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

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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 activated Ras producing *UAS-Ras1^{V12}* transgene caused early (~23-25 Hr pupation) and late pupal death, respectively. Co-expression of *UAS-hsrω-RNAi* transgene or *EP3037* (lncRNA *hsrω* gene's GAL4-driven over-expressing allele) with *sev-GAL4>UAS-Ras1^{V12}* advanced death to 23-25 Hr after pupation. We show that the normal ecdysone surge was absent in 23 Hr old *GMR-GAL4>UAS-Ras1^{V12}* pupae or those co-expressing *sev-GAL4>UAS-Ras1^{V12}* with *hsrω-RNAi* or *EP3037*. Microarray and immunostaining data revealed Dilp8 and several members of JNK pathway to be elevated in >16 Hr old *GMR-GAL4>UAS-Ras1^{V12}*, *sev-GAL4>UAS-Ras1^{V12} hsrω-RNAi* and *sev-GAL4>UAS-Ras1^{V12} EP3037* pupae. Down- or up-regulation of hsrω transcripts exaggerates Ras signalling resulting in elevated JNK signalling and consequent more Dilp8 secretion by the affected eye discs. We show for the first time that the intriguing early pupal death following expression of certain transgenes by the predominantly eye-specific *GMR-GAL4* or *sev-GAL4* drivers activates JNK-mediated Dilp8 secretion which reduces post-pupal ecdysone and thus leads to pupal death.

Summary Statement

GMR-GAL4 or *sev-GAL4* driven expression of activated Ras in *Drosophila* eye discs triggers enhanced JNK-Dilp8 activity, resulting in reduced post-pupation ecdysone and pupal death.

Introduction

An earlier study in our laboratory (Ray and Lakhotia, 1998) indicated that *Ras* mutant alleles genetically interact with the *hsrω* long non-coding RNA (lncRNA) gene (Lakhotia, 2011). With a view to explore this interaction further, we initiated studies using the GAL4-inducible activated form of DRas1 (*Ras1*^{V12}, Karim et al., 1996) in conjunction with *hsrω-RNAi* transgene or an *EP* allele of *hsrω* (Mallik and Lakhotia, 2009a). 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 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 (Firth et al., 2006; Morris et al., 2006; Mallik and Lakhotia, 2009a). Such lethality following expression of certain transgenes under these two drivers is unexpected since these GAL4 drivers are generally believed to be expressed mostly in developing eye cells (Freeman, 1996; Bowtell et al., 1991; Maxiner et al., 1998; Firth et al., 2006; Ray and Lakhotia, 2015) and it is known that even a complete absence of eyes has no effect on viability of pupae and emergence of adult flies (Sang and Jackson, 2005). We recently reported that, besides the commonly known expression in eye disc cells, the *GMR-GAL4* and *sev-GAL4* drivers express in several other cell types, including specific neurones in the central nervous system as well (Ray and Lakhotia, 2015). This 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 is one of the eight insulin-like signal peptides in *Drosophila* (Nassel et al., 2013) which is secreted by damaged or inappropriately growing imaginal discs and has been reported to delay metamorphosis by inhibiting ecdysone synthesis (Garelli et al., 2012; Colombani et al., 2012). Our study shows, for the first time, an involvement

of Ras signalling and hsrω lncRNAs, presumably via the JNK signalling, in initiating Dilp8 secretion which disrupts post-pupal ecdysone synthesis and thereby causes early pupal death.

Results

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 (Karim et al., 1996). The *sev-GAL4>Ras1^{V12}* pupae developed normally like wild type (Fig. 1A, C) 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 (Mallik and Lakhotia, 2009a) in *sev-GAL4>Ras1^{V12}* expressing pupae, development appeared normal till 8-9 Hr after pupa formation (APF) (Fig. 1C and D) but further development was affected, resulting in death of ~ 96% (N =1177) of them by about 23-25 Hr APF while the few survivors died as late pupae. In the case of co-expression of the *EP3037* over-expressing allele of *hsrω* (Mallik and Lakhotia, 2009a) with *sev-GAL4>Ras1^{V12}*, 42% (N =1109) died as early pupae (~23-25 Hr APF) while the remaining died as late pupae. 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. 1G, H, K and L). Such dying pupae showed a demarcation between head, thorax and abdomen, but their Malpighian tubules, which in *sev-GAL4>Ras1^{V12}*, as in wild type, become abdominal by this stage (Fig. 2A), remained thoracic (Fig. 2B, C). Malpighian tubules in the early dying 23-24 Hr old pupae retained a comparatively longer anterior segment (Fig. 2D-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 (Ray and Lakhotia, 2015). 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; but in 23-24 Hr old wild type and *sev-GAL4>Ras1*^{V12} pupae, all, except the anterior-most pair, had disappeared (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 (Ray and Lakhotia, 2015), also persisted till 23-24 Hr APF when *hsrω-RNAi* or *EP3037* was co-expressed with *Ras1*^{V12} (not shown).

