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Opposing transcriptional and post-transcriptional roles for Scalloped in binary Hippo-dependent neural fate decisions

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ABSTRACT

The Hippo tumor suppressor pathway plays many fundamental cell biological roles during animal development. Two central players in controlling Hippo-dependent gene expression are the TEAD transcription factor Scalloped (Sd) and its transcriptional co-activator Yorkie (Yki). Hippo signaling phosphorylates Yki, thereby blocking Yki-dependent transcriptional control. In post-mitotic *Drosophila* photoreceptors, a bistable negative feedback loop forms between the Hippo-dependent kinase Warts/Lats and Yki to lock in green vs blue-sensitive neuronal subtype choices, respectively. Previous experiments indicate that *sd* and *yki* mutants phenocopy each other's functions, both being required for promoting the expression of the blue photoreceptor fate determinant *melted* (*melt*) and the blue-sensitive opsin Rh5. Here, we demonstrate that Sd ensures the robustness of this neuronal fate decision via multiple antagonistic gene regulatory roles. In Hippo-positive (green) photoreceptors, Sd directly represses both *melt* and *Rh5* gene expression through defined TEAD binding sites, a mechanism that is antagonized by Yki in Hippo-negative (blue) cells. Additionally, in blue photoreceptors, Sd is required to promote the translation of the Rh5 protein through a 3′UTR-dependent and microRNA-mediated process. Together, these studies reveal that Sd can drive context-dependent cell fate decisions through opposing transcriptional and post-transcriptional mechanisms.

1. Introduction

Ensuring that the correct complement of genes remains on or off in any given cell type is an essential feature of multicellular organisms. This is particularly critical in the peripheral nervous system, where exclusive sensory receptor expression is necessary for selective and specific activation of a given sensory neuron. Such exclusion is well-established in the visual system of most animals, where individual photoreceptors (PRs) express a single opsin photopigment and repress the expression of others to prevent sensory overlap (Mazzoni et al., 2004). The gene regulatory mechanisms underlying this mutual exclusion, however, are still under investigation.

The *Drosophila* eye has long served as a powerful model to understand the functions and architecture of gene regulatory networks underlying PR subtype cell fate specification (Cook and Desplan, 2001; Viets et al., 2016). Each of the approximately 750 individual eye units (ommatidia) in the *Drosophila* compound eye contains 8 PRs (Fig. 1A). Based on the specific opsin that is expressed in the R8 photoreceptor, two major ommatidial subtypes, pale (p) and yellow (y), are present in the adult eye. Pale ommatidia are primarily defined based on the expression of the blue-sensitive opsin, Rhodopsin 5 (Rh5), while yellow ommatidia express the green-sensitive opsin, Rh6. These ommatidial subtypes are randomly distributed through the eye in a 30:70 blue:green ratio (Fig. 1B), and are established and maintained through a bistable negative feedback loop between two signaling molecules: the pleckstrin homology-containing protein Melted (Melt) and the Hippo signaling kinase Warts (Wts, aka Lats) (Fig. 1C) (Mikeladze-Dvali et al., 2005).

Wts is a core component of the Hippo kinase complex that phosphorylates and inactivates the transcriptional co-activator Yorkie (Yki) (Huang et al., 2005; Justice et al., 1995; Misra and Irvine, 2018; Pan, 2010; Xu et al., 1995). Hippo signaling is best understood in the context of growth regulation, where Wts and Yki function in a homeostatic feedback loop: Wts blocks Yki function and Yki initiates its own inactivation by promoting Hippo pathway gene expression (Dong et al., 2007;

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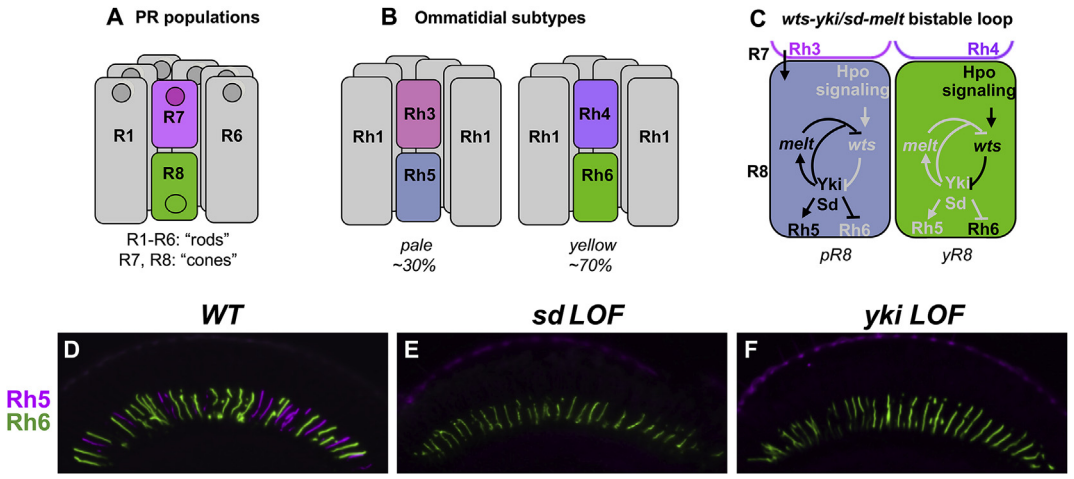

Fig. 1. *sd* and *yki* are required for ommatidial subtype specification.
(A) Schematic of an individual ommatidium with its 8 photoreceptors (PRs): six "rod-like" outer PRs (R1-R6, grey) and two "cone-like" inner PRs (R7, pink and R8, green). (B) Two main ommatidial subtypes [pale (p) and yellow (y)] and corresponding Rhodopsin (Rh) expression patterns. (C) A *melt-yki/sd-wts* bistable loop specifies pR8 (*melt*) vs. yR8 (*wts*) fates. An instructive pR7-to-pR8 signal (arrow from pink pR7) activates *melt* in yR8s. Genes or proteins that are expressed and/or active are represented by black font; genes or proteins that are not expressed and/or active are denoted by grey font. (D–F) Retina cryosections stained for Rh5 (magenta) and Rh6 (green) in control (D), *sd* knockdown (LOF) (E, *lGMR-GAL4 > UAS-sd^{RNAi}*) *or yki* knockdown (LOF) (F, lGMR-*GAL4 > UAS-yki^{RNAi}*) retinas.

Genevet et al., 2010; Hamaratoglu et al., 2006; Oh and Irvine, 2008). In contrast, in post-mitotic PR fate decisions, Yki promotes the expression of the *wts* repressor, *melt*, generating a double-negative "on/off" feedback loop between *wts* and Yki that ensures two stably maintained fate choices (Fig. 1C) (Jukam et al., 2013). In green PRs, Hippo signaling promotes the expression of green fate determinants (*wts* and *Rh6*), and prevents the expression of Yki-dependent blue fate determinants (*melt* and *Rh5*) (Mikeladze-Dvali et al., 2005). In blue PRs, Yki promotes *melt,* thereby repressing *wts* and inhibiting Hippo signaling, further promoting Yki-dependent activation of blue fate effectors and suppression of green fate effectors (Fig. 1C) (Jukam et al., 2013). Thus, Wts-positive (Yki-inactive) cells adopt the default green/*wts*/Rh6 fate, while Wts-negative (Yki-active) cells acquire the blue/*melt*/Rh5 fate.

