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Binary Regulation of Hippo Pathway by Merlin/NF2, Kibra, Lgl, and Melted Specifies and Maintains Post-mitotic Neuronal Fate

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Abstract

Patterning the *Drosophila* retina for color vision relies on post-mitotic specification of photoreceptor subtypes. R8 photoreceptors express one of two light-sensing Rhodopsins, Rh5 or Rh6. This fate decision involves a bistable feedback loop between Melted, a PH-domain protein, and Warts, a kinase in the Hippo growth pathway. Here, we show a subset of the Hippo pathway —Merlin(Mer), Kibra(Kib), and Lethal(2)giant larvae(Lgl), but not Expanded or Fat--is required for Warts expression and activity in R8 to specify Rh6 fate. Melted represses *warts* transcription to disrupt Hippo pathway activity and specify Rh5 fate. R8 Hippo signaling therefore exhibits ON-or-OFF regulation, promoting mutually exclusive fates. Furthermore, Mer and Lgl are continuously required to maintain R8 neuronal subtypes. These results reveal a role for Mer, Kib, and Lgl in neuronal specification and maintenance, and show that the Hippo pathway is re-implemented for sensory neuron fate by combining canonical and non-canonical regulatory steps.

Keywords

binary cell fate; color vision; photoreceptor; rhodopsin; Hippo pathway; neural development; neural maintenance; Merlin; Warts; Kibra; Lgl

Introduction

In neural development, neurons progressively differentiate towards terminal fates, culminating in the post-mitotic specialization of subtypes with subtle, yet critical differences. For example, sensory neurons of the same class often express one sensory receptor from among several options to endow each neuron with a precise functional identity. While neural progenitor specification (Jessell, 2000) and the role of transcription factors in generating neural diversity (Hobert, 2008) are well studied, the mechanisms by which signaling pathways control terminal neuronal subtype specification events, like mutually exclusive sensory receptor expression, are less well understood. Here, we use terminal differentiation of R8 photoreceptor subtypes in the *Drosophila* eye as a model to study how an otherwise equipotent precursor cell uses a signaling pathway to instruct one of two alternate fates.

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The *Drosophila* compound eye comprises ~800 ommatidia, or unit eyes, that each contains 8 photoreceptor neurons (Hardie, 1985) which express light-sensitive Rh proteins and transmit visual information to the brain. Six outer photoreceptors (R1–R6) express Rh1 and, like vertebrate rods, mediate motion detection and dim light vision (Yamaguchi et al., 2008). Two inner photoreceptors, R7 and R8, are involved in color vision, analogous to vertebrate cones, and detect light by expressing one of four Rhs of different wavelength sensitivity that define ommatidial subtypes (reviewed in Rister and Desplan, 2011). In 'yellow' (y) ommatidia, yR7 expresses long-UV-sensitive Rh4 and yR8 expresses green-Rh6, while in 'pale' (p) ommatidia, pR7 expresses short-UV-Rh3 and pR8 expresses blue-Rh5. 'y' and 'p' subtypes are distributed stochastically in a roughly 70% 'y' to 30% 'p' ratio (Figure 1A). R7 and R8 coordinate their subtype identities in two sequential post-mitotic fate decisions. First, stochastic expression of the transcription factor gene *spineless* (*ss*) in ~70% of R7 cells induces yR7 fate and *rh4* (Wernet et al., 2006). pR7s without *ss* express *rh3* and instruct the underlying R8 to become pR8 and express *rh5*. The remaining yR8s express *rh6* by default (Chou et al., 1999).

Two proteins previously known as growth regulators--the NDR Ser/Thr kinase Warts (Wts), and a PH-domain containing protein, Melted (Melt)--control post-mitotic specification of R8 subtypes (Figure 1B) (Mikeladze-Dvali et al., 2005). *melt* is necessary and sufficient for pR8 fate and *rh5* expression (Figure 1E) while *wts* is necessary and sufficient for yR8 and *rh6* (Figure 1F). *wts* and *melt* repress each other's transcription in a double negative, bistable feedback loop that directs robust expression of either *rh5* or *rh6* in R8. The bistable loop ensures an unambiguous R8 fate decision in response to an instructive signal from the R7 cell.

Wts is a tumor suppressor and nexus of the Hippo pathway, which coordinates the balance between proliferation and apoptosis (Figure 1C) (reviewed in Pan, 2010; Halder and Johnson, 2011). Wts inhibits growth by phosphorylating the transcriptional co-activator Yorkie (Yki) in the cytoplasm, preventing it from entering the nucleus (Huang et al., 2005; Dong et al., 2007; Oh and Irvine, 2008) where Yki would otherwise interact with transcription factors such as Scalloped (Sd) or Homothorax (Hth) (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008, Peng et al., 2009) to upregulate pro-proliferation and anti-apoptosis target genes.

Wts is activated upstream by another Ser/Thr kinase, Hippo (Hpo), bound by the scaffolding protein Salvador (Sav) and co-factor Mob-as-tumor suppressor (Mats) (Harvey and Tapon, 2007). Further upstream are several independent or semi-redundant input branches (Figure 1C): FERM domain proteins Expanded (Ex) and Merlin (Mer) (Hamaratoglu et al., 2006; Pellock et al., 2006; Tyler and Baker, 2007) appear to act with the WW-domain protein Kibra (Kib) to activate Hpo (Baumgartner et al., 2010, Genevet et al., 2010, Yu et al., 2010). The membrane protein Crumbs (Crb) mediates upstream activation of Ex (Chen et al., 2010; Ling et al., 2010; Robinson et al., 2010), and Ex can also directly inhibit Yki (Badouel et al., 2009, Oh et al., 2009). Another input consists of the atypical cadherin, Fat, and the kinase Discs overgrown (Dco), which act through the unconventional myosin Dachs to control Wts protein levels (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006; Tyler and Baker, 2007). Finally, apical-basal polarity regulators Lethal giant larvae (Lgl) and atypical protein kinase C (aPKC) act antagonistically to influence the Hippo pathway by unknown mechanisms, possibly by affecting the inhibition of Hpo by dRassf (Polesello et al., 2006; Grzeschik et al., 2010).

Mutations in *fat*, *lgl*, *crb*, *ex*, *mer*, *hpo*, *sav*, *mats*, and *wts*, or over-expression of *yki*, cause overgrowth in developing tissues, while pathway activation or *yki* depletion blocks proliferation and induces cell death (Pan, 2010). Many Hippo pathway members have

orthologs implicated in human cancers, in particular, the *mer* ortholog, *NF2*, whose mutations cause Neurofibromatosis type 2 (MacCollin et al., 1993).

The Hippo pathway also regulates non-growth processes in *Drosophila*, implying that pathway regulation can be heterogeneous and context-dependent. The Hippo pathway functions in autophagic cell death in salivary glands (Dutta and Baehrecke, 2008), apico-basal polarity (Hamaratoglu et al., 2009; Genevet et al., 2009), establishment and maintenance of dendritic arborizations (Emoto et al., 2006), and differentiation of the optic lobe neuroepithelia (Reddy et al., 2010). In these processes, *wts* is believed to be ubiquitously expressed and controlled post-transcriptionally to mediate its activation.

R8 subtype specification is the only known cellular process in which *wts* is controlled transcriptionally through a genetic interaction with Melt. Melt modulates the TOR/Insulin signaling pathway in fat metabolism (Teleman et al., 2005), yet mutants in other TOR/Ins pathway genes do not exhibit R8 phenotypes (Mikeladze-Dvali et al., 2005), suggesting that Melt acts independently to regulate R8 subtypes. Whether Wts acts with its canonical signaling partners besides Hpo and Sav for cell fate determination, and how diverse biological processes such as tissue growth and sensory receptor expression can re-use the same signaling modules, is not known.

Here, we report that Wts is regulated by the integration of canonical (tumor suppressor pathway) and non-canonical mechanisms for R8 subtype specification. *mer*, *kib*, *lgl*, and the *hpo/sav/mats/wts* core complex specify yR8 subtypes while Ex and Fat upstream inputs are not involved. *mer* and *kib* are required for *wts* expression and to repress *melt* such that the state of the *wts-melt* transcriptional feedback loop is coupled to upstream Hippo pathway signaling. Conditional inactivation of *mer* or *lgl* in adult flies reveals that continuous Hippo pathway activity also maintains mutually exclusive Rh5 and Rh6 expression, and *melt* can disrupt Hippo signaling long after R8 subtype specification has occurred. Therefore, constitutive and continuous activation by upstream Hippo inputs keep Wts “ON” to specify yR8, while repression of *wts* transcription by Melt acts as a circuit breaker to switch pathway activity “OFF” to specify pR8. Such regulation allows Hippo pathway activity to depend on the presence or absence of Wts, which ensures binary output of Rh5 and Rh6.

Results

To test whether the upstream Hippo growth network regulates Wts in R8 photoreceptor subtype specification, we genetically manipulated Mats, Hpo, Sav, Ex, Kib, Mer, Fat, Fj, Dco, Dachs, dRassf, aPKC, and Lgl and assayed for changes in the ratio of R8 subtypes as defined by the expression of Rh5 and Rh6.

The Hippo “core complex” module is functionally conserved in R8 subtype specification

Wts, Hpo, Sav, and Mats physically interact to form the “core complex” of Hippo growth signaling (reviewed in Zhao et al., 2010). Yet core complex members can behave differently in distinct contexts, as Hpo activates another NDR-kinase, Tricornered (Trc), instead of Wts, to establish dendritic fields of larval body wall neurons (Emoto et al., 2006). In R8, mutations in *hpo* and *sav* phenocopy *wts* mutants (Mikeladze-Dvali et al., 2005). To confirm that the core complex functions canonically during R8 photoreceptor subtype specification, we analyzed *mats* and performed epistasis among *mats*, *wts*, and *hpo*.

We generated *wts*, *hpo*, or *sav* whole mutant eyes with the FLP/FRT system using *eyeless-FLP* (*ey-FLP*) and a chromosome containing a cell lethal mutation and *GMR-hid* that eliminates all non-homozygous mutant cells in the eye (Stowers and Schwartz; 1999; Newsome et al., 2000). In these mutants, all R8s adopted the pR8 subtype fate and expressed

Rh5 (Figures 1F and 1N; and Figure S1D-E) (Mikeladze-Dvali et al., 2005). As homozygous *mats* mutant eyes do not fully differentiate photoreceptors (Lai et al., 2005), we examined late *mats* function in the retina by expressing *mats-RNAi* with either the *lGMR-Gal4* driver, in cells posterior to the morphogenetic furrow (Wernet et al., 2003), or a combination of *lGMR-Gal4* and *ey-Gal4* in the entire eye. Three different *mats-RNAi* transgenes resulted in loss of Rh6 and expansion of Rh5 (Figures 1G and 1N; Figure S1A-B and S1F). Thus, all four members of the Hippo core complex are required to specify yR8.

