The Hippo pathway effector Yki downregulates Wg signaling to promote retinal differentiation in the Drosophila eye

Erika Wittkorn¹, Ankita Sarkar¹, Kristine Garcia¹, Madhuri Kango-Singh¹,²,³,* and Amit Singh¹,²,³,*

ABSTRACT

The evolutionarily conserved Hippo signaling pathway is known to regulate cell proliferation and maintain tissue homeostasis during development. We found that activation of Yorkie (Yki), the effector of the Hippo signaling pathway, causes separable effects on growth and differentiation of the Drosophila eye. We present evidence supporting a role for Yki in suppressing eye fate by downregulation of the core retinal determination genes. Other upstream regulators of the Hippo pathway mediate this effect of Yki on retinal differentiation. Here, we show that, in the developing eye, Yki can prevent retinal differentiation by blocking morphogenetic furrow (MF) progression and R8 specification. The inhibition of MF progression is due to ectopic induction of Wingless (Wg) signaling and Homothorax (Hth), the negative regulators of eye development. Modulating Wg signaling can modify Yki-mediated suppression of eye fate. Furthermore, ectopic Hth induction due to Yki activation in the eye is dependent on Wg. Last, using Cut (Ct), a marker for the antennal fate, we show that suppression of eye fate by hyperactivation of yki does not change the cell fate (from eye to antenna-specific fate). In summary, we provide the genetic mechanism by which yki plays a role in cell fate specification and differentiation—a novel aspect of Yki function that is emerging from multiple model organisms.

KEY WORDS: Drosophila eye, Hippo signaling, Wingless, Growth regulation, Patterning, Retinal differentiation

INTRODUCTION

Growth regulatory pathways play an important role during organogenesis to regulate patterning, growth and differentiation (Raff, 1996; Baker, 2001; Tumaneng et al., 2012; Verghese et al., 2012, 2013; Pichaud, 2014). These pathways crosstalk with each other, and are re-utilized to generate complexity and diverse cell types in different organisms (Pires-daSilva and Sommer, 2003; Jukam and Desplan, 2011; Jukam et al., 2013). The Hippo signaling pathway, an evolutionarily conserved pathway, is involved in organ size regulation and regeneration, and in diseases like cancer (Kango-Singh and Singh, 2009; Zhao et al., 2011; Staley and Irvine, 2012; Halder and Camargo, 2013; Verghese et al., 2013). Initially identified in genetic screens in Drosophila, the pathway comprises a core kinase cascade and multiple upstream regulators that converge on the regulation of nuclear availability of the Yorkie (Yki) oncogene (the homolog of mammalian YAP/TAZ) (Huang et al., 2005; Edgar, 2006; Pan, 2010; Halder and Johnson, 2011; Verghese et al., 2013; Yu and Guan, 2013). The core kinase cascade comprises the two serine/threonine kinases Hippo (Hpo, the Drosophila homolog of mammalian MST1/2) (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003) and Warts (Wts, the Drosophila homolog of the mammalian NDR family proteins LATS1/2) (Justice et al., 1995; Xu et al., 1995), and their cognate adaptor proteins Salvador (Sav, the Drosophila homolog of mammalian WW45) (Kango-Singh et al., 2002; Tapon et al., 2003) and Mob as tumor suppressor (Mats, the Drosophila MOB homolog) (Lai et al., 2005). The upstream kinase Hpo binds Sav, and regulates the activity of the downstream Wts and Mats complex via phosphorylation. The Wts/Mats complex regulates Yki activity via phosphorylation, which leads to cytoplasmic sequestration and degradation of Yki. Yki is also regulated by phosphorylation-independent mechanisms where it binds Expanded (Ex), Hpo or Wts, and is localized to the plasma membrane, preventing its nuclear translocation (Oh and Irvine, 2010; Verghese et al., 2013; Yu and Guan, 2013).