Yki, a YES-associated protein (YAP), is a transcriptional co-activator that does not bind DNA itself, but instead requires a DNA-binding partner (Huang et al., 2005). The primary binding partners for Yki/Yap factors are members of the TEAD family of transcription factors (Cao et al., 2008; Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). In *Drosophila*, the single TEAD family member is encoded by Scalloped (Sd) (Campbell et al., 1992). Sd/TEAD and Yki/YAP can physically interact and together activate TEAD-site-containing reporter expression *in vitro* (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). Furthermore, in ectopic *yki* conditions, *sd*/TEAD is essential for *yki*/YAP to induce tissue overgrowth and activate target gene expression. However, *in vivo*, *sd* mutants do not phenocopy *yki* growth phenotypes and *sd* mutants do not show changes in *yki* target gene expression (Wu et al., 2008). These data suggest that Sd and Yki use distinct mechanisms to control tissue size. Studies aimed at addressing this conundrum have shown that in developing wing, eye, and follicle cells, Sd functions as a transcriptional repressor under "Hippo-on" conditions to inhibit cell growth, and that in "Hippo-off" cells, Yki antagonizes Sd repression to promote growth regulatory genes (Koontz et al., 2013). This suggests that Sd and Yki can play opposite roles during growth.

In post-mitotic PRs, we have previously shown that *sd* mutants phenocopy *yki*'s knockdown phenotype in PR subtype fate specification: both *sd* and *yki* are necessary to promote blue PR fate and inhibit green PR fate (Jukam et al., 2013) (Fig. 1D–F). Combined, these findings suggest that *sd* and *yki* function together in this cell fate specification event. In the study presented here, we investigated the molecular basis underlying this interaction. We find that Sd plays roles at both the transcriptional and post-transcriptional level to ensure blue vs green PR subtype fate decisions. At the transcriptional level, Sd directly represses blue fate effector gene expression in Hippo (Wts)-positive green PRs, and Yki antagonizes this repression in Hippo (Wts)-negative blue PRs. This is consistent with previously reported antagonism between Sd and Yki (Koontz et al., 2013). In addition to this function, we find that Sd promotes blue fate through a post-transcriptional, microRNA (miRNA)-dependent process in Wts-negative blue PRs, revealing a cooperative interaction with Yki in promoting blue PR fate. Together, our new findings elucidate a multi-tiered regulatory network involving the *Drosophila* TEAD transcription factor that functions at both the transcriptional and post-transcriptional level to precisely specify neuronal subtype fate.

2. Materials and methods

2.1. Cloning and generation of transgenic flies

The *pattB-pRh5-LacZ-3′UTR^{SV40}* reporter was made by subcloning the *Rh5* promoter *(pRh5 -269/+50)* into the *pattB-lacZ* vector (Bischof et al., 2007). *pattB-melt450-nLacZ* was previously described (Jukam et al., 2013). Site-directed mutants of Sd binding sites were generated by PCR using the QuikChange Site-directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. The primers for mutating the Sd binding sites are as follows, with mutant sequences capitalized, and underlined sequences from the pattB-LacZ vector:

Rh5ΔSd1_forward: gattctttgtaTGCGCtgaatctaaacttttttcacgctgac
Rh5ΔSd1_reverse: gattcaGCGCAtacaaagaatctacgcttttatttatatataatttac
Rh5ΔSd2_forward: cgcctcagTGCGCccttaagctgcggctaagac
Rh5ΔSd2_reverse: gcttaaggGCGCActgaggcggtcaggttacgacag
Rh5ΔSd3_forward: ggattatccttcctgTGCGCcacacgcaccccaaggtc
Rh5ΔSd3_reverse: gcgtgtgGCGCAcaggaaggataatcccaacgtc
Rh5ΔSd4_forward: gaggccaTGCGCtcgacctgcaaaggaaactag
Rh5ΔSd4_reverse: ggtcgaGCGCAtggcctccgagtcacctatggtctg
meltΔSd1_forward: gcgcgTGCGCacttttctgccagctcttttc
meltΔSd1_reverse: ggcagaaaagtGCGCAcgcgcggctgtcgcgccatc
meltΔSd2_forward: cagctcttttGCGCGcatgttttttttttagcgcccacg
meltΔSd2_reverse: catgCGCGCaaaagagctggcagaaaagtc
meltΔSd3_forward: caaaagacctgGCGCAcacgaaggtccaaagaactc
meltΔSd3_reverse: cgtgTGCGCcaggtcttttgattagatgag
meltΔSd4_forward: caagGCGCAaccggtgctatcagtctag
meltΔSd4_reverse: ctagactgatagcaccggtTGCGCcttggccaacttgccgtcgag

To generate the *pattB-pRh5-nLacZ-3′UTR^{Rh5}* construct, the *Rh5*

promoter (−269/+50), the nuclear LacZ coding sequence, and the 127 bp *Rh5* 3′UTR were assembled 5′ to 3′ into the vector pattB (Bischof et al., 2007). The *Rh5* 3′UTR was PCR-amplified from genomic DNA that was purified from *yw*^{67}*; Sp/CyO; TM2/TM6B* flies using the primers gaatgcggccgcTGGTACAATTGTCAGATTAACG (5′) and ggggtaccAATGCCAACTTTTTTTGG- TTTG (3′) (restriction sites underlined). Resulting constructs were injected into ΦX-51C flies using site-specific integration with standard techniques (Rainbow Transgenic Flies).

2.2. Drosophila stocks

UAS-sd$^{RNAi(N+C)}$ and *UAS-yki*^{RNAi} lines (Zhang et al., 2008) were kindly provided by Jin Jiang, University of Texas Southwestern. *UAS-yki*^{RNAi} experiments were performed using the *yki*^{RNAi} line from Dr. Jiang recombined with the *yki*^{RNAi} line GD40497 (Vienna Drosophila Research Center) (Jukam et al., 2013). *UAS-Dicer2* lines from the Bloomington Drosophila Stock Center (BDSC #60008 and #60009) were included to increase RNAi efficiency (Lee et al., 2004). *Dicer-1* RNAi (TRiP^{HMS02594}, BDSC #42901), *loqs* RNAi (TRiP^{HMS00089}, BDSC #34780) and *drosha* RNAi (TRiP^{HMS00064}, BDSC #33657) were also obtained from BDSC. *pWIZ-wΔ13* (a *white* gene RNAi line) was kindly provided by Richard Carthew, Northwestern University, to reduce autofluorescence due to eye pigmentation (Lee and Carthew, 2003). Unless otherwise indicated, wild-type flies refer to *yw*^{67}*; Sp/CyO; TM2/TM6B* flies. All lines were balanced with this stock to ensure similar genetic backgrounds. When a genotype was heterozygous on the third chromosome, TM2 flies were analyzed rather than TM6B flies, as TM6B carries a truncation mutation in the *Rh6*-encoding gene (Cook et al., 2003). All RNAi experiments were performed with heterozygous RNAi and *longGMR-GAL4* as well as *UAS-Dicer2*. The *longGMR-GAL4* (long Glass Multiple Reporter) transgene is expressed in all PRs (Wernet et al., 2003) and additional retinal cells posterior to the morphogenetic furrow. When appropriate, *UAS-luciferase* (BDSC #35788) was used to normalize for the number of UAS-transgenes. Flies were raised on standard cornmeal-molasses media at 25 °C with 12 h:12 h light:dark cycles.

