Activated Ras expression in eye discs with altered levels of hsrω lncRNA causes JNK-induced Dilp8 secretion and reduces post-pupal ecdysone leading to early pupal death in *Drosophila*

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Running title: Dilp8 and early pupal death

Abstract

We examined reasons for pupal death following expression of certain transgenes with predominantly eye-disc expressing *GMR-GAL4* or *sev-GAL4* drivers in *Drosophila*. *GMR-GAL4* or *sev-GAL4* driven expression of *UAS-Ras1*^{V12} transgene, producing activated Ras, resulted in early (~25-30 Hr pupation) and late pupal death, respectively. Co-expression of *UAS-hsrω-RNAi* transgene or *EP3037* to down or up-regulate, respectively, hsrω lncRNAs with *sev-GAL4>UAS-Ras1*^{V12} advanced death to 25-30 Hr after pupation. The normal post-pupation ecdysone surge was absent in 24 Hr old *GMR-GAL4>UAS-Ras1*^{V12} pupae or those co-expressing *sev-GAL4>UAS-Ras1*^{V12} with *hsrω-RNAi* or *EP3037*. Interestingly, exogenous ecdysone substantially suppressed their early death when provided for 12 Hr, beginning at 8-9 Hr after pupation. Microarray, qRT-PCR and immunostaining data revealed significantly elevated levels of Dilp8 and several members of JNK pathway genes but down-regulation of some of the ecdysone biosynthesis pathway genes in early dying pupae but not so much at their late third instar stage. Altered hsrω transcript levels in activated Ras expression background triggered greater Dilp8 secretion in early pupae. The consequent reduction in post-pupal ecdysone leads to early pupal death. This study explains for the first time the cause of early pupal death following expression of certain transgenes in developing eyes, which are otherwise dispensable for survival, and highlights the global consequences of deregulated signalling in one tissue through downstream events in other tissues.

Keywords: *GMR-GAL4, sev-GAL4,* Eiger, Halloween genes, lncRNA

1. Introduction

An earlier study in our laboratory [1] indicated that *Ras* mutant alleles genetically interact with the *hsrω* long non-coding RNA (lncRNA) gene [2]. With a view to explore this interaction further, we initiated studies using the GAL4-inducible activated form of DRas1 ($Ras1^{V12}$) [3] in conjunction with *hsrω-RNAi* transgene or an over-expressing *EP* allele of *hsrω* [4]. We found that *GMR-GAL4* or *sev-GAL4* driven expression of activated Ras led to early or late pupal death, respectively. Interestingly, while the early pupal death following *GMR-GAL4* driven $Ras1^{V12}$ expression was not affected by altered levels of hsrω lncRNAs, the late pupal death following *sev-GAL4* driven expression of activated Ras was advanced to early pupal lethality when hsrω nuclear transcripts were either down- or up-regulated.

Several earlier studies have also reported pupal lethality when certain transgenes are expressed using the *GMR-GAL4* or *sev-GAL4* drivers [4, 5]. Pupal lethality following expression of such transgenes under these two mostly eye-specific GAL4 drivers [6-9], has been intriguing since even a complete absence of eyes has no effect on viability of pupae and emergence of adult flies [10]. Our recent [11] finding that, in addition to the well-known expression in eye disc cells, the *GMR-GAL4* and *sev-GAL4* drivers also express in several other cell types including specific neurons in the central nervous system raised the possibility that the pupal death may be due to the transgene's ectopic expression in some of these other cells.

In the present study, therefore, we examined the cause of early pupal death following expression of activated Ras in a background where the hsrω transcripts were down- or up-regulated. We found that ecdysone levels were significantly reduced in the early dying pupae. Further analyses revealed that levels of several members of the JNK signalling pathway and Dilp8 were up-regulated in the early dying pupae. Dilp8, one of the eight insulin-like signal peptides in *Drosophila* [12], is secreted by damaged or inappropriately growing imaginal discs to delay metamorphosis by inhibiting ecdysone synthesis [13, 14]. Our study shows, for the first time, an involvement of Ras signalling and hsrω lncRNAs, presumably via the JNK signalling, in initiating Dilp8 secretion after the larval metamorphosis. This disrupts post-pupal ecdysone synthesis and thereby causes early pupal death. Such effects highlight the global consequences of deregulated signalling in one tissue through downstream events in other tissues.

2. Material and Methods

2.1. *Fly stocks*

All fly stocks and crosses were maintained on standard agar cornmeal medium at 24 ± 1^0C. The following stocks were obtained from the Bloomington Stock Centre (USA): w^{1118}*; sev-GAL4; +* (no. 5793), w^{1118}*; UAS-GFP* (no. 1521), w^{1118}*; UAS-rpr* (no. 5824), w^{1118}*; UAS-Tak1* (no. 58810), ecd^1 (no. 218) and w^{1118}*; UAS-*$Ras1^{V12}$ (no. 4847). The other stocks, viz., w^{1118}*; GMR-GAL4*[7], w^{1118}*; UAS-hsrω-RNAi*^{3} [15], w^{1118}*; EP3037/TM6B* [15], w^{1118}*; GMR-GAL4; UAS-hsrω-RNAi*^{3}, w^{1118}*; GMR-GAL4; EP3037/TM6B,* w^{1118}*; Sp/CyO;* dco^2 *e/TM6B* and w^{1118}*; UAS-127Q* [16], were available in the laboratory. The *UAS-hsrω-RNAi*^{3} is a transgenic line for down regulating the hsrω-nuclear transcripts while the *EP3037* allele over-expresses *hsrω* gene under a GAL4 driver [15]. The *UAS-RafRBDFLAG* stock [17] was provided by Dr S Sanyal (Emory University, USA). Using these stocks, appropriate crosses were made to generate following stocks:

a) w^{1118}; *sev-GAL4 UAS-GFP; dco*^{2} *e/TM6B*
b) w^{1118}; *sev-GAL4 UAS-GFP; UAS-hsrω-RNAi*^{3}
c) w^{1118}; *sev-GAL4 UAS-GFP; EP3037/TM6B*
d) w^{1118}; *UAS-GFP; UAS-Ras1*^{V12}
e) w^{1118}; *sev-GAL4 UAS-GFP; ecd*^{1}
f) w^{1118}; *UAS-RafRBDFLAG; UAS-Ras1*^{V12}*/TM6B*

Some of these were used directly or were further crossed to obtain progenies of following genotypes as required:

a) w^{1118}; sev-GAL4 *UAS-GFP/UAS-GFP; dco*^{2} *e/+*
b) w^{1118}; *sev-GAL4 UAS-GFP/UAS-GFP; dco*^{2} *e/UAS-Ras1*^{V12}
c) w^{1118}; *sev-GAL4 UAS-GFP/UAS-GFP; UAS-hsrω-RNA*$^{i3/}$*UAS-Ras1*^{V12}
d) w^{1118}; *sev-GAL4 UAS-GFP/UAS-GFP; EP3037/ UAS-Ras1*^{V12}
e) w^{1118}; sev-GAL4 *UAS-GFP/UAS-RafRBDFLAG; dco*^{2} *e/+*
f) w^{1118}; *sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; dco*^{2} *e/UAS-Ras1*^{V12}
g) w^{1118}; *sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; UAS-hsrω-RNAi*^{3}*/UAS-Ras1*^{V12}
h) w^{1118}; *sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; EP3037/UAS-Ras1*^{V12}
i) w^{1118}; *GMR-GAL4/UAS-GFP*
j) w^{1118};*GMR-GAL4/UAS-GFP; +/ UAS-Ras1*^{V12}
k) w^{1118};*GMR-GAL4/UAS-GFP; UAS-hsrω-RNAi*^{3}*/ UAS-Ras1*^{V12}
l) w^{1118};*GMR-GAL4/UAS-GFP; EP3037/ UAS-Ras1*^{V12}
m) w^{1118}; *GMR-GAL4/UAS-rpr*
n) w^{1118}; *GMR-GAL4/UAS-Tak1*
o) w^{1118}; *GMR-GAL4/UAS-127Q*.

The w^{1118}, *dco*^{2} *e* and *UAS-GFP* markers are not mentioned while writing genotypes in Results while the *UAS-hsrω-RNAi*^{3} transgene [15] is referred to as *hsrω-RNAi*.

For conditional inhibition of ecdysone synthesis using the temperature-sensitive *ecd*^{1} mutant allele [18, 19], the freshly formed *sev-GAL4 UAS-GFP; ecd*^{1} pupae, reared from egg-laying till pupation at 24 ± 1^{0}C, were transferred to 30 ± 1^{0}C for further development.

Flies of desired genotypes were crossed and their progeny eggs were collected at hourly intervals. Larvae that hatched during a period of 1 Hr were separated to obtain synchronously growing larvae. Likewise, larvae that began pupation during a period of 1 Hr were separated to obtain pupae of defined age (expressed as Hr after pupa formation or Hr APF). Actively moving late third instar larvae and pupae of desired ages were dissected in Poels' salt solution (PSS) [20] and tissues fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, 130 mm NaCl, 7 mm Na_2HPO_4, 3mm KH_2PO_4,pH 7.2) for 20 min. After three 10 min washes in 0.1% PBST (PBS + 0.1% Triton-X-100), the tissues were counterstained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, 1µg/ml) and mounted in DABCO for imaging the GFP expression.

2.2. Whole organ immunostaining

Brain and eye discs from *sev-GAL4 UAS-GFP/UAS-GFP; dco*^{2}*e/+, sev-GAL4 UAS-GFP/UAS-GFP; dco*^{2} *e/UAS-Ras1*^{V12}*,sev-GAL4 UAS-GFP/UAS-GFP; UAS-hsrω-RNAi/UAS-Ras1*^{V12}*,* and *sev-GAL4 UAS-GFP/UAS-GFP; EP3037/UAS-Ras1*^{V12} actively migrating late third instar larvae and pupae of desired age were dissected out in PSS and immediately fixed in freshly prepared

4% paraformaldehyde in PBS for 20 min and processed for immunostaining as described earlier [21]. The following primary antibodies were used: mouse monoclonal anti-Broad-core (DSHB, 25E9.D7) at 1:50 dilution, rabbit monoclonal anti-p-JNK (Promega) at 1:100 dilution and rat anti-Dilp8 (gifted by Dr. P. Léopold, France) [14] at 1:50 dilution. Appropriate secondary antibodies conjugated either with Cy3 (1:200, Sigma-Aldrich, India) or Alexa Fluor 633 (1:200; Molecular Probes, USA) or Alexa Fluor 546 (1:200; Molecular Probes, USA) were used to detect the given primary antibody. Chromatin was counterstained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride, 1µg/ml). Tissues were mounted in DABCO antifade mountant for confocal microscopy with Zeiss LSM Meta 510 using Plan-Apo 40X (1.3-NA) or 63X (1.4-NA) oil immersion objectives. Quantitative estimates of the proteins in different regions of eye discs were obtained with the help of Histo option of the Zeiss LSM Meta 510 software. All images were assembled using the Adobe Photoshop 7.0 software.

