Identification of Genes Involved in the Differentiation of R7y and R7p Photoreceptor Cells in Drosophila

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ABSTRACT The R7 and R8 photoreceptor cells of the Drosophila compound eye mediate color vision. Throughout the majority of the eye, these cells occur in two principal types of ommatidia. Approximately 35% of ommatidia are of the pale type and express Rh3 in R7 cells and Rh5 in R8 cells. The remaining 65% are of the yellow type and express Rh4 in R7 cells and Rh6 in R8 cells. The specification of an R8 cell in a pale or yellow ommatidium depends on the fate of the adjacent R7 cell. However, pale and yellow R7 cells are specified by a stochastic process that requires the genes spineless, tango and klumpfuss. To identify additional genes involved in this process we performed genetic screens using a collection of 480 P{EP} transposon insertion strains. We identified genes in gain of function and loss of function screens that significantly altered the percentage of Rh3 expressing R7 cells (Rh3%) from wild-type. 36 strains resulted in altered Rh3% in the gain of function screen where the P{EP} insertion strains were crossed to a sevEP-GAL4 driver line. 53 strains resulted in altered Rh3% in the heterozygous loss of function screen. 4 strains showed effects that differed between the two screens, suggesting that the effect found in the gain of function screen was either larger than, or potentially masked by, the P{EP} insertion alone. Analyses of homozygotes validated many of the candidates identified. These results suggest that R7 cell fate specification is sensitive to perturbations in mRNA transcription, splicing and localization, growth inhibition, post-translational protein modification, cleavage and secretion, hedgehog signaling, ubiquitin protease activity, GTPase activation, actin and cytoskeletal regulation, and Ser/Thr kinase activity, among other diverse signaling and cell biological processes.

KEYWORDS Photoreceptor Cell fate Gene inactivation Ectopic expression Mutant screen

Color vision in most organisms is dependent upon the expression of spectrally distinct visual pigments (opsins) in different photoreceptor cells (Jacobs 1981; Nathans et al. 1986; Wikler and Rakic 1990). The organization of the retinal mosaic reflects a variety of developmental mechanisms, including regional specialization, stochastic, and precise cell-cell adjacency (Vieira et al. 2016). Drosophila melanogaster is capable of color vision and is a useful experimental system for examining the developmental programs that produce photoreceptor cells having different color sensitivities (Quinn et al. 1974; Spatz et al. 1974; Chou et al. 1996; Chou et al. 1999; Tang and Guo 2001; Cook et al. 2003; Wernet et al. 2003; Mikaeladze-Dvali et al. 2005). The compound eye consists of ~800 ommatidia, which each contain a pair...
of R7 and R8 photoreceptors cells that mediate polarization sensitivity and color vision (Fortini and Rubin 1991; Yamaguchi et al. 2010).

Two main ommatidial subtypes were identified based on pale or yellow fluorescence when illuminated with blue light (Kirschfeld et al. 1978; Franceschini et al. 1981), and contain R7pale/R8pale (R7p/R8p) or R7yellow/R8yellow (R7y/R8y) cell pairs expressing Rh3/Rh5 or Rh4/Rh6, respectively (Chou et al. 1996; Papatsenko et al. 1997; Chou et al. 1999). The R7y and R7p photoreceptor cells are distributed randomly (Bell et al. 2007) and thought to be generated stochastically through the action of spineless (ss), tango (to) and khumpfas (khuf) (Wernet et al. 2006; Thanawala et al. 2013; Johnston and Desplan 2014; Anderson et al. 2017). The premise behind this stochastic cell-fate mechanism is that variation in the expression of ss in individual R7 cells in the developing pupal eye leads to the formation of R7y and R7p cells. This is consistent with the idea that regulators of this process may be rate-limiting or define dosage-sensitive steps in the formation of R7y cells. Cell-to-cell variation in gene expression may arise as the result of promoter architecture (Jones et al. 2014), transcriptional or translational bursting (Macdams and Arkin 1997; Singh and Soltani 2013), cell-cell interactions (Axelrod 2010), epigenetic differences between cells (Kar et al. 2017) and other processes. To identify additional regulators of R7 photoreceptor cell differentiation, we conducted Gain of Function (GOF) and Loss of Function (LOF) genetic screens to identify genes that govern this process. Here we show that mutations in many additional genes regulate the proportion of R7y and R7p cells.

