Expression of *hsr ω -RNAi* transgene prior to heat shock specifically compromises accumulation of heat shock-induced Hsp70 in *Drosophila melanogaster*

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Abstract A delayed organismic lethality was reported in *Drosophila* following heat shock when developmentally active and stress-inducible noncoding *hsr ω -n* transcripts were down-regulated during heat shock through *hs-GAL4*-driven expression of the *hsr ω -RNAi* transgene, despite the characteristic elevation of all heat shock proteins (Hsp), including Hsp70. Here, we show that *hsr ω -RNAi* transgene expression *prior* to heat shock singularly prevents accumulation of Hsp70 in all larval tissues without affecting transcriptional induction of *hsp70* genes and stability of their transcripts. Absence of the stress-induced Hsp70 accumulation was not due to higher levels of Hsc70 in *hsr ω -RNAi* transgene-expressing tissues. Inhibition of proteasomal activity during heat shock restored high levels of the induced Hsp70, suggesting very rapid degradation of the Hsp70 even during the stress when *hsr ω -RNAi* transgene was expressed ahead of heat shock. Unexpectedly, while complete absence of *hsr ω* transcripts in *hsr ω ⁶⁶* homozygotes (*hsr ω -null*) did not prevent high accumulation of heat shock-induced Hsp70, *hsr ω -RNAi* transgene expression in *hsr ω -null* background blocked Hsp70 accumulation. Nonspecific RNAi transgene expression did not affect Hsp70 induction. These observations reveal that, under certain conditions, the stress-induced Hsp70 can be selectively and rapidly targeted for proteasomal degradation even during heat shock. In the present case, the selective degradation of Hsp70 does not appear to be due to down-regulation of the *hsr ω -n* transcripts per se; rather, this may be an indirect effect of the expression of *hsr ω -RNAi* transgene whose RNA products may titrate away some RNA-

binding proteins which may also be essential for stability of the induced Hsp70.

Keywords lncRNA · Proteasome · *hsr ω* · CHIP · Hsc70

Introduction

Heat shock response is one of the most conserved cellular cascades that effectively protect cells and the organism from adverse environmental conditions like physiologically high temperature, oxidative stress, cytotoxins, etc. (Craig 1985; Lindquist 1986; Feder and Hofmann 1999). While the classical hallmark of the cell stress response has been the rapid induction of different families of heat shock or stress proteins, multiple long noncoding RNAs (lncRNAs) are now also known to be involved in the cell stress response (Lakhotia 2012; Place and Noonan 2014). The *93D/hsr ω* gene of *Drosophila*, with its multiple transcripts, is the earliest known lncRNA gene having significant roles in development and in cell stress response (Lakhotia and Mukherjee 1982; Mohler and Pardue 1982; Lakhotia 2011). The *hsr ω* gene comprises a proximal region (~2.6 kb), with two exons, E1 (~475 bp) and E2 (~750 bp), an intron (~700 bp), and a distal >5-kb region carrying short tandem repeats of 280 bp, unique to the locus (Lakhotia 2012); till recently, it was believed to produce two primary transcripts, viz., *hsr ω -n1* (*hsr ω -RB*) and *hsr ω -pre-c* (*hsr ω -RC*) from which the intron is spliced out to produce the *hsr ω -n2* (*hsr ω -RG*) and *hsr ω -c* (*hsr ω -RA*) transcripts, respectively (Lakhotia 2011). Out of these four *hsr ω* transcripts (two primary and two processed), the *hsr ω -c* is cytoplasmic, while the other three are nuclear. Recent annotation at the Flybase (<http://www.flybase.org>) indicates that the *hsr ω* gene is longer than previously believed and produces additional transcripts. Little is known about the newly

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annotated *hsrw* transcripts although other studies in our lab (Mustafa and Lakhotia unpublished) confirm the presence of these new transcripts (*hsrw*-RD and *hsrw*-RF) and that the nearly 21 kb *hsrw*-RF transcript is heat shock inducible. A very small 23-bp long translatable open reading frame (ORF) is present in the *hsrw*-c of *Drosophila melanogaster*, although its translation product has not yet been identified (Fini et al. 1989).

The nucleus-limited *hsrw*-n transcripts (*hsrw*-RB and RG on Flybase) interact with several RNA processing proteins and organize the nucleoplasmic omega speckles (Prasanth et al. 2000; Jolly and Lakhotia 2006; Onorati et al. 2011; Singh and Lakhotia 2015). The omega speckles function as storage sites for several RNA processing proteins, which are dynamically released from or sequestered by the *hsrw*-n transcripts according to cellular needs (Lakhotia et al. 1999; Lakhotia 2011; 2012; Jolly and Lakhotia 2006; Singh and Lakhotia 2015). The *hsrw*-null individuals are poorly viable and are thermosensitive (reviewed in Lakhotia 1989; 2011). A recent study from our laboratory (Lakhotia et al. 2012) showed that conditional down- or up-regulation of the *hsrw* nuclear transcripts through *hs*-*GAL4*-driven activation of *UAS-hsrw-RNAi* transgene (Mallik and Lakhotia 2009) or of *EP* (Brand and Perrimon 1993) alleles of *hsrw*, respectively, during heat shock results in delayed organismic lethality in spite of the characteristic elevation in cellular levels of the different HSPs, including the Hsp70. The delayed organismic lethality in these genotypes during recovery was correlated with the absence of omega speckles and a slow and incomplete restoration of the hnRNPs on developmentally active gene loci during recovery after heat shock (Lakhotia et al. 2012). It is known (Mallik and Lakhotia 2011) that global activation of *UAS-hsrw-RNAi* transgene by *Act-GAL4* driver also disrupts the omega speckles. Therefore, we wanted to see how the disruption of omega speckles in unstressed cells through down- or up-regulation of *hsrw* transcripts affects their stress response. Accordingly, in the present study, we have examined heat shock response in tissues where *hsrw* transcripts were down- or up-regulated sometime before the cells were exposed to heat shock. We found that expression of *UAS-hsrw-RNAi* transgene in unstressed cells severely affected the cellular levels of Hsp70 during heat shock as well as during subsequent recovery. Interestingly, expression of the *UAS-hsrw-RNAi* transgene did not affect heat shock-induced transcription, transport, and stability of the hsp70 messenger RNAs (mRNAs) but enhanced rapid degradation of the synthesized Hsp70 through proteasomal pathway even when the cells were under stress.

Materials and methods

Fly strains

All flies were cultured on standard cornmeal-agar food medium at 24 ± 1 °C. *Oregon R*⁺ strain was used as wild type (WT).

