The BEAF-32 insulator protein is required for Hippo pathway activity in the terminal differentiation of neuronal subtypes

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Abstract

The Hippo pathway is critical for not only normal growth and apoptosis but also cell fate specification during development. What controls Hippo pathway activity during cell fate specification is incompletely understood. In this research report, we identify the BEAF-32 insulator protein as a regulator of Hippo pathway activity in *Drosophila* photoreceptor differentiation. Though morphologically uniform, the fly eye is composed of two subtypes of R8 photoreceptor neurons defined by expression of light-detecting Rhodopsin proteins. In one R8 subtype, active Hippo signaling induces Rhodopsin6 (Rh6) and represses Rhodopsin5 (Rh5) whereas in the alternate subtype, inactive Hippo signaling induces Rh5 and represses Rh6. The activity state of the Hippo pathway in R8 is determined by the expression of *warts*, a core pathway kinase, which interacts with the growth regulator *melted* in a double negative feedback loop. We show that the *BEAF-32* insulator is required for expression of *warts* and repression of *melted*. Furthermore, *BEAF-32* plays a second role downstream of Warts to induce Rh6 and prevent Rh5 fate. *BEAF-32* is dispensable for Warts feedback, indicating that *BEAF-32* differentially regulates *warts* and Rhodopsins. Loss of *BEAF-32* does not noticeably impair the functions of the Hippo pathway in eye growth regulation. Our study identifies a context-specific regulator of Hippo pathway activity in post-mitotic neuronal fate, and reveals a developmentally specific role for a broadly expressed insulator protein.

Introduction

The Hippo signaling pathway is a critical regulator of growth and apoptosis in organ size control (Irvine and Harvey, 2015; Yu et al., 2015; Zhao et al., 2011). However, proliferation-independent roles for the pathway during animal development have also been discovered. The identification of the Hippo pathway as a regulator of R8 photoreceptor subtype specification in *Drosophila* was among the first examples of a mitosis-independent role for the Hippo pathway in determining cell fate (Mikeladze-Dvali et al., 2005). More recently, the pathway has been shown to regulate dendritic field tiling in neurons (Emoto et al., 2006), cell differentiation in pre-implantation embryos (Cockburn et al., 2013; Nishioka et al., 2009), neuroblast differentiation upon cell cycle exit (Reddy et al., 2010), and hematopoiesis (Milton et al., 2014), among others. Because R8 photoreceptors are post-mitotic neurons and are not competent to divide, they are an excellent system in which to elucidate context-specific mechanisms of Hippo pathway function (Hsiao et al., 2013; Jukam and Desplan, 2011; Jukam et al., 2013). How the Hippo pathway is regulated differently in division and differentiation is incompletely understood. Here, we describe the BEAF-32 insulator protein as a regulator of the Hippo pathway in cell fate specification in the developing *Drosophila* retina.

The fly eye is composed of ~800 ommatidia (unit eyes); each ommatidium contains eight photoreceptors named R1-R8 (Hardie, 1985). The outer photoreceptors, R1-R6, express the broad spectrum-detecting Rhodopsin1 (Rh1) and function in motion detection (Heisenberg and Buchner, 1977; Yamaguchi et al., 2008; Wardill et al., 2012). The inner photoreceptors, R7 and R8, are specialized for color vision, with some contribution from R1-R6 (Schnaitmann et al., 2013). Though morphologically uniform, the fly eye is composed of two main types of ommatidia defined by expression of color-sensing Rhodopsins (Rhs) in the inner PRs (Rister et al., 2013). In the “pale” (**p**) subtype, **p**R7s express Rhodopsin3 (Rh3) and **p**R8s express Rhodopsin5 (Rh5) (**Fig. 1A**). In the “yellow” (**y**) subtype, **y**R7s express Rhodopsin4 (Rh4) and **y**R8s express Rhodopsin6 (Rh6) (**Fig. 1B**). The ommatidial subtypes are stochastically distributed throughout the eye in a **p**:**y** ratio of ~35:65 (**Fig 1C, F**).

The specification of ommatidial subtypes is determined in R7s by the stochastic ON/OFF expression of the PAS-bHLH transcription factor Spineless (Ss) (Johnston and Desplan, 2014; Wernet et al., 2006). The ON/OFF state of Ss determines R8 subtype fate through an inductive signal (Chou et al., 1996; Papatsenko et al., 1997) that results in mutually exclusive R8 expression of the Hippo pathway kinase Warts (Wts) and the growth regulator Melted (Melt). In **p**R7s lacking Ss, Rh3 is expressed in R7s and a signal from R7s triggers activation of *melt* and repression of *wts,* leading to Rh5 expression in **p**R8s (**Fig. 1A**) (Mikeladze-Dvali et al., 2005). In **y**R7s expressing Ss, Rh4 is expressed in R7s and the signal is repressed, causing the default state of *melt* repression and *wts* activation leading to Rh6 expression (**Fig. 1B**). The double-negative feedback loop between *wts* and *melt* controls the presence or absence of Wts downstream of the constitutively active upstream Hippo pathway (Jukam and Desplan, 2011). Wts negatively regulates Yorkie (Yki), which acts with a network of photoreceptor-specific transcription factors to transduce Hippo pathway output into expression of Rh5 or Rh6 (Jukam et al., 2013).

Here, we identify the BEAF-32 insulator protein as a critical regulator of Wts and Hippo pathway activity in R8 subtype specification. *BEAF-32* is required for the expression of *wts* and also functions downstream of Wts to regulate Rhs, but does not noticeably affect growth. Finally, we demonstrate that *BEAF-32* is differentially required for Hippo pathway positive feedback and Rh expression. The role of BEAF-32 in post-mitotic determination of photoreceptor subtypes suggests that insulators have highly specific functions in development.

Results and Discussion

BEAF-32 is a regulator of the Hippo pathway controlling Rh expression

To identify transcription factors that regulate the Hippo pathway and R8 subtype specification, we conducted an *in vivo* RNAi screen for genes whose knockdown caused a change in the proportion of R8s expressing a *rh5-LexA, lexAOP-GFP* transcriptional reporter (*rh5>>GFP*) (Vasiliauskas et al., 2011), (i.e. low Hippo pathway activity) (**Fig. 1D**). We screened 652 lines targeting transcription factor genes, which resulted in 113 lethal phenotypes, 155 eye morphology phenotypes, and one line with a dramatic increase in Rh5.

In the screen, we identified *BEAF-32* as a positive regulator of the Hippo pathway. RNAi knockdown of *BEAF-32* caused a dramatic increase in the proportion of R8s that express *rh5>>GFP* (**Fig. 1E**). *BEAF-32* RNAi also caused an increase in R8s that express Rh5 protein and a decrease in R8s expressing Rh6 protein (**Fig. 1G, H, L**).

BEAF-32 (Boundary element-associated factor of 32kDa; hereafter referred to as BEAF) is one of several known *Drosophila* insulator proteins, including dCTCF, GAGA factor, Su(Hw), Zw5, CP190, and mod(mdg4). BEAF binds preferentially near promoters at several thousand sites in the genome (Emberly et al., 2008; Jiang et al., 2009; Negre et al., 2011; Yang et al., 2012) and generally promotes gene expression. Two *BEAF* isoforms, *BEAF-32A* and *BEAF-32B*, are identical except for the 80 amino acid DNA-binding domain; however, *BEAF-32B* appears to be the dominant isoform (Jiang et al., 2009; Roy et al., 2007). Though BEAF binds throughout the genome, homozygous *BEAF* mutants, null for both isoforms ($BEAF^{AB\text{-}KO}$), are viable, suggesting that *BEAF* is required for specific developmental processes like R8 subtype specification.

