Altered hsrω lncRNA levels in activated Ras background further enhance Ras activity in *Drosophila* eye and induces more R7 photoreceptors

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Abstract

To examine role of hsrω nuclear lncRNAs in Ras signaling cascade, we down- or up-regulated these transcripts in eye discs of *Drosophila* expressing *sev-GAL4* driven activated ras^{V12} transgene. The *sev-GAL4* driven Ras^{V12} transgene expression dependent late pupal lethality and extra R7 photoreceptors in ommatidia, were significantly enhanced when levels of hsrω lncRNA were down/up-regulated. This was associated with enhanced p-MAPK expression, reduced Yan levels, and greater association of RafRBDFLAG with Ras, indicating elevated Ras activation which was both cell autonomous and non-autonomous. RNAseq analysis revealed significant increase in expression of certain sno/sn/scaRNAs and some RNA processing genes in *sev-GAL4>*Ras^{V12} which was further modulated when hsrωRNA levels were co-altered. Down-regulation of hsrωRNAs elevated positive modulators of Ras signaling while their up-regulation reduced expression of negative modulators of Ras signaling, and thus both conditions have similar outcome. Further enhancement of activity of hyperactive Ras following changes in hsrω lncRNA levels in cell autonomous as well as non-autonomous manner emphasizes roles of lncRNAs in cell signaling during development and disease conditions associated with hyperactive Ras pathway mutants.

Summary

Our findings highlight roles of hsrω lncRNA in conditionally modulating the important Ras signaling pathway and provide evidence for cell non-autonomous Ras signaling in *Drosophila* eye discs.

Introduction

Evolution of multi-cellularity and the associated division of labour between different cell types has necessitated inter-cellular signaling pathways with complex network of regulatory circuits. The evolution of biological complexity has also been paralleled by substantial increase in the non-coding component in diverse genomes. There is increasing realization in recent years that the large varieties of short and long non-coding RNAs (lncRNA) have great roles in cell signaling and gene regulation (Mattick and Makunin, 2006; Geisler and Coller, 2013; Huang et al., 2013; Morris and Mattick, 2014; Jose, 2015; Lakhotia, 2016; Lakhotia, 2017; Peng et al., 2017). The lncRNAs like acal, mrhl, CRNDE, LncND, and URHC have been reported to regulate JNK, Wnt, insulin, Notch and ERK/MAPK signaling pathways, respectively (Arun et al., 2012; Ellis et al., 2014; Xu et al., 2014; Ríos-Barrera et al., 2015; Katsushima et al., 2016). Besides their roles in developmental regulation (Katsushima et al., 2016; Kotake et al., 2016; Lakhotia, 2017; Misawa et al., 2017; Zhang et al., 2017), diverse lncRNAs have been reported to interact with components of the various signaling pathways in cancer, activating or repressing their strength and thus affecting outcomes like cell proliferation or apoptosis (Liu et al., 2015; Wang et al., 2015). An earlier study from our laboratory (Ray and Lakhotia, 1998) showed that mutant alleles of *ras* (ras^{E62K} and ras^{D38N}) dominantly enhanced the lethality due to nullisomic condition of the *hsrω* gene, which produces multiple lncRNAs (Lakhotia, 2011; Lakhotia, 2016). The RAS/RAF/MAPK signaling pathway regulates many developmental pathways as it affects cell division, proliferation, growth as well as death, besides its major roles in many human cancers (Fernández-Medarde and Santos, 2011; Pylayeva-Gupta et al., 2011). Ectopic expression of activated Ras causes hyperplastic growth of the concerned organ in *Drosophila* also (Karim and Rubin, 1998; Prober and Edgar, 2000).

In the present study, we further examined interaction between the *hsrω* gene and the Ras signaling pathway. We found that down- or up-regulation of hsrω nuclear lncRNAs, through *sev-GAL4* driven RNAi or its over-expression, respectively, exaggerates the phenotypes following ectopic expression of activated Ras producing *UAS-*Ras^{V12} transgene in developing eye discs. The *sev-GAL4* driven expression of Ras^{V12} is known to cause ommatidial derangement and rough eyes due to increase in number of R7 photoreceptor cells (Karim et al., 1996). Intriguingly, our results show that reduced as well as enhanced levels of hsrω lncRNAs in *sev-GAL4>*Ras^{V12} expressing eye discs enhances Ras signaling due to substantial increase in levels of activated Ras and phosphorylated MAPK (p-MAPK) in cell autonomous as well as non-autonomous manner. This resulted in further increase in number of the R7 photoreceptors, with down-regulation of hsrω lncRNAs being more effective than its up-regulation. We found presence of activated Ras and its downstream component (RafRBDFLAG, Ras binding domain of Raf protein tagged with FLAG) in cells that do not express the *sev-GAL4>UAS-*Ras^{V12} and *UAS-RafRBDFLAG* transgenes, indicating the possibility that activated Ras complex itself can move from the source cell to the adjacent cells, leading to cell non-autonomous Ras signaling. With a view to understand how changes in levels of hsrω lncRNAs enhance Ras signaling activity, we examined

changes in transcriptomes of eye discs expressing activated Ras alone or in background of altered levels of hsrω RNAs. Unexpectedly, levels of transcripts of none of the known members of Ras/Raf/MAPK signaling pathway were found to be significantly affected by changes in hsrω transcripts levels in activated Ras expression background. Interestingly, several sn/snoRNAs along with a scaRNAs and some members of the RNA processing machinery were differentially expressed in activated Ras background and were further affected when the hsrω RNA levels were down-or up-regulated. In addition, while down-regulation of *hsrω* activity resulted in up-regulation of a few positive modulators of Ras signaling pathway, up-regulation of these transcripts caused down-regulation of the negative regulators of Ras/Raf/MAPK pathway, and thus resulting in increase in Ras activity in either cases.

The present study thus shows that expression of activated Ras not only affects activity of other protein coding genes but also causes altered expression of many non coding RNAs involved in RNA processing. An over- or under-expression of the nuclear lncRNAs produced by the *hsrω* gene, which is known to affect dynamics of hnRNPs and other proteins associated with omega speckles (Prashant et al, 2000; Lakhotia et al, 2012; Singh and Lakhotia 2015, 2016), seems to modulate levels of diverse RNA processing components and of certain other Ras signaling modulators. Together, these enhance the signaling cascade in cell autonomous as well as non-autonomous manner. The cell non-autonomous Ras signaling seems to correlate with Ras activity, with increase in activity inducing more of cell non-autonomous signaling, possibly through direct transfer of activated Ras complex to neighbouring cells which themselves do not express activated Ras.

Results

Alterations in hsrω RNA levels aggravate pupal lethality and enhance the rough eye phenotype due to *sev-GAL4* driven expression of activated Ras in eye discs

We used *UAS-Ras*^{V12} transgene (Karim et al., 1996), which upon GAL4 driven expression produces mutant Ras that does not undergo RasGTP to RasGDP transformation and can be active even in absence of activation by the upstream receptor tyrosine kinase (RTK). The *sev-GAL4* driven ectopic expression of *UAS-Ras*^{V12} leads to additional R7 rhabdomeres and rough eye morphology (Karim et al., 1996). To alter levels of the >10kb nuclear lncRNAs produced by *hsrω* gene, the *UAS-hsrωRNAi* transgene was used to down-regulate the hsrω nuclear lncRNAs (Mallik and Lakhotia, 2009) while for up-regulating this gene, the GAL4 inducible *EP3037* (Liao et al., 2000; Mallik and Lakhotia, 2009) allele was used. In some experiments *hsrω*^{66}, which is a near null allele (Johnson et al., 2011) of *hsrω* gene was also used to down-regulate hsrω RNA transcripts.