Three *hsrw-RNAi* lines (Mallik and Lakhotia 2009), viz., *w*; +/+; *UAS-hsrw-RNAi*³/*UAS-hsrw-RNAi*³, *w*; *UAS-hsrw-RNAi*²/*UAS-hsrw-RNAi*²; +/+, and *w*; *UAS-hsrw-RNAi*(2X)/*UAS-hsrw-RNAi*(2X); +/+ were used for down-regulation of *hsrw* transcripts under globally expressed *Actin5C-GAL4* (Ekengren et al. 2001) referred to here as *Actin-GAL4* or salivary gland (SG)-specific *Sgs3-GAL4* (Cherbas et al. 2003) or the differentiating eye disc-specific *GMR-GAL4* (Ellis et al. 1993; Ray and Lakhotia 2015) driver. The *UAS-hsrw-RNAi* transgene carries the *hsrw* gene's 280-bp repeat unit (Lakhotia 2011) in the *SympUAST-w* vector which causes both strands to transcribe when activated by a GAL4 driver and thus causes down-regulation of the repeat-containing *hsrw*-n transcripts (Mallik and Lakhotia 2009). Most of the studies were carried out with *w*; +/+; *UAS-hsrw-RNAi*³/*UAS-hsrw-RNAi*³ line, referred to as *hsrw-RNAi*. The *w*; +/+; *EP3037/EP3037* line carries an EP element (Brand and Perrimon 1993) at -144 position from the *hsrw* gene's major transcription start site (www.flybase.org) and was used for the GAL4-driven over-expression of *hsrw* transcripts. The *w*; +/+; *hsrw*⁶⁶/*hsrw*⁶⁶ (Johnson et al. 2011; Lakhotia et al. 2012) stock was used as *hsrw*-null. The *hsrw*⁶⁶ allele carries a deletion of ~1.6 kb of the promoter region (including the first nine bases corresponding to the exon-1 of *hsrw* gene) and thus does not produce any of the *hsrw* transcripts (Johnson et al. 2011; Lakhotia et al. 2012). To see the effect of transgenic expression of *hsrw*-c transcripts, *Sp/CyO*; *UAS-pre-c1.9/UAS-pre-c1.9* transgenic stock was used in which complementary DNA (cDNA) from the 1.9 kb *hsrw*-pre-c (*hsrw*-RA) transcript is placed under the *UAS* promoter and the transgene is inserted on chromosome 3 (Akanksha 2012). To see if expression of *UAS-hsrw-RNAi* under *Act-GAL4* driver causes any change in levels of the various Hsc70 proteins which may affect Hsp70 induction following heat shock, expression of YFP-tagged Hsc70Cb (115570, Kyoto DGGR) was examined in +/+; *Hsc70Cb-YFP*/+ and *ActGAL4*/+; *Hsc70Cb-YFP/hsrw-RNAi* larvae obtained by appropriate fly crosses.

Three other RNAi transgenic lines, viz., *Egfr-RNAi* (31525, Bloomington) with a 443-bp insert, *Hsp60C-RNAi* with a 504-bp insert (Sarkar and Lakhotia 2005), and *Hsp60D-RNAi* with a 671-bp insert (Arya and Lakhotia 2008), were also used to examine the effect of *Act-GAL4*-driven expression of these RNAi transgenes on Hsp70 induction. Appropriate crosses were made to obtain *Act-GAL4*/+; *Egfr-RNAi*/+ or *Act-GAL4/Hsp60C-RNAi*; +/+ or *Act-GAL4*/+; *Hsp60D-RNAi*/+ progeny larvae.

Heat shock and recovery

Actively wandering late third-instar larvae of desired genotypes were transferred in batches to prewarmed microfuge tubes lined with moist filter paper and submerged in a water bath maintained at 37 ± 1 °C for the desired duration (see

“Results” section). Control samples of larvae of comparable age and genotypes were kept in microfuge tubes containing moist filter papers at 24 ± 1 °C in parallel. The control/heat-shocked third-instar larvae were either immediately dissected or transferred to vials containing standard food medium for recovery at 24 ± 1 °C. For assessing viability of the control/heat-shocked larvae, numbers of those pupating and finally emerging as adult flies were counted.

SDS-PAGE and western blotting

Following heat shock or the desired period of recovery from heat shock, either total late third-instar larvae or specific larval tissues were dissected out in Poels’ salt solution (PSS, Lakhotia and Tapadia 1998) and immediately lysed in boiling sodium dodecyl sulfate (SDS) sample buffer for 10 min. In one set, *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN, calpain inhibitor, Sigma-Aldrich, India) was used for inhibiting the proteasomal activity (Zhou et al. 1996) during heat shock. For this purpose, SGs of late third-instar larvae of desired genotype (see “Results” section) were dissected out in PSS and transferred to 37 °C for 1 h either in fresh PSS as control or in PSS containing 10 mM ALLN before lysis in the SDS sample buffer. Following lysis, proteins were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting as described earlier (Prasanth et al. 2000). Primary antibodies used were rabbit anti-Hsp60 (1:500 dilution, Stressgen), rat anti-Hsp70 (7Fb, 1:1000 dilution, Velazquez and Lindquist 1984), rat anti-Hsc/Hsp70 (7.10.3, 1:500 dilution, Palter et al. 1986; Zatsepina et al. 2001), mouse anti-Hsp90 (1:500 dilution, Morcillo et al. 1993), and mouse anti- β -tubulin (E7, 1:100 dilution, Sigma-Aldrich, India). Corresponding secondary antibodies were HRP-tagged anti-rabbit, anti-rat, and anti-mouse (1:1500 dilution, Bangalore Genei, India). The signal was detected using the Supersignal West Pico Chemiluminescent Substrate kit (Pierce, USA). For each treatment and genotype, western blotting was repeated at least three times. For reprobing a blot with another antibody, following the detection of the first antibody binding, the blot membrane was kept in stripping buffer (100 mM 2-mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl pH 6.8) at 50 °C for 30 min on a shaker followed by processing for detection with the desired second antibody.

Tissue immunofluorescence

Following heat shock/recovery, tissues were dissected out from larvae of the desired genotypes and processed for immunostaining as described earlier (Prasanth et al. 2000). Primary antibodies used for detection of Hsp70 and the P-body-specific GW182 protein were rat 7Fb (1:100 dilution, Velazquez and Lindquist 1984) and mouse anti-GW182 (1:200 dilution, Ding and Han 2007), respectively. Cy3-

conjugated anti-rat or anti-mouse antibodies (1:200 dilution, Sigma-Aldrich, India) were used as secondary antibodies as required.

Polytene chromosome squash preparation and immunostaining

To examine the puffing of heat shock genes after heat shock/recovery, SGs from actively wandering late third-instar larvae of the desired genotypes were dissected out in $1 \times$ phosphate-buffered saline (PBS), fixed in aceto-methanol for 30 s, and stained with aceto-orcein for 10 min, following which, the SGs were squashed in 45 % acetic acid. Preparations were examined by phase-contrast microscopy.

To examine the distribution of RNA Pol II on polytene chromosomes, heat-shocked SGs from larvae of desired genotype were dissected out in $1 \times$ PBS, squashed, and processed as described earlier (Singh and Lakhotia 2012). Active RNA Pol II (hyperphosphorylated form) on these chromosomes was detected with the mouse H5 antibody (1:50 dilution, Bregman et al. 1995) and Cy3-labeled anti-mouse (1:200 dilution, Sigma-Aldrich, India) secondary antibody.

RNA-RNA in situ hybridization

To localize the hsp70 transcripts in intact tissue, RNA-RNA in situ hybridization was carried out with digoxigenin-labeled hsp70 riboprobe generated by in vitro transcription of the *pPW18* clone as described (Sharma and Lakhotia 1995). SGs from late third-instar larvae were dissected out in RNase-free $1 \times$ PBS following heat shock/recovery and processed for RNA/RNA in situ hybridization as earlier (Lakhotia et al. 2001). The hybridization signal was detected with rhodamine-conjugated anti-dig antibody (1:200 dilution, Roche, Germany).