METHODS & MATERIALS

Drosophila stocks and genetics

All stocks were maintained in humidified incubators on standard cornmeal/molasses/agar media at 25°C. Stocks used in the experiments described here were obtained from the Bloomington Drosophila Stock Center and included w^{1118} (FBst0003605) and w^{118}, P[w+mW.hs = sevEP-GALA4.Bi]/7 (FBst0005793) (w; sevEP-GALA4). The sevEP-GALA4 strain was selected because it is expressed at a higher level and is more cell type specific than P[GAL4-Hsp70.sev] (FBpt0000379), which is driven by the Hsp70 promoter and is thought to have sevenless independent activity (Bailey 1999; Therrien et al. 1999). The 480 P[EP] (FBpt001317) (Rorth 1996) transposon insertion strains used in the screen are listed in Supplemental Table S1. Additional stocks used in Table 2 include w^{ss}; P[act>myc] /TM3, P[act>myc = HB-lacZ]/GS2, SB (FBst0078357) and w^{+}; P[w+mC = UAS-ss.A5]A1 / SM6a (FBst0078354).

Screen Design

The goal of this project was to identify genes that alter the percentage of Rh3 expressing R7 cells (Rh3%). This was undertaken in two screens. First, a gain of function (GOF) screen was performed by crossing each P[EP] strain to w; sevEP-GALA4 flies. Second, a heterozygous loss of function (LOF) screen was performed by crossing each P[EP] strain to w^{1118} flies. For each cross 8-10 female F1 progeny were used to determine the number of R7 photoreceptor cells expressing Rh3 and Rh4 (Rh3%). In an additional analysis, we calculated the difference (Diff) between the two screens (Diff = GOF – LOF) for each P[EP] insertion strain. This was designed to identify effects found in the GOF screen that are larger than, or potentially masked by, the effect from the P[EP] insertion alone, as observed in the heterozygous LOF screen.

Description of phenotypes scored

Immunofluorescence of R7 photoreceptor cells was performed on dissociated ommatidia, prepared as described (Hardie et al. 1991; Ranganathan et al. 1991). Rh3 and Rh4 were detected by immunohistochemistry using mouse monoclonal antibodies as previously described (Chou et al. 1996; Chou et al. 1999). Antibodies were diluted in antibody dilution buffer (3% normal goat serum, 0.03% triton X-100, and 0.1% bovine serum albumin in PBS). Dilutions used for indirect immunofluorescence were as follows: 1:20 anti-Rh3 mouse monoclonal (clone 2B1, IgG1); 1:10 anti-Rh4 mouse monoclonal (clone 11E6, IgG1). For direct immunofluorescence, directly conjugated anti-Rh3-Alexa Fluor 488 was used at 1:50 (Earl and Britt 2006). Secondary antibodies and other immunological reagents were obtained from Jackson ImmunoResearch Laboratories, Inc. and Molecular Probes. Images were collected using an Axioskop2 Plus / AxioCamHRC microscope (Carl Zeiss, Inc.; Thornwood, NY).

Statistical analyses

Dissociated ommatidia were examined by fluorescence microscopy and the number of R7 cells expressing Rh3 or Rh4 were counted in each sample. To account for varying number of R7 cells examined for each cross, logistic regression with Rh3 expression (yes/no) as the outcome at the individual R7 cell-level was used to examine the association between a specific mutant in the GOF screen and the probability of a cell expressing Rh3. An odds ratio was constructed to compare the odds of a cell expressing Rh3 in a specific mutant to the odds of a cell expressing Rh3 in all other mutants from the same chromosome. We adjusted for multiple testing by applying a False Discovery Rate (FDR) correction across all comparisons (all mutant strains from all chromosomes) (Benjamini and Hochberg 1995). An FDR threshold of 0.10 was used to identify mutant strains associated with the differences in the probability of an R7 cell expressing Rh3. The same model was used to determine if the probability of Rh3 expression for a specific mutant strain is significantly altered in the LOF screen. Effect size is reported as a standardized percent Rh3, which is the difference between the mutant strain specific percent Rh3 and the median percent Rh3 for all mutant strains on that chromosome. Cells with inconclusive calls were not considered in the analysis.

We also examined whether the difference in the probability of Rh3 expression between the GOF screen and the LOF screen for a specific mutant strain was significantly lower/higher than other mutant strains on the same chromosome. A logistic regression model for each mutant strain was estimated using an indicator for the specific mutant strain being tested (yes/no), type of mutation (GOF or LOF) and the interaction effect between these two factors. The interaction effect from this model was used to determine if the effect of a particular P[EP] allele differed between the two screens (GOF or LOF). An FDR correction was used for multiple testing and a threshold of 0.10 was used to identify candidate genes.

