30* clone which specifically detects the hsrω-n RNA, was followed by immunostaining with the Hrb87F (P11, 1:10 dilution) antibody as described earlier (Prasanth *et al.* 2000). Cy3-conjugated anti-mouse secondary antibody (1:200, Sigma-Aldrich, India) was used to detect the primary antibody. Chromatin was counterstained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, 1 µg/mL). Samples were examined under an E800 Nikon fluorescence microscope using a 60× (1.4 NA, plan-apo) oil immersion objective.

All images were assembled using Adobe Photoshop 7.0.

3. Results

3.1 *GAL4-driven expression of the hsrω-RNAi transgene or the EP alleles affects hsrω transcripts as expected*

In order to confirm that *GAL4*-driven expression of the *hsrω-RNAi* transgene ablates the hsrω-n RNA while expression of *EP93D* and *EP3037* elevates these transcripts, semi-quantitative RT-PCR was performed with RNA isolated from third instar larvae carrying two copies of

hsrω-RNAi or *EP93D* or *EP3037* together with one copy of the ubiquitously expressed *Act5C-GAL4* driver using the hsrω-n-transcript specific LP and R1 primers (figure 2A). As shown in figure 1, compared with *Act5C-GAL4/CyO; +/+* (lane 1) third instar control larvae, those co-expressing two copies of the *hsrω-RNAi* transgene (lane 2) showed a distinct reduction (38.3%) in cellular levels of the hsrω transcripts. Among the two *EP* lines (figure 1), *Act5C-GAL4*-driven expression of *EP3037* (lane 4) resulted in greater enhancement (408.5%) of hsrω transcript levels than following *EP93D* expression (229.8%, lane 3). G3PDH levels were used as internal control to confirm that variation in the level of hsrω-n transcripts in the different samples was not due to variations in loading. These alterations in the cellular levels of hsrω-n transcripts using the *Act5C-GAL4* driver are in agreement with those noted earlier (Mallik and Lakhotia 2009a) with the eye-specific *GMR-GAL4* driver.

3.2 *A novel non-coding large hsrω transcript, also associated with the omega speckles, is generated by splicing of the nucleus-limited hsrω-n RNA*

So far it has been known that the >10-kb-large nucleus-limited hsrω-n transcript, which corresponds to the entire transcription unit, is not spliced (Garbe *et al.* 1986; reviewed in Lakhotia 2003). Our present results, however, revealed that the large nuclear hsrω-n transcript also exists in spliced as well as unspliced forms. As shown in figure 2B (lane 1), RT-PCR of total RNA isolated from wild-type third instar larval eye discs, using the *hsrω* exon 1 forward primer (P1) and a reverse primer corresponding to the spacer region located between the second exon and the repeat sequences (R1, figure 2A) in the *hsrω* gene, unexpectedly generated two PCR amplicons (figure 2B, 1.8 kb and 1.1 kb, respectively). Since the size difference between the two amplification products correlated exactly with size of the 700 bp intron (figure 2A) present in the hsrω-n transcript, the gel containing the PCR products was subjected to sequential Southern hybridization using DIG-labelled probes corresponding to the two exons (*pJG10*) and the intron (*pDRM18*), respectively. As shown in figure 2B, lane 2, hybridization signals corresponding to both the bands shown in the gel in lane 1 (figure 2B) were obtained using the *pJG10* probe, which detects the two exons only. On the other hand, only a single hybridization signal (figure 2B, lane 3), corresponding to the larger (1.8 kb) PCR amplicon, was seen following hybridization with the DIG-labelled intron-specific *pDRM18* probe. These results indicate that the hsrω-n transcripts exist in two forms, one unspliced and the second with the 700 bp intron spliced out. We named these two transcripts as hsrω-n1 and hsrω-n2, respectively.

To determine if the novel hsrω-n2 splice variant transcript is, like the earlier known unspliced hsrω-n1 transcript (Prasanth *et al.* 2000), also associated with omega speckles, hnRNP complexes from wild-type third instar larval eye disc homogenates were immunoprecipitated. The Hrb87F antibody immunoprecipitated complex was subjected to RT-PCR using the hsrω-n-specific primers (P1 and R1) that distinguish between the hsrω-n1 and hsrω-n2 variant forms of the nuclear hsrω-n transcripts. As shown in figure 2C, RT-PCR, using the P1 and R1 primers, with total RNA extracted from the Hrb87F immunoprecipitated complexes from wild-type larval eye discs produced two amplicons (1.8Kb and 1.1Kb, respectively), reflecting a physical association of the hsrω-n1 as well as hsrω-n2 transcripts with the Hrb87F protein. This suggests that both the transcripts are present in omega speckles.

The levels of the hsrω-n2 transcripts are also affected, in a manner comparable with the above noted levels of hsrω-n1 transcripts, by the *GAL4*-driven expression of *hsrω-RNAi* transgene or any of the two *EP* alleles (data not shown).

3.3 *GAL4-mediated targeted expression of the hsrω-RNAi transgene or the EP alleles has diverse phenotypic consequences*

We targeted expression of the *hsrω-RNAi* transgene or the *EP93D* or *EP3037* alleles to different tissues at various stages of development using several *GAL4* drivers. As noted below, depending upon the domain of expression of the *GAL4* driver, specific effects were seen in each case following RNAi-mediated depletion or EP-mediated over-expression of the hsrω transcripts and the effects were, in most cases, proportional to the number of copies of the *hsrω-RNAi* transgene or the *EP* alleles being driven (*see* table 1 for summary).

3.3.1 *dpp-GAL4:* The *GAL4* in this driver line is prominently expressed in the morphogenetic furrow of eye discs and in a stripe of cells at the anterior-posterior (AP) boundary in other discs of third instar larvae (Staehling-Hampton *et al.* 1994). *dpp-GAL4*-mediated expression of one copy of either *hsrω-RNAi* or *EP93D* or *EP3037* did not show any discernable external eye phenotype (N ~ 300 for each genotype). However, pseudo-pupil images revealed mild disorganization of photoreceptor neurons (not shown). When two copies of the *hsrω-RNAi* transgene or any of the *EP* alleles of the gene were expressed, few survived to adulthood and those that emerged had severe disorganization of photoreceptors in eyes (figure 3A).