RNA isolation, RT-PCR, and RT-qPCR

Following heat shock/recovery, 20 pairs of SG were dissected out from late third-instar larvae of the desired genotype and total RNA was isolated using the TRI reagent as per the manufacturer’s (Sigma-Aldrich, India) instructions. RNA pellets were resuspended in nuclease-free water. The cDNA was synthesized as earlier (Lakhotia et al. 2012), and PCR was carried out for G3PDH transcripts, as loading control, with 5’-CCACTGCCGAGGAGGTCAACTA-3’ as the forward and 5’-GCTCAGGGTGATTGCGTATGCA-3’ as the reverse primers. The hsp70 cDNA was amplified using 5’-AGGGTCAGATCCACGACATC-3’ as forward and 5’-CGTCTGGGTTGATGGATAGG-3’ as reverse primers. The thermal cycling parameters included an initial denaturation at 94 °C for 4 min followed by 25 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. Final extension was at 72 °C for

10 min. The PCR products were electrophoresed on 2.0 % agarose gel with a 100-bp DNA ladder marker (Bangalore Genei, India). In each case, the RT-PCR was carried out with at least two independently prepared RNA samples. RNA isolated as above was also processed for real-time quantitative PCR (RT-qPCR) for Hsp70 and G3PDH transcripts, using the above primers and SYBR Green dye on 7500 Real-Time PCR System (Applied Biosystems) with the 7500 software v2.0.4.

Microscopy

Aceto-orcein-stained polytene chromosome preparations were examined under Nikon E800 microscope with $\times 100$ oil immersion phase-contrast objective. Images were captured using DS-Fi1c Nikon camera. Fluorescence imaging of immunostained or RNA/RNA in situ hybridized tissues was carried out with LSM 510 Meta Zeiss laser scanning confocal microscope, using appropriate filters/dichroics for the given fluorochrome. Each immunostaining and in situ hybridizations were carried out at least twice. All images were assembled using Adobe Photoshop 7.0.

Results

Globally altered developmental expression of *hsw* transcripts causes delayed lethality after heat shock treatment

We used the globally active *Act-GAL4* driver (Ekengren et al. 2001) to either down-regulate the *hsw*-n transcripts through expression of the *hsw-RNAi* transgene or up-regulate *hsw* gene using the *EP3037* allele (Mallik and Lakhotia 2009). To examine effects of constitutively altered expression of *hsw* gene on thermotolerance, actively wandering third-instar WT, *Act-GAL4/CyO*; +/+, *Act-GAL4/CyO*; *hsw-RNAi*, and *Act-GAL4/CyO*; *EP3037* larvae were heat shocked at 37 °C for 1 h and their further development at 24 °C was monitored till emergence of flies. As shown in Fig. 1, while almost all parallel control (unstressed) wild type (WT) and *Act-GAL4/CyO*; +/+ larvae pupated and emerged as healthy flies, only 87 ± 3 % of *Act-GAL4/CyO*; *hsw-RNAi* larvae and 15 ± 2 % of *Act-GAL4/CyO*; *EP3037* control (unstressed) larvae emerged as adult flies (also see Mallik and Lakhotia 2011). Following 1-h heat shock, all WT and *Act-GAL4/CyO*; +/+ larvae pupated and most of them emerged as flies (Fig. 1). However, heat shock caused larval as well as pupal lethality in *Act-GAL4/CyO*; *hsw-RNAi* line resulting in only about 20 % fly emergence (Fig. 1). The *Act-GAL4/CyO*; *EP3037* larvae were more thermosensitive since although ~ 95 % of the heat-shocked larvae pupated, none of them emerged as flies (Fig. 1).

Fig. 1 Global down- or up-regulation of *hsw* transcripts prior to heat shock enhances thermosensitivity. Graphical presentation of survival of control and heat-shocked third-instar larvae of different genotypes (noted on X-axis) to subsequent pupal and adult stages (pupae, solid-filled bars and adult, blank bars). Y-axis shows the % survivors, while X-axis shows the genotypes together with the total number (in parentheses) of control or heat-shocked larvae examined

In the earlier study (Lakhotia et al. 2012) where *hs-GAL4* was used to down-regulate *hsw*-n transcripts (through *hsw-RNAi* transgene) or up-regulate *hsw* (through the *EP3037* allele), cells carried the *hsw* transcripts and omega speckles as expected for the given genotype till the point when heat shock was applied, which also activated the *hs-GAL4*-driven expression of *hsw-RNAi* or *EP3037*. On the other hand, in case of *Act-GAL4*-driven *hsw-RNAi* or *EP3037* genotypes, levels of *hsw* transcripts and, therefore, the organization of omega speckles were globally affected since long before the exposure to heat shock (Mallik and Lakhotia 2011). In order to understand if alterations in levels of *hsw* transcripts prior to thermal stress affect induction of heat shock proteins, we examined levels of Hsp83, Hsp70, and Hsp60 in *Act-GAL4>hsw-RNAi* or *Act-GAL4>EP3037* third-instar larvae after heat shock and during recovery following heat shock.

Expression of *Act-GAL4>hsw-RNAi*, but not *Act-GAL4>EP3037*, specifically affects levels of Hsp70 after heat shock and during recovery from heat shock

Western blotting was carried out with total larval proteins from WT, *Act-GAL4>hsw-RNAi*, and *Act-GAL4>EP3037* after 1-h heat shock to detect levels of Hsp83, Hsp70, and Hsp60. As expected (Lindquist and Craig 1988; Singh and Lakhotia 2000), Hsp83 was elevated after heat shock while, as reported earlier for WT (Lakhotia and Singh 1996), Hsp60 remained comparable in control and heat-shocked samples from each of the genotypes (Fig. 2a). Further, as expected, the heat shock-inducible Hsp70 was completely absent in control cells of all the genotypes and its levels were elevated

Fig. 2 Expression of *hsrw*-RNAi transgene prior to heat shock greatly suppresses accumulation of Hsp70 after heat shock and during recovery. **a** Western blot detection of Hsp83, Hsp70, and Hsp60 proteins from whole larvae in unstressed (Con) and after 1 h heat-shocked (HS) late third-instar WT larvae (Oregon R⁺, A) and those with down- (*Act-GAL4>hsrw*-RNAi, B) or up-regulated (*Act-GAL4>EP3037*, C) levels of *hsrw* transcripts. **b** Western detection of Hsp70 (upper row) in whole larval proteins in different genotypes (mentioned above each lane) after 1-h heat shock; the lower row shows levels of β-tubulin in corresponding samples. **c** Western detection of Hsp70 (upper row) from SG of late third-

instar larvae in different genotypes (mentioned above each lane) after 1-h heat shock; the lower row shows levels of β-tubulin in corresponding samples. **d** Western blot showing Hsp70 (upper row) in third-instar larvae of different genotypes (noted above each lane, abbreviations are same as in a) after 1-h heat shock at 37 °C or 2-, 4-, or 8-h recovery following 1-h heat shock. The lower row shows levels of β-tubulin in corresponding samples. Mean (±SE of three replicates) normalized Hsp70 levels (ratio of Hsp70 band density to that of β-tubulin in the given lane) in different genotypes and conditions are noted below the corresponding lanes in the blots in b–d

