RESEARCH ARTICLE

The ZO-1 protein Polychaetoid as an upstream regulator of the Hippo pathway in Drosophila

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Abstract

The generation of a diversity of photoreceptor (PR) subtypes with different spectral sensitivities is essential for color vision in animals. In the Drosophila eye, the Hippo pathway has been implicated in blue- and green-sensitive PR subtype fate specification. Specifically, Hippo pathway activation promotes green-sensitive PR fate at the expense of blue-sensitive PRs. Here, using a sensitized triple heterozygote-based genetic screening approach, we report the identification of the single Drosophila zonula occludens-1 (ZO-1) protein Polychaetoid (Pyd) as a new regulator of the Hippo pathway during the blue- and green-sensitive PR subtype binary fate choice. We demonstrate that Pyd acts upstream of the core components and the upstream regulator Pez in the Hippo pathway. Furthermore, we found that Pyd represses the activity of Su(dx), a E3 ligase that negatively regulates Pez and can physically interact with Pyd, during PR subtype fate specification. Together, our results identify a new mechanism underlying the Hippo signaling pathway in post-mitotic neuronal fate specification.

Author summary

The Hippo signaling pathway was originally discovered for its critical role in tissue growth and organ size control. Its evolutionarily conserved roles in various biological processes, including cell differentiation, stem cell regeneration and homeostasis, innate immune biology, as well as tumorigenesis, have been subsequently found in other species. During the development of the Drosophila eye, the Hippo pathway promotes green- and represses blue-sensitive photoreceptor (PR) subtype fate specification. Taking advantage of this binary PR fate choice, we screened Drosophila chromosomal deficiency lines to seek new regulators of the Hippo signaling pathway. We identified the Drosophila membrane-associated ZO-1 protein Pyd as an upstream regulator of the Hippo pathway to specify PR
subtypes. Our results have demonstrated that Pyd represses Su(dx)’s activity in the Hippo pathway to specify PR subtypes. Our results demonstrate a new mechanism underlying the Hippo signaling pathway in post-mitotic neuronal fate specification.

Introduction

Generating neuronal diversity during the development of a sensory organ is a prerequisite for the organ to perceive and discriminate various external stimuli. For example, the perception of color relies on comparing the outputs of multiple light-sensing photoreceptor (PR) subtypes with different spectral sensitivities [1–3]. During development, the fate of sensory neurons is progressively restricted toward terminal differentiation, finally generating diverse neuronal subtypes. Although the role of transcriptional regulations during neuronal terminal differentiation has been extensively studied [4,5], the details of how specific signaling pathways influence this process are not well understood. Here we use the blue- and green-sensitive PR binary fate decisions in the *Drosophila* eye as a model to understand the role of the Hippo pathway in post-mitotic neuronal terminal differentiation.

The *Drosophila* eye is a powerful model to understand the principles of neuronal development [1,6–8]. The *Drosophila* compound eye contains ~750 individual units, ommatidia, each of which consists of eight PRs: the outer PRs R1-R6 and the inner PRs R7-R8 (Fig 1A). There are two main subtypes of ommatidia: pale (p) and yellow (y) ommatidia, present in the adult *Drosophila* eye (Fig 1B). The outer photoreceptors R1-R6 in both p and y ommatidia express the broad spectrum light sensitive opsin Rhodopsin 1 (Rh1) and are responsible for dim light vision and motion detection. However, the inner R7 and R8 cells express Rhodopsins with different spectral sensitivities, making them capable of performing color vision [9]. In p ommatidia, R7s express UV-sensitive Rh3 and R8s express blue-sensitive Rh5, while in y ommatidia, R7s express UV sensitive Rh4 and R8s express green-sensitive Rh6 (Fig 1B). The p vs. y fate decision is first made in R7s via the stochastic activation of the transcription factor Spineless in yR7s during mid-pupation [11]. R7s that do not express Spineless (i.e. the pR7s) instruct their underlying R8s to adopt pale R8 (pR8) fate through Activin and BMP signaling [12]. R8s that do not receive the pR7 signals default to yellow R8 (yR8) fate [13,14]. The effectors involved in p vs. y fate decision in R8s involve two proteins—Melted (Melt) and Warts (Wts) [15] (Fig 1C). *melt* encodes a pleckstrin homology domain-containing protein [16], while *wts* encodes a serine/threonine kinase that is a core component in the Hippo signaling pathway [17–19]. *melt* expression is activated in pR8s by the pR7-driven Activin and BMP signals and its expression leads to the transcriptional repression of *wts*. Conversely, *wts* represses *melt* expression in yR8s by suppressing the activity of the transcriptional coactivator Yorkie (Yki), the downstream effector of the Hippo pathway. Yki is necessary for *melt* expression in pR8s. Therefore, *wts*, *melt* and Yki form a double negative regulatory loop to ensure pR8 vs. yR8 subtype fate decision (Fig 1C) [10]. Yki, together with its DNA-binding partner Scalloped (Sd), regulates the output of the regulatory loop to promote the expression of blue-sensitive Rh5 and prevent the expression of green-sensitive Rh6 [20].

The Hippo pathway was originally discovered in *Drosophila* for its pivotal roles in tissue growth and organ size control [21]. Its critical and conserved roles in mammals have been subsequently identified in a wide range of biological processes, including stem cell regeneration and homeostasis, innate immune biology, cell differentiation, as well as tumorigenesis [19,22–24]. The components of the Hippo pathway can be classified into three categories: the core kinase complex, the downstream effectors and the upstream regulators. The core kinase
complex contains the kinases Wts [17,18], Hippo (Hpo) [25–29], and Mob as tumor suppressor (Mats) [30], as well as the scaffold protein Salvador (Sav) [31,32]. Hpo phosphorylates Wts, affiliated by Sav and Mats, and Wts phosphorylates and inhibits the ability of Yki to enter the nucleus [33–35].