2.3. Immunohistochemistry

Cryosections and antibody stainings were performed as previously described (McDonald et al., 2010; Wernet et al., 2003; Xie et al., 2007). Briefly, fly heads were embedded and frozen in OCT, sectioned (10 μm), fixed in 4% formaldehyde/PBS, and then washed 3 × 10 min with PBX (PBS+0.3% Triton X-100). The slides were incubated with primary antibodies overnight at 4 °C in BNXS buffer (1 x PBX, 0.1% BSA, 0.05% saponin), washed 4 × 10 min with PBX, and incubated for 90 min at room temperature with secondary antibodies diluted in BNXS buffer. After 4 × 10min PBX washes, samples were mounted with Fluoromount (Sigma). Antibody dilutions were: mouse anti-Rh5 (1:1000; S. Britt) (Chou et al., 1996); rabbit anti-Rh6 (1:2000, C. Desplan) (Tahayato et al., 2003); rabbit anti-Spalt (1:100) (Xie et al., 2007); anti-chicken LacZ (1:1000; Abcam). AlexaFluor 488-, 555- and 647-conjugated secondary antibodies (1:1500; Invitrogen) were used. Digital images were obtained with an Apotome deconvolution system (Zeiss), and processed with Axiovision 4.5 (Zeiss) and Adobe Photoshop 7.0 software. Spalt-positive R8 cells were analyzed for Rh5, Rh6 or LacZ staining from at least 3 individual animals and 2 experimental replicates (total R8 n's > 400), and statistical significance relative to controls was performed using a two-tailed, unpaired *t*-test.

2.4. Luciferase reporter assays

Luciferase reporter constructs *Rh5* (−269/+50)*-Luc* and expression vectors pAc-Otd, pAc-Sd, and pAc-Yki were previously described (Jukam et al., 2013; Xie et al., 2007). Sd site-mutated luciferase reporters *Rh5ΔSd3-Luc* and *Rh5ΔSd1234-Luc* were generated by subcloning respective mutated *Rh5* promoters from pattB into the pGL3 basic vector (Promega). *Drosophila* S2 cells (Invitrogen) were maintained in HyQ SFX-Insect media (Hyclone) at room temperature. 1×10^6 cells were plated in 12-well tissue culture dishes (Corning) 24 h prior to transfection with 1.5 μL Fugene HD (Roche) and 125 ng pGL3 reporter, 125 ng pAc-LacZ, and 250 ng total pAc-expressing vectors. Luciferase assays were performed as previously described (Jukam et al., 2013; Xie et al., 2007). Samples were transfected in triplicate for each experiment, each experiment was performed at least three independent times and statistical significance relative to controls was performed using a two-tailed, unpaired *t*-test. Data from single representative experiments are shown.

2.5. RT-PCR analysis

For RT-PCR, retinas were dissected from adult flies, and total RNA was extracted using Trizol (Life Technologies) and treated with DNAse I (Promega) to remove any residual DNA. The quality of the purified RNA was analyzed with an Agilent 2100 Bioanalyzer. First-strand synthesis and PCR amplification was performed using a QIAGEN OneStep RT-PCR Kit with the following primers: *Rh5*: forward TGGTACTTGTCAAAGATTAACG, reverse: AACTCTTTCAGTTCATTGCACG; *GAPDH1*: forward CAAGAACGCTGACATCCTGA, reverse CAGAAGTCACAAACGCCTCA; *LacZ*: forward CGCCATCTGCTGCACGCGGAAG, reverse: TTGACACCAGACCAACTGGTAATG.

3. Results

3.1. Scalloped and Yorkie play distinct roles in photoreceptor subtype specification

Genetically, *sd* and *yki* are necessary for blue PRs to be specified from an equicompetent blue/green R8 PR precursor state. Specifically, loss of *sd* or *yki* by either mutation or RNAi leads to loss of blue fate markers (the blue-sensitive Rh5 opsin protein and *melt* reporter activity) and concomitant expansion of green PR markers (Rh6 opsin protein and the *wts*-LacZ reporter) in all R8 PRs (Jukam et al., 2013) (Fig. 1D–F). To further characterize how *sd* and *yki* control blue fate decisions, we first analyzed *Rh5* gene expression in control, *yki* knockdown and *sd* knockdown retinas using RT-PCR. *orthodenticle* mutant retinas (*otd*^{uvi}) were used as a control, as *otd* is essential for the expression of the *Rh5* gene in *Drosophila* (Tahayato et al., 2003). Consistent with this, *Rh5* mRNA was not detected in *otd*^{uvi} retinas (Fig. 2A and B). *Rh5* mRNA was also not detected in *yki* knockdown retinas (Fig. 2A and B), confirming the requirement of *yki* for *Rh5* gene expression. Surprisingly, however, we still detected *Rh5* mRNA in *sd* knockdown retinas compared to controls (Fig. 2A), albeit at ~50% of wild-type expression levels (Fig. 2B and data not shown). These findings suggest that although *sd* and *yki* are both required for Rh5 protein expression, they can play different roles in the transcriptional control of *Rh5* gene expression.

3.2. Scalloped directly represses Rh5 expression in green R8s

Previous studies described a minimal *Rh5* promoter (*pRh5*, -269/+50) that recapitulates endogenous Rh5 protein expression in the *Drosophila* retina (Jukam et al., 2013; McDonald et al., 2010; Tahayato et al., 2003; Xie et al., 2007) (Fig. 2C and D). To test whether Sd and Yki regulate *Rh5* gene expression through this regulatory region, we analyzed *pRh5* reporter expression in *sd* and *yki* knockdown retinas. Similar to *Rh5* mRNA (Fig. 2A), *pRh5-LacZ* expression was eliminated with *yki* knockdowns (Fig. 2F and G) but maintained in *sd* knockdowns (Fig. 2E). We also noted expansion of *pRh5-LacZ* to almost all R8s (97 ± 1%) in *sd* knockdowns (Fig. 2G). Because green PR fate is expanded to all R8s in *sd* knockdowns (Fig. 1E), these data suggest that Sd is not necessary for *Rh5* expression, but instead, is required to repress *Rh5* gene expression in green PRs.

Given that Sd is a TEAD family specific DNA-binding transcription factor (Campbell et al., 1992; Xiao et al., 1991), we next analyzed the