3.3.2 *elav-GAL4:* In this driver line, *GAL4* is expressed in all cells of the peripheral (PNS) as well as the central (CNS) nervous system (Lin and Goodman 1994). About 4% of progeny (N=303) carrying one copy of *elav-GAL4*, but no responder transgene, were found to die as undifferentiated

Table 1. Phenotypic effects of *GAL4*-driven misexpression of the *hsr*ω gene (for details, see text)

	Phenotype with *hsr*ω*-RNAi*		Phenotype with *EP93D*		Phenotype with *EP3037*	
GAL4 driver	One copy	Two copies	One copy	Two copies	One copy	Two copies
dpp-GAL4 (homozygous lethal)	Mild disorganization of photoreceptors	Few surviving flies which show severely disorganized photoreceptors	Mild disorganization of photoreceptors	Few surviving flies which show severely disorganized photoreceptors	Mild disorganization of photoreceptors (figure 3A)	Few surviving flies which show severely disorganized photoreceptors
elav-GAL4 (~4% die during pupal stage)	Pupal death rescued; adults with normal lifespan	NA	Pupal death not rescued	NA	Slightly enhanced (12%) pupal death	NA
ptc-GAL4	NE	Substantial lethality	Extra/missing scutellar bristles	Notum and scutellar bristle defects	Changes in scutellar bristle polarity	Extra/missing scutellar bristles
ap-GAL4 (homozygous lethal)	Dose-dependent rescue of the lethality of *ap-GAL4* homozygotes. Wings in flies are ribbon-like out growths (figure 3B)		NA		NA	
P{GawB}l(2)C805^{C805} (homozygous lethal)	NE	~23% pupae die; weak but fertile adults	~21% pupae die; more female survivors; all flies are weak	~65% pupae die; surviving flies very weak (figure 3D)	Pupal lethality; emerging flies very weak	Pupal lethality; emerging flies very weak; males sterile and with sex-comb defects (figure 3C)
GMR-GAL4/GMR-GAL4 (flies exhibit rough eyes)	Eye degeneration in flies carrying two copies of *GMR-GAL4* rescued in *hsr*ω*-RNAi* dose dependent manner		*EP93D* copy number dependent enhancement in roughening of the eye surface		Mild enhancement in eye roughening	Early stage lethality
Act5C-GAL4 (homozygous lethal)	Majority die before adult stage (greater death with two copies of *hsr*ω*-RNAi*. The sex ratio of the survivors skewed in favor of males		Majority die before adult stage in *EP93D* dose dependent manner. More female survivors than males		Majority die before adult stage in *EP3037* dose dependent manner. More female survivors than males	
UAS-lamC/Act5C-GAL4 (~80% die as larvae and the rest as early pupae)	Time of death delayed to early or late pupae; a few reach the pharate stage but with severely malformed head and thorax (figure 3E)	NA	NA	NA	NA	NA

NA, not analysed; NE, no observable effect.

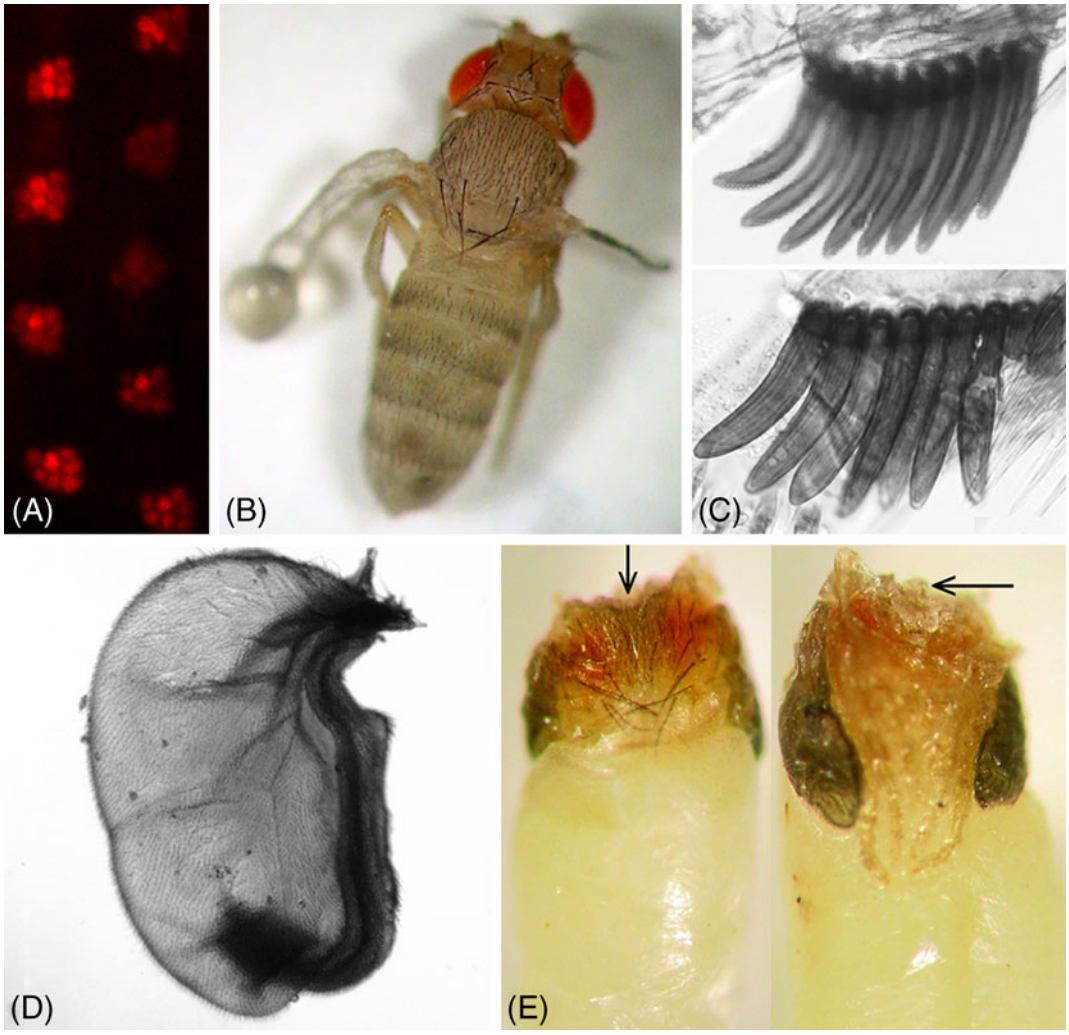

Figure 3. Illustrative phenotypes following misexpression of the *hsr*ω gene (**A**) Pseudopupil image of eye from a *dpp-GAL4/+; EP3037/+* fly. (**B**) Ribbon-like wing seen in *ap-GAL4/ap-GAL4; hsr*ω*-RNAi/hsr*ω*-RNAi* adult fly. (**C**) Compared with the wild-type-like sex combs in w^{1118}*; P{GawB}l(2)C805*^{C805}*/CyO; +/+* (upper panel), the w^{1118}*; P{GawB}l(2)C805*^{C805}*/CyO; EP3037/EP3037* male flies (lower panel) exhibit abnormalities in the sex comb with distortion and malformation of teeth. (**D**) Blistered wings seen in *P{GawB}l(2)C805*^{C805}*/CyO; EP93D/EP93D* adult fly. (**E**) Severe head capsule defects seen in dorsal (left panel) and ventral (right panel) views of *w; UAS-lamC/Act5C-GAL4; hsr*ω*-RNAi/+* pharate dissected out from pupal case.