2.3. Measurement of ecdysone levels

Fifteen pupae each of appropriate stages and desired genotypes were collected in 1.5 ml tubes and stored at -70°C in methanol. They were homogenized in methanol and centrifuged at 13000 rpm following which the pellets were re-extracted in ethanol and air dried [22]. The dried extracts were thoroughly dissolved in EIA buffer at 4°C overnight prior to the enzyme immunoassay. The 20E-EIA antiserum (#482202), 20E AChE tracer (#482200), precoated (Mouse Anti-Rabbit IgG) EIA 96-Well Plates (#400007), and Ellman's Reagent (#400050) were obtained from Cayman Chemical (USA), and assays were performed according to the manufacturer's instructions.

2.4. Microarray Analysis

RNA was isolated from 16-17 Hr old *sev-GAL4>UAS-GFP*, *sev-GAL4>Ras1*^{V12}, *sev-GAL4>Ras1*^{V12}*hsrω*-RNAi and *sev-GAL4>Ras1*^{V12}*EP3037* pupae using TriReagent (Sigma-Aldrich) as per manufactures instructions. Microarray analysis of these RNA samples was performed on Affimetrix Drosophila Genome 2.0 microarray chips for 3' IVT array following the Affymetrix GeneChip Expression Analysis Technical manual using the GeneChip 3' IVT Plus Reagent Kit, Affymetrix GeneChip® Fluidics station 450, GeneChip® Hybridization oven 645 and GeneChip®Scanner 3000. Summary of the expression levels for each gene in the four genotypes was obtained from the Affymetrix Transcription analysis console and was subjected to Gene ontology search using David Bioinformatics software (https://**david**.ncifcrf.gov).The microarray data have been deposited at GEO repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80703).

2.5. Real Time Quantitative Reverse transcription–PCR (RT-qPCR)

Total RNAs were isolated from eye discs of third instar larvae and appropriate pupal stages of the desired genotypes using TriReagent as per the manufacturer's (Sigma-Aldrich) instructions. First-strand cDNAs were synthesized as described earlier [15]. The prepared cDNAs were subjected to real time PCR using forward and reverse primer pairs as listed in Table 1. Real time qPCR was performed using 5µl qPCR Master Mix (Syber Green, Thermo Scientific), 2 picomol/µl of each primer per reaction in 10 µl of final volume in ABI 7500 Real time PCR machine.

Table 1. Forward and reverse primers used for qRT-PCR for quantification of different transcripts

Transcripts and Primers		SEQUENCE (5'-3')	AMPLICON SIZE
Spookier	*Forward*	TGCGACTGGTCTCAATTACAA	164 bp
	Reverse	TCACATGGATATCCCGAAGAA	
Phantom	*Forward*	CTTGCATTTCCGAGACGATG	150bp
	Reverse	CCACTGGGTCCATGTGGATA	
Tak1-like 1	*Forward*	ACCGATTGTTCACCGCGATA	229bp
	Reverse	TTGACGTGTCATCAGCTCCC	
Dilp 8	*Forward*	GGAGTGGGCACAAATAAGGT	153bp
	Reverse	TCTTTGGAAGTTGCCTCATTGG	
Eiger 2	*Forward*	GACGCGAACGGACGTTTAAA	228bp
	Reverse	TTTGCGATAAAGGCACAGCG	
Gadd45	*Forward*	GGGATCCCTCTTCTGCCTGA	121bp
	Reverse	GTCGTCCACCTTGATCACGT	
Puckered	*Forward*	TACAAGTCGCTCTCCCTGCT	196bp
	Reverse	GGGTGCTTAATCCCACAGAA	
G3PDH	*Forward*	AGGCGTTTGTGACTTCTGGA	129bp
	Reverse	TGTCCTCCAGACCCTTGTTC	

3. Results

3.1. Expression of *sev-GAL4* driven activated Ras leads to late pupal lethality which is advanced by altered levels of hsrω transcripts

We used *sev-GAL4* driver to express the *UAS-Ras1*^{V12} transgene which produces activated Ras and thus triggers the downstream signalling cascade without the need for ligand binding [3].The *sev-GAL4>Ras1*^{V12} pupae developed normally like wild type (Fig. 1A, E and I) till mid-pupal stages but a majority (~88%, N =1058) of them died subsequently (Fig. 1B, F and J); the remaining pupae eclosed with rough eyes due to the extra R7 photoreceptor. Interestingly, when levels of hsrω RNA were lowered by co-expression of *hsrω-RNAi* transgene [15] in *sev-GAL4>Ras1*^{V12} expressing pupae, development appeared normal till 8-9 Hr after pupa formation (APF) (Fig. 1C, G and K) but further development was affected, resulting in death of ~ 96% (N =1177) of them by about 25-30 Hr APF; the few survivors died as late pupae. Following co-expression of the *EP3037* over-expressing allele of *hsrω* [15] with *sev-GAL4>Ras1*^{V12}, 42% (N =1109) died as early pupae (~25-30 Hr APF) while the remaining died as late pupae (Fig. 1D, H and L). Anterior part of all the early dying pupae appeared empty with necrotic patch/es at the eye region being visible by 23-24 Hr APF (Fig. 1K and L). Such dying pupae showed a demarcation between head, thorax and abdomen, but their Malpighian tubules, which become abdominal by this stage in *sev-GAL4>Ras1*^{V12} and in wild type (Fig. 2A), remained thoracic (Fig. 2B, C). Malpighian tubules in the early dying 23-24 Hr old pupae also retained a comparatively longer anterior segment (Fig. 2E and F) like that seen in the 14-15 Hr old *sev-GAL4>Ras1*^{V12} or wild type (not shown) pupae with their cells being far less compactly arranged than in 23-24 Hr *sev-GAL4>Ras1*^{V12} pupae (Fig. 2G-I). Interestingly, the salivary glands, which normally disappear by this stage of pupal development, also persisted beyond 15-16 Hr APF stage (Fig. 2J-L) in pupae co-expressing *sev-GAL4>Ras1*^{V12}and *hsrω-RNAi* or *EP3037*. The other remarkable early pupal phenotype that persisted in the early dying pupae was the *sev-GAL4* driven *UAS-GFP* expression in the 7 pairs of dorso-medially placed neurons in each of the

posterior neuromeres in abdominal ganglia [11]. In *sev-GAL4>Ras1*^{V12} pupae (Fig. 2M), like that in wild type, all the 7 pairs of these dorso-medial neurons showed *UAS-GFP* expression at 8-9 Hr APF. As reported earlier [11], all of these, except the anterior-most pair, disappeared in 23-24 Hr old wild type and *sev-GAL4>Ras1*^{V12} pupae (Fig. 2P). However, all the 7 pairs of the dorso-medial neurons continued to show the *UAS-GFP* expression in 23-24 Hr old *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* or *sev-GAL4>Ras1*^{V12} *EP3037* pupae (Fig. 2Q, R). In addition to these dorso-medial pairs, several other GFP expressing neurons that are scattered in lateral sides of the abdominal ganglia and which disappear by 23-24 Hr APF in wild type and *sev-GAL4>Ras1*^{V12} pupae [11], also persisted till 23-24 Hr APF when *hsrω-RNAi* or *EP3037* was co-expressed with *Ras1*^{V12} (not shown).

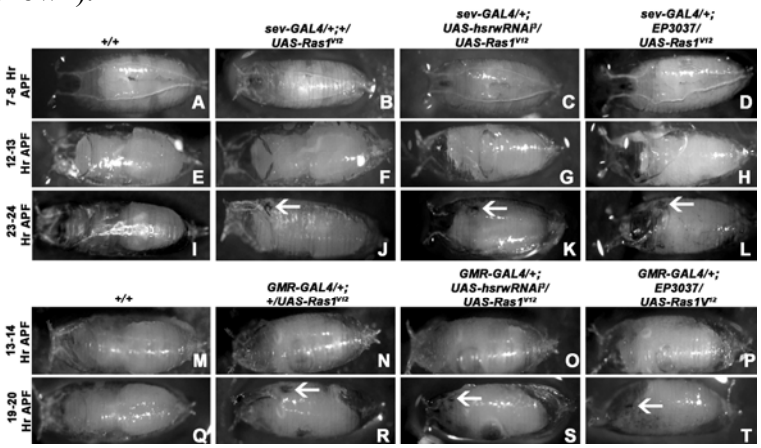

Fig. 1. *sev-GAL4* or *GMR-GAL4* driven expression of activated Ras together with altered hsrω-n transcript levels leads to early pupal lethality accompanied by absence of prepupal to pupal metamorphic changes. (**A-T**) pupae of different stages (noted on left of each row) in different genotypes (noted on top of the columns). White arrows in **J-L** and **R-T** indicate the necrotic patch near eye region