Statistical analysis of specific genotypes

Comparisons of the proportions (percentages) of opsin expression in different genetic backgrounds were performed with a z-score and are shown in Table 2 (Fleiss et al. 2003). The z-score was calculated using the equation:

\[
    z = \frac{p_2 - p_1}{\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \left(1 + \frac{1}{n_1} + \frac{1}{n_2}\right)^{\frac{1}{2}}
\]

where \(p_1\) and \(p_2\) are proportions of marker expression in each of the two different genotypes under comparison. \(n_1\) and \(n_2\) are number of
ommatidia counted for each genotype. \( p_{avg} = \) average proportion for both genotypes combined. \( q_{avg} = 1-p_{avg} \) The significance of the difference between the two proportions was determined from the normal distribution as a two-tailed test.

**Network analysis**

esyn (version 2.0) (Bean et al. 2014) was used to construct networks involved in R7 cell fate by extracting known genetic and physical interactions among candidate genes from the mutant screens and \( s_s \), \( t_g o \), \( s_s \) and \( k_l u \), genes known to be involved in R7 cell fate specification (based on FlyBase, version FB2018.04). Networks were restricted to include only connections that involved at least one candidate gene.

**Validating candidate genes**

To validate selected candidates, we tested \( P\{EP\} \) strains as homozygotes as described above. A standardized \( R_h 3\% \) was calculated for the Homozygous Loss of Function (HomLOF) data and compared to the median \( R_h 3\% \) for each chromosome from the Heterozygous Loss of Function (HetLOF) screen.

**Data availability**

The data described in this manuscript is available as Supplemental Tables S1, S2 and S3 available at figshare: https://doi.org/10.25387/g3.12994292

**RESULTS**

**Screen design**

The genetic screens described here were developed to identify new genes involved in the cell fate decision that regulates \( R_h 3 \) vs. \( R_h 4 \) expression in R7p and R7y photoreceptors cells, respectively. Three genes, \( s_s \), \( t_g o \) and \( k_l u \) are required for this process (Wernet et al. 2006; Thanawala et al. 2013; Johnston and Desplan 2014; Anderson et al. 2017) and we have shown that the cell fate decision between R7p and R7y also depends upon the Epidermal growth factor receptor (Egfr) (Birkholz et al. 2009a). We used the \( P\{EP\} \) transposable element (Rorth 1996), taking advantage of its utility 1) as a modular GAL4 system for over- or mis-expression in a Gain of Function (GOF) Screen (Figure 1 and 2) as a single P-element mutagenesis experiment performed as a heterozygous Loss of Function (LOF) Screen. The rationale for these screens is that the stochastic expression of \( s_s \) and the specifcation of R7y or R7p photoreceptor cells may be dependent upon the precise expression level of a regulator that is rate-limiting for this process. Such a regulator could potentially be identified by its effect on R7 photoreceptor cell differentiation when the concentration, expression or gene dosage of the regulator is altered within the system.

Our approach using both a traditional GOF and a heterozygous LOF screen is based on the observation that \( s_s \) is required for expression of \( R_h 4 \) in R7 cells. Homozygous \( s_s \) mutant patches show a dramatic decrease in \( R_h 4 \) expressing R7 cells and a corresponding increase in \( R_h 3 \) expressing R7 cells (Wernet et al. 2006). In addition, overexpression of \( s_s \) leads to expression of \( R_h 4 \) in most or all R7 cells (Wernet et al. 2006; Johnston and Desplan 2014). Finally, \( s_s \) mutants show a dosage effect, in that loss of a single copy of \( s_s \) leads to an increase in \( R_h 3 \) expressing R7 cells from 35 to 44%, an increase of 9% from wild-type controls (Johnston and Desplan 2014). These findings suggest that genetic screens designed to increase or decrease expression of rate limiting regulators of \( R_h 3 \) and \( R_h 4 \) expression may identify additional genes involved in this process.

**Screen results**

In the GOF screen, each \( P\{EP\} \) target line was crossed to the \( w; s_e v-E P-G A L 4 \) driver line (Figure 1). In this strain, GAL4 is expressed in the pattern of \( s_e v e s s e n l e s s \) protein and is produced dynamically in the 3rd instar larval eye-antenna imaginal disc, posterior to the morphogenetic furrow in R7 photoreceptors and other cells (Tomlinson et al. 1987; St Pierre et al. 2002; Ray and Lakhota 2015). The Target lines are transgenic strains that carry independent insertions of the \( P\{EP\} \) transposon at different locations in the fly genome. The \( P\{EP\} \) transposon contains a series of yeast Upstream Activating Sequence sites (UAS sites, vertical bars in a block) that are the target of GAL4 binding. Upon mating of a Driver line carrying \( s_e v-E P-G A L 4 \) to a single Target line carrying a unique \( P\{EP\} \) insertion, the offspring will express GAL4 in R7 photoreceptors. GAL4 will bind to and activate transcription from UAS sites of the adjacent endogenous gene or genomic region. In this manner, an endogenous gene may be expressed under the control of the \( s_e v e s s e n l e s s \) enhancer. Figure modified from (Rorth et al. 1998). Drosophila illustration modified from (Commons 2017).