or fully differentiated pupae. Interestingly, co-expression of one copy of *hsr*ω*-RNAi* rescued the pupal death due to the *elav-GAL4* insertion so that all emerged as adult flies (N= 807) with a normal life span (median life span = 36 days) and without any external phenotype.

In progeny expressing one copy of *EP93D* under the *elav-GAL4* driver (N=476), ~3.0% died as undifferentiated or fully differentiated pharates. The eclosing *elav-GAL4/+; EP93D/+* flies had near normal median lifespan (37.2 days). On the other hand, 12% (N=275) of the progeny expressing one copy of *EP3037* under the *elav-GAL4* driver died during pupal stage. The eclosing flies (88%) had normal median lifespan of 47 days.

Progeny expressing two copies of *hsr*ω*-RNAi* or *EP93D* or *EP3037* under the pan-neuronal *elav-GAL4* driver were not examined.

3.3.3 *ptc-GAL4:* The *GAL4* expression in this line is seen in anterior cells adjacent to the AP boundary in the wing disc, in lamina glia and in subretinal glia cells of the optic stalk and eye discs, in the optic lobes and in the developing genital discs (Hinz *et al.* 1994). Expression of one copy of *hsr*ω*-RNAi* had no observable phenotypic effects. However, expression of two copies of the transgene resulted in substantial lethality.

Expression of one copy of *EP93D* under the *ptc-GAL4* driver resulted in extra or missing scutellar bristles in flies,

while with two copies of the *EP93D* allele, the notum was also deformed in addition to the extra or missing scutellar bristles. One copy of *EP3037* caused changes in scutellar bristle polarity, while with two copies of this allele, extra or missing scutellar bristles were also seen (not shown).

3.3.4 *ap-GAL4:* The GAL4 expression in this driver is seen in the dorso-ventral compartment of wing disc due to insertion of the *P[GAL4]* element in the *apterous* gene; however, this insertion inactivates the *apterous* gene and thus results in recessive lethality (Calleja *et al.* 1996). Interestingly, expression of one or two copies of *hsrω-RNAi* transgene resulted in a dose-dependent rescue of the lethality due to *P[GAL4]* insertion mediated inactivation of the *apterous* gene. Wings in surviving flies, however, were ribbon-like outgrowths without any identifiable structures (figure 3B).

Effect of *ap-GAL4* expression in conjunction with the *EP93D* or *EP3037* alleles was not examined.

3.3.5 *P{GawB}l(2)C805^{C805}:* This *GAL4* driver is expressed in larval ring gland, histoblasts, gut and Malpighian tubules and in adult male accessory glands, testis sheath and cyst cells (Hrdlicka *et al.* 2002). Expression of one copy of the *hsrω-RNAi* transgene under this driver did not show any discernable phenotypic effect, but with two copies of the *hsrω-RNAi* transgene, 22.8% (N =105) of the progeny died during the pupal stage. The remaining *P{GawB}l(2)C805^{C805}/CyO; hsrω-RNAi/hsrω-RNAi* survivors were very weak but fertile.

Expression of one copy of *EP93D* under this driver resulted in death of 20.4% (N=186) pupae. The sex ratio of the surviving adults was skewed in favour of females (1:1.8). On the other hand, amongst the *P{GawB}l(2) C805^{C805}/CyO; EP93D/EP93D* (N=101) progeny, 13.9% died prior to differentiation and 51.5% died as fully differentiated pharates. The surviving progeny flies with one copy of *EP3037* (N=309) were very weak, but in the progeny with two copies (N=60) of the *EP3037* allele, males were sterile and displayed malformed sex comb with reduced number of teeth (figure 3C). A small number of *P {GawB}l(2)C805^{C805}/CyO; EP93D/EP93D* and *P{GawB}l (2)C805^{C805}/CyO; EP3037/EP3037* flies exhibited abnormally everted blistered wings (figure 3D). Similar effects were also seen with the *P{w[+mW.hs] = GawB}c825 GAL4* driver (data not presented). However, expression of either the *hsrω-RNAi* or *EP93D* or *EP3037* transgene using this driver did not result in any sex-comb defects as the domain of expression of the *P{w[+mW.hs] = GawB}c825 GAL4* driver was restricted to the amnioserosa, adult ovary and adult male accessory glands and seminal vesicles (Manseau *et al.* 1997; Hrdlicka *et al.* 2002).

3.3.6 *GMR-GAL4:* This line shows strong expression of *GAL4* in all cells behind the morphogenetic furrow in the developing eye discs (Hay *et al.* 1994). As reported earlier (Mallik and Lakhotia 2009b), expression of the *hsrω-RNAi* transgene (N=236) rescued eye degeneration in a dose-dependent manner in flies carrying two copies of *GMR-GAL4*. Augmentation of hsrω transcript levels using two copies of *EP93D* (N=240) enhanced the rough eye phenotype of flies carrying two copies of the *GMR-GAL4* transgene, while expression of two copies of the *EP3037* allele under control of *GMR-GAL4* resulted in complete lethality during early stages of development (Mallik and Lakhotia 2009b).

3.3.7 *Act5C-GAL4:* In this driver line, the GAL4 protein is ubiquitously expressed under the *Actin5C* promoter. Expression of the *hsrω-RNAi* transgene or any of the two *EP* alleles (the *EP93D* and *EP3037*) resulted in dose-dependent high incidence of larval and pupal death (see below). However, *Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi* and *Act5C-GAL4/CyO; EP93D/EP93D* stocks could be established since the surviving flies in each case remained fertile. With a view to examine effect of *Act5C-GAL4*-driven expression of the *hsrω-RNAi* transgene, two separate crosses were made: in one, both the parents were homozygous for the *hsrω-RNAi* transgene, while in the other, both parents were heterozygous for the *hsrω-RNAi* transgene and the *TM6B* balancer. The latter cross would generate progeny with one as well as two copies of the *hsrω-RNAi* transgene. Two copies of *EP3037* in the presence of *Act5C-GAL4* resulted in greater lethality and sterility. Therefore, *Act5C-GAL4/CyO; EP3037/TM6B* stock was established and used.