Similar to *BEAF* RNAi, homozygous null *BEAF* mutants ($BEAF\text{-}32^{AB\text{-}KO}$) (Roy et al., 2007) (**Fig. 1I, L**) and flies with the *BEAF* null mutant allele over a 105 kb deficiency completely lacking the *BEAF* locus (**Fig. 1J, L**) displayed an increase in Rh5 and decrease in Rh6 expression. An independent *BEAF* mutant allele caused by a P-element insertion ($BEAF\text{-}32^{NP6377}$) placed over the deficiency showed similar changes in the Rh5:Rh6 ratio (NP/def; **Fig. 1L**). All three *BEAF* mutant conditions and *BEAF* RNAi displayed significant increases

in Rh5 and decreases in Rh6, and any phenotypic variability among these is likely due to differences in genetic background. A genomic fragment containing the *BEAF* gene locus (Roy et al., 2007) rescued the mutant phenotype, restoring the normal Rh5:Rh6 ratio (**Fig. 1K, L**), indicating that the Rh phenotype is specifically due to loss of *BEAF*. RNAi-mediated knockdown of other insulator genes (*CTCF*, *Cp190*, *mod(mdg4),* *su(Hw),* and *Trl/GAGAfactor*) did not significantly increase Rh5 in the retina (**Fig. S1**). Therefore, the regulation of Rhs in R8s by insulators is likely restricted to *BEAF* and not a general property of insulator function.

BEAF acts in R8s downstream of R7 signaling to control Rhs

We next determined the cellular focus of *BEAF* activity. Consistent with previous reports that *BEAF* is expressed in all cells of the fly (Roy et al., 2007), a 900 bp *BEAF* promoter drove a *BEAF-GFP* transgene in all photoreceptors, including all R8s (**Fig. 2A**). Photoreceptor-specific expression of a dominant negative BEAF protein lacking the DNA-binding domain and containing only the protein-binding BED domain (*all PRs>dom neg*) (Gilbert et al., 2006) induced an increase in Rh5 and decrease in Rh6 (**Fig. 2B, D**). Whole-retina clones of *BEAF* null or P-element insertion mutants displayed changes in Rh expression (**Fig. 2C, D**) similar to those in viable whole-animal *BEAF* mutants. *BEAF* null mutant clones displayed upregulation of Rh5 and loss of Rh6 compared to wild type clones (**Fig. 2E, F**). Thus, *BEAF* is required for proper expression of Rh5 and Rh6 in R8 photoreceptor neurons of the eye.

The most upstream trigger for R8 subtype fate is the stochastic ON/OFF expression of Ss in R7s. Expression of Ss represses an unknown signal to R8s, resulting in Wts expression, active Hippo signaling, and Rh6 expression (**Fig. 1B**). In the absence of Ss, the signal induces repression of *wts*, leading to inactive Hippo signaling and Rh5 expression (**Fig. 1A**). Ss was expressed at a similar frequency in *BEAF* null mutant clones as wild type clones (**Fig. 2G, H**), indicating that *BEAF* is not required for Ss expression. Rh3 and Rh4, targets of Ss regulation in R7s (Thanawala et al., 2013; Wernet et al., 2006), were expressed at similar ratios in *BEAF* null mutant and wild type clones (**Fig. 2I, J**). Thus, *BEAF* is not required for Ss expression or R7 subtype specification.

We next showed that *BEAF* acts downstream of Ss and the signal to control R8 subtypes. Ectopic expression of Ss in all R7s from a BAC transgene (Johnston and Desplan, 2014) repressed the signal to R8s, causing nearly all R8s to adopt yR8 fate and express Rh6 (**Fig. 2K, M, O**). Ectopic Ss expression in R7s in *BEAF* null mutants displayed increased Rh5 and decreased Rh6 expression (**Fig. 2L, M, P**), showing that Ss requires *BEAF* activity to control R8 subtype. Genetic ablation of R7s in *sevenless* (*sev*) mutants removed the signal from R7s to R8s, causing nearly all R8s to acquire yR8 fate and express Rh6 (**Fig. 2Q, S, T**). *sev*; *BEAF null* double mutant R8s primarily expressed Rh5 (**Fig. 2R, S, U**), showing that the default Hippo activity ON state of R8s requires *BEAF* to activate Rh6 and repress Rh5. Altogether, these data indicate that *BEAF* acts in R8s downstream of the signal from R7s to control R8 subtype specification (**Fig. 2V**).

BEAF binds genes encoding the Hippo pathway members and *melt*

To explore how *BEAF* regulates the R8 regulatory network, we examined five independent ChIP datasets (4 ChIP-chip, 1 ChIP-seq) available from the modENCODE consortium (Negre et al., 2011). We identified strong peaks (**Fig. 3A**; red diamonds) that are likely direct binding sites for BEAF and weak peaks (**Fig 3A**; empty diamonds) that may be the result of DNA looping and insulator-insulator interaction (Liang et al., 2014). Strong BEAF binding peaks were present for all the core members of the Hippo pathway (*hpo, sav, mats, wts*), upstream regulators known to function in R8 (*kib, mer, apkc, lgl*), and output regulators (*yki, sd*) (**Fig. 3A**). BEAF also bound *melt*, part of the critical *melt/wts* bistable feedback loop (**Fig. 3A**). Although BEAF weakly bound *ss*, the fate trigger in R7s, and *otd*, a general activator of Rh3 and Rh5 and repressor of Rh6, we did not detect any defects indicative of changes in *ss* (**Fig. 2G-J**) or *otd* (**Fig. 1D-J, 2E**) expression in *BEAF* mutants. Additionally, BEAF does not bind at loci of the other photoreceptor-restricted transcription factors that regulate R8 rhodopsins (*sens, pph13,* and *tj*). The absence of the BEAF consensus DNA binding sequence (CGATA) in the *rh5* and *rh6* promoter regions, which are sufficient to induce their subtype specific expression, is consistent with a model wherein BEAF does not regulate Rh5 or Rh6 expression through direct binding. Together, these binding profiles suggest that

BEAF could regulate R8 subtype fate by controlling aspects of the Hippo pathway or expression of *melt* and *wts*.

BEAF* is required for repression of *melt* and activation of *wts

Since BEAF bound *melt* and *wts*, which are in a transcriptional double-negative feedback loop critical for R8 subtype specification, we examined the role of *BEAF* in their regulation. In **p**R8s, *melt* represses *wts* expression to activate Rh5 and repress Rh6 (**Fig. 1A**) (Mikeladze-Dvali et al., 2005). In **y**R8s, *wts* is expressed to repress Rh5 and induce Rh6 (**Fig. 1B**). *BEAF* null mutants display loss of Rh6 and gain of Rh5 expression, suggesting that *melt* is up-regulated and *wts* expression is down-regulated. Indeed, *melt* (*melt-lacZ*) is de-repressed (**Fig. 3B**) and *warts* expression (*warts-lacZ*) is lost (**Fig. 3C**) in R8s in *BEAF* null mutant clones, indicating that *BEAF* is required for the repression of *melt* and activation of *warts* expression (**Fig. 3D**).

***BEAF* is required downstream of Wts and Melt for regulation of Rhs**

The Rh phenotype observed in *BEAF* mutants could be caused simply by de-repression of *melt* and loss of *wts* expression (**Fig. 3D**). Alternatively, *BEAF* could play other roles in the pathway and the *BEAF* mutant phenotype could be due to misregulation of additional downstream genes. In *melt* mutants, *wts* is expressed and the Hippo pathway is active, inducing nearly all R8s to express Rh6 and lose Rh5 (**Fig. 4A, C**) (Mikeladze-Dvali et al., 2005). Removing *BEAF* in *melt* mutants caused up-regulation of Rh5 and down-regulation of Rh6 (**Fig. 4B, C**) as compared to *melt* single mutants, suggesting that *BEAF* acts downstream of or in parallel to *melt* to control Hippo pathway activity and Rh expression state (**Fig. 4F**).