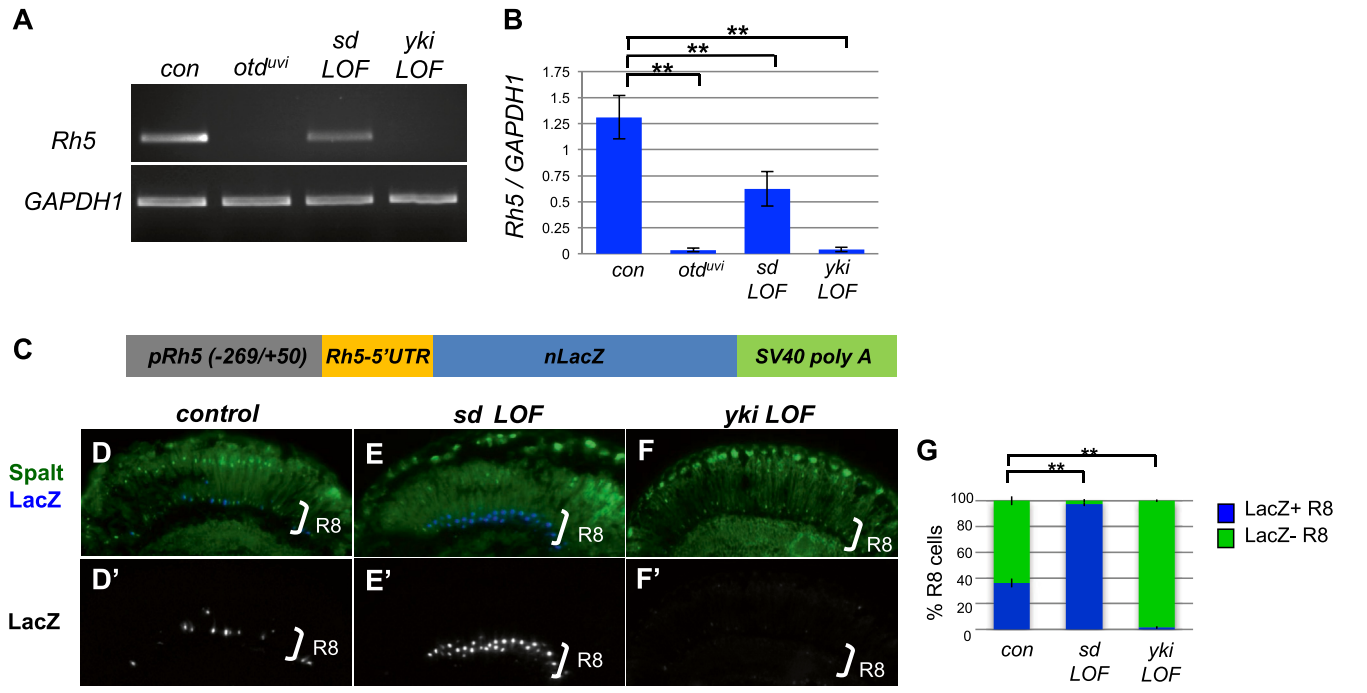

Fig. 2. *sd* and *yki* differentially regulate blue PR specification.
(A) RT-PCR for *Rh5* mRNA in control, otd^{uvi} (otd^{uvi}; *Sp/CyO, TM2/TM6B*); *sd* knockdown (*lGMR-GAL4* > $UAS\text{-}sd^{RNAi}$), *or yki* knockdown (*lGMR-GAL4* > $UAS\text{-}yki^{RNAi}$) retinas. otd^{uvi} was used as a negative control, as it is known to directly activate *Rh5* gene expression (Tahayato et al., 2003). *Rh5* mRNA is detected in wild type and *sd* knockdown retinas, but is absent in *yki* knockdowns. (B) Quantification of *Rh5* expression. The level of *Rh5* mRNA was normalized to *GAPDH1* levels. Control: 1.31 ± 0.21; otd^{uvi}: 0.03 ± 0.02; *sd* LOF: 0.62 ± 0.17; *yki* LOF: 0.03 ± 0.02. (C) Schematic of the *pRh5-nLacZ* reporter carrying the minimal wild-type *Rh5* promoter (−269/+50). (D–F) Retina cryosections stained for Spalt (green) and LacZ (blue) in control (D, *lGMR-GAL4*), *sd* knockdown (E, *lGMR-GAL4* > $UAS\text{-}sd^{RNAi}$) *or yki* knockdown (F, *lGMR-GAL4* > $UAS\text{-}yki^{RNAi}$) retinas, showing *pRh5-nLacZ expression*. Spalt labels all R7 and R8 nuclei. LacZ protein is observed in ~36% R8 cells ($36 \pm 3\%$) in control retinas (B, B′), while it is in most R8 cells ($97 \pm 1\%$) in *sd* knockdown retinas (C, C′). There are very few LacZ-expressing R8s present in *yki* knockdown retinas ($1.5 \pm 0.9\%$) (D, D′). (G) Quantification of *pRh5* reporter-expressing R8s. From left to right in graph: *pRh5* in wild type retinas (n = 460 R8s), in *sd* knockdown retinas (n = 536) and *in yki* knockdown retinas (n = 610). Error bars represent SD; **p < 0.01.

minimal *Rh5* promoter (*pRh5*) sequence for potential Sd/TEAD binding sites (Sandelin, 2004), and identified four (Sd1-4) (Fig. 3A and Fig. S1). Mutation of all four candidate sites (*pRh5ΔSd1234*) resulted in a similar expansion into most R8s ($94 \pm 3\%$) (Fig. 3B and E) as was observed for wild type *pRh5* reporter expression in *sd* knockdown retinas (Fig. 2G). Mutating the strongest Sd consensus site (*pRh5ΔSd3*, Fig. S1) (Sosinsky et al., 2003) showed a similar expansion ($93 \pm 1\%$) (Fig. 3C and E). In contrast, *Rh5* promoters in which the site with the next strongest consensus site (Sd2, *pRh5ΔSd2*) or the two weakest consensus sites (Sd1 and Sd4, *pRh5ΔSd14*) were mutated showed restricted reporter expression

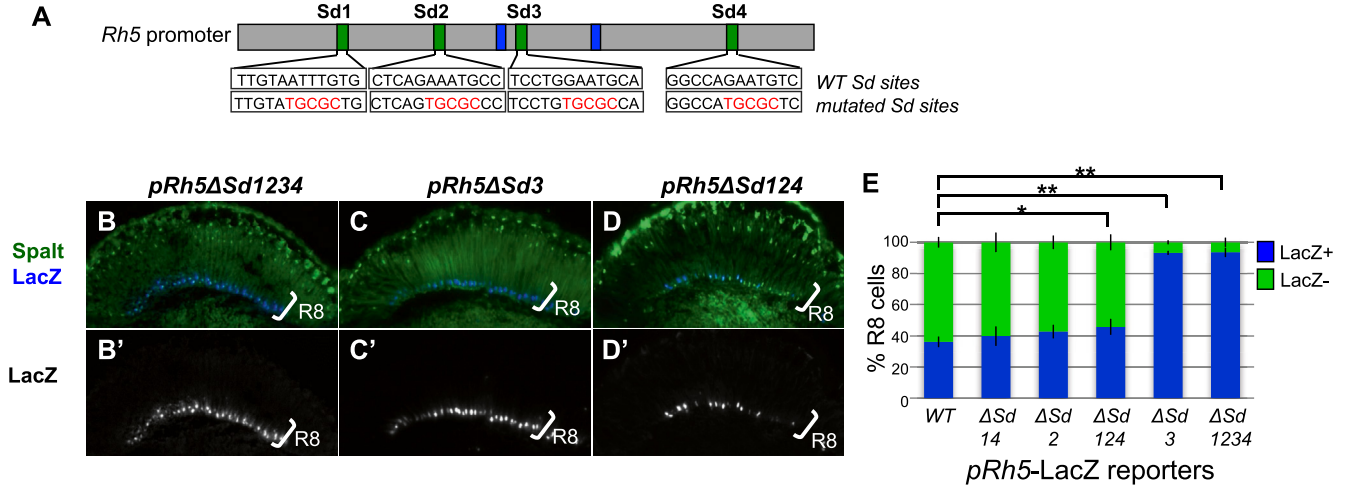

Fig. 3. Sd represses *Rh5* transcription through Sd/TEAD binding sites.
(A) Schematic of the *Rh5* promoter (−269/+50) showing the Sd binding sites (green boxes) as well as previously described Otd binding sites (blue boxes) (Tahayato et al., 2003). (B–D) Retina cryosections stained for Spalt (green) and LacZ (blue), or LacZ alone in wild type retinas, showing *pRh5ΔSd1234*(B), *pRh5*ΔSd3 (C) or *pRh5ΔSd124* (D) reporter expression. (E) Quantification of *pRh5* reporter expression in R8s (LacZ+ = blue; LacZ- = green). From left to right in graph: wild type *pRh5* (*pRh5WT* ($36 \pm 3\%$, n = 807), *pRh5ΔSd14* ($39 \pm 6\%$ n = 460), *pRh5ΔSd2* ($42 \pm 4\%$, n = 509), *pRh5ΔSd124* ($45 \pm 5\%$, n = 621), *pRh5ΔSd3* ($93 \pm 1\%$, n = 605) and *pRh5ΔSd1234* ($94 \pm 7\%$, n = 770). Error bars represent SD; * p < 0.05, **p < 0.01.