Expression of *hpo* in all photoreceptors (*GMR-hpo*) induced Rh6 expression in all R8s (Figure S1G) (Mikeladze-Dvali et al., 2005). wts^{P1} hypomorphic mutant eyes suppressed the *hpo* mis-expression phenotype, as about half of R8s expressed Rh5 (Figure S1H). *mats-RNAi* also suppressed the "all Rh6" *hpo* gain-of-function phenotype, as *ey, lGMR>matsRNAi; GMR-hpo* retinas contained both Rh5 and Rh6 (~80% of R8s expressed Rh5; 60% Rh5+Rh6, and ~20% only Rh6) (Figure S1I). Similarly, removing *hpo* from *lGMR>wts* retinas reduced the proportion of R8s that expressed Rh6 exclusively (data not shown). Thus, *wts*, *hpo*, and *mats* are required for each other's ability to promote yR8 fate and Rh6, consistent with functional conservation of the Hippo core complex in R8 subtype specification.

mer and *kib* are required to specify R8 photoreceptor subtypes independently of upstream Hippo pathway regulators Ex and Fat

To test whether upstream regulators of *wts* act in R8, we first generated whole *mer* mutant eyes. Hypomorphic mer^{3} led to Rh5 expression in almost all (>99%) R8 cells, with concomitant loss of Rh6 (Figure 1H). Similar results were obtained with null alleles (mer^{1}, mer^{2}, mer^{4}), in mer^{4} mutant clones, or after eye-specific expression of *mer* RNAi (Figure 1N). Wild-type *UAS-mer* expressed in all photoreceptors using *lGMR-Gal4* rescued the mer^{4} mutant phenotype. We next analyzed mutants for Kib, which acts in a complex with Mer and Ex to promote Hippo pathway activity. kib^{del} null mutant retinas contained mostly Rh5-expressing R8s, indicating that *kib* is also required for yR8 fate. Eye-specific *kib* RNAi expression (*ey+lGMR-Gal4; UAS-kib-RNAi, UAS-Dcr2*) led to similar results (Figure 1I). Thus, *mer* and *kib* are required to specify yR8 fate and induce Rh6 expression.

We also generated eyes mutant for *ex*, which acts upstream of *wts* for growth regulation, in part redundantly with *mer* (McCartney et al., 2000; Hamaratoglu et al., 2006). Three different alleles of *ex* (ex^{e1}, ex^{AP49}, ex^{AP50}) did not significantly affect the R8 subtype ratio (Figures 1J and 1N; Figures S2A-B). To test for redundancy with *mer*, we removed one copy of *mer* in homozygous *ex* mutant clones and again observed no detectable difference in the Rh5:Rh6 ratio (Figures S2C-C″), consistent with *mer* functioning non-redundantly in the Hippo pathway to specify R8 subtypes. Several other studies have suggested that *mer* has functions separate from *ex* in the Hippo pathway (Pellock et al., 2006; Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006), but to our knowledge this is the first example where *mer* mutant phenotypes perfectly match *hpo*, *sav*, and *wts* mutant phenotypes with *ex* completely dispensable (Figure 1N). Null or strong alleles of *fat* (fat^{8}, fat^{Grv}, fat^{fd}), *dco* (dco^{3}), *dachs* (d^{GC13}), and *dRASSF* ($dRASSF^{X16}$, $dRASSF^{44.2}$), or ex^{e1}, $fat^{Gr\text{-}v}$ double mutants, also all had normal pR8:yR8 ratios (Figures 1K-N; Figures S2D-D′). Therefore, the Mer/Kib upstream branch of the Hippo pathway is required for R8 subtype specification, while the Fat/Fj/Dco/Dachs pathway, Ex, and dRASSF are not involved.

Mer requires its BB-domain and C-terminus for activity in R8 subtype specification

For viability, Mer requires the N-terminal FERM domain, as well as portions of the protein C-terminal half (LaJeunesse et al., 1998). We tested whether these domains are also required for Mer in R8. Mer constructs missing either the C-terminal half (*UAS-mer*$^{\Delta C}$) or the 7-aa

"Blue Box" sequence (^{170}YQMTPEM^{177}) in the FERM domain (*UAS-mer$^{\Delta BB}$*) were ectopically expressed in photoreceptors with *lGMR-GAL4*. Almost all R8s expressed Rh5 and not Rh6 (Figures 2C and 2G), suggesting that these proteins act as dominant negatives. Furthermore, *mer$^{\Delta BB}$* and *mer$^{\Delta C}$* constructs expressed in *mer* whole-mutant eyes failed to rescue the loss of yR8s (>99% of R8s expressed Rh5) (Figure 2D–G). Thus, the 7-aa Blue-Box domain, which is perfectly conserved in human NF2, and the C-terminal half of the protein are essential for Mer function in R8 subtype specification.

Mer acts autonomously in R8 for subtype specification

The R8 *mer* mutant phenotype is not due to general photoreceptor fate defects as *mer* retinas did not aberrantly express the cell fate markers Spalt (R7 & R8) and Rh1 (R1–R6) (Figures S3D-D′ and S3G-G′). As pR8 fate depends on signaling from pR7, *mer* could affect R7 subtype specification, and only indirectly affect R8 *rh* expression. However, Rh3 and Rh4 protein expression was normal in *mer* or *kib* mutant retinas (Figures 2J-J ‴; Figures S3A-B ′), leading to extensive Rh4/Rh5 (yR7/pR8) mis-coupling (Figure 2I) never observed in wild-type.

To assess whether *mer* acts cell-autonomously in R8, we mis-expressed the dominant negative *UAS-mer$^{\Delta BB}$* using *PanR8-Gal4*, which is expressed exclusively in all R8s starting at ~70% pupation, after subtypes are specified (Mikeladze-Dvali et al., 2005). *PanR8>Mer$^{\Delta BB}$* retinas exhibited a dramatic gain of Rh5 (Figure 2K-K′), and partial loss of Rh6 (Figure 2K″), leading many R8s to co-express Rh5 and Rh6 (Figures 2H and 2K). This is likely because the PanR8 driver is expressed late, after Rh6 has been expressed. Rh6 protein levels appeared lower in R8 cells that co-expressed Rh5 (Figure 2K ″), confirming that yR8 subtype fate had been re-specified to pR8. Thus, *mer* is specifically required in R8 to regulate R8 subtype fate.

Kib and the core complex are necessary and sufficient to specify yR8 fate

We next tested if Mer was sufficient to induce yR8 fate in wild-type animals by using *lGMR-Gal4* to mis-express either a full length (*UAS-mer^{+}*) or a 35-amino acid truncated protein (*UAS -mer$^{1-600}$*) proposed to be an activated form (LaJeunesse et al., 1998). Neither Mer construct affected R8 subtypes (Figures 2B and 2L). In contrast, *lGMR>kib* retinas exhibited a complete fate transformation and all R8s expressed Rh6 (Figure 2M), similar to mis-expression of *wts*, *sav*, or *hpo* (Mikeladze-Dvali et al., 2005). Thus, *kib* is necessary and sufficient to induce yR8 fate.

An activated, membrane-tethered Mats containing a myristoylation sequence (*UAS-Myr-matsGFP*) (Ho et al., 2010) driven with *lGMR-GAL4* also induced Rh6, with a corresponding loss of Rh5 (Figure 2N). Late expression of *Myr-matsGFP* with *PanR8-Gal4* was also sufficient to reprogram pR8 subtypes to express Rh6 (Figure 2H). In contrast, expression of a dominant negative construct with a mutated myristoylation sequence (*lGMR-Gal4, UAS-Myr^{G2A}-matsGFP*) induced Rh5 (Figure S1C). Therefore, *kib* and all four core complex members are sufficient to induce yR8 subtypes. These results also support the idea that membrane localization of core complex proteins is important for their activation (Ho et al., 2010). *mer* might be permissively required for Hippo pathway activation in R8, as *mer* is necessary, but not sufficient, to induce the yR8 fate when ectopically expressed at high levels.

Wts and Sav require Mer to induce the yR8 subtype

In the tumor suppressor pathway, Mer acts upstream of Wts to promote Wts activity (Hamaratoglu et al., 2006). However, removing *mer* in clonal patches of *GMR-wts* retinas completely suppressed the ability of *wts* to activate Rh6 and led to a *mer* mutant phenotype

(>99% of R8s expressed Rh5) (Figures 3A-A ″). All R8s in neighboring *GMR-wts* only tissue expressed Rh6 exclusively (Figure 3A ″). Similar results were obtained in *mer* mutant clones when *sav* (*mer; GMR-sav*) or *sav* and *wts* (*mer; GMR-sav, wts*) were mis-expressed in all photoreceptors (Figures 3B-B″ and data not shown). Therefore, *mer* might act downstream of the pathway, or it might act upstream but be required for the ability of *sav* and *wts* to regulate R8 subtype fate.

To address these possibilities, we used Hpo to activate the pathway independently of *mer*. If *mer* acts upstream of *hpo* and *wts*, as in growth signaling, activated Hpo should suppress the *mer* phenotype. Hpo can auto-activate when expressed at high levels (Praskova et al., 2004). Indeed, *lGMR-hpo* led all R8s to express Rh6 (data not shown, see Figure S1G) and suppressed the *mer* phenotype, inducing Rh6 in most R8s (Figure 3C). Activated Mats (*lGMR>Myr-mats*) also suppressed the *mer* mutant phenotype (Figures 3D–E). These results suggest that *mer* acts upstream of the *hpo*/*sav*/*mats*/*wts* core complex in R8, as in growth control. Moreover, because *mer* is required for *wts* and *sav* activity in R8, even when *wts* and *sav* are expressed at high levels, the Mer upstream branch is a critical activator of Wts for R8 subtype specification.

ex and *dRassf* are not required for R8 subtype specification

Intriguingly, two upstream genes whose functions are not required for R8 subtype determination, *ex* and *dRassf*, were sufficient to alter R8 subtypes when mis-expressed. *lGMR>dRassf* induced Rh5 which led to partial co-expression of Rh5 and Rh6 (Figure S4B), while *lGMR>ex* mimicked Hippo pathway activation and induced gain of Rh6 and loss of Rh5 (Figure S4C). *ex* and *dRassf* are likely sufficient to artificially perturb the Hippo pathway by performing their canonical activities—Ex to activate Hpo or sequester Yki, dRassf to inhibit Hpo—when ectopically expressed at high levels. This suggests that normal Ex and dRassf protein function is not prohibited in R8 subtypes; rather, they might be sequestered from the Wts/Hpo complex, or not highly expressed in post-mitotic R8s. Indeed, Ex levels are lower posterior to the morphogenetic furrow and in pupation relative to the larval eye disc (Milton et al., 2010). Supporting this hypothesis, we were unable to detect a difference in Ex immunoreactivity between wild-type and *ex* mutant clones in adult photoreceptors (data not shown), and temporal RNA-seq data from *Drosophila* modENCODE indicate that *dRassf* reaches its lowest expression level during late pupation (Graveley et al., 2010).