We next tested whether *wts* and the Hippo pathway require *BEAF* to regulate Rh expression. Mis-expression of Wts and Salvador (Sav, an upstream positive regulator of Wts) in wild type clones (*BEAF*+) induced Rh6 in all R8s (**Fig. 4D, E**). *BEAF* null mutant clones generated in retinas simultaneously mis-expressing Wts and Sav in all photoreceptors resulted in the up-regulation of Rh5 and loss of Rh6 (**Fig. 4D, E**). *BEAF* null mutant clones in retinas with mis-expression of Wts alone displayed similar phenotypes (data not shown). Thus, *BEAF* is required for Hippo pathway activity to promote the Rh6 R8 fate.

Altogether, our epistasis analysis indicates that *BEAF* acts downstream of or in parallel to *wts* and *melt* to regulate Rh5 and Rh6 expression (**Fig. 4F**), as well as upstream of *wts* and *melt* to regulate their expression (**Fig. 3D**).

Yki but not Sd requires *BEAF* function to regulate Rhs

The Yorkie (Yki) and Scalloped (Sd) transcription factors are heterodimerization partners that regulate Hippo pathway target genes downstream of Wts (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). In **p**R8s with Hippo pathway OFF, Yki/Sd are active and induce Rh5 and repress Rh6 (**Fig. 1A**). In **y**R8s with active Hippo signaling, Yki is inactive and Rh6 is expressed, whereas Rh5 is repressed (**Fig. 1B**). *yki* null mutant cells are eliminated via apoptosis and cannot be examined in adult eyes, but strong expression of RNAi can effectively knockdown *yki* function in the retina (Jukam et al., 2013). RNAi knockdown of *yki* caused a loss of Rh5 and gain of Rh6 in all R8s (**Fig. 4G, I**). Retinas with simultaneous RNAi knockdown of *yki* and *BEAF* displayed up-regulation of Rh5 and loss of Rh6 (**Fig. 4H, I**) relative to *yki-RNAi* alone. With the caveats inherent to RNAi-based epistasis analysis, we conclude that *BEAF* is required downstream of or in parallel to *yki* to control Rh5 and Rh6 (**Fig. 4L**).

Sd is present in all R8s and appears to play a permissive role in Rh regulation. *sd* mutants display Rh6 in all R8s and completely lose Rh5 (Jukam et al., 2013). Whereas wild type clones (*sd*+) in homozygous *BEAF* null mutant tissue up-regulated Rh5 and lost Rh6, *sd* mutant clones in *BEAF* mutant tissue expressed Rh6 and lost Rh5 in most R8s (**Fig. 4J, K**), suggesting that Sd acts downstream of *BEAF* to regulate Rhs. These data are consistent with a model wherein Yki, but not Sd, requires *BEAF* to regulate Rhs, suggesting that Yki and Sd may have separable roles in R8 subtype specification (**Fig. 4L**). Given the strong but incomplete phenotypic suppression in the above epistasis, however, we cannot exclude more complicated models.

Positive feedback regulation of *wts* expression is independent of *BEAF*

We next tested *BEAF* for a role in the positive network-level feedback that is a feature of the R8 Hippo pathway, but not the Hippo growth pathway (Jukam et al., 2013). Wts and Yki cross-regulate in a double negative feedback

loop, in which Wts phosphorylates Yki to inactivate it in **y**R8s and Yki down-regulates transcription of *wts* in **p**R8s (**Fig. 1A-B**). Thus, Wts activates its expression by inhibiting its repressor Yki (**Fig. 4P**). In wild type R8s, *wts* (i.e. *wts-lacZ*) is expressed in **y**R8s to generate an active Hippo pathway and Rh6 expression. Ectopic expression of *wts* (*GMR-wts*) caused all R8s to express *wts* (Mikeladze-Dvali et al., 2005). Since *BEAF* is required for *wts* expression in otherwise wild-type **y**R8s, we predicted that *BEAF* would be required for positive feedback regulation of *wts* when *wts* was ectopically expressed. However, retinas with ectopic *wts* expression in *BEAF* null mutants displayed *wts-lacZ* expression in all R8s (**Fig. 4M**), suggesting that *BEAF* is not required for the positive feedback regulation of *wts* (**Fig. 4O, P**). One interpretation of this result is that *BEAF* is required only for initiation of *wts* expression and not its maintenance. Alternatively, the wild type *wts/yki* feedback loop could be near a threshold that is highly sensitive to *BEAF* regulation, whereas ectopic expression of *wts* biases the regulation strongly towards *wts* expression overcoming the absence of *BEAF*. Consistent with a role for *BEAF* downstream of *wts* and *yki* to regulate opsins, we still observe loss of Rh6 in most *BEAF* mutant cells that express *wts-lacZ* (**Fig. 4M, N**).

Conclusion

We have shown that the BEAF insulator protein is required for a post-mitotic neuronal fate decision in *Drosophila* photoreceptors. BEAF regulates Hippo pathway activity to control R8 subtype fate and Rhodopsin expression (**Fig. 4Q**): First, *BEAF* regulates *wts* and *melt* expression by acting upstream. Second, *BEAF* is required for the Hippo pathway to promote Rh6 and repress Rh5. We also demonstrate that *BEAF* acts downstream of or in parallel to Yki for regulation of Rhodopsins. Finally, we show that this regulation of Rhodopsins is independent of *wts* feedback. It appears likely that BEAF regulates cell specification by permissively promoting Hippo pathway activity and wts expression to specify the default yR8 fate.

Despite its role in regulating the Hippo pathway in post-mitotic neuronal fate, *BEAF* appears to be dispensable for Hippo growth signaling in the eye because homozygous null mutants are viable, exhibit no gross external morphological defects, and show no dramatic differences in eye clone size or pupal interommatidial cell number as compared to wild-type tissue (**Fig. 2E, G, I, 3B, C, 4D, Fig. S2**). Additionally, *BEAF* depletion did not suppress the under-proliferation in *yki*-RNAi eyes despite suppressing *yki-RNAi* Rhodopsin phenotypes (**Fig. 4G, H**). This differential regulation of the Hippo pathway in R8s compared to growth is consistent with other transcriptional regulators of R8 subtypes (*ewg*, *tj*) having minimal or no proliferation defects (Hsiao et al., 2013; Jukam et al., 2013). It is possible that BEAF regulates the Hippo pathway indirectly in R8, by acting on yet-to-be-discovered R8-specific Hippo pathway regulators. Alternatively, *BEAF* may play a larger role in R8s because of Hippo pathway positive feedback, and have less effect in Hippo growth signaling where homeostatic regulation through negative feedback may compensate for the absence of BEAF. The compensation of ectopic Wts on *wts* expression, but not opsin control, in *BEAF* mutants is consistent with such a model.

Non-CTCF insulators appear to be restricted to arthropods, and among several fly species examined (*Anopheles gambiae, Apis mellifera, or Tribolium castaneum*), *BEAF* was present exclusively in the *Drosophila* genus (Heger et al., 2013; Schoborg and Labrador, 2010). We speculate that conserved signaling modules of the Hippo pathway in growth control may be co-opted for

cell fate specification by regulatory factors like *BEAF* that are unique to dipterans.