Multiple upstream inputs that feed into the core of the Hippo pathway in tissue growth have been identified in recent years. In *Drosophila*, these upstream inputs include the atypical cadherins Fat and Dachs [36–39], the cell adhesion molecule Echinoid (Ed) [40], the complex formed by the FERM-domain protein Expanded (Ex), Merlin (Mer) [41] and the WW-domain
protein Kibra (Kib) [42,43], as well as the cell polarity determinants Crumbs (Crb), Lethal giant larvae (Lgl) and Scribble (Scrib) [44–47]. These upstream inputs act redundantly in tissue growth [41,42]. For example, growth defect in the imaginal disc carrying kib or mer mutations is much weaker compared to those carrying the mutations of the core components of the Hippo pathway. In contrast, double mutations for ex and mer or kib cause severe growth phenotype as demonstrated in hpo or wts mutations [41,42,48]. Interestingly, among these upstream regulators, mer, kib and lgl regulate the Hippo pathway in yR8 fate decision, while fat, dachs and ex are not involved in this process [49]. Additionally, crb is not required for the activation of the Hippo pathway during R8 subtype fate decisions [50]. Therefore, the pale and yellow binary fate assay in the Drosophila retina provides an efficient model with less upstream complication to understand the upstream regulation of the Hippo pathway.

Taking advantage of the pR8 and yR8 binary fate assay, we generated and carried out a sensitive and efficient genome-wide screening to identify the new regulators of the Hippo pathway. We identified the Drosophila ZO-1 protein Pyd as a new upstream regulator of the Hippo pathway. Using loss- and gain-of-function studies, we show that Pyd is required and sufficient to promote green-sensitive yR8 fate and repress blue-sensitive pR8 fate. We additionally determined the roles in PR subtype fate specification for pez and suppressor of deltex (su(dx)), the upstream regulators of the Hippo pathway in the Drosophila midgut epithelium [51,52]. Using epistasis analyses, we revealed that Pyd acts upstream of the core components and the upstream regulator Pez in the Hippo pathway, while it may function in parallel to Kib to repress Su(dx)’s activity to specify R8 subtypes. Together, our study identifies a new upstream regulator of the Hippo pathway that functions in post-mitotic neuronal fate specification.

Results

A triple heterozygote-based screening to identify new regulators of the Hippo pathway

A complementation test is generally used to determine whether or not two mutations define the same or different genes [53]. In most cases, if two recessive mutations that cause similar phenotypes are not in the same genes, the two mutations can be complemented by the corresponding wild type alleles in the F1 double heterozygotes and therefore manifest the wild type phenotype. However, when these mutations are in the genes of the same signaling pathway, they can fail to complement each other. Instead, the double heterozygote can exhibit a mutant phenotype [53,54]. To test whether this non-complementation between the genes in the same pathway can be used to screen the Drosophila deficiency collections [55,56] to identify new components of the Hippo signaling pathway, we performed complementation assays for pR8 and yR8 subtype fate specification between a mutation of wts, the core component of the Hippo pathway, and mutations in other genes in the pathway. Compared to the ratios of pR8 (Rh5+: 33.9 ± 3.6%) and yR8 (Rh6+: 58.5 ± 3.6%) in wild type flies (Fig 1D and 1H), flies heterozygous for a hypomorphic wts enhancer trap line wts288 [15] (wts288/+)(Rh5+: 36.5 ± 3.4%, Rh6+: 65.3 ± 2.3%), a null kib allele kib1 [43] (kib1/+)(Rh5+: 34.7 ± 2.3%, Rh6+: 65.3 ± 2.3%), or the sav deficiency mutation Df(3R)BSC803 (referred to as savdf hereafter) (savdf/+)(Rh5+: 33.0 ± 3.7%, Rh6+: 67.0 ± 3.7%) did not show any statistical difference in the ratios of pR8 and yR8 (Figs 1H and S1A–S1D), while the heterozygous mer1 flies [48] (mer1/+)(Rh5+: 40.1 ± 4.1%, Rh6+: 59.9 ± 4.1%) (Figs 1E and 1H)
and S1G and S1H). There was a higher increase in pR8s at the expense of yR8s in wtsZn/mer3 heterozygous retinas (Rh5+: 56.2±1.9%, Rh6+: 43.8±1.9%) and in mer3/kib1 heterozygous retinas (Rh5+: 65.7±9.9%, Rh6+: 34.3±9.9%) (Figs 1F and 1H and S1F) compared to heterozygous mer3 (mer3/+) mutants and other double heterozygous flies (Figs 1H and S1F). These results showed the heterozygous mutations of the genes in the Hippo pathway can enhance each other’s R8 subtype phenotype, but the effects are too subtle for large-scale screening.

We therefore generated a wtsZn-kib1 chromosome by recombining wtsZn and kib1 mutations and designed a triple heterozygote-based phenotype enhancement assay. We analyzed pR8 and yR8 subtypes in wtsZn-kib1/mer3 and wtsZn-kib1/sav1 flies, respectively. The number of pR8s was significantly increased in all wtsZn-kib1/mer3 (Rh5+: 89.0±3.7%) and wtsZn-kib1/sav1 flies (Rh5+: 90.3±2.1%) flies. In contrast, yR8s were dramatically decreased in these flies (Rh6+: 11.0±3.7% and 9.7±2.1%, respectively) (Figs 1G and 1H and S1F). These results indicated that the wtsZn-kib1 heterozygous flies provide a sensitive genetic background, into which introducing one copy of a loss-of-function mutation in a gene of the Hippo pathway to generate triple heterozygous flies can dramatically affect R8 subtypes. Therefore, the triple heterozygote-based assay can be used as an efficient and sensitive tool to screen the Drosophila chromosomal deficiencies to identify new regulators of the Hippo pathway.