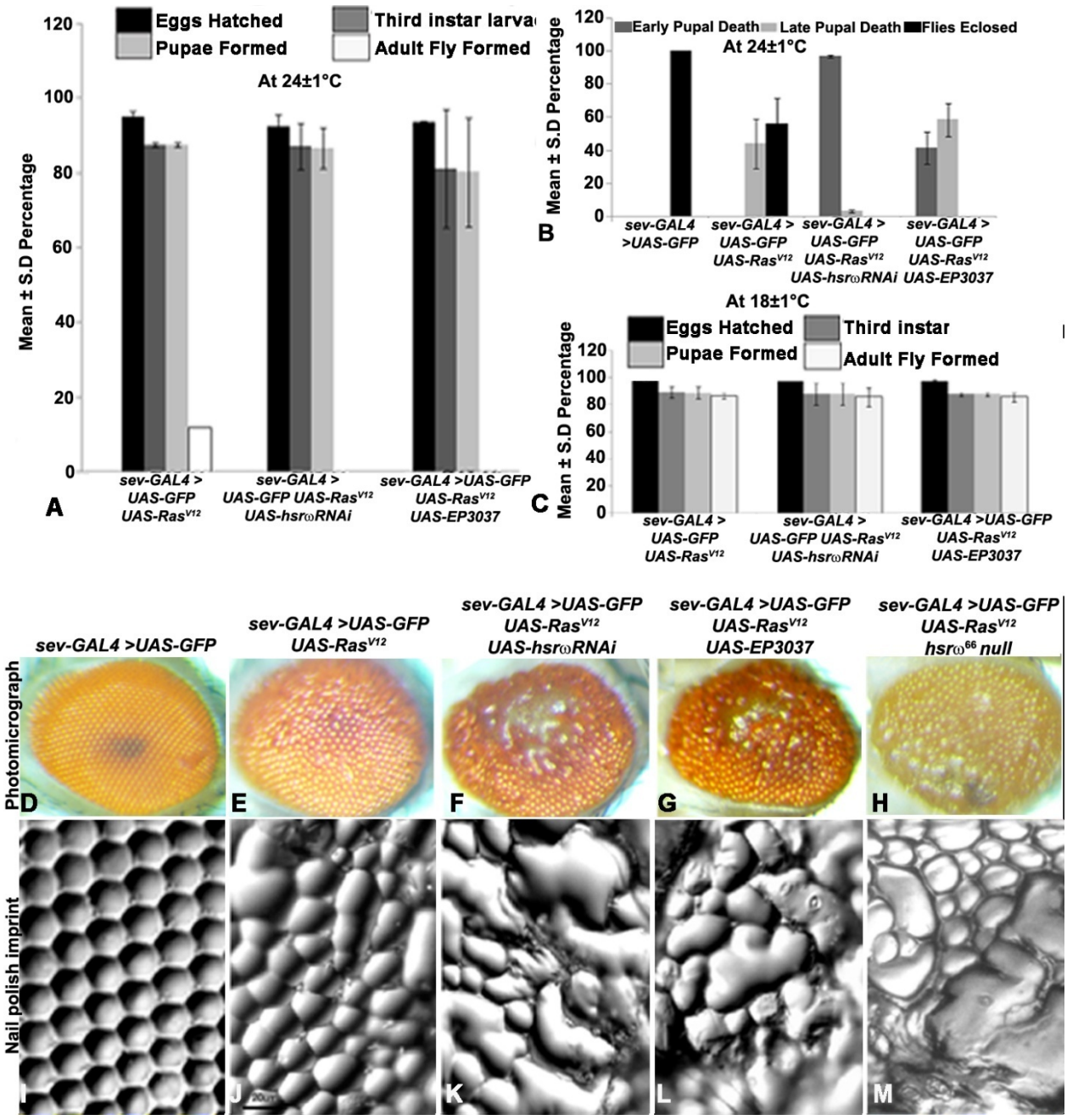

Fig. 1. Alterations in levels of hsrω RNAs enhance the pupal lethality and roughening of eyes due to *sev-GAL4* driven expression of activated Ras in developing eyes of *Drosophila*. **A-C** Histograms showing mean percent (± S.D.) survival (Y-axis) of different genotypes (X-axis) till different stages of development (**A**) and mean percent survival of pupae when reared at 24±1°C (**B**) and mean percent (± S.D.) survival (Y-axis) of different genotypes (X-axis) till different stages of development when reared at 18±1°C (**C**), **D-H** Photomicrographs and **I-M** nail polish imprints of adult eyes of individuals of different genotypes, noted above each column, reared at 18±1°C (**D-G** and **I-L**) or at 24±1°C (**H** and **M**).

At 24±1°C, only 11-12% of *sev-GAL4* driven Ras^{V12} expressing pupae eclosed as adult flies with rough eye phenotype together with de-pigmented patches and occasional black spots in eyes (Fig 1A). Most of those failing to emerge died as pharates (Fig 1B). When either *hsrωRNAi* or *EP3037* was co-expressed in *sev-GAL4* driven Ras^{V12} background, none of the pupae eclosed, with a majority dying as early pupae (Fig. 1A, B), indicating that changes in hsrω transcripts enhanced the effects of ectopic expression of activated Ras. With a view to examine the adult eye phenotypes, the above three genotypes were grown at 18±1°C, since the strength of the GAL4 driven expression is reduced at lower temperatures (Brand et al., 1994; Mondal et al., 2007). When reared at 18±1°C, more than 80% flies eclosed in each case, with no early pupal lethality (Fig. 1C). In this case also, *sev-GAL4* driven activated Ras expression caused roughening of eyes (Fig. 1I, J) compared to normal eye (Fig. 1D, E). Interestingly, those co-expressing *hsrωRNAi* or *EP3037* and Ras^{V12} showed greater roughening of eyes and ommatidial fusion than in *sev-GAL4>*Ras^{V12} eyes (Fig. 1F, G, K, L).

The *sev-GAL4* driven activated Ras expression in the $hsr\omega^{66}$, reared at 24±1°C, also resulted in greater roughening of eyes with fusion of ommatidia (Fig. 1H, M).

When third instar larval eye discs of these genotypes, grown at 24±1°C, were immunostained with anti-Elav to mark the neuronal photoreceptor cells in eye discs, it was evident that both down- or up-regulation of hsrω transcripts in *sev-GAL4* driven activated Ras expression background further enhanced the number of photoreceptor cells and consequently the ommatidial disarray (compare Fig 2A-B with Fig 2C-E). In agreement with the reported expression of *sev-GAL4* driver (Ray and Lakhotia, 2015), the *sev-GAL4* directed *UAS-GFP* transgene, present in all these genotypes, was expressed in a subset of photoreceptor cells, and in the two future cone cells. Accordingly, the average number of GFP+ve and Elav+ve cells (photoreceptor cells with the Sevenless expression) in each *sev-GAL4>UAS-GFP* ommatidium varied between 3-4, that of GFP+ve and Elav-ve (future cone cells) between 1-2 cells while the GFP-ve and Elav+ve rhabdomeres (photoreceptor cells without the Sevenless expression) varied between 3-4. Identification of these three classes of cells in third instar larval eye discs from *sev-GAL4>UAS-*Ras^{V12} larvae showed a small but specific increase only in GFP+ve and Elav+ve cells (Fig 2K), i.e., *sev-GAL4* expressing photoreceptor cells. Interestingly, the numbers of GFP+ve and Elav+ve cells in eye discs that had altered levels of hsrω RNAs in *sev-GAL4>UAS-*Ras^{V12} background, showed a much greater increase (Fig. 2K), with the most pronounced increase being in *sev-GAL4>UAS-*Ras^{V12} *UAS-hsrωRNAi* genotype (Fig 2C, K).

It may be noted that, in agreement with an earlier report (Mallik and Lakhotia, 2011), *sev-GAL4* driven expression of *UAS-hsrωRNAi* or *EP3037* in normal wild type Ras expression background did not cause any roughening of eyes (data not presented).

Thus alterations in levels of hsrω transcripts in activated Ras expression background enhanced the number of *sev-GAL4* expressing neuronal cells but not of the non-neuronal *sev-GAL4* expressing future cone cells.

The additional photoreceptors in eye discs with altered levels of hsrω transcripts in activated Ras expression background are R7 type

The Ras/Raf/MAPK signaling dependent differentiation of R7 photoreceptor, the last one of the 8 photoreceptors to differentiate, is initiated by binding of the Boss ligand to the Sevenless receptor tyrosine kinase (RTK) (Tomlinson and Struhl, 2001; Mavromatakis and Tomlinson, 2016). Of the multiple R7 precursor cells, only one cell in which the Boss ligand binds with and activates the RTK, which in turn activates the downstream Ras by converting the GDP-bound Ras to active GTP-bound Ras, differentiates into R7 in normal development. Activation of Ras initiates signaling cascade involving a series of phosphorylation reactions culminating in phosphorylation of MAPK and its nuclear translocation, which eventually triggers R7 differentiation (Karin and Hunter, 1995). Since the Ras^{V12} does not need ligand binding for activation, *sev-GAL4>UAS-*Ras^{V12} expression directly drives differentiation of two or more R7 photoreceptor cells per ommatidium. To confirm that the additional GFP+ve and Elav+ve photoreceptor cells seen in the experimental genotypes indeed belonged to the R7 lineage, eye discs from larvae of different genotypes were immunostained with Runt antibody. In wild type third instar larval eye discs, each developing ommatidium shows two Runt-positive photoreceptors, viz. R7 and R8 (Edwards and Meinertzhagen, 2009; Tomlinson et al., 2011). Since the R7 and R8 photoreceptors are present one above the other in each ommatidium, a given optical section shows either the R7 or R8, which could be further distinguished because the *sev-GAL4>UAS-GFP* is expressed only in R7. In wild type discs, R7 cells formed a well arranged pattern of rows of cells with one Runt and GFP-positive rhabdomere in each developing ommatidium (Fig 2F). Most of the ommatidia in eye discs expressing *sev-GAL4* driven activated Ras showed two Runt as well as GFP-positive R7 photoreceptor cells (Fig 2G), resulting in derangement of the regular pattern. Down-regulation of hsrω RNA in the same background led to about four R7 photoreceptors in each ommatidium with severely disarrayed ommatidial pattern (Fig 2H, L). Up-regulation of hsrω RNA through *EP3037* expression in activated Ras background also increased the number of R7 photoreceptors to about three per ommatidium (Fig 2I, L), which is less than that in *sev-GAL4>UAS-*Ras^{V12} *UAS-hsrωRNAi* eye discs. Down-regulating hsrω RNA level using the $hsr\omega^{66}$ allele too, resulted in derangement of photoreceptor array in developing eye discs (Fig 2E) due to increase in neuronal cells (Fig 2K), which were confirmed by Runt staining to be R7 photoreceptors (Fig 2J, L).