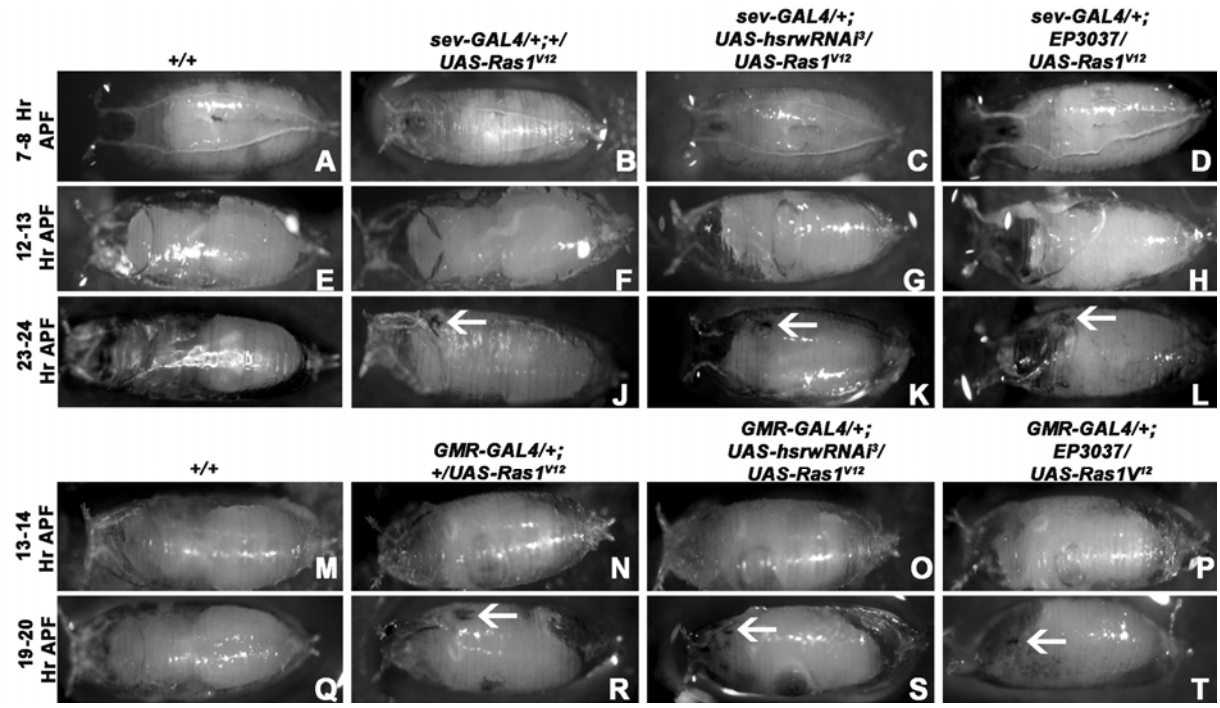

Fig. 1. *sev-GAL4* or *GMR-GAL4* driven expression of activated Ras together with altered hsrω-n transcript levels leads to early pupal lethality accompanied by absence of prepupal to pupal metamorphic changes. (**A-T**) pupae of different stages (noted on left of each row) in different genotypes (noted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