Insulators were classically defined as proteins that bind particular DNA sequences to either interfere with promoter-enhancer interactions or prevent chromatin-state position effects from affecting transgenes (Gaszner and Felsenfeld, 2006). This definition has expanded to include proteins that mediate chromosomal interactions to regulate 3-D chromatin organization and global gene expression (Bushey et al., 2009; Phillips-Cremins and Corces, 2013; Wood et al., 2011). Despite these studies, surprisingly few roles for insulator proteins in specific biological processes in flies have been characterized, including the regulation of oogenesis (Hsu et al., 2015; Roy et al., 2007; Soshnev et al., 2013) and spermatogenesis (Soltani-Bejnood et al., 2007; Thomas et al., 2005). Our result that *BEAF* regulates Hippo pathway activity for terminal differentiation of R8 neuronal subtypes, but has no observed effect on general growth control or other photoreceptor fate, indicates that broadly expressed insulators can have exquisitely specific functions in development.

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Materials and Methods

Drosophila genetics and transgene descriptions

Flies were raised on corn meal-molasses-agar medium under standard laboratory conditions. $y^1, w^{67};+;+$ flies were considered "wild-type" and used as a control for *rh* expression. All experiments were conducted at 25'C unless otherwise noted.

lGMR-Gal4 (long Glass Multiple Reporter) contains a pentamerized 38 bp Glass binding site and is expressed in all photoreceptors and some other retina cells posterior to the morphogenetic furrow (Wernet et al., 2003). *ey-Gal4* drives transgene expression in eye primordium and eye imaginal discs. *UAS-Dicer2(Dcr2)* is co-expressed to increase RNAi processing efficiency (Dietzl et al., 2007). *warts-lacZ* contains a P-element inserted into *warts* locus (Justice et al., 1995). *melt-lacZ* contains the 1st intron of melted cloned upstream of *nls:lacZ* (Mikeladze-Dvali et al., 2005).

BEAF-32$^{AB\text{-}KO}$ is semi-viable (Roy et al., 2007). The original *BEAF-32*$^{AB\text{-}KO}$ stock possibly contained a second-site mutation that caused rhabdomere defects, and the chromosome was cleaned during recombination to FRT42D. After recombination, the resulting FRT42D *BEAF-32*$^{AB\text{-}KO}$ flies contained normal rhabdomeres. *BEAF-32*^{NP6377} is a null allele caused by a P-element insertion (Gurudatta et al., 2012). We also recombined this allele onto an FRT42D chromosome, which removed the lethality and growth defects previously described on the chromosome (Gurudatta et al., 2012). The deficiency *Df(2R)BSC429* (Bloomington stock #24933) contains a 105 kb FLP/FRT-derived deletion that completely removes the *BEAF* coding sequence, in addition to several other genes. Placing *BEAF-32*$^{AB\text{-}KO}$ trans-heterozygous over a second, ~250 kb deficiency (*Df(2R)BSC858*; stock#27928) gave similar results.

Homozygous mutant adult eyes ("whole eye mutant clones") were generated using the FLP/FRT system (Xu and Rubin, 1993). FLP recombinase expressed under control of the *eyeless* (*ey*) promoter (*ey-FLP*) (Newsome et al., 2000) induced recombination of FRT chromosomes containing a cell lethal mutation and GMR-hid to remove all non-mutant eye tissue (Stowers et al., 2000). Mutant clones were made using FRT-FLP mediated recombination

between the mutant chromosome and an otherwise wild-type chromosome containing P[*w*+, *ubi-GFP*].

RNAi Screen

Transcription factors were defined according to the FlyTF database (Adryan and Teichmann, 2006), which includes manual curation from the literature and computationally generated structure homologies. The data set identifies 1052 candidate DNA binding proteins, including 753 proposed as transcription factors (~450 site specific). Another 299 genes did not meet their criteria, but had transcription related Gene Ontology annotations (Adryan and Teichmann, 2006; Adryan and Teichmann, 2007).

UAS-RNAi fly lines were obtained from a genome-wide library of *Drosophila* RNAi at the Vienna *Drosophila* RNAi Center (VDRC) (Dietzl et al., 2007). Each transgenic line contained a 300-500 bp inverted hairpin construct under the control of a 10X multimerized UAS promoter. We tested several Gal4 drivers, including *lGMR-Gal4* (strongly expressed in all photoreceptors after the morphogenetic furrow), *sens-Gal4* (strongly expressed in R8 and weakly and variably expressed in other photoreceptors), and *ey-Gal4+lGMR-Gal4* (*ey-Gal4* is strongly expressed in the early eye primordium and disc), with or without co-expression of *UAS-Dicer2* (*Dcr2*). Dcr2 is thought to enhance RNAi processing efficiency in cell types more refractory to UAS-RNAi such as adult neurons (Dietzl et al., 2007).

UAS-Dcr2; *ey-Gal4*, *lGMR-Gal4* was determined to be the optimal Gal4 driver because it induced RNAi phenotypes very similar to phenotypes of a gene's respective loss-of-function mutant for 7 of 7 known R7 and R8 subtype regulators: Rh6 was lost with *warts, merlin, mats*, and *sav* RNAi, while Rh5 was lost with *melt-RNAi*. *spineless-RNAi* flies had expansion of Rh3 into all R7 photoreceptors. In addition, *dve-RNAi* resulted in an increase in Rh5 expression, in the outer photoreceptors. An independent paper describing a role for Dve in restricting R8 Rhs from R1-R6 was in preparation during the screen and published (Johnston et al., 2011). Second, *otd-RNAi* completely removed *rh5* expression, consistent with a requirement for Otd for *rh5* transcription and direct binding to the *rh5* promoter. These positive controls demonstrate that expression of UAS-RNAi under control of *UAS-Dcr2*; *ey-Gal4*, *lGMR-Gal4* is an

effective tool to induce loss-of-function developmental phenotypes detectible in adult photoreceptors.

The screen was performed by crossing UAS-RNAi males to virgin female reporter-driver lines. To maximize virgin collection, the female driver stock contained a *hs*-hid transgene on the Y-chromosome (Dietzl et al., 2007). We performed two 30 minute heat-shocks spaced 8 hours apart at 37'C in late larval and early pupal stages to eliminate males.

R8 subtypes were assessed for defects in F1 progeny by examining for a change in the proportion of R8s expressing a *rh5-LexA::VP16*, *lexAOP::GFP* (*rh5>>GFP*) transcriptional reporter. The LexA/lexAOP binary expression system was used to amplify GFP levels while keeping the reporter Gal4-independent (Lai and Lee, 2006; Vasiliauskas et al., 2011).

Water Immersion Protocol for visualizing *rh5>>GFP*

GFP was visualized in living adult flies by neutralizing the cornea using water immersion technique (Pichaud and Desplan, 2001). 10-12 flies of the appropriate F1 genotype were placed on a streak of clear nail polish (Wet'N'Wild brand) perpendicular to the straight edge with one retina facing up, in the middle of a 10cm Petri dish. The dish was placed on the microscope stage and water added to submerge flies. Images of *rh5>>GFP* were taken with SPOT camera mounted on a fluorescent microscope with the Nikon Plan Fluor 40X objective lens immersed in water. About 30-40 ommatidia are visible in the same focal plane. If *rh5>>GFP* appeared in greater than 60% of ommatidia of at least two flies, the genotype was later dissected and Rh5 and Rh6 visualized with antibodies. During the pilot screen it was discovered that the ey-Gal4 + lGMR-Gal4 exhibited, on occasion, an increase in the Rh6:Rh5 ratio (from 70:30 to 85:15). Therefore we only assayed subtype phenotypes that increase the Rh5 R8 proportion.