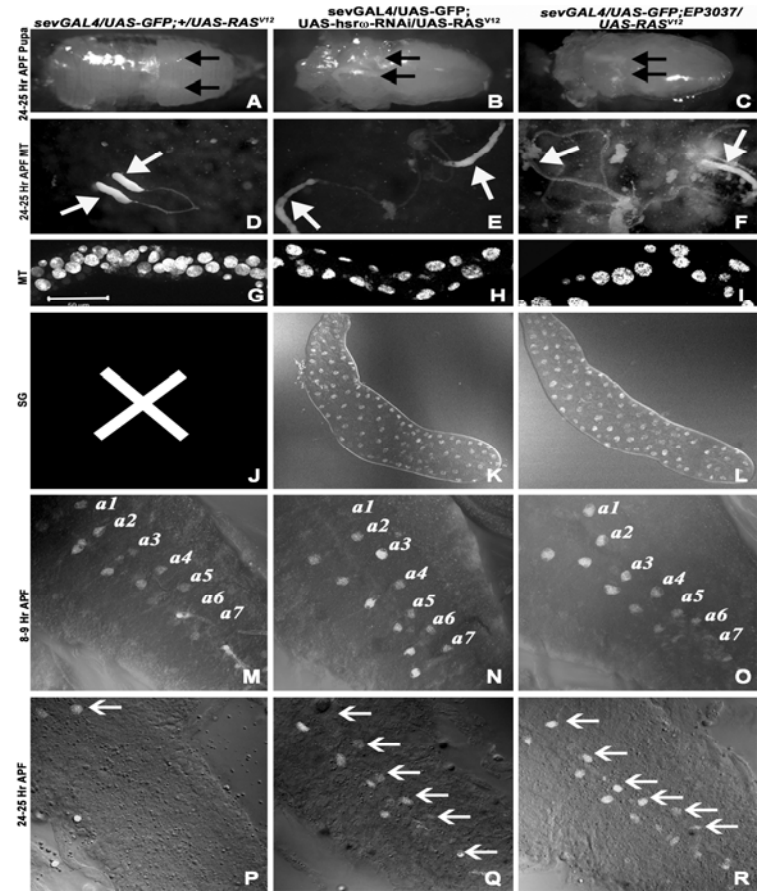

Fig. 2. The prepupal to pupal metamorphic changes in internal organs are affected in early dying pupae. 24-25 Hr old pupae (**A-C**) and dissected out anterior Malpighian tubules (**D-F**) of genotypes noted above each column.White arrows in **D-F**indicate the distal segments of anterior Malpighian tubules. (**G-L**) Confocal optical sections of DAPI stained Malpighian tubules (**G-I**) and salivary glands (**J-L**) of 24-25 Hr pupae; a cross is marked at **J** as salivary glands are no longer seen at 24-25 Hr APF in *sev-GAL4>Ras1*^{V12}. (**M-R**) Confocal projections of ventral ganglia showing *sev-GAL4*>UAS-GFP expressing (white) dorso-medial segmental paired neurons (arrows) at 8-9 Hr APF (**M-O**) and at 24-25 Hr APF (**P-R**) stages in different genotypes noted above each column.

It may be noted that the small proportion of pupae which do not die at early pupal stage, show normal developmental changes comparable to those seen in wild type or *sev-GAL4>Ras1*^{V12} pupae of comparable age. However, their subsequent development gets affected so that they fail to eclose and die as late pupae.

The *GMR-GAL4* expression is generally similar to that of the *sev-GAL4*, except in eye discs where the *sev-GAL4* expression is limited to a few rhabdomeres in each ommatidium while the *GMR-GAL4* expression is much more extensive, being expressed in nearly all cells of the eye disc behind the morphogenetic furrow [11]. Correspondingly, *GMR-GAL4* driven expression of *UAS-Ras1*^{V12} resulted in 100% early pupal lethality with developmental defects setting in by 8-9 Hr APF. Unlike the 13-14 or 19-20 Hr old wild type pupae (Fig. 1M, Q) or *GMR-GAL4>UAS-GFP* pupae (not shown), those expressing *GMR-GAL4>Ras1*^{V12} did not show proper demarcation of head, thorax and abdominal regions (Fig. 1N and R). Co-expression of *EP3037* or *hsrω-RNAi* in *GMR*-GAL4>*UAS-Ras1*^{V12}expressing individuals did not affect the time of pupal death as all of them died at the early stage with similar phenotypes (Fig. 1N-P, R-T). A necrotic patch appeared by 19-20 Hr APF at each of prospective adult eye site (Fig. 1R, S, T). The early disappearing dorso-medial pairs of neurons in the abdominal ganglia also continued to show GFP expression till 20-24 Hr APF in the dying *GMR-GAL4>UAS-Ras1*^{V12}pupae (see Fig. 3I-L)..

3.2. Expression of *GMR-GAL4* driven activated Ras leads to early pupal lethality which is not affected by altered levels of hsrω transcripts

3.3. Ecdysone signalling at early pupal stages is compromised in the early dying pupae

The above phenotypes indicated that the early pupal changes that follow the ecdysone pulse beginning at 8-10 Hr APF [23] were absent in pupae that would die around 25-30 Hr APF. Therefore, we examined the distribution/expression of Broad, one of the early responders to ecdysone signalling [24, 25], by immunostaining of late third instar larval and pupal (12-25 Hr APF) ventral ganglia of different genotypes with a Broad-core antibody which recognizes all the isoforms of Broad [26]. The Broad positive ventral ganglia cells from third instar larval (not shown) and early pupal stages (12-13 Hr APF) of all the genotypes showed similar intense and uniform nuclear fluorescence for Broad with little cytoplasmic signal (Fig. 3A-D). However, beginning at 16 Hr APF, the distribution of Broad in *sev-GAL4>UAS-GFP* and *sev-GAL4>Ras1*^{V12} ventral ganglia changed remarkably. Instead of its uniform nuclear distribution seen in younger pupae, Broad became restricted to a few intense peri-nuclear granules (Fig. 3E and F) and at the same time, its presence in cytoplasm was stronger than in earlier stages. Interestingly, ventral ganglia in pupae co-expressing *hsrω-RNAi* or *EP3037* with *Ras1*^{V12} under the *sev-GAL4* driver did not show these changes since even at 24-25 Hr APF, most Broad positive cells showed more or less uniform pan-nuclear distribution of Broad with low presence in cytoplasm (Fig. 3G, H). A few nuclei in the 24-25 Hr old pupal ventral ganglia co-expressing *sev-GAL4* driven *Ras1*^{V12} and *hsrω-RNAi* or *EP3037* showed Broad in a punctate or granular form, but these puncta were distributed across the nuclear area rather than being typically peri-nuclear as seen in the same age *sev-GAL4>UAS-GFP* and *sev-GAL4>Ras1*^{V12} pupal ventral ganglia (Fig. 3).

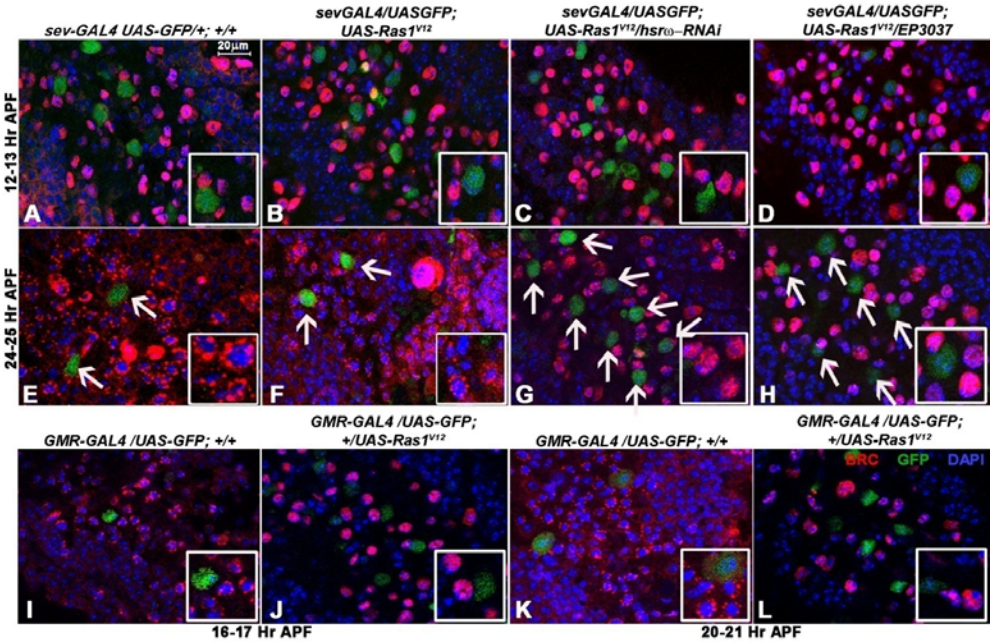

Fig. 3. Distribution of Broad protein in ventral ganglia cells at 16-17 Hr or later APF is affected in early dying pupae. (**A-H**) Confocal optical sections showing parts of ventral ganglia from 12-13 Hr (**A-D**) and 24-25 Hr (**E-H**) old pupae immunostained with anti-Broad-core (red) and counterstained with DAPI (blue); GFP (green) marks the *sev-GAL4* expressing neurons (also indicated by white arows). Genotypes are noted above each column. (**I-L)** Confocal optical sections of parts of ventral ganglia from 16-17 Hr (**I, J**) and 20-21 Hr (**K, L**) old pupae (genotypes noted above each image) immunostained with anti-Broad-core (red), counterstained and with DAPI (blue); GFP (green) marks the *GMR-GAL4* expressing neurons. Insets show higher magnification images of a few Broad expressing cells. Scale bar in (**A**) indicates 20µm and applies to all images.

The *GMR-GAL4>UAS-GFP* expressing ventral ganglia cells from 16-17 and 20-21 Hr old pupae (Fig. 3I, K) showed the Broad protein as noted above for corresponding age *sev-GAL4>UAS-GFP* and *sev-GAL4>Ras1^{V12}* pupae. However, in the ventral ganglia of individuals expressing *GMR-GAL4>Ras1^{V12}*, the Broad protein retained its uniformly strong pan-nuclear distribution in most cells (Fig. 3J, L) as in the 12-13 Hr APF or younger pupae; the cytoplasmic presence of Broad was also low in these cells.

Interestingly, none of the *sev-GAL4>UAS-GFP* expressing neurons in the ventral ganglia (marked by white arrows in Fig 2E-H), showed Broad expression, neither at 8-9 Hr nor at 24-25 Hr APF stage (Fig. 3). Thus they do not seem to respond to ecdysone through the Broad signalling pathway.

The absence of redistribution of Broad protein in 16 Hr or older pupae that would die at about 25-30 Hr APF further indicated a failure of the post-pupation ecdysone signalling. This was confirmed by immunoassay for 20-hydroxyecdysone. It was seen that ecdysone levels in 8-9 Hr old pupae expressing *sev-GAL4* driven activated Ras alone or together with *hsrω-RNAi* transgene or *EP3037* allele were comparable to those in similar age *sev-GAL4>UAS-GFP* pupae (Fig. 4). However, the 24-25 Hr APF stage pupae that were co-expressing *hsrω-RNAi* or *EP3037* with *Ras1^{V12}* under the *sev-GAL4* driver did not display the increase in ecdysone levels that is characteristically seen in those expressing the *sev-GAL4* driven *UAS-GFP* or only activated Ras or only the *hsrω-RNAi* transgene or *EP3037* (Fig. 4).

The 24-25 Hr APF stage pupae expressing activated Ras under the *GMR-GAL4* driver also did not show the expected increase in ecdysone levels at this stage (data not presented).