In the heterozygous LOF screen, each \( P\{EP\} \) strain was crossed to the \( w^{118} \). The median of \( R_h 3\% \) for each chromosome of the LOF screen are 49.5%, 44.0% and 45.3% for chromosomes X, 2 and 3 respectively, Figure 2A. In the heterozygous LOF screen, each \( P\{EP\} \) strain was crossed to \( w^{118} \). The median of \( R_h 3\% \) for each chromosome of the LOF screen are 44.0%, 38.0% and 39.1% for chromosomes X, 2 and 3 respectively, Figure 2B.

We also examined whether the difference in \( R_h 3\% \) between the GOF screen and the LOF screen for a specific mutant strain was significantly lower/higher than other mutant strains on the same chromosome. This was designed to identify effects found in the GOF screen that are larger than, or potentially masked by, the effect of the \( P\{EP\} \) insertion alone, as observed in the heterozygous LOF
We calculated the difference (Diff) between the two screens (Diff = GOF - LOF) for each P\(^{EP}\) insertion strain. The median of Rh3\(^3\) Diff for each chromosome are 5.0\%, 5.9\% and 5.9\% for chromosomes X, 2 and 3 respectively, Figure 2C. The medians for Rh3\(^3\) for different chromosomes in the GOF and LOF screens, and the Diff comparison, likely vary because of the genetics and varying sources of the chromosomes used to generate the hopped P\(^{EP}\) strains. The median Rh3\(^3\) of the Diff comparison is non-zero and we believe that this results from an effect of the strains carrying a copy of only the P\(^{EP}\) transposon in the LOF screen vs. carrying a copy of both the P\(^{EP}\) and sevEP-GAL4 transposons in the GOF screen. Performing a non-parametric Kruskal-Wallis test for differences between group medians, there are significant differences across chromosomes in the median percentage of cells expressing Rh3\(^3\) in the GOF and LOF screens and Diff comparison (p-values 6.3 \times 10^{-41}, 1.9 \times 10^{-32} and 6.7 \times 10^{-4}, respectively).

Based on the genomic site of the P\(^{EP}\) insertion, we identified 36 candidate genes in the GOF screen (Figure 2A; Supplemental Table S2), 53 in the LOF screen (Figure 2B; Supplemental Table S2) and 4 from the Diff comparison (Figure 2C; Supplemental Table S2) having an FDR < 0.10. Some genes appeared in multiple screens resulting in 69 unique candidate genes in total (Figure 2D). Of the 36 candidate genes from the GOF screen, 21 (58.3\%) exhibited an increase in Rh3\(^3\)\% compared to other GOF mutants from the same chromosome. Likewise, of the 53 candidate genes from the LOF screen, 32 (60.4\%) showed an increase in Rh3\(^3\)\% compared to other LOF mutants on the same chromosome. Of the 4 mutants identified in the Diff comparison, 3 (75\%) indicated a difference in Rh3\(^3\) levels between GOF and LOF screens that were greater than the Diff median for that chromosome.

**Top candidates**

For a more qualitative examination, we prioritized 10 of the 69 candidate genes (Table 1). These 10 include the 4 candidate genes from the Diff comparison and the top 3 candidates based on p-value from the GOF and LOF screens. In 5 of the 10 candidate genes, the LOF
mutant did not result in a significant change in Rh3%, but the GOF screen did. In 4 of these 5, (IGF-II mRNA-binding protein (Imp), grauzone (grau), Tyrosylprotein sulfotransferase (Tps), and Furin 2 (Fur2)), Rh3% increased with GOF. GOF of the other gene, Small ribonucleoprotein particle protein Smd2 (Smd2), led to a decrease in Rh3%. In 5 of the remaining mutant strains, two (Tao and Phospholipase A2 group III (Gillsplz2)) had a significant decrease in Rh3% in the LOF mutants, but the magnitude of the decrease did not change significantly with GOF. Both Actin-related protein 3 (Arp3) and Rho GTase activating protein at 18B (RhoGAP18B) had a significant increase in Rh3% in their respective LOF mutants, but for both mutations, that increase was dampened with GOF. For both genes, the GOF screen and the Diff comparison were suggestive (unadjusted p-values between 0.03 and 0.11) of a difference, but none of the four comparisons reached statistical significance after multiple testing correction. For the final gene in Table 1, G protein β-subunit 13F (Gβ13f), LOF causes a slight up-regulation in Rh3%, while GOF caused a slight down-regulation in Rh3%. Both comparisons were nominally significant but failed to pass multiple testing correction, but the Diff comparison was statistically significant.