(42 ± 4% and 39 ± 6%, respectively), similar to the expression driven by the wild type *Rh5* promoter (36 ± 3%) (Figs. S2A, S2B, and 3E). Combinatorial mutations of all three weak Sd sites (*pRh5ΔSd124)* led to slight but significant expansion in reporter expression (45 ± 5%) (Fig. 3D and E). Together, these results suggest that the Sd3 site is key for the Sd-dependent *Rh5* repression in green PRs, while the other three sites together play an auxiliary role in this process.

In cell-based luciferase assays, Sd and Yki synergize with Otd to activate the −269/+50 *pRh5* promoter (Jukam et al., 2013). The above results, however, suggest that *in vivo*, Sd may repress *Rh5* gene expression in Yki-negative green PRs through the same regulatory region. To test if Sd was sufficient to repress *Rh5* promoter activity, we measured *pRh5*-dependent luciferase reporter activity in cultured *Drosophila* S2 cells in the presence or absence of Otd and Sd. As previously reported (Jukam et al., 2013; McDonald et al., 2010; Tahayato et al., 2003; Xie et al., 2007), Otd was sufficient to activate *Rh5* promoter activity in this system (Fig. S3A). Sd, on the other hand, significantly repressed Otd-mediated transactivation (Fig. S3A). Sd had no effect on basal *pRh5* expression (Fig. S3A). Consistent with Sd directly repressing Rh5 promoter expression, mutating all four candidate TEAD/Sd sites (*pRh5ΔSd1234*) abolished Sd-mediated repression *in vitro* (Fig. S3B). Mutating the third Sd site (*pRh5ΔSd3*), however, still resulted in significant Sd-dependent repression *in vitro* (Fig. S3B), in contrast to the relief of repression observed *in vivo*. We postulate that Sd levels are limiting *in vivo*, but when present at sufficient levels *in vitro*, can still repress *Rh5* gene expression through weaker binding sites. Combined, the *in vivo* and *in vitro* studies suggest that Sd is not required for promoting *Rh5* promoter activity in Yki-active blue R8s, but instead, represses Rh5 expression in Yki-inactive green R8s.

3.3. Yki antagonizes Sd repression but is not required for basal Rh5 gene expression

As shown in Fig. 2, Yki is required for *Rh5* gene expression in blue-sensitive R8 PRs. Previous studies have suggested that Yki can function as a Sd-dependent co-activator (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008) or an anti-repressor (Koontz et al., 2013). To test between these models in the context of PR fate decisions, we performed epistasis experiments with *sd* and *yki*. We found that similar to *sd* knockdowns (Fig. 2E), and opposite to *yki* knockdowns (Fig. 4A), *pRh5-LacZ* reporter expression was expanded to >90% R8 PRs (92 ± 3%) in *yki* and *sd* double knockdowns (Fig. 4B and D). These data indicate that *sd* functions downstream of *yki* to repress *Rh5*. Consistent with this, the *pRh5ΔSd3* reporter remains expressed in >90% R8 cells (91 ± 2%) in *yki* knockdowns (Fig. 4C and 4D). These data suggest that Yki is not essential for *Rh5* transcriptional activation but instead, antagonizes Sd-dependent repression in blue R8 PRs (Fig. 4E).

3.4. Yki antagonizes Sd repression of the subtype fate determinant gene melt

Besides the blue-sensitive *Rh5* opsin, Sd and Yki function together to promote the expression of the blue cell fate determinant *melt* (Fig. 1C). Our previous studies have shown that the expression of *melt* in blue PRs is regulated by a 450 bp enhancer, *melt450* (Jukam et al., 2013). As with the *pRh5* enhancer, analysis of the *melt450* enhancer revealed four putative Sd/TEAD binding sites (Fig. 5A and Fig. S1). Mutating these sites (*meltΔSd1234*) led to expansion of reporter expression into ~90% R8 cells (90 ± 2%) (Fig. 5B-5C, 5F). *meltΔSd1234* reporter expression was also retained in *yki* knockdowns retinas (Fig. 5E), in contrast to the wild-type reporter, whose expression is lost in *yki* knockdown retinas (Fig. 5D–5F). Therefore, *yki* is not required for *melt* expression in the absence of Sd site-dependent repression. Together, our data suggest that Sd represses both the blue R8 fate determinant *melt* and effector *Rh5* in green (*wts*+) PRs, while Yki antagonizes this Sd-dependent repression activity to allow their expression in blue (*wts*-) PRs.

3.5. The Rh5 3′UTR mediates post-transcriptional regulation of blue opsin protein expression

The above results indicate that neither Sd nor Yki are necessary for *Rh5* gene transcriptional activation, yet both are required for Rh5 protein expression (Fig. 1D–F), suggesting a role for post-transcriptional regulation. Given the importance of the 3′UTR in post-transcriptional regulation, we tested the possibility that the *Rh5* 3′UTR is required for Rh5 protein expression *in vivo*. The *pRh5* reporter described thus far carries the 3′ UTR of the SV40 T-antigen (Bischof et al., 2007). To test a potential role for the endogenous *Rh5* 3′UTR, we generated a new *Rh5* reporter construct that included the *Rh5* 3′UTR sequence (*pRh5-nLacZ-3′UTR^{Rh5}*, Fig. 6A) and analyzed its expression in wild type and *sd* knockdown retinas.

We first tested the transcription of the reporter by RT-PCR. *LacZ* transcription from the *pRh5-nLacZ-3′UTR^{Rh5}* reporter was detected in both control and *sd* knockdown eyes, but not in *yki* knockdown eyes (Fig. 6B and 6C), similar to *Rh5* mRNA expression (Fig. 2A and 2B). Thus, adding the *Rh5* 3′UTR did not affect the transcription of the new *pRh5-nLacZ-3′UTR^{Rh5}* reporter. The level of LacZ protein (β-galactosidase) expression from the *pRh5-nLacZ-3′UTR^{Rh5}* reporter, however, mirrored