Identification of pyd as a new PR subtype fate determinant

Taking advantage of the wtsZn-kib1-based triple heterozygote assay, we screened 112 deficiency lines from the Bloomington deficiency Kit, aiming to identify new regulators of the Hippo pathway. The deletions of these 112 deficiencies cover the 61B1 to 88C9 region on the third chromosome (S2A and S2B Fig) [55,56]. When crossed with the wtsZn-kib1 chromosome, most of these deficiencies didn’t significantly affect R8 subtype specification (the range of pR8 ratio is 32%–45%, and yR8 ratio is 55%–68%). However, we found that the deficiency Df(3R)ED5330 significantly increased pR8 ratio at the expense of yR8s (Rh5+: 56.9±3.2%, Rh6+: 43.1±3.2%), compared to the adjacent deficiencies (Rh5+ range: 35.0%–37.9%, Rh6+: 65.0%–62.1%) (Fig 2A–2C and 2E). Further analyses of other deficiencies with smaller deletions in this area led us to the deficiency Df(3R)pyd ex147 (referred to as pyd ex147) [57] (Fig 2A, 2D and 2E), in which partial sequences of two genes, pyd and CG8379, are deleted (Fig 2A). All deficiencies with the deletions of these sequences enhanced the wtsZn-kib1 phenotype to promote pR8 and repress yR8 subtype fate specification (Rh5+ range: 52.2%–55.2%, Rh6+: 47.8%–44.8%) (Fig 2E), suggesting either pyd or CG8379 is a PR subtype fate determinant. While there is no mutant allele or RNA interference (RNAi) reagent available for CG8379, we analyzed two pyd null mutations, pyd ex180 and pyd ex147 [57,58]. pR8 cells were significantly expanded and yR8 cells were significantly reduced in both pyd ex180 (Rh5+: 95.1±2.2%, Rh6+: 4.9±2.2%) and pyd ex147 (Rh5+: 94.6±3.4%, Rh6+: 5.4±3.4%) mutant flies (Fig 2F–2H). Additionally, the homozygous pyd112 flies (pyd112/pyd112) or the heteroallelic flies for pyd112 and pyd ex180 or another pyd mutation pyd A [59] demonstrated a similar phenotype (S3B, S3C, S3F and S3H–S3J Fig). Furthermore, knock-down of pyd in all photoreceptor cells using IMGR-GAL4 to drive the expression of independent pyd RNAi constructs phenocopied the null pyd mutations (Figs 3A, 3B, 3D, and S3E and S3G). Together, these results demonstrate that Pyd is a PR subtype fate determinant in the Drosophila eye and is required to promote yR8 and repress pR8 subtype fate specification.

Pyd cell-autonomously regulates the Hippo pathway for R8 subtype fate specification

Because the R8 subtype fate depends on both R7- and R8-dependent events [11,15], we next tested whether Pyd regulates pR8 and yR8 ratios in a cell-autonomous manner. For this
purpose, we first used the *sevenless*-GAL4 (*sev-GAL4*) and the *senseless*-GAL4 (*sens-GAL4*) drivers to knock down *pyd* in R7 and R8 cells, respectively. The two drivers have been previously used to drive the expression of UAS-RNAi constructs and have significantly reduced the expression of their target genes in R7 (*sev-GAL4*) or R8 cells (*sens-GAL4*) [10]. We found knock-down of *pyd* in R8 cells (*sens-GAL4>*pyd*RNAi) significantly increased pR8 ratio (Rh5+: 90.2 ± 3.0% vs. 33.5 ± 1.5% in control) at the expense of yR8s (Rh6+: 9.8 ± 0.3% vs. 66.5 ± 1.5% in control) (Fig 3C, 3D and 3M). In contrast, knock-down of *pyd* in R7 cells did not affect pR8 and yR8 subtype fates (Fig 3E, 3F and 3M). These data suggest Pyd functions cell-autonomously to regulate R8 subtype fate specification. We further confirmed this by knocking down *pyd* in *sevenless* (*sev*) mutant flies. The R7 cell is missing in *sev* mutant flies, and most R8s express Rh6 [13] (Rh5+: 3.5±1.2%, Rh6+: 96.5±1.2% in *sev* flies) (Fig 3C and 3M). Knock-down of *pyd* in *sev* mutant flies caused the similar phenotype (Rh5+:87.6±2.2% Rh6+: 12.4±2.2%) (Fig 3H and 3M) with *pyd* knock-down in wild type flies (Fig 3B). All these results confirm that Pyd functions in R8 cells to specify pR8 and yR8 subtypes.

The phenotype enhancement between *pyd* and *wts/Zn-kin* mutations suggests *pyd* is a regulator of the Hippo signaling pathway. To confirm that *pyd* regulates R8 subtype fate specification via regulating Hippo signaling, we analyzed R8 subtypes in *yki* knock-down retinas in *pyd* loss-of-function (LOF) background. Yki is the downstream effector of the Hippo pathway and
is required for pR8 subtype fate specification: yki knock-down resulted in a dramatic loss of pR8s and an expansion of yR8s (Rh5+: 0.9±0.5%, Rh6+: 99.1±0.5%) [10] (Fig 3I and 3M). We found knock-down of yki in pR8 LOF retinas suppressed pR8 phenotype and led to a loss of pR8 cells (1.2±0.6%) and an expansion of yR8s (98.8±0.6%) (Fig 3J and 3M), suggesting yki is downstream of pyd during R8 phenotype. The transcription factor Sd is required to recruit Yki to DNA and regulate its target genes expression during R8 fate decisions. The transcription factor Sd is required for Hippo signaling to promote yR8 and repress pR8 subtype fate specification.

**Pyd regulates wts and melt expression in R8 cells**

Previous findings have shown that a key step in dictating pR8 (Rh5-positive) vs. yR8 (Rh6-positive) fate is through the transcriptional activation of melt and wts in pR8s and yR8s, respectively [15]. To determine whether pyd functions upstream of the melt-wts regulatory loop, we first analyzed wts and melt expression in pyd knock-down retina by using an enhancer trap line for wts (wts-nLacZ, aka wts\(ex\)) [15] and an expression reporter for melt (melt450-nLacZ) [10]. wts\(ex\) was expressed in ~65% of R8s (yR8s, 65.2±3.6% of R8s) in wild type retinas (Fig 4A and 4E). However, its expression was lost in most of R8s in pyd knock-down retina (5.2±2.1% of R8s) (Fig 4B and 4E). In contrast, melt expression was expanded into most R8s in pyd
knock-down retinas (91.9±3.2% of R8s) (Fig 4D and 4F), compared to its expression in ~35% of R8s in wild type retinas (34.9±3.5%) (Fig 4C and 4F). Therefore, these results indicate Pyd is required for wts expression and melt repression in yR8 cells.

As pyd plays a similar role with wts to repress melt expression, we tested whether pyd is also transcriptionally expressed in both pR8 and yR8 subtypes, and its expression is not regulated by wts or melt.

Pyd functions upstream of the core components of the Hippo pathway for PR subtype fate specification

To further understand how Pyd regulates the Hippo pathway to specify PR subtypes, we performed epistasis assays for Pyd and the core Hippo components in pR8 and yR8 subtype fate specification. Misexpression of wts, hpo or sav in wild type retinas was sufficient to induce yR8 fate and repress pR8 fate in most or all R8 cells [15,49] (Rh5+: 7.1±2.5%, Rh6+: 92.9±2.5% in IGMG>R>wt retinas; Rh5+: 0%, Rh6+: 100% in IGMG>R>hpo retinas; Rh5+: 0%, Rh6+: 100% in GMR>sav retinas) (Fig 5B, 5E and 5I). We found that misexpression of these core genes wts, hpo or sav in pyd mutant retinas had the same abilities to promote yR8 and repress pR8 subtype fate specification with their misexpression in wild type retinas: with the misexpression of wts, hpo, or sav, all or most of pR8s in pyd mutant retinas adopted to yR8 subtype fate (Rh5+:
Polychaetoid functions as an upstream regulator of the Hippo pathway.