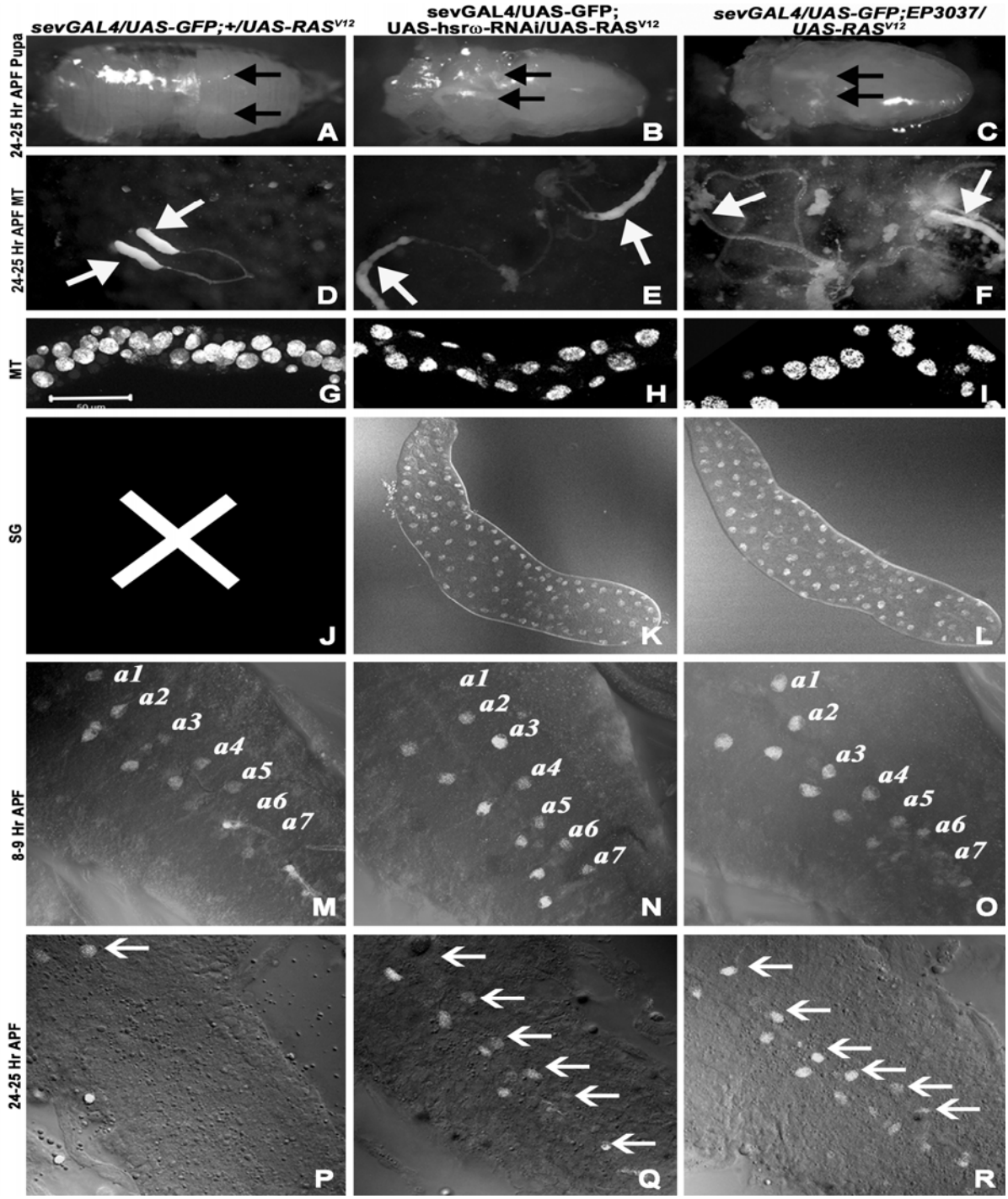

Fig. 2. The prepupal to pupal metamorphic changes in internal organs are affected in early dying pupae. 24-25 Hr old pupae (**A-C**) and dissected out anterior Malpighian tubules (**D-F**) of genotypes noted above each column. White arrows in **D-F** indicate the distal segments of anterior Malpighian tubules. Confocal optical sections of DAPI stained Malpighian tubules (**G-I**) and salivary glands (**J-L**) of 24-25 Hr pupae; a cross is indicated at **J** as salivary glands are no longer seen at 24-25 Hr APF in *sev-GAL4>Ras1*^{V12}. 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.

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

GMR-GAL4 driven expression of *UAS-Ras1^{V12}* resulted in 100% early pupal lethality with developmental defects setting in by 13-14 Hr APF (Fig. 1N). Unlike the 13-14 Hr old wild type pupae (Fig. 1M), those expressing *GMR-GAL4>Ras1^{V12}* did not show proper demarcation of head, thorax and abdominal regions (Fig. 1N and R). A necrotic patch appeared by 19-20 Hr APF at each of prospective adult eye site. The early disappearing dorso-medial pairs of neurons in the abdominal ganglia continued to show GFP expression till 20-24 Hr APF in the dying *GMR-GAL4>UAS-Ras1^{V12}* pupae (Fig. 3I-L). 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 same early stage with similar phenotypes (Fig. 1N-P, R-T).

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 (Handler 1982) were absent in pupae that would die around 23-24 Hr APF. Therefore, we examined the distribution/expression of Broad, one of the early responders to ecdysone signalling (Zollman et al., 1994; Zhou et al., 2009), 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 (Emery et al., 1994). The Broad positive ventral ganglia cells from third instar larval (not shown) and early pupal stages (12-13 Hr APF) of all 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 so that instead of its the uniform nuclear distribution, it became restricted to a few intense peri-nuclear granules (Fig. 3E and F) and at the same time, the cytoplasmic staining for Broad became higher than in earlier stages. Interestingly, ventral ganglia co-expressing *hsrω-RNAi* or *EP3037* with *Ras1^{V12}* under the *sev-GAL4* driver did not show this change and even at 24-25 Hr APF (Fig. 3G, H) continued to show the younger pupal stage pattern (Fig. 3C, D) of more or less uniform pan-nuclear distribution of Broad protein; the cytoplasmic presence of Broad in 24-25 Hr APF in these genotypes also remained very low. It may be mentioned that a few cells in the 24-25 Hr old

pupal ventral ganglia co-expressing *sev-GAL4* driven *Ras1*^{V12} and *hsrω-RNAi* or *EP3037* showed a punctate or granular, rather than uniform, presence of Broad protein in nuclei. However, these puncta were mostly distributed across the nuclear area rather than being typically peri-nuclear as in the same age *sev-GAL4>UAS-GFP* and *sev-GAL4>Ras1*^{V12} pupal ventral ganglia (Fig. 3).

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 *GMR-GAL4>Ras1*^{V12} ventral ganglia cells, the Broad protein retained its uniformly strong pan-nuclear distribution (Fig. 3J, L) as in the 12-13 Hr 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 BR-C expression, neither at 8-9 Hr nor at 24-25 Hr APF stage (Fig. 3).