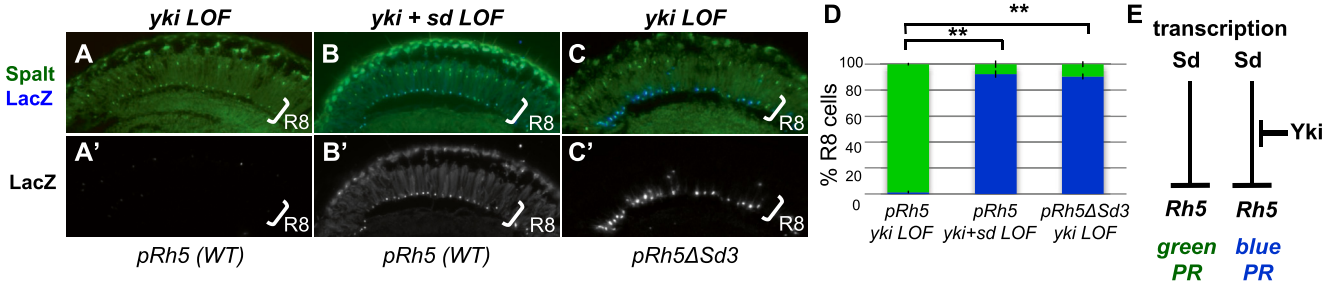

Fig. 4. Yki suppresses Sd-mediated *Rh5* repression.
(A–B) Retina cryosections stained with antibodies against Spalt (green) and LacZ (blue) for *pRh5-LacZ* reporter expression in *yki* knockdown retinas (A, *lGMR-GAL4 > UAS-yki^{RNAi} + UAS-Luciferase*) and *yki* and *sd* double-knockdown retinas (B, *lGMR-GAL4 > UAS-sd^{RNAi} + UAS-yki^{RNAi}*). LacZ protein-positive R8 cells were rarely detected in *yki* knockdown retinas (1.5 ± 1%) (A, A′), while LacZ protein was in almost all R8 cells in *yki + sd* knockdown retinas (92 ± 3%) (B, B′). (C) Retina cryosections stained with antibodies against Spalt (green) and LacZ (blue) for *Rh5ΔSd3*-reporter expression in *yki* knockdown retinas (*lGMR-GAL4 > UAS-yki^{RNAi}*) shows its expression in almost all R8 cells (91 ± 2%) (C, C′). (D) Quantification of *pRh5* reporter expression in R8s. From left to right in graph: wild type *pRh5* (*pRh5*) in *yki* knockdown retinas (n = 564), *pRh5 in yki* and *sd* double knockdown *retinas* (n = 498) and *pRh5ΔSd3* in *yki* knockdown retinas (n = 520). Error bars represent SD; **p < 0.01. (E) A model showing that Sd and Yki act antagonistically in regulation of *Rh5* transcription. At the transcriptional level, Sd represses *Rh5* transcription, while Yki is required for *Rh5* transcription by antagonizing the Sd repressor activity.

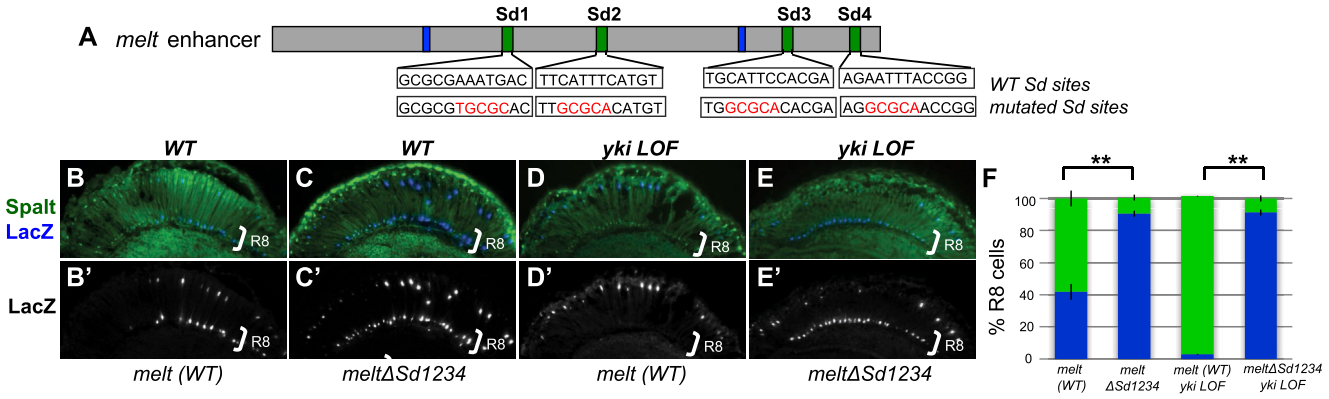

Fig. 5. Sd directly represses *melt* transcription in green R8s.
(A) Schematic of the 450bp *melt* enhancer showing the Sd binding sites (green bars) and previously described Otd binding sites (blue bars) (Jukam et al., 2013). (B–E) Retina cryosections stained with antibodies against Spalt (green) and LacZ (blue) to show the wild type and Sd site-mutated *melt* enhancer reporters expression in wild type retinas (B and C) and *yki* knockdown retinas (D). Wild type *melt* enhancer reporter is expressed in a subset of R7 and R8 cells in wild type retinas (41 ± 5%) (B, B′). The expression of the Sd site-mutated *melt* enhancer reporter (*meltΔsd1234*) is expanded to most R8 cells (90 ± 2%) (C, C′). *meltΔsd1234* reporter expression is not affected in *yki* knockdown retinas (91 ± 2%) (E, E′), compared to the wild type *melt* reporter, which expression is lost in R8 cells in *yki* knockdown retinas (3 ± 0.2%) (D, D′). (F) Quantification of *melt* enhancer reporter expression in R8s. From left to right in graph: wild type *melt* enhancer in wild type retinas (*melt (WT)*, (n = 750), *meltΔSd1234 in* wild type retinas (n = 420), *melt (WT)* in *yki* knockdown retinas (n = 561), and *meltΔSd1234* in *yki* knockdown retinas (n = 465). Error bars represent SD; **p < 0.01.

endogenous Rh5 protein expression: LacZ was detected in wild type eyes (Fig. 6D), but was absent from *sd* knockdown retinas (Fig. 6E). These findings lead to the conclusion that the 3′UTR of the *Rh5* gene is essential for its post-translational regulation in blue *vs*green PRs.

3.6. microRNA processing genes are required for Sd-mediated Rh5 post-transcriptional regulation

3′UTRs often contain regulatory regions for microRNAs (miRNAs) that post-transcriptionally influence gene expression. To investigate if