Data in table 2 indicate that *Act5C-GAL4*-driven depletion or overexpression of the hsrω transcripts results in significantly enhanced early death (table 2, rows 3 and 4) of the *hsrω-RNAi*- or *EP93D*- or *EP3037*- expressing individuals since the observed numbers of pupae in each case were much less than expected. In crosses between adult flies carrying the *TM6B* balancer chromosome (table 2, columns 4 and 6), tubby (carrying one copy of the *hsrω-RNAi* transgene or the *EP3037* allele and the *TM6B* balancer chromosome, respectively) and non-tubby (with two copies of *hsrω-RNAi* transgene or of the *EP3037* allele, respectively) pupae were expected in a 2:1 ratio. However, in the *Act5C-GAL4/CyO; hsrω-RNAi/TM6B* self-cross, the ratio of tubby: non-tubby pupae was highly skewed in favour of the tubby progeny (table 2, row 4), suggesting a dose-dependent effect of the *hsrω-RNAi* transgene expression on larval viability. In the case of *Act5C-GAL4/CyO; EP3037/TM6B* self-cross also, the ratio of tubby (one copy of the *EP3037* allele) to non-tubby (two copies of *EP3037* allele) was in favour of the former (table 2; also see below).

Since only a very small number of the embryos survived to pupal stage in the above crosses, any death during pupae

Table 2. *Act5C-GAL4*-mediated global misexpression of hsrω transcripts results in early lethality

Survival of progeny	Crosses				
	Act5C-GAL4/CyO; +/+ × Act5C-GAL4/CyO; +/+	*Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi × Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi*	*Act5C-GAL4/CyO; hsrω-RNAi/TM6B × Act5C-GAL4/CyO; hsrω-RNAi/TM6B*	*Act5C-GAL4/CyO; EP93D/EP93D × Act5C-GAL4/CyO; EP93D/EP93D*	*Act5C-GAL4/CyO; EP3037/TM6B × Act5C-GAL4/CyO; EP3037/TM6B*
1. Total no. of eggs examined	1130	2006	1096	1160	1100
2. % fertilized eggs	87.9	86.1	87.5	90.3	88.1
3. % embryos hatched	72.7 (75.0)	71.2 (75.0)	40/2 (56.25)	78.8 (75.0)	59.9 (56.25)
4. % pupae obtained[a]	43.9 (50.0)	31.5 (50.0)	16.2 (37.5)	34.1 (50.0)	21.1 (37.5)
5. Ratio of tubby:non-tubby pupae	NA	NA	5.5:1.0 (2.0:1.0)	NA	2.2:1.0 (2.0:1.0)

The *CyO* or *TM6B* and *Act5C-GAL4* homozygotes are known to die as early embryos and first instar larvae, respectively (*www.flybase.org*). Accordingly, the values in parentheses in rows 3 and 4 indicate the expected percentage of survivors following the early embryonic (*CyO* or *TM6B*) or 1st instar larval (*Act5C-GAL4*) death of homozygous progeny in the given cross; the expected ratio of tubby to non-tubby pupae in columns 4 and 6 of row 5 are indicated in parentheses. [a]The % values in row 4 were calculated with respect to the total fertilized eggs (row 2); NA, not applicable.

to adult development was examined separately, starting with a larger number of progeny pupae (table 3). Data presented in table 3 revealed that with a larger sample size (row 1) also, a greater number of *hsrω-RNAi/TM6B* or *EP3037/TM6B* pupae were present than those homozygous for the *hsrω-RNAi* transgene or the *EP3037* allele in the *Act5C-GAL4/CyO; hsrω-RNAi/TM6B* or *Act5C-GAL4/CyO; EP3037/TM6B* self-crosses, respectively. A significant proportion of pupae expressing two copies of either the *hsrω-RNAi* transgene or the *EP* alleles, especially *EP3037*, died as fully differentiated pharates (table 3, row 3). Most of the surviving *Act5C-GAL4/CyO; EP3037/EP3037* flies were nearly sterile, but the *Act5C-GAL4/CyO; EP3037/TM6B* flies were fertile, although their fecundity was less than normal (data not presented).

Interestingly, *Act5C-GAL4*-driven global reduction in cellular levels of the hsrω transcripts was found to result in greater death of females, while overexpression of either of the *EP* (*EP93D* or *EP3037*) alleles significantly reduced the proportion of male flies that survived to the adult stage (table 3, rows 5 and 6). The male to female ratio, when tested with the χ^2 test, was found to be highly significantly different ($P<0.025$ or 0.005; *see* table 3) from the expected 1:1 ratio in each of these crosses. Since the sex of pupating larvae was not examined, the present data do not provide information about the time of sex-specific death following depletion or overexpression of the hsrω transcripts.

3.3.8 *UAS-lamC/Act5C-GAL4; +/+:* *Act5C-GAL4*-driven ubiquitous overexpression of a full-length *lamin C* cDNA causes 83.2% progeny (N=167) to die as early larvae while the others die soon after pupation. Interestingly, co-expression of a single copy of the *hsrω-RNAi* transgene with *UAS-lamC* under control of the *Act5C-GAL4* driver delayed the early larval lethality so that 42.8% (N=154) of such progeny died as undifferentiated pupae while 48.1% died as differentiated pharates. Also, 93.2% (N=69) of the pharates showed severe defects in the head capsule (figure 3E) in which except for the presence of two red pigmented eye lobes other head structures and a distinct boundary between the head and thorax were absent. Intriguingly, the few flies (9.1%) co-expressing the lamin C and *hsrω-RNAi* transgenes that emerged were all females.

Effects of ubiquitous expression of two copies of the *hsrω-RNAi* transgene or of any of the two *EP* alleles in lamin C overexpression background were not examined.

