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Opposite Feedbacks in the Hippo Pathway for Growth Control and Neural Fate

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Abstract

Signaling pathways are re-used for multiple purposes in plant and animal development. The Hippo pathway in mammals and *Drosophila* coordinates proliferation and apoptosis via the co-activator and oncogene, YAP/Yorkie (Yki), which is homeostatically regulated through negative feedback. In the *Drosophila* eye, cross-repression between the Hippo pathway kinase, LATS/Warts (Wts), and growth regulator, Melted, generates mutually exclusive photoreceptor subtypes. Here, we show that this all-or-nothing neuronal differentiation results from Hippo pathway positive feedback: Yki both represses its negative regulator, *warts*, and promotes its positive regulator, *melted*. This post-mitotic Hippo network behavior relies on a tissue-restricted transcription factor network—including a conserved Otx/Orthodenticle-Nrl/Traffic Jam feedforward module—that allows Warts-Yki-Melted to operate as a bistable switch. Altering feedback architecture provides an efficient mechanism to co-opt conserved signaling networks for diverse purposes in development and evolution.

Core signaling pathways are re-used for different purposes during development, allowing extraordinary cell-type diversity (1). For example, the TGF- β , Notch, RTK/MAPK, and Wnt signaling pathways each act repeatedly, from embryogenesis to adulthood, to coordinate tissue patterning, growth and specification throughout the animal. The Hippo pathway is best known for its role in growth control in both flies and mammals, where it regulates the balance between division and death in mitotic cells (2). But the Hippo pathway also regulates post-mitotic events such as photoreceptor subtype specification in the *Drosophila* eye (3, 4). How the same signaling network can be regulated for context-appropriate outcomes as diverse as proliferation and differentiation is not well understood.

The *Drosophila* eye comprises about 800 unit eyes (ommatidia), each containing eight photoreceptors (R1-R8) (5). Two main ommatidial subtypes are defined by light-sensing

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Supplementary Materials

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Materials and Methods

Figs. S1-S13

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Rhodopsin (Rh) proteins expressed in the color vision photoreceptors, R7 and R8: ‘p’ ommatidia, with UV-sensitive Rh3 in R7 and blue-Rh5 in R8, and ‘y’ ommatidia with longer UV-Rh4 in R7 and green-Rh6 in R8 (Fig. 1A and reviewed in (6)). **p** and **y** subtypes are distributed randomly in the retina in a **p:y** ratio of ~30:70, following stochastic expression of the transcription factor Spineless in the R7 of subtype **y** (**yR7s**). **pR7s**, which lack Spineless, signal to underlying R8s to induce **pR8/Rh5** fate, whereas the remaining R8s become **yR8/Rh6** by default (6). **p** vs. **y** fate in R8s is established by a bistable transcriptional feedback loop between Melted (Melt), a pleckstrin homology-domain protein that specifies **pR8/Rh5** fate, and Wts, a kinase in the Hippo pathway that specifies **yR8/Rh6** fate (Fig. 1B-C) (3).

In its canonical role as a tumor suppressor, Wts is activated by the Hippo kinase (Hpo) and phosphorylates Yki, the *Drosophila* ortholog of the human oncogene YAP, to sequester Yki in the cytoplasm (2). In the absence of Hippo signaling, non-phosphorylated Yki enters the nucleus and binds as a co-activator to transcription factors like Scalloped (Sd), Homothorax (Hth) and Mothers against Dpp (Mad) (7-11) to activate target proliferation and anti-apoptotic genes. Yki can also induce its negative regulators *expanded*, *merlin*, *kibra*, or *dmyc* to provide negative feedback onto itself during growth control (12-14).

Here we show that in post-mitotic R8s, as in growth, Yki and its DNA-binding partner Sd mediate transcriptional output of the Hippo pathway. However, the R8 regulatory architecture is fundamentally different, as Yki promotes positive feedback onto itself. This regulation requires a tissue-specific transcription factor network that includes Orthodenticle (Otd) and Traffic Jam (Tj), orthologs of the mammalian photoreceptor determination genes Crx and Nrl (15), respectively, as well as Pph13 and Gfi1/Senseless (Sens). This network generates the post-mitotic context for the Hippo pathway to regulate an all-or-nothing fate decision and ensure robust terminal differentiation of sensory neuron subtypes.

Yki and Sd regulate R8 subtype specification

To test whether Yki functions in R8 neurons, we manipulated *yki* and assayed Rh5 and Rh6 expression. *yki* null mutant eye progenitor cells do not divide and are eliminated by apoptosis (16). We therefore used Gal4 drivers to express *yki*-targeted RNA interference (RNAi) in post-mitotic photoreceptors. Knockdown of *yki* in all photoreceptors early (*IGMR>yki^{RNAi}*), only in adults (using Gal80^{ts}), or in all R8s (and some R1-R6) (*sens>yki^{RNAi}*) caused almost all R8s to express Rh6, whereas Rh5 was nearly absent (Fig. 1D and fig. S1A, C). Conversely, over-expressing wild-type or activated *yki/YAP* (*yki^{S168A}* or human *YAP^{S127A}*) (17, 18) in all photoreceptors (*IGMR>yki*) transformed almost all R8s into Rh5-expressing **pR8s** (Fig. 1D and fig. S5C). Ectopic Yki did not require the **pR7** signal to induce **pR8** fate since Yki induced Rh5 even in the absence of R7s (*sev*; *GMR>yki*) (fig. S2A). Furthermore, mis-expressing *yki* only in **yR8s** after the fate decision (*Rh6>yki*) also induced Rh5 (fig. S2C). *yki* manipulations did not affect general neuronal fate, specific photoreceptor fate, or expression of other Rhodopsins (fig. S2B, D-F). Thus, *yki* is necessary and sufficient in R8s to specify **pR8/Rh5** and prevent **yR8/Rh6** subtypes.