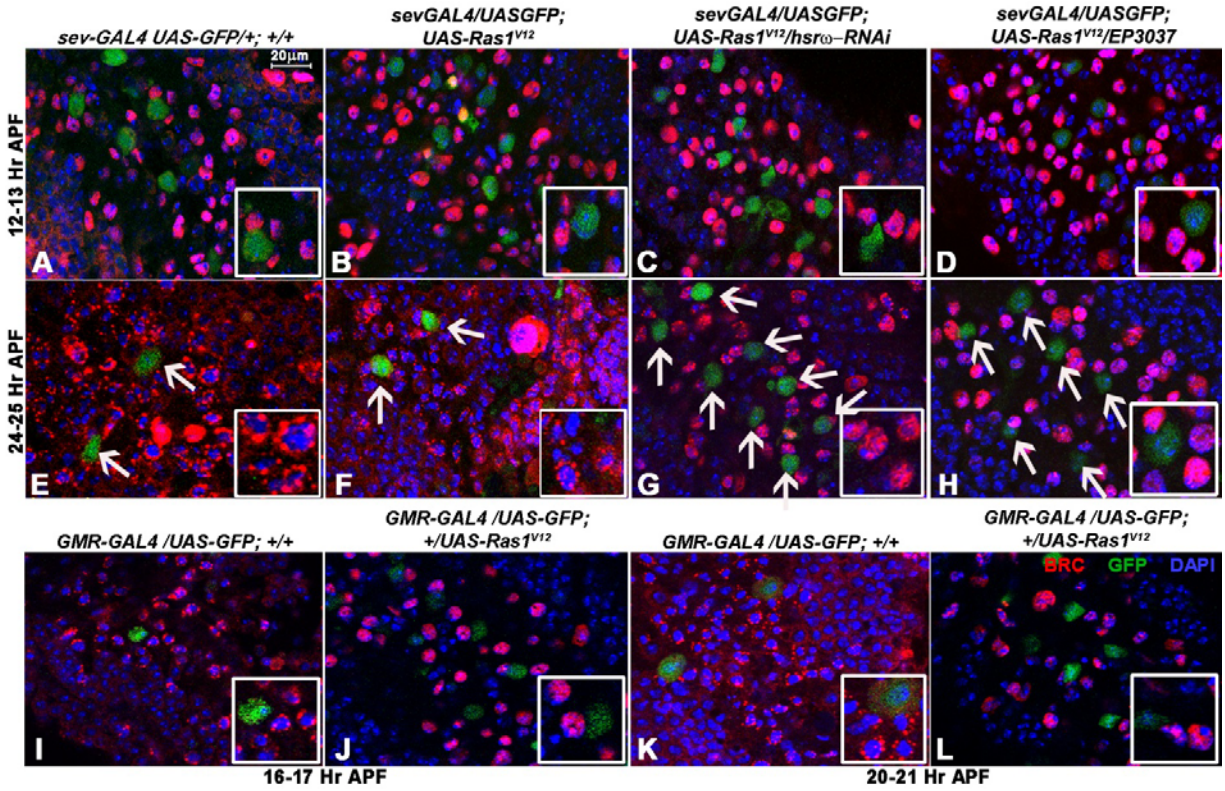

Fig. 3 Distribution of Broad protein in ventral ganglia cells at 16-17 Hr or later APF is affected in early dying pupae. (**A-H**) Confocal optical sections showing parts of ventral ganglia from 12-13 Hr (**A-D**) and 24-25 Hr (**E-H**) old pupae immunostained with anti-Broad-core (red) and counterstained 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 failure to redistribute the Broad protein in 16 Hr or older pupae that would die at about 23-25 Hr APF further indicated a failure of the post-pupation ecdysone signalling. An immunoassay for 20-hydroxyecdysone levels revealed that ecdysone levels in 8-9 Hr APF old pupae in those expressing *sev-GAL4* driven activated Ras alone or together with *hsrω-RNAi* transgene or *EP3037* allele were comparable with 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).

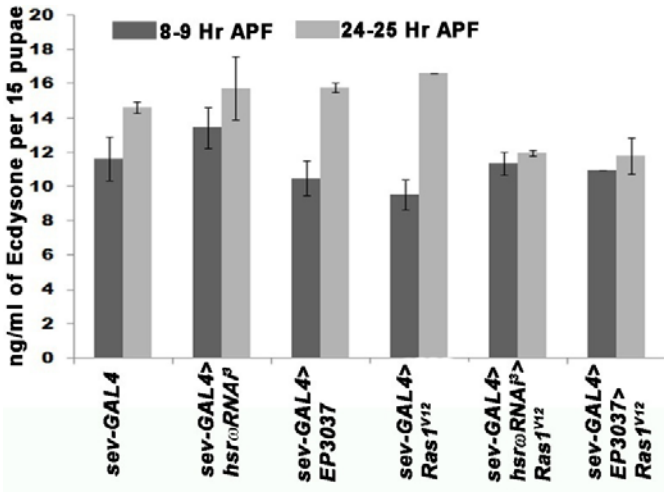

Fig. 4. Histogram showing mean (±S.E. of three independent replicates) levels of 20 hydroxyecdysone (Y axis) in different genotypes (X-axis) at 8-9 Hr APF (dark grey) and at 24-25 Hr APF stages (light grey). Each genotype also carries *UAS-GFP* transgene.

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 inhibited the scheduled elevation in ecdysone levels during early pupal stages so that in the absence of critical level of ecdysone signalling, these pupae do develop further and die.

Indirect 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 (Garen et al., 1977), which inhibits production of ecdysone when grown at the restrictive temperature (29-30°C). The *ecd*^{1} larvae were reared at 24°C and the freshly formed white prepupae were transferred to 30°C to conditionally inhibit further ecdysone synthesis. When examined at 24-25 Hr APF, these pupae showed all the developmental defects, including death (data not presented) as noted above for 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.

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

We recently showed (Ray and Lakhotia, 2015) that contrary to the conventional belief, the *GMR-GAL4* as well as the *sev-GAL4* also express, besides the eye discs, 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 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*, we undertook a microarray analysis to examine global trasncriptional changes in 16-17 Hr old pupae of different genotypes.

Total RNAs extracted 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 1, 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 focussed 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 Table1). 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 1), 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 (Ragab et al., 2011, Hauling et al., 2014), while the hsrω transcripts have roles in stress responses, cell death and several other pathways (Mallik and Lakhotia, 2009b: Lakhotia, 2011, 2016). 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 1). The down-regulation of lipid and steroid synthesis pathway genes agrees with the above noted reduced levels of ecdysone in the early dying pupae.

Some recent reports (Garelli et al., 2012; Colombani et al., 2012) have suggested that damage to or slow growth of imaginal discs affect ecdysone synthesis through up regulation of Dilp8 levels and JNK signalling. Therefore, we examined the microarray data for levels of transcripts of some of the genes involved in ecdysone, insulin and JNK pathways through pair wise comparisons of microarray data for different genotypes (Table 2), taking >1.5 fold difference as significant. In one set (columns 1-3 in Table 2), 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 2), 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 (Huang et al., 2008) 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 in these genotypes. The down-regulation of 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 the *shadow* gene product converts 2-deoxyecdysone into ecdysone (Warren et al., 2002). 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 elevate 20-hydroxyecdysone levels since its substrate, ecdysone, would be limiting due to down-regulated shadow transcripts in *sev-GAL4>Ras1^{V12} hsrω-RNAi* and *sev-GAL4>Ras1^{V12} EP3037*genotypes.