3.4 *Altered levels of hsrω transcripts affect omega speckles*

The nuclear hsrω-n transcripts co-localize with a variety of hnRNPs to form nucleoplasmic structures called omega

Table 3. Ubiquitous expression of the *hsrω-RNAi* or *EP* transgenic constructs under control of the constitutively expressed *Act5C-GAL4* results in sex-specific lethality

Survival of progeny	Crosses (and progeny classes)							
	Act5C-GAL4/CyO; +/+ × Act5C-GAL4/CyO; +/+	*Act5C-GAL4/2CyO; hsrω-RNAi/hsrω-RNAi × Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi*	*Act5C-GAL4/CyO; hsrω-RNAi/TM6B × Act5C-GAL4/CyO; hsrω-RNAi/TM6B*		*Act5C-GAL4/CyO; EP93D/EP93D × Act5C-GAL4/CyO; EP93D/EP93D*	*Act5C-GAL4/CyO; EP3037/TM6B × Act5C-GAL4/CyO; EP3037/TM6B*		
			Act5C-GAL4/CyO; hsrω-RNAi/TM6B	*Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi*		*Act5C-GAL4/CyO; EP3037/TM6B*	*Act5C-GAL4/CyO; EP3037/EP3037*	
1. Total no. of pupae examined	1243	597	2518	513	611	1900	806	
2. No. (%) of dead undifferentiated pupae[a]	12 (1.0%)	22 (3.7%)	216 (8.6%)	49 (9.6%)	6 (1.0%)	16.0 (0.8%)	127 (15.8%)	
3. No. (%) of dead differentiated pupae[a]	8 (0.6%)	48 (8.0%)	158 (6.3%)	87 (16.9%)	146 (23.9%)	53 (2.8%)	566 (70.2%)	
4. No. (%) of males surviving as adults[a]	615 (49.5%)	297 (49.7%)	1156 (45.9%)	211 (41.1%)	187 (30.6%)	873 (45.9%)	26 (3.2%)	
5. No. (%) of females surviving as adults[a]	608 (48.9%)	230 (38.5%)	988 (39.2%)	166 (32.3%)	272 (44.5%)	958 (50.4%)	87 (10.8%)	
6. Male:female ratio in the surviving adults	1.0:1.0[†]	1.3:1.0[††]	1.2:1.0[††]	1.3:1.0[†††]	1.0:1. 5[††]	1.0:1.1[†††]	1.0:3.3[††]	

[a] The % values in rows 2–5 were calculated with respect to the total number of pupae examined in each case (row 1).
[†] $P>0.5$; [††] $P<0.005$; [†††] $P<0.025$ (χ^2 test).

speckles (Prasanth *et al.* 2000). To examine the status of hnRNPs associated with omega speckles following mis-expression of the *hsr*ω gene, Malpighian tubules from third instar larvae expressing the *hsrω-RNAi* transgene or the *EP93D* or *EP3037* alleles under control of the *Act5C-GAL4* driver were subjected to FRISH with DIG-labelled *pDRM30* antisense riboprobe followed by immunostaining with the P11 antibody, which recognizes the *Drosophila* Hrb87F protein (Saumweber *et al.* 1980). In Malpighian tubule nuclei from *Act5C-GAL4/CyO; +/+* (experimental control) larvae (figure 4A–C), the hsrω-n transcripts were distributed as small speckles in the nucleoplasm and as a single large cluster on chromatin, presumably representing the *hsr*ω gene locus (figure 4B, arrowhead). In agreement with the earlier report (Prasanth *et al.* 2000), immunostaining of these Malpighian tubules with anti-Hrb87F revealed that in addition to a diffuse staining over chromatin regions, Hrb87F co-localized with hsrω-n-RNA-containing omega speckles in the nucleoplasm (figure 4C). The site of *93D* transcription was also strongly decorated by the Hrb87F antibody (figure 4C). *Act5C-GAL4*-driven expression of the *hsrω-RNAi* transgene (figure 4D–F) led to a severe reduction in the number of omega speckles in the nucleoplasm (figure 4E); likewise, hybridization at the site of transcription (figure 4E, arrowhead) was also much less. Interestingly, in parallel with the absence of hsrω-n RNA speckles, hnRNP speckles were also not visible in these nuclei (figure 4F). The majority of the Hrb87F in *Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi* Malpighian tubule nuclei was present in homogenous and diffuse form in the nucleoplasm (figure 4F). On the other hand, *EP*-mediated up-regulation of hsrω transcript levels in *Act5C-GAL4/CyO; EP93D/EP93D* (figure 4G–I) or in *Act5C-GAL4/CyO; EP3037/EP3037* (figure 4J–L) Malpighian tubule nuclei resulted in a different distribution of both hsrω-n transcripts and Hrb87F. In contrast to the abundant fine nucleoplasmic hnRNP speckles seen in *Act5C-GAL4/CyO; +/+* (figure 4C) larval Malpighian tubule nuclei, the number of hnRNP speckles in hsrω overexpressing cells was reduced (figure 4I and L), but the few hsrω-n RNA/hnRNP speckles seen in these nuclei were generally larger than those in the control genotype (figure 4, compare I and L with C). In these nuclei, the hsrω-n transcripts and the Hrb87F protein were present as large aggregates at the site of transcription (figure 4H and K).

4. Discussion

We used the UAS-GAL4 binary expression system (Brand and Perrimon 1993) to either down-regulate or overexpress the large non-coding hsrω transcripts in *Drosophila* in a spatio-temporally regulated manner and examined the