multifold in heat-shocked WT as well as the *Act-GAL4>EP3037* samples. Surprisingly, however, samples from heat-shocked *Act-GAL4>hsrw*-RNAi larvae showed barely detectable Hsp70 (Fig. 2a). To confirm if the absence of heat shock-inducible Hsp70 in cells carrying *hsrw*-RNAi transgene was due to some effect of the chromosome carrying the transgene or of the *Act-GAL4/CyO* chromosomes, levels of Hsp70 were also examined in heat-shocked +/+; *hsrw*-RNAi (undriven) and *Act-GAL4/CyO*; +/+ larval samples. Larvae of both these genotypes displayed the characteristic high levels of induced Hsp70 as in WT (Fig. 2b), ruling out any effect of these chromosomes. To further confirm a role of *hsrw*-RNAi transgene expression in suppressing the induction of Hsp70 in heat-shocked cells, Hsp70 levels were also examined in two other independent *hsrw*-RNAi transgenic lines viz. *hsrw*-RNAi² and *hsrw*-RNAi(2X) in which the *hsrw* 280 bp repeat transgene is inserted on chromosome 2 (Mallik and Lakhota 2009). These transgenes were driven by the *Sgs3-GAL4* driver which specifically expresses in third-instar larval salivary glands (SGs) soon after the ecdysone release (Beckendorf and Kafatos 1976; Cherbas et al. 2003). Western blotting of SG proteins from late third-instar larvae showed that as expected, the inducible Hsp70 was strongly present following 1-h heat shock in +/+; *Sgs3-GAL4*/+

(no responder transgene), *hsrw*-RNAi²/+; +/+ (undriven transgene), and *hsrw*-RNAi(2X)/+; +/+ (undriven transgene) larval SG, but not in those expressing any of the *hsrw*-RNAi transgene under the *Sgs3-GAL4* driver (*hsrw*-RNAi²/+; *Sgs3-GAL4*/+ and *hsrw*-RNAi(2X)/+; *Sgs3-GAL4*/+). In both cases, levels of Hsp70 were significantly low (Fig. 2c). Together, these results confirmed that cells which express GAL4-driven *hsrw*-RNAi transgene prior to heat shock fail to show the heat shock-induced elevation in levels of Hsp70.

Western blotting for Hsp70 after different periods of recovery from heat shock showed comparable levels of Hsp70 in WT and *Act-GAL4>EP3037* larval protein samples. Interestingly, the *Act-GAL4>hsrw*-RNAi samples continued to show significantly reduced levels of Hsp70 after 2-, 4-, and 8-h recovery from 1-h heat shock (Fig. 2d), although the levels were marginally higher than immediately after heat shock.

In order to get cell-type-specific information about the heat shock induction of Hsp70 in cells expressing *hsrw*-RNAi transgene, immunostaining was carried out with the 7Fb antibody in late third-instar larval tissues of WT, *Act-GAL4>hsrw*-RNAi, and *Act-GAL4>EP3037* immediately after heat shock and during recovery. Hsp70 was present in cytoplasm, nucleus, and cell membrane in SG and fat body cells of WT and *Act*-

GAL4>EP3037 larvae (Fig. 3 (a, a', c, c', d, d', f, f')) after heat shock as well as after 2-h recovery. However, Hsp70 was barely detectable in cytoplasm or nucleus after heat shock or during recovery in SG and fat body cells from *Act-GAL4>hsr ω -RNAi* larvae (Fig. 3 (b, b' e, e')).

In agreement with earlier reports (Lakhotia and Singh 1989; Singh and Lakhotia 1995; Lakhotia et al. 2002; Roberts and Feder 1999), heat shock induced Hsp70 only in the stellate cells with complete absence in the principal cells of WT (Fig. 3 (g)) and *Act-GAL4>EP3037* (Fig. 3 (i)) larval

Malpighian tubules (MT), while during recovery from heat shock, it was abundantly present in stellate as well as the principal cells in both genotypes (Fig. 3 (g', i')). Interestingly, Hsp70 was completely absent in *Act-GAL4>hsr ω -RNAi*-expressing MT stellate as well principal cells after heat shock (Fig. 3 (h)) while during recovery, only a very weak presence of Hsp70 was noted in principal cells with almost complete absence in stellate cells (Fig. 3 (h')).

Heat shock-induced Hsp70 is present in midgut imaginal cells of WT (Fig. 3 (j)) and *Act-GAL4>EP3037* larvae (Fig. 3

Fig. 3 Expression of *hsr ω -RNAi* transgene prior to heat shock suppresses Hsp70 after heat shock and recovery in different larval tissues. Confocal projection merged images (DAPI—red; Hsp70—green) of three consecutive optical sections corresponding to mid nuclear regions showing localization of heat shock-inducible Hsp70 in salivary glands (SG, a–c'), fat body (FB, d–f'), Malpighian tubule (MT, g–i'), and gut (j–l') cells after 1-h heat shock (a–c, d–f, g–i, j–l) and after 2-h recovery from the 1-h heat shock (a'–c', d'–f', g'–i', j'–l') in different genotypes (noted above each column). Arrows and arrowheads in g–i' indicate principal

and stellate cells, respectively, while in j–l', they indicate endoreplicated and imaginal cells, respectively. m–p Immunolocalization of Hsp70 (green) in 1 h heat-shocked wild type (m, n) and *GMR-GAL4>hsr ω -RNAi* (o, p) third-instar larval eye imaginal discs showing Hsp70 expression in entire eye imaginal disc of the wild type but not in *GMR-GAL4>hsr ω -RNAi*, where it is barely detectable behind the morphogenetic furrow. n, p Magnified views of regions behind the morphogenetic furrow in m, o, respectively

(l)), although the endoreplicated midgut cells did not show any Hsp70 staining immediately after heat shock. During recovery from heat shock, in addition to the imaginal cells, a very low level of Hsp70 was also detectable in the endoreplicated cells in WT (Fig. 3 (j')) and *Act-GAL4>EP3037* larvae (Fig. 3 (l')). As in other tissues, the levels of Hsp70 were comparable in WT and *Act-GAL4>EP3037* larval midgut cells after heat shock (Fig. 3 (j, l)) and recovery (Fig. 3 (j'–l')). In contrast, a very weak staining for Hsp70 was seen in *Act-GAL4>hsrw-RNAi*-expressing midgut imaginal cells after heat shock (Fig. 3 (k)), while during recovery, the staining was slightly enhanced (Fig. 3 (k')) but was still significantly less than in corresponding WT or *Act-GAL4>EP3037* cells.

The tissue-specific *GMR-GAL4* driver, which expresses only behind the morphogenetic furrow of eye imaginal disc cells (Ray and Lakhota 2015), was used to drive *hsrw-RNAi* transgene in larval eye discs. Immunostaining for Hsp70 in WT and *GMR-GAL4>hsrw-RNAi* late larval eye discs after heat shock revealed that unlike the WT discs in which Hsp70 is strongly expressed throughout the eye-antennal disc, Hsp70 was hardly expressed behind the morphogenetic furrow (Fig. 3 (m–p)) in *GMR-GAL4>hsrw-RNAi* discs. Remarkably, the areas anterior to the morphogenetic furrow, where the *GMR-GAL4* is not expressed (Ray and Lakhota 2015), the *GMR-GAL4>hsrw-RNAi* eye-antennal discs showed as strong presence of Hsp70 as in WT.