Comparison of GOF and LOF screen results with analyses of spineless

As an internal reference for the GOF screen, we expressed ss under the control of the sevEP-GAL4 driver line (Strain # 4, Table 2). Previous studies demonstrated that overexpression of ss under the control of the P{longGMR-GAL4} or the P{GAL4-ninaE.GMR} driver lines leads to expression of Rh4 in almost all R7 and many R1-6 photoreceptor cells (Wernet et al. 2006). By contrast, our experiments with the more restricted sevEP-GAL4 driver line showed no statistically significant change in Rh3 and Rh4 expression in R7 cells compared to the P{VAS-ss} responder line alone (Strain # 3, Table 2). This discrepancy likely reflects a difference in the location and/or timing of G4M4 expression in the driver lines. Despite the failure of sevEP-GAL4 driven ss expression to alter Rh3% in the screening paradigm, 36 statistically significant candidates were identified in the GOF screen.

As mentioned in the introduction, the rationale for the heterozygous LOF screen is the prediction that loss of a single copy of a rate limiting or dosage sensitive regulator of R7 cell differentiation would alter Rh3%. Loss of a single copy of ss has been shown to increase the proportion of R7 cells that express Rh3 from 35 to 44% (9% change) (Johnston and Desplan 2014). This effect, observed in Df(3R) Exel7330 heterozygotes, is comparable to the maximum effect we observed for heterozygous P[EP] insertions in the LOF screen (Table 1, Figure 2B). However, our comparison of heterozygous ssD115.7 (Strain # 6) animals to w1118 (Strain # 5) controls showed a 5.6% increase in Rh3%, that is not statistically significant (Table 2). The discrepancy between the two results may be due to the use of differing alleles or genetic backgrounds. Df(3R)Exel7330 contains a 125Kb deletion removing approximately 20 genes (FBar00383138), whereas ssD115.7 is a 10Kb intragenic deletion within ss (Duncan et al. 1998). Furthermore, the previous study did not include specific experimental values sufficient for statistical comparison with relevant controls, or with our results. Thus, the 53 statistically significant candidates identified in the heterozygous LOF screen exceed expectations.

Potential interactions between candidates and known regulators

As a first step in defining the relationship between the 69 candidate genes identified in the screen and the well characterized mediators of R7 photoreceptor cell differentiation and the regulation of Rh3 vs. Rh4 expression, ss, tgo and klu, we examined the known genetic and physical interactions between these genes. Of the 69 candidate genes, 66 were included in the esyN database. 38 candidate genes have known interactions with another candidate(s) or an interaction with another gene 1 link away from another candidate(s).

This analysis identified 85 additional genes that interact physically or genetically with the candidates identified in the screen and ss, tgo or klu. These additional genes have known interaction with two or more candidate genes (Figure 3). Most candidate genes are in a single large network where the genes expanded (ex) and pebbled (peb) were the most highly connected (i.e., hub genes). This large network contains the known regulators of R7 photoreceptor cell specification ss, tgo and klu. Although ss, tgo and klu do not have known direct genetic or protein interactions with any of the candidate genes identified in the screen, there are numerous secondary interactions between candidates throughout the network, including peb, imp, inflamed (if), slit (slit), TNF-receptor-associated factor 4 (Traf4) and others. A much smaller network consists of candidate genes CG15514 and jim l fellows