Yki is a co-factor of DNA-binding transcription factors such as Sd, Hth, or Mad (7-11) to activate Hippo target genes. *hth* or *mad* loss-of-function (*IGMR>hth^{DN}* (19) and *IGMR+ey>mad^{RNAi}*) (fig. S3D) did not noticeably affect Rh5 or Rh6. In contrast, retinas with *sd* loss-of-function (*sd^B*, *sd^C*, *sd^{47M}*, or *IGMR>sd^{RNAi}*), like *yki* loss-of-function eyes, expressed Rh6 in almost all R8s with a corresponding loss of Rh5 (Fig. 1F and fig. S3E) and did not affect other photoreceptor subtypes (fig. S3A-C). *sd* mutants or *sd^{RNAi}* also suppressed the *yki* gain-of-function phenotype (*sd*; *IGMR>yki*) (Fig. 1F and fig. S3E-F),

indicating that *sd* is required for *yki* function and likely encodes the Yki partner required for Rh5 and Rh6 regulation.

We next confirmed that *yki* acts canonically downstream of Wts to regulate Rhodopsin expression. In yR8s, Merlin (Mer) constitutively activates Hippo signaling to promote Wts activity (4). Their loss (*mer^d* or *wts^{RNAi}*) led to Rh5 expansion, but *yki^{RNAi}* suppressed these phenotypes (Fig. 1G and fig. S4A); furthermore, ectopic *yki* (*GMR-yki*) strongly suppressed Hippo pathway-induced Rh6 (*GMR-wts*, *GMR-hpo*, or *GMR-wts+sav*) (fig. S4B-C). We also tested whether *yki* and *melt* require each other to activate Rh5. Strong (*ey+IGMR>yki^{RNAi}*) or even mild (*yki^{B5/+}*) *yki* loss-of-function suppressed the ability of ectopic *melt* to induce Rh5 (fig. S4D). However, ectopic *yki* still induced Rh5 when *melt* was absent, even when expressed late in yR8s (*Rh6>yki; melt*) (Fig. 1H), consistent with *yki* functioning downstream of Melt and the Hippo pathway to regulate Rhodopsins. This regulation occurred through Wts-dependent Yki phosphorylation and inactivation because mis-expression of dominant negative kinase-dead (KD) forms of Wts or Hpo in the retina (*IGMR>hpo^{KD}* or *IGMR>wts^{KD}*) (3) or constitutive gain-of-function alleles that reduce phosphorylation by Wts (*yki^{Dbo1}*, *yki^{Dbo2}*) (20) resulted in Rh5 expression in >70% of R8s (fig. S5 and ref. 3). Thus, the molecular relationship among Hippo pathway members in growth also exists in R8 fate.

Yki creates network-level Hippo pathway positive feedback by regulating *wts* and *melt*

We next assessed whether *yki*-dependent feedback exists with its upstream regulators in R8s by removing retinal *yki* and *sd* function and assaying expression of *wts* (yR8s/Rh6) and *melt* (pR8s/Rh5). *yki^{RNAi}* or *sd^{47M}* mutant clones caused *wts-lacZ* expansion and *melt-lacZ* loss in most R8s (Fig. 2A-B, D), mirroring the gain of Rh6 and loss of Rh5. Conversely, activated *yki* (*yki^{Dbo1/+}*, *IGMR>yki*, or *IGMR>yki^{S168A}:GFP*) expanded *melt-lacZ* into >85% of R8s, with correspondingly decreased *wts-lacZ* (Fig. 2C, fig. S6A-C), and this function required *sd* (fig. S6A-C). Therefore, in post-mitotic photoreceptors Yki promotes its own activity with positive network-level feedback by activating *melt* and repressing its direct negative regulator, *wts*—a regulation opposite from that in growth control.

Yki/Sd could regulate the *wts-melt* cross-repression by activating *melt*, repressing *wts*, or both. To determine the feedback mechanism, we first performed epistasis analysis between *yki* and *wts* while monitoring *melt* expression. In *wts^{RNAi}* retinas, *melt* expanded into all R8s, yet when *yki* was simultaneously removed (*IGMR>wts^{RNAi}+yki^{RNAi}*), *melt* expression was lost (Fig. 2D). Conversely, ectopic *wts* failed to repress *melt* in the presence of ectopic *yki* (fig. S6C). Thus, *yki* does not activate *melt* by repressing *wts*; rather, it acts downstream of *wts* to promote *melt*.

If Yki repressed *wts* exclusively by inducing *melt*, *wts* should be de-repressed in *melt* mutants even if upstream Hippo signaling is inactive. However, *wts-lacZ* was absent in *mer;melt* double mutant R8s (fig. S6D), suggesting that a *melt* independent factor(s) represses *wts* in *mer* mutants. We hypothesized that the factor(s) includes Yki. Indeed, *melt* failed to repress *wts* in the absence of *yki* or *sd* (Fig. 2D), and ectopic *yki* largely retained the ability to repress *wts* expression in the absence of *melt* (*melt; GMR>yki*) (Fig. 2E). Thus, Yki not only functions downstream of *wts* to activate *melt*, but also functions downstream of *melt* to repress *wts*.