Upstream of the kinase cascade, multiple genes impact the output of the Hippo pathway, e.g. genes regulating extracellular matrix and cytoskeleton, apical-basal polarity, planar cell polarity and growth factor signaling (Gusche et al., 2010; Boggiano and Fehon, 2012; Staley and Irvine, 2012; Yu and Guan, 2013). Hyperactivation of the Hippo pathway (e.g. by overexpression of Hpo or Ex) induces apoptosis, leading to the formation of smaller organs and inactivation of Yki (Harvey et al., 2003; Udan et al., 2003; Wu et al., 2003; Verghese et al., 2012, 2013); however, its downregulation (e.g. by loss of function of ex, hpo, sav, wts and mats) results in overgrowth and activation of Yki. Activated Yki binds to its cognate transcription factor(s) [Scalloped (Sd), Homothorax (Hth), Teashirt (Tsh)] and translocates to the nucleus to induce transcriptional expression of its target genes (Harvey et al., 2003; Jia et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003, 2008; Gouliev et al., 2008; Zhang et al., 2008; Peng et al., 2009). Hippo pathway target genes include genes that regulate cell proliferation, cell survival or cell growth to limit organ size, e.g. cyclin E, cyclin A, cyclin B, E2f1, Drosophila inhibitor of apoptosis 1 (Diap1), bantam microRNA and Myc. In addition, several upstream components such as kibra, ex, crumbs (crb) and four-jointed (ff) are regulated via a feedback mechanism to maintain steady-state Hippo signaling (Verghese et al., 2013). Thus, the Hippo pathway responds to multiple upstream regulatory inputs by transcriptionally regulating a battery of genes in a tissue- and context-dependent manner.

Drosophila imaginal discs are an excellent model with which to study the role of signaling pathways in growth and patterning. Drosophila eyes are derived from a set of eye imaginal discs (Poulson, 1950; Cohen, 1993) that grow during larval stages, and each give rise to an adult compound eye comprising 800 unit eyes or ommatidia (Ready et al., 1976; Wolff and Ready, 1993; Kumar,
2011; Singh et al., 2012; Tare et al., 2013a). Each ommatidium is made up of ~20 cells, including eight photoreceptor neurons and non-neuronal cells such as pigment cells, cone cells and bristles (Ready et al., 1976; Held, 2002; Rognant and Treisman, 2009; Kumar, 2011, 2013; Singh et al., 2012). Cell fate specification and differentiation in the developing eye field are regulated by a group of genes referred to as retinal determination (RD) genes (Pappu and Mardon, 2004; Rognant and Treisman, 2009; Kumar, 2011; Singh et al., 2012; Burgy-Roukala et al., 2013). These are twin of eyeless (toy), eyeless (ey), eyeogone (eyg), twin of eyeogone (toe), Optix, eyes absent (eya), sine oculis (so), dachshund (dac) and ophthalmosa (opt) (Bonini et al., 1993; Hanson et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994; Seimiya and Gehring, 2000; Jang et al., 2003). Among these, the Drosophila Pax6 homolog Eya is required for eye field specification, whereas downstream genes eya, so and dac are required for retinal determination and differentiation (Kumar, 2011; Burgy-Roukala et al., 2013). Loss of function of RD genes blocks early eye development, and misexpression of these genes can reprogram other tissues to form ectopic eyes (Pappu and Mardon, 2004; Burgy-Roukala et al., 2013).

During early third instar, following a period of proliferation, a synchronous wave of differentiation is initiated at the posterior margin of the eye imaginal disc and is referred to as the morphogenetic furrow (MF) (Ready et al., 1976; Wolff and Ready, 1993). The MF moves from posterior margin of the eye imaginal disc to anterior and results in differentiation of retinal precursor cells into photoreceptor (PR) neurons (Ready et al., 1976; Wolff and Ready, 1993; Kumar, 2013). The PR clusters are regularly spaced and comprise eight (R1-R8) photoreceptor neurons. The proneural genes of the achaete-scute complex (AS-C) and aortal (ato)-encoding basic HLH proteins, play important roles in PR differentiation (Jarman et al., 1994; Bertrand et al., 2002; Tanaka-Matatsuki and Du, 2008). The PR cluster formation involves the selection of R8 founder neuron and subsequent recruitment of additional photoreceptor precursors in the order R2/5, R3/4 and R1/6/7 (Ready et al., 1976; Wolff and Ready, 1993).