Table 1 Selected Mutants with Significant Differences in R7 cells expressing Rh3

| Allele    | Chr | Screen with FDR < 0.10 | Gain of Function Rh3% (GOF) | Loss of Function Rh3% (LOF) | Difference (Diff) | EP Orientation | EP Position |
|-----------|-----|------------------------|-----------------------------|----------------------------|-------------------|----------------|------------|
| SmD2EP3399 | 3   | GOF & Diff             | −5.29% (2.25E-05)           | 0.94%                       | −5.89%            | forward        | 5' end     |
| TpseEP1218 | X   | GOF & Diff             | 7.80% (8.76E-11)            | 2.10%                       | 6.11%             | forward        | 5' end     |
| Fur2EP1493 | X   | GOF & Diff             | 7.06% (3.96E-07)            | −1.39%                      | 8.45%             | reverse        | 5' end     |
| G13EP1071  | X   | Diff                   | 2.54%                       | −2.31%                      | 6.27%             | reverse        | 5' end     |
| grauEP688  | 2   | GOF                    | 6.94% (2.14E-06)            | 2.94%                       | 4.18%             | forward        | 5' end     |
| impEP740   | X   | GOF                    | 8.10% (1.42E-07)            | 2.59%                       | 5.59%             | forward        | 1st intron |
| Arp3EP3640 | 3   | LOF                    | 2.87%                       | 7.03% (1.82E-05)            | −3.82%            | forward        | 5' end     |
| RhoGAP18BEP1326 | X | LOF          | 3.11%                       | 6.93% (1.35E-05)            | −3.40%            | reverse        | mid or 1st intron |
| TaoEP455   | X   | GOF & LOF              | −8.20% (2.17E-09)           | −6.08%                      | −1.70%            | forward        | 5' end     |
| Gillsplz2EP1516 | X | GOF & LOF            | −8.54%                      | −7.63% (1.35E-08)           | −0.94%            | reverse        | 5' end     |

The Table shows the top candidate alleles identified in the study, the Chromosome (Chr) containing the P[EP] insertion and the screens for these alleles showing significant effects. Abbreviations: False Discovery Rate (FDR), Standardized Percent of Rh3 expressing R7 cells (Rh3%), Gain of Function (GOF), heterozygous Loss of Function (LOF), Difference between primary screens (Diff = GOF - LOF). The table includes the top 3 candidates from the GOF and LOF screens, and 4 candidates showing a significant Diff comparison. EP Orientation refers to the direction of transcription from the P[EP] insertion relative to the indicated gene. EP Position refers to the position of the P[EP] insertion relative to the transcript of the indicated gene. The Standardized Rh3% refers to the difference between the mutant specific Rh3% and the median Rh3% across all mutants on the same chromosome for that screen. Shaded cells indicate measurements that meet an FDR < 0.10 and the p-value of the highest statistical significance for that mutant strain is also shown.
of the genome and are biased with respect to the regions targeted of an endogenous gene. The GOF screen is predicated on the assumption that the P{EP} element is inserted in a region 5' of the transcription initiation site of the endogenous gene, as shown in Figure 1. Ideally, the cross of P{EP} and P[sevEP-GALA] strains would produce offspring expressing the full-length protein of the encoded gene in the pattern of P[sevEP-GALA]. This is not necessarily the case because of the orientation and position of the P{EP} insertion. As indicated in Table 1, several of the P{EP} lines carry insertions that are in the opposite orientation of the transcribed gene (reverse EP orientation), Fur2EP1493, GB13EP1071, RhoGAP1EP1326 and GIIIisPla2EP1516. For these insertion lines and for similarly oriented strains within the screen, statistically significant GOF effects may not be due to expression of the indicated gene, and the lack of a significant effect for a particular P{EP} line is not a sufficient test of the indicated gene. Furthermore, the position of the P{EP} insertion in an intron or within the gene is also not a test of expression of the full-length gene product, as in the case of Imp57P. These caveats also apply to a portion of the 29 additional insertion lines showing a statistically significant change in Rh3% in the GOF screen that are indicated in Supplemental Table S2.

The P{EP} strain library was generated by P-element transposition, which has been used as a method of mutagenesis for many years (Rubin et al. 1982). Each P{EP} strain is associated with a specific genomic location, based on sequenced flanking regions, and annotated within Flybase as an allele of one or more genes. The heterozygous LOF screen is predicated on the assumption that insertion of the P{EP} element may cause loss of function of the annotated gene. This too is not necessarily the case and is also dependent on the specific insertion site. Associated caveats include: 1) many of the P{EP} lines are alleles of essential genes, but are homologous viable, suggesting that they have a partial loss of function (hypo-morphs), 2) the sequenced insertion sites may be in regions of the gene that do not affect function in any way, 3) the insertion site may reside between two genes and affect the function of both, one, or neither gene as in the case of GIIIisPla2EP1516, Traf6EP1516, 3) the observed phenotypes may be independent of the P{EP} insertion. This could occur because of additional mutations in the backgrounds of the strains that were used for P{EP} mobilization or that resulted from the mobilization event itself. In addition, male recombination (Hiraizumi 1971), or other mutations or chromosome aberrations could occur that are unlinked to the P{EP} insertion site (Hiraizumi et al. 1973; Simmons and Lim 1980). Also, the 411 insertion strains that lack a phenotype in the GOF, LOF screens or the Diff comparison do not serve to exclude those nearby genes from involvement in the regulation of Rh3 expression. Rather the function of the study was simply to identify those insertions strains that do show a phenotype. 