These results showed that expression of activated Ras in conjunction with *hsrω-RNAi* or *EP3037* under the *sev-GAL4* driver or expression of activated Ras alone under the *GMR-GAL4* driver does not affect ecdysone levels till ~8 Hr APF but the scheduled elevation in ecdysone levels after this stage [23] is inhibited.

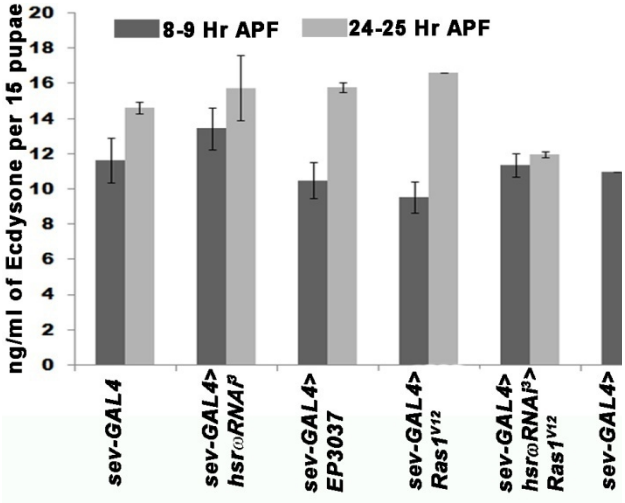

Fig. 4. **The expected increase in ecdysone levels in post 8-9 Hr pupae does not occur in pupae that are destined to die early.** Histogram showing mean (±S.E. of two independent replicates) levels of 20 hydroxyecdysone (Y axis) in different genotypes (X-axis) at 8-9 Hr APF (dark grey) and at 24-25 Hr APF stages (light grey). Each genotype also carries *UAS-GFP* transgene.

Further support for reduced levels of ecdysone being responsible for the early pupal death was obtained by examining phenotypes of otherwise wild type early pupae in which the ecdysone levels were conditionally down-regulated using the temperature sensitive ecd^1 allele [18, 19]. This temperature-sensitive mutation inhibits production of ecdysone when grown at the restrictive temperature (29-30°C). The ecd^1 larvae were reared at 24°C and 0-1 Hr old white prepupae were transferred to 30°C to conditionally inhibit further ecdysone synthesis. About 50% of these pupae showed early death by 25-30 Hr APF while the remaining ones died as late pupae (Table 2). The dying 24-25 Hr old ecd^1 pupae, reared since beginning of pupation at 30°C, showed persistence of the dorso-medial pairs of segmental neurons in ventral ganglia, less compact arrangement of Malpighian tubule cells (Fig 5A, B) and the other developmental defects seen in pupae co-expressing *sev-GAL4* driven activated Ras and *hsrω-RNAi* or *EP3037*. Thus we believe that the thwarted metamorphosis and subsequent death of early pupae expressing $Ras1^{V12}$ under *GMR-GAL4* or co-expressing $Ras1^{V12}$and *hsrω-RNAi* or *EP3037* under the *sev-GAL4* driver is a consequence of sub-threshold levels of ecdysone after 8 Hr of pupal development.

3.4. Externally provided ecdysone partially rescues the early pupal death

To further confirm that the early pupal death that occurs when $Ras1^{V12}$ and *hsrω-RNAi* or $Ras1^{V12}$ and *EP3037* are co-expressed under the *sev-GAL4* driver is a consequence of sub-threshold levels of ecdysone, we exposed 8-9 Hr old pupae of different genotypes to exogenous ecdysone by incubating them in 1 µg/ml 20-hydroxy-ecdysone solution for 12 Hr, following which they were taken out and allowed to develop further. To confirm that such exogenous exposure to ecdysone is effective, we used ecd^1 mutant pupae as control. Fresh 0-1 Hr old ecd^1 white prepupae, grown through the larval period at 24°C, were transferred to 30°C to block further ecdysone synthesis. After 8 hr at 30°C, they were transferred to ecdysone solution for 12 Hr at 30°C so that while the endogenous ecdysone synthesis remains inhibited, it can be available in the medium of incubation. After the 12 Hr ecdysone treatment, they were returned to 30°C for further development. As noted above and from the data in Table 2 and Fig. 5, about 50% ecd^1 pupae maintained at 30°C from 0-1 Hr prepupa stage onwards without any exogenous ecdysone

died by 25-30 Hr APF. However, there was a 2-fold decrease in the early death of *ecd*^{1} pupae maintained at 30°C but exposed to exogenous ecdysone from 8 to 20 hr APF (Table 2), although none of them eclosed as flies. Significantly, the prepupal to pupal metamorphic changes in these surviving pupae occurred normally so that the dorso-medial pairs of segmental neurons in ventral ganglia and Malpighian tubules displayed the expected changes (Fig. 5C, D). The survival of a significant proportion of *ecd*^{1} pupae, maintained at 30°C while being exposed to exogenous ecdysone, clearly indicated that the moulting hormone penetrated the early pupal case in at least some of them and rescued the absence of endogenous ecdysone in *ecd*^{1} pupae at the restrictive temperature.

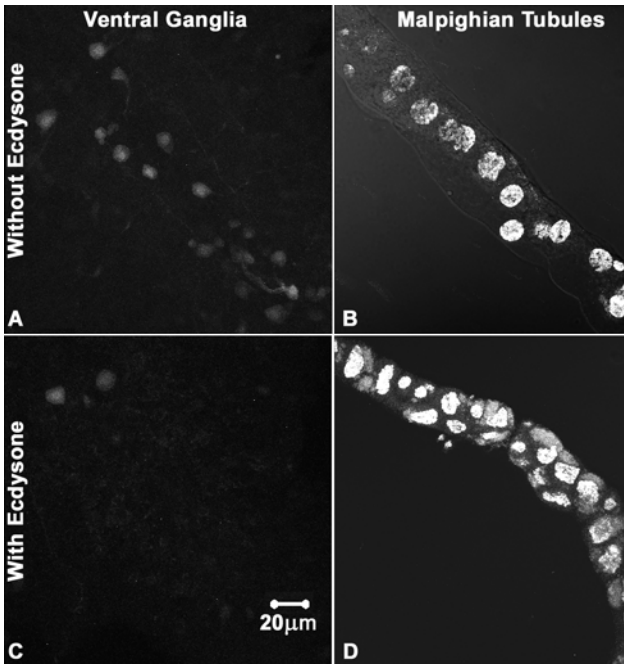

Fig. 5. Exogenous ecdysone promotes prepupal to pupal metamorphic changes in *ecd*^{1} mutant pupae maintained at the restrictive temperature (30^{0}C). (**A**, **C**) Confocal projection images showing *sevGAL4>UAS-GFP* (white) expression in 24-25 Hr old *ecd*^{1} mutant pupae maintained at 30^{0}C since the beginning of pupation without (**A**) or with (**C**) exposure to exogenous ecdysone between 8-20 Hr APF. (**B**, **D**) Confocal projection images of DAPI-stained (white) Malpighian tubules of 24-25 Hr old *ecd*^{1} mutant pupae maintained at 30^{0}C since the beginning of pupation without (**B**) or with (**D**) exposure to exogenous ecdysone between 8-20 Hr APF. The scale bar in **C** applies to all the images.

Table 2. Exogenous ecdysone suppresses early pupal death in *sev-GAL4>UAS-GFP; ecd*^{1} (maintained at restrictive temperature from 0 Hr prepupa onwards), *Sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *Sev-GAL4>Ras1*^{V12} *EP3037* pupae

Treatment	**Genotype**								
	***sev-GAL4>UAS-GFP; ecd*^{1} at 30^{0}C**			***sev-GAL4>Ras1*^{V12} *hsrω-RNAi* at 24^{0}C**			***sev-GAL4>Ras1*^{V12} *EP3037* at 24^{0}C**		
	N	**Early Pupal Death**	**Late Pupal Death**	**N**	**Early Pupal Death**	**Late Pupal Death**	**N**	**Early Pupal Death**	**Late Pupal Death**
a. Control	**43**	**54%**	**46%**	**49**	**82%**	**18%**	**38**	**71%**	**29%**
b. Ecdysone treatment*	**48**	**27%**	**73%**	**62**	**58%**	**42%**	**50**	**32%**	**68%**
Fold change in time of death (a/b)		**2 Fold decrease**			**1.4 Fold decrease**			**2.2 Fold decrease**	

* 8 Hr old pupae incubated in ecdysone (1µg/ml) containing PBS for 12 Hr at the indicated temperature following which they were bloated dry and monitored for their subsequent development.

Significantly, when 8 Hr old pupae co-expressing *sev-GAL4* driven activated Ras and *hsrω-RNAi* or activated Ras and *EP3037* were exposed to exogenous ecdysone as above, there was a near 2-fold increase in those continuing to develop to late stages, although none of them emerged as

flies (Table 2). The somewhat lesser increase (1.4 fold, Table 2) in survival of the ecdysone exposed *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* pupae beyond the early stage seems to be related to the fact that a larger proportion of them die early (see above). All pupae surviving beyond the early stages displayed all the prepupal to pupal metamorphic changes (not shown).

Together, these results confirm that *sev-GAL4* driven activated Ras and *hsrω-RNAi* or activated Ras and *EP3037* expression somehow inhibits ecdysone release that occurs after 8-9 Hr pupal stage and therefore, such pupae fail to undergo the expected metamorphic changes.

3.5. Co-expression of *sev-GAL4>Ras1*^{V12} and *hsrω-RNAi* or *EP3037* down-regulated steroid biosynthesis pathway genes while up regulating Dilp8 and some JNK pathway genes

We recently showed [11] that contrary to the conventionally believed eye-disc specific expression of *GMR-GAL4* and *sev-GAL4* drivers, they also express in several other tissues, including a set of neurons in the central nervous system (also see above). However, neither of these drivers express in prothoracic glands where ecdysone is primarily synthesized. Therefore, to identify the signals that may affect ecdysone levels following co-expression of *sev-GAL4* driven *Ras1*^{V12} and *hsrω*-RNAi or *EP3037*, total RNAs from 16-17 Hr old pupae of different genotypes (*sev-GAL4>UAS-GFP, sev-GAL4>Ras1*^{V12}, *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12} *EP3037*) were used for a microarray based transcriptome analysis. As noted in Table 3, a pair wise comparison between different genotypes revealed significant up- or down-regulation of many genes belonging to diverse GO categories. In order to narrow down the genes which may be causally associated with the early pupal death, we focused on those genes that were commonly up- or down-regulated in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12} *EP3037* samples when compared with their levels in *sev-GAL4>Ras1*^{V12} (columns 1 and 2 in Table 3). The commonly up-regulated groups of genes in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12} *EP3037* larvae included, among many others (Table 3), those associated with stress responses, cell and tissue death pathways, autophagy, toll signalling, innate immune system etc. Some of these seem to be primarily related to activated Ras or altered *hsrω* expression since the Ras/MAPK pathway is known to regulate immune response [27, 28] while the hsrω transcripts have roles in stress responses, cell death and several other pathways [29-31]. More interestingly, those commonly down-regulated in both the early pupal death associated genotypes (*sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12}*EP3037*) included lipid and steroid biosynthesis processes, besides some of the immune response related genes (Table 3). The down-regulation of lipid and steroid synthesis pathway genes agrees with the above noted reduced levels of ecdysone in the early dying pupae.