Our microarray data 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 2). Interestingly, when compared with *sev-GAL4>Ras1^{V12}*, the increase is 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 nearly 100% death of former as early pupae compared to ~42% in case of *sev-GAL4>Ras1^{V12} EP3037*.

Like the Dilp8, transcripts of *tobi* (*target of brain insulin*) were also significantly up-regulated (Table 2) when *sev-GAL4>Ras1^{V12}* was expressed in conjunction with *hsrω-RNAi* or *EP3037*. Recently, Garelli et al (2015) showed 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.

As the data in Table 2 reveal, levels of different members of the JNK signalling pathway showed considerable variability from no effect to up- or down-regulation in different genotypes. 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, most 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 2). 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.

The elevated levels of Dilp8 transcripts revealed by the microarray data were validated by real time QRT-PCR with total RNA isolated from 8-9 Hr pupal eye discs of different genotypes. In agreement with microarray data, levels of Dilp8 transcripts were about 2-fold elevated in *sev-GAL4>Ras1^{V12} hsrω-RNAi* and *sev-GAL4>Ras1^{V12} EP3037* than in eye discs expressing only the activated Ras (data not shown).

Elevation in levels of Dilp8 in the early dying pupae was further confirmed by confocal microscopy following immunostaining of eye discs with anti-Dilp8. 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). Compared to the third instar stage, the 8-9 Hr APF stage eye discs showed stronger presence of Dilp8 in the photoreceptor cells of eye discs in all the four genotypes. Interestingly, the peripodial cells of early pupal eye discs (Fig. 5A-D) co-expressing *sev-GAL4>Ras1^{V12}* and *hsrω-RNAi* showed a much stronger Dilp8 staining (Fig. 5C) when compared with that in *sev-GAL4>UAS-GFP* (Fig. 5A) or *sev-GAL4>Ras1^{V12}* (Fig. 5B) pupal discs. Similarly, the peripodial cells of early pupal *sev-GAL4>Ras1^{V12} EP3037* eye discs also showed elevated Dilp8 staining (Fig. 5D) although this was less than that in similar age *sev-GAL4>Ras1^{V12} hsrω-RNAi* eye discs.

Since our microarray 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) in late third instar eye discs of these genotypes. All the photoreceptor cells in *sev-GAL4>UAS-GFP* (Fig. 5E) 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. 5F). Interestingly, co-expression of *hsrω-RNAi* transgene with *sev-GAL4>Ras1^{V12}* resulted in a very large increase in p-JNK staining (Fig. 5G) in all photoreceptors cells irrespective of GFP expression. Up regulation of hsrω transcripts in *sev-GAL4>Ras1^{V12}* expressing eye discs was associated with a marginal increase in p-JNK levels when compared with *sev-GAL4>UAS-GFP* (Fig. 5H). Immunostaining for p-JNK in late larval eye discs expressing *GMR-GAL4* driven GFP (control, Fig. 5M) or *Ras1^{V12}* (Fig. 5N) 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.5O) in photoreceptor cells of different genotypes, obtained using the Histo tool on projection images of 12 optical sections through the photoreceptor arrays in each disc, confirm the significant increase in p-JNK in *sev-GAL4>Ras1^{V12} hsrω-RNAi* and *sev-GAL4>Ras1^{V12} EP3037* eye discs.

To further show that 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 (Morris et al., 2006). 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 (Mallik and Lakhotia, 2009a), 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 (Silverman et al., 2003; Geuking et al., 2009) and in agreement with our other observations, its *GMR-GAL4* driven expression led to early pupal lethality.

To confirm 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 (Freeman et al., 2010). We examined the effect of *sev-GAL4* driven co-expression of *RafRBDFLAG* with *Ras1^{V12}* without or with *hsrω-RNAi* or *EP3037* 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.5I-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 3. 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.