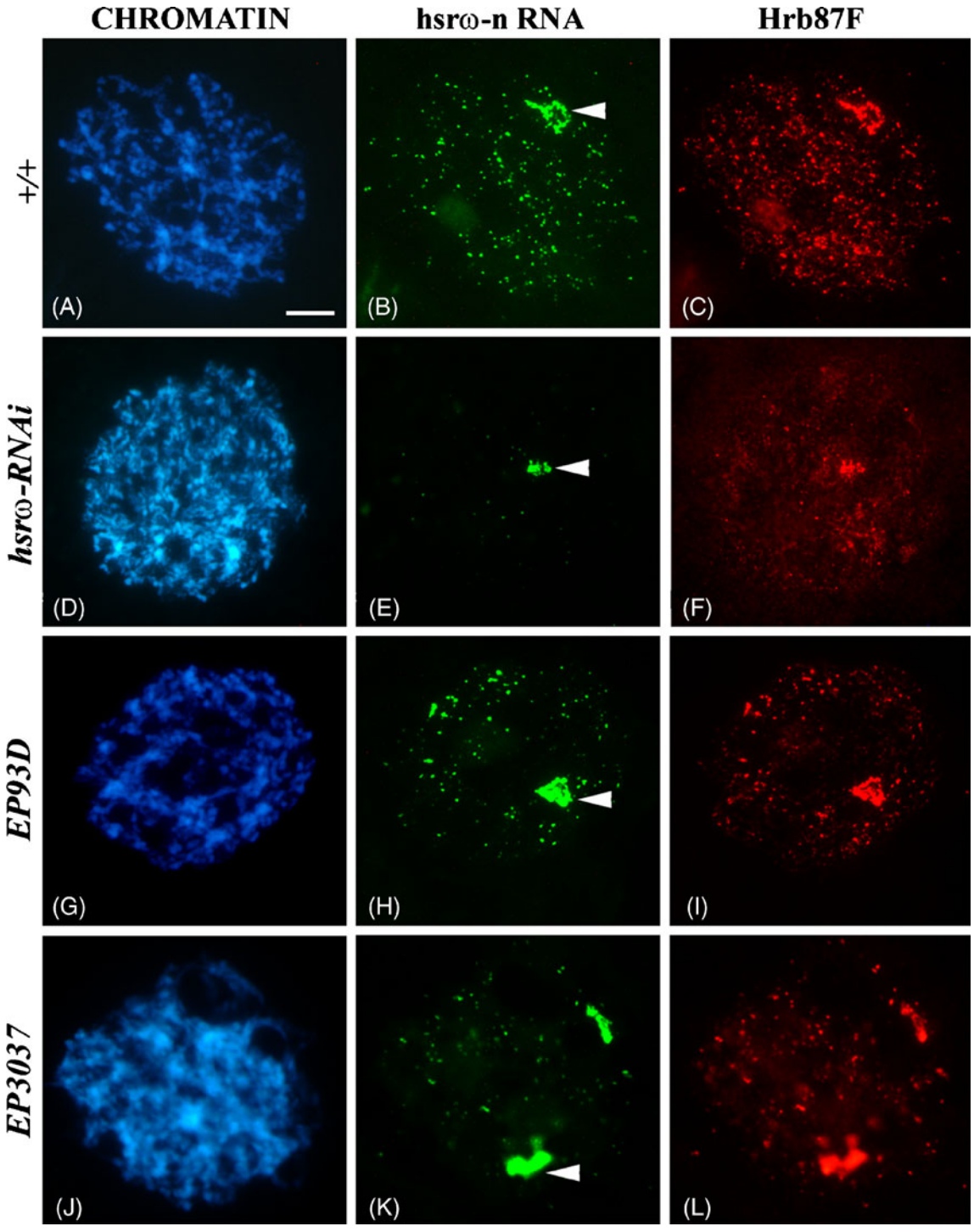

Figure 4. Combined FRISH of *pDRM30* riboprobe to hsrω-n RNA (green, **B**, **E**, **H**, **K**) and immunostaining with the Hrb87F antibody (red, **C**, **F**, **I**, **L**) in Malpighian tubules from *Act5C-GAL4/CyO; +/+* (**A**–**C**), *Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi* (**D**–**F**), *Act5C-GAL4/CyO; EP93D/EP93D* (**G**–**I**), and *Act5C-GAL4/CyO; EP3037/EP3037* (**J**–**L**). Note that the pattern of distributions of the hsrω-n RNA speckles and the Hrb87F hnRNP are identical to each other in all the genotypes examined. Arrowheads in **B**, **E**, **H** and **K** represent the putative chromosomal site of the *hsrω* gene in each nucleus. The nuclei (column 1) were counterstained with DAPI (blue, **A**, **D**, **G**, **J**). Scale bar = 5 μm.

consequences thereof. We showed (figure 1) that following ubiquitous expression of the *hsrω-RNAi* transgene or the *EP* alleles under the *Act5C-GAL4* driver, the endogenous levels of the hsrω transcripts are depleted or enhanced, respectively, as expected. Since the hsrω-n transcript is exclusively nuclear (Garbe *et al.* 1986; Lakhotia and Sharma 1996) and this was effectively down-regulated by expression of the *hsrω-RNAi* transgene (present data and also *see* Mallik and Lakhotia 2009a, b, 2010), it appears that, as in human cells (Robb *et al.* 2005), nuclear RNA-induced silencing complexes (RISC) are present in *Drosophila* cells as well and can efficiently knock down the target nuclear RNA. Indirect evidence for the presence of nuclear RNAi machinery in *Drosophila* is also provided by the fact that localization of HP1 and heterochromatin formation (Pal-Bhadra *et al.* 2004) and contacts between targets of Polycomb group (PcG) proteins (Grimaud *et al.* 2006) are mediated by the RNAi machinery.

During the course of this study, we discovered a novel ncRNA produced by the *hsrω* locus. It has so far been believed (reviewed in Lakhotia and Sharma 1996; Lakhotia 2003) that unlike splicing of the 1.9 kb hsrω-pre-c transcript to produce the 1.2 kb cytoplasmic hsrω-c transcript, the intronic region in the >10-kb-large nuclear hsrω-n transcript is not spliced out. The present results, however, using a novel and informative set of PCR primers, revealed that the 700-bp-long intronic region is spliced out from some of the >10 kb large nuclear hsrω-n transcripts as well and that both the unspliced and spliced forms of the large nuclear transcript remain detectable. The present studies further suggest that, like its precursor, this large spliced product too is likely to be an integral part of the omega speckles since immunoprecipitation of hnRNP complexes pulls down this species of RNA along with the earlier known hsrω-n transcript. It may be noted that using a different set of primers, Prasanth *et al.* (2000) also reported the presence of a shorter (0.5 kb) PCR amplification product corresponding to the exons of the *hsrω* gene in hnRNP immunoprecipitated complexes but ascribed it to the possibility of presence of some of the cytoplasmic 1.2 kb hsrω-c transcripts in the immunoprecipitate. In light of the present finding, it is likely that the spliced form noted by Prasanth *et al.* (2000) was in fact due to the spliced product of the hsrω-n transcript. In this context, recent results by Ji and Tulin (2009), wherein they showed that splicing of the intron from the hsrω-pre-c transcripts is inhibited in poly (ADP-ribose) glycohydrolyase (PARG) null mutants, also need to be re-evaluated. Functional significance of the splice variants of hsrω-n (hsrω-n1 and hsrω-n2) transcripts requires further studies.