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**Table 2 R7 Photoreceptor Opsin Expression in Different Genetic Backgrounds**

| Strain # | Genotype Brief description | Rh3% (n) | Statistical Comparison |
|----------|----------------------------|----------|------------------------|
| 1        | \(w^{118}\) Control        | 35.5 (363)|                        |
| 2        | \(w^{118}; P^{w^{118}+}\) sevEP-GAL4.B7 / + (sevEP-GALA) driver line | 35.1 (427) | NSDF #1 |
| 3        | \(w^{118} / w; P^{w^{118}+}= UAS-ss.A5A1 / + (UAS-ss) responder line | 45.0 (329) | SDF #1 P = 0.01 |
| 4        | \(w^{118} / w; P^{w^{118}+}= UAS-ss.A5A1 / + (UAS-ss) responder line | 51.9 (391) | NSDF #3 |
| 5        | \(w^{118} / Y / (\text{al})\) Control | 30.6 (214) | NSDF #1 |
| 6        | \(w^{118} / Y; ss^{D1527} + / (\text{al})\) ss heterozygote | 36.2 (472) | NSDF #5 |

Statistical comparisons of strains were carried out as described in the Methods; Percent of Rh3 expressing R7 cells (Rh3%), n= the number of ommatidia counted. As indicated, the observed percentages were Not Significantly Different From (NSDF, P ≥ 0.05) or Significantly Different From (SDF) the strain indicated, at the p value shown by a two tailed test. Tested animals were female unless otherwise indicated.

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(Rh3%) connected by MOB kinase activator 4 (Mob4). Only a single link is shown between a pair of genes even if multiple types of connections have been identified (e.g., a suppressing genetic interaction and a physical interaction). The 85 additional connecting genes in the network were not evaluated in the mutant screen, except for transcriptional Adaptor 2b (Ada2b), and scalloped (sd) (insertions P{EP} EP3412 and P{EP}sdEP1088, respectively), which did not reach statistical significance. Eight candidate genes (ex, phe, fat facets (ff), Sin3A, Imp, sli, Tao and highwire (hiw)) are highly embedded within the network and have connections to 10 or more other genes. These interaction networks suggest numerous mechanisms for regulation of ss, tgo and klu by the genes identified in the screens. SupplemenTable S3 lists the genes in the network and the total number of connections for each gene.

**Candidate gene validation**

As an additional step in validating the effects of mutations on Rh3%, we examined a subset of insertion strains as homozygotes. The rationale for the heterozygous LOF (HetLOF) screen was based upon the assumption that reduction in the level of a rate-limiting regulator of R7 photoreceptor differentiation (even in heterozygotes) would demonstrate a change in Rh3%. If this was true, then our expectation is that animals homozygous for the insertion (Homozygous Loss of Function, HomLOF) would have a further reduction in that critical regulator and would demonstrate a larger change in Rh3%. Figure 4 shows that for the viable homozygotes tested, the change in Rh3% is more extreme than in either the LOF (HetLOF) or GOF screens in most cases, as shown for the dramatic increase of Rh3% in sliEP397 (sli) mutants compared to cn bw controls (Figure 4 and Figure 5).

**DISCUSSION**

The genetic screens described here were designed to identify transposon insertion strains that showed alterations in the stochastic differentiation of R7 photoreceptor cells expressing Rh3 or Rh4. Potential alterations were defined as significant Rh3% change in the GOF or LOF screens. The screens identified 69 unique candidate genes. Many of the genes identified in the screen have previously identified protein-protein and genetic interaction between themselves and the known regulators of R7 cell differentiation, klu, tgo and ss. Preliminary validation of candidates by examination of homoyzgous insertions (HomLOF) showed substantially increased effects on Rh3%.