8.8±3.1%, Rh6+: 91.2±3.1% in IGMR->wts, pyd LOF retinas; Rh5+: 0%, Rh6+: 100% in IGMR->hpo, pyd LOF retinas; Rh5+: 0%, Rh6+: 100% in GMR->sav, pyd LOF retinas) (Fig 5C, 5D, 5F and 5I). To further confirm the epistasis of Pyd and Wts, we performed pyd and melt double LOF experiments. wts expression in R8s is derepressed in melt mutant flies [15]. We knocked down melt in pyd mutant (pyd+/+/pyd112) flies or knocked down pyd in melt mutant (melt11) flies. Loss of melt in both experiments suppressed the phenotype caused by loss of pyd (Rh5+: 0.4±0.6%, Rh6+: 99.5±0.6% in IGMR->meltRNAI flies; Rh5+: 0.5±0.8%, Rh6+: 99.5±0.8% in IGMR->meltRNAI, pyd LOF flies) (Figs 5G–5I and S5A–S5F). Together, these results indicate that Pyd genetically acts upstream of or in parallel to the core components of the Hippo pathway.
The expression of pyd in both pR8 and yR8 subtypes indicates that the Pyd protein at its endogenous level in pR8 cells might not be sufficient to induce yR8 fate. In order to test whether the core components of the Hippo pathway are necessary for Pyd-mediated yR8 fate specification, we tested whether overexpression of Pyd in all PR cells by using a strong GMR-GAL4 driver [60] (GMR-GAL4>UAS-pyd) can induce yR8 fate specification. We found that pyd overexpression induced Rh6 expression in most R8 cells (Rh6+: 89.9±3.1%). Rh5 expression was only observed in a small proportion of R8s in pyd overexpression retinas (Rh5+: 11.1±3.1%) (Fig 5K and 5P). We then knocked down wts, hpo, sav or mats in pyd-overexpressing retinas, and found that knocking down any of these genes abolished the overexpressed Pyd’s ability to promote yR8 and suppress pR8 fate specification (Rh5+: 93.0±3.7%, Rh6+: 4.1±3.7%, Rh5 and Rh6 co-expression (coRh5Rh6): 2.9±1.1% in GMR>UAS-pyd GOF flies; Rh5+: 72.5±4.1%, Rh6+: 10.4±4.1%, coRh5Rh6: 17.1±1.2% in GMR>UAS-hpoRNAi, pyd GOF flies; Rh5+: 80.5±3.6%, Rh6+: 9.0±3.6%, coRh5Rh6: 10.5±3.1% in GMR>UAS-savRNAi, pyd GOF flies; Rh5+: 83.5±3.4%, Rh6+: 6.2±3.4%, coRh5Rh6: 10.3±1.8% in GMR>UAS-matsRNAi, pyd GOF flies) (Fig 5L–5P), suggesting these Hippo core components are necessary for Pyd to promote yR8 and inhibit pR8 subtype fate specification. Together, these epistasis experiments suggested Pyd genetically functions upstream of the core component genes in the Hippo pathway for R8 subtype fate decisions.

**Pyd functions upstream of pez for R8 subtype fate specification**

Pyd was previously shown to directly interact with the E3 ubiquitin ligase Su(dx) in regulating the size of the Drosophila ovary stem cell niche [58]. Additionally, Su (dx) targets and degrades Pez during intestinal stem cell proliferation [52]. Pez is the *Drosophila* homolog of Protein tyrosine phosphatase non-receptor type 14 (PTPN14) and functions as a partner of Kib. Both Pez and Kib are required for the activity of the Hippo pathway to restrict intestinal stem cell proliferation in the *Drosophila* midgut epithelium [51]. However, the roles of both Pez and Su (dx) in post-mitotic PR subtype fate specification have not been explored. Here, we first analyzed pR8 and yR8 subtypes in heteroallelic *pez*/*pez* mutant flies. Almost all R8s expressed Rh5 (97.5±1.2%), the pR8 fate marker, in *pez*/*pez* mutant flies at the expense of Rh6 (2.5±1.2%) (Fig 6B and 6G). Furthermore, misexpression of *pez* (IGMR>pez) led to a significant increase in Rh6-expressing yR8s (94.6±2.5%) and a reduction in Rh5-expressing pR8s (5.4±2.5%) (Fig 6C and 6G). These results demonstrate that *pez* is necessary and sufficient to promote yR8 and repress pR8 subtype fate specification in the *Drosophila* eye. We then determined the genetic relationship between *pyd* and *pez*. Misexpression of *pez* was able to suppress the phenotype caused by *pyd* mutations (Rh5+: 6.1±2.9%, Rh6+: 93.9±2.9% in IGMR>pez, pyd LOF retinas) (Fig 6D and 6G), similar with misexpression of the core components of the Hippo pathway. Further, we knocked down *pez* in *pyd*-overexpressing eyes and found that loss of *pez* repressed the phenotype caused by overexpressed Pyd (Rh5+: 90.2±3.9%, Rh6+: 9.8±3.9% in GMR>UAS-pydRNAi>pez RNAi) (Fig 6E–6G), indicating Pez is required for Pyd to promote yR8 and repress pR8 subtype fate. Together, these results indicated that Pez acts downstream of Pyd in the Hippo pathway to specify R8 subtype fate specification.

**Pyd suppresses Su(dx) in R8 subtype fate specification**

Su(dx) plays an opposite role to Pyd in regulating the size of the ovary stem cell niche [58]. In order to investigate the functional relationship of the two proteins in R8 subtype fate specification, we first tested whether Su(dx) plays any role in PR subtype fate specification by knocking down *su(dx)* in retinas. Knock-down of *su(dx)* (IGMR>UAS-su(dx)RNAi) led to an opposite phenotype to loss of *pez*: Rh6-expressing yR8s were significantly increased (86.8±3.1%) at the
expense of Rh5-expressing pR8s (13.2±3.1%) (Fig 6H and 6N). We additionally used su(dx) mutations su(dx)$^2$ and su(dx)$^{32}$ to generate heteroallelic su(dx)$^2$/su(dx)$^{32}$ mutant flies and analyzed R8 subtypes in these flies. su(dx)$^2$/su(dx)$^{32}$ retinas showed a similar R8 subtype phenotype (Rh5+: 15.2±4.3%, Rh6+: 84.8±4.3%) with su(dx) knock-down retinas (Fig 6I and 6N).