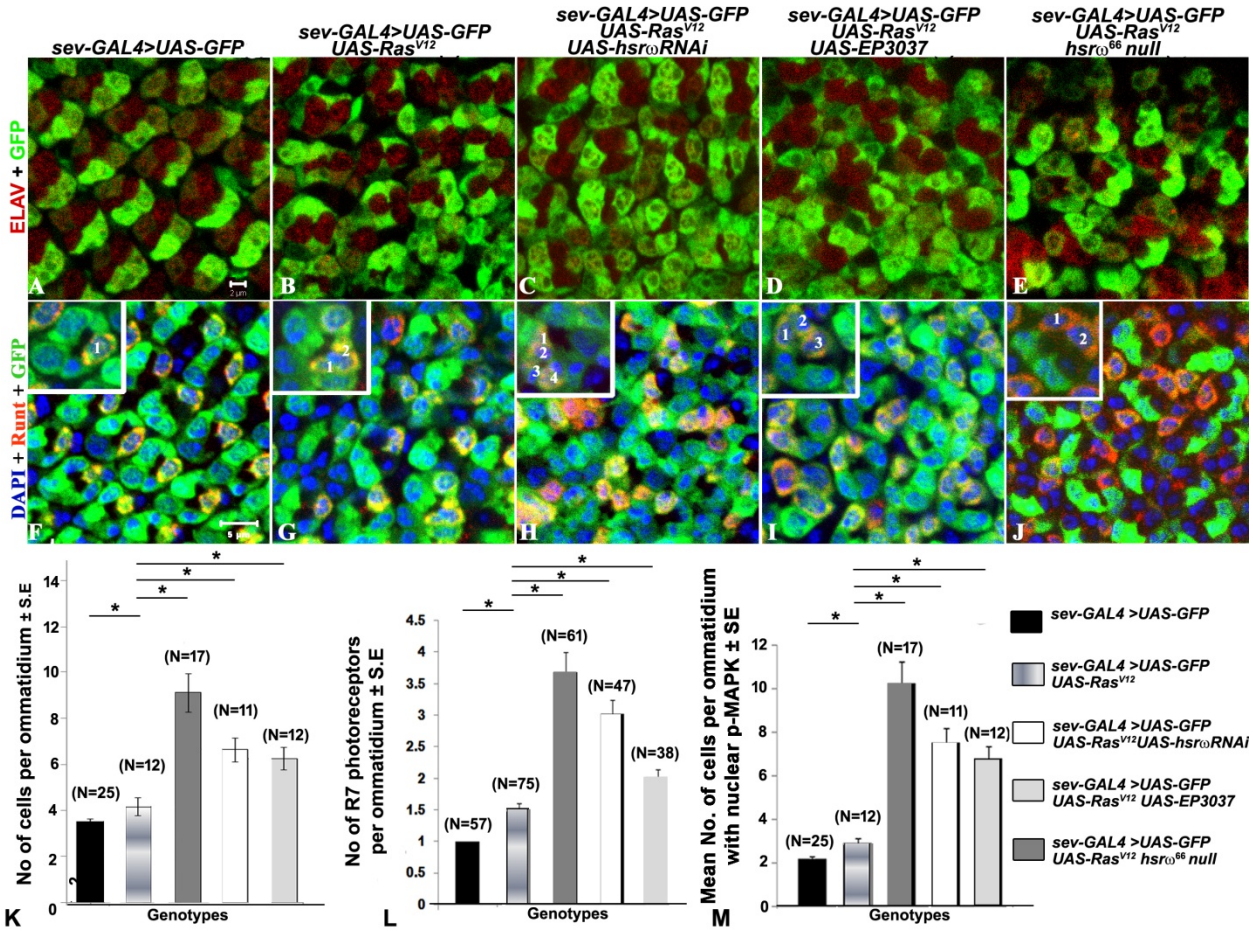

Fig. 2. Changes in hsrω RNA levels in activated Ras expressing background, promotes recruitment of more cells to R7 photoreceptor/neuronal fate. A-J Confocal optical sections of third instar larval eye discs of different genotypes (noted above each column) showing photoreceptor cells in developing ommatidia marked by Elav staining (red, **A-E**) and the Runt positive R7 photoreceptors (red, **F-J**); the *sev-GAL4>GFP* expressing cells are green in **A-J**; counterstaining with DAPI (blue, **F-J**). Insets in **F-J** show single representative ommatidium, with the Runt and GFP+ve cells being numbered. Scale bar in **A** denotes 2µm and in **F** denotes 5µm and applies to **A-E** and **F-J**, respectively. **K-M** Histogram showing mean numbers (± S.E., Y-axis) of neuronal cells per ommatidium in different genotypes (**K**), mean number of R7 photoreceptor cells per ommatidium (**L**) and mean number of cells with nuclear p-MAPK per ommatidium (**M**) in third instar larval eye discs in different genotypes (X-axis) with each bar denoting separate genotype as mentioned in the key on right; the number (**N)** of ommatidia examined is mentioned above each bar.

Altered hsrω RNA levels further enhance Ras signaling in eye discs ectopically expressing activated Ras in cell autonomous as well as non-autonomous manner

The above noted increase in number of R7 photoreceptors in each ommatidium in eye discs with altered levels of hsrω transcripts in activated Ras expression background suggests further

increase in Ras signaling. In order to measure the level of Ras signaling, we examined distribution of p-MAPK as phosphorylation of MAPK and its nuclear translocation is a measure of active Ras signaling (Karin and Hunter, 1995). In addition, we also examined levels of Yan, a transcription factor which is negatively regulated by Ras signaling (Brunner et al., 1994; O'Neill et al., 1994).

In the normally developing control eye discs, only a few cells in each ommatidium showed nuclear p-MAPK localization (Fig 2M, 3A). Expression of activated Ras led to greater number of cells showing nuclear p-MAPK staining (Fig 3B, 2M) besides an overall increase in p-MAPK presence. When hsrω RNA levels were either down- (Fig 3C) or up-regulated (Fig 3D), the number of cells with nuclear p-MAPK showed a steep increase (Fig 2M, 3C-D) with concomitant increase in overall p-MAPK levels in the eye discs. Interestingly, not only the cells expressing *sev-GAL4* driver, identified by the *UAS-GFP* expression, but GFP-negative, and thus non *sev-GAL4* expressing cells (marked by arrows in Fig 3B-D) also showed higher p-MAPK levels. This suggested a non-autonomous Ras signaling.

In control *sev-GAL4>UAS-GFP* third instar larvae, the Yan transcription factor is widely expressed in eye discs posterior to the morphogenetic furrow (MF), with especially stronger presence in the MF itself (marked by arrows in Fig 3E-H). The Yan staining progressively declined from anterior to posterior region of eye discs, where it was sparsely present in differentiated photoreceptors. Following *sev-GAL4* driven expression of activated Ras a small but perceptible decrease in Yan expression along the antero-posterior axis of the discs and at the MF was noted (Fig 3F). Down- or up-regulation of hsrω RNA levels in the *sev-GAL4* driven activated Ras expression background further reduced the expression of Yan in the eye discs (Fig 3G-H), including in the MF cells. Since the *sev-GAL4* driver has no expression at the MF (Ray and Lakhotia 2015), the distinct reduction in Yan staining all over the eye disc, including the MF, clearly indicates a cell non-autonomous Ras signaling, which was further enhanced when levels of hsrω non-coding transcripts were reduced or elevated.

With a view to ascertain if above changes that suggested elevated Ras signaling in the test genotypes were associated with enhanced Ras expression and/or with a higher proportion of Ras being in an active form, we co-immunostained developing eye discs of wandering third instar control larvae of different genotypes for Ras and RafRBDFLAG since the *UAS-RafRDBFLAG* construct (Freeman et al., 2010), which expresses the FLAG-tagged active Ras binding domain of Raf, binds only with active Ras. Fig. 3I-L presents confocal images of immunostainied eye discs while the Fig. 4 shows results of quantification of mean intensity levels of Ras, RafRBDFLAG and co-localization of Ras and RafRBDFLAG, to provide an estimate of active Ras levels in eye discs of different genotypes.

Following expression of *sev-GAL4* driven *UAS-RafRBDFLAG* in developing eye discs of wandering third instar control larvae with normal developmental Ras expression, little co-localization of the RafRBDFLAG with the native Ras was detectable (Fig 3I). In contrast, FLAG

tagged RafRBD was substantially co-localized with the Ras in eye discs expressing *sev-GAL4* driven activated Ras, as expected with the presence of Ras in active form (Fig 3J). Down- or up-regulation of hsrω RNAs in this background clearly enhanced the number of cells that showed distinctly co-localized RafRBDFLAG and Ras (Fig 3K, L). Interestingly, as may be noted from Fig 3J-L, eye discs co-expressing *sev-GAL4* driven *UAS-Ras^{V12}* and *UAS-hsrωRNAi* or *EP3037* had a greater number of GFP-ve cells, adjoining the GFP+ve cell, which also showed colocalized Ras and RafRBDFLAG. Since neither RafRBDFLAG nor activated Ras was expressed in the non *sev-GAL4* expressing GFP-ve cells, their co-localization in such cells reflects movement of activated Ras complex from *sev-GAL4* expressing cells to the neighbouring cells.

With a view to know if the increased colocalization seen in greater numbers of cells in eye discs expressing activated Ras without or with altered hsrω RNA levels reflected equal or differential elevation in levels of total Ras, activated Ras and RafRBDFLAG proteins, we used the Histo option of the Zeiss Meta 510 Zen software to quantify the Ras, FLAG, DAPI and GFP fluorescence signals in these genotypes. As seen in Fig. 4A, the total Ras is expectedly higher in *sev-GAL4>UAS-Ras^{V12}* than in *sev-GAL4>UAS-GFP* eye discs. The more than 2 times further increase in Ras staining in discs co-expressing *UAS-hsrωRNAi* or *EP3037* with activated Ras, clearly shows that the under- or over-expression of hsrω transcripts in activated Ras over-expression background further enhanced Ras levels. The increase in GFP staining in *sev-GAL4>UAS-Ras^{V12} UAS-hsrωRNAi* correlates with the earlier noted greater increase in *sev-GAL4* driven GFP expressing cells in these eye discs. The more or less comparable levels of DAPI fluorescence in the samples of eyes discs in different genotypes indicates that the increase in Ras or GFP activity in specific genotypes is not due to a general increase in number of cells in some genotypes. It is significant that the levels of RafRBDFLAG protein showed the expected increase in *sev-GAL4>UAS-Ras^{V12}* than in *sev-GAL4>UAS-GFP* eye discs but co-expression of *UAS-hsrωRNAi* or *EP3037* with activated Ras was not associated with further increase in the FLAG staining (Fig. 4A). In order to determine how much of the enhanced levels of Ras in *sev-GAL4>UAS-Ras^{V12}* and more so in *sev-GAL4>UAS-Ras^{V12} UAS-hsrωRNAi* and *sev-GAL4>UAS-Ras^{V12} EP3037* eye discs was in activated form, we examined colocalization of Ras and RafRBFFLAG fluorescence signals (Fig. 4B). In agreement with the ectopic expression of activated Ras in *sev-GAL4>UAS-Ras^{V12}* eye discs, nearly 40% of RafRBDFLAG was associated with Ras compared to only about 5% of the FLAG signal being associated with Ras in *sev-GAL4>UAS-GFP* discs. Interestingly, co-expression of *UAS-hsrωRNAi* or *EP3037* in *sev-GAL4>UAS-Ras^{V12}* eye discs resulted in further increase in association of Ras and RafRBDFLAG proteins, indicating that a greater proportion of Ras in these cells is in activated form with which the RafRBDFLAG can bind.