A role for Lgl and aPKC in R8 subtype specification

We next tested *lgl*, a neoplastic tumor suppressor and apico-basal cell polarity regulator that genetically interacts with the Hippo pathway in the developing eye disc (Grzeschik et al., 2010). lgl^{4} mutant clones contained R8s that expressed Rh5 almost exclusively, with little or no Rh6 (Figures 4A-A″). *lgl-RNAi* also led to induction of Rh5 and loss of Rh6 (*ey, lGMR>*lgl^{RNAi}: 80.6% Rh5, 11.7% Rh6, 7. 7% co-expression), as did a conditional allele (lgl^{ts3}) at the non-permissive temperature (Figures 4B–D and 4G). The *lgl* mutant defect is specific to R8, as R7 and outer photoreceptor Rh expression was unaffected (Figures S3C-C′ and S3F-F′). Loss of *lgl*, or RNAi targeting another apico-basal polarity regulator, *bazooka*, severely disrupted photoreceptor polarity and rhabdomere morphology, as measured by F-actin localization (Figures S5A-A ″). But only *lgl* affected the Rh5:Rh6 ratio, suggesting that the *lgl* mutant yR8 specification defect is not due to general cell polarity aberrations.

We investigated another polarity regulator, aPKC, which acts antagonistically to Lgl (Lee et al., 2006), and is genetically linked to the Hippo growth pathway (Grzeschik et al., 2010). Over-expression of an “activated” aPKC targeted to the plasma membrane with a CAAX prenylation motif (*lGMR>*$aPKC^{CAAX\text{-}WT}$) (Sotillos et al., 2004) disrupted photoreceptor

polarity, and converted all R8s to pR8 (Figure 4F) without affecting R7 Rhs (Figure S5B). aPKC kinase function is required for this phenotype, as over-expression of a membrane tethered kinase-dead version (*lGMR>aPKC*$^{CAAX\text{-}KD}$) did not strongly increase Rh5 (Figure 4E).

Wts requires Lgl to specify yR8 in parallel to Mer

Because *lgl*, *mer*, and *wts* have identical Rh phenotypes, we examined genetic interactions between *lgl* and core complex members, *wts* and *sav*. *lgl* mutant clones suppressed the ability of ectopic Wts to induce yR8 as most R8s expressed Rh5 in *lgl; GMR-wts* tissue (Figures 3H-H″) indicating that *lgl*, like *mer*, is required for Wts activity. But when *sav* was co-overexpressed with *wts* in *lgl* mutants, all R8s expressed Rh6 (Figures 4I-I″). Thus, the ability of Wts to become activated in *lgl* mutant R8s is sensitive to levels of *sav*. Furthermore, as *mer* can fully suppress the *GMR-sav, wts* overexpression phenotype while *lgl* cannot (compare Figures 3B-B ″ to 4I-I ″), Lgl might promote Wts activity in parallel to the Mer/Kib branch.

lgl mutants and *aPKC* gain-of-function have identical R8 phenotypes, suggesting that aPKC also influences the Hippo pathway to induce pR8 fate. Wts mis-expression significantly suppressed Rh5 induction but not the polarity phenotype of activated *aPKC* (Figure S5C), consistent with aPKC acting upstream or in parallel to Wts. These data are consistent with Lgl and aPKC acting upstream of Wts, likely in parallel to Mer/Kib, to regulate the core complex, although other possibilities cannot be excluded.

Upstream Hippo pathway regulators control *wts* and *melt* expression

In R8, *melt* represses *wts* while *wts* represses *melt* transcription and *wts* is the ‘output’ of the bistable feedback loop for Rh expression (Mikeladze-Dvali et al., 2005). To test the epistatic relationship between *mer* and *melt*, we generated retinas double mutant for *mer* and *melt* (Figures 5A–C) which phenocopied *mer* single mutants: most R8 cells expressed Rh5 and less than 1% expressed Rh6 (Figure 5C). Similarly, loss of *lgl* suppressed the *melt* phenotype, as *lgl* mutant clones in *melt* mutant retinas expressed only Rh5 (Figures 5D-D″). Thus, like *wts*, *mer* and *lgl* are required for the *melt* phenotype, consistent with a role in R8 to activate Wts genetically downstream of *melt*.

To determine if upstream Hippo signaling influences the transcriptional feedback loop between *wts* and *melt*, we asked whether loss of *mer* affected *wts* or *melt* transcription. *wts-lacZ* expression was lost in *mer* mutant clones, and instead all R8s expressed *melt-lacZ* (Figures 5E–F″). Similar results were obtained for *kib* mutants, with slightly less expressivity (Figures 5G-G″ and data not shown). These data are consistent with a model wherein the upstream Mer/Kib branch represses *melt* and promotes *wts* transcription by keeping Wts protein active. In the absence of *mer* or *kib*, Wts protein kinase is inactive and unable to promote its own expression through the bistable feedback loop. Therefore, transcriptional feedback between *wts* and *melt* is coupled to the “ON/OFF” state of the entire upstream Mer/Kib activation branch of the Hippo pathway.

Mer and Hippo pathway are first required in R8 at ~40% pupation

The genetic program that defines p- and y- ommatidial subtypes begins in early pupation with stochastic expression of *ss* in R7 cells (Wernet et al., 2006) followed by an R7-to-R8 signal that induces R8 subtypes, and then Rh5 and Rh6 expression around 70–80% pupation (Earl and Britt, 2006). To examine temporal dynamics of the Hippo pathway in R8 subtype specification, we took advantage of a temperature sensitive *mer*^{ts} allele, which at the non-permissive temperature behaves genetically as to a null allele (MacDougall et al., 2001). To define the earliest requirement for *mer*, we moved flies to the non-permissive temperature at

10% pupation to remove *mer* function, and then shifted them permanently to the permissive temperature (18°C) to restore *mer* function at successively later time points. When *mer* was inactivated until up to ~38% pupation, we observed a wild-type Rh5:Rh6 ratio in R8 (Figure 6A). Yet when inactivation persisted until 44% pupation, the proportion of R8 cells expressing Rh5 increased, and continued to increase as *mer* function was removed for longer periods (Figure 6A). Complete transformation into pR8 was achieved when *mer* function was restored later than ~70% pupation, when R8 subtypes are fully established. Therefore, between 38 and 44% pupation (or even later as it might take time for wild-type *mer* function to recover), *mer* and upstream Hippo signaling become required in presumptive yR8 photoreceptors. Subtypes appear fully specified after 70–80% pupation, when *melt*/Rh5 and *wts*/Rh6 are expressed in p- or y- R8 subtypes, respectively (Figure 6B).

Mer, Lgl, and Hippo pathway signaling are continuously required to maintain yR8 photoreceptor neuron subtypes

We next attempted to identify the latest point at which *mer* is required by using mer^{ts} to remove *mer* function at successively later time points. Even when we inactivated *mer* as late as 90% pupation—after Rh5 and Rh6 are expressed—retinas always exhibited some de-repression of Rh5 into yR8s. Retinas from mer^{ts} adult flies reared at 18°C and shifted to 29°C for 7 days (when they were 5–10 days old) also showed an increase in Rh5 expressing R8s, which often co-expressed Rh6, revealing that Rh5 is de-repressed into yR8 cells as R8s shift to pR8 fate upon loss of *mer* (Figure 6H). Strikingly, when mer^{ts} flies were kept at 29°C for 19 days, almost all R8s expressed Rh5, and often co-expressed Rh6 (Figures 6E-E‴ and 6H; compare to 6C-D‴). Thus, in addition to its role in yR8 establishment, the *mer* upstream branch of the Hippo pathway is also required to maintain yR8 fate in adult flies.

To determine whether yR8 fate maintenance was specific to the Mer branch, or if it involved the entire Hippo pathway, we performed a similar experiment using lgl^{ts}. Homozygous lgl^{ts} flies reared at 18°C until several days post-eclosure and shifted to 29°C for 16 days de-repressed Rh5 into the yR8 subtype, which co-expressed Rh5 and Rh6 in 14.4% of R8s (Figures 6G-G‴). Control lgl^{ts} flies reared continuously at 18°C or lgl^{ts}/+ flies shifted to 29°C as adults exhibited a wild-type Rh5:Rh6 ratio (Figures 6F-F‴ and 6I). Thus, two different upstream regulators, *mer* and *lgl*, are required to specify as well as maintain yR8 subtypes, suggesting that the entire R8 Hippo pathway is required to keep Wts protein active for the life of the neuron.

Melt is sufficient to re-specify adult yR8 into pR8

As *wts* expression and Rh6 (*i.e.*, yR8) are the default state for R8 in the absence of an R7 signal, the role of *melt* in specifying pR8 appears to be as an “OFF” switch that can disrupt Hippo pathway activity (Figures 7A-A′). This model predicts that ectopic Melt should still be able to turn off Hippo pathway activity in adult photoreceptors. To test this hypothesis, we mis-expressed Melt long after R8 subtype specification, starting in 7 day old flies using an inducible “flp-out” cassette (*hs-FLP; GMR-FRT-w+, STOP-FRT-Gal4*). This led almost all R8s in the dorsal half of the retina to express Rh5 while ~70% still contained Rh6 (Figures 7B–C′). This phenotype is similar to late removal of *mer* or *lgl* function and demonstrates that *melt* is sufficient to abrogate Hippo signaling in yR8, to allow reactivation of Rh5 even in adult flies.

Discussion

This work describes a mechanism for a post-mitotic neuronal subtype decision wherein a complex signaling pathway is regulated both canonically and non-canonically to instruct either of two cell states. The control point for Hippo pathway signaling in R8 is the

expression or repression of Wts, whose activity is promoted by constitutive upstream Hippo pathway activity (Figure 7D). Several lines of evidence support this model. First, two upstream inputs, *mer/kib* and *lgl*, and the core complex *of hpo*/*sav*/*mats*/*wts* are required for R8 subtype specification and all have identical Rh phenotypes. Second, *mer* is required for Wts activity and expression, indicating the entire Hippo pathway is coupled to the *wts-melt* transcriptional feedback loop and binary fate mechanism. Third, *mer* and *lgl* temperature-sensitive experiments showed that upstream Hippo pathway signaling is continuously required to maintain yR8 fate. Finally, Hippo signaling can be abrogated long after the fate decision by late induction of *melt*, which re-specifies pR8 fate, demonstrating that *melt* can disrupt active Hippo signaling.

Given that *wts* acts genetically downstream of *melt* in R8 subtype specification, why engage transcriptional regulation of *wts* instead of simply inactivating Wts kinase? Regulating signal transduction members by transcription might be an efficient way to arrest signaling permanently, especially if the regulation is mediated by a bistable loop, a common genetic motif used by cells to transition from one cell state to another, often irreversibly (reviewed in Ferrell, 2002). It is thus well suited to promote mutually exclusive gene expression and switch-like fate decisions (reviewed in Jukam and Desplan, 2010). Regulation of *wts* by *melt* acts as an “OFF” switch for Hippo pathway activity, and the bistable feedback loop reinforces this cell state transition, despite the activity of multiple upstream Hippo regulators.

Hippo pathway and R8 subtype specification dynamics

These results deepen our understanding of key temporal steps in R8 subtype specification. Ectopic *kib*, *mats*, and *hpo* can induce the yR8 fate in *all* R8s, but require Wts for their function, indicating that some basal level of Wts must be present initially in all R8s. Such Wts is likely dispensable for yR8 specification until at least 40% pupation, when *mer* is first required.

wts is de-repressed in *melt* mutants, and *melt* is de-repressed in *wts* mutants. Thus, general factor(s) must be competent to activate transcription of *wts* and *melt* in all R8s, independent from the R7 signal that coordinates R7/R8 subtypes. This activation would be restricted to mutually exclusive R8s by the bistable feedback loop. In presumptive yR8s, this general activator might boost Wts levels to allow sustained activation by constitutive upstream Hippo signaling. In presumptive pR8, the R7 signal could induce a further increase in *melt* expression or protein activity that allows Melt to inactivate the Hippo pathway and repress *wts*. Alternatively, the R7 signal might inhibit upstream activators of the Hippo pathway like Mer or Lgl, which likely act near the membrane and are candidates for substrates that mediate reception of the R7 signal. Melt could then be activated by loss of Wts, and reinforce the loss in Wts activity by transcriptionally repressing *wts*.