Since damage to or slow growth of imaginal discs is reported [13, 14] to affect ecdysone synthesis through up regulation of Dilp8 levels and JNK signalling, we examined the microarray data for levels of transcripts of genes involved in ecdysone, insulin and JNK pathways through pair wise comparisons of different genotypes (Table 4), taking >1.5 fold difference as significant. In one set (columns 1-3 in Table 4), we compared transcript levels of specific genes in *sev-GAL4>UAS-GFP* (control) with those in *sev-GAL4>Ras1*^{V12}, *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* or *sev-GAL4>Ras1*^{V12} *EP3037* genotypes while in another set (columns 4 and 5 in Table 4), transcript levels in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* or *sev-GAL4>Ras1*^{V12} *EP3037* were compared with those in *sev-GAL4>Ras1*^{V12} early pupae.

Three (*spookier, phantom* and *shadow*) of the five 20-hydroxy-ecdysone synthesis related Halloween genes [32] showed consistent down-regulation in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and

sev-GAL4>Ras1^{V12} EP3037 genotypes when compared with *sev-GAL4>Ras1^{V12}* pupae, while *shade* did not show any significant change and *disembodied* displayed up-regulation. The down-regulation of spookier, phantom and shadow transcripts in 16-17 Hr old *sev-GAL4>Ras1^{V12}hsrω-RNAi* and *sev-GAL4>Ras1^{V12}EP3037* pupae correlates with the above noted (Fig. 4) reduced ecdysone levels since while the *spookier* and *phantom* genes act at early stages in ecdysone biosynthesis [32], the *shadow* gene product converts 2-deoxyecdysone into ecdysone [33].

Our microarray analysis of total pupal RNA further revealed that of the eight known Dilps (Dilp1-8), only the levels of Dilp8 were significantly up-regulated in *sev-GAL4>Ras1^{V12} hsrω-RNAi* and *sev-GAL4>Ras1^{V12} EP3037* genotypes when compared with *sev-GAL4>UAS-GFP* or *sev-GAL4>Ras1^{V12}* (Table 4). Interestingly, when compared with *sev-GAL4>Ras1^{V12}*, the increase in Dilp8 transcripts in *sev-GAL4>Ras1^{V12}hsrω-RNAi* was about 2 folds greater than in *sev-GAL4>Ras1^{V12}EP3037* (Table 2). This seems to correlate with the earlier noted (section **3.1**) much higher frequency of death of former as early pupae than in case of *sev-GAL4>Ras1^{V12} EP3037*.

Like the Dilp8, transcripts of *tobi* (*target of brain insulin*) were also significantly up-regulated (Table 4) when *sev-GAL4>Ras1^{V12}* was expressed in conjunction with *hsrω-RNAi* or *EP3037*. It has recently [34, 35] been shown that the Dilp8 action on ecdysone synthesis in prothoracic gland may involve the neuronal relaxin receptor Lgr3. Our microarray data indicated that the Lgr3 transcripts were marginally elevated in *sev-GAL4>Ras1^{V12}hsrω-RNAi* expressing early pupae but not in *sev-GAL4>Ras1^{V12}EP3037* individuals.

Levels of different members of the JNK signalling pathway showed considerable variability from no effect to up- or down-regulation in different genotypes (Table 4). Interestingly, a greater number of the JNK pathway genes were up regulated in *sev-GAL4>Ras1^{V12} hsrω-RNAi* when compared with *sev-GAL4>UAS-GFP* or *sev-GAL4>Ras1^{V12}* genotypes. On the other hand, several of the JNK pathway genes appeared down regulated in *sev-GAL4>Ras1^{V12} EP3037* genotypes when compared with *sev-GAL4>UAS-GFP* or *sev-GAL4>Ras1^{V12}* genotypes (Table 4). However, genes like *eiger*, *Gadd45* and *Takl1* were significantly up regulated in *sev-GAL4>Ras1^{V12} hsrω-RNAi* as well as in *sev-GAL4>Ras1^{V12} EP3037* genotypes. On the other hand, the *Tak1* was down-regulated in both. It is noteworthy that the fold change for each of these genes was greater in case of *sev-GAL4>Ras1^{V12} hsrω-RNAi* than in *sev-GAL4>Ras1^{V12}* EP3037.

Table 3. Major pathways affected in individuals co-expressing *sev-GAL4* driven activated Ras (*Ras1*^{V12})and *EP3037*or activated Ras and *hsrω-RNAi* when compared with those expressing *sev-GAL4* driven activated Ras only.

Pathways commonly up-regulated in *sev-GAL4>Ras1*^{V12}*>EP3037* and *sev-GAL4>Ras1*^{V12}*>hsrω-RNAi*	**Pathways commonly down-regulated in *sev-GAL4>Ras1*^{V12}*>EP3037* and *sev-GAL4>Ras1*^{V12}*>hsrω-RNAi***	**Pathways down-regulated in *sev-GAL4>Ras1*^{V12}*>EP3037* but up-regulated in *sev-GAL4>Ras1*^{V12}*>hsrω-RNAi***	**Pathways down-regulated only in *sev-GAL4>Ras1*^{V12}*>EP3037***	**Pathways up-regulated only in *sev-GAL4>Ras1*^{V12}*>hsrω-RNAi***
GO:0006030: chitin metabolic process	GO:0008610: lipid biosynthetic process	GO:0007160: cell-matrix adhesion	GO:0051174: regulation of phosphorus metabolic process	GO:0042180: cellular ketone metabolic process
GO:0006022: aminoglycan metabolic process	GO:0005976: polysaccharide metabolic process	GO:0031589: cell-substrate adhesion	GO:0019220: regulation of phosphate metabolic process	GO:0006575: cellular amino acid derivative metabolic process
GO:0016052: carbohydrate catabolic process	GO:0006694: steroid biosynthetic process	GO:0007155: cell adhesion	GO:0055114: oxidation reduction	GO:0019471: 4-hydroxyproline metabolic process
GO:0005976: polysaccharide metabolic process	GO:0002376: immune system process	GO:0010631: epithelial cell migration	GO:0009101: glycoprotein biosynthetic process	GO:0043436: oxoacid metabolic process
GO:0006950: response to stress	GO:0045087: innate immune response	GO:0007165: signal transduction	GO:0046068: cGMP metabolic process	GO:0006082: organic acid metabolic process
GO:0009266: response to temperature stimulus		GO:0007188: G-protein signalling, coupled to cAMP nucleotide second messenger	GO:0035103: sterol regulatory element binding protein cleavage	GO:0019752: carboxylic acid metabolic process
GO:0009308: amine metabolic process		GO:0007187: G-protein signalling, coupled to cyclic nucleotide second messenger	GO:0007552: metamorphosis	GO:0007254: JNK cascade
GO:0005975: carbohydrate metabolic process		GO:0007166: cell surface receptor linked signal transduction	GO:0006334: nucleosome assembly	GO:0031098: stress-activated protein kinase signalling pathway
GO:0006629: lipid metabolic process		GO:0031644: regulation of neurological system process	GO:0031497: chromatin assembly	GO:0007600: sensory perception
GO:0045087: innate immune response		GO:0050890: cognition	GO:0006281: DNA repair	GO:0006816: calcium ion transport
GO:0006955: immune response		GO:0035272: exocrine system development	GO:0065004: protein-DNA complex assembly	GO:0007568: aging
GO:0008063: Toll signalling pathway		GO:0022008: neurogenesis	GO:0006259: DNA metabolic process	
GO:0008219: cell death			GO:0006325: chromatin organization	
GO:0035070: salivary gland histolysis			GO:0032933: SREBP-mediated signalling pathway	
GO:0048102: autophagic cell death			GO:0008593: regulation of Notch signalling pathway	
GO:0016271: tissue death			GO:0010627: regulation of protein kinase cascade	
GO:0007559: histolysis			GO:0045165: cell fate commitment	
GO:0012501: programmed cell death			GO:0008356: asymmetric cell division	
GO:0006865: amino acid transport			GO:0007164: establishment of tissue polarity	
GO:0006508: proteolysis				
GO:0006582: melanin metabolic process				

GO:0006570: tyrosine metabolic process			GO:0051605: protein maturation by peptide bond cleavage GO:0007218: neuropeptide signalling pathway GO:0006836: neurotransmitter transport GO:0001505: regulation of neurotransmitter levels GO:0007269: neurotransmitter secretion GO:0007090: regulation of S phase of mitotic cell cycle GO:0007346: regulation of mitotic cell cycle GO:0045132: meiotic chromosome segregation	

Table 4. Transcription of several genes in Steroid and Ecdysone biosynthesis, Insulin and JNK pathways is affected when levels of hsrω transcripts are altered along with the expression of *sev-GAL4>Ras1^{V12}*