In order to see if the above noted increase in Ras and activated Ras levels was associated with increased transcription of the resident *Ras* and/or *UAS-Ras^{V12}* transgene, we examined the levels of Ras transcripts derived from these two sources using semi-quantitative RT-PCR with primers designed to differentiate between these two transcripts (see Supplementary Methods). The

normal resident *Ras* gene transcripts remained more or less comparable in all the four genotypes (*sev-GAL4>UAS-GFP*, *sev-GAL4>UAS-Ras^{V12}*, *sev-GAL4>UAS-Ras^{V12} hsrωRNAi* and *sev-GAL4>UAS-Ras^{V12} EP3037*) and likewise, the transcripts derived from the *Ras^{V12}* transgene remained similar in *sev-GAL4>UAS-Ras^{V12}* and those co-expressing *hsrωRNAi* or *EP3037* with *UAS-Ras^{V12}* (see Supplementary Fig S1 A and B). This indicated that the elevated Ras activity in the latter two genotypes with altered hsrω RNA levels is unlikely to be due to increased transcription of *UAS-Ras^{V12}* transgene or the resident *Ras* gene. As noted later, the RNA-seq data also did not show any significant increase in Ras transcripts in *sev-GAL4>UAS-Ras^{V12}* eye discs co-expressing *hsrωRNAi* or *EP3037* compared to those expressing only activated Ras.

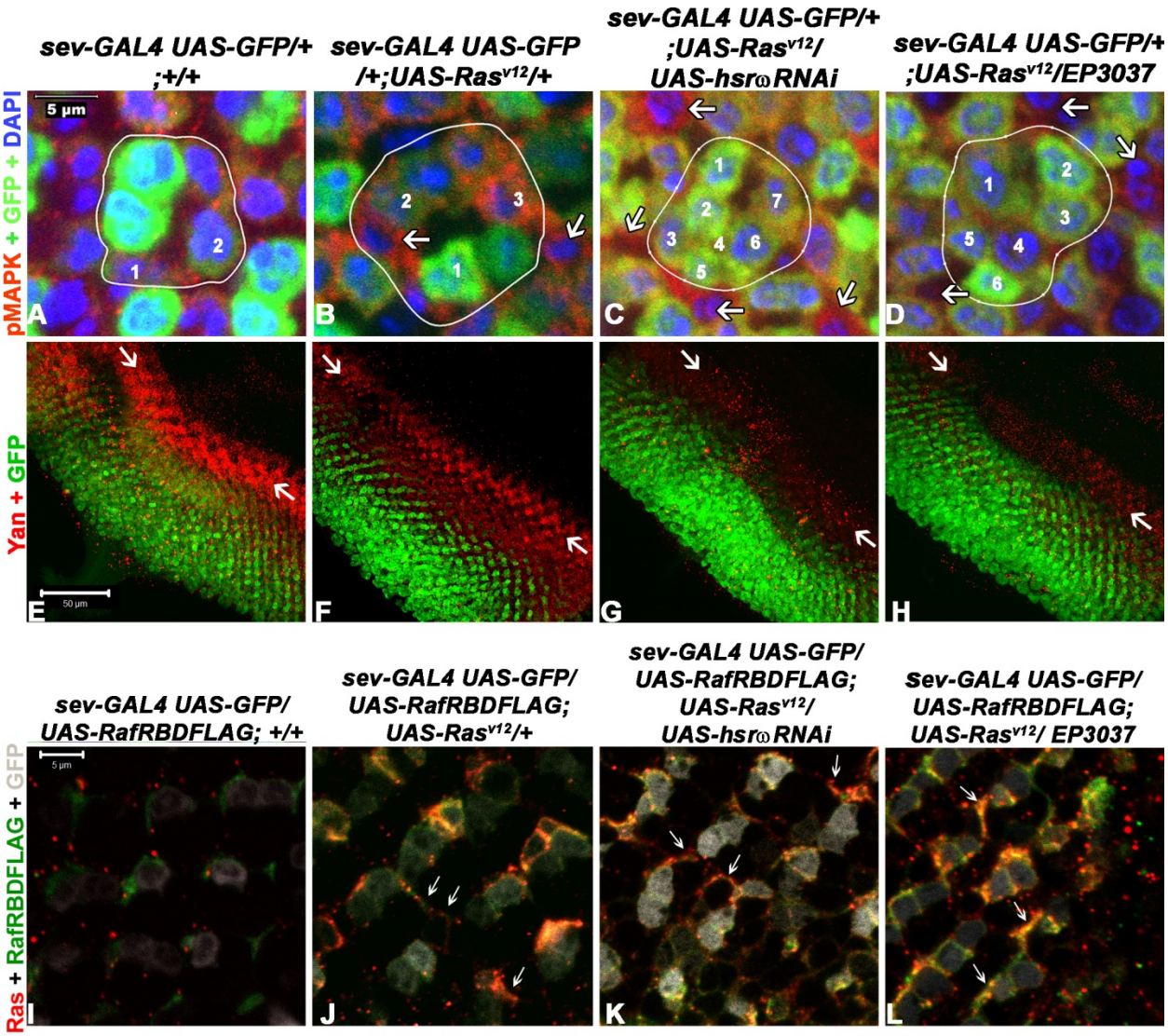

Fig. 3. Alteration in hsrω RNA levels leads to increase in cell autonomous as well as non-autonomous Ras signaling. A-D Confocal optical sections of parts of third instar larval eye discs of different genotypes (noted above each column) immunostained with p-MAPK antibody (red); *sev-GAL4* driven GFP expressing cells are green (counterstained with DAPI, blue); a

representative developing ommatidium in each is demarcated by white line. Some of the non-GFP expressing cells but with distinct p-MAPK staining in **B-D** are indicated by white arrows. Scale bar in **A** denotes 5µm and applies to **A-D. E-H** Confocal optical sections of third instar larval eye discs of different genotypes (noted above each column) showing immunostaining for Yan (red) and *sev-GAL4* driven GFP fluorescence (green); white arrows mark the morphogenetic furrow. Scale bar in **E** denotes 50µm and applies to **E-H. I-L** Confocal optical sections of parts of third instar larval eye discs of different genotypes (noted above each column) immunostained with Ras (red) and FLAG (green) antibodies, the *sev-GAL4>GFP* expressing cells are grey. Scale bar in **I** denotes 5µm and applies to **I-L**

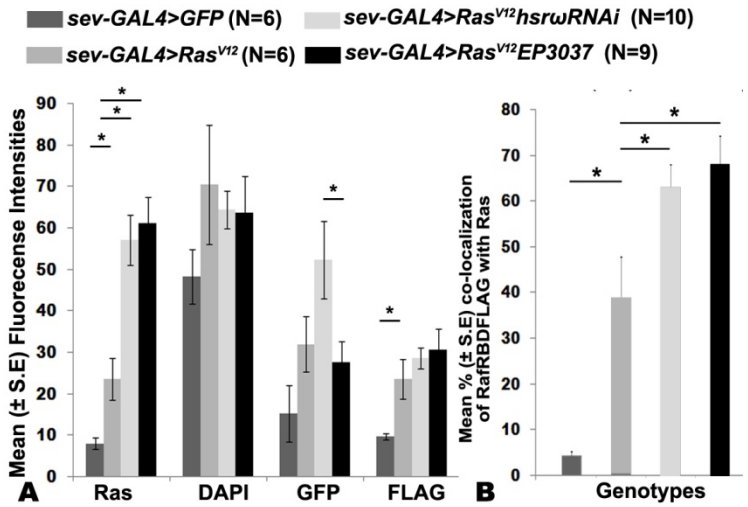

Fig. 4. Alterations in hsrωRNA levels in activated Ras background further enhance levels of active Ras. A Histograms showing intensities (Y-axis) of Ras, DAPI, GFP and RafRBDFLAG fluorescence (X-axis) in third instar larval eye discs of different genotypes. **B** Colocalization of RafRBDFLAG and Ras, expressed as mean percent (± S.E, Y-axis) of RafRBDFLAG protein associated with Ras, which indicates relative levels of activated Ras in different genotypes (X-axis). Number of eye discs observed (**N**) for each genotype is given in parenthesis in the key at top. A horizontal line connecting specific pairs of histogram bars and the * mark indicate that the given pair of means are significantly different ($P \leq 0.05$) on Student's 't'-test.

Down- or up-regulation of hsrω transcripts commonly affects many RNA processing components in activated Ras background but differentially affects positive and negative modulators of Ras signaling

With a view to understand how the down- and up-regulation of levels of hsrω lncRNAs similarly enhance Ras signaling activity in cells ectopically expressing activated Ras, we undertook transcriptome analysis of total cellular RNA in eye discs of different genotypes. The eye discs from *sev-GAL4>UAS-GFP* late third instar larvae served as control. We also examined changes in transcriptome following *sev-GAL4* driven down- or up-regulation of hsrω transcripts

in normal Ras background. Although detailed results of this analysis will be presented elsewhere, in the present we have used this data whenever required to compare with changes that are relevant in the context of their expression in activated Ras expression background.