***Act-GAL4*-driven expression of *hsrw-RNAi* transgene does not affect heat shock-induced transcriptional activation of *hsp70* genes or stability of their transcripts**

It is possible that the greatly reduced level of Hsp70 in heat-shocked and recovering cells that have been expressing *hsrw-RNAi* transgene prior to heat shock is due to a failure to induce the *hsp70* genes at 87A and 87C and/or to rapid turnover of the induced *hsp70* transcripts. Therefore, we examined induction of the 87A and 87C puffs, which harbor multiple copies of *hsp70* genes (Goldschmidt-Clermont 1980), in SG polytene nuclei and localization of active RNA Pol II thereon; in addition, we also examined level and distribution of *hsp70* transcripts in different genotypes by RT-PCR and in situ hybridization.

Examination of the heat shock-induced puffing of the 87A and 87C loci in SG polytene chromosomes revealed formation of puffs in WT (Fig. 4a), *Act-GAL4>hsrw-RNAi* (Fig. 4e) and *Act-GAL4>EP3037* (Fig. 4i) although unlike the nearly equal-sized twin puffs at the 87A and 87C sites in WT and *Act-GAL4>EP3037* cells, the 87A puff was distinctly smaller than 87C puff in *Act-GAL4>hsrw-RNAi* (Fig. 4e) cells. Remarkably, while the 87A and 87C puffs were fully regressed in WT ($n=28$, Fig. 4b, c) and *Act-GAL4>EP3037* cells ($n=32$, Fig. 4j and k) by 2–4-h recovery from heat shock,

the *Act-GAL4>hsrw-RNAi* nuclei continued to show puffing at these sites even after 4 h of recovery ($n=36$, Fig. 4f, g). These puffs appeared regressed in *Act-GAL4>hsrw-RNAi* after 8-h recovery from heat shock, ($n=30$, Fig. 4h).

Since, in certain cases, puff formation and transcriptional induction can be uncoupled from each other (Winegarden et al. 1996), we also examined the distribution of phosphorylated (active) form of RNA Pol II on polytene chromosome spreads from heat-shocked SG of the above three genotypes. The distribution of RNA Pol II on 87A and 87C puffs was similar in WT (Fig. 5a), *Act-GAL4>hsrw-RNAi* (Fig. 5b), and *Act-GAL4>EP3037* (Fig. 5c) polytene chromosomes.

Comparison of the *hsp70* mRNAs through semiquantitative RT-PCR also revealed that levels of *Hsp70* transcripts under unstressed condition (control) or after heat shock or after 2-h recovery from heat shock in *Act-GAL4>hsrw-RNAi* SG were comparable, respectively, to those in WT and *Act-GAL4>EP3037* SG (Fig. 5d). This was further confirmed by real-time qPCR which showed that the levels of *hsp70* transcripts in unstressed WT and *Act-GAL4>hsrw-RNAi* larvae were similarly very low, and in both the genotypes, 1-h heat shock caused ~300-fold increase in *hsp70* transcripts (data not presented).

Cellular distribution of *hsp70* transcripts was examined through RNA-RNA FISH with *hsp70* mRNA-specific riboprobe in larval SG of the above three genotypes after 1-h heat shock and after 2-h recovery from heat shock. The patterns of cellular distribution and intensity of the in situ hybridization signals in the three genotypes were comparable. In all the three genotypes, these transcripts were most abundant in cytoplasm immediately outside the nuclear envelope with mild presence in nucleoplasm during heat shock as well as after 2-h recovery (Fig. 5e). The twin puffs at 87A and 87C also showed comparable hybridization signals in all genotypes immediately after heat shock as well as after 2-h recovery (Fig. 5f). These observations suggest that the transcription and transport of *hsp70* mRNAs from nucleus to cytoplasm are not affected by the expression of the *hsrw-RNAi* transgene prior to heat shock.

Together, these results show that the steady-state levels of heat shock-induced *hsp70* transcripts are similar in WT and *Act-GAL4>hsrw-RNAi*-expressing tissues.

We also immunolocalized the P-body-specific GW182 protein in SG and MT cells after 1-h heat shock and after 2-h recovery from heat shock to see if *hsrw-RNAi* expression prior to heat shock affected the P-bodies which are important in RNA turnover during cell stress (Lin et al. 2008). We did not find any detectable difference in the distribution or numbers of GW182 protein bodies between WT, *Act-GAL4>+*, *Act-GAL4>hsrw-RNAi*, and *Act-GAL4>EP3037* genotypes (Fig. 6). This suggests that processing of transcripts after heat shock by P-bodies in *Act-GAL4>hsrw-RNAi* cells is not affected and therefore may not be responsible for the reduced

Fig. 4 *Act-GAL4*-driven expression of *hsrw-RNAi* transgene results in delayed regression of heat shock-induced puffs during recovery. Parts of SG polytene chromosome spreads from third-instar larvae showing 93D (star), 87C (arrowhead), and 87A (arrow) loci on 3R after 1-h heat

shock (a, e, i) and after 2-h (b, f, j), 4-h (c, g and k), or 8-h (d, h, l) recovery from 1-h heat shock in different genotypes noted on left side of each row. The scale bar in a represents 10 μ m and applies to all images

levels of heat shock-induced Hsp70 in cells with down-regulated *hsrw* transcripts.

Limited accumulation of Hsp70 in heat-shocked cells already expressing *hsrw-RNAi* transgene is not due to high levels of Hsc70 proteins

It is possible that the *Act-GAL4*-driven continued expression of *hsrw-RNAi* transgene during development may generate some kind of cell stress which may elevate levels of some or all Hsc70 proteins which in turn may prevent further accumulation of Hsp70. To examine this, we used the 7.10.3 antibody, which recognizes all the Hsc70 cognates as well as the stress-induced Hsp70 and Hsp68 proteins (Palter et al. 1986; Zatsepina et al. 2001), to determine levels of Hsc70/Hsp70 in western blots of control and 1 h heat-shocked protein samples from WT and *Act-GAL4>hsrw-RNAi* transgene-expressing late third-instar larvae. As shown in Fig. 7a, the levels of Hsc70/Hsp70, detected with the 7.10.3 ab, were comparable in control samples from both genotypes, but after heat shock, while the signal in WT samples was significantly increased, that in the *Act-GAL4>hsrw-RNAi* transgene-expressing larval samples showed only a marginal increase. Since the 7.10.3 ab also recognizes the stress-induced Hsp70, the limited increase in the intensity of ab binding in heat-shocked *Act-GAL4>hsrw-RNAi* larval samples appears to be due to the poor accumulation of Hsp70. This was confirmed

by reprobing the blot with the 7Fb antibody to specifically identify the stress-inducible Hsp70. In agreement with the above results, the induction of Hsp70 in the *Act-GAL4>hsrw-RNAi* transgene-expressing larvae was much less than that in WT larval samples (Fig. 7a).

We further examined expression of YFP-tagged Hsc70Cb in unstressed and stressed +/+; *Hsc70cb-YFP/+* and *ActGAL4/+; Hsc70cb-YFP/hsrw-RNAi* larval SG and found that levels of this Hsc70 species were comparable in both the genotypes under control as well as after 1-h heat shock (Fig. 7b). Similar results were obtained with other larval tissues (not shown).

Together, these results show that the long-term expression of *Act-GAL4>hsrw-RNAi* transgene does not elevate the levels of Hsc70 in unstressed or stressed cells.