Mer, Lgl, and the Hippo pathway in neuronal maintenance

Neural subtypes, once specified, appear to require active genetic programs to maintain their fate (Lesch and Bargmann, 2010). A second role for the Hippo pathway in R8, in addition to specifying yR8, is to maintain *exclusion* of the opposite fate in old R8 neurons, by continuously repressing *rh5* in adult yR8 subtypes. When *mer* or *lgl* was removed late, the yR8 character was partially lost, as many R8 cells contained both Rh5 and Rh6. Photoreceptors must coordinate sensory receptor expression with hard-wired axon projections to the visual processing centers (reviewed in Sanes and Zipursky, 2010), so the functional identity of the R8 neuron should be stable or the fly risks sensory confusion.

Context-Specificity and Mer, Kib, and Lgl protein function in the Hippo signaling network

Upstream inputs and downstream transcriptional targets of Wts signaling in development are context-dependent (Pellock et al., 2006; Cho et al., 2006; Peng et al., 2009; Milton et al., 2010, Grusche et al., 2010). Evidence that Kib can bind separately to either Ex or Mer, as well as synergistically in a complex to both, implies that Ex, Kib, and Mer act semi-redundantly in growth control (Genevet et al., 2010; Grusche et al., 2010). Our results demonstrate that Mer/Kib can operate as a branch completely independent from Ex upstream of Wts for R8 specification.

mer and *lgl* mutants phenocopy *wts* mutants with respect to R8 subtype specification. If Mer and Lgl do not act in a linear pathway or complex, why would Hippo signaling require two non-redundant upstream inputs to keep Wts protein active? Mer/Kib and Lgl might simply provide complementary means to promote Wts function in R8. The protein-protein interactions between Mer, Kib and Sav (Yu et al., 2010) and Kib and Wts (Genevet et al., 2010) support a localization or scaffolding role for Mer and Kib in activation of Hpo and Wts, perhaps by recruiting Hpo, Sav, and Mats near the membrane where Hpo can phosphorylate Wts. Membrane tethered Mats promotes Wts activation in our system and in growth (Ho et al., 2010). Kib, like Mats, Hpo, Sav, or Wts, is sufficient to induce the yR8 subtype. Dramatically increasing the expression level of *kib*, *hpo*, *mats*, *sav*, or *wts* might promote synergistic localization and binding of all six proteins near the membrane, resulting in phosphorylated and active Wts.

Lgl could function to inhibit a negative pathway regulator, such as the dSTRIPAK PP2A phosphatase complex that acts on Hpo (Ribeiro et al., 2010). Lgl would then promote activation of Wts by preventing inactivation of Wts or Hpo. Such regulation would be useful where a continuously active Hippo pathway is necessary, as in the maintenance of yR8 subtype fate. Alternatively, the intracellular polarity roles of Lgl and aPKC might generically affect localization of Hippo pathway components. Perhaps the molecular signal from R7 that promotes Melt and represses Wts activity shares features with non-autonomous signals that affect polarity regulators of Hippo signaling in dividing cells.

R8 subtypes as a model for studying Mer/NF2 function

Several reports suggest that Hippo pathway signaling might underlie some pathology of neurofibromatosis type 2 (NF-2). *Mer/NF2* can regulate contact-dependent growth inhibition and nuclear localization of the Yki ortholog and oncogene, YAP, in human meningioma cell lines (Striedinger et al., 2008), as well as genetically interact with YAP during tumorigenesis in the mouse liver (Zhang et al., 2010). Other studies implicated mis-regulated EGFR signaling in NF-2 mutant tumors (Benhamouche et al., 2010) or suggested that Hippo pathway-like overgrowth phenotypes in *mer; ex* double mutants are a secondary consequence of EGFR mis-regulation in *Drosophila* (Maitra et al., 2006). Our results support a role for Mer upstream of the Hippo pathway in the context of R8 subtype specification. The binary assay of Rh5 or Rh6 in R8 photoreceptors can be used to dissect Mer protein function in Hippo signaling without complications from redundant upstream regulators of Wts.

Re-use of signaling pathways in development

The modular re-use of signaling pathway components for different purposes in development is widespread, and is likely a key molecular conduit for the evolution of cellular and morphological diversity (Wilkins, 2002). The uncoupling of *mer*, *kib*, *lgl* and the core Hippo signaling cassette from growth control allows the genes to contribute to a broader array of developmental decisions. It will be interesting to see if the Lgl/Mer/Kib/Hpo/Sav/Mats/Wts

module is regulated by Melt in other post-mitotic fate specification events during neural development.

Experimental Procedures

Drosophila Stocks

For *Drosophila* genotypes and fly strains, see Supplemental Experimental Procedures.

Drosophila Genetics and Induction of Mutant Tissue

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. For details on Gal4 drivers, generating whole-mutant retinas, the *mer* and *lgl* temperature sensitive experiments, and late induction of *melt*, please see Supplemental Experimental Procedures.

Immunostaining and Imaging

Adult retina dissections were performed as described (Mazzoni et al., 2008). Briefly, retinas were dissected in PBS over ice and fixed in PBS + 4% paraformaldehyde for 20 min at room temperature. After three washes in PBT (PBS + 0.2% Triton-X), the lamina was removed and retinas incubated overnight at room temperature with primary antibodies diluted in PBT. After three rinses and one 30min wash in PBT, retinas were incubated in secondary antibody diluted in PBT for 4–6 hours at room temperature, followed by four washes in PBT. Retinas were mounted using SlowFade (Molecular Probes, Invitrogen, Eugene, OR) on glass slides with coverslip.

Antibodies and dilutions were as follows: mouse anti-Rh3 (1:100, gift form S. Britt, University of Colorado), rabbit anti-Rh4 (1:100, gift from C. Zuker, Columbia University), mouse anti-Rh5 (1:200, S Britt), rabbit anti-Rh6 (1:2000), rabbit anti-Sal (1:100, gift from B. Mollereau, Ecole Normale Superieure-Lyon), goat anti-β-gal (1:5000, Biogenesis), rabbit anti-β-gal (1:5000, Cappel), sheep anti-GFP (1:1000, AbD Serotec), rabbit anti-GFP (1:1000, Invitrogen), mouse anti-Elav (1:40, DSHB). All secondary antibodies were Alexa Flour (488, 555, or 647)-conjugated made in donkey (1:800, Molecular Probes). Alexa Fluor 488 coupled Phalloidin (1:150, Invitrogen) was used to visualize actin to outline photoreceptor rhabdomeres.

All fluorescent images were taken with a Leica TCS SP2 or SP5 confocal laser scanning microscope.

Quantification and Statistics

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. A two-tailed, unpaired t-test was used to calculate statistical significance when appropriate. 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. All error bars in figures are ± one standard deviation around the mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Badouel C, Gardano L, Amin N, Garg A, Rosenfeld R, Le Bihan T, McNeill H. The FERM-domain protein Expanded regulates Hippo pathway activity via direct interactions with the transcriptional activator Yorkie. Dev Cell. 2009; 16:411–420. [PubMed: 19289086]

Baumgartner R, Poernbacher I, Buser N, Hafen E, Stocker H. The WW domain protein Kibra acts upstream of Hippo in Drosophila. Dev Cell. 2010; 18:309–316. [PubMed: 20159600]

Benhamouche S, Curto M, Saotome I, Gladden AB, Liu CH, Giovannini M, McClatchey AI. Nf2/Merlin controls progenitor homeostasis and tumorigenesis in the liver. Genes Dev. 2010; 24:1718–1730. [PubMed: 20675406]

Bennett FC, Harvey KF. Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signaling pathway. Curr Biol. 2006; 16:2101–2110. [PubMed: 17045801]

Chen CL, Gajewski KM, Hamaratoglu F, Bossuyt W, Sansores-Garcia L, Tao C, Halder G. The apical-basal cell polarity determinant Crumbs regulates Hippo signaling in Drosophila. Proc Natl Acad Sci U S A. 2010; 107:15810–15815. [PubMed: 20798049]

Cho E, Feng Y, Rauskolb C, Maitra S, Fehon R, Irvine KD. Delineation of a Fat tumor suppressor pathway. Nat Genet. 2006; 38:1142–1150. [PubMed: 16980976]

Chou WH, Huber A, Bentrop J, Schulz S, Schwab K, Chadwell LV, Paulsen R, Britt SG. Patterning of the R7 and R8 photoreceptor cells of Drosophila: evidence for induced and default cell-fate specification. Development. 1999; 126:607–616. [PubMed: 9895309]

Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders RA, Maitra A, Pan D. Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell. 2007; 130:1120–1133. [PubMed: 17889654]

Dutta S, Baehrecke EH. Warts is required for PI3K-regulated growth arrest, autophagy, and autophagic cell death in Drosophila. Curr Biol. 2008; 18:1466–1475. [PubMed: 18818081]

Earl JB, Britt SG. Expression of Drosophila rhodopsins during photoreceptor cell differentiation: insights into R7 and R8 cell subtype commitment. Gene Expr Patterns. 2006; 6:687–694. [PubMed: 16495161]

Emoto K, Parrish JZ, Jan LY, Jan YN. The tumour suppressor Hippo acts with the NDR kinases in dendritic tiling and maintenance. Nature. 2006; 443:210–213. [PubMed: 16906135]

Ferrell JE Jr. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. Curr Opin Cell Biol. 2002; 14:140–148. [PubMed: 11891111]

Genevet A, Polesello C, Blight K, Robertson F, Collinson LM, Pichaud F, Tapon N. The Hippo pathway regulates apical-domain size independently of its growth-control function. J Cell Sci. 2009; 122:2360–2370. [PubMed: 19531586]

Genevet A, Wehr MC, Brain R, Thompson BJ, Tapon N. Kibra is a regulator of the Salvador/Warts/Hippo signaling network. Dev Cell. 2010; 18:300–308. [PubMed: 20159599]

Goulev Y, Fauny JD, Gonzalez-Marti B, Flagiello D, Silber J, Zider A. SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in Drosophila. Curr Biol. 2008; 18:435–441. [PubMed: 18313299]

Graveley BR, Brooks AN, Carlson JW, Duff MO, Landolin JM, Yang L, Artieri CG, van Baren MJ, Boley N, Booth BW, et al. The developmental transcriptome of Drosophila melanogaster. Nature. 2011; 471:473–479. [PubMed: 21179090]

Grusche FA, Richardson HE, Harvey KF. Upstream regulation of the hippo size control pathway. Curr Biol. 2010; 20:R574–582. [PubMed: 20619814]

Grzeschik NA, Parsons LM, Allott ML, Harvey KF, Richardson HE. Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. Curr Biol. 2010; 20:573–581. [PubMed: 20362447]

Halder G, Johnson RL. Hippo signaling: growth control and beyond. Development. 2011; 138:9–22. [PubMed: 21138973] Rister J, Desplan C. The retinal mosaics of opsin expression in invertebrates and vertebrates. Dev Neurobiol. 2011

Hamaratoglu F, Gajewski K, Sansores-Garcia L, Morrison C, Tao C, Halder G. The Hippo tumor-suppressor pathway regulates apical-domain size in parallel to tissue growth. J Cell Sci. 2009; 122:2351–2359. [PubMed: 19531584]

Hamaratoglu F, Willecke M, Kango-Singh M, Nolo R, Hyun E, Tao C, Jafar-Nejad H, Halder G. The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. Nat Cell Biol. 2006; 8:27–36. [PubMed: 16341207]

Hardie, RC. Functional organization of the fly retina. In: Ottoson, D., editor. Progress in Sens Physiol. 5. Springer; Berlin, Heidelberg, New York, Toronto: 1985. p. 1-79.