The *sev-GAL4* driven expression of activated Ras in eye discs resulted in differential expression of many genes, with 374 genes being down-regulated and 138 up-regulated, when compared with those in *sev-GAL4>UAS-GFP* eye discs (List 1 in Fig 5A and B, Supplementary Table S1, sheet1). Besides the expected increase in levels of transcripts of genes involved in cell growth, proliferation and differentiation, transcripts of many genes involved in RNA biosynthesis, metabolism and processing were also found to be up-regulated when compared with *sev-GAL4>UAS-GFP* eye discs (Supplementary Table S1, sheet2).

As expected, the levels of Ras transcripts were significantly higher in *sev-GAL4>UAS-Ras*^{V12} than in *sev-GAL4>UAS-GFP*. In agreement with the above noted RT-PCR results (Supplementary Fig. S1A, B), the RNA seq data also showed that levels of Ras transcripts did not show any further significant increase when hsrω RNA levels were down- or up-regulated in *sev-GAL4* driven activated Ras expression background (see, Supplementary Table S1, sheet 2). The RNA seq data also revealed levels of transcripts of the genes that act directly downstream in the Ras signaling cascade were not further up-regulated in eye discs co-expressing *sev-GAL4* driven *UAS-Ras*^{V12} and *hsrωRNAi* or *EP3037*.

A search of the RNA seq data on the basis of GO terms showed that eye discs with *sev-GAL4* driven activated Ras expression showed up-regulation of several genes involved in R7 cell differentiation (e.g., *salm, ten-m, cadn, svp, dab, nej*) and also of certain genes involved in photoreceptor development (e.g., *rno, doa, pdz-gef, jeb, atx2* and *csn4*) (see Supplementary Table S1, sheet2). However, none of these genes showed any further change when *hsrωRNAi* or *EP3037* was co-expressed with activated Ras, except for *svp*, whose transcripts were further up-regulated in eye discs co-expressing *sev-GAL4* driven activated Ras and *hsrωRNAi* but not in those co-expressing activated Ras and *EP3037* (see later). Results of a real-time qRT-PCR analysis for levels of transcripts of many of the genes, which are part of Ras signaling cascade, in different genotypes (see Supplementary Fig. S1 C) validated the RNA seq data as none of them were found to show differential expression in eye discs expressing activated Ras along with changes in hsrω RNA levels compared to those expressing activated Ras alone.

In order to understand the basis for the unexpected similar enhancing effect of down- or up-regulation of hsrω transcripts on the Ras signaling in eye discs that were expressing activated Ras under the *sev-GAL4* driver, we looked for genes that were commonly down- or up- regulated following expression of *hsrωRNAi* or *EP3037* in *sev-GAL4* driven activated Ras expression background (encircled in red and white, respectively, in Fig. 4A and B). The group that was down-regulated by activated Ras expression and further down-regulated when *hsrωRNAi* or *EP3037* was co-expressed included only one gene (encircled in red in Fig 5A), while the group that was up-regulated following activated Ras expression and further up-regulated when hsrω

RNA levels were reduced or elevated included three genes (encircled in red in Fig. 5B). The single gene in the first group, *CG13900*, codes for a splicing factor 3b subunit involved in mRNA splicing via spliceosome (U2 snRNP), while the three genes up regulated on Ras activation and further up-regulated on co-alterations in hsrω RNA levels were *CG15630*, *Hsp70Bb* and *CG14516*. On the basis of their described roles in the Flybase, however, none of these appeared to be directly involved in modulating Ras pathway. The *CG13900* gene encoding a splicing factor, however, may be involved in post-transcriptional modulation of activities of other genes, including those in the Ras pathway.

When we looked for genes that were commonly down or up-regulated following *hsrωRNAi* or *EP3037* co-expression in *sevGAL4>Ras^{V12}* expressing eye discs compared to those of *sevGAL4>Ras^{V12}* but which showed no change in *sevGAL4>Ras^{V12}* as compared to *sevGAL4>UAS-GFP* control eye discs, a total of 88 genes were found to be commonly down-regulated (encircled in white in Fig. 5A, and Fig. 6A-C) and 45 (encircled in white in Fig. 5B, and Fig. 6D) commonly up-regulated. One group among the 88 commonly down-regulated genes comprised of several snoRNAs, snRNA and scaRNA. As shown in Fig. 6A, the genes in the sno/scaRNAs group were significantly up-regulated by activated Ras expression but co-expression of either *hsrωRNAi* or *EP3037* with activated Ras led to their significant down-regulation. It is important that, except one, none of these sno/scaRNAs are significantly affected when *hsrωRNAi* or *EP3037* is expressed under the *sev-GAL4* in normal Ras background (Fig. 6A). Interestingly, *sev-GAL4* driven *hsrωRNAi* or *EP3037* expression in normal Ras background affects some other sno/scaRNAs which are not affected by activated Ras expression (data not presented). Six genes, associated with the GO term Transcription factors, were significantly up-regulated following *sev-GAL4* driven activated Ras expression but all of them were commonly down-regulated when *hsrωRNAi* or *EP3037* were co-expressed (Fig. 6B). Among these, the Ssb-c31a is a protein that binds with Raf (Friedman et al., 2011) and is reported to be a negative regulator of transcription (Lacoste et al., 1995).

The other genes among these commonly down-regulated 88 genes (Fig. 6C) belonged to diverse GO terms without any apparent and known association with Ras signaling. One of these genes, the *dlc90F* encodes a dynein light chain protein, which is reported to bind to Raf (Friedman et al., 2011) and to be a positive regulator of FGF signaling (Zhu et al., 2005); however, not much is known about its role in Ras signaling.

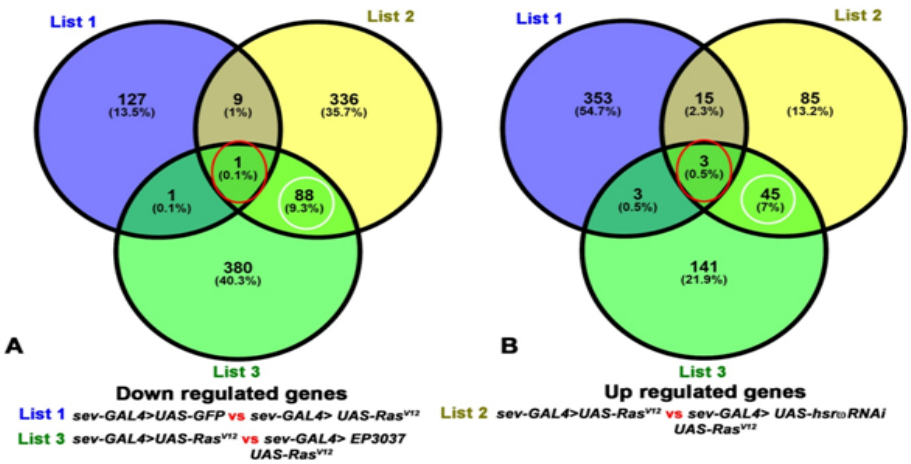

Fig. 5. Total RNA sequencing revealed changes in levels of transcripts of several genes following expression of activated Ras alone or along with changes in hsrωRNA levels. A-B Venn diagrams showing numbers of genes down- or up-regulated in third instar larval eye discs with either decrease (list 2) or increase (list 3) in levels of hsrω RNA achieved by driving *UAS-hsrωRNAi* or *EP3037*, respectively, under *sev-GAL4* driver along with activated Ras expression as compared to control eye discs (*sev-GAL4>UAS-Ras^{V12}*, List 1). Gene numbers in red circles in **A** and **B** denote genes commonly down- or up- regulated in *sev-GAL4>UAS-hsrωRNAi UAS-Ras^{V12}* and *sev-GAL4>EP3037 UAS-Ras^{V12}* compared to *sev-GAL4>UAS-Ras^{V12}* and which also show significant changes in *sev-GAL4>UAS-Ras^{V12}* when compared to *sev-GAL4>UAS-GFP* eye discs. Numbers in white circles in **A** and **B** denote genes differentially regulated in *sev-GAL4>UAS-hsrωRNAi UAS-Ras^{V12}* and *sev-GAL4> EP3037 UAS-Ras^{V12}* compared to *sev-GAL4>UAS-Ras^{V12}* but which show no change in *sev-GAL4>UAS-Ras^{V12}* when compared to *sev-GAL4>UAS-GFP* eye discs.

The group of 45 genes that were significantly up-regulated by co-expression of *hsrωRNAi* or *EP3037* in activated Ras background (Fig. 6D) included diverse genes, none of which seem to be directly involved in Ras signaling pathways. However, the *kuz* (*kuzbanian*) gene, encoding a metalloprotease, is expressed in developing ommatidia with roles in neuroblast fate determination, round-about (Robo) signaling and neuron formation (Sotillos et al., 1997; Udolph et al., 2009; Coleman et al., 2010). Therefore, this may be one potentially important gene which is up-regulated only when hsrω RNA levels are altered in activated Ras expressing eye discs in parallel with increased numbers of R7 photoreceptors.

Finally, we looked at genes that were differentially affected when *hsrωRNAi* or *EP3037* were co-expressed with activated Ras. Again, these genes belonged to different pathways (see Supplementary Table S1, sheets 4-7) but one group that appeared significant was that of positive and negative modulators of photoreceptor differentiation (Fig. 7). The Gene ontology search results revealed that down-regulation of hsrω transcripts in activated Ras background enhanced levels of R7 cell fate determining genes, namely, *phly, svp, rau* and *socs36E* compared to only activated Ras expressing eye discs (Fig 7 and Supplementary Table S1, sheet4).