Act-GAL4-driven *hsrw-RNAi* transgene expression compromises the posttranslational stability of Hsp70 during heat shock and recovery

Since above results indicated normal activation, transport, and stability of the heat shock-induced hsp70 transcripts in *Act-GAL4>hsrw-RNAi*-expressing cells, we next asked if the very low level of Hsp70 in these cells was due to posttranslational stability of the synthesized Hsp70. To see if the translated Hsp70 is rapidly degraded so that after 1-h heat shock, this protein is no longer detectable in *Act-GAL4>hsrw-RNAi*-

Fig. 5 *Act-GAL4*-driven expression of *hsrw-RNAi* transgene does not affect the heat shock-induced activation of *hsp70* genes or the stability and transport of *hsp70* mRNAs. **a–c** Immunolocalization of RNA pol II (bright white) on polytene chromosome (grey) spreads from heat-shocked wild type (**a**), *Act-GAL4>hsrw-RNAi* (**b**), and *Act-GAL4>EP3037* (**c**) SG showing presence of RNA pol II on 93D (star), 87C (arrowhead), and 87A (arrow) loci on 3R after 1-h heat shock. **d** Semiquantitative estimation of Hsp70 transcript levels in SGs of third-instar larvae by RT-PCR from different genotypes in control, after 1-h heat shock, and

after 2-h recovery from heat shock. G3PDH (lower lane) amplicons served as loading control. **e** RNA-RNA FISH with *hsp70*-mRNA-specific riboprobe showing distribution of *hsp70* mRNA (bright white fluorescence) in SG cells of late third-instar larvae of different genotypes (noted above each column and explained below **f**) after 1-h heat shock or after 2-h recovery following heat shock. **f** Magnified views of parts of nuclei from corresponding upper images in **e** showing *hsp70* RNA hybridization signals (arrowheads) at the twin *hsp70* gene loci

expressing cells, we exposed WT, *Act-GAL4>hsrw-RNAi*, and *Act-GAL4>EP3037* larval SGs for 5 or 10 min to 37 °C heat shock prior to western detection of Hsp70. The Hsp70 protein was nearly undetectable in any of the genotypes after 5-min heat shock (Fig. 7c), but after 10-min heat shock, this protein was distinctly present in WT and *Act-GAL4>EP3037* but not in *Act-GAL4>hsrw-RNAi* protein samples (Fig. 7c). These results indicate that the near absence of induced Hsp70 in *Act-GAL4>hsrw-RNAi* cell is a feature that starts with the very early stage of heat shock response.

Therefore, we next examined if the stress-induced Hsp70 is rapidly degraded in *Act-GAL4>hsrw-RNAi* cells even during heat shock. To examine the posttranslational stability of Hsp70 protein, the SGs of actively wandering third-instar larvae of different genotypes were heat shocked in PSS (control)

or in PSS containing the ALLN proteasome inhibitor (Zhou et al. 1996). It is significant that unlike the very low levels of Hsp70 in *Act-GAL4>hsrw-RNAi* SG heat shocked in PSS, the *Act-GAL4>hsrw-RNAi* SG heat shocked in the presence of proteasomal inhibitor showed Hsp70 to be as abundant as in the other two genotypes (lane B—ALLN in Fig. 7d).

As reported earlier (Lakhota et al. 2002), heat shock induces *hsp70* mRNA in stellate as well as principal cells of MT, but while stellate cells show Hsp70 protein after heat shock, the principal cells show Hsp70 only during recovery from heat shock. Immunostaining of WT MT for Hsp70 after heat shock in absence or presence of proteasome inhibitor (Fig. 7e–h) revealed that inhibition of proteasomal activity had no effect on Hsp70 in principal cells, although in stellate cells, its level was higher following heat shock in presence of the

Fig. 6 P-bodies are not affected by *Act-GAL4*-driven expression of *hsrw-RNAi* transgene. Immunolocalization of GW182 protein (bright white dots) in SG (a–h) and MT (i–p) cells after 1-h heat shock (a–d, i–l) and after 2-h recovery from 1-h heat shock (e–h, m–p) in different genotypes mentioned above each lane. Chromatin (red) is stained with DAPI

proteasomal inhibitor (Fig. 7f). As noted above, Hsp70 was not seen in any of the *Act-GAL4*>*hsrw-RNAi* MT cells after heat shock (Fig. 7g), but in the presence of proteasome inhibitor, Hsp70 was detectable, although at lower level than in WT, in *Act-GAL4*>*hsrw-RNAi* MT stellate cells; the principal cells were still negative (Fig. 7h).

These results show that the near absence of Hsp70 in *Act-GAL4*>*hsrw-RNAi* cells following heat shock is because of its proteolytic degradation through proteasome machinery as soon as it is synthesized.

Expression of *hsrw-RNAi* transgene in *hsrw-pre-c* over-expression or in *hsrw*-null background also suppresses heat shock-induced Hsp70 accumulation

Expression of *hsrw-RNAi* transgene is expected to down-regulate the *hsrw-n* transcripts without affecting the smaller cytoplasmic *hsrw-c* transcript (Mallik and Lakhotia 2011). However, to see if the observed effect on Hsp70 accumulation following *hsrw-RNAi* transgene expression may be due to some perturbations in levels of the cytoplasmic *hsrw-c* transcripts which have been earlier speculated (Fini et al. 1989; Lakhotia 2011) to have some role in ribosome-mediated translational regulation, we introduced the *UAS-hsrw-pre-c*

transgene in the *Act-GAL4*>*hsrw-RNAi* background so that the 1.2 kb *hsrw-c* transcript levels remain high. Interestingly, however, as seen in the western blot in Fig. 8a, even when *hsrw-RNAi* and *hsrw-pre-c* transgenes were co-expressed with the *Act-GAL4* driver, level of Hsp70 in heat-shocked cells was as low as in only *hsrw-RNAi*-expressing tissues (Fig. 8a).

Since expression of *hsrw-RNAi* transgene down-regulates the *hsrw-n* transcripts (Mallik and Lakhotia 2009), we examined if a complete absence of all *hsrw* transcripts in *hsrw*⁶⁶ homozygotes (Johnson et al. 2011; Lakhotia et al. 2012) also affects accumulation of Hsp70 in heat-shocked cells. Surprisingly, Hsp70 was typically induced by heat shock in *hsrw*⁶⁶ homozygotes (Fig. 8a). Next, we examined the effect of expression of *Act-GAL4*>*hsrw-RNAi* transgene in *hsrw*⁶⁶ background. For this purpose, we generated *Act-GAL4/hsrw-RNAi*²; *hsrw*⁶⁶/*hsrw*⁶⁶ and *Act-GAL4/hsrw-RNAi*²; *hsrw*⁶⁶/*TM6B* third-instar larvae and examined induction of Hsp70 following heat shock. Intriguingly and unexpectedly, *Act-GAL4*-driven expression of *hsrw-RNAi*² transgene in *hsrw*⁶⁶ homozygous background also resulted in as low accumulation of Hsp70 in heat-shocked cells as when it was expressed in WT or *hsrw*⁶⁶/*TM6B* backgrounds (Fig. 8a).