Harvey K, Tapon N. The Salvador-Warts-Hippo pathway - an emerging tumour- suppressor network. Nat Rev Cancer. 2007; 7:182–191. [PubMed: 17318211]

Ho LL, Wei X, Shimizu T, Lai ZC. Mob as tumor suppressor is activated at the cell membrane to control tissue growth and organ size in Drosophila. Dev Biol. 2010; 337:274–283. [PubMed: 19913529]

Hobert O. Regulatory logic of neuronal diversity: terminal selector genes and selector motifs. Proc Natl Acad Sci U S A. 2008; 105:20067–71. [PubMed: 19104055]

Huang J, Wu S, Barrera J, Matthews K, Pan D. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila Homolog of YAP. Cell. 2005; 122:421–434. [PubMed: 16096061]

Jessell TM. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet. 2000 Oct; 1(1):20–9. [PubMed: 11262869]

Jukam D, Desplan C. Binary fate decisions in differentiating neurons. Curr Opin Neurobiol. 2010; 20:6–13. [PubMed: 20022236]

Lai ZC, Wei X, Shimizu T, Ramos E, Rohrbaugh M, Nikolaidis N, Ho LL, Li Y. Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. Cell. 2005; 120:675–685. [PubMed: 15766530]

LaJeunesse DR, McCartney BM, Fehon RG. Structural analysis of Drosophila merlin reveals functional domains important for growth control and subcellular localization. J Cell Biol. 1998; 141:1589–1599. [PubMed: 9647651]

Lee CY, Robinson KJ, Doe CQ. Lgl, Pins and aPKC regulate neuroblast self-renewal versus differentiation. Nature. 2006; 439:594–598. [PubMed: 16357871]

Lesch BJ, Bargmann CI. The homeodomain protein hmbx-1 maintains asymmetric gene expression in adult C. elegans olfactory neurons. Genes Dev. 2010; 24:1802–1815. [PubMed: 20713521]

Ling C, Zheng Y, Yin F, Yu J, Huang J, Hong Y, Wu S, Pan D. The apical transmembrane protein Crumbs functions as a tumor suppressor that regulates Hippo signaling by binding to Expanded. Proc Natl Acad Sci U S A. 2010; 107:10532–10537. [PubMed: 20498073]

MacCollin M, Mohney T, Trofatter J, Wertelecki W, Ramesh V, Gusella J. DNA diagnosis of neurofibromatosis 2. Altered coding sequence of the merlin tumor suppressor in an extended pedigree. JAMA. 1993; 270:2316–2320. [PubMed: 8230593]

MacDougall N, Lad Y, Wilkie GS, Francis-Lang H, Sullivan W, Davis I. Merlin, the Drosophila homologue of neurofibromatosis-2, is specifically required in posterior follicle cells for axis formation in the oocyte. Development. 2001; 128:665–673. [PubMed: 11171392]

Maitra S, Kulikauskas RM, Gavilan H, Fehon RG. The tumor suppressors Merlin and Expanded function cooperatively to modulate receptor endocytosis and signaling. Curr Biol. 2006; 16:702–709. [PubMed: 16581517]

Mazzoni EO, Celik A, Wernet MF, Vasiliauskas D, Johnston RJ, Cook TA, Pichaud F, Desplan C. Iroquois complex genes induce co-expression of rhodopsins in Drosophila. PLoS Biol. 2008; 6:e97. [PubMed: 18433293]

McCartney BM, Kulikauskas RM, LaJeunesse DR, Fehon RG. The neurofibromatosis-2 homologue, Merlin, and the tumor suppressor expanded function together in Drosophila to regulate cell proliferation and differentiation. Development. 2000; 127:1315–1324. [PubMed: 10683183]

Mikeladze-Dvali T, Wernet MF, Pistillo D, Mazzoni EO, Teleman AA, Chen YW, Cohen S, Desplan C. The growth regulators warts/lats and melted interact in a bistable loop to specify opposite fates in Drosophila R8 photoreceptors. Cell. 2005; 122:775–787. [PubMed: 16143107]

Milton CC, Zhang X, Albanese NO, Harvey KF. Differential requirement of Salvador-Warts-Hippo pathway members for organ size control in Drosophila melanogaster. Development. 2010; 137:735–743. [PubMed: 20110315]

Newsome TP, Asling B, Dickson BJ. Analysis of Drosophila photoreceptor axon guidance in eye-specific mosaics. Development. 2000; 127:851–860. [PubMed: 10648243]

Oh H, Irvine KD. In vivo regulation of Yorkie phosphorylation and localization. Development. 2008; 135:1081–1088. [PubMed: 18256197]

Oh H, Irvine KD. In vivo analysis of Yorkie phosphorylation sites. Oncogene. 2009; 28:1916–1927. [PubMed: 19330023]

Pan D. The hippo signaling pathway in development and cancer. Dev Cell. 2010; 19:491–505. [PubMed: 20951342]

Pellock BJ, Buff E, White K, Hariharan IK. The Drosophila tumor suppressors Expanded and Merlin differentially regulate cell cycle exit, apoptosis, and Wingless signaling. Dev Biol. 2007; 304:102–115. [PubMed: 17258190]

Peng HW, Slattery M, Mann RS. Transcription factor choice in the Hippo signaling pathway: homothorax and yorkie regulation of the microRNA bantam in the progenitor domain of the Drosophila eye imaginal disc. Genes Dev. 2009; 23:2307–2319. [PubMed: 19762509]

Polesello C, Huelsmann S, Brown NH, Tapon N. The Drosophila RASSF homolog antagonizes the hippo pathway. Curr Biol. 2006; 16:2459–2465. [PubMed: 17174922]

Praskova M, Khoklatchev A, Ortiz-Vega S, Avruch J. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. Biochem J. 2004; 381:453–462. [PubMed: 15109305]

Reddy BV, Rauskolb C, Irvine KD. Influence of fat-hippo and notch signaling on the proliferation and differentiation of Drosophila optic neuroepithelia. Development. 2010; 137:2397–2408. [PubMed: 20570939]

Ribeiro PS, Josue F, Wepf A, Wehr MC, Rinner O, Kelly G, Tapon N, Gstaiger M. Combined functional genomic and proteomic approaches identify a PP2A complex as a negative regulator of Hippo signaling. Mol Cell. 2010; 39:521–534. [PubMed: 20797625]

Rister J, Desplan C. The retinal mosaics of opsin expression in invertebrates and vertebrates. Dev Neurobiol. 2011 May 9.10.1002/dneu.20905

Robinson BS, Huang J, Hong Y, Moberg KH. Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein Expanded. Curr Biol. 2010; 20:582–590. [PubMed: 20362445]

Sanes JR, Zipursky SL. Design principles of insect and vertebrate visual systems. Neuron. 2010; 66:15–36. [PubMed: 20399726]

Silva E, Tsatskis Y, Gardano L, Tapon N, McNeill H. The tumor-suppressor gene fat controls tissue growth upstream of expanded in the hippo signaling pathway. Curr Biol. 2006; 16:2081–2089. [PubMed: 16996266]

Sotillos S, Diaz-Meco MT, Caminero E, Moscat J, Campuzano S. DaPKC-dependent phosphorylation of Crumbs is required for epithelial cell polarity in Drosophila. J Cell Biol. 2004; 166:549–557. [PubMed: 15302858]

Stowers RS, Schwarz TL. A genetic method for generating Drosophila eyes composed exclusively of mitotic clones of a single genotype. Genetics. 1999; 152:1631–1639. [PubMed: 10430588]

Striedinger K, VandenBerg SR, Baia GS, McDermott MW, Gutmann DH, Lal A. The neurofibromatosis 2 tumor suppressor gene product, merlin, regulates human meningioma cell growth by signaling through YAP. Neoplasia. 2008; 10:1204–1212. [PubMed: 18953429]

Teleman AA, Chen YW, Cohen SM. Drosophila Melted modulates FOXO and TOR activity. Dev Cell. 2005; 9:271–281. [PubMed: 16054033]

Tyler DM, Baker NE. Expanded and fat regulate growth and differentiation in the Drosophila eye through multiple signaling pathways. Dev Biol. 2007; 305:187–201. [PubMed: 17359963]

Wernet MF, Labhart T, Baumann F, Mazzoni EO, Pichaud F, Desplan C. Homothorax switches function of Drosophila photoreceptors from color to polarized light sensors. Cell. 2003; 115:267–279. [PubMed: 14636555]

Wernet MF, Mazzoni EO, Celik A, Duncan DM, Duncan I, Desplan C. Stochastic spineless expression creates the retinal mosaic for colour vision. Nature. 2006; 440:174–180. [PubMed: 16525464]

Wilkins, AS. The Evolution of Developmental Pathways. Sinauer; Sunderland, MA: 2002.

Willecke M, Hamaratoglu F, Kango-Singh M, Udan R, Chen CL, Tao C, Zhang X, Halder G. The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. Curr Biol. 2006; 16:2090–2100. [PubMed: 16996265]

Willecke M, Hamaratoglu F, Sansores-Garcia L, Tao C, Halder G. Boundaries of Dachsous Cadherin activity modulate the Hippo signaling pathway to induce cell proliferation. Proc Natl Acad Sci U S A. 2008; 105:14897–14902. [PubMed: 18809931]

Wu S, Liu Y, Zheng Y, Dong J, Pan D. The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. Dev Cell. 2008; 14:388–398. [PubMed: 18258486]

Yamaguchi S, Wolf R, Desplan C, Heisenberg M. Motion vision is independent of color in Drosophila. Proc Natl Acad Sci U S A. 2008; 105:4910–4915. [PubMed: 18353989]

Yu J, Zheng Y, Dong J, Klusza S, Deng WM, Pan D. Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. Dev Cell. 2010; 18:288–299. [PubMed: 20159598]

Zhang L, Ren F, Zhang Q, Chen Y, Wang B, Jiang J. The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Dev Cell. 2008; 14:377–387. [PubMed: 18258485]

Zhang N, Bai H, David KK, Dong J, Zheng Y, Cai J, Giovannini M, Liu P, Anders RA, Pan D. The Merlin/NF2 tumor suppressor functions through the YAP oncoprotein to regulate tissue homeostasis in mammals. Dev Cell. 2010; 19:27–38. [PubMed: 20643348]

Zhao B, Li L, Lei Q, Guan KL. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. Genes Dev. 2010; 24:862–874. [PubMed: 20439427]

Highlights

- *mer*, *kib*, and *lgl* specify yR8 photoreceptor neurons and induce Rh6 expression.
- *mer* and *kib* are required for *warts* expression and *melted* repression in R8.
- *mer* and *lgl* activity are continuously required to maintain the yR8 fate in adults.
- *melted* is sufficient to disrupt active Hippo pathway signaling in R8.