The initiation and progression of the MF also requires signaling morphogens [e.g. hedgehog (hh), decapentaplegic (dpp)] and other transcription factors. In the developing eye, dpp expression is restricted to a stripe straddling the MF as it traverses across the eye disc, and serves as an excellent marker for the MF (Chanut and Heberlein, 1997; Kumar, 2013). An important function of Dpp is to repress the expression of Wingless (Wg), a negative regulator of the MF (Ma and Moses, 1995; Treisman and Rubin, 1995; Burke and Basler, 1996). Wg, a signaling morphogen, serves as a ligand for the highly conserved Wg/WNT signaling pathway. Wg is required for nuclear localization of β-catenin homolog Armadillo (Arm) (Aberle et al., 1997; Seto and Bellen, 2004; Geissler and Zach, 2012; Swarup and Verheyen, 2012). In the absence of Wg, Armadillo (Arm) is phosphorylated by Shaggy kinase (Sgg), which leads to its retention in the cytoplasm and its eventual degradation, which prevents spatial expression of Wg target genes (Swarup and Verheyen, 2012). In the developing eye imaginal disc, wgs is involved in several diverse functions of cell proliferation, differentiation and cell death. Wg regulates expression of Hh, a homeodomain-containing transcription factor (Moskow et al., 1995; Rieckhof et al., 1997; Kurant et al., 1998; Pai et al., 1998; Bessa et al., 2002), to suppress eye fate and thereby define the boundary of the developing eye. Hh is expressed anterior to the MF and acts as a negative regulator of retinal differentiation: loss of hth results in eye enlargement, whereas gain-of-function of hth suppresses the eye development (Pai et al., 1998; Pichaud and Casares, 2000). tsh encodes a nuclear protein with zinc-finger motifs and plays diverse functions during development (Fasano et al., 1991; Roder et al., 1992; de Zulueta et al., 1994; Mathies et al., 1994; Erkner et al., 1999; Wu and Cohen, 2000; Singh et al., 2002, 2004). During eye development, tsh requires Wg signaling to suppress eye fate by induction of hth (Singh et al., 2002, 2004, 2012; Tare et al., 2013a). Thus, eye differentiation is orchestrated by the concerted activities of signaling morphogens and their pathways that regulate MF progression.

Several components of the Hippo pathway show general growth defects but also specific defects in eye development, for example, loss of ex causes reduction in the eye size and duplication of antennae (Boedigheimer and Laughon, 1993). Similarly, loss of ft is reported to cause loss of neurons in the eye and a marked delay in MF progression (Silva et al., 2006; Tyler and Baker, 2007; Napoli et al., 2011). We, therefore, tested the effects of loss of Hippo signaling on eye development and differentiation to investigate whether differentiation defects occur in all or certain components of the Hippo pathway. We found that Yki activation caused separable effects on growth and differentiation of the eye. Expression of Yki and its activated forms (YkiS168A and YkiS3A) results in increased proliferation and suppression of retinal differentiation or delayed differentiation in the eye disc. Here, we present many pieces of evidence to show that Yki activation regulates retinal differentiation in the developing fly retina. Activation of Yki can suppress eye fate by blocking MF progression and by downregulation of expression of RD genes. Furthermore, Wg signaling is ectopically activated during this process. Thus, our data suggest a role for the Hippo pathway effector Yki not only in the proliferative phase, but also in patterning and differentiation during organogenesis.

RESULTS

Downregulation of the Hippo pathway (e.g. by loss of function of wts) throughout the developing imaginal discs, or in somatic clones causes defects in growth and differentiation. Loss-of-function clones of wts, a core kinase of the Hippo signaling pathway, exhibit loss of retinal differentiation on both dorsal (D) and ventral (V) eye margins (supplementary material Fig. S1). However, the mechanism by which Hippo signaling is involved in eye development is yet to be understood. Therefore, to test the role of Hippo signaling in the developing Drosophila eye, we used bi-Gal4 to misexpress transgenes that either downregulate or upregulate the Hippo pathway. The bi-Gal4 expression is limited to the DV margins (both anterior and posterior to the MF) of the developing eye imaginal disc (Calleja et al., 1996; Tare et al., 2013b). This expression domain of bi-Gal4 allows assessment of effects of misexpression on both the differentiation of photoreceptor neurons (posterior to the MF) and as well as in undifferentiated retinal precursor cells (anterior to the MF) (Tare et al., 2013b).