If ectopic Yki can repress *wts* independently of *melt*, what is the role of *melt*? Although all R8s expressed Rh6 in *melt* mutants, up to 20% of R8s still co-expressed Rh5 in 1-day old adults, which decreased to <8% after three weeks (fig. S7). Because no such delay was

observed when *yki* was removed, and because *yki* activation consistently induces Rh5 in 100% of R8s, we infer that the transient Rh5 expression in young *melt* mutant flies reflects transient Yki activity. The above data suggest that Melt and Yki are temporally separated: Melt is not required to initiate **pR8** fate in response to the **pR7** signal, but instead likely consolidates Wts inactivity and Yki activity immediately after the fate decision to ensure robust Yki function in **pR8**s.

Altogether, these data indicate that Yki regulates both *wts* and *melt* to promote **pR8** specification, and reveal that Yki network-level positive feedback occurs through two mechanisms (Fig. 2F): (i) a Wts-Yki double negative feedback loop, wherein Wts inactivates Yki biochemically whereas Yki represses *wts* transcription; and (ii) a double positive feedback loop between Melt and Yki, wherein Yki activates *melt* expression, and Melt promotes Yki (or inhibits the Hippo pathway to activate Yki). This combination ensures a complete switch from the default (**yR8**) to the induced (**pR8**) fate.

The R8 Hippo network is distinct from the Hippo growth regulation network

To evaluate the context-specificity of the R8 Hippo network, we asked whether *wts* transcriptional regulation is specific to post-mitotic R8 cells. We used the G-TRACE lineage reporter (21) to simultaneously label historical (GFP) and contemporary (RFP) *wts-Gal4* expression and found that GFP was only co-expressed with RFP in **yR8**s (Fig. 2G). This indicates that *wts-Gal4* was not expressed earlier in mitotic **pR8** progenitor cells, and instead is activated post-mitotically to control R8 subtype specification.

We also asked whether the novel Hippo regulatory relationships in R8—*melt* repressing *wts*, *yki/sd* repressing *wts* and activating *melt*—also exist in growth contexts. First, we examined *wts-lacZ* expression following Hippo pathway manipulation in the posterior compartment of the larval wing disc using an *engrailed (en)-Gal4* driver. Unlike in R8s, neither *yki^{RNAi}* nor *wts* mis-expression increased *wts-lacZ* expression. Furthermore, in third instar larval eye discs, we did not detect up-regulation of *wts-lacZ* in *sd* mutant clones (fig. S8A) or of *melt-lacZ* in *wts* mutant clones (fig. S8B). This suggests that the Hippo pathway does not regulate the expression of *wts* or *melt* in dividing epithelial cells of the wing or eye disc.

We next tested whether the Wts-Yki-Melt regulatory circuit exists in growth control by manipulating *melt* and assaying the Yki growth target Ex. Whereas ectopic *yki* or *wts^{RNAi}* (which phenocopy *melt* gain-of-function in R8) autonomously induced Ex levels when expressed in the wing, ectopic *melt* did not increase Ex protein or *ex* transcription (*ex-lacZ*) (fig. S8D). Although *en>melt* adult wings were slightly larger than *en-Gal4* control wings, strong over-expression of *melt* in the developing and adult eye did not noticeably affect eye size or morphology (fig. S8C). This difference is likely due to *melt*'s known role in the wing as a growth promoter in the Insulin/TOR pathway (22), which is dispensable for *melt*-dependent R8 subtype determination (3). Collectively, these experiments indicate that the regulatory architecture and transcriptional feedback mechanism of the Hippo network differ between R8 subtype specification and growth regulation in at least two mitotically active tissues.

A conserved feedforward Otd-Tj (Crx–Nrl) module regulates photoreceptor subtypes

Given the differences in Yki-mediated feedback in growth and R8 fate, we investigated how the Wts-Yki-Melt regulatory circuit is established specifically in R8s but not in growth. Because *melt* is a context-specific inhibitor of the Hippo pathway, we focused on mechanisms underlying *melt* regulation in the eye. We generated serial deletions of the *melt*

first intron (4kb), previously shown to confer expression in **pR8s** (3), and identified a 450bp (*melt450-lacZ*) element that drives reporter expression in **pR8s** (and **pR7s**) and responds appropriately to *wts* in R8 (Fig. 3A-B and fig. S9A-B). *melt450* contains two conserved K₅₀ homeodomain (HD) binding sites whose mutations abolished reporter expression (Fig. 3A-B and fig. S9A), suggesting that a K₅₀ factor directly promotes *melt* expression. A good candidate was the pan-photoreceptor K₅₀ HD transcription factor, Otd (23), which directly activates *Rh5* in **pR8s** and controls proper **pR8:yR8** ratios (24-26) (fig. S9C). Consistent with *otd* being required for *melt* transcription, *melt-lacZ* was lost from all R8s when *otd* was removed (*sens>otd^{RNAi}*) (Fig. 3B), and *melt450-lacZ* was lost in eye-specific *otd^{uvi}* mutants (fig. S9B). Moreover, Otd was sufficient to activate a *melt450-luciferase* (*luc*) reporter 3-fold in cultured *Drosophila* S2 cells, requiring intact K₅₀ sites (Fig. 3C). Thus, Otd directly activates expression of both the fate determinant, *melt*, and its downstream output, *Rh5*, generating feedforward regulation to promote **pR8** fate (Fig. 3C).