Genes	Mean Fold changes in transcript levels between different pairs of genotypes				
	sev-GAL4> *Ras1^{V12}* **vs** ***sev-GAL4>UAS-GFP***	***sev-GAL4>*** *Ras1^{V12}* ***hsrω-*** **RNAi vs** ***sev-GAL4>UAS-GFP***	***sev-GAL4>*** *Ras1^{V12}* ***EP3037*** **vs** ***sev-GAL4>UAS-GFP***	***sev-GAL4>*** *Ras1^{V12}* ***hsrω-*** **RNAi vs** ***sev-GAL4>*** *Ras1^{V12}*	***sev-GAL4>*** *Ras1^{V12}* ***EP3037*** **vs** ***sev-GAL4>*** *Ras1^{V12}*
			Ecdysone Biosynthesis Pathway		
Spok	2.55	-1.24	-1.81	-3.17	-4.62
phm	1.74	-1.18	-1.75	-2.06	-3.04
dib	-1.24	4.63	1.91	5.73	2.37
sad	1.65	-1.31	-1.65	-2.16	-2.71
shd	-1.11	1.35	-1.16	1.5	-1.05
			Insulin Pathway		
Dilp1	1.21	1.48	-1.9	1.22	-2.31
Dilp 2	-1.75	-1.11	-1.76	1.58	-1.01
Dilp 3	1.65	1.28	-1.33	-1.29	-2.19
Dilp 4	-1.09	1.59	-1.23	1.73	-1.13
Dilp 5	-1.07	1.6	-1.92	1.71	-1.8
Dilp 6	-1.24	1.18	-1.63	1.47	-1.32
Dilp 7	-1.21	1.36	-1.28	1.65	-1.05
Dilp 8	1.48	6.56	2.79	4.43	1.88
Tobi	N. S.	8.08	2.83	5.34	1.87
Lgr3	1.13	1.84	1.09	1.63	-1.04
			JNK Pathway		
bsk	1.71	1.96	-1.17	1.15	-2
CYLD	1.59	2.12	1.01	1.33	-1.58
dpp	1.27	2.49	-1.43	1.95	-1.82
egr	2.86	5.66	2.1	1.98	-1.36
Gadd45	2.08	5.71	2.74	2.74	1.32
hep	1.28	1.56	-1.4	1.37	-1.59
Jra	1.48	2.31	1.08	1.56	-1.37
kay	-1.15	1.68	-1.49	1.93	-1.3
Mnn1	1.36	1.68	-1.61	1.23	-2.19
msn	1.11	2.16	-1.43	1.94	-1.59
NLaz	1.21	2.86	1.57	2.37	1.3
PSR	1.16	1.11	-1.77	-1.04	-2.04
puc	1.11	2.4	1.09	2.16	-1.02
pyd	-1.92	2.15	1.13	4.12	2.16
Rala	-3.29	-1.1	-2.91	3	1.13
raw	1.52	4.31	1.63	2.83	1.07
scaf	1.86	1.52	-1.3	-1.22	-2.41
Src42A	1.27	1.56	-1.22	1.23	-1.56
Src64B	1.94	3.2	1.26	1.65	-1.54
Tak1	1.6	-1.1	-2.27	-1.75	-3.62
Takl1	-1.58	7.84	2.59	12.37	4.08
Traf-like	-1.46	1.68	-1.05	2.45	1.39
wnd	1.06	-1.08	-1.71	-1.15	-1.82

Note: the gray highlighted values indicate similar up-or down regulation in genotypes co-expressing *hsrω-RNAi* or *EP3037* with *Ras1^{V12}*

We validated some of the microarray data by qRT-PCR with RNA samples from late larval and early pupal (8-9 Hr APF) eye discs and/or prothoracic glands (Fig. 6). In agreement with the microarray data for whole pupal RNA, qRT-PCR of 8-9 Hr old pupal eye disc RNA confirmed that transcripts of the JNK signalling pathway genes like *tak1-like 1, eiger 2, Gadd45* and *puckered* were significantly up-regulated in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12} *EP3037* when compared with same age *sev-GAL4>Ras1*^{V12} eye discs (Fig. 6). Interestingly, the late third instar larval eye discs did not show as much fold difference for these transcripts (Fig. 6).

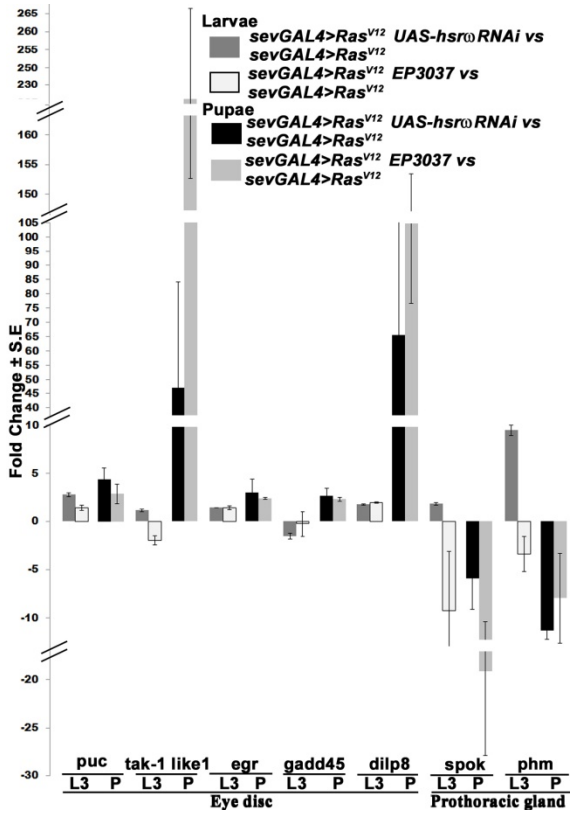

Fig. 6. In early dying pupal genotypes, transcript levels of some JNK pathway, Dilp8 and Haloween genes change significantly at early pupal but not late larval stages. Histogram showing qRT-PCR based mean (±S.E.) fold changes (Y-axis) in levels of different transcripts (X-axis) in eye discs or prothoracic glands of *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12} *EP3037* late 3rd instar larvae (L3) or 8-9 Hr old pupae (P) when compared with those expressing only activated Ras under *sev-GAL4* driver.

Similarly, qRT-PCR for Dilp8 transcripts from late third instar larval and 8-9 Hr pupal eye discs of different genotypes revealed that, in agreement with microarray data for total pupal RNA, Dilp8 transcripts were greatly (65-120 folds) elevated in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12}*EP3037* pupal eye discs when compared with those expressing only the activated Ras (Fig. 6). On the other hand, levels of Dilp8 transcripts in late third instar larval eye discs showed only a marginal up-regulation in *sev-GAL4>Ras1*^{V12}*hsrω-RNAi* and *sev-GAL4>Ras1*^{V12}*EP3037* eye discs (Fig. 6).

Down-regulation of spookier and phantom transcripts in *sev-GAL4>Ras1*^{V12}*hsrω-RNAi* and *sev-GAL4>Ras1*^{V12}*EP3037* genotypes at early pupal stage was also validated by qRT-PCR using RNA from 8-9 hr old pupal prothoracic glands. Compared to *sev-GAL4>Ras1*^{V12}, these two transcripts showed much greater reduction in *sev-GAL4>Ras1*^{V12}*hsrω-RNAi* and *sev-GAL4>Ras1*^{V12}*EP3037* pupal (Fig 6) than in larval prothoracic glands. Intriguingly, *sev-GAL4>Ras1*^{V12}*hsrω-RNAi* larval eye discs showed up-regulation of spookier and phantom transcripts (Fig. 6).

Elevation in levels of Dilp8 in the early dying pupae, but not in late third instar eye discs was also confirmed by immunostaining of eye discs with anti-Dilp8 and confocal microscopy. Dilp8 was nearly absent in photoreceptor cells of late third instar larval eye discs of all genotypes (not shown). However, a very faint expression of Dilp8 was seen in the cytoplasm of peripodial cells of late third instar larval eye discs of all the genotypes (not shown). Interestingly, the 8-9 Hr APF stage eye discs showed stronger presence of Dilp8 in photoreceptor cells of all the four genotypes than in the third instar stage. The peripodial cells of early pupal eye discs (Fig. 7A-D) co-expressing *sev-GAL4>Ras1*^{V12} and *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12}*EP3037* showed much stronger Dilp8 staining (Fig. 7C, D) than in *sev-GAL4>UAS-GFP* (Fig. 7A) or *sev-GAL4>Ras1*^{V12} (Fig. 7B) pupal discs.

Since our above microarray and qRT-PCR data indicated enhanced JNK signalling following co-expression of *sev-GAL4>Ras1*^{V12} and *hsrω-RNAi* transgene or *EP3037*, we also examined levels of phosphorylated JNK (p-JNK, Basket) in late third instar eye discs of these genotypes. All the photoreceptor cells in *sev-GAL4>UAS-GFP* (Fig. 7E) eye discs, independent of the expression of GFP, showed low levels of p-JNK in their cytoplasm. Photoreceptor cells from *sev-GAL4>Ras1*^{V12} larval discs showed slightly elevated p-JNK staining (Fig. 7F). Interestingly, co-expression of *hsrω-RNAi* transgene with *sev-GAL4>Ras1*^{V12} resulted in a much greater increase in p-JNK staining (Fig. 7G) in all photoreceptors cells irrespective of GFP expression. Up regulation of hsrω transcripts in *sev-GAL4>Ras1*^{V12} expressing eye discs was also associated with increase in p-JNK levels when compared with *sev-GAL4>UAS-GFP* (Fig. 7H), although the increase was less than that in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* eye discs.

Immunostaining for p-JNK in late larval eye discs expressing *GMR-GAL4* driven GFP (control, Fig. 7M) or *Ras1*^{V12} (Fig. 7N) revealed that the p-JNK levels were very high in discs expressing *GMR-GAL4* driven *Ras1*^{V12}, which correlates with their death as early pupae. Since co-expression of *hsrω-RNAi* transgene or *EP3037* allele did not alter the early pupal death in *GMR-GAL4* driven *Ras1*^{V12} expressing individuals, we did not examine p-JNK levels in their eye discs. Data on the relative quantities of p-JNK (Fig. 7O) in photoreceptor cells of different genotypes, obtained using the Histo tool of the LSM510 Meta software on projection images of 12 optical sections through the photoreceptor arrays in each disc, confirmed the significant increase in p-JNK in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12}*EP3037* eye discs.

To further know if the early pupal lethality observed above is related to JNK activation, we examined effects of *GMR-GAL4* driven expression of *UAS-rpr* or *UAS-127Q* or *UAS-Tak1* on pupal lethality and eye phenotypes. Like the *GMR-GAL4>Ras1*^{V12}, *GMR-GAL4* driven expression of *UAS–rpr* also caused early pupal death [5]. Interestingly, their eye imaginal discs, again like those of *GMR-GAL4>Ras1*^{V12} larvae, showed enhanced p-JNK level (not shown). On the other hand, *GMR-GAL4* driven expression of *UAS-127Q* did not cause early pupal death although, as known from earlier studies [15], resulted in rough eye phenotype in adult flies; in agreement with normal survival of *GMR-GAL4>UAS-127Q* pupae, their eye imaginal discs did not show elevated p-JNK (not shown). Tak1 is a known up-regulator of JNK signalling [36, 37], and in agreement with our other observations, *GMR-GAL4* driven expression of Tak1 led to early pupal lethality.