BEAF binding analysis

BEAF-32 ChIP data for *Drosophila Melanogaster* were obtained from five available studies from modENCODE (http://www.modencode.org). For the purpose of this analysis, the preprocessed peak calls available in the modENCODE's 'dmel-interpreted-1' FTP directory were used. The midpoint of

each identified ChIP peak regions was used to annotate the gene diagrams, based on the FlyBase dm3 gene annotation on the UCSC Genome Browser (genome.ucsc.edu). Strong peaks were defined as having greater than 2.5 fold enrichment. Weak peaks were defined as having less than 2.5 fold enrichment. The scripts used for analysis can be found at https://github.com/pdeford/beaf32-hippo-chip .

Antibodies

Antibodies and dilutions used were as follows: mouse anti-Rh3 (1:10) (gift from S. Britt, University of Colorado), rabbit anti-Rh4 (1:100) (gift from C. Zuker, Columbia University), mouse anti-Rh5 (1:200) (Chou et al., 1996) rabbit anti-Rh6 (1:2000) (Tahayato et al., 2003), guinea pig anti-Ss 2.21 (1:200) (gift from Y.N. Jan, University of California, San Francisco) (Kim et al., 2006), rat anti-ElaV (1:50) (DSHB), sheep anti-GFP (1:500) (AbD Serotec), mouse anti-Dlg, and goat anti-β-gal (Biogenesis). All secondary antibodies were Alexa Flour(488, 555, or 647)-conjugated (1:400) made in donkey (Molecular Probes).

Antibody staining and imaging

Adult or staged pupal retinas were dissected as described (Hsiao et al., 2012). Briefly, retinas were dissected and immediately fixed for 15 minutes with 4% paraformaldehyde at room temperature. After lamina removal, retinas were rinsed two times in PBX (PBS + 0.2% Triton-X) and washed in PBX for >2 hours. Retinas were incubated overnight with primary antibodies diluted in PBX at room temperature and then rinsed two times in PBX and washed in PBX for >4 hours. Retinas were incubated 4-6 hours or overnight with secondary antibodies diluted in PBX at room temperature and then rinsed two times in PBX and washed in PBX for >2 hours. Adult retinas were mounted in Slowfade (Molecular Probes) and pupal retinas in Vectashield (Vectorlabs) on glass slides with coverslip. Images were acquired using a Leica TCS SP5, Zeiss 710, or Zeiss 780 confocal microscope. Objectives were 10X, 20X, or 60X. Images were processed in Photoshop (Adobe) or ImageJ. Brightness or Contrast adjustments, if any, were simple linear adjustments made to the entire image, in accordance with journal guidelines. Figures were prepared using Illustrator

(Adobe).

Quantification of Photoreceptor Subtypes

Confocal images were taken and the number of R8 cells that expressed Rh5, Rh6, both, or neither were counted. The percentage of R8s expressing Rh5 (%Rh5) was calculated for each retina, and mean %Rh5 of all retinas within a genotype was used to compare across genotypes. Retinas were scored if there were 75 or more ommatidia present in a single focal plane. Most retinas contained ~200-300 ommatidia in a single image. For all genotypes, more retinas were observed than quantified to confirm a particular phenotype. Means and standard deviations for all experiments can be found in Supplemental Table 1.