Further, we found that misexpression of su(dx) (IGMR>su(dx)$^{RNAi}$) caused an increase in pR8s (71.1±4.8%) and a reduction in yR8s (28.9±4.8%) (S6A–S6C Fig). Therefore, Su(dx) and Pyd play opposite roles in R8 subtype fate decisions.

Previous yeast two-hybrid assays and co-immunoprecipitation (co-IP) tests in the Drosophila S2 cells have shown that Pyd and Su(dx) can interact with each other and form a complex. To explore the functional relationship of the two interacting proteins during R8 subtype fate.

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**Fig 6.** *pez* and *su(dx)* specify R8 subtypes downstream of *pyd*. (A-F) Cryosections of adult eyes stained for Rh5 (red) and Rh6 (green) in control (IGMR-GAL4) (A), *pez* mutant (*pez$^+/pez^*$) (B), *pez* misexpression (IGMR> *pez*) (C), *pez* misexpression in pyd LOF (IGMR> *pez*, pydT$^{s135}$/pydT$^{s135}$) (D), knock-down of *pez* (GMR> *pez*RNAi+$^*$Luc) (E) and knock-down of *pez* in pyd overexpression (GMR> *pyd*+$^*$peyRNAi) (F) flies. (G) Quantification of Rh5 and Rh6-expressing R8s in the eyes with the indicated genotypes. y axis presents proportion of R8s that express Rh5 (red) or Rh6 (green). NS: not significant. **P < 0.001. Error bars represent standard deviation. IGMR-GAL4: n = 10 retinas, n = 1822 R8s; *pez$^+/pez^*$: n = 6 retinas, n = 967 R8s; IGMR> *pez*: n = 6 retinas, n = 1201 R8s; IGMR> *pez*, pyd LOF: n = 6 retinas, n = 1261 R8s; IGMR> *pez*RNAi+$^*$Luc: n = 6 retinas, n = 1001 R8s; GMR>pydT$^{s135}$/pydT$^{s135}$: n = 5 retinas, n = 936 R8s. (H-M) Cryosections of adult eyes stained for Rh5 (red) and Rh6 (green) in *su(dx)$^+$ knock-down (H, IGMR> su(dx)$^{RNAi}$), *su(dx)$^+$ mutant (*su(dx)$^2$/su(dx)$^{32}$), *pyd* LOF (pydT$^{s135}$/pydT$^{s135}$) (I), *su(dx)$^+$ knock-down in pyd LOF (IGMR> su(dx)$^{RNAi}$, pydT$^{s135}$/pydT$^{s135}$) (K), *su(dx)$^+$ GOF (GMR> *su(dx)$^{+}$Luc) (L) and *su(dx)$^+$, *pyd* double GOF (GMR> *su(dx)$^{+}$+pyd) (M) flies. (N) Quantification of Rh5- and Rh6-expressing R8s in the eyes with the indicated genotypes. y axis presents proportion of R8s that express Rh5 (red) or Rh6 (green). **P < 0.001. Error bars represent standard deviation. IGMR-GAL4: n = 10 retinas, n = 1822 R8s; IGMR>su(dx)$^{RNAi}$: n = 6 retinas, n = 903 R8s; su(dx)$^2$/su(dx)$^{32}$: n = 6 retinas, n = 1038 R8s; pyd LOF: n = 10 retinas, n = 1902 R8s; GMR>su(dx)$^{RNAi}$, pyd LOF: n = 6 retinas, n = 980 R8s; GMR>su(dx)$^{+}$+Luc: n = 4 retinas, n = 823 R8s; GMR>su(dx)$^{+}$+pyd: n = 4 retinas, n = 802 R8s.
specification, we performed epistasis analyses for pyd and su(dx). We knocked down su(dx) in pyd mutant flies and found that knock-down of su(dx) suppressed the increase of the number of pR8s and the decrease of yR8s caused by pyd mutations (Rh5+: 14.1±3.5%, Rh6+: 85.9±3.5% in lGMR>su(dx)RNAi, pyd LOF retinas; compared to Rh5+: 95.1±2.2%, Rh6+: 4.9±2.2% in pyd LOF retinas) (Fig 6J, 6K and 6N). These results suggest that Pyd genetically functions upstream of Su(dx) and that the pR8 expansion phenotype in pyd mutant flies depends on the presence of functional Su(dx). We then looked into the effect of Pyd on the misexpressed Su(dx). As shown above, misexpression of Su(dx) leads to an expansion of pR8s at the expense of yR8s. We found that overexpression of Pyd suppressed the ability of Su(dx) to increase pR8s and decrease yR8s (Rh5+: 18.7±4.0%, Rh6+: 81.3±4.0% retinas in GMR>pyd+su(dx) retinas; compared to Rh5+: 71.1±4.3%, Rh6+: 28.9±4.3% in GMR>su(dx) retinas) (Fig 6L–6N). Considering that Pyd and Su(dx) can physically interact with each other and form a complex, these double LOF and double GOF results indicate Pyd acts through suppressing Su(dx) to regulate R8 subtype fate specification.

Pyd functions genetically in parallel to Kib to specify R8 subtypes

Kib can bind to Pez and the two proteins function together to regulate Hippo signaling in the Drosophila midgut epithelium [51]. To test whether Kib also genetically functions downstream of Pyd to specify R8 subtypes, we misexpressed kib in pyd mutant retinas. Similar to pez, kib misexpression was also able to suppress pyd mutant phenotype (Rh5+: 1.8±1.2%, Rh6+: 98.2±1.2% in lGMR>kib, pyd LOF retinas) (Fig 7B, 7C and 7I), suggesting pyd functions upstream of or in parallel to Kib in R8 subtype fate specification. We then tested whether Pyd’s ability to induce yR8 subtype requires Kib. We knocked down kib in pyd-overexpressing retinas (GMR>pyd+kibRNAi) and found that knock-down of kib was not able to affect the overexpressed Pyd to promote yR8 and repress yR8 subtype fate (Rh5+: 8.2±4.2%, Rh6+: 88.3±4.2%, coRh5Rh6: 3.5±2.5% in GMR>kibRNAi, pyd GOF flies) (Figs 7D–7F and 7I and S7A–S7D), although kib knock-down in wild type flies is sufficient to repress yR8 fate specification (Rh5+: 92.1±3.7%, Rh6+: 3.4±3.7%, coRh5Rh6: 4.5±2.7% in GMR>kibRNAi flies (Figs 7E and 7I, and S7A and S7D)), indicating the overexpressed Pyd is able to circumvent knock-down of kib to specify R8 subtypes. Collectively, these results suggest that Pyd and Kib may function genetically in parallel to each other during R8 subtype fate specification.