To ascertain that the JNK activation in eye discs co-expressing *sev-GAL4* driven *Ras1*^{V12} with *hsrω-RNAi* or *EP3037* is dependent upon the activated Ras expression, we co-expressed *UAS-RafRBDFLAG*. The RafRBDFLAG construct acts as a dominant negative suppressor of Ras signalling [17]. We examined the effect of *sev-GAL4* driven co-expression of *RafRBDFLAG*

with *Ras1*^{V12} without or with *hsrω-RNAi* or *EP3037* expression on pupal survival and p-JNK levels in eye discs of late 3rd instar larvae. Interestingly, neither the p-JNK levels were elevated nor was there any pupal lethality when the elevated Ras signalling was suppressed by co-expression of *RafRBDFLAG*. The p-JNK staining in all these cases (Fig.7I-L, O) was in fact lower than that seen in *sev-GAL4>UAS-GFP* larval eye discs.

The pupal survival, adult eye phenotype (when surviving as adults) and p-JNK staining patterns in the different genotypes examined in our study are summarized in Table 5. Together, these observations clearly show that high level of ectopic expression of activated Ras is associated with enhanced p-JNK levels and early pupal death, except when *RafRBDFLAG* is co-expressed to reduce the Ras signalling.

Table 5. Ras signalling dependent elevated p-JNK levels correlate with early pupal lethality

Genotype	**Pupal survival**	**Eye phenotype**	**p-JNK levels in 3rd instar disc**
GMR-GAL4>Ras1^{V12}	Early pupal death	NA	Very high
GMR-GAL4>rpr	Early pupal death	NA	High
GMR-GAL4>Tak1	Early pupal death	NA	Very high
GMR-GAL4>127Q	normal	Severe rough eye	Normal
sev-GAL4>UAS-GFP	Normal	Normal	Normal
sev-GAL4>Ras1^{V12}	88% Late pupal death; 12 % emerge as flies	Surviving flies with rough eyes	Higher than *sev-GAL4>UAS-GFP* but less than *sev-GAL4> Ras1*^{V12}*EP3037*and *sev-GAL4> Ras1*^{V12}*hsrω-RNAi*
sev-GAL4>Ras1^{V12} *hsrω-RNAi*	96% Early pupal death; 4% late pupal death	NA	Very high
sev-GAL4>Ras1^{V12} *EP3037*	42% Early pupal death; 58% late pupal death	NA	Higher than *sev-GAL4> Ras1*^{V12} but less than *sev-GAL4> Ras1*^{V12} *hsrω-RNAi*
sev-GAL4>RAFRBDFLAG	Normal	Normal	Low
sev-GAL4>Ras1^{V12} *RAFRBDFLAG*	Normal	Rough eye	Low
sev-GAL4>Ras1^{V12} *RAFRBDFLAG hsrω-RNAi*	Normal	Severe rough eye	Low
sev-GAL4>Ras1^{V12} *RAFRBDFLAGEP3037*	normal	Severe rough eye	Low

NA = not applicable as no adults emerged in these genotypes

Shaded cells indicate correlation between early pupal death and the observed enhanced p-JNK levels in eye discs

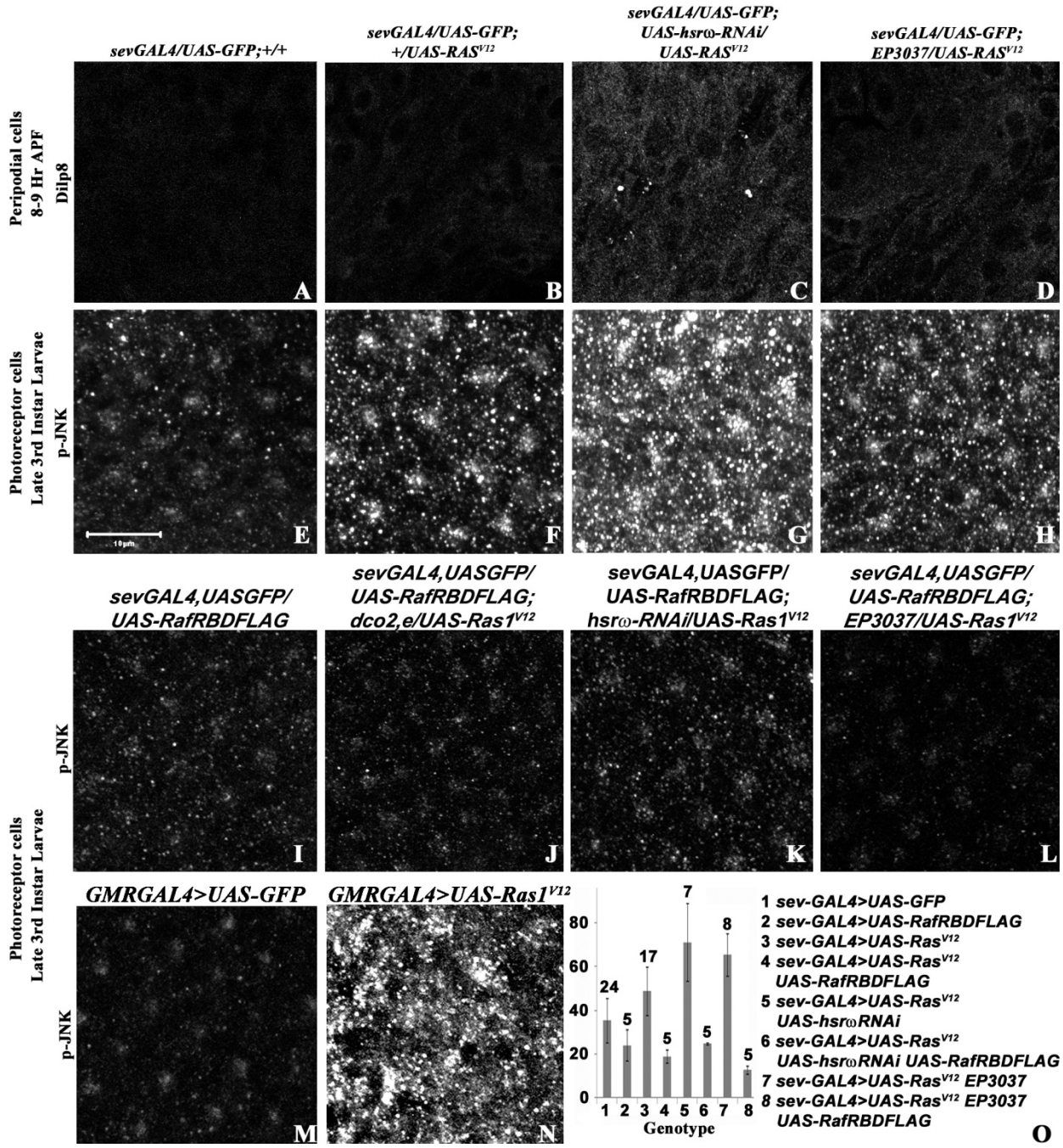

Fig. 7. **Expression of Dilp8 and phosphorylated JNK (p-JNK) is enhanced in eye discs of early dying pupae.** (**A-D)** Confocal optical sections showing expression of Dilp8 (white) in peripodial cells of 8-9 Hr APF stage eye discs of different genotypes (noted on top of each column); (**E-L)** Confocal projection images of third instar larval eye disc photoreceptor cells showing p-JNK (white) in different genotypes (noted above the columns). (**M-N**) Confocal projections showing p-JNK (white) in photoreceptor cells of third instar larval eye discs expressing GFP (**M**) or activated Ras (**N**) under *GMR-GAL4* driver. Genotypes of discs are noted above each column. Scale bar in **E** denotes 10μm and applies to all confocal images**.** (**O**)Histogram showing mean (± S. E.) fluorescence intensity (Y-axis) of p-JNK in the eye discs of different genotypes (1-8, identified on right side); number on top of each bar indicates the number of eye discs examined.

4. Discussion

Present study examines possible reasons for the unexpected pupal death when certain transgenes are expressed using the *GMR-GAL4* or *sev-GAL4* drivers, which are predominantly active in eye discs. We found that the ecdysone surge that normally occurs beyond 8-12 Hr APF [23] was absent in the early dying pupae and in agreement with this decrease in ecdysone levels in the early dying pupae, our microarray and qRT-PCR data revealed down regulation of several of the genes involved in ecdysone bio-synthesis. Together, these suggest that the observed early pupal death in some of the examined genotypes is due to sub-threshold levels of post-pupal ecdysone so that the required metamorphic changes in >8-12 Hr old pupae fail to occur and the organisms die. Our finding that conditional inhibition of ecdysone synthesis during early pupal stage in the temperature sensitive ecd^1 mutant also results in early and late pupal death like that in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* or *sev-GAL4>Ras1*^{V12} *EP3037* genotypes, and that this can be significantly suppressed by exogenous ecdysone provided during 8 to 20 Hr APF, clearly indicate that the reduced level of ecdysone after the 8-12 Hr APF stage is responsible for early pupal death in these genotypes. The fact that all of them do not survive beyond the early stage may be related to variability in penetration of ecdysone through the pupal case and other biological variables.

In agreement with the reduced levels of ecdysone in pupae, our microarray and qRT-PCR data clearly show that key genes like *spookier* and *phantom*, which are involved in the early steps in biosynthesis of ecdysone, are indeed down-regulated in early pupal prothoracic glands but not at late third instar stage. The unaltered levels of the *shade* gene transcripts, which encode Cyp314a1 that adds a hydroxyl group to ecdysone in the final step, may not help maintain 20-hydroxyecdysone levels since its substrate, ecdysone, would be limiting due to down-regulated spookier, phantom and shadow transcripts in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12} *EP3037* genotypes. Moreover, the *shade* gene product converts ecdysone to 20-hydroxy-ecdysone in fat bodies (Huang et al 2008; Sakagi et al, 2016). Therefore, the factors that affect prothoracic gland's activity may not alter the levels of shade transcripts in fat body. The observed elevation in levels of disembodied transcripts (Table 4) in the microarray analysis of total pupal RNA may appear incongruent, since the Disembodied protein acts between Spookier and Phantom in ecdysone synthesis and, therefore, is expected to be also down-regulated like the other ecdysone-biosynthesis pathway genes. However, it is to be noted that the microarray data is derived from total pupal RNA rather than from only the prothoracic glands. It is also possible that *disembodied* may express in some other tissues as well, since it can be regulated independent of *spookier* [38].