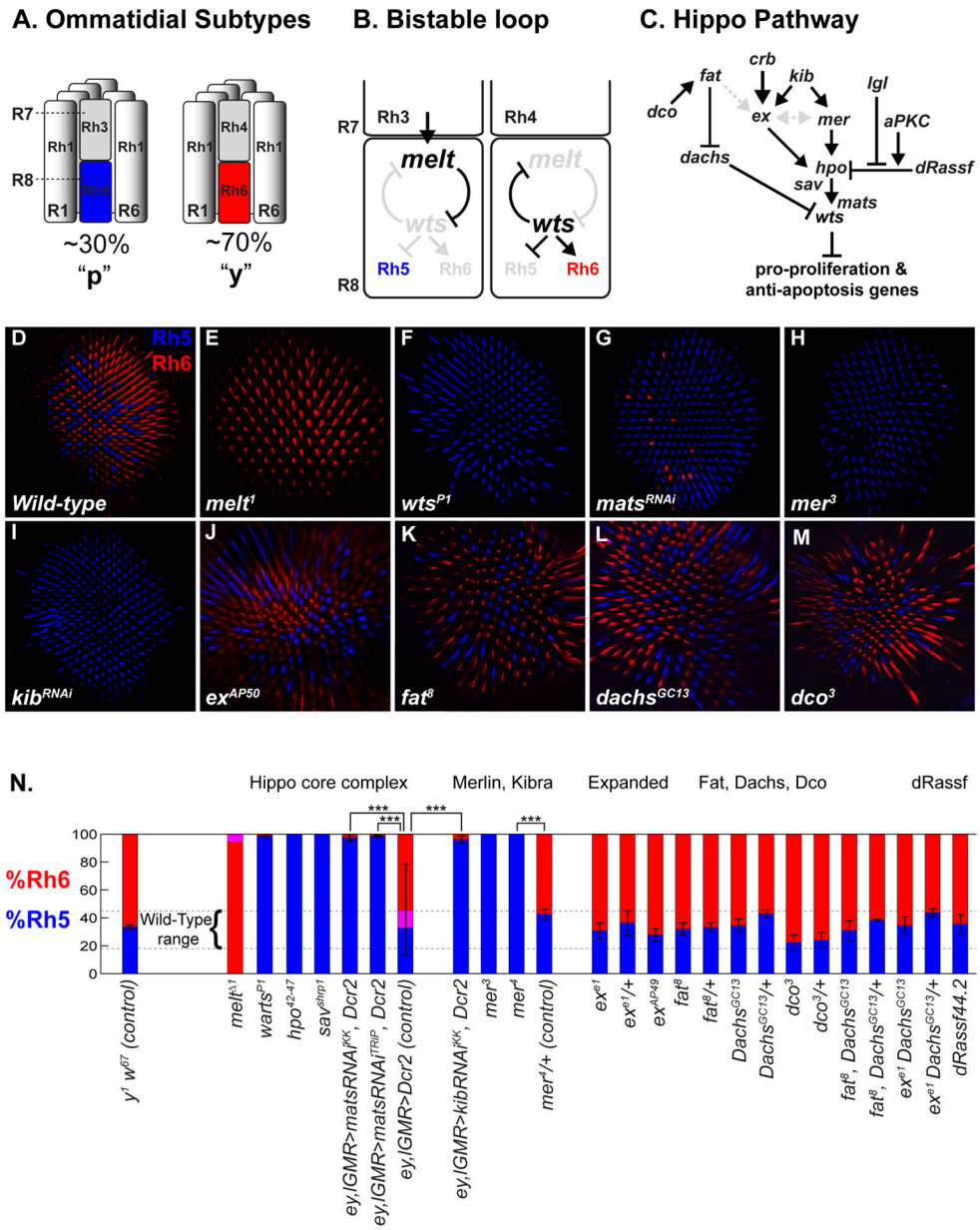

Figure 1. A Merlin/Kibra branch of the Hippo pathway regulates R8 subtypes specification

(A) R7/R8 Rhodopsin pairing defines ommatidial subtypes. Outer photoreceptors (R1–R6) express *rh1*, whereas R7/R8 primarily express either *rh3*/*rh5* or *rh4*/*rh6*.

(B) A bistable feedback loop regulates mutually exclusive Rh5 and Rh6 expression in R8: *warts* and *melted* are expressed in **y-** and **p**-R8, respectively, and repress each other's transcription.

(C) The Hippo signaling network regulates proliferation and cell death. Upstream inputs converge onto the Hpo/Sav/Mats/Wts "core complex" to regulate Wts activity.

(D–M): Confocal images of adult retinas stained with Rh5 (blue) and Rh6 (red) antibodies that label R8 subtypes. (E–F, H, J–M) are whole mutant retinas. Genotypes in Supp Methods.

(D) wild-type. Note the roughly 70:30 ratio of Rh6:Rh5.

(E) *melt*^{1}

(F) *warts*^{P1}
(G) retina expressing *mats-RNAi*
(H) *mer*^{3}
(I) retina expressing *kib-RNAi*. All R8s in *kib*^{del} null mutants also express Rh5, but the *kib*^{del} chromosome contains a mutation in *rh6* preventing analysis of Rh6 expression (data not shown).
(J) *ex*^{AP50}
(K) *fat*^{8}
(L) *dachs*^{GC13}
(M) *dco*^{3}
(N) Quantification of R8 subtypes in Hippo pathway mutants. Graph presents proportion R8s (y-axis) that contain Rh5 (blue), Rh6 (red), or co-expression (pink). Dashed lines indicate the wild-type range of 20%–45% Rh5 based on counts in wild-type or balancer strains of various genetic backgrounds (data not shown). Heterozygote controls contained either a wild-type chromosome (chr), or, for mutants on chr X, FM7c; chr II, the CyO balancer; chr III, TM2 or TM6B. Mean %Rh5 were compared with a two-tailed, unpaired t-test. (***) $p<0.001$. wild-type: n=9 retinas, N=3055 ommatidia, *mats-RNAi*: n=8, N=1561; *kib-RNAi*: n=11, N=3214; *ex*^{e1}: n=8, N=1869; all others genotypes: n≥4, N≥800.

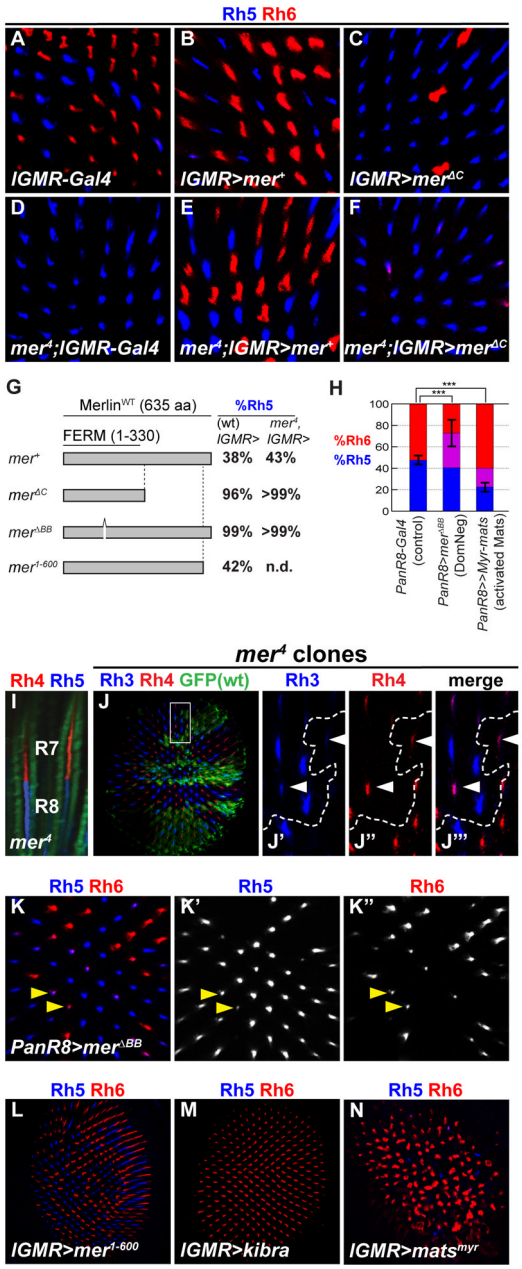

Figure 2. Merlin acts specifically in R8 and requires the FERM domain and C-terminus to regulate yR8 photoreceptor subtypes

(A–F, J–N) adult retinas stained for Rh5 (blue) and Rh6 (red). Full genotypes listed in Supplemental Methods.

(A) *lGMR-Gal4* control

(B) *lGMR>mer+*

(C) *lGMR>mer$^{\Delta C}$*

(D) *mer^{4}*; *lGMR-Gal4* (negative control for rescue experiments)

(E) *mer^{4}*; *lGMR>mer^{+}* (rescue of R8 subtype specification)

(F) *mer^{4}; lGMR> mer$^{\Delta C}$*

(G) Merlin deletion constructs (LaJeunesse et al., 1998) mis-expressed in wild-type and *mer*^{4} mutant retinas. Columns indicate percent of R8s that express Rh5. n.d.= not determined.
(H) Proportion of R8s that express Rh5 (blue), Rh6 (red), or both (purple) in *PanR8>mer*$^{\Delta BB}$ (comparing %Rh5; n=8 retinas, N= 2329 ommatidia) *PanR8>Myr-mats*(comparing %Rh6; n=7, N=2355) and *PanR8-Gal4* controls (n=7, N=2131). Two-tailed, unpaired t-test; *** denotes $p<0.001$.
(I) Side view of *mer*^{4} mutant ommatidia showing Rh4 (red) and Rh5 (blue) mis-coupling in the same ommatidium. Alexa-488 conjugated Phalloidin (green) stains actin and labels rhabdomeres (outer photoreceptors in image).
(J-J″) *mer*^{4} mutant clones (absence of GFP) stained with antibodies for R7 Rhs, Rh3 (blue) and Rh4 (red). (J′-J″) “Dorsal third” yR7 ommatidia, where Rh3 and Rh4 are co-expressed (white arrowheads) (Mazzoni et al. 2008). White box in panel (J) indicates area shown in adjacent panels. Dashed line indicates clone boundary.
(K-K″) *PanR8>mer*$^{\Delta BB}$ (K′) Rh5 (K″) Rh6. Arrows show co-expression of Rh5 and Rh6. Note the decrease in Rh6 intensity in co-expressing cells (K″).
(L) *lGMR>mer*$^{1-600}$
(M) *lGMR>kib*
(N) *lGMR>Myr-mats-GFP* (activated *mats* containing a Myristoylation sequence) The disorganized ommatidia and altered rhabdomere structure in some R8s is likely due to earlier growth functions of *mats* (Ho et al., 2010).