Kib was previously shown to block the Su(dx)-induced Pez degradation in the cultured Drosophila S2 cells [52]. We therefore asked whether Kib is able to suppress Su(dx)’s activity in R8 subtype fate specification. To test this, we misexpressed Kib in Su(dx)-misexpressing retinas. Compared to most R8s expressing Rh5 in Su(dx)-misexpressing retinas (Rh5+: 71.1±4.8%, Rh6+: 28.9±4.8%) (Fig 7G and 7I), kib misexpression suppressed Su(dx)’s activity, leading most R8s expressing Rh6 at the expense of Rh5 (Rh5+: 2.1±1.9%, Rh6+: 97.9±2.1%) (Fig 7H and 7I). These results suggested that Kib, similar with Pyd, can suppress Su(dx)’s activity in R8 subtype fate specification.

Discussion

In this study, we designed a sensitized genetic screen using a triple heterozygote-based PR subtype phenotype enhancement assay to identify novel regulators of the Hippo pathway in the Drosophila eye. Taking advantage of this genome-wide screening, we identified the Drosophila ZO-1 protein Pyd as a new PR subtype fate determinant. We demonstrated Pyd is an upstream regulator of the Hippo signaling pathway and is required for the pathway to promote yR8 and repress pR8 PR subtype fate specification. We also determined the roles of Pez and Su(dx) in R8 subtype fate specification and found they play opposite roles in this process (Fig 8), as they
act in intestinal stem cell proliferation. Previous reports have shown that Pyd and Su(dx) can physically interact with each other. We found that Pyd and Su(dx) act antagonistically during R8 subtype fate specification (Fig 8). Further, our pyd and su(dx) double LOF and double GOF results have indicated that the R8 subtype phenotype in pyd LOF retinas depends on the presence of Su(dx), and on the other hand, the overexpressed Pyd represses Su(dx)'s activity to promote pR8 and inhibit yR8 fate specification. Considering that Su(dx) can induce Pez degradation [52], Pyd may be required for Hippo signaling by antagonizing Su(dx)'s activity and therefore stabilizing Pez (Fig 8). Interestingly, it is the WW domain of the Su(dx) protein that interacts with both Pez and Pyd. Therefore, it is possible that Pyd competes with Su(dx) to bind and stabilize Pez. Our data also showed that Kib suppresses Su(dx)'s activity during R8 subtype fate specification, consistent with the previous report that Kib can block Su(dx)-induced Pez degradation [52]. However, Kib was not shown to interact with Su(dx) and it can’t decrease the binding between Su(dx) and Pez [52]. Therefore, Pyd and Kib may use different mechanisms to stabilize Pez: Pyd competes with Su(dx) for Pez binding, while Kib directly binds to Pez. Since loss of pyd or kib lead to significant expansion of pR8s and
reduction of yR8s, both of the two mechanisms is required in wild type retinas. However, over-expression of any one of pyd and kib can circumvent loss of another gene (Fig 7), suggesting the two mechanisms might function independently (Fig 8). It will be of interest and important to test this model using biochemical approaches in future studies and explore whether and how Pyd directly competes with Su(dx).

The Hippo pathway was originally discovered in Drosophila and its evolutionarily conserved roles in various biological processes have been subsequently found in mammals [19,34,61–68]. However, the regulatory mechanisms upstream of the signaling pathway are less understood. Most of the core components of the Hippo pathway were first isolated as a result of their overgrowth phenotypes in mosaic mutant-based screens [69]. However, this strategy is not efficient to identify the upstream components of the pathway because the overgrowth defects caused by mutations of the upstream genes is much weaker compared to those induced by mutations of the core components due to the redundant roles of the upstream components in tissue growth control [42,43,51,70]. Interestingly, Fat, Expanded as well as Crumbs are not required for the activation of Hippo signaling during R8 subtype fate specification [49], making the upstream regulation of the Hippo pathway during R8 subtype fate decisions less complicated. Additionally, Hippo-dependent R8 subtype fate specification can be precisely quantified. Taking advantage of these features, we generated a sensitive genetic

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**Fig 8. Model: the role of Pyd in R8 subtype fate specification.** Pyd represses Su(dx) and may function in parallel to Kib to promote yR8 and repress pR8 subtype fate specification. Pyd may antagonize the Su(dx)-mediated Pez degradation. See Discussion for detail. Genes or proteins that are inactive or not expressed are denoted by grey font, while those that are expressed and/or active are represented by blue, green or black font. Model is based on the data in this study and the previous works [10,15,49,52].

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background with double heterozygous wts and kib mutations that affect R8 subtypes modestly, but is able to significantly change pR8 and yR8 subtypes when coupled with one more mutation in a gene of the Hippo pathway. This sensitive genetic tool makes it possible to perform a genome-wide screening by testing the activities of the Drosophila deficiency lines to affect R8 subtypes. Notably, previous studies have demonstrated that Mer physically interacts with Wts and Kib [42,71]. Our results showed the R8 subtype phenotype was enhanced more in heterozygous wts\textsuperscript{Zn}-mer\textsuperscript{3} or kib\textsuperscript{1}-mer\textsuperscript{3} flies than in wts\textsuperscript{Zn}-kib\textsuperscript{1}, kib\textsuperscript{1}-sav\textsuperscript{df} or wts\textsuperscript{Zn}-sav\textsuperscript{df} flies. Therefore, the quantitative phenotype enhancement assays for R8 subtypes have a potential application to predict the physical interactions between the components of the Hippo pathway.