Because Otd is expressed in all photoreceptors, we posited that other factor(s) act with Otd to regulate the Hippo pathway in R8. We performed a photoreceptor-specific RNAi screen and identified *traffic jam* (*tj*), a basic leucine zipper (bZIP) transcription factor (27). *tj* knockdown in all photoreceptors, or a null *tj* allele we generated (*tj⁻¹*), each led to loss of Tj protein and a significantly reduced Rh5:Rh6 ratio (Fig. 4A-C and fig. S10B-C) without affecting R7 opsins (fig. S10D), suggesting that Tj is required for **pR8** fate.

Tj retinal expression preceded Rhodopsin expression and was restricted to all R7s and R8s from ~60 hours after pupal formation (APF) into adulthood (Fig. 4F and fig. S10A). *tj* knockdown in R8 (*sens>tj^{RNAi}*) phenocopied *tj⁻¹* mutants, whereas R7 knockdowns (*sev>tj^{RNAi}*) had wild-type Rh5:Rh6 ratios (fig. S10B-C, E), indicating that *tj* autonomously affects R8 fate.

Consistent with Tj's role in promoting **pR8** fate, *melt-lacZ* was lost and *wts-lacZ* was expanded into most *tj* mutant R8s (Fig. 4D and fig. S11A). Epistasis experiments revealed that *tj* both promotes *melt*, independently from *wts* (*wts^{p1}; IGMR>tj^{RNAi}; melt-lacZ*) (Fig. 4D and fig. S11A), and is necessary for *melt* to fully repress *wts* (*tj⁻¹; IGMR>melt; wts-lacZ*) (Fig. 4D and fig. S11A). Furthermore, although Rh5 was uniquely expressed in all R8s in *melt* gain-of-function or *wts* loss-of-function retinas, simultaneous removal of *tj* in either situation resulted in Rh5:Rh6 co-expression in most R8s (Fig. 4E and fig. S11B). This indicates that *tj* functions downstream of *wts* and *melt* to repress Rh6, but does not activate Rh5. Combined, these experiments reveal that Tj controls **pR8** fate through three distinct mechanisms: Tj promotes *melt*, represses *wts*, and represses *Rh6* (Fig. 4E, right).

Tj is the single *Drosophila* ortholog of the four mammalian MAF-bZIP transcription factors (28). One MAF factor—Nrl (Neural Retina Leucine zipper)—is a target of the Otd orthologs, Otx2 and Crx, and functions synergistically with Crx in the mouse retina to promote rod photoreceptor formation at the expense of cones (29-31). Given that *otd* and *tj* also promote one photoreceptor fate (**pR8s**) at the expense of another (**yR8s**), we examined the genetic relationship between *otd* and *tj* in the fly retina. Otd was unaffected in *tj^{RNAi}* retinas (fig. S11C), but Tj was absent in *otd^{uvi}* mutants (Fig. 4F). Thus, Otd promotes *tj* in fly photoreceptors, analogous to how Otx2/Crx promotes Nrl in mammalian rods. Otd-dependent **pR8** gene expression, however, is not just a consequence of activating *tj*, as re-supplying *tj* in *otd^{uvi}* mutants did not restore *melt* (fig. S11D-E). Hence, *otd* acts upstream of *tj*, but both are required to promote *melt* expression. Thus, similar to Crx and Nrl regulation of mammalian photoreceptor fate, *otd* and *tj* form a coherent feedforward loop that promotes **pR8** fate (Fig. 4H).

This model predicts that Otd and Tj cooperate to promote *melt*. Indeed, although no MAF consensus DNA binding sites were detected in the *melt450* enhancer, Tj was sufficient to induce *melt450-luc* 12-fold and synergistically increased Otd-dependent activation from ~3-fold to 35-fold in S2 cells (Fig. 4G). Similar to our *in vivo* results, Tj did not induce *Rh5* expression in S2 cells, but did repress *Rh6* promoter activity (Fig. 4G). Combined, our *in vivo* and *in vitro* results uncover a second and conserved feedforward system in pR8s, wherein Otd induces *tj*, and Otd and Tj together activate *melt* expression in pR8s.

Yki requires the conserved Otd-Tj module to induce pR8 fate

Since Otd and Tj are expressed in all R8s, whereas Yki/Sd function is biochemically restricted to pR8s by Wts, we asked whether these transcriptional regulators integrate to promote pR8 fate. Consistent with *otd* and *tj* being essential for *melt* activation, *yki* misexpression failed to induce *melt* in *otd^{uvi}* or *tj^{RNAi}* eyes (Fig. 5A). Moreover, *yki* failed to activate *Rh5* in *otd^{uvi}* flies, or repress *Rh6* in *tj^{RNAi}* eyes (fig. S12A). Thus, *yki* requires *otd* and *tj* activity to exert its pR8 specification functions: (i) *yki* requires *otd* and *tj* to induce *melt*, (ii) *yki* requires *otd* to activate *Rh5*, and (iii) *yki* requires *tj* to repress *Rh6* (Fig. 5B, right).