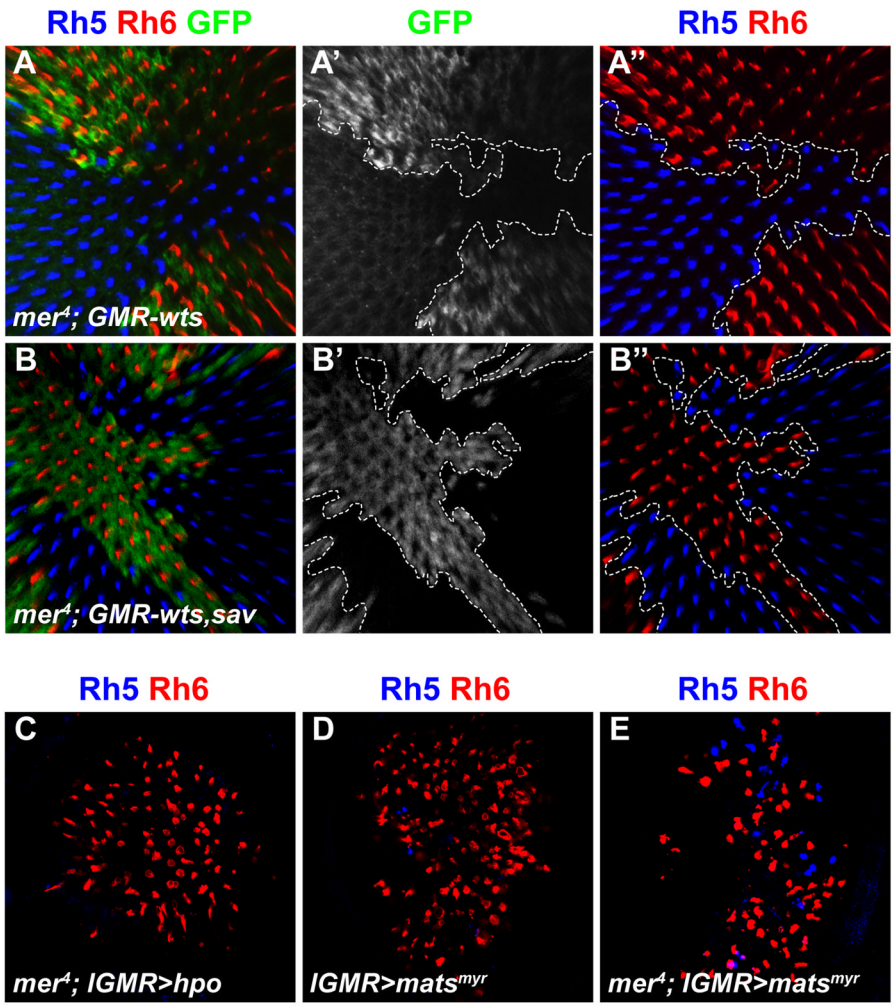

Figure 3. Warts requires Merlin to induce yR8 subtype

(A–B″) all depict adult retina stained with antibodies for Rh5 (blue), Rh6 (red), and GFP (green). Dashed lines indicate clonal boundaries.

(A) *mer*^{4}; *GMR-wts* (*mer*^{4} mutant clones (marked by absence of GFP) in retina that mis-expresses *wts* in all photoreceptors.) Note the presence of only Rh6 in *GMR-wts* tissue when *mer* is wild-type (GFP^{+}), and absence of Rh6 when *mer* is removed (non-GFP).

(A′) GFP only.

(A″) Rh6 and Rh5 only.

(B) *mer*^{4}; *GMR-sav, wts* (*mer*^{4} mutant clones marked by absence of GFP in retinas that mis-expresses *sav* and *wts* in all photoreceptors).

(B′) GFP only.

(B″) Rh6 and Rh5 only.

(C–E) Adult retinas stained with antibodies for Rh5 (blue) and Rh6 (red).

(C) *mer*^{4}; *lGMR>hpo*. Compare to Figure 2D (*mer*^{4} mutants) and Figure S1H (*GMR-hpo*). Experiment was performed at 18 °C as *lGMR>hpo* flies do not eclose at 25 C. (D) *lGMR>Myr-mats*

(E) *mer*^{4}; *lGMR>Myr-mats*. Compare to *mer*^{4} mutants in Figure 2D.

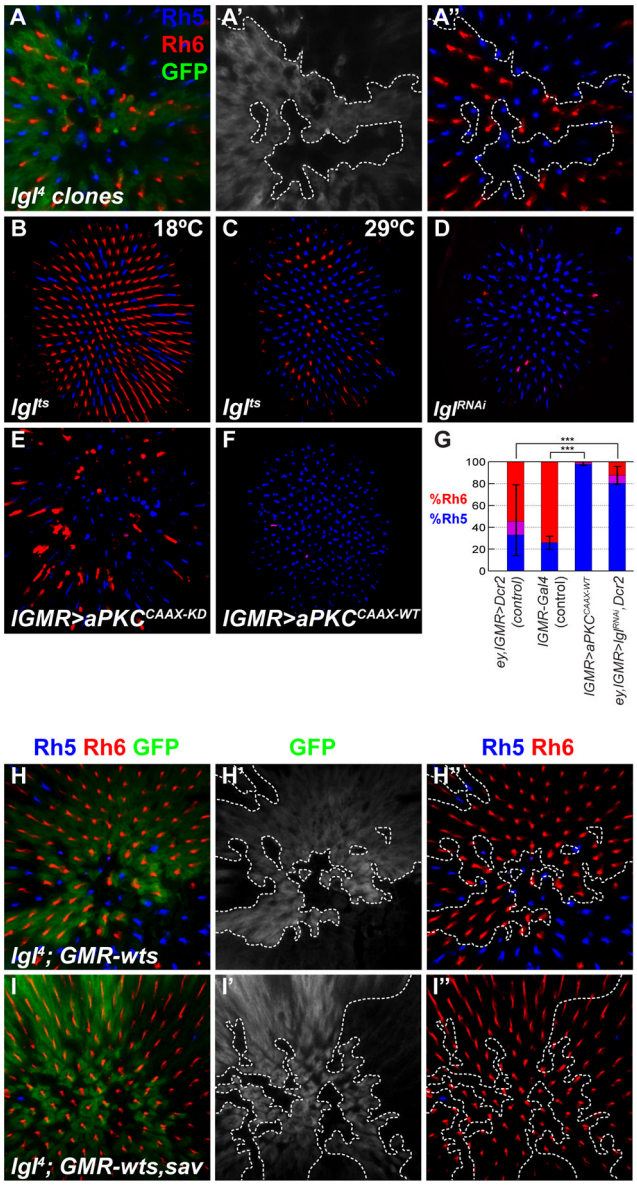

Figure 4. Lgl is required and aPKC is sufficient to specify yR8 subtype

(A–F, H–I″) Adult retinas stained for Rh5 (blue), Rh6 (red), GFP (green). Dashed lines indicate clonal boundaries.

(A-A″) *lgl*^{4} clones (non-GFP)

(A′) GFP only

(A″) Rh6 and Rh5 only. Dashed line indicates clone boundary.

(B) *lgl*^{ts}3 raised at 18°C

(C) *lgl*^{ts3} shifted to 29°C during early pupation

(D) retina expressing *lgl-RNAi*

(E) *lGMR>aPKC*$^{CAAX\text{-}KD}$(F) *lGMR>aPKC*$^{CAAX\text{-}WT}$ (active)

(G) Proportion of R8 that express Rh5 (blue), Rh6 (red), or both (purple) in listed genotypes. For all genotypes: n≥5, N≥1100; %Rh5 means compared with two-tailed, unpaired t-test; ***p<0.001;*p<0.05.

(H) *lgl*^{4} clones in *GMR-wts* retina background.

(H′) GFP only
(H″) Rh5 and Rh6 only
(I) *lgl*^{4} clones in *GMR-wts, sav* background.
(I′) GFP only
(I″) Rh5 and Rh6 only

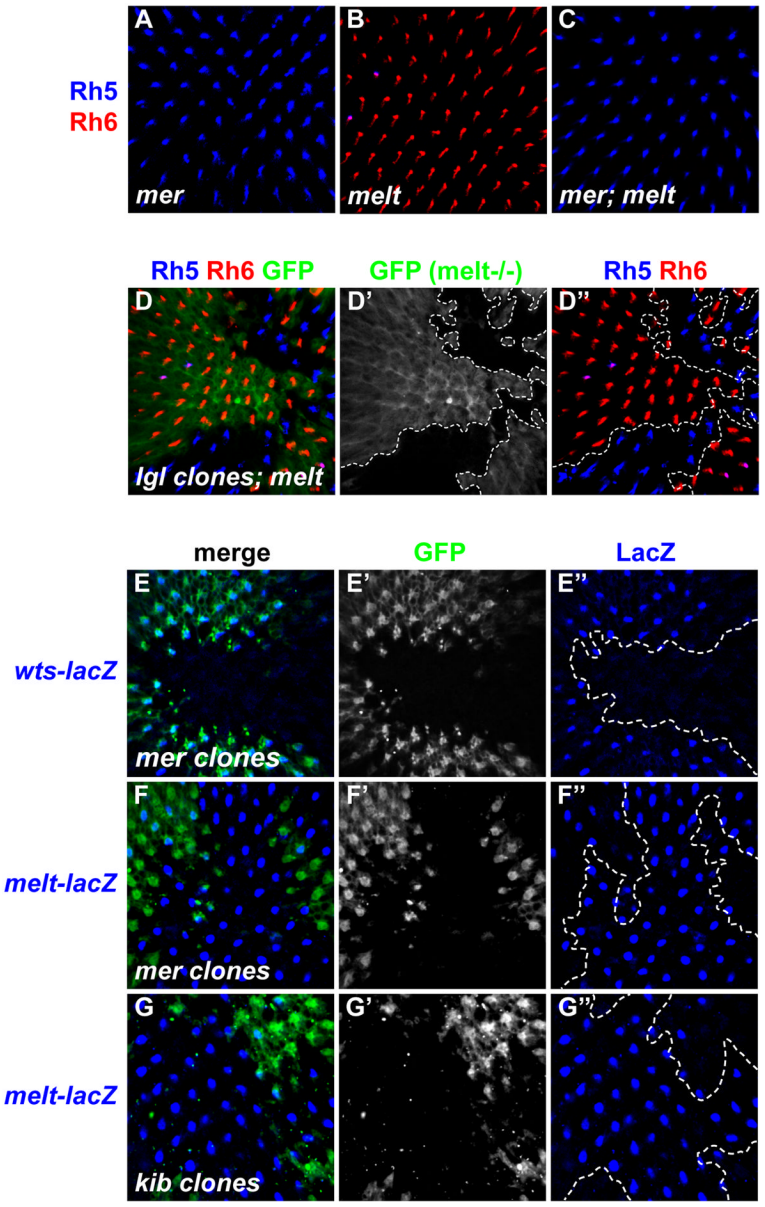

Figure 5. Merlin and Kibra regulate the expression of Warts and Melted in R8

(A–C) Adult retinas stained for Rh5 (blue) and Rh6 (red).