Yki activation regulates eye development

We first blocked Hippo signaling by misexpression of three different yki transgenes: full-length yki tagged with GFP (UAS-yki GFP) and two constitutively active yki transgenes (UAS-ykiS168A and UAS-ykiS3A) (Oh and Irvine, 2008, 2009), to test its role in retinal differentiation. In the wild-type eye imaginal disc, cell outlines were identified by the expression of Discs large (Dlg), and all photoreceptor neurons were detected by the expression of pan-neuronal protein Elav (Fig. 1A). Misexpression of full-length yki GFP (bi>yki GFP) resulted in a significant suppression of eye fate on the DV margins of the developing eye field (Fig. 1C,D; supplementary material Table S1). A few GFP-positive cells showed Elav expression in the bi region (Fig. 1C), and the corresponding adult...
Fig. 1. Hippo signaling is required for eye development. (A,B) Wild-type eye imaginal disc and compound eye of adult fly are shown. (A) Eye imaginal disc is stained for the membrane-specific marker Discs large (Dig, green) and the pan-neural marker Elav (red), which marks the retinal neuron-specific fate. (C,E,G) Eye discs in which bi-Gal4 drives misexpression of (C) full-length yki (bi>yki-GFP), and its hyper activated forms (E) ykiS168A (bi>ykiS168A) and (G) bi>ykiS358 (bi>ykiS358). The resulting adult eyes are shown in D,F,H respectively. Hyperactivated Yki resulted in suppression of the eye fate on both dorsal and ventral margins of (C,E,G) eye imaginal disc and (D,F,H) the adult eye. (C,E) GFP (blue) marks the bi-Gal4 driver domain in the eye disc. (I,J) Misexpression of ykiRNAi on DV margins (bi>dicer+ykiRNAi(N+C)) resulted in enlargement of the eye field at both dorsal and ventral margins of the (I) eye disc and in the (J) adult. The orientation of all imaginal discs is identical with posterior towards the left and dorsal upwards.

Eye was highly reduced along the DV margins (Fig. 1D) when compared with the wild-type eye (Fig. 1B). Misexpression of ykiS168A (bi>ykiS168A) or UAS-ykiS358 (bi>ykiS358) at the DV margin resulted in a significantly stronger suppression of eye-specific fate in the eye imaginal disc (Fig. 1E,G; supplementary material Table S1) and the adult eye (Fig. 1F,H) respectively. By contrast, misexpression of UAS-ykiRNAi (N+C) (bi>ykiRNAi (N+C)) resulted in the enlargement of eye field at DV margins (Fig. 1J). Thus, transgenes expressing full-length or activated forms of Yki showed significant overgrowth and problems in the MF progression and differentiation of the photoreceptors (Fig. 1C,E,G).

We then tested phenotypes of modulating Hippo (Hpo) levels on photoreceptor differentiation (Fig. 2). Misexpression of hpoRNAi using bi-Gal4 (bi>hpoRNAi) resulted in the enlargement of eye field along the DV margins, as evident from the extension of Elav expression domains (Fig. 2A; supplementary material Table S1). In comparison with the wild-type adult eye (Fig. 1B), bi>hpoRNAi formed a significantly larger adult eye (Fig. 2B). By contrast, misexpression of hpo on the DV margin (bi>hpo) resulted in a highly reduced or ‘no-eye’ phenotype as seen in the eye imaginal disc (Fig. 2C) as well as the adult eye (Fig. 2D). This decrease in size and number of photoreceptors may be attributed to the induction of cell death by hyperactivation of the Hippo pathway (Tapon et al., 2002; Udan et al., 2003; Verghese et al., 2012). Therefore, we analyzed gain-of-function ‘flp-out’ clones of hpo (hsFLP>hpo), marked by GFP generated at late second instar and analyzed within 12 h show a few cell size clones that do affect eye fate, as seen in the (E) eye disc and (F) the adult eye.