To test this integration molecularly, we analyzed the ability of Yki+Sd to influence Otd and Tj-dependent activation of *melt* and *Rh5* in S2 cells. Yki+Sd weakly activated *melt* (~3-fold), additively increased Tj-dependent activation (from 8-fold to 12-fold), and synergistically increased Otd-dependent activation of *melt* (from ~3-fold to 20-fold) (Fig. 5B). However, the highest *melt* activation was observed with Otd, Tj, Sd, and Yki together (60-fold), consistent with the requirement of all four factors for pR8 fate *in vivo*. Similarly, Yki and Sd minimally activated the *Rh5* promoter (~3-fold), but largely increased Otd-dependent activation (from 60- to 125-fold) (Fig. 5B). Although the K₅₀/Otd sites were necessary for expression of *melt* and *Rh5*, mutating potential Sd sites in the *Rh5* promoter did not decrease reporter expression *in vivo*, suggesting that Yki/Sd-dependent activation of *Rh5* occurs indirectly. These studies support the model that Otd, Tj, and Yki/Sd cooperate to promote pR8-specific gene expression.

Altogether, our results indicate that the pR8 state depends on two overlapping feedforward regulatory networks: (i) Otd directly promotes *melt* and *Rh5* expression (Fig. 3A-C) (24, 25). Melt then further promotes *Rh5* by antagonizing the Hippo pathway and promoting Yki activity; (ii) Otd promotes *tj* expression, and Tj and Otd then synergistically induce *melt*, while Tj also represses *wts* and *Rh6*. Because Yki requires Otd and Tj to induce *melt* (which ultimately promotes Yki), Otd/Tj provide a critical transcriptional context for Yki positive feedback in R8.

Photoreceptor- and R8-restricted transcription factors promote yR8 fate.

We next investigated the mechanisms controlling the yR8 ‘default’ state (active Hippo pathway and *Rh6*). Sens, an R8-restricted zinc finger transcription factor necessary early for R8 specification (32), and later for terminal R8 differentiation (25, 32, 33), and Pph13, a pan-photoreceptor Q₅₀ homeodomain transcription factor, are both essential for *Rh6* expression (34). Thus, we tested whether these factors also promote yR8 fate. Removing *sens* late (*sens>sens^{RNAi}*) reduced *wts* expression (fig. S13A), indicating that Sens functions in yR8s to promote *wts* and *Rh6* expression (Fig. 5C). Sens did not, however, strongly affect pR8 fate as *Rh5* was only mildly affected (fig. 5C). In *pph13^{hazy}* null mutant retinas, not only were *wts* and *Rh6* expression lost, but *melt* was also expanded into most R8s (Fig. 5D-E). In addition, the *Rh6* promoter required the Pph13/Q₅₀ HD binding site for its *in vivo* activity (fig. S13C), supporting the notion that Pph13 binds to and activates the *Rh6*

promoter (34). Rh5 protein was difficult to assess due to *pph13*'s role in forming rhabdomeres, where Rh5 protein localizes. Nevertheless, *Rh5-GFP* remained expressed (fig. S13B), confirming that Otd, but not Pph13, is required for *Rh5* promoter activity (34). Therefore, *pph13* regulates **y**R8 fate determinants (it promotes *wt*s and represses *melt*) and directly activates Rh6 (Fig. 5E), in another feedforward loop resembling Otd/Tj/Melt and Otd/Melt/Rh5 regulation in the alternate subtype.

Since both Sens and Pph13 are expressed in all R8s, what prevents these factors from activating **y**R8 gene expression in **p**R8s? Tj plays this role for Rh6, as Pph13 strongly activates *Rh6* promoter activity (~80-fold) and Tj represses this activation (Fig. 5F). Since Rh5 persists in *sens* and *pph13* mutants, these factors are likely to be permissive to promote *wt*s and *Rh6* in all R8s.

Together, these results indicate that the R8 Hippo network topology requires Otd, Tj, Pph13, and Sens activity to permissively promote both subtypes in all R8s. Such regulation endows any R8 with competence to respond to the stochastically expressed signal from **p**R7. Ultimately, the Yki-Wts-Melt feedback module provides the instructive switch that decides between default (**y**R8) and acquired (**p**R8) states (Fig. 6A).

Discussion

A fundamental strategy in animal development is to re-purpose the same signaling pathways for a diversity of functions. We identified a tissue-specific transcription factor network that enables the otherwise homeostatic Hippo growth control pathway to act as a bistable switch for terminal cell fate. This alteration in network level properties—such as positive *vs.* negative feedback—within biochemically conserved pathways is an efficient means to re-use a signaling network in contexts as distinct as proliferation and terminal differentiation.

How is the R8-specific Hippo regulatory circuit achieved? The two interlinked positive feedback loops (one with *wt*s, one with *melt*) provide the R8 Hippo pathway with multiple points of potential regulation. Context-specific expression of *wt*s and *melt* is defined by overlapping expression of four transcription factors: Otd, Tj, Pph13, and Sens (Fig. 6A). Otd and Pph13 are expressed in all photoreceptors and generate a permissive context that endows the initially equipotent R8s with the competence to become either subtype: Otd promotes *melt/Rh5* whereas Pph13 promotes *wt*s/*Rh6* expression. This competence is further restricted by expression of Tj in R7 and R8, and Sens in R8s, which ensures that *melt* and *wt*s cross-regulation is restricted to R8s. Importantly, it is the status of Yki activity and resulting feedback that assures the outcome of **p** *vs.* **y** fate: in **p**R8s, Yki functions with Otd and Tj to promote *melt* and *Rh5*; in **y**R8s, *wt*s inhibits Yki, preventing *melt* and *Rh5* expression and allowing 'default' *wt*s and *Rh6* expression by Pph13 and Sens. Each of these four transcription factors regulates a partially overlapping subset of R8 subtype fate genes, and together, the network cooperates at multiple regulatory nodes to provide the specific context for repurposing the Hippo pathway.