Several earlier studies have shown that the *hsrω* gene is transcribed in almost all cells of the organism (Bendena *et al.* 1991; Mutsuddi and Lakhotia, 1995; Lakhotia *et al.* 2001). Nullosomy for *hsrω* gene resulting from two small partially overlapping deficiencies (*Df(3R)e*^{gp4} and *Df(3R)GC14*) in trans-heterozygous condition causes substantial lethality (Mohler and Pardue 1984; Lakhotia and Ray 1996; Lakhotia 2003). However, the mere presence of a ncRNA in cells cannot be a direct demonstration of 'function'. The results obtained with the *hsrω*-nullosomic genotypes were also compromised by the fact that several genes flanking the *hsrω* locus were present in monosomic condition, and therefore, contribution of the monosomic condition of the flanking genes to the organismic lethality remained uncertain. The different phenotypes observed in the present study provide a clear demonstration of the involvement of this non-coding gene in critical functions in diverse cell types since these phenotypes can be causally ascribed to the specific down- or up-regulation of only the hsrω-n transcripts. Absence of a discernable phenotypic effect following *ptc-GAL4* or *P{GawB}l(2)C805*^{C805}-driven expression of one copy of the *hsrω-RNAi* transgene but a distinct phenotype with two copies of the same suggests that these drivers could not down-regulate the hsrω transcripts critically enough in the first case. It is notable that in other cases also the phenotypic effect of *hsrω-RNAi* or *EP* expression generally correlated with the number of copies of each construct in the genome and that the effects of RNAi on a given phenotype were in most cases the reverse of overexpression. Further, the phenotypic effects following down- or up-regulation of the hsrω transcripts by the different *GAL4* drivers always correlated with the domain of expression of the given driver. Thus, ubiquitous down-regulation as well as overexpression of cellular levels of the hsrω transcripts resulted in substantial organismal lethality, while tissue-specific misexpression of the gene led to specific and distinct defects depending on the extent of modulation of hsrω transcripts and the developmental stage and tissue in which the *hsrω-RNAi* or the *EP* constructs were activated. All these establish that the observed phenotypes were indeed due to specific effects of the altered levels of these non-coding transcripts in target cells.

Unlike most other *GAL4* drivers used in this study, *dpp-GAL4*-driven expression of the *hsrω-RNAi* transgene or of the *EP* alleles caused nearly similar phenotypes (disorganized photoreceptors with one copy or substantial lethality with two copies). Similar phenotypic consequences of down- or up-regulation of the hsrω-n transcripts may, in the first instance, suggest a non-specific effect of these transcripts. However, this similarity in the phenotypes may relate to the fact that the *dpp* expressing domain is critical for generating a very specific signalling field that induces other cells to differentiate in a context-dependent manner (Schwank and Basler 2010). Consequently, it is likely that any disturbance in this signaling by any factor would have similar consequences. As discussed later, down- or up-regulation of the hsrω transcripts is expected to affect nuclear RNA metabolism and thus disrupt the Dpp signaling.

It is interesting that some of the *GAL4* drivers, with limited or global domain of expression, caused lethality at larval and/or pupal stages following RNAi or overexpression of the hsrω transcripts. However, in most cases (table 1), the lethality was not 100%. It has been noted earlier also that complete absence of the *hsrω* gene due to trans-heterozygosity of overlapping deletions does not result in 100% lethality (Mohler and Pardue 1984; Lakhotia and Ray 1996). However, while the few surviving *hsrω*-null adult flies were infertile and died in a few days (Mohler and Pardue 1984; Lakhotia and Ray 1996), the *Act5C-GAL4* driven under- or overexpressing progeny that survived to adulthood, not only had a near normal phenotype and longevity, but were also fertile, albeit with a varyingly reduced fecundity in different genotypes (data not presented). A comparable result was obtained with *elav-GAL4*-driven expression of either of the *EP* lines since the surviving flies were fertile and had normal life span. The normal viability and good fertility of some of the progeny in these cases suggest that either the *Act5C-GAL4*- or *elav-GAL4*-driven expression in some proportion of progeny in each case is not strong enough to critically alter the levels of the hsrω transcripts or there exist 'back up' pathways that take care of the critical *hsrω* functions at least in certain proportion of individuals. As a working hypothesis, we favour the latter possibility because the *Act5C-GAL4* or *elav-GAL4*-driven expression of other responder transgenes has generally not been found to vary as much. Further, it is interesting to note from the data in tables 2 and 3 that the pre-adult lethality in the stock established as *Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi* was less than in the first generation of *Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi* homozygotes produced in the *Act5C-GAL4/CyO; hsrω-RNAi/TM6B* cross. We have also noted that since the time of establishment of the *Act5C-GAL4/CyO; hsrω-RNAi/hsrω-RNAi* and *Act5C-GAL4/CyO; EP93D/EP93D* stocks, they have become somewhat better established stocks although certain degree of lethality of the progeny continues in each generation. This may suggest a rapid selection or activation of the 'backup' pathways through epigenetic or other means. The nature and population dynamics of the possible 'back up' for *hsrω* functions need further study.

The two *EP* lines used for overexpression of the *hsrω* gene produced varying phenotypic consequences. As also noted earlier (Mallik and Lakhotia 2009a, b, 2010), *GAL4*-driven expression of the *EP3037* allele produced higher levels of the hsrω-n transcripts (figure 1) and accordingly resulted in most cases in more severe phenotypes than the *EP93D*. These differences are presumably related to the different sites of insertion of the *EP*-element in the promoter region (130 bp in *EP93D* and 144 bp in *EP3037*; *see* Mallik and Lakhotia 2010) of the *hsrω* gene so that the degree of enhancement of the hsrω transcript levels in the two cases varies in response to the specific *GAL4* driver, which in turn would varyingly affect the cell type's activity. Furthermore, we have also noted that even in absence of any *GAL4* driver, the *EP3037* line is weaker than the *EP93D* line, which may reflect different consequences of the site of insertion of the *EP* transposon.

In agreement with previous observations with *hsrω* nullosomics (Lakhotia and Ray 1996; Lakhotia *et al.* 1999), the present observations also show that levels of these transcripts affect sex-specific viability since global reduction in levels of hsrω transcripts with *Act5C-GAL4* driver resulted in death of more female than the male progeny, while overexpression with *Act5C-GAL4* or *P{GawB}l(2)C805*^{C805} driver caused greater mortality of males. Mutations in many of the genes regulating sex determination and dosage compensation in *Drosophila* exhibit similar sex-specific lethality. Previous studies (Samuels *et al.* 1994; Lakhotia *et al.* 1999) have shown that the key sex-determining and RNA-binding Sxl protein gets localized exclusively to the 93D locus after heat shock. Sxl, in addition to regulating its own splicing, also regulates the sex-specific alternative splicing of a number of pre-mRNAs involved in sex determination and dosage compensation pathways (Penalva and Sanchez 2003). Further studies are needed to know if perturbation in the levels of hsrω transcripts following expression of the *hsrω-RNAi* transgene or the *EP* alleles causes sex-specific lethality by affecting the Sxl pathway.