In view of the above unexpected results, we further examined if the absence of Hsp70 in *Act-GAL4*>*hsrw-RNAi* heat-

Fig. 7 Hsc70 levels are not increased in *hsrw-RNAi* transgene-expressing cells, but post-translational stability of the heat shock-induced Hsp70 is compromised. **a** Western blot showing sequential detection of Hsc70/Hsp70 and Hsp70 using 7.10.3 and 7Fb antibodies, respectively, in control and heat-shocked WT (A) and *Act-GAL4>hsrw-RNAi* (B) larvae. **b** Confocal projection images of YFP fluorescence in control (Con) and heat-shocked (HS) SG from *Hsc70cb-YFP/+* and *ActGAL4/+; Hsc70cb-YFP/hsrw-RNAi* larvae (genotypes indicated above the image panels). **c** Western blot showing Hsp70 (upper row) in third-instar larval proteins from different genotypes (A, B, and C genotypes, marked above each lane and expanded below **d**, after 5- and 10-min heat shock at 37 °C. **d** Levels of Hsp70 in SG of different genotypes following 1-h heat shock in PSS or in PSS containing the proteasome inhibitor ALLN. β -Tubulin was used as loading control (lower lanes) in three western blots in **a**, **c**, **d**. **e–h** Immunolocalization of Hsp70 (green) in MT after 1-h heat shock in PSS (**e**, **g**) or in PSS containing ALLN (**f**, **h**) in wild type (**e**, **f**) or *ActGAL4>hsrw-RNAi* (**g**, **h**). Nuclei are stained with DAPI (red). Arrowheads indicate the stellate cells while asterisks denote principal cells in **e–h**

shocked cells was a consequence of some effect of the process of RNAi itself. For this purpose, we used three other RNAi transgenic lines, viz., *Egfr-RNAi* (31525, Bloomington), *Hsp60D-RNAi* (Arya and Lakhotia 2008), and *Hsp60C-RNAi* (Sarkar and Lakhotia 2005), each of which carries a relatively long RNAi insert (see “Materials and methods” section). Crosses were set to obtain *Act-GAL4/+; Egfr-RNAi/+* or *Act-GAL4/Hsp60C-RNAi; +/+* or *Act-GAL4/+; Hsp60D-*

RNAi/+ larvae which were heat shocked for 1 h, and the total larval proteins were processed for western detection of Hsp70. Parallel unstressed control and WT samples were also examined. As shown in Fig. 8b, the Hsp70 was as strongly induced by heat shock in all the three RNAi transgene-expressing larvae as in WT. Thus, the noninduction of Hsp70 in *hsrw-RNAi* transgene-expressing cells does not appear to be a consequence of general RNAi activity.

Fig. 8 Expression of *hsr ω -RNAi* transgene in *hsr ω -pre-c* over-expression or even in *hsr ω -null* background also suppresses heat shock-induced Hsp70 accumulation. **a** Western blots showing levels of Hsp70 in third-instar larval proteins from different genotypes (noted above each lane) after 1 h of heat shock at 37 °C. **b** Western blot showing typical induction of Hsp70 and Hsp68 (detected with 7Fb ab) in larvae expressing *Egfr-RNAi* or *hsp60D-RNAi* or *hsp60C-RNAi* transgenes under the *Act-GAL4* driver (genotypes marked above each lane). The lower rows in all blots show levels of β -tubulin in corresponding samples

Discussion

The noncoding *hsr ω* gene of *D. melanogaster* is developmentally expressed in almost all cell types and is one of the most highly induced genes following heat shock (Lakhotia 2011). The nuclear transcripts of *hsr ω* gene, *hsr ω -n1*, and *hsr ω -n2* are essential for organization of the omega speckles which are believed to regulate the availability of various hnRNPs and certain other RNA-binding proteins (Prasanth et al. 2000; Mallik and Lakhotia 2011; Lakhotia 2011; Singh and Lakhotia 2015). In an earlier study (Lakhotia et al. 2012), it was seen that when the *UAS-hsr ω -RNAi* transgene or an EP allele of *hsr ω* (*EP93D* or *EP3037*) was activated during heat shock using the *hs-GAL4* driver, the major heat shock genes and stress proteins like Hsp70 and Hsp83 were characteristically induced, yet all the individuals exhibited delayed death during recovery. This delayed death was correlated with the delayed restoration of RNA Pol II, HP1, and hnRNPs to the developmentally active gene loci when *UAS-hsr ω -RNAi* transgene or an EP allele of *hsr ω* was expressed during heat shock (Lakhotia et al. 2012).

In the present study, we used *Act-GAL4* or *Sgs-GAL4* or *GMR-GAL4* drivers, instead of the *hs-GAL4* driver, to alter the levels of *hsr ω* transcripts much before the tissues experienced heat shock. In these cases also, as in earlier study (Lakhotia et al. 2012), we have seen a delayed restoration of hnRNPs, etc. to their normal chromosomal sites during recovery from heat shock (data not shown), which may be the major factor

for the observed delayed death. The most striking effect of expression of the *hsr ω -RNAi* transgene, but not of the *EP3037* allele, prior to heat shock is the near complete absence of Hsp70 in heat-shocked cells. The former would down-regulate levels of *hsr ω -n* transcripts while the latter would result in their elevated levels (Mallik and Lakhotia 2009). The present results show that cells with down-regulated levels of *hsr ω* transcripts fail to elevate the levels of Hsp70 when heat shocked, although Hsp83 showed the expected increase.

Normally, the stress-inducible Hsp70 shows the most robust increase in stressed cells and continues to remain so for a few hours even after the stress condition is withdrawn (Shi et al. 1998). Only in certain WT tissues, like the MT and midgut polytene cells, this protein's inducibility by heat shock follows a different pattern (Lakhotia and Singh 1989, 1996; Lakhotia et al. 2002; Lakhotia and Prasanth 2002; Roberts and Feder 1999). However, the Hsp70 accumulation after heat shock failed to follow the pattern characteristic of even these tissues when the *UAS-hsr ω -RNAi* transgene was expressed prior to heat shock.

The continued puffing at the Hsp70-encoding 87A and 87C sites even after 4-h recovery from heat shock in *Act-GAL4*>*hsr ω -RNAi*-expressing SG also appears to be a consequence of the greatly reduced levels of Hsp70 in these cells since an optimal level of Hsp70 is required to autoregulate the stress-induced heat shock genes, including the *hsp70* gene copies (DiDomenico et al. 1982; Stone and Craig 1990;

Gong and Golik 2006). Several earlier studies from our laboratory showed that a subnormal induction of the 93D puff harboring the *hsrw* gene during heat shock affects puffing at the 87A and 87C sites in a condition-specific manner (reviewed in Lakhotia 1989, 2011). In the present study also, we noted that the 87A puff was generally smaller than the 87C puff in heat-shocked SGs that were already expressing the *UAS-hsrw-RNAi* transgene. However, the unequal puffing of the 87A and 87C puffs is unlikely to be responsible for the drastically reduced levels of Hsp70 in these cells. Our observations on in situ localization of active RNA Pol II and *hsp70* transcripts on the heat shock-induced 87A and 87C puffs and elsewhere show that heat shock-induced active transcription of the *hsp70* genes and transport of *hsp70* mRNAs from nucleus to cytoplasm are not affected by expression of *UAS-hsrw-RNAi* under the *Act-GAL4* driver. Since levels of heat shock-induced *hsp70* transcripts, as detected by RT-PCR, remained comparable and immunostaining for a P-body-specific GW182 protein did not reveal any significant difference in the distribution of P-bodies in different genotypes, it is unlikely that the *hsp70* transcripts are prematurely degraded when the *UAS-hsrw-RNAi* transgene is expressed prior to heat shock under *Act-GAL4* or *Sgs3-GAL4* or *GMR-GAL4* drivers. This is also supported by our observation that even 10 min after heat shock, these glands failed to show the characteristic high induction of Hsp70.