While other instances of pathways with both positive and negative feedback exist, these are conceptually different from R8 Hippo regulation. For example, in Sprouty (hSpry) regulation of Ras/MAPK-mediated EGFR signaling, EGFR induces hSpry2 expression but hSpry2 inhibits EGFR function (negative feedback); however, hSpry2 also promotes EGFR activity by preventing Cbl-dependent EGFR inhibition (positive feedback) (35, 36). hSpry2 positive feedback is likely coupled to its negative feedback to fine-tune the length and amplitude of receptor activation (36). In contrast, the opposite Hippo pathway feedbacks occur in vastly different cell types (mitotic epithelial cells *vs.* post-mitotic neurons), and both forms of feedback cannot co-exist in R8 since Yki's repression of *wt*s expression

(positive feedback) would make Yki up-regulation of Hippo regulators (negative feedback) inconsequential.

Gaining positive feedback or losing negative feedback within Hippo signaling could permit oncogenesis. Indeed, the Yki ortholog, YAP, is an oncogene (37, 38) and is amplified in multiple tumors, and LATS1/2 (Wts) down-regulation is associated with non-small cell lung carcinomas, soft tissue sarcoma, metastatic prostate cancers, retinoblastoma, and acute lymphoblastic leukemia (39). Otx and MAF factors are also oncogenic in a number of tissues (40, 41). Thus, understanding the regulatory networks identified here in other contexts will be crucial for deciphering how normal signaling pathways can go awry.

Our findings also reveal that a Crx/Otd-Nrl/Tj feedforward module plays a conserved role in post-mitotic photoreceptor fate specification in both flies and mammals. Both induce one photoreceptor fate at the expense of another, and both regulate opsins with a feedforward loop wherein Crx/Otd activates Nrl/Tj expression and Crx-Nrl or Otd-Tj synergistically activate downstream targets (31). Given such deep evolutionary conservation, this module may be critical for generating photoreceptor diversity in other complex visual systems.

This work has two main implications. First, although positive feedback is well documented in other switch-like, irreversible cell fate decisions such as in *Xenopus* oocyte maturation or cell cycle entry (42–44), our work suggests that positive feedback could have a broad role in terminal neuronal differentiation, which often requires permanent fate decisions to maintain a neuron's functional identity. Second, the changes in network topology in R8 photoreceptors allows a finely tuned growth control pathway to be used as a switch in a permanent binary cell fate decision. Context-specific regulation allows the feedback architecture to change in an otherwise conserved signaling module. This may be a general mechanism to endow signaling networks with new systems properties and diversify cell fates in development and evolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Yki and Sd instruct mutually exclusive R8 neuron subtypes

(A) Main photoreceptor subtypes and Rhodopsin coupling in the *Drosophila* eye.

(B) Confocal images of whole-mounted, wild-type adult retinas. Left: R8 subtypes visualized with antibodies to Rh5 (blue) and Rh6 (red); right: R8 subtypes labeled with transcriptional reporters for *melt* (–gal antibody, magenta) and *wt* (GFP antibody, green). Scale bar, 25µm.

(C) Hippo pathway regulation of R8 subtypes. R7 signals to R8 to induce pR8s (*melt* and Rh5). yR8 cells express *wt* and Rh6. *wt* and *melt* act in a double negative transcriptional feedback loop. Additional Hippo pathway members required to specify yR8 fate include the entire “core complex” (Wts, the Hpo kinase, the adapter protein Salvador (Sav), and Wts co-factor, Mats) and the upstream regulators Lethal Giant Larvae (Lgl), the FERM-domain protein and NF-2 ortholog, Merlin (Mer), and the WW-domain protein, Kibra (Kib) (4). atypical Protein Kinase C (aPKC) antagonizes yR8 fate. Black arrows and lines represent genetic regulatory interactions.

(D) *yki* is necessary and sufficient to specify pR8 fate. *yki* knock-down (*yki^{RNAi}*) (left). Pan-photoreceptor expression of activated Yki (right), induced by *GMR-flp*/FRT mediated excision of a transcriptional STOP between *tubulin* promoter and *yki^{S168A}* (*GMR-flp, tub-FRT-STOP-FRT-yki^{S168A}*). Scale bar, 25µm.

(E) Effect of *yki* manipulations on percentage of R8 cells expressing Rh5 or Rh6 (y-axis). Yki is necessary (left) and sufficient (right) to induce Rh5. Wild-type range is ~20-40% Rh5. From left to right in graph: *sens-Gal4* (n=10, N=2998 R8 neurons), *sens>yki^{RNAi}* (n=8, N=2112), *y, w* (n=14 retinas, N=2790), *GMR-yki* (n=4, N=510). Error bars are ± standard deviation (SD); two-tailed, unpaired Student’s t-test; ** denotes p < 0.01, *** denotes p < 0.001.

(F) *sd* is required for pR8 fate. Left: *sd^{47M}* mutant clone (GFP absence). The total number of ommatidia was not reduced, indicating R8 cells were mis-specified into yR8 rather than pR8 cells being lost. Right: Yki mis-expression in *sd* mutant background.

(G) Yki acts downstream of the Hippo pathway and *melt* to control Rh5 and Rh6. Left: *mer^d* (left); right: *IGMR>yki^{RNAi}* suppress the *mer^d* phenotype. Scale bar, 50μm.