In case of over-expression of hsrω RNA in activated Ras background, none of the above four genes involved in R7 differentiation were up-regulated. However, several other negative regulators of Ras signal transduction pathway like *bru, klu, mesr4, cdep, epac,, nfat, ptp-er, cg43102, rhogap1a, rhogef2, spri, exn* etc were down regulated (Supplementary Table S1, sheet 7, and Fig 7) in *sev-GAL4>Ras^{V12} EP3037* eye discs. On the basis of their GO term classification (David Bioinformatics), genes like *bru, cdep, sos, pdz-gef, cg43102, rhogap1a, rhogef2, spri, exn* in this group are involved in Ras guanyl nucleotide exchange factor activity while the other genes like *klu, mesr4, nfat, ptp-er* affect small GTPase mediated signal transduction. Being negative-regulators, their down-regulation by co-expression of activated Ras and *EP3037* would lead to further enhanced Ras activity. It is interesting to note that in normal developmental Ras activity background, the *sev-GAL4* driven expression of *UAS-hsrωRNAi* or *EP3037* did not exert comparable differential effects on expression of these positive and negative modulators of Ras signaling since as shown in the last two columns in Fig. 7, these genes were either not affected or were commonly down regulated in both conditions.

Among the other groups of genes that showed differential changes upon down or up-regulation of the hsrω transcripts in activated Ras expressing eye discs, was a group involved in ribosome biogenesis that was down regulated in activated Ras expressing discs with down-regulated hsrω transcripts. Expression of these genes was, however, not affected when *EP3037* was co-expressed with activated Ras (Supplementary material S2 sheets 6-7).

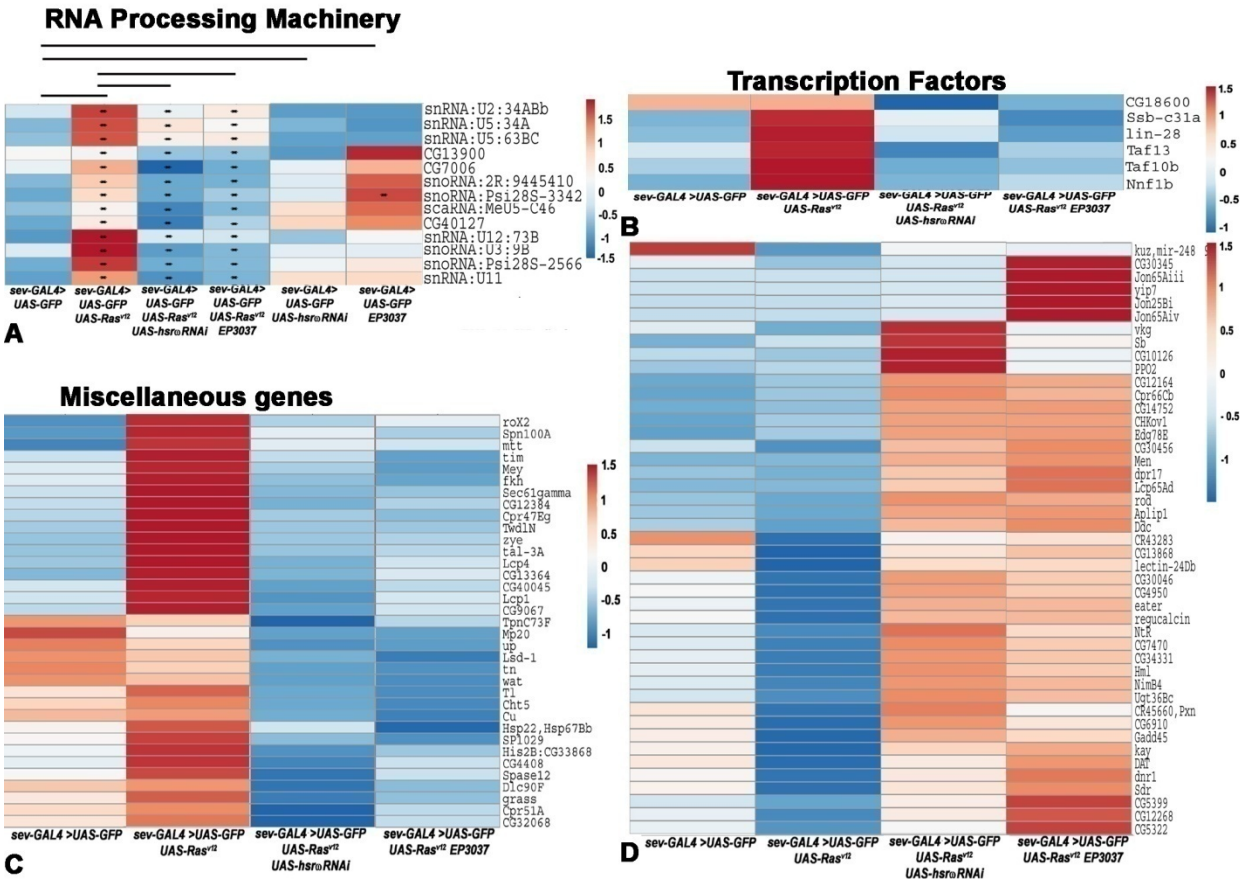

Fig. 6. Many gene transcripts, which are up-regulated on Ras activation, show opposite changes in their levels after hsrω RNA levels are reduced or elevated. A-C Heat maps of FPKM values of different transcripts (noted on right of each row) belonging to RNA processing machinery (**A**), gene transcription (**B**) and other diverse Gene Ontology groups (**C**) in different genotypes (noted at base of each column), which are significantly down-regulated in activated Ras and altered hsrω RNA levels when compared to only activated Ras expressing eye discs. **D.** Heat maps showing FPKM values of different transcripts (noted on right of each row) belonging to diverse Gene Ontology groups in different genotypes (noted at base of each column) which are significantly up-regulated in eye discs when hsrω RNA levels are altered in activated Ras background compared to only activated Ras expressing eye discs. Asterisks in **A** indicate significant differences ($p \leq 0.05$) between the compared genotypes (indicated by black lines at top of the panel).

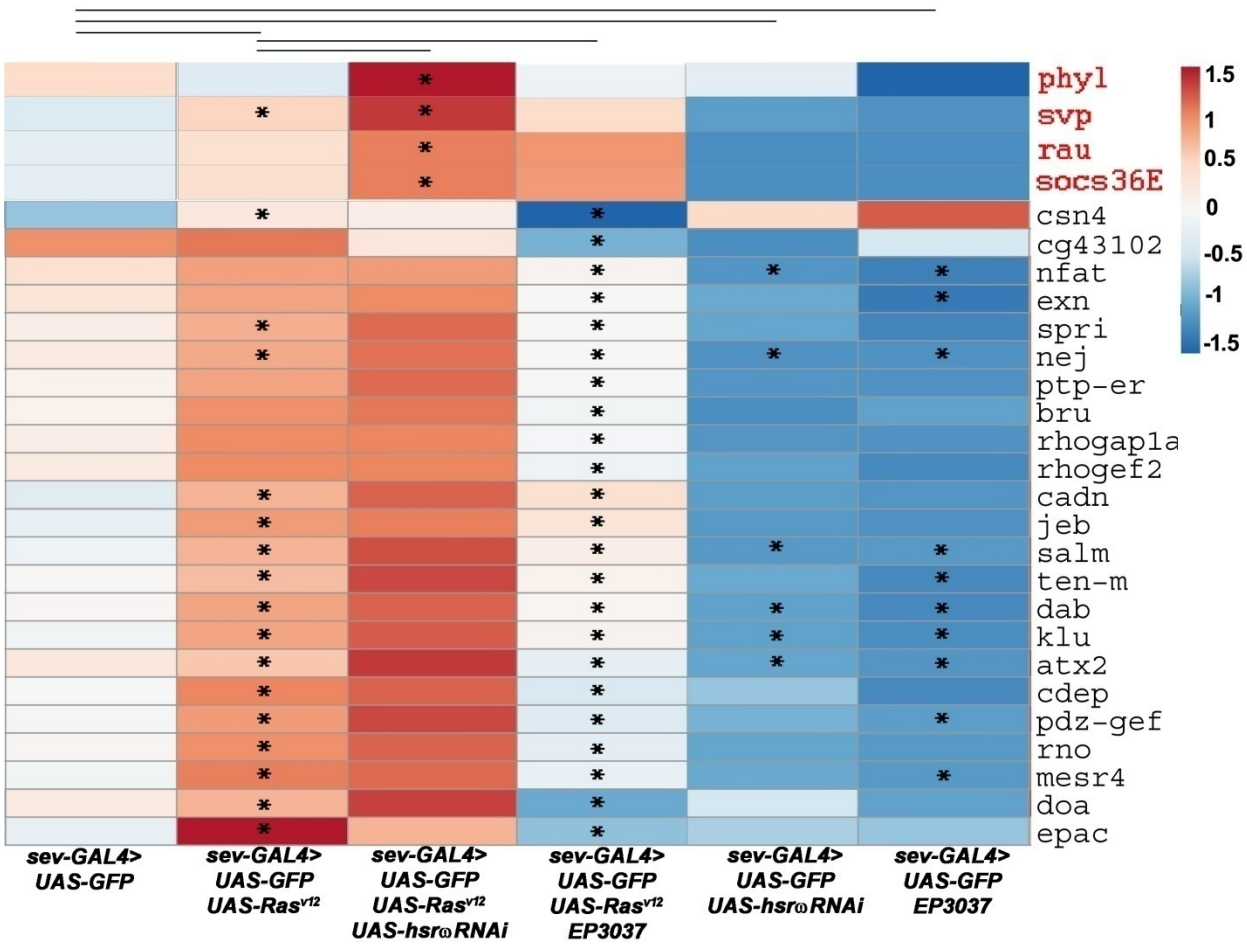

Fig. 7. Transcripts of positive Ras modulators are up-regulated while negative modulators are down-regulated upon down- and up-regulation, respectively, of hsrω transcripts in activated Ras expression background. Heat maps of FPKM values of gene transcripts involved in photoreceptor differentiation (noted on right of each row) which are significantly up- or down-regulated in eye discs when hsrω RNA levels are altered in activated Ras background compared to only activated Ras expressing eye disc. The four genes up-regulated in *sev-GAL4>Ras^{V12} hsrωRNAi*, marked in red letters, are potential positive modulators of Ras signaling. The last two columns, representing the FPKM values obtained in *sev-GAL4>hsrωRNAi* and *sev-GAL4>EP3037*, respectively, show that the opposing effects are seen only in activated Ras expression background. Asterisks indicate significant differences ($p \leq 0.05$) between the compared genotypes (indicated by black lines at the top).