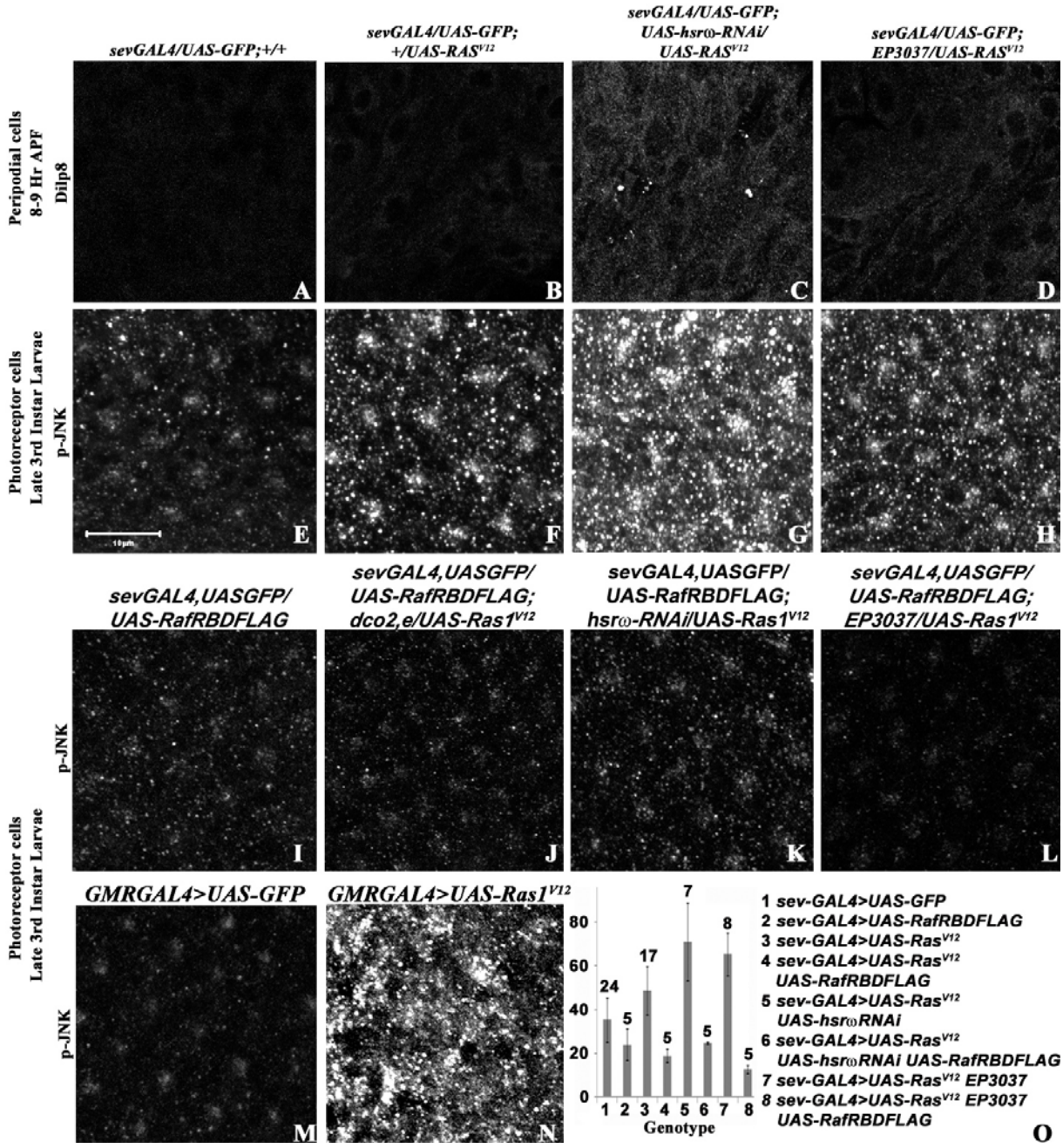

Fig. 5. **Expression of Dilp8 and phosphorylated JNK (p-JNK) is enhanced in eye discs of early dying pupae.** (**A-D)** Confocal optical sections showing expression of Dilp8 (white) in peripodial cells of 8-9 Hr APF stage eye discs of different genotypes (noted on top of each column); (**E-L)** Confocal 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.

Discussion

Present study examines possible reasons for the unexpected pupal death when certain transgenes are directed to be expressed with the *GMR-GAL4* or *sev-GAL4* drivers which are predominantly expressed in eye discs. We found that the ecdysone surge that normally occurs after 8-12hr APF (Handler, 1982) was absent in the early dying pupae and in agreement with this decrease in ecdysone levels in the early dying pupae, our microarray 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, involving expression of activated Ras under *GMR-GAL4* or *sev-GAL4* driver, is because of sub-threshold levels of ecdysone so that the required metamorphic changes fail to occur and the organism dies.

Since the ecdysone synthesis and release from prothoracic gland is regulated by neuro-endocrine signals emanating from the central nervous system (Heinrich et al., 1987; Reitz et al., 2009; Niwa and Niwa 2014), it remained possible that the ectopic expression of activated Ras in certain neurons in brain and ventral ganglia that express *sev-GAL4* and *GMR-GAL4* drivers (Ray and Lakhotia, 2015), may affect ecdysone synthesis and/or release. However, our results with conditional inhibition of ecdysone using the temperature sensitive ecd^{1} mutant allele (Garen et al., 1977) revealed that persistence of the *sev-GAL4* and *GMR-GAL4* expressing CNS neurons in the early dying pupae is a consequence, rather than a cause, of the reduced ecdysone signalling following *sev-GAL4* driven co-expression of activated Ras with reduced or elevated levels of hsrω transcripts or the *GMR-GAL4* driven expression of activated Ras. Our finding that unlike the *sev-GAL4* driven expression of activated Ras, that following *GMR-GAL4* driven expression by itself led to early pupal death also suggests that the reduced ecdysone signalling is because of some signals generated in the eye discs rather than in brain or ventral ganglia since the expression of these two drivers in brain and abdominal ganglia is essentially similar (Ray and Lakhotia, 2015). On the other hand, the *sev-GAL4* driver expresses only in a subset of photoreceptor cells while the *GMR-GAL4* has a much wider expression in nearly all cells posterior to the morphogenetic furrow in the developing eye discs (Freeman, 1996; Bowtell et al., 1991; Maxiner et al., 1998; Firth et al., 2006; Ray and Lakhotia, 2015). Therefore, *GMR-GAL4* driven expression of activated Ras is expected to cause more extensive effects on the developmental programme in eye discs. In parallel with this, the signal/s emanating from eye discs expressing activated Ras, therefore, are expected to be stronger than when expressed with

sev-GAL4. Apparently, co-expression of *hsrω-RNAi* or *EP3037* with *sev-GAL4> Ras1^{V12}* enhances the outgoing signal/s from the discs. Indeed our other observations (M. Ray and S. C. Lakhotia, in preparation) 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 also to a greater extent, so that the cumulative effect of activated Ras expression may become nearly as strong as that of the *GMR-GAL4>Ras1^{V12}* expression. Since expression of *GMR-GAL4>Ras1^{V12}* by itself results in early pupal lethality, co-expression of *hsrω-RNAi* or *EP3037* does not further advance the time of death.