(H) Ectopic expression of *yki* in the opposite subtype with *Rh6-Gal4* induces Rh5 in *melt²¹* mutants. *Rh6-Gal4* expressing cells co-labeled by expression of GFP (green). Right: Rh5 channel only. Note: Except where noted, in all manuscript figures Rh5 and Rh6 are labeled in blue (Rh5) and red (Rh6).

Fig. 2. Yki and Sd regulate *wts* and *melt* expression in Hippo pathway positive feedback

(A) *yki^{RNAi}* retinas (top-right) contain *wts-lacZ* (green) in almost all R8s, compared to about two-thirds of R8s in wild-type controls (top-left); *melt-lacZ* (blue) is absent from most R8s in *yki^{RNAi}* retinas (bottom-right) compared with controls (bottom-left). Top panels: antibodies to γ -gal (*wts-lacZ*) and Rh6 (red). Bottom panels: γ -gal (*melt-lacZ*), Rh5 (green) and Rh6 (red). Images at R8 nuclei focal plane. Scale bar, 10 μ m.

(B) *sd* mutant clones (absence of GFP) labeled with antibodies to γ -gal to mark *wts-lacZ* (top, green) and *melt-lacZ* (bottom, magenta) transcriptional reporters. Dashed circles in top panels show wild-type R8s without *wts-lacZ*, whereas *sd* mutant R8s contain *wts-lacZ*. Dashed lines indicate clone boundary. Bottom panel also stained for Rh6. Scale bar, 10 μ m.

(C) A heterozygous *yki* gain-of-function allele is sufficient to induce *melt* expression in most R8s. Images in focal plane of R8 nuclei. Antibodies to γ -gal (blue), Rh5 and Rh6. Scale bar, 10 μ m.

(D) Sagittal sections of adult eyes with nuclei stained for the R8 marker, Sens (blue) and *wts* (γ -gal; green) or *melt* (γ -gal; magenta) expression. Left: *melt* requires *yki* and *sd* to repress *wts*. *wts-lacZ* is absent from R8s in *IGMR>melt* retinas, but de-repressed when *yki* or *sd* are simultaneously removed (bottom two panels). Right: *melt-lacZ* is expressed in most R8s in *IGMR>wts^{RNAi}* retinas, but is lost when *yki* or *sd* are removed. White bracket denotes R8 layer. Scale bar, 50 μ m.

(E) Top: *melt; IGMR>yki:GFP* adult retina labeled for Rh5 and Rh6 (left). Yki can repress *wts* (γ -gal, green in right) in most R8s in the absence of *melt*. Right panels shows R8 nuclear layer. A minority of R8s still express *wts*, but most do not. Compare to *melt* mutant retina, where *wts* is expressed in almost all R8s (bottom right). Scale bar, 25 μ m.

(F) Model of Wts-Yki-Melt feedback circuit in R8 subtypes. Regulatory arrows are genetic and show transcriptional control, except for Wts inhibition of Yki, which is biochemical and post-transcriptional. Dashed lines indicate non-mutually exclusive interactions.

(G) Lineage-tracing experiment for *wt^s-Gal4* expression in R8 subtypes using G-TRACE (see Methods). Adult retinas stained for: RFP (red), GFP (green), and phalloidin (blue). RFP alone labels contemporary, adult *wt^s-Gal4* expression. GFP labels *wt^s-Gal4* expression lineage. Right two panels are grayscale of RFP and GFP, respectively. Dashed circles show that **p**R8 cells did not express *wt^s-Gal4* in their history. Scale bar, 10μm.

Fig. 3. The photoreceptor regulator Otd promotes *melt* expression in pR8s

(A) Schematic diagram of *melt* locus and genomic DNA fragments tested for pR8 expression. Blue boxes: K₅₀/Otd binding sites (TAATCC). Red Xs: Otd site mutations. (B) Top-left: A 4 kb intronic *melt-lacZ* reporter expresses in subset of Sal-positive R8 cells and distal non-neuronal cells ('NC'). Bottom-left: a 450 bp *melt* enhancer is expressed in pR8s (and some pR7s). Top right: Adult *otd* loss-of-function retinas immunostained for -gal (magenta) and the R7/R8 marker Sal (green). Bottom-right: Mutation of two K₅₀/Otd binding sites in the *melt450* enhancer (*melt450 K50-lacZ*) abolishes reporter expression. Scale bar, 50 μm.

(C) Luciferase reporter assays in S2 cells. y-axis: relative luciferase units (RLU) normalized to a control that represents cells transfected with empty expression vector (=1 RLU). Otd activates the *melt450* enhancer ~3-fold, but does not activate *melt450 K50*. Error bars are ±SD; n=3; ** p < 0.01. Bottom box: Otd and Melt form a feedforward loop to promote Rh5 expression (pR8 fate).

Fig 4. Tissue-restricted transcription factors control *melt* and Rhodopsins with regulatory logic conserved in mammalian eye development

(A) Retinas expressing *tj^{RNAi}* lose Rh5 and gain Rh6. Scale bar, 50μm.

(B) Diagram of the *tj* locus. *tj¹* was generated by imprecise excision of a P-element (triangle) in the 5' UTR. The *tj¹* null mutant deletion is indicated by the black line.