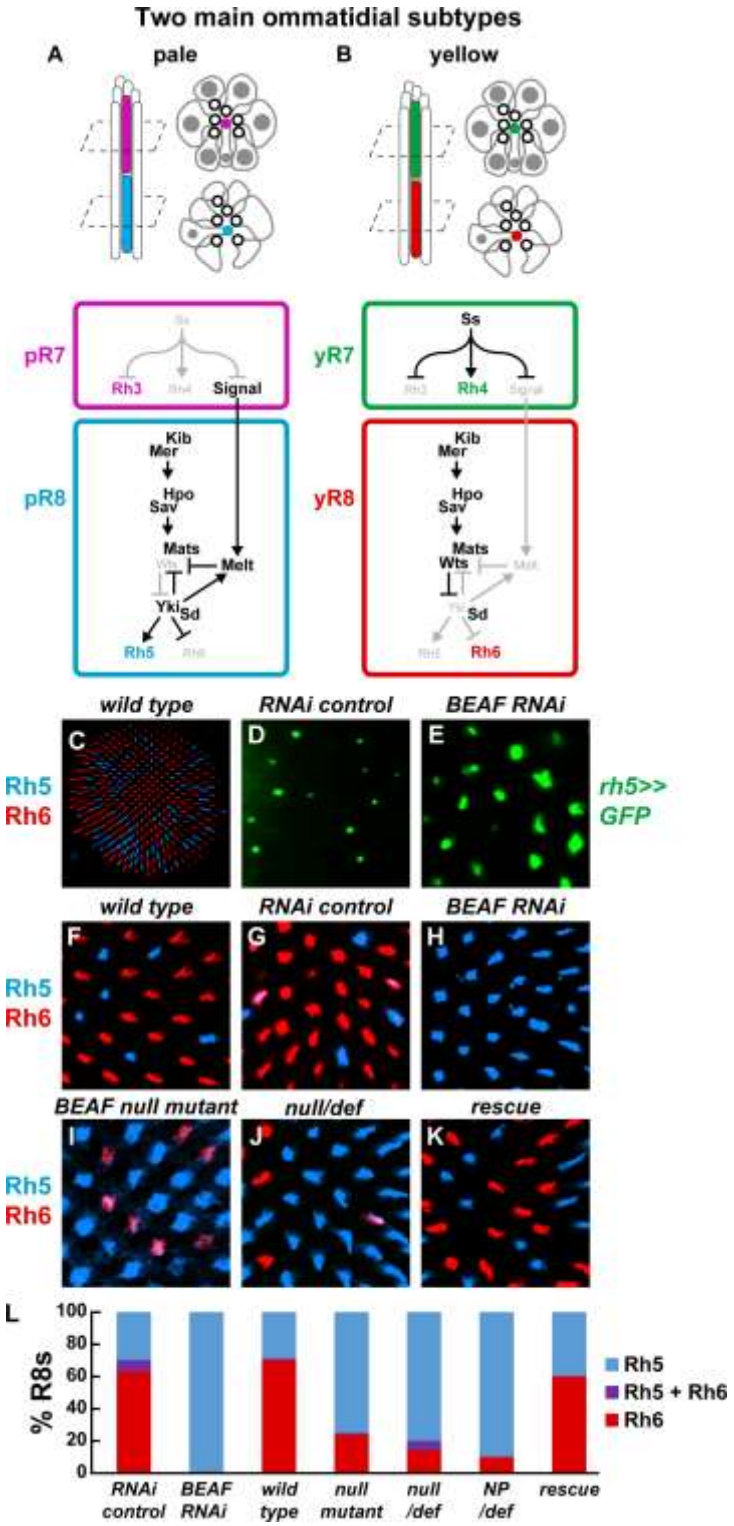
Drosophila Genotypes and Stocks

Short Genotype	Complete Genotype	Fig	Source
wild type	*yw; sp/CyO; +*	1C, F, L, 2D	
RNAi control (rh5>>GFP)	*UAS-Dcr2*; *ey-Gal4*, *lGMR-Gal4 / +; rh5-LexA*,*lexAOP-GFP /+*	1D	Dietzl et al., 2007 Vasiliauskas et al., 2011
BEAF RNAi (rh5>>GFP)	*yw, UAS-Dcr2*; *ey-Gal4*, *lGMR-Gal4 / +*; *rh5-LexA*, *lexAOP-GFP / UAS-BEAF-RNAi*	1E	Dietzl et al., 2007 Vasiliauskas et al., 2011 VDRC
RNAi control	*yw, UAS-Dcr2; ey-Gal4*, *lGMR-Gal4 / CyO; +*	1G, L	Dietzl et al., 2007
BEAF RNAi	*yw, UAS-Dcr2; ey-Gal4*, *lGMR-Gal4 / +; UAS-BEAF-RNAi / +*	1H, L	VDRC
BEAF Null mutant	*yw; BEAF-32$^{AB\text{-}KO}$ / BEAF-32$^{AB\text{-}KO}$; +*	1I, L	Roy et al., 2007
BEAF Null/Df	*BEAF-32$^{AB\text{-}KO}$ / BEAF-32$^{Df(2R)BSC429}$; +*	1J, L	Roy et al., 2007 Bloomington (#24933)
BEAF rescue	*yw; BEAF-32$^{AB\text{-}KO}$ / BEAF-32$^{AB\text{-}KO}$; gBF*	1K, L	Roy et al., 2007
BEAF-GFP	*yw; +; GFBF*	2A	Roy et al., 2007
all PRs>dom neg	*yw; lGMR-Gal4/ +; UAS-BEAF-BID/ +*	2B, D	Gilbert et al., 2006
BEAF whole eye clone	*yw, ey-FLP*; *FRT42D BEAF-32$^{AB\text{-}KO}$ / FRT42D GMR-hid, cl; +*	2C, D	
BEAF mutant clones	*yw, ey-FLP*; *FRT42D BEAF-32$^{AB\text{-}KO}$ / FRT42D ubi-GFP; +*	2E-J	
ss gof	*yw; +; ss^{48C04}* (*ss transgene 6*)	2K, M	Johnston and Desplan, 2014
BEAF; ss gof	*yw; +; BEAF-32$^{AB\text{-}KO}$ / BEAF-32$^{AB\text{-}KO}$; ss^{48C04}*	2L, M	Roy et al., 2007 Johnston and Desplan, 2014

sev	*sev, yw; +; +*	2Q, S	
sev; BEAF	*sev, yw; BEAF-32^{AB-KO} / BEAF-32^{AB-KO}; +*	2R, S	
BEAF mutant clones; melt-lacZ	*yw, ey-FLP*; *FRT42D BEAF-32^{AB-KO} / FRT42D ubi-GFP ; melt-lacZ / +*	3B	Mikeladze-Dvali et al., 2005
BEAF mutant clones; wts-lacZ	*yw, ey-FLP*; *FRT42D BEAF-32^{AB-KO} / FRT42D ubi-GFP; wts-lacZ / +*	3C	Roy et al., 2007 Justice et al., 1995
melt	*yw; +; melt$^{\Delta 32}$ / melt$^{\Delta 32}$*	4A, C	Mikeladze-Dvali et al., 2005
BEAF; melt	*yw; BEAF-32^{AB-KO} / BEAF-32^{AB-KO} ; melt$^{\Delta 32}$ / melt$^{\Delta 2}$*	4B, C	Roy et al., 2007 Mikeladze-Dvali et al., 2005
BEAF clones; all PRs>wts, sav	*yw, ey-FLP*; *FRT42D BEAF-32^{AB-KO} / FRT42D ubi-GFP; GMR-wts, GMR-sav / +*	4D, E	Tapon et al., 2002
yki RNAi	*yw, UAS-Dcr2; ey-Gal4*, *lGMR-Gal4 / / yki-RNAi$^{(N+C)}$; +*	4G, I	Zhang et al., 2008
BEAF RNAi; yki RNAi	*yw, UAS-Dcr2; ey-Gal4*, *lGMR-Gal4 / / yki-RNAi$^{(N+C)}$; UAS-BEAF-RNAi / +*	4H, I	VDRC Zhang et al., 2008
sd clones; BEAF	*yw, ey-FLP, ubiGFP FRT19A / sd^{47M}; BEAF-32^{AB-KO} / BEAF-32$^{Df(2R)BSC429}$; +*	4J, K	Wu et al., 2008 Roy et al., 2007
BEAF; GMR-wts / wts-lacZ	*yw; BEAF-32^{AB-KO} / BEAF-32^{AB-KO} ; GMR-wts, GMR-sav / wts-lacZ*	4M, N	Roy et al., 2007 Tapon et al., 2002 Justice et al., 1995

wts-lacZ	Justice et al., 1995; Xu et al., 1995
GMR-wts	Tapon et al., 2002
GMR-wts GMR-sav	Tapon et al., 2002
rh5-LexA, *lexAOP-GFP*	Lai and Lee, 2006; Vasiliauskas et al., 2011;
BEAF^{A-KO}	Roy et al., 2007
BEAF^{AB-KO}	Roy et al., 2007
BEAF$^{Df(100\ kb)}$	Bloomington Stock Center
BEAF$^{Df(200\ kb)}$	Bloomington Stock Center
UAS-BEAF-BID (DomNeg)	Gilbert et al., 2006
BEAF genomic fragment (*gBF*)	Roy et al., 2007
BEAF-GFP fusion (*GFBF*), under control of *BEAF* promoter	Roy et al., 2007
ey-FLP, P[ubiGFP] FRT19A	Jessica Treisman
ey-Gal4 lGMR-Gal4	Dietzl et al., 2007
UAS-Dcr2	Dietzl et al., 2007

Figures



1. *BEAF* is required for yR8 subtype specification.

(A-B) Schematic showing eight photoreceptors and a cross-section of their rhabdomeres, the membranous structures containing Rhodopsin (Rh) proteins, within an ommatidium. Gray indicates cell bodies and nuclei. White circle with black outlines indicate outer photoreceptor rhabdomeres. Colored rhadomeres indicate R7 (top) and R8 (bottom). Below is the regulatory network controlling Rh expression in R7 (top) and R8 (bottom).

A. Pale "**p**" ommatidial subtype.

B. Yellow "**y**" ommatidial subtype

C. Retina showing Rh5 and Rh6 expression in stochastic and mutually exclusive R8 subsets. R8 subtypes are visualized by Rh5 (**p**R8, blue) and Rh6 (**y**R8, red) antibodies in all panels unless otherwise noted.