Recent studies have unravelled significant role of the Dilp8 in coordinating imaginal tissue growth and ecdysone release from the prothoracic gland (Garelli et al., 2012, 2015; Colombani et al., 2012; Demay et al., 2014; Katsuyama et al., 2015). Damaged or abnormally growing discs are believed to secrete Dilp8 which transiently delays synthesis and/or secretion of ecdysone to postpone metamorphosis (Garelli et al., 2012; Colombani et al., 2012). Ectopic expression of activated Ras is known to disrupt development of eye discs (Karim et al., 1996). Eye development is more severely affected when the ectopic expression of *Ras1^{V12}* is driven by the *GMR-GAL4* than by the *sev-GAL4* driver (Karim et al., 1996; Maxiner et al., 1998; Firth and Baker, 2006; M. Ray and S. C. Lakhotia, unpublished). The enhanced levels of Dilp8 transcripts in all the genotypes that exhibited early pupal death and the high levels of Dilp8 in peripodial cells of eye discs in such genotypes confirm that the disruptions in development of eye discs that follow *sev-GAL4>Ras1^{V12}* expression in background of down- or up-regulated levels of hsrω transcripts cause these discs to activate Dilp8 synthesis and lead to its secretion via the peripodial cells.

It is reported earlier (Garelli et al., 2012, 2015; Colombani et al., 2012; Demay et al., 2014; Katsuyama et al., 2015; Zimmermann et al., 2015) that damage to developing discs activates JNK signalling which in turn activates Dilp8. In agreement, our microarray 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, specially of the Eiger transcripts, whose protein product activates the JNK signalling (Igaki et al., 2002; Zhang et al., 2010). It is interesting that while levels of Tak1 transcript, whose product specifically activates the JNK pathway (Takatsu et al., 2000) were not affected in any of the genotypes examined in

our microarray analysis, those of its homolog Takl1 (Manning et al., 2002) were highly elevated 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 larvae.

It is intriguing that while transcripts of most members of the JNK pathway are up-regulated in *sev-GAL4>Ras1^{V12} hsrω-RNAi* early pupae, many of them appear down-regulated in *sev-GAL4> Ras1^{V12} EP3037*. These differences may reflect other network effects of the down-or up-regulated levels of hsrω transcripts which are involved in multiple pathways and, therefore, have pleiotropic effects (Lakhotia, 2011; Mallik and Lakhotia, 2011). These differences may also be responsible for the differences in the extent of early pupal death between the two genotypes.

An earlier study in our lab (Mallik and Lakhotia, 2009b) showed that down regulation of hsrω transcripts suppressed JNK activation while their over-expression enhanced JNK signaling in eye discs. The present microarray 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} hsrω-RNAi* transgene co-expressed but when *EP3037* was expressed with *Ras1^{V12}*, transcripts of most of the JNK pathway genes showed a significant down-regulation while a few were elevated but to a lesser extent (Table 2). It is likely that these differences in JNK activation in the present and earlier study (Mallik and Lakhotia, 2009b) relate to, besides the effects of developmental stage (larva vs pupa) and eye disc vs total larval RNA samples, the expression of activated Ras. Such different effects of the genetic background reflects the involvement of hsrω lncRNAs in multiple regulatory pathways (Lakhotia, 2011; Mallik and Lakhotia, 2011)

Many earlier studies in *Drosophila* and mammalian cells have shown that activated Ras can trigger Eiger (TNF) mediated JNK signalling (Minden et al., 1994; Uhlirova et al., 2005; Wu et al., 2010; Ohsawa et al., 2012; Khoo et al., 2013; Miles et al., 2011; Andersen et al,2015; Jones et al., 2016). The complete nullification of the effects of activated Ras by co-expression of RafRBDFLAG, which acts as a dominant negative suppressor of Ras signalling (Freeman et al., 2010), further confirmed that the elevated Ras-signalling mediated damage to eye imaginal discs indeed triggers the cascade of events starting with JNK-mediated elevated secretion of Dilp8 in early pupae which in turn reduces ecdysone levels and consequently results in early pupal death.

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 damaged

eye discs. Our study reveals, for the first time, that the intriguing pupal death observed following predominantly eye-specific *GMR-GAL4* or *sev-GAL4* driven expression of certain transgenes, is because of activation of the JNK-mediated Dilp8 secretion and reduced ecdysone synthesis/release. Earlier studies on Dilp8 effects on *Drosophila* development (Garelli et al., 2012, 2015; Colombani et al., 2012; Demay et al., 2014; Katsuyama et al., 2015; Zimmermann et al., 2015) have so far shown only a delay in pupation. In our study, however, the pupation was not affected. Instead, the pre-pupal ecdysone pulse was reduced due to elevated Dilp8 levels after the late third instar stage. As the organism cannot postpone the development at the early pupal stage, death ensues. Apparently, the *GMR-GAL4* or *sev-GAL4* driven expression of activated Ras does not cause as much damage during the 3rd instar stage to warrant Dilp8 secretion to delay the pupation. It would be interesting to examine if the late pupal death seen in several genotypes also involves Dilp8 signalling.

Material and Methods

Fly stocks

All fly stocks and crosses were maintained on standard agar cornmeal medium at 24±1^{0}C. The following stocks were obtained from the Bloomington Stock Centre (USA): w^{1118}*; sev-GAL4;* + (no. 5793; Bailey 1999), 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* (Freeman, 1996), w^{1118}*; UAS-hsrω-RNAi*^{3} (Mallik and Lakhotia, 2009a), w^{1118}*; EP3037/TM6B* (Mallik and Lakhotia, 2009a), 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* (Kazemi-Esfarjani and Benzer, 2000), 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 (Mallik and Lakhotia, 2009a). The *UAS-RafRBDFLAG* stock (Freeman et al., 2010) 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} and *dco*^{2} *e* markers are not mentioned while writing genotypes in Results while the *UAS-hsrω-RNAi*^{3} transgene (Mallik and Lakhotia, 2009a) is referred to as *hsrω-RNAi*.