(C) Quantification of Rh5 and Rh6-expressing R8s. From left to right in graph: control (*otd-GAL4*): (n=5, N=997), *otd>tj^{RNAi}* (n=4, N=898), *tj¹* (n=6 retinas, N=1230 R8 neurons). Error bars are SD; ** p < 0.01.

(D) Tj regulates *wts* and *melt*. Quantification of *wts* and *melt*-expressing R8s. From left to right: control (y w): (n=4, N=920), *IGMR>tj^{RNAi}* (n=7, N=1411), *IGMR>melt* (n=4, N=980), *IGMR>melt+tj^{RNAi}* (n=4, N=892), *IGMR>wts^{RNAi}* (n=5, N=912), *IGMR>tj^{RNAi}+ wts^{RNAi}* (n=4, N=760); error bars are SD; **p<0.01. Diamonds denote complete loss of *melt-lacZ*.

(E) Tj regulates Rh6 downstream of *wts* and *melt*. From left to right: y w control (n=6, N=1185), *IGMR>tj^{RNAi}* (n=8, N=1675), *IGMR>melt* (n=5, N=1081), *IGMR>melt+tj^{RNAi}* (n=5, N=1021), *IGMR>wts^{RNAi}* (n=4, N=782), *IGMR>tj^{RNAi}+ wts^{RNAi}* (n=4, N=774). %Rh5 compared; error bars are SD. ** p < 0.01. Right diagram: Tj regulates *wts*, *melt*, and *Rh6*.

(F) Tj is expressed in R7 and R8, but not in *otd* mutants. Adult sections, top-left: wild-type retinas labeled for the neuronal marker Elav (blue), the R7/R8 marker Sal (green), and Tj (red). Bottom-left: Tj alone in grayscale in wild-type retinas. Right: *otd* mutants (*otd^{UVJ}*) lose Tj (red); Sal (green). Scale bar, 50μm.

(G) Luciferase reporter assays in S2 cells. From top-left: Tj activates *melt450* expression ~12-fold, while Otd and Tj synergistically activate *melt450* ~37-fold; bottom left: mutating K50/Otd sites in *melt450* abolishes Otd-dependent, but not Tj-dependent activation. Top-right: Otd, but not Tj, activates *Rh5* promoter expression. Bottom right: Tj can repress *Rh6*. Error bars are ±SD; n=3.

(H) A OTX/CRX/Otd—MAF/NRL/Tj coherent feedforward motif instructs photoreceptor fate in flies and mammals. Crx(Otd) promotes expression of Nrl(Tj), and both promote a specific photoreceptor fate and Rhodopsin expression. Left, mammalian motif; right, fly motif.

Fig. 5. The Hippo pathway requires photoreceptor specification factors to regulate R8 subtypes
(A) Yki requires Otd and Tj to induce Melt. Adult sections of (top): *IGMR>yki* (left) and *old^{uv}*; *IGMR-GAL4>UAS-yki* (right) retinas labeled for *melt-lacZ* (–gal, magenta) and Sal (green), or (bottom): *IGMR>UAS-yki* (left), and *IGMR>UAS-tj^{RNAi}+UAS-yki* labeled for *melt-lacZ* (magenta) and Sens (blue). Scale bar, 50 μm.
(B) Yki, Sd, Otd, and Tj synergistically activate *melt* and *Rh5*. Luciferase reporter assays for *melt* and *Rh5* enhancer activity in S2 cells. Cells were transfected with indicated combinations of Otd, Tj, Yki and Sd. Right: Yki requires Otd and Tj to activate *melt*, and Yki requires Otd to activate *Rh5*. Error bars are ±SD; n=3.
(C) *sens* late-mutant retinas (see Methods) lose Rh6 and *wts* expression; some Rh5 remains.
(D) *pph13* mutant retinas lose *wts* expression. *wts>GFP* remains expressed in R8s of the Dorsal Rim Area (DRA) R8s (bracket) at the margin of the retina, but is absent in yR8s. Scale bars, 50 μm.
(E) Left: *pph13* mutants gain *melt-lacZ* (blue) in most R8s. Right: Pph13 is required for *wts* and Rh6 expression, and to specify yR8 fate. Scale bar, 10 μm
(F) Tj reduces Pph13-mediated activation of *Rh6* promoter. Error bars are ±SD; n=3.

Fig 6. Tissue-restricted transcription factors promote re-use of Hippo pathway with positive feedback for post-mitotic neuronal fate

(A) Model; Top: A functionally conserved cassette of genes from Kib/Mer to Yki/Sd is re-wired for a context-specific purpose through changes in network-level feedback. Comparison of network-level feedback switch in Hippo pathway for growth vs. post-mitotic R8 fate. In growth control (left), at least four negative feedbacks onto Yki generate homeostatic regulation for Yki's growth promoting function. In post-mitotic R8 fate specification, positive feedback onto Yki induces an all-or-nothing decision to become pR8 and express Rh5. Four tissue-specific transcription factors (red ovals), including the conserved Otd-Tj (OTX-NRL) module, are co-expressed only in R8 in the eye and generate the permissive context for a Wts-Yki-Melt regulatory circuit and Yki positive feedback. Gray ovals are Hippo growth pathway genes not involved in R8 subtype specification. Orange ovals are genes involved in both contexts. Yellow ovals are genes that create the R8 feedback mechanism, and are yellow in the growth pathway for comparison. Bottom: Effect of Hippo pathway regulatory interactions on network-level feedback onto Yki, in growth control (left; negative feedback) or post-mitotic neural differentiation (right, positive feedback).