ZO-1 proteins are crucial for the formation and maintenance of tight junctions in vertebrate cells [72]. While in Drosophila cells, which lack tight junctions [73], Pyd is associated with both adherens and septate junctions [74,75]. ZO-1 proteins are members of the membrane-associated guanylate kinase (MAGUK) family and contain a GUK (guanylate kinase) domain, three PDZ domains, and a SH3 domain [58]. The cellular localization and the presence of multiple protein-protein interaction domains suggest the ZO-1 proteins may play important roles in coupling the extracellular signals to intracellular signaling pathways. A previous study in cultured cells have found that the transiently expressed ZO-1 protein can interact with the carboxy-terminal PDZ binding motif of TAZ, a downstream effector of the Hippo pathway in mammals, via its first PDZ domain [76]. Whether Pyd interacts with Yki, the Drosophila homolog of YAP/TAZ, hasn’t been explored. However, our result that knock-down of wts is sufficient to suppress the phenotype in pyd overexpression retinas suggests the interaction between Yki and Pyd, if there is any, does not play a significant role for the cytoplasmic retention of Yki and thus inhibiting its activity as a transcription co-activator. Additionally, Pyd has been previously implicated in the regulation of the Notch pathway in context-dependent manners [59,77,78]. However, the Notch pathway has not been shown to cell-autonomously regulate R8 subtype fate specification in the Drosophila eye. Our results in this study provide evidence that Pyd is a regulator of the Hippo signaling pathway and functions as an upstream regulator of the pathway for PR subtype fate decisions. Considering its interactions with junctions and cytoskeleton proteins, Pyd might function as a scaffold to organize other components of the Hippo pathway at the plasma membrane to form functional complexes. Furthermore, genetic or direct interactions between Pyd and some transmembrane proteins have been previously reported [79,80]. Given that Pyd functions upstream of the Hippo pathway during R8 subtype fate decisions, it will be of great interest to test the role of these Pyd-interacting transmembrane proteins for R8 subtype fate decisions and investigate whether any of them acts as a transmembrane receptor in Hippo signaling. Notably, Mer plays key roles to recruit the core Hippo components to apical membrane area [81]. Pyd and its transmembrane partner may be required for Mer membrane associations.

The R8 terminal differentiation into pR8 or yR8 subtype fate occurs in the late pupal stage and is dependent on the activation and deactivation of the Hippo signaling pathway [82]. As a negative regulator of the Hippo pathway, melt is expressed in a subset of R8s from 40% pupation [10] and is indispensable to transcriptionally repress wts expression and de-activate Hippo signaling [49], allowing these R8s to adopt the pR8 subtype fate. In this study, we determined that the E3 ligase Su(dx) as another negative mediator of the Hippo pathway for R8 subtype fate specification. Su(dx) was shown to degrade Pez and therefore inactivate Hippo signaling in midgut epithelium [52], indicating Su(dx) inactivates Hippo signaling by a different mechanism with Melt-mediated transcriptional repression of wts. It is possible that Su(dx) is present in a subset of R8s at 40–50% APF stage and its presence reduces the default Hippo signaling and thus results in elevated Yki activity which, together with the transcription factors
Otd, Traffic jam and Scalloped [10,20], initiates the melt-wts bistable loop to activate melt and repress wts expression, and finally leads to the generation of pR8 subtype.

Materials and methods

Drosophila stocks

The following fly lines were used: pyd\textsuperscript{ex180}, pyd\textsuperscript{ex147}, UAS-GFP-pyd [58], kib\textsuperscript{1}, pez\textsuperscript{1}, pez\textsuperscript{2}, UAS-kib, UAS-pez [43,51], wts\textsuperscript{Zn} (wts-n LacZ) [15,17], melt\textsuperscript{A1} [16], sd\textsuperscript{kk} [83], IMGR-GAL4 [84], UAS-pyd\textsuperscript{RNAi-#450} [79], sens88-GAL4 [11], yki\textsuperscript{Is} [33], UAS-hpo [26], GMR-sav [32], pWIZ-w\textsuperscript{Δ13} (a white gene RNAi line) [85], UAS-pyd\textsuperscript{RNAi} (KK105581), UAS-yki\textsuperscript{RNAi} (KK109756), UAS-sd\textsuperscript{RNAi} (KK108877), UAS-wts\textsuperscript{RNAi} (KK101055), UAS-hpo\textsuperscript{RNAi} (KK101704), UAS-sav\textsuperscript{RNAi} (KK107562), UAS-mats\textsuperscript{RNAi} (KK100140) and UAS-kib\textsuperscript{RNAi} (KK108510) were from the Vienna Drosophila Resource Center (VDRC). UAS-pyd\textsuperscript{RNAi} (HMS00263) UAS-wts, UAS-su(dx), su(dx)\textsuperscript{2}, su(dx)\textsuperscript{32}, sev\textsuperscript{14}, UAS-su(dx)\textsuperscript{RNAi} (HMS005478), UAS-pez\textsuperscript{RNAi} (HMS00862), UAS-kib\textsuperscript{RNAi} (HMC03256), UAS-Dicer2, sev-GAL4, GMR-GAL4 [60], UAS-Luciferase, Df(3R)BSC803 (sav\textsuperscript{df}), Df(3R)ED6096, Df(3R)BSC466, Df(3R)ED5330, Df(3R)Exel6150, Df(3R)BSC478, Df(3R)BSC506, Df(3R)BSC566, Df(3R)BSC6152, and Df(3R)pyd\textsuperscript{B12} were from the Bloomington Drosophila Stock Center (BDSC). p[GawB]NP4419, p[GawB]NP7518, p[GawB]NP0961, and p[GawB]NP4414 were from the Kyoto Stock Center. lGMR-GAL4, pWIZ-w\textsuperscript{Δ13} and UAS-Dicer2 lines were recombined onto a single chromosome for use in RNAi-mediated knockdown experiments [10]. UAS-pez and pyd\textsuperscript{ex180}, both on the 3\textsuperscript{rd} chromosome, were recombined and used for misexpression of pez in pyd LOF flies. All flies and crosses were raised on standard cornmeal-molasses media at 25 °C with 12 hr:12hr light-dark cycles except that GMR-GAL4\textgreater UAS-su(dx) (for Fig 6L and 6M) were in room temperature (22 °C).

Immunohistochemistry

Fly head cryosections, dissection for whole mount retinas, and antibody staining were performed as previously described with modifications [82,86]. Adult fly heads were embedded and frozen in OCT and sectioned (12 μm) using the Cryostat CM1850 (Leica). The samples were then fixed in 4% paraformaldehyde/ PBS, and washed 3x10 min with PBX (PBS + 0.3% Triton X-100), incubated with primary antibodies overnight at 4 °C in antibody dilution buffer (PBX + 1% BSA), washed 4x10 min with PBX, and incubated 90 min at room temperature with secondary antibodies diluted in antibody dilution buffer. After 4x10min PBX washes, samples were mounted in anti-fade reagent, and imaged. Antibody dilutions were: rabbit Salm (1:150) [82]; mouse Rh5 (1:1000) [87]; rabbit Rh6 (1:100, this study); chicken LacZ (1:1000, Abcam). Alexa Fluor 488, 555 and 655-conjugated secondary antibodies (1:1500, Invitrogen) were used. Digital images were obtained with an Apotome deconvolution system (Zeiss), and processed with Axiovision 4.5 (Zeiss) and Adobe Photoshop software. Quantifications for the longitudinal sections were performed by counting at least 800 ommatidia from four or more individual flies per genotype, and only sections that include both R7 and R8 layers were counted. Quantifications for the tangential sections use one section for each retina to avoid repeatedly counting the same ommatidia. Retinas for quantifying the whole mount staining are from at least three flies per genotype.