Discussion

Present study was initiated to examine interactions between long non-coding hsrω RNAs and Ras signaling cascade. Although, some studies have implicated oncogenic Ras to influence expression of several long non coding transcripts (Rotblat et al 2011, Kotake 2016, Zhang et al 2017 Jiang et al., 2017; Jinesh et al., 2017), regulation of Ras signaling cascade by lncRNAs is

not yet known. Earlier study from our lab showed that a *ras* mutant allele enhanced *hsrω*-null phenotype (Ray and Lakhotia, 1998). The present study shows that alterations in levels of hsrω transcripts exaggerate phenotypes that follow when activated Ras is ectopically expressed in developing eye discs of *Drosophila* larvae. The *UAS-Ras^{V12}* transgene (Karim et al., 1996) has been widely used for examining consequence of ectopic expression of ligand-independent activated Ras. Its expression in developing eye discs under the *sev-GAL4* driver is known to disrupt ommatidial arrays because of recruitment of additional cells to R7 photoreceptor path (Karim et al., 1996). Our results clearly show that reduced as well as over-expression of hsrω transcripts in activated Ras background significantly enhanced the number of R7 photoreceptor per ommatidium unit. As revealed by detection of p-MAPK, Yan and activated Ras associated RafRBDFLAG in eye discs, the increase in R7 photoreceptors was distinctly correlated with the enhanced Ras activity levels in *sev-GAL4>hsrωRNAi UAS-Ras^{V12}* and *sev-GAL4>EP3037 UAS-Ras^{V12}* genotypes.

Of the multiple transcripts produced by *hsrω* gene (http://flybase.org; Lakhotia, 2011; Lakhotia, 2017), the >10kb nuclear transcripts are primarily involved in biogenesis of omega speckles and thus affect the dynamic exchanges of hnRNPs and other omega speckle associated proteins between different nuclear compartments (Lakhotia et al., 2012; Singh and Lakhotia, 2015; Lakhotia, 2016; Singh and Lakhotia, 2016; Lakhotia, 2017). The GAL4 induced expression of *hsrωRNAi* transgene and of the *EP3037* allele of *hsrω* are known to primarily lower and elevate, respectively, levels of the larger and nucleus-limited transcripts of *hsrω* gene (Mallik and Lakhotia, 2009; Mallik and Lakhotia, 2011). Either of these conditions have comparable consequences for dynamics of omega speckles and their associated proteins, which are involved in RNA processing (Lakhotia et al., 2012; Singh and Lakhotia, 2015). Such common consequences for dynamics of several important members of RNA processing machineries may be responsible for the rather unexpected commonality in enhancing the Ras signaling cascade when the hsrω nuclear transcript levels were down or up-regulated in the background of ectopic expression of activated Ras.

The down- or up-regulation of hsrω transcripts apparently did not further enhance the transcription of *UAS-Ras^{V12}* transgene and the other downstream Ras-signaling pathway genes since their transcript levels did not show any significant elevation in *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* or *sev-GAL4>Ras^{V12}EP3037* over those in *sev-GAL4>Ras^{V12}* eye discs. Therefore, the greatly enhanced Ras signaling seems to be mediated through post-transcriptional events. It is notable that in spite of the similar levels of Ras transcripts in *sev-GAL4>Ras^{V12} UAS-hsrωRNAi*, *sev-GAL4>Ras^{V12}EP3037* and *sev-GAL4>Ras^{V12}* eye discs, the levels of total as well as activated Ras, as revealed by immunostaining and RafRBDFLAG association (Fig. 3I-L and Fig 4), appeared to be more enhanced in the former two genotypes. This clearly suggests effects at post-transcriptional levels.

It is significant that down- or up-regulation of hsrω transcripts in wild type Ras background does not affect ommatidial differentiation since the eyes in *sev-GAL4>UAS-hsrωRNAi* or *sev-*

GAL4>EP3037 flies are normal with no extra rhabdomere (Mallik and Lakhotia, 2011). Immunostaining for Ras and RafRBDFLAG in *sev-GAL4>UAs-GFP* or *sevGAL4>UAS-hsrωRNAi* or *sev-GAL4>EP3037* eye discs also did not show any difference between them (data not shown). Apparently, ectopic over-expression of activated Ras makes the cells detectably sensitive to alterations in hsrω transcript levels. This may have implications for a role of the hsrω transcripts in modulating any aberrant Ras activity during normal development.

The significant increase in Ras activity seen in *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* or *sev-GAL4> Ras^{V12} EP3037* eye discs seems to be due to modulation of activities of other regulators of the Ras signaling cascade. Our data suggest that *sev-GAL4>Ras^{V12} UAS-hsrωRNAi* eye discs have significantly elevated levels of transcripts of some of the genes (*phly, svp, rau* and *socs36E*) whose products have been identified as positive modulators of Ras activity in R7 differentiation. The *phly* encodes a nuclear receptor, which acts downstream of Ras and is responsible for R7 fate determination (Chang et al., 1995). The Svp is an orphan nuclear receptor responsible for transforming cone cells to R7 photoreceptor cells in conjunction with activated Ras (Begemann et al., 1995; Kramer et al., 1995). Rau is known to sustain RTK signaling downstream of Htl or Egfr via two Ras association (RA) domains, which have a binding preference for GTP-loaded Ras to maintain its activity via a positive feedback loop (Sieglitz et al., 2013). Svp expression is also known to increase Rau expression (Sieglitz et al., 2013). The Socs36E too can amplify Ras/MAPK signaling in precursors of R7 cells (Almudi et al., 2010). Thus, up-regulation of these genes in eye discs with decreased hsrω RNA levels in activated Ras expression background correlates with the observed greater increase in R7 photoreceptors than when activated Ras is expressed alone. While none of these positive modulators showed enhanced activity in *sev-GAL4>Ras^{V12}UAS-EP3037* eye discs, transcripts of another set of genes (*bru, klu, mesr4, cdep, epac, nfat, ptp-er, cg43102, rhogap1a, rhogef2, spri, exn* etc), which can act as negative modulators of the Ras cascade, were actually down regulated. On the basis of their GO term classification (David Bioinformatics), some of these genes, viz., *bru, cdep, sos, pdz-gef, cg43102, rhogap1a, rhogef2, spri, exn*, are involved in Ras guanyl nucleotide exchange factor activity (Lee et al., 2002; Jékely et al., 2005; Yan and Perrimon, 2015)) while others like *klu, mesr4, nfat, ptp-er* are negative regulators of small GTPase mediated signal transduction (Huang and Rubin, 2000; Brachmann and Cagan, 2003; Ashton-Beaucage et al., 2014). In either cases, therefore, the net result would be up-regulation of Ras activity as actually seen in *sev-GAL4>UAS-hsrωRNAiUAS-Ras^{V12}and sev-GAL4>UAS-EP3037 UAS-Ras^{V12}* eye discs. The action of positive regulators can be expected to be stronger than that of the negative modulators and this may be one of the factors for the greater enhancement in Ras signaling in *sev-GAL4> Ras^{V12}UAS-hsrωRNAi* than in *sev-GAL4> Ras^{V12}UAS-EP3037* eye discs.

The >10kb long nuclear lncRNAs, whose levels are primarily altered by the GAL4 driven expression of *hsrωRNAi* transgene or the *EP3037* allele (Mallik and Lakhotia, 2009, 2011), are essential for biogenesis of the omega speckles and consequently for the dynamic movement of the omega speckle associated hnRNPs, other RNA binding proteins and some of the nuclear

matrix proteins between different sub-cellular compartments (Mallik and Lakhotia, 2011; Lakhotia, et al 2012; Singh and Lakhotia, 2015). Any disturbance in the intra-cellular dynamicity of these proteins would affect their functions, which in turn would impinge upon a large variety of gene activity at transcriptional and post-transcriptional levels because of the central roles of different hnRNPS in prost-transcriptional processing of diverse RNAs (Han et al., 2010; Piccolo et al., 2014). Present results suggest that besides acting through omega speckle associated RNA-binding proteins, the hsrωRNAs may also directly or indirectly affect different small ncRNAs since certain snoRNAs, snRNAs and scaRNAs, which are highly up regulated in activated Ras background but were found to be significantly down-regulated when the hsrω transcripts were either up- or down-regulated. Such alterations in levels of the small ncRNAs may also contribute to the enhanced Ras signaling in these genotypes. Although the most widely appreciated function of snoRNAs is their roles in maturation of rRNAs, through extensive 2'-O-methylation and pseudouridylation modifications, guided, during or after their transcription (Henras et al., 2015; Sloan et al., 2017), especially by C/D box and H/ACA box snoRNAs, respectively (Dragon et al., 2006; Dieci et al., 2009), they have other regulatory roles as well, including modifications of some snRNAs (Falaleeva and Stamm, 2013; Dupuis-Sandoval et al., 2015; McMahon et al., 2015).The Cajal body associated scaRNAs are essential for proper functioning and maturation of snRNAs, which in turn are critical for appropriate processing of mRNAs (Darzacq et al., 2002; Kiss et al., 2002; Richard et al., 2003; Kiss, 2004; Deryusheva and Gall, 2009). The cumulative consequences of alterations in these diverse small RNAs (sca, sn and snoRNAs) on different gene's transcriptional and translational activities can be very extensive. It would be interesting to examine the possibility that some of these small ncRNAs are part of an auto-inhibitory loop in Ras signaling such that their down regulation results in further enhanced Ras activation. Loss of two snoRNAs, SNORD50A and SNORD50B, in human cells has been shown to be associated with increased levels of active K-Ras leading to hyperactivated Ras/ERK signaling (Siprashvili et al., 2016). Homologs of these two snoRNAs are not yet identified in flies.