For conditional inhibition of ecdysone synthesis using the temperature-sensitive *ecd*^{1} mutant allele (Garen et al., 1977), 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). The late third instar actively moving larvae and pupae of desired ages (expressed as after pupa formation or

APF) were dissected in Poels' salt solution (PSS, Tapadia and Lakhotia, 1997) 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.

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-$Ras1^{V12}$, sev-GAL4 UAS-GFP/UAS-GFP; EP3037/UAs-$Ras1^{V12}$,* from actively migrating late third instar larvae and pupae of desired age were dissected out in PSS and immediately fixed in freshly prepared 3.7% paraformaldehyde in PBS for 20 min and processed for immunostaining as described earlier (Prasanth et al., 2000). 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, rat anti-Dilp8 (gifted by Dr. P. Léopold, France, Colombani et al., 2012) 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 on different regions of eye discs were obtained with the help of Histo options of the Zeiss LSM Meta 510 software. All images were assembled using the Adobe Photoshop 7.0 software.

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 13000rpm following which the pellets were re-extracted in ethanol and air dried. The dried extracts were thoroughly dissolved in EIA buffer at 4°C overnight prior to the enzyme immunoassay. 20E-EIA antiserum (#482202), 20E AChE tracer (#482200), precoated (Mouse Anti- Rabbit IgG) EIA 96-Well Plates (#400007), and Ellman's Reagent (#400050) were from

Cayman Chemical (USA), and assays were performed according to the manufacturer's instructions.

Microarray Analysis

RNA was isolated from 16-17 Hr *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 laboratory methods described in 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).

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

Total RNA was isolated from eye disc of third instar larvae and appropriate pupal stage of the desired genotype using TriReagent as per the manufacturer's (Sigma-Aldrich) instructions. First-strand cDNA was synthesized as described earlier (Mallik and Lakhotia, 2009a). cDNA prepared was subjected to PCR using Dilp8 specific primers (forward primer 5'GAGAGCTCCAGTGTCATGCT-3' and reverse primer 5'-GGAGTGGGCACAAATAAGGT-3') and G3PDH primers (forward primer 5'-CCACTGCCGAGGAGGTCAACTA-3'and reverse primer 5'GCTCAGGGTGATTGCGTATGCA-3'). Real time PCR was performed using 5 µl qPCR Master Mix (Syber Green, Thermo Scientific), 2.5 picomol/µl of each primer per reaction set in 10 µl of final volume in ABI 7500 Real time PCR machine.

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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

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Data availability

The microarray data are being deposited at GEO (http://www.ncbi.nlm.nih.gov/geo/).

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Table 1. 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 only activated Ras

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:0006022: aminoglycan metabolic process GO:0016052: carbohydrate catabolic process GO:0005976: polysaccharide metabolicprocess GO:0006950: response to stress GO:0009266: response to temperature stimulus GO:0009308: amine metabolic process GO:0005975: carbohydrate metabolic process GO:0006629: lipid metabolic process GO:0045087: innate immune response GO:0006955: immune response GO:0008063: Toll signaling pathway GO:0008219: cell death GO:0035070: salivary gland histolysis GO:0048102: autophagic cell death GO:0016271: tissue death GO:0007559: histolysis	GO:0008610: lipid biosynthetic process GO:0005976: polysaccharide metabolic process GO:0006694: steroid biosynthetic process GO:0002376: immune system process GO:0045087: innate immune response	GO:0007160: cell-matrix adhesion GO:0031589: cell-substrate adhesion GO:0007155: cell adhesion GO:0010631: epithelial cell migration GO:0007165: signal transduction GO:0007188: G-protein signaling, coupled to cAMP nucleotide second messenger GO:0007187: G-protein signaling, coupled to cyclic nucleotide second messenger GO:0007166: cell surface receptor linked signal transduction GO:0031644: regulation of neurological system process GO:0050890: cognition GO:0035272: exocrine system development GO:0022008: neurogenesis	GO:0051174: regulation of phosphorus metabolic process GO:0019220: regulation of phosphate metabolic process GO:0055114: oxidation reduction GO:0009101: glycoprotein biosynthetic process GO:0046068: cGMP metabolic process GO:0035103: sterol regulatory element binding protein cleavage GO:0007552: metamorphosis GO:0006334: nucleosome assembly GO:0031497: chromatin assembly GO:0006281: DNA repair GO:0065004: protein-DNA complex assembly GO:0006259: DNA metabolic process GO:0006325: chromatin organization GO:0032933: SREBP-mediated signaling pathway GO:0008593: regulation of Notch signaling pathway GO:0010627: regulation of	GO:0042180: cellular ketone metabolic process GO:0006575: cellular amino acid derivative metabolic process GO:0019471: 4-hydroxyproline metabolic process GO:0043436: oxoacid metabolic process GO:0006082: organic acid metabolic process GO:0019752: carboxylic acid metabolic process GO:0007254: JNK cascade GO:0031098: stress-activated protein kinase signaling pathway GO:0007600: sensory perception GO:0006816: calcium ion transport GO:0007568: aging

GO:0012501: programmed cell death GO:0006865: amino acid transport GO:0006508: proteolysis GO:0006582: melanin metabolic process GO:0006570: tyrosine metabolic process			protein kinase cascade GO:0045165: cell fate commitment GO:0008356: asymmetric cell division GO:0007164: establishment of tissue polarity GO:0051605: protein maturation by peptide bond cleavage GO:0007218: neuropeptide signaling 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 2. 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 $Ras1^{V12}$

Table 3. 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} RAFRBDFLAG EP3037	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