Despite the intriguing results of the screens, a series of caveats related to the use of the P{EP} insertions lines should be noted. P-elements insert in a biased manner within genomic hot-spots, preferentially into gene promoters and replication origins (Spradling et al. 2011). Therefore, insertions do not represent a random sample
Despite these caveats, the current study contributes significantly to our understanding of the specification of R7 photoreceptor cells and the regulation of Rh3 and Rh4 expression. Many of the genes identified in the screen have previously identified protein-protein and genetic interaction between themselves and the known regulators of R7 cell differentiation, klu, tgo and ss. Preliminary validation of candidates by examination of homozygous insertions (HomLOF) showed substantially increased effects on Rh3%. The candidate genes identified encode proteins comprising a broad range of biological functions. Of the selected genes highlighted in Table 1, SmD2 encodes an RNA binding protein that is involved in pre-mRNA splicing (Mount and Salz 2000). Tfst shares homology with tyrosyl-protein sulfotransferases (Gaudet et al. 2011), localizes to the Golgi apparatus and is involved in protein secretion (Bard et al. 2006). Fur2 encodes a proprotein convertase that mediates ligand activation through protein cleavage (Kunnapuu et al. 2009). Gb13F encodes a G-protein β-subunit that mediates G-protein coupled receptor signaling and modulates hedgehog signaling (Li et al. 2018). Grau encodes a zinc-finger transcription factor (Chen et al. 2000). Imp encodes an mRNA binding protein that promotes and regulates transcript targeting, and plays a role in axonal remodeling, synaptogenesis and oogenesis (Boylan et al. 2008; Medioni et al. 2014). Arp3 encodes an actin related protein that is required for myoblast fusion and axonal arborization and synapse formation (Richardson et al. 2007; Koch
et al. 2014). RhoGAP18B encodes a GTPase activating protein that plays a role in the behavioral response to ethanol and neuromuscular junction formation (Laviolette et al. 2005; Rothenfluh et al. 2006). Tao encodes a mitogen-activated protein kinase kinase kinase and acts together with hippo (hpo) to activate warts (wts) -mediated repression of yorkie (yki) (Poon et al. 2011). GIIIspla2 encodes a phospholipase A2 that interacts genetically with the E3 ubiquitin ligase parkin (Cha et al. 2005).

The major findings of the current work are: 1) the results provide strong support for the hypothesis that Rh3% is sensitive to gene dosage effects through loss of function or gain of function as might be expected for a stochastic, cell-autonomous biological process, 2) numerous genes in addition to klu, tgo and ss, are likely to play a role in regulating this process, 3) the identification of cell-cell signaling and cell surface molecules suggests that R7 cell subtype specification may not be exclusively cell-autonomous, but likely involves inputs from other cell types or tissues. Interestingly, the interaction network defined by the candidate genes contains several genes previously shown to influence specification of Rh3 or Rh4 expression in R7 photoreceptor cells (Rh3%) as well as the inductive signal that is thought to coordinate the expression of opsin genes in adjacent R7 and R8 photoreceptor cells within individual ommatidia. These include (Egfr) (Birkholz et al. 2009a) that is required in both processes, as well as Merlin (Mer), wts, hpo, kibra, yki (Jukam and Desplan 2011; Jukam et al. 2013) and thickveins (tkv) (Wells et al. 2017) that coordinate opsin gene expression in R7 and R8 cells. Furthermore, one of the candidate genes identified in the screen, Fur2, has also been shown to regulate the induction of Rh5 expression in R8 cells (Wells et al. 2017), although this could be secondary to the effect on Rh3 expression in R7 cells shown in the current study. These results suggest numerous mechanisms through which processes in the R7 cell responsible for R7y vs. R7p cell fate may couple to inductive signaling that specifies paired R8y and R8p cell fates, respectively. Further validation of candidate genes using precise excision to test phenotypic reversion, additional mutant alleles, and RNAi will provide definitive evidence of their involvement. The initial analyses here are insufficient to determine the precise mechanism of action of the identified candidate genes. They could potentially function cell autonomously within the R7 photoreceptor cell or play roles outside of the R7 cell. The candidate genes may interact genetically with, or be independent of, ss. Finally, the genes identified in this study may play an instructive or a permissive role in R7 cell differentiation. Additional experiments will be required to further validate the candidate genes and resolve these mechanistic questions.

Figure 4 Homozygous Loss of Function validation of selected candidates from mutant screens. The bar graph shows the standardized Rh3% for animals in the Heterozygous Loss of Function (HetLOF, red bars) and Gain of Function (GOF, blue bars) screens. Rh3% for animals in the Homozygous Loss of Function (HomLOF) validation are shown in green. Rh3% of HomLOF were examined for 28 selected candidates. Four of these mutants (Arp3, Autophagy-related 2, (Atg2), fat, and lov) were homozygous lethal and are not included in the figure. A standardized Rh3% was calculated for the HomLOF data using the Rh3% median for each chromosome from the HetLOF screen.

Figure 5 Homozygous loss of slit dramatically increases Rh3%. A) The white-eyed, but otherwise wild-type cn bw retina with R7 cells stained for Rh3 (green) or Rh4 (red). This eye section has 31% Rh3 (n = 232). B) Flies homozygous for the slitEP937 insertion (identified in our screen) have 60.4% Rh3 (n = 618).
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