Polyclonal antibody production

Polyclonal antiserum against Rh6 was generated against a KLH-conjugated peptide from the Rh6 deduced amino acid sequence (CLACGKDLTSDSRTQAT corresponding to amino
acids 344–361). Peptide synthesis, KLH-conjugation, rabbit immunizations and bleeds were performed by GenScript (Piscataway, NJ).

Supporting information

S1 Text. A list of Drosophila genotypes used in this study.

S1 Fig. R8 subtype phenotype enhancement in double and triple heterozygotes with Hippo pathway mutations. (A-I) Cryosections of adult eyes immunostained for Rh5 (red) and Rh6 (green) in control, heterozygous, double heterozygous and triple heterozygous flies with Hippo pathway mutations. Compared to control and heterozygous mutations (A-E), double heterozygous Hippo pathway mutations slightly increased Rh5-expressing R8s and reduced Rh6-expressing R8s (F-H). In flies with triple heterozygous Hippo pathway mutations, the number of the Rh5-expressing R8s were dramatically increased at the expense of the Rh6-expressing R8s (I). See Fig 1 for the quantification of R8 subtypes in these genotypes.

S2 Fig. Diagram of the phenotype enhancement screening using the sensitized wtsZn-kib flies. (A) Diagram of deficiencies on the third chromosome (from 61B1 to 88C9, 112 deficiencies from the Bloomington Deficiency Kit in this area) that are used in the R8 subtype determinant screening. (B) Diagram showing the fly crossing strategy in the screening.

S3 Fig. Pyd is required to specify R8 photoreceptor subtypes. (A-E) Adult eye cryosections immunostained for Rh5 (red) and Rh6 (green) in control, pyd mutant or pyd knock-down eyes. (A) Control, (B) pydB12/pydB12, (C) pydB12/pyd4, (D) IGMR>pydRNAi-HMS00263 and (E) IGMR>pydRNAi-#450. (F) Adult eye tangential cryosection immunostained for Rh5 (red) and Rh6 (green) in pyd LOF (pydx180/pydB12) flies. (G) Quantification of R8 subtypes in pyd mutants and pyd knock-down eyes. Error bars represent standard deviation. NS: not significant. ** p < 0.001. Control: n = 10 retinas, n = 1822 R8s; pydB12/pydB12: n = 4 retinas, n = 820 R8s; pydB12/pyd4: n = 5 retinas, n = 909 R8s; IGMR>pydRNAi-HMS00263: n = 5 retinas, n = 840 R8s; IGMR>pydRNAi-#450: n = 5 retinas, n = 806 R8s. (H-I) Retina whole mount staining for control (I) and pyd LOF (pydx180/pydB12) eyes. (I) Quantification of R8 subtypes in control and pyd LOF eyes (the retina whole mount staining). ** p < 0.001. Control: n = 3 retinas, n = 725 R8s; pydx180/pydB12: n = 3 retinas, n = 524 R8s.

S4 Fig. Knock-down of sd suppresses pyd mutant phenotype. (Related to Fig 3). (A-B) Adult eye cryosections immunostained for Rh5 (red) and Rh6 (green) in knock-down of sd (A, otd-GAL4>UAS-sdRNAi) and knock-down of sd in pyd LOF (B, otd-GAL4>UAS-sdRNAi, pydx180/pydB12) flies.

S5 Fig. Loss of function of melt suppresses pyd mutant phenotype. (Related to Fig 5). (A-C) Adult eye cryosections immunostained for Rh5 (red) and Rh6 (green) in knock-down of melt (A, otd-GAL4>UAS-meltRNAi), pyd LOF (B, pydx180/pydB12), and knock-down of melt in pyd LOF (C, otd-GAL4>UAS-meltRNAi, pydx180/pydB12) flies. (D-F) Adult eye tangential cryosections immunostained for Rh5 (red) and Rh6 (green) in melt mutant (D, meltΔ), pyd knock-down (E, otd-GAL4>UAS-pydRNAi-KK), and knock-down of pyd in melt mutant (F, meltΔ, otd-GAL4>UAS-pydRNAi-KK) flies.
S6 Fig. Misexpression of su(dx) induces pR8 subtype fate at the expense of yR8. (A–B) Adult eye cryosections immunostained for Rh5 (red) and Rh6 (green) in control (A) and su (dx) misexpression eyes. (A) Control (IGMR-GAL4), (B) su(dx) misexpression (IGMR-GAL4>su(dx)). (C) Quantification of R8 subtypes in su(dx) misexpression eyes. Error bars represent standard deviation. ** p < 0.001. IGMR-GAL4: n = 10 retinas, n = 1822 R8s; IGMR>su(dx): n = 6 retinas, n = 816 R8s.

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S7 Fig. Kib is not required for the overexpressed Pyd to promote yR8 and suppress pR8 fate specification. (Related to Fig 7). (A–C) Adult eye cryosections immunostained for Rh5 (red) and Rh6 (green) in kib knock-down (A, GMR>kibRNAi-HMC03256+Luc), pyd overexpression (B, GMR>pyd+Luc) and kib knock-down in pyd overexpression (C, GMR>pyd+kibRNAi-HMC03256) flies. UAS-Luciferase (UAS-Luc) was used as a control to balance the number of UAS sites. (D) Quantification of the Rh5- and Rh6-expressing R8s in the eyes with the indicated genotypes. NS: not significant. Error bars represent standard deviation. GMR>kibRNAi-HMC03256+Luc: n = 4 retinas, n = 901 R8s; GMR>pyd+Luc: n = 6 retinas, n = 912 R8s; GMR>pyd+kibRNAi-HMC03256+Luc: n = 4 retinas, n = 921 R8s.

(TIF)

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Polychaetoid functions as an upstream regulator of the Hippo pathway

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