D. *rh5>>GFP* was expressed in a subset of R8s in RNAi controls. Visualized by water immersion (see methods).

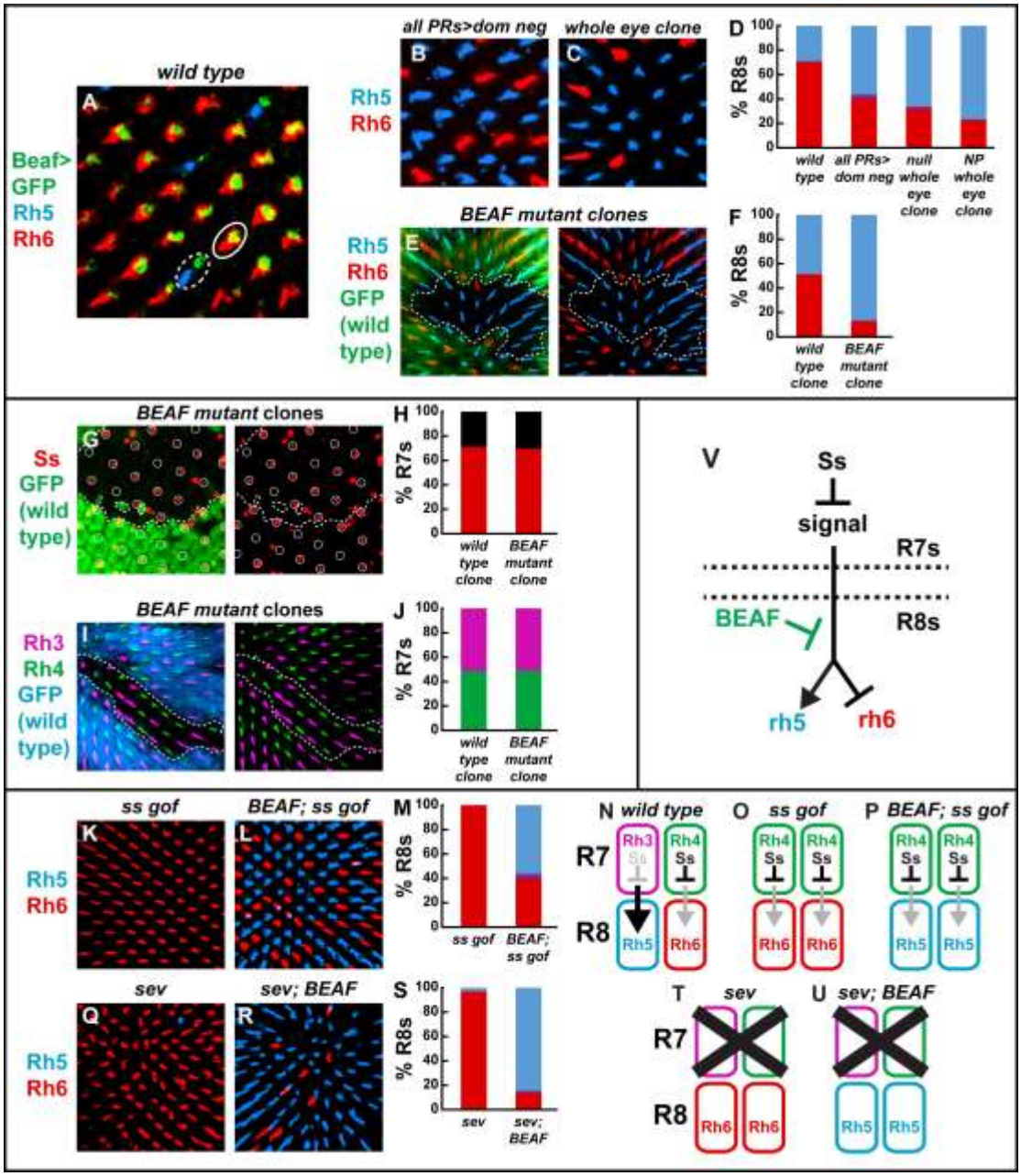
E. *rh5>>GFP* was expressed in most R8s when *BEAF* is knocked down by RNAi.

F-G. Rh5 and Rh6 were expressed in R8 subsets in wild-type (F) or RNAi Gal4 control (G) retinas.

H-J. Most R8s contained Rh5, and few contained Rh6, in retinas expressing *BEAF-RNAi* (H) or homozygous mutant for $BEAF^{AB\text{-}KO}$ (I) or $BEAF^{AB\text{-}K0}$ over a deficiency covering the *BEAF* locus (J).

J. A *BEAF* genomic fragment restored normal Rh5 and Rh6 expression in *BEAF* homozygous null mutants.

K. Quantification of phenotypes.



2. *BEAF* acts in R8s downstream of R7 signaling to control Rhs

A. *BEAF-GFP* under control of the *BEAF* promoter was expressed in both R8 subtypes. Rh5-expressing **p**R8 (dashed circle); Rh6-expressing **y**R8 (solid circle).

B-D. Rh5-expressing R8s increased and Rh6-expressing R8s decreased when a BEAF dominant negative construct is expressed specifically in

photoreceptors (B); A similar phenotype is observed in whole eye *BEAF* null mutant clones (C). (D) Quantification of B-C.

E-F. *BEAF* null mutant clones (GFP-) contained more R8s with Rh5 as compared to wild-type or heterozygous tissue (GFP+). Dotted line represents clone boundary in all panels unless otherwise noted. (F) Quantification of E.

G-H. Ss was expressed stochastically with similar frequency in *BEAF* null (GFP-) and control (GFP+) tissue in pupal retinas. R7 cells are circled. (H) Quantification of G).

I-J. The Rh3 and Rh4 expression ratio was normal in *BEAF* null mutant clones (GFP-). (J) Quantification of I.

K. Ectopic Ss expression in all R7s induced Rh6 and inhibited Rh5 expression in nearly all R8s.

L. Ectopic Ss expression in the absence of BEAF resulted in increased Rh5- and decreased Rh6-expressing R8s.

M. Quantification of K-L.

N-P. Schematics depicting (G-H).

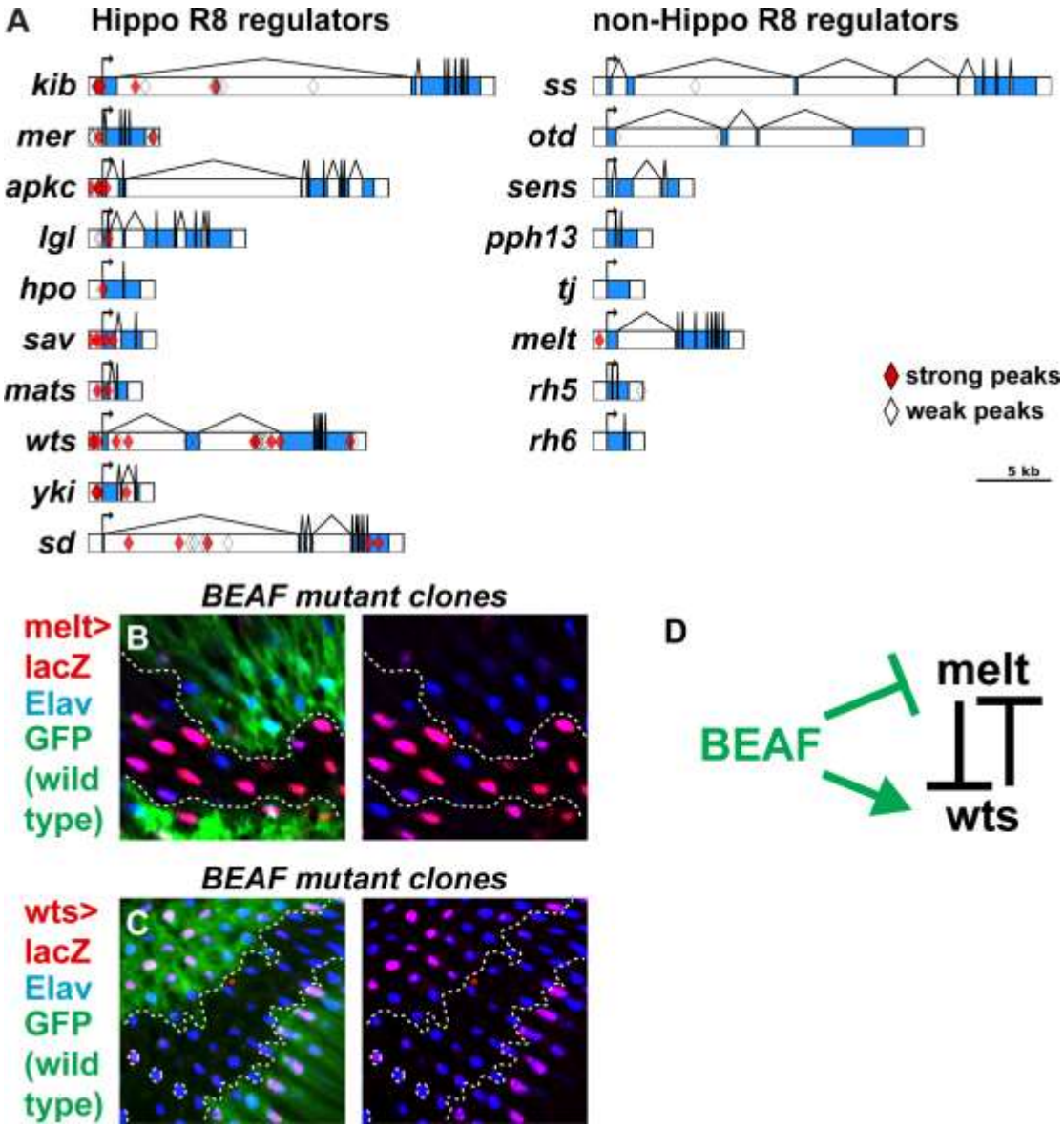
Q. Genetic ablation of R7s (and hence the signal to R8) in *sev* mutants caused expression of Rh6 and loss of Rh5 in nearly all R8s.