(A) hemizygous mer^{ts}/Y mutants kept at the non-permissive temperature from early pupation.

(B) $melt^{1}$.

(C) mer^{ts}/Y; $melt^{1}$ double mutants.

(D-D″) lgl^{4} clones in $melt^{1}$ mutant background. Rh5 (blue), Rh6 (red), GFP (green). $melt^{1}$ tissue is GFP-positive; lgl^{4}; $melt^{1}$ double mutant tissue is GFP-negative. Dashed lines indicate clonal boundaries.

(E–G″) Adult retinas showing either *mer* or *kib* mutant clones (GFP-negative) stained with β-gal antibody that labels nuclearly localized *warts*- or *melt-lacZ*. Confocal image taken in the R8 nucleus layer focal plane. In (E), (F), and (G), lacZ-positive R8 cells that overlap GFP represent wild-type R8 nuclei. Dashed lines indicate clonal boundaries.

(E) *mer*^{4} clones (GFP-negative) and *warts-LacZ*. Note the presence of *warts-LacZ* expression in wild-type tissue (GFP+).
(E′) grayscale of GFP in (E)
(E″) *wts-lacZ* of (E).
(F) *mer*^{4} mutant clones (GFP-negative) and *melt-lacZ.* Note that *melt-lacZ* expression is present in roughly a third of wild-type R8 cells, but in almost all *mer*^{4} mutant R8s.
(F′) grayscale of GFP in (F)
(F″) *melt-lacZ* of (F)
(G) *kib*^{del} mutant clones (GFP-negative) and *melt-lacZ.*
(G′) grayscale of GFP in (G)
(G″) *melt-lacZ* of (G).

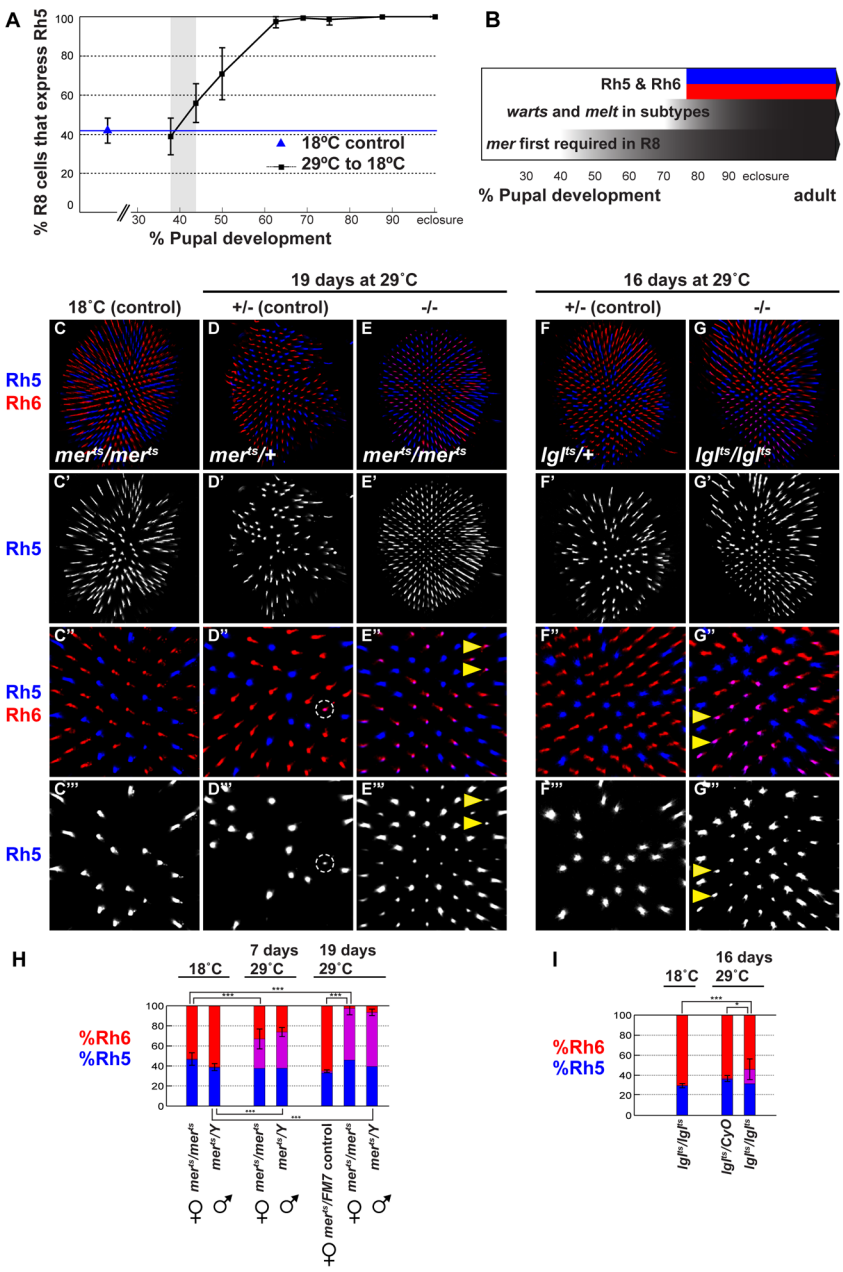

Figure 6. Continuous Merlin and Lgl signaling is required to maintain the yR8 subtype fate

(A) *mer* is first required for yR8 specification at ~38–44% pupation (shaded gray bar). Graph shows proportion of R8s expressing Rh5 in adult retinas of mer^{ts} flies where *mer* function was restored after shift from the restrictive to permissive temperature at various time points during pupation (black squares). (B) R8 subtype specification timeline during development. *mer* is required as early as ~40% pupation, followed by R8 subtype-specific expression of *wts* and *melt*, which define **y-** and **p**-R8, respectively, and induce Rh6 and Rh5 expression around 70–80% pupation. R8 subtypes and mutually exclusive Rh expression are maintained in adults.

All images in (C-G‴) are adult retinas stained for Rh5 (blue) and Rh6 (red).

(C-C‴) homozygous mer^{ts} flies reared at the permissive temperature.

(C′) Rh5 of (C).

(C″) close up of (C).
(C‴) Rh5 of (C′)
(D) heterozygous *mer*^{ts}/+ control flies shifted to the restrictive temperature (29°C) for 19 days during adulthood.
(D′) Rh5 of (D)
(D″) close up of (D). Rh5 and Rh6 are co-expressed in fewer than 2% of R8 cells (Dashed circle).
(D‴) Rh5 of (D″). Dashed circle indicates the rare yR8 that has weakly de-repressed Rh5.
(E) homozygous *mer*^{ts} flies shifted to the restrictive temperature (29°C) for 19 days during adulthood.
(E′) Rh5 of (E). (E″) close-up of (E′). Arrowheads indicate examples of Rh5 de-repressed in the former yR8 subtype.
(E‴) Rh5 of (E″).
(F) heterozygous *lgl*^{ts}/+ flies shifted to the restrictive temperature for 16 days during adulthood.
(F′) Rh5 of (F).
(F″) close-up of (F′).
(F‴) Rh5 of (F″).
(G) homozygous *lgl*^{ts} flies shifted to the restricted temperature for 16 days during adulthood.
(G′) Rh5 of (G).
(G″) close-up of (G′). Many Rh6-expressing cells co-express Rh5 (pink); examples indicated by arrowheads.
(G‴) Rh5 of (G″).
(H) Proportion of R8s that express Rh5 when *mer* function is removed in adult flies, as compared to controls. y-axis shows percent exclusively Rh5 (blue), exclusively Rh6 (red), and Rh5/Rh6 co-expression (purple). Two-tailed, unpaired t-test; (***) denotes $p<0.0001$.
(I) Proportion of R8s that express Rh5 when *lgl* function is removed in adult flies, as compared to controls. y-axis shows percent exclusively Rh5 (blue), exclusively Rh6 (red), and Rh5/Rh6 co-expression (purple). Two-tailed, unpaired t-test; (***) denotes $p<0.001$. (*) denotes $p<0.05$.

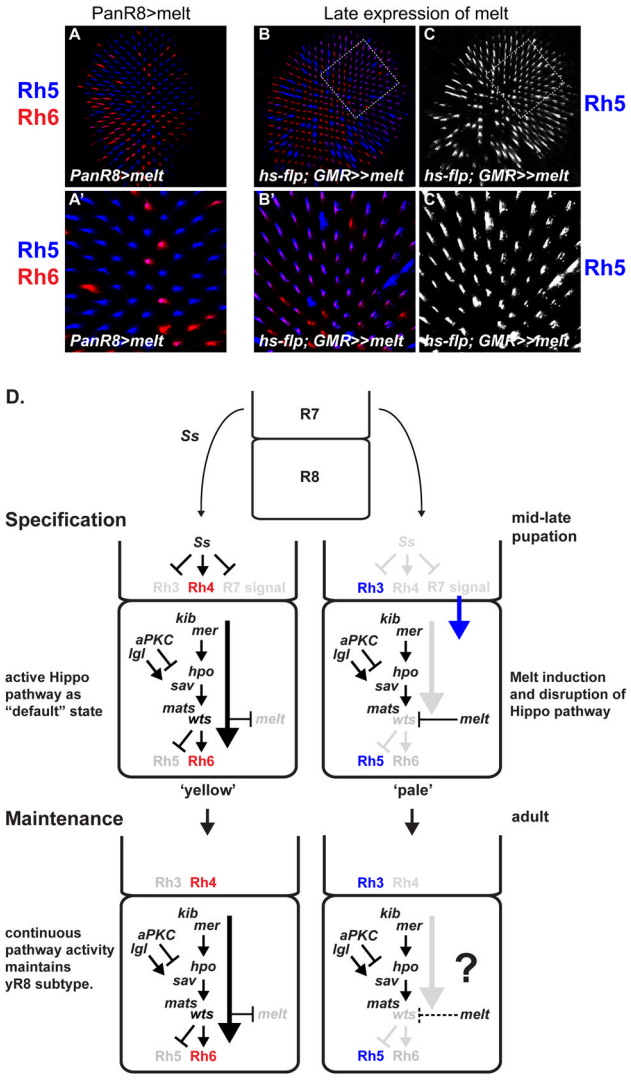

Figure 7. Model for regulation of Hippo pathway signaling in a post-mitotic fate decision

(A–C′) All panels show adult retinas stained for Rh5 (blue) and Rh6 (red).

(A) *PanR8>melt. melt* expressed immediately after subtype-specific Rh expression can reset the bistable feedback loop and induce the pR8 fate.

(A′) close up of (A). Note increase in Rh5-only expressing pR8s and decrease in Rh6-expressing yR8 cells.

(B) ectopic *melt* expressed in adult flies (see methods).

(B′) close-up of (B). Note that Rh6 staining appears weaker in cells that co-express Rh5 and Rh6.

(C) Rh5 of (B).

(C′) Rh5 of (B′).

(D) Model of Hippo pathway regulation for binary fate specification of R8. Constitutive and continuous upstream pathway signaling by Mer, Kib, and Lgl promotes yR8 fate, while repression of *warts* by Melt disrupts Hippo signaling to specify pR8 fate. Genes in black or colored font are expressed and active in that particular cell; genes in gray font are not expressed or not active.