Since the ecdysone synthesis and release from prothoracic gland is regulated by neuro-endocrine signals emanating from the central nervous system [39, 40], it is possible that the ectopic expression of activated Ras in certain neurons in brain and ventral ganglia that express *sev-GAL4* and *GMR-GAL4* drivers [11] may affect ecdysone synthesis. However, our results that the *sev-GAL4* and *GMR-GAL4* expressing CNS neurons persist in case of conditional inhibition of ecdysone using the temperature sensitive ecd^1 mutant allele [18, 19] and disappear in individuals escaping early death by exogenous ecdysone indicate that persistence of these CNS neurons in the early dying pupae is a consequence, rather than a cause, of the reduced ecdysone signalling. It is to be noted in this context that the *ecd* gene's primary role in ecdysone biosynthesis during late larval/pupal stage is in the prothoracic gland [41, 42]. Further support for the primary role of eye discs in the early pupal death in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* or *sev-GAL4>Ras1*^{V12}

EP3037 genotypes is provided by the fact that unlike the almost comparable expression of these two drivers in brain and abdominal ganglia [11], that in the eye discs is significantly different. While the *sev-GAL4* driver expresses only in a subset of photoreceptor cells, the *GMR-GAL4* has a much wider expression in nearly all cells posterior to the morphogenetic furrow [6-9, 11]. Ectopic expression of activated Ras is known to disrupt development of eye discs [3] and as reported earlier [3, 6, 9], we (M. Ray and S. C. Lakhotia, in preparation) also found that eye development is much more severely affected when *GMR-GAL4* drives expression of $Ras1^{V12}$. Our other observations (M. Ray and S. C. Lakhotia, in preparation) further show that unlike in discs expressing only *sev-GAL4>*$Ras1^{V12}$, co-expression of *sev-GAL4>*$Ras1^{V12}$ with *hsrω-RNAi* or *EP3037* results in enhanced Ras expression which spreads non-autonomously to neighbouring cells to a much greater extent. Consequently, the cumulative effect of activated Ras expression may become nearly as strong as that following *GMR-GAL4>*$Ras1^{V12}$ expression. The more severe damage in eye discs in all these conditions is accompanied by the early pupal death. Together, these considerations lead us to suggest that the early pupal death that occurs when $Ras1^{V12}$ alone is expressed under *GMR-GAL4* or when it is co-expressed with *hsrω-RNAi* or *EP3037* under *sev-GAL4* is most likely related to the over-expression of $Ras1^{V12}$ in eye discs.

Recent studies have unraveled significant role of the signal peptide Dilp8 in coordinating imaginal tissue growth and ecdysone synthesis in the prothoracic gland [13, 14, 43, 44]. Damaged or abnormally growing discs are believed to secrete Dilp8 to transiently delay the synthesis of ecdysone [13, 45]. The enhanced levels of Dilp8 transcripts in early pupal eye discs and Dilp8 protein in their peripodial cells in all the genotypes that exhibited early pupal death confirm that the disruptions in development of eye discs that follow *GMR-GAL4>*$Ras1^{V12}$ expression or *sev-GAL4>*$Ras1^{V12}$ expression in background of down- or up-regulated levels of hsrω transcripts cause the pupal discs to activate Dilp8 synthesis and lead to its secretion via the peripodial cells. It may be noted that the Dilp8 antibody used in this study may not recognize active Dilp8. However, since the levels of Dilp8 transcripts as well as Dilp8 protein increase in eye discs of early pupae that are destined to die, we expect that levels of active Dilp8 would also increase proportionately.

A recent study [46] has not reported any lethality at larval or pupal stages following global over-expression of Dilp8 using the ubiquitously expressed *tubulin* promoter. These results may appear in conflict with our finding that elevated Dilp8 levels in eye discs correlate with early pupal death. However, it is possible that the *tubulin* promoter may not have enhanced Dilp8 to the required critical level to affect the early pupal ecdysone synthesis. Alternatively, a global expression of Dilp8 may not have the same consequence in terms of viability as that of expression in a specific tissue since as reported by Vallejo et al [46], global expression delays pupation and causes adults to be overweight because every tissue is equally affected. On the other hand, our experimental conditions severely disturb the relative levels of Dilp8 only in specific cell types. Such disbalance may affect synthesis of ecdysone in early pupae and thus lead to the observed early pupal death.

It is reported earlier [13, 14, 34, 43, 44, 47] that damage to developing discs activates JNK signalling which in turn activates Dilp8. Many earlier studies in *Drosophila* and mammalian cells have shown that activated Ras can trigger Eiger (TNF) mediated JNK signalling [45, 48-54]. The complete nullification of the effects of activated Ras by co-expression of RafRBDFLAG, which acts as a dominant negative suppressor of Ras signalling [17], further confirmed that the elevated Ras-signalling mediated damage to eye imaginal discs indeed

triggered the JNK signalling. In agreement, our microarray, qRT-PCR and immunostaining data from *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12} *EP3037* early pupae revealed up-regulation of several members of the JNK signalling pathway, whose protein product activates the JNK signalling [55, 56]. It is interesting that while levels of Tak1 transcripts, whose product specifically activates the JNK pathway [57] were not affected in any of the genotypes examined, those of its homolog Takl1 [58] were very highly up-regulated in *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* as well as *sev-GAL4>Ras1*^{V12} *EP3037* pupae. This may also contribute to elevated JNK signalling in eye discs of these pupae.

An earlier study in our lab [29] showed that down-regulation of hsrω transcripts suppressed JNK activation while their over-expression enhanced JNK signalling in eye discs. The present data on the other hand show a reverse trend since many of the JNK pathway genes appeared up regulated when *sev-GAL4* driven *Ras1*^{V12} and *hsrω-RNAi* transgenes co-expressed but when *EP3037* was expressed with *Ras1*^{V12}, transcripts of most of the JNK pathway genes showed a significant down-regulation although a few were elevated but to a lesser extent (Table 4). It is likely that these differences in JNK activation in the present and earlier study [29] relate to the expression of activated Ras. Since the hsrω lncRNAs are involved in multiple regulatory pathways [30, 31, 59], it is not surprising that consequences of alterations in their levels are different in varying genetic and developmental backgrounds. Such contextual effects may also underlie the apparently paradoxical finding that both up- and down-regulation of *hsrω* expression has similar effects in the background of ectopic expression of activated Ras. This may be because the up- as well as down-regulation of some of the *hsrω* transcripts disturbs the dynamics of hnRNP and other omega speckle associated proteins [60, 61] which would have cascading effects. It is likely that such dys-regulated dynamics of some key regulatory molecules like hnRNPs etc have common consequence for Ras and JNK signalling in the present situation. Details of these events remain to be understood.

Together, present results show that altered levels of the hsrω transcripts exaggerate Ras signalling resulting in elevated JNK signalling and consequently Dilp8 secretion by the affected cells. Our finding that the early pupal death observed following elevated levels of Dilp8 in eye discs is novel since the literature available so far [13, 14, 34, 43, 44, 47] has suggested that the major role of Dilp8 is to delay ecdysone synthesis, which provides more time to the 3rd instar larvae to attain a certain minimal level of development before they move onto the pupal stage. Our qRT-PCR data on levels of different transcripts in eye discs and prothoracic glands (Fig. 6) reveal that the levels of transcripts of the JNK-pathway, Dilp8 and ecdysone biosynthesis pathway (*spookier* and *phantom*) genes were not as much affected in third instar larvae as in the early pupal tissues. Other studies on polarity genes and other mutants that drive tumor growth, which delays and finally prevents pupation, have reported about 100 fold increase in Dilp8 transcript levels [62, 63]. In the present case, although the p-JNK levels were elevated in late third instar *sev-GAL4>Ras1*^{V12} *hsrω-RNAi* and *sev-GAL4>Ras1*^{V12} *EP3037* eye discs, the Dilp8 levels in larval eye discs showed only ~2-fold increase which may not be enough to inhibit ecdysone biosynthesis in prothoracic glands. On the other hand, Dilp8 levels in the early pupal eye discs of early dying pupal genotypes were very high. This correlates with the substantial reduction in transcript levels of some of the Haloween genes in pupal prothoracic glands. Therefore, it appears that the damage caused by *GMR-GAL4* or *sev-GAL4* driven expression of activated Ras during the 3rd instar stage remains below the threshold required for significantly elevating Dilp8 secretion and, therefore, the larva pupates as usual. However, as the *GMR-GAL4* or *sev-GAL4* driven elevated Ras-JNK signalling continues even after pupation, Dilp8 levels

increase and suppress the next ecdysone peak that occurs in >8-9 Hr APF. Since at this stage, the organism cannot postpone development, death ensues. It would be interesting to see if the observed late pupal death following *GMR-GAL4* or *sev-GAL4* driven expression of some transgenes also relates to Dilp8 and ecdysone signalling.

5. Conclusions

Our study reveals that the intriguing pupal death observed following predominantly eye-specific *GMR-GAL4* or *sev-GAL4* driven expression of activated Ras is because of activation of the JNK-mediated Dilp8 secretion and reduced ecdysone synthesis after pupation. These results also provide an explanation for the early pupal death following expression of certain transgenes in developing eyes, which are otherwise dispensable for survival. Present study highlights the communication between epithelia and the CNS and other organs so that deregulation of epithelial signalling results in global consequences through downstream events in other tissues.

Acknowledgements

We thank the Bloomington *Drosophila* Stock Center (USA) for providing fly stocks, Developmental Studies Hybridoma Bank (DSHB, Iowa, USA) for the anti-Broad-Core and Dr. P. Leopold (France) for the anti-Dilp8. We thank the DBT-BHU Interdisciplinary School of Life Sciences for the Microarray and real time PCR facilities.

Competing interests

Authors declare no conflicting interests

Author contributions

MR and SCL planned experiments, analyzed results and wrote the manuscript. MR carried out the experimental work and collected data.

Funding

This work was supported by a research grant (no. BT/PR6150/COE/34/20/2013) to SCL by the Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi. SCL was also supported by the Raja Ramanna Fellowship of the Department of Atomic Energy, Govt. of India, Mumbai and is currently supported as Senior Scientist by the Indian National Science Academy (New Delhi). MR was supported by the Council of Scientific & Industrial Research, New Delhi through a senior research fellowship.

Data availability

The microarray data have been deposited at GEO repository (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80703).

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