R. *sev; BEAF null* double mutants displayed upregulation of Rh5 and down-regulation of Rh6.

S. Quantification of Q-R.

T-U. Schematics describing (Q-R).

V. Model for how *BEAF* acts in R8s, downstream of R7 signaling to control Rhs.



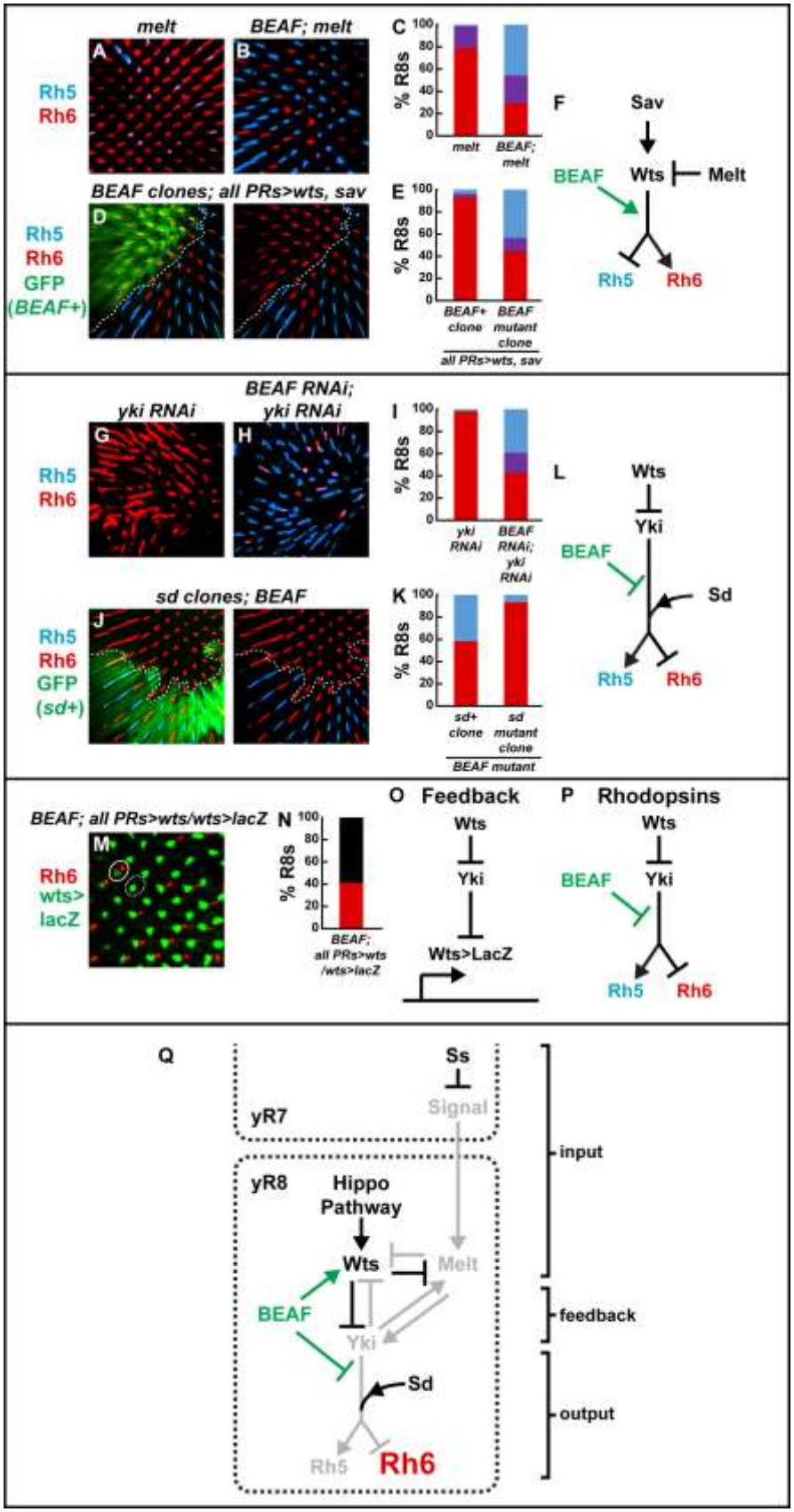
3. *BEAF* regulates R8 subtypes by promoting *warts* expression and preventing *melt* expression.

A. Summary of BEAF modEncode ChIP binding data at loci of R8 Hippo pathway genes (left) and non-Hippo R8 subtype regulators (right). Diamonds are ChIP-chip peak centers (red diamonds are strong peaks; empty diamonds are weak peaks); exons are blue; non-coding sequence is white.

B. *melt-lacZ* was upregulated in BEAF null mutant clones (GFP-) as compared to control tissue (GFP+).

C. *wts-lacZ* was lost in BEAF null mutant clones (GFP-) as compared to control tissue (GFP+).

D. Model for BEAF regulation of *wts* and *melt* expression.



4. *BEAF* is essential for Hippo Pathway regulation of Rhodopsins, but not positive feedback, in R8 subtype specification.

A. *melt* mutants contained Rh6 in almost all R8s.

B. Rh5 was upregulated in *BEAF null; melt* double mutants.

C. Quantification of A-B.

D. *GMR-wts+sav* induced Rh6 and inhibited Rh5 expression in otherwise wild-type R8s (GFP+). *BEAF* null mutant clones (GFP-) in *GMR-wts+sav* retinas showed increased Rh5 and decreased Rh6 expression.

E. Quantification of D.

F. Schematic showing how *BEAF* acts downstream of *wts* and *melt* to regulate Rhodopsins.

G. *yki-RNAi* retinas displayed Rh6 expression in all R8s.

H. *BEAF-RNAi* + *yki-RNAi* resulted in up-regulation of Rh5 and down-regulation of Rh6.

I. Quantification of G-H.

J. *sd* mutant clones (GFP-) in whole-eye *BEAF* null mutant background (GFP+) showed Rh6 in all R8s.

K. Quantification of H.

L. Model for *BEAF* regulation of Rhodopsin output downstream of Yki and upstream of Sd.

M. *wts-lacZ* (Green) was expressed in all R8s in *BEAF null*;*GMR-wts* retinas. Circle shows Rh6 and *wts-lacZ* co-expressed; dotted circle shows R8 expressing *wts-lacZ* but not Rh6. Note: R8 nuclei are in a different cell region than Rh-containing rhabdomeres.

N. Quantification of M. Red indicates % R8s expressing Rh6; black indicates % R8s lacking Rh6 (presumably expressing Rh5).

O-P. *BEAF* is dispensable for Hippo pathway feedback, but not Hippo pathway regulation of Rhodopsins.

Q. Working model for *BEAF* regulation of R8 subtypes. Arrows indicate genetic regulation.

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