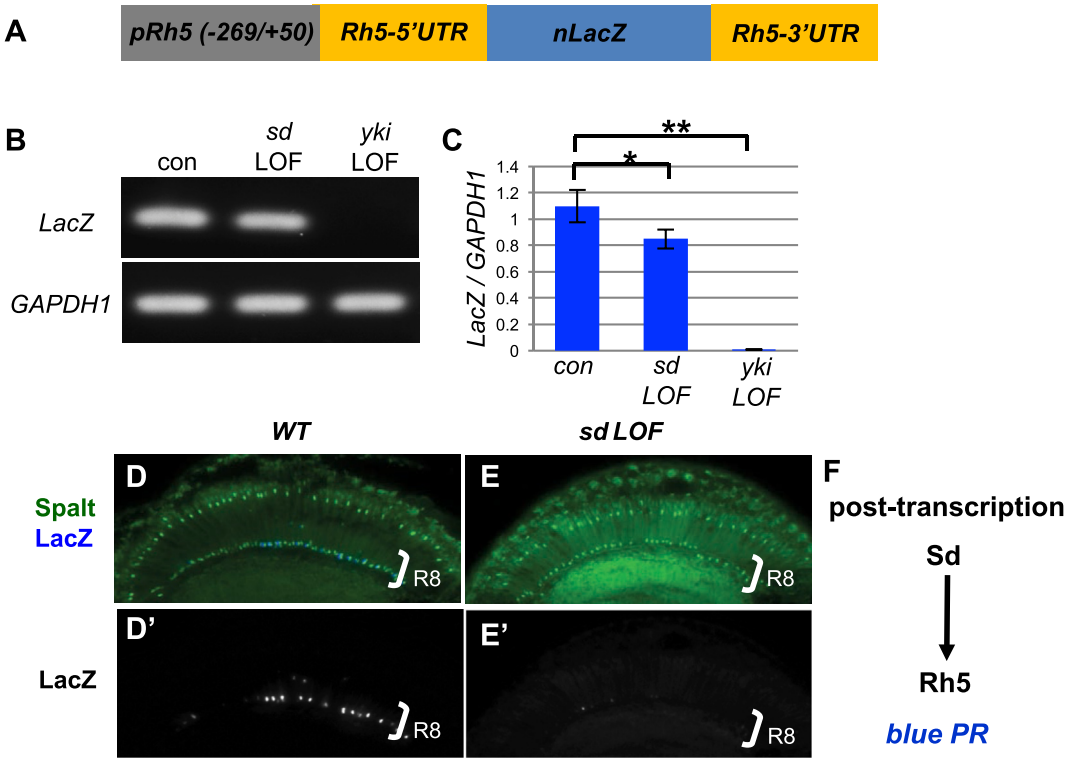

Fig. 6. The *Rh5* 3′ UTR mediates *Rh5* post-transcriptional regulation.
(A) Schematic of the Rh5-n*LacZ-3′UTR* reporter, carrying the wild-type *Rh5* promoter (−269/+50) and its 147 bp 3′UTR. (B) RT-PCR for *LacZ* mRNA in control (*lGMR-GAL4*), *sd* knockdown (*lGMR-GAL4 > UAS-sd^{RNAi}*), *or yki* knockdown (*lGMR-GAL4 > UAS-yki^{RNAi}*) retinas. *LacZ* mRNA is detected in wild type and *sd* knockdown retinas, but not in *yki* knockdown retinas. (C) Quantification of the *LacZ*mRNA expression from (B), normalized to *GAPDH1*. Error bars represent SD; *p < 0.05; **p < 0.01. (D–E) Retina cryosections stained with antibodies against Spalt (green) and LacZ (blue) to show expression of *Rh5-nLacZ-3′UTR^{Rh5}* reporter in wild type and *sd* knockdown retinas. *Rh5-nLacZ-3′UTR^{Rh5}* reporter is expressed in a subset of R8 cells in wild type retinas (D, D′), the same pattern as *Rh5-nLacZ-3′UTR^{SV40}* (Fig. 2D). The expression of *Rh5-nLacZ-3′UTR^{Rh5}* reporter is rarely detected by LacZ staining in *sd* knockdown retinas (E,E′), compared to the pan-R8 expression of *Rh5-nLacZ-3′UTR^{SV40}* in *sd* knockdown retinas (Fig. 2C). (F) A model showing Sd is required for Rh5 expression at the post-transcriptional level.

miRNA genes are involved in Rh5 post-transcriptional regulation, we tested the effects of manipulating the miRNA processing factors Drosha, Dicer-1 and Loquacious (Loqs). Drosha cleaves primary miRNA transcripts into hairpin-shaped pre-miRNAs (Fukuda et al., 2007; Lee et al., 2003; Martin et al., 2009), while Dicer-1 is necessary for processing pre-miRNAs into functional miRNAs (Lee et al., 2004). Loqs is a partner of Dicer-1 and is generally required for efficient miRNA procession (Fukunaga et al., 2012; Lim et al., 2016). Knockdown of the *drosha*, *Dicer-1* or *loqs* genes alone in the *Drosophila* eye did not cause obvious changes in blue and green R8 fate specification (Fig. 7A–D and Fig. S4). However, double knockdown of *sd* and either *drosha*, *Dicer-1* or *loqs* led to significant co-expression of Rh5 and Rh6 protein in R8 cells ($73 \pm 14\%$, $50 \pm 2\%$ and $40 \pm 4\%$ co-expression, respectively) (Fig. 7F–H and Fig. S4), compared to a small amount of co-expression R8 cells ($7 \pm 4\%$) in *sd* knockdown eyes (Fig. 7E and Fig. S4). These results indicate that knocking down the miRNA machinery significantly promotes the expression of Rh5 protein, corroborating a scenario in which Sd regulates Rh5 post-transcriptionally through a pathway involving miRNAs.

4. Discussion

The mutually exclusive expression of sensory receptor genes in sense organs is essential to prevent sensory input overlap in the mature organism. Here, we show that, in the fly retina, the TEAD factor Sd achieves this in blue and green PRs using two different mechanisms: direct transcriptional repression of the blue fate determinant *melt* and blue *Rh5* opsin genes in green photoreceptors, and relief of post-transcriptional control of the *Rh5* mRNA in blue photoreceptors. In addition, Yki, a major Sd cofactor, antagonizes Hippo-specific and Sd-dependent repression of *melt* and *Rh5* to promote blue PR fate. Thus, Sd and Yki play multiple roles to ensure a robust bistable cell fate decision in post-mitotic sensory neurons.

The antagonistic relationship between Sd repression and Yki derepression is similar to the model previously proposed in cell cycle control (Koontz et al., 2013). Nevertheless, the mechanisms by which Sd represses gene expression in green PRs remains unknown. In cell growth, for instance, repression is mediated in part through Tgi, a Tondu domain containing protein, which Yki competes with to alleviate repression (Koontz et al., 2013). However, we did not detect a significant change in Rh5 protein or reporter expression with knockdown of *Tgi* in PRs (data not shown), suggesting the existence of another Sd co-repressor in this system. Indeed, a zinc finger protein Nerfin-1 was recently identified as a Tgi-independent Sd co-repressor that participates in Hippo-dependent cell growth and competition during *Drosophila* eye development (Guo et al., 2019). Our preliminary studies showed that knockdown of *nerfin-1* led to an expansion of Rh5-expressing blue PRs at the expense of green PRs (data not shown), comparable to the expanded expression of Sd site mutants in the *melt* and *Rh5* reporters. Therefore, Nerfin-1 is very likely to be at least one Sd co-repressor during blue- and green PR fate specification in the *Drosophila* eye. Combined, these findings suggest Sd repression activity is a general mechanism in controlling the output of the Hippo pathway.

If the role of Sd in green PRs were solely to repress *Rh5* transcription, then *Rh5* mRNA levels might be expected to be elevated in *sd* mutants relative to controls. Instead, we observed a ~50% reduction (Fig. 2A and 2B). This observation could reflect two possibilities, which are not mutually exclusive. First, based on our previous (Jukam et al., 2013) and unpublished findings that Otd cooperates with Yki to activate *Rh5* in Hippo-negative blue PRs, we expect that in *sd* mutants, where all R8s switch to Hippo-positive (and hence Yki-inactive) green PRs, *Rh5* activation in green PRs would be reduced. Second, since our current studies suggest a new role for miRNAs in the post-transcriptional control of *Rh5*, it is possible that *Rh5* mRNA stability is affected in *sd* mutants.