Earlier studies (Prasanth *et al.* 2000; Lakhotia 2003) demonstrated that the nucleus-limited hsrω-n transcripts act as the organizer for sequestering a variety of hnRNPs and other related RNA-processing proteins that are not engaged in active transcriptional activities in the form of omega speckles. The present observations provide further evidence for this role since a strong correlation between the levels of hsrω transcripts and the size and numbers of omega speckles was seen in the *GAL4*-induced *hsrω-RNAi* or the *EP93D* and *EP3037* constructs (also see Mallik and Lakhotia 2009a). Omega speckles regulate the dynamics of a variety of RNA-binding proteins that are involved in nuclear RNA processing (Lakhotia *et al*. 1999; Prasanth *et al*. 2000; Jolly and Lakhotia 2006). Any perturbation in these essential events is expected to have wide-ranging consequences on the downstream processes that are critically dependent on appropriate RNA processing and transport. It is interesting that while in most cases, hsrωRNAi had deleterious consequences, *elav-GAL4*- or *ap-GAL4*- or *GMR-GAL4*-driven expression of the *hsrω-RNAi* transgene actually appeared 'beneficial', since either the lethality or eye phenotype associated with these *GAL4* drivers were varyingly suppressed by the RNAi. In earlier reports also we had noted that induced apoptosis or the neurodegeneration following expression of proteins with expanded polyQ stretches (Mallik and Lakhotia, 2009a, b, 2010) were also suppressed by hsrω-RNAi. In the cases of induced apoptosis or polyQ-mediated neurodegeneration, the suppression of the respective phenotypes following co-expression of the

*hsrω-RNA*i transgene was found to result indirectly from the enhanced availability of hnRNPs, etc., following down-regulation of the hsrω transcripts and consequent disappearance of the omega speckles (Mallik and Lakhotia 2009b, 2010). Reasons for the observed lethality or eye damage due to homozygosity of the *ap-GAL4* or *GMR-GAL4* transgene, respectively, are not well understood, but the rescue of these phenotypes by hsrω-RNAi is likely to be related to altered hnRNP metabolism. This, however, needs further analysis.

Induced overexpression of lamin C causes cell death through activation of the canonical caspase cascade in *Drosophila* (Stuurman *et al*. 1999; Gurudatta *et al.* 2010). Additionally, it not only leads to depletion of cellular levels of HP1 but also reduces HP1 binding at the chromocenter (Gurudatta *et al.* 2010). Thus, the early death of individuals in which the *Act5C-GAL4* driver causes global overexpression of lamin C can be ascribed to widespread apoptosis induced by the high levels of lamin C (Gurudatta *et al*. 2010). We have shown earlier (Mallik and Lakhotia 2009b) that depletion of hsrω transcripts through conditional RNAi substantially inhibited caspase-mediated induced apoptosis in *Drosophila*. Thus, co-expression of *hsrω-RNAi* may prevent apoptosis induced by excess lamin C in many cells and this may delay the death of lamin-C-overexpressing individuals. The lamin A/C speckles are known to co-localize with certain splicing factors and mediate the spatial organization of splicing factor compartments (Jagatheesan *et al*. 1999; Kumaran *et al*. 2002); further, a proper organization of nuclear lamina is essential for appropriate gene activity in different cell types (Kind and van Steensel 2010). Therefore, the severe perturbations in nuclear architecture that follow overexpression of lamin C is expected to disrupt normal development and differentiation. In this context, it is likely that the disappearance of omega speckles following hsrω-RNAi enhances the availability of the various RNA-binding proteins and this may partly circumvent the expected sequestration of the splicing factors by the overabundance of lamin A/C. Thus, hsrω-RNAi can not only prevent apoptosis and delay the organismal death but may also facilitate normal cell differentiation in spite of lamin overexpression so that at least some (9%) of these individuals develop as phenotypically normal adult flies.

The apparently 'beneficial' effects of down-regulation of hsrω transcripts are seen only under conditions of severe pathology of cells, e.g. induced apoptosis (Mallik and Lakhotia 2009b), expanded polyQ-induced neurodegeneration (Mallik and Lakhotia 2009a), or lethality following inactivation of the *ap* gene due to insertion of the *ap-GAL4* transgene or the severe abnormalities and lethality following overexpression of lamin C. It appears that in all these cases, disappearance of the omega speckles results in an increased availability of the various RNA-binding proteins, which otherwise remain sequestered in the omega speckles, and this may neutralize some or all of the severe pathological state. On the other hand, the cellular homeostasis in a normal cell would be disturbed by any reduction or enhancement of these transcripts and thus would result in abnormal differentiation.

The different *GAL4* drivers used in our study do not share a common target, neither cell type nor a particular class of cell function, and yet in each case domain-specific phenotypic consequences follow the targeted under- or overexpression of hsrω transcripts. We suggest that such diverse and wide-ranging phenotypic consequences of altered levels of a ncRNA are to be viewed in the context of pivotal role of the hsrω-n transcripts in organizing the omega speckles and, thereby, regulating activities of hnRNPs and several other regulatory factors (Lakhotia *et al.* 1999; Prasanth *et al.* 2000; Jolly and Lakhotia, 2006). With the multifarious activities mediated by hnRNPs (Han *et al* 2010), it is expected that disruption in their nuclear distribution would have cascading consequences. Recent studies further show that these non-coding transcripts also modulate, directly or indirectly, activities and/or levels of members of the cell death machinery, JNK-signalling, proteasome and histone acetyltransferase like CBP (Mallik and Lakhotia 2009b, 2010). Other studies in our laboratory (Lakhotia *et al*. 2011) reveal that optimal levels of the hsrω transcripts are essential for restoration of various hnRNPs, HP1 and RNA pol II to their normal nuclear sites during recovery from heat shock. Other results (Onorati *et al.* 2011) reveal a direct interaction of these non-coding transcripts with the ISWI, an important component of chromatin remodelling complexes. With such key and diverse roles in cell regulation, it is not surprising that under- as well as overexpression of these non-coding transcripts affects normal development and differentiation. Emergence of this new functional dimension of RNA activity further challenges the conventional belief that ncRNA molecules are 'junk'. It is evident that an understanding of the intricate regulatory circuits governing gene expression that orchestrate the precise organization of living organisms will require an in-depth analysis and understanding of the diverse non-coding transcripts.

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