Several ribosomal protein genes show reduced expression following activation of the Ras pathway (Friedman et al., 2011). It remains to be examined if the observed reduction in some of the ribosomal proteins gene transcripts in *sev-GAL4> Ras^{V12}UAS-hsrωRNAi* eye discs has any effect on the very high Ras activity in these eye discs. Likewise, further studies are needed to identify roles, if any, of altered expression of genes like *CG13900*, *dlc90F* and *kuz* in further elevating the Ras signaling in the experimental genotypes.

An interesting finding of the present study was the marked increase in cell non-autonomous Ras signaling. The *sev-GAL4* driven activated Ras as well as RafRBDFLAG transgenes were expressed only in a specific subset of cells in the eye discs. Therefore, the presence of RafRBDFLAG bound Ras in adjacent non *sev-GAL4* expressing cells is unexpected and suggests that the activated Ras-Raf complex can move out of the source cells to neighbouring cells. The great reduction in Yan expression even in MF region, where the *sev-GAL4* driver is not at all expressed, when activated Ras expression is accompanied by altered hsrω transcript levels also

provides strong evidence for existence of cell non-autonomous Ras signaling. Although several studies (Uhlirova et al., 2005; Yan et al., 2009; Parry and Sundaram, 2014; Takino et al., 2014; Enomoto et al., 2015) have indicated cell non autonomous Ras signaling, only one study reported transfer of GFP tagged H-Ras to T cells from the antigen-presenting cells (Goldstein et al., 2014). Our results also strongly indicate movement of the activated Ras complex across to neighbouring cells.

Our findings, besides highlighting roles of non coding part of the genome in modulating important signaling pathway like Ras, also unravel new insights into the working of Ras signaling cascade itself. The observed non-autonomous spread of Ras signaling and increase in Ras activity by hsrω lncRNAs are also significant in view of the fact that activated Ras/Raf mutations are implicated in diverse malignancies. Although some earlier studies indicated roles of certain lncRNAs in ERK/MAPK activation in cancer (Xu et al., 2014; Kotake et al., 2016; Zhang et al., 2017), their roles in enhancing Ras activity itself has not been known. Future studies on interactions between the diverse small nc and lncRNAs and signaling pathways like Ras are expected to unravel new dimensions of cellular networks that regulate and determine the basic biological processes of cell proliferation, differentiation and death on one hand, and roles in cancer on the other.

Materials and Methods

Fly stocks

All fly stocks and crosses were maintained on standard agar-maize powder-yeast and sugar food at 24±1^{0}C. The *w^{1118}; sev-GAL4; +* (no. 5793; Bailey 1999), *w^{1118}; UAS-GFP* (no. 1521), *w^{1118}; UAS-Ras^{V12}* (no. 4847) stocks were obtained from the Bloomington Stock Centre (USA):. For targeted (Brand and Perrimon, 1993) down-regulation of the hsrω transcripts, *UAS-hsrω-RNAi^3* transgenic line was used, while its up regulation was achieved through the GAL4 inducible *EP3037* over expressing *hsrω* allele (Mallik and Lakhotia, 2009). These two lines are referred to in the text as *UAS-hsrωRNAi* and *EP3037*, respectively. For hsrω-null condition, the *w: hsrω^{66}/hsrω^{66}* stock (Johnson et al., 2011) was used. 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 finally obtain progenies of the following genotypes:

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- Ras^{V12}

c) w^{1118}; sev-GAL4 UAS-GFP/UAS-GFP; UAS-hsrωRNAi/UAS-Ras^{V12}

d) *w^{1118}; sev-GAL4 UAS-GFP/UAS-GFP; EP3037/UAS-Ras^{V12}*

e) *w^{1118}; sev-GAL4 UAS-GFP/UAS-GFP; hsrω^{66}/hsrω^{66} UAS-Ras^{V12}*

f) w^{1118}; sev-GAL4 *UAS-GFP/UAS-RafRBDFLAG; dco*^{2} *e*/+

g) w^{1118}; *sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; dco*^{2} *e/UAS-Ras*^{V12}

h) w^{1118}; *sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; UAS-hsrωRNAi/UAS-Ras*^{V12}

i) w^{1118}; *sev-GAL4 UAS-GFP/UAS-RafRBDFLAG; EP3037/UAS-Ras*^{V12}

The w^{1118}, *dco*^{2} and *e* markers are not mentioned while writing genotypes in Results.

Lethality Assay

For lethality assay, freshly hatched 1st instar larvae of *sev-GAL4>UAS-GFP*, *sev-GAL4>Ras*^{V12}, *sev-GAL4>UAS-Ras*^{V12}*UAS-hsrωRNAi* and *sev-GAL4>UAS-Ras*^{V12}*EP3037* were collected during one hour interval and gently transferred to food vials containing regular food and reared at 24±1°C or at 18±1°C. The total numbers of larvae that pupated and subsequently emerged as flies were counted for at least three replicates of each experimental condition and/or genotypes.

Photomicrography of adult eyes

For examining the external morphology of adult eyes, flies of the desired genotypes were etherized and their eyes photographed using a Sony Digital Camera (DSC-75) attached to a Zeiss Stemi SV6 stereobinocular microscope or using Nikon Digital Sight DS-Fi2 camera mounted on Nikon SMZ800N stereobinocular microscope.

Nail polish imprints

Flies to be examined for organization of ommatidial arrays were anaesthetized and decapitated with needle and the decapitated head was briefly dipped in a drop of transparent nail polish placed on a slide. It was allowed to dry at RT for 5-10 min. The dried layer of nail polish was carefully separated from the eye tissue with fine dissecting needles and carefully placed on another clean glass slide with the imprint side facing up, and finally flattened by gently placing a cover slip over it as described earlier (Arya and Lakhotia, 2006). The eye imprints were examined using 20X DIC optics.

Whole organ immunostaining

Eye discs from actively migrating late third instar larvae of desired genotypes 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: rat monoclonal anti-Elav (DSHB, 7E8A10, 1:100), rabbit monoclonal anti-Ras (27H5, Cell signaling, 1:50), mouse anti-FLAG M2 (Sigma-Aldrich, India, 1:50), rabbit p-MAPK (Phospho-p44/42 MAPK (Thr202, Tyr204), D13.14.4E, Cell signaling, 1:200), mouse anti-Yan (8B12H9, Developmental Studies Hybridoma Bank, Iowa, 1:100) and guinea pig anti-Runt, a gift by Dr. K. Vijaya Raghavan, India, (Kosman et al., 1998) at 1:200 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 antibodies. 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. When required, quantitative estimates of proteins in different regions of eye discs and their co-localization were obtained with the Histo option of Zeiss LSM Meta 510 software. All images were assembled using Adobe Photoshop 7.0 software.

Next Generation RNA sequencing

Total RNAs were isolated from 30 pairs of eye discs from *sev-GAL4>UAS-GFP*, *sev-GAL4>UAS-hsrωRNAi*, *sev-GAL4>EP3037*, *sev-GAL4>UAS-Ras^{V12}*, *sev-GAL4>UAS-hsrωRNAi UAS-Ras^{V12}* and *sev-GAL4>EP3037 UAS-Ras^{V12}* third instar larvae using Trizol (Invitrogen, USA) reagent as per manufacture's protocol. 1µg of the isolated RNA was subjected to DNAase treatment using 2U of TurboTM DNase (Ambion, Applied Biosystem) enzyme for 30 min at 37°C. The reaction was stopped using 15mM EDTA followed by incubation at 65°C for 5-7 min and purification on RNAeasy column (Qiagen). The purified RNA samples were processed for preparations of cDNA libraries using the TruSeq Stranded Total RNA Ribo-Zero H/M/R (Illumina) and sequenced on HiSeq-2500 platform (Illumina) using 50bp pair-end reads, 12 samples per lane and each sample run across 2 lanes. This resulted in a sequencing depth of ~20 million reads in each case. Triplicate biological samples were sequenced in each case. The resulting sequencing FastQ files were mapped to the *Drosophila* genome (dm6) using Tophat with Bowtie. The aligned SAM/BAM file for each was processed for guided transcript assembly using Cufflink 2.1.1 and gene counts were determined. Mapped reads were assembled using Cufflinks. Transcripts from all samples were subjected to Cuffmerge to get final transcriptome assembly across samples. In order to ascertain differential expression of gene transcripts between different samples, Cuffdiff 2.1.1 was used (Trapnell et al., 2012). A P-value <0.05 was taken to indicate significantly differentially expressing genes between the compared genotypes. Genes differentially expressed between experimental and control genotypes were categorized into various GO terms using DAVID bioinformatics Resources 6.8 (Huang et al., 2009) https://david.ncifcrf.gov for gene ontology search. In order to find out distribution of differentially expressing genes into various groups, Venn diagrams and Heat maps were prepared using the Venny2.1 and ClustVis softwares, respectively (Metsalu and Vilo, 2015).

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

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