Specific function of the 1.2 kb cytoplasmic *hsrw-c* transcript during development or in stress response is not known; however, its small translatable ORF has been suggested to have some role in translational activities in cells (Fini et al. 1989; Lakhotia 2012). Although earlier studies (Mallik and Lakhotia 2009) indicated that expression of the *hsrw* 280-bp repeat sequence present in the *hsrw-RNAi* transgene does not affect the levels of *hsrw-c* transcripts, it remains possible that the levels of *hsrw-pre-c* and, therefore, of the *hsrw-c* transcripts too may be affected due to the process of RNAi amplification (Zhang and Ruvkun 2012), which in turn may affect translatability of the *hsp70* transcripts. Therefore, we also examined heat shock-induced accumulation of Hsp70 in cells co-expressing *UAS-hsrw-RNAi* and *UAS-hsrw-pre-c* transgenes. Since in this case, also Hsp70 levels remained as low as in only *hsrw-RNAi* transgene-expressing cells, we believe that the potentially reduced levels of *hsrw-pre-c* and *hsrw-c* transcripts are unlikely to be a reason for the observed low levels of Hsp70 in the heat-shocked *hsrw-RNAi*-expressing cells. Our studies also show that global expression of *hsrw-RNAi* transgene does not affect the basal levels of different Hsc70 proteins in unstressed cells. The 7.10.3 ab detects cognate as well as the induced members of the Hsp70 family in *Drosophila* (Palter et al. 1986; Zatsepina et al. 2001). In agreement with results obtained with the 7Fb ab, which detects only the stress-inducible Hsp70 (Velazquez and Lindquist 1984; Palter et al. 1986; Zatsepina et al. 2001), the

7.10.3 ab signal in heat-shocked samples from *hsrw-RNAi* transgene-expressing larvae was much less intense than in WT. Thus, autoregulation of Hsp70 induction by enhanced levels of Hsc70s does not appear to be a reason for noninduction of Hsp70 in *hsrw-RNAi* background.

Our present observations suggest that the absence or very little presence of Hsp70 in cells that have been expressing the *UAS-hsrw-RNAi* transgene before they were exposed to the thermal stress is due to specific degradation of Hsp70 by the proteasomal machinery since, when the proteasome inhibitor was present during heat shock, the Hsp70 accumulated in *UAS-hsrw-RNAi* transgene-expressing cells to the same extent as in other genotypes. Earlier studies have indeed shown that expression of *UAS-hsrw-RNAi* transgene enhances proteasomal activity (Mallik and Lakhotia 2010). It is intriguing that the improved proteasomal activity specifically removes the stress-induced Hsp70 but not any other HSP. It is known that Hsp70 follows a different pathway for degradation than other HSPs (Zhou et al. 1996; Silver and Noble 2012) and, therefore, might be singularly targeted in *hsrw-RNAi* transgene-expressing cells during heat shock and recovery. It is intriguing that Hsp70 does not accumulate in stressed cells only when the *UAS-hsrw-RNAi* transgene is activated sometime before the heat shock (present results), but its accumulation remains unaffected when this transgene is activated along with heat shock (Lakhotia et al. 2012).

The stress-induced Hsp70 is known to be rapidly removed by proteasomal degradation in WT cells recovering from the stress following its ubiquitination by the carboxy terminus of Hsp70-binding protein (CHIP). This protein plays a dual role in the heat shock response by ubiquitination of misfolded proteins and presenting them to Hsp70 during stress, and when misfolded proteins are no longer available during recovery, CHIP ubiquitinates Hsp70 for proteasomal degradation (Ballinger et al. 1999; Qian et al. 2006). It remains to be seen if CHIP is involved in the unusually rapid and premature degradation of Hsp70 or some other factors are at play in cells that express the *UAS-hsrw-RNAi* transgene prior to heat shock.

Our findings that *hsrw*-null (*hsrw*⁶⁶ homozygous) condition does not affect the heat shock-induced massive accumulation of Hsp70 but expression of *UAS-hsrw-RNAi* transgene in *hsrw*-null background affects Hsp70 levels after heat shock were completely unexpected. The first condition may suggest that a complete absence of *hsrw* gene since fertilization, as in *hsrw*⁶⁶ homozygotes may not affect the Hsp70 accumulation. However, absence of heat shock-induced Hsp70 protein when the *hsrw-RNAi* transgene is expressed in *hsrw*-null background is very perplexing since these cells do not have any target for the RNAi, as none of the *hsrw* transcripts is present. This raises the intriguing possibility that the observed effect may not be a consequence of down-regulation of the *hsrw* transcripts per se but may be a consequence of this transgene's expression itself. The *hsrw-RNAi* transgene construct includes

a monomer of the 280-bp tandem repeats at the *hsr ω* gene, taken from the *pDRM30* clone (Hogan et al. 1995) together with ~80 bp of flanking vector sequence (Mallik and Lakhotia 2009). The *SympUAS* vector (Giordano et al. 2002) would cause transcription of both strands of the 280 bp plus the flanking vector sequence when GAL4 is available. The 280-b repeat sequence of the *hsr ω -n* transcripts binds with several proteins (Lakhotia 2011; Onorati et al. 2011). It remains possible that binding of some protein/s to the sense and/or anti-sense strands over-produced by this transgene construct may lead to functional depletion of that/those protein/s, which in turn may affect stability of the heat shock-induced Hsp70. It is known that excessive production of some RNAs or triplet repeat expansion in certain RNAs sequesters specific proteins resulting in dysregulation of their activities and thus proteinopathies (Lenzken et al. 2014; Walsh et al. 2015). Our results with three other RNAi transgenic lines (*Act-GAL4>Egfr-RNAi* or *Act-GAL4>Hsp60C-RNAi* or *Act-GAL4>Hsp60D-RNAi*), each of which includes a long (>400 bp) RNAi construct, show that the absence of inducible Hsp70 in *hsr ω -RNAi* transgene-expressing background is specific for expression of the *hsr ω -RNAi* transgene. The protein-binding activities of each of the two strands produced by the *SympUAS-hsr ω -RNAi* transgene construct when activated by GAL4 need to be examined. The dsRNA-dependent protein kinase, protein kinase R (PKR), has been implicated in determining the stability of hsp70 mRNA in mammalian cells such that PKR-null cells do not show significant accumulation of Hsp70 when exposed to cell stress (Zhao et al. 2002). Although our results suggest that the heat-shocked-induced hsp70 transcripts are not destabilized by *hsr ω -RNAi* transgene expression, it would be interesting to examine if the PKR homologues in *Drosophila* affect Hsp70 protein's stability.

The present observations appear to be the first to show selective instability of heat shock-induced Hsp70 so that it gets targeted to proteasomal degradation as soon as synthesized while the other induced heat shock proteins remain stable. This adds a new dimension to the complexity of regulation of the cell stress response. This study also brings out additional issues that may need to be considered when applying experimental RNAi approach. Normally, one expects the sense strand of the RNAi construct to get degraded. However, if it does not happen and if the RNA sequence can bind with some proteins, unexpected consequences may also follow.

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