Yki activity is required for RD gene expression

The human Pax6 homolog, Ey, is required for eye-field specification, and is one of the earliest genes expressed throughout the early first instar larval eye imaginal disc (Halder et al., 1995; Pappu and Mardon, 2004; Burgy-Roukala et al., 2013). During third instar larval stage, Ey is downregulated to allow eye differentiation to proceed; Ey expression is therefore restricted anterior to the MF in the cells that
develop into head cuticle (Fig. 3A), (Halder et al., 1998). The RD gene eya, which acts downstream of ey, is expressed in a broad stripe in the differentiated cells posterior to the MF (Fig. 3B) (Bonini et al., 1993) and dac is expressed in two stripes—directly anterior and posterior to the MF (Fig. 3C) (Mardon et al., 1994). Misexpression of yki3SA (bi>yki3SA) resulted in an upregulation of Ey (Fig. 3D), and strong suppression of Eya (Fig. 3E) and Dac (Fig. 3F) at the DV margin. It is expected that Ey should be present if there is no retinal differentiation on the DV margin (Fig. 3D). To test whether these results could be extrapolated to other domains of the eye, we misexpressed yki3SA at the dorsoventral (DV) margin (Fig. 3D). To test whether these results could be expected that Ey should be present if there is no retinal differentiation on the DV margin (Fig. 3D). To test whether these results could be extrapolated to other domains of the eye, we misexpressed yki3SA (bi>yki3SA) and generated ‘flip-out’ clones (Act>yki3SA+GFP) in the eye imaginal disc. Misexpression of yki3SA using dpp-Gal4 (dpp>yki3SA), which drives expression on the posterior margin of the developing eye imaginal disc (marked by GFP reporter), resulted in the complete suppression of eye fate, as evidenced by the absence of Elav expression. In dpp>GFP+yki3SA eye imaginal disc, Ey (Fig. 3G), Eya (Fig. 3H) and Dac are downregulated (Fig. 3I). Gain-of-function ‘flip-out’ clones of yki (GFP-positive) resulted in the suppression of eye fate, as evidenced by the lack of Elav positive nuclei in the clones (Fig. 3J,J′,K′,L″). Anterior to the MF, Ey was downregulated in the clones (Fig. 3J,J′). The gain-of-function clones of yki3SA strongly suppressed Eya (Fig. 3K,K′) and Dac (Fig. 3L,L″) expression. We observed that stochastic overexpression of yki3SA in the cells at disc margin using Gal4 drivers that induce expression very early during disc development, e.g. dpp-Gal4 or bi-Gal4, resulted in the induction of Ey but suppression of photoreceptor differentiation. However, overexpression of yki3SA later in the early to mid-second instar in ‘flip-out’ clones, resulted in the downregulation of Ey and in suppression of differentiation. Furthermore, the clone sizes ranged from large to small, reflecting whether the clone was induced during the late first or late second instar of larval development. Thus, suppression of differentiation by downregulation of RD genes (eya, dac) is a consistent phenotype of yki3SA overexpression. It is possible that hyperactivation of yki suppresses the R8 specification, and thus results in the observed defects in differentiation.

Activated Yki blocks retinal differentiation

Expression of Scabrous (Sca) and Atonal (Ato) – the two early markers for R8 specification (Fig. 4A,B) (Baker et al., 1990; Mlodzik et al., 1990; Jarman et al., 1994) were analyzed when Hippo signaling was downregulated or Yki was hyperactivated. Misexpression of yki3SA using bi-Gal4 driver (bi>yki3SA) resulted in the complete loss of Sca (Fig. 4C), Ato (Fig. 4D) and Elav expression at the DV margin. It suggests that the cells expressing hyperactivated yki not only suppress the RD genes, but also downregulate the markers for R8 photoreceptor specification and differentiation. These effects were confirmed by misexpression of yki using dpp-Gal4 driver (dpp>yki3SA; Fig. 4E,F) and in random ‘flip-out’ gain-of-function clones of yki3SA (marked by GFP reporter) (Fig. 4G,H) in the eye imaginal disc. Thus, hyperactivation of yki blocks differentiation of the R8 cells. As R8 specification and differentiation are associated with morphogenetic furrow (MF) progression, the requirement for yki3SA in MF progression was tested.

Yki is required for MF progression

In the developing eye, Dpp and Hh signaling is required for normal development and MF progression (Ready et al., 1976; Wolff and...
Fig. 4. Activation of yki blocks retinal differentiation in the developing eye disc. Panels show the expression pattern of (A) Scaborous (Sca, green) and (B) Atonal (Ato, green), the two markers for R8 specification in the wild-type developing eye disc. Misexpression of yki3SA (bi>yki3SA) led to the suppression of MF at both the dorsal and ventral margins (white arrowheads). The eye field (boundary marked by a white dotted line) is formed at the DV margin but retinal differentiation is suppressed. The orientation and magnification are identical for all imaginal discs.