In terms of the post-transcriptional control of *Rh5*, we demonstrated that the *Rh5* 3′ UTR was required to prevent its co-expression with Rh6 in *sd* knockdown green PRs. In addition, the simultaneous knockdown of *sd*

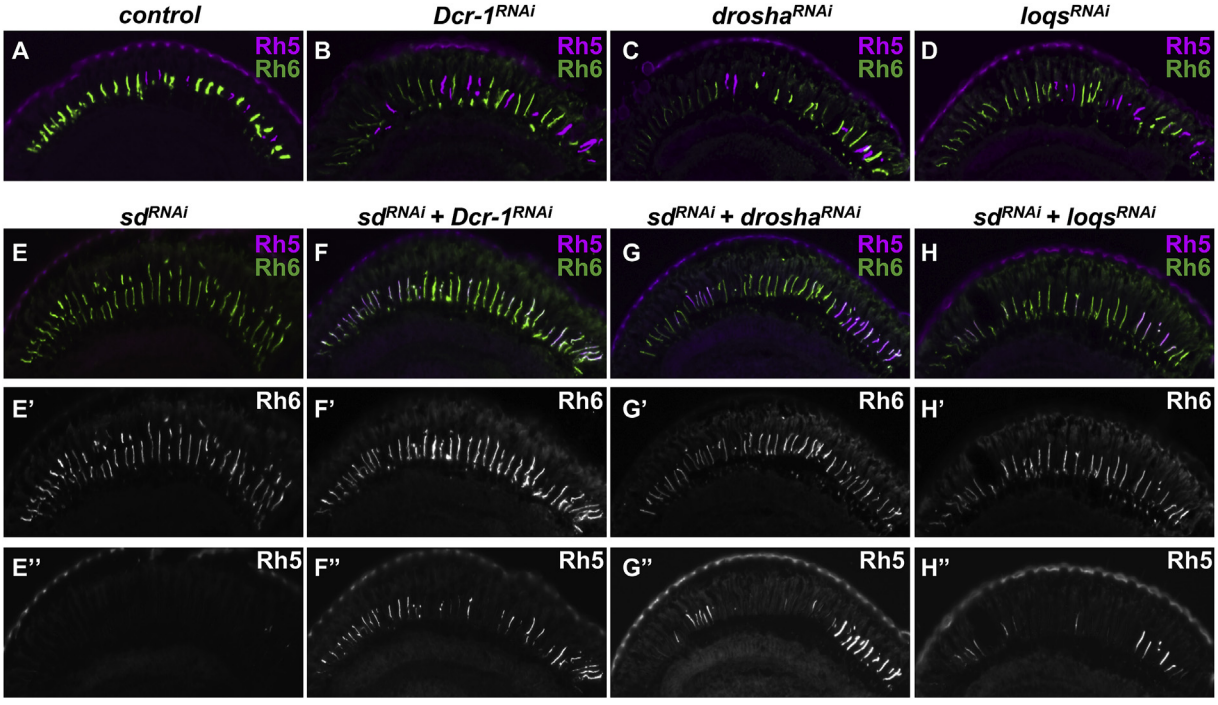

Fig. 7. miRNA processing genes are required for Sd-mediated Rh5 post-transcriptional regulation.
(A–H) Retina cryosections stained with antibodies against Rh5 (magenta, or grayscale in E″-H″) and Rh6 (green, or grayscale in E′-H′) in which *sd* (E–H) and/or components of the miRNA processing machinery (*Dcr-1* [B,F], *drosha* [C,G], or *loqs* [D,H]) have been knocked down with *lGMR-GAL4*. *UAS-Luciferase* was used as a control in A-E to balance the number of the UAS sites. In the presence of Sd, Rh5 and Rh6 are mutually exclusive in a ~30:70 ratio (A–D), whereas *sd* knockdown in the presence of miRNA processing gene knockdowns leads to expansion of Rh5 and co-expression with Rh6 in green PRs (F–H, $73 \pm 14\%$, $50 \pm 2\%$ and $40 \pm 4\%$ Rh5 and Rh6 co-expression, respectively), compared to the co-expression being rarely detected in *sd* only knockdown retinas ($7 \pm 4\%$) (E). [see Fig. S4 for quantification and statistical analysis].

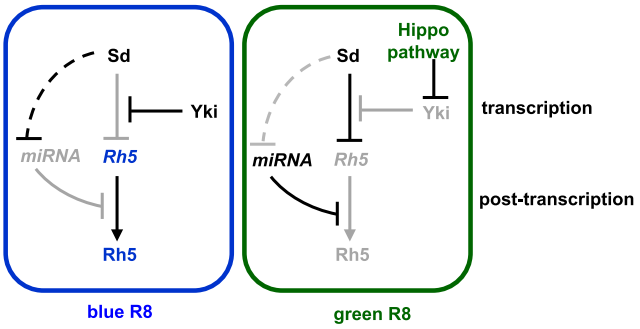

Fig. 8. Model of Sd and Yki cooperatively specifying blue PR subtype fate. Sd and Yki play distinct roles in blue and green PR subtype fate specification. At the transcriptional level, Sd represses *Rh5* (and *melt*) gene transcription in Hippo-positive green PRs, while nuclear-localized Yki in blue PRs antagonizes Sd repression. In green PRs, active Hippo signaling inactivates Yki by preventing its entry into the nucleus. Besides the transcriptional repression by Sd, it is also involved in promoting Rh5 protein expression in blue PRs by repressing miRNA-dependent targeting of the *Rh5 3′UTR*, showing a cooperative role with Yki in promoting blue PR fate. Grey font denotes genes or proteins that are not expressed or not active.

and miRNA processing machinery genes led to Rh5 protein de-repression (and co-expression with Rh6) in a substantial subset of green R8 cells. Together, these data suggest miRNA-dependent regulation of *Rh5* depends on Sd, either directly or indirectly. We posit that, as a transcription factor, Sd prevents the transcription of *Rh5*-directed miRNA genes (Fig. 8). However, follow-up studies will be important for defining the complete repertoire of miRNA-dependent events involved in this Hippo-directed cell fate decision. For example, possible differences in an *pRh5* reporter and endogenous Rh5 protein were reported in retinas mutant for the transcription factor *PvuII-PstI homology 13* (*pph13*) (Jukam et al., 2013). While this disparity could be due to the rhabdomere defects observed in *pph13* mutants (Mishra et al., 2010), there is potential for a role for Pph13 in *Rh5* post-transcriptional regulation. Finally, it is possible that the *Rh5* 3′UTR recruits other non-coding RNAs or proteins to regulate its expression.

Combined, the bimodal functions of Sd in Yki-vs Wts-positive cells form a feedforward regulatory module in post-mitotic PR fate decisions, robustly preventing sensory receptor overlap. Feedforward modules between transcription factors and miRNAs have been previously reported in neuronal differentiation and other biological processes (Alon, 2007; Chang et al., 2007; Li et al., 2009; O'Donnell et al., 2005). For example, the proto-transcription factor c-Myc can directly activate E2F1 transcription (Leung et al., 2008), but also limit E2F1 translation by activating miR-175p and miR-20a (O'Donnell et al., 2005). In contrast to the c-Myc-miRNAs-E2F1 activation module, which fine-tunes a proliferative signal in dividing cells (El Baroudi et al., 2011), however, the Sd-miRNA-Rh5 repression module ensures a robust ON-OFF switch in the terminal PR differentiation process. If similar mechanisms take place during Hippo-dependent cell growth remains to be determined.

Whether *yki* is also involved in Sd's post-transcriptional control in blue PRs remains unresolved, as *yki* itself is essential for blue PR fate, and hence, Rh5-expressing cells. Previous studies have demonstrated that Yki is important for the activation of at least one miRNA to promote cell growth (i.e. *bantam*) (Oh and Irvine, 2011). However, in the case of *Rh5* regulation, the miRNA must be repressed in Yki-expressing cells, rather than activated. In this context, it is worth noting that the Yki ortholog YAP has been shown to mediate widespread miRNA suppression in tumor cells (Hippo-negative) by sequestering an RNA helicase p72/DDX-17, a regulatory component of microRNA-processing machinery (Koontz et al., 2013; Mori et al., 2014). Comparably, our results suggest that the miRNA(s) is/are inactive in Yki-positive blue PRs in order to allow Rh5 protein expression. These findings raise the possibility that YAP/Yki- and TEAD/Sd-dependent regulation of miRNA biogenesis is a universal mechanism in control of the Hippo signaling pathway in tissue growth and neuronal cell fate decisions.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.06.022.

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