Yki activation leads to ectopic induction of Hth and Tsh

Wg is known to activate hth - a negative regulator known to suppress eye fate in the developing eye imaginal disc (Pichaud et al., 1998). Co-expression of yki with dTCFDNS (bi>yki+dTCFDNS) (Fig. 6D,E) or with Sgg (bi>yki+sgg3SA) (Fig. 6F,G) resulted in a significant rescue of retinal differentiation (marked by Elav) in the bi domain of the eye disc (supplementary material Table S1). The resulting eye imaginal disc (Fig. 6D,F) and the associated adult phenotype displayed a wild-type eye (Fig. 6E,G). The eye disc associated with bi>yki+sgg3SA displayed ectopic Elav expression due to the extension of the Elav-positive cells along the DV margin (Fig. 6F), in comparison with the wild-type eye (Fig. 1A). These results demonstrate that, normally, Hippo signaling negatively regulates Wg signaling during retinal differentiation in the developing eye imaginal disc.

Yki activation induces Wingless signaling to regulate MF progression

In the developing eye, Wg serves as a negative regulator of eye fate, and is known to block MF progression (Ma and Moses, 1995; Treisman and Rubin, 1995; Kumar, 2011). Therefore, we tested whether downregulation of Hippo signaling affected Wg levels in regions where retinal differentiation was suppressed. Misexpression of hyperactivated yki3SA using bi-Gal4 (bi>yki3SA) (Fig. 6B,B') or in 'flip-out' clones (Fig. 6C,C'; supplementary material Table S1) induced ectopic Wg expression. These observations suggest that upregulation of Wg signaling may be the mechanism by which misexpression of yki3SA suppresses MF progression. We therefore tested effects of modulating levels of Wg signaling in the cells misexpressing yki3SA. Activation of Wg signaling ultimately activates the downstream transcription factor dTCF, which in turn induces Wg target genes (Swarup and Verheyen, 2012). Therefore, we tested two antagonists of Wg signaling pathway using transgenes that expressed either a dominant-negative form of Drosophila T-cell Factor (UAS-dTCFDNS) (van de Wetering et al., 1997) or the activated form of shaggy (UAS-sgg3SA), the negative regulator of the pathway (Hazelett et al., 1998). Co-expression of yki with dTCFDNS (bi>yki+dTCFDNS) (Fig. 6D,E) or with Sgg (bi>yki+sgg3SA) (Fig. 6F,G) resulted in a significant rescue of retinal differentiation (marked by Elav) in the bi domain of the eye disc (supplementary material Table S1). The resulting eye imaginal disc (Fig. 6D,F) and the associated adult phenotype displayed a wild-type eye (Fig. 6E,G). The eye disc associated with bi>yki+sgg3SA displayed ectopic Elav expression due to the extension of the Elav-positive cells along the DV margin (Fig. 6F), in comparison with the wild-type eye (Fig. 1A). These results demonstrate that, normally, Hippo signaling negatively regulates Wg signaling during retinal differentiation in the developing eye imaginal disc.
Casares, 2000; Singh et al., 2002; Tare et al., 2013a). Furthermore, Tsh can induce Wg and Hth to suppress the eye fate (Singh et al., 2002; Tare et al., 2013a). We tested Hth and Tsh expression levels in the biyki3SA eye discs. In wild-type eye disc, Hth is expressed in the cells anterior to the MF that are not destined to form the head cuticle, in the peripodial membrane of the eye disc, and in a proximal ring in the antennal region of the eye-antennal imaginal disc (Fig. 7A). Gain-of-function of hth blocks differentiation of the photoreceptor neurons (van de Wetering et al., 1997; Pai et al., 1998; Bessa et al., 2002; Singh et al., 2002). Wild-type Tsh expression is also seen directly anterior to the MF (Fig. 7B). Misexpression of activated yki either by using bi-Gal4 (bi>yki3SA) (Fig. 7C,D) or dpp-Gal4 (dpp>yki3SA) (Fig. 7E,F) caused ectopic induction of Hth and Tsh along with complete loss of Elav (Fig. 7C,D). These results were further verified in ‘flp-out’ clones misexpressing UAS-yki3SA, which showed a loss of Elav-positive photoreceptors (Fig. 7G,H), as well as an upregulation of Hth (Fig. 7G,G′) and Tsh expression (Fig. 7H,H′). Overall, these data showed that activated Yki induced ectopic expression of Hth and Tsh to suppress retinal differentiation in the eye.

**Activated Yki suppresses eye fate by a Wg-dependent mechanism**

To test whether activated Yki suppresses the eye fate by Wg-mediated induction of Hth and Tsh, we blocked Wg signaling in the bi domain where Yki is activated (bi>yki+sgg) and then tested expression of Wg, Hth and Tsh. We found that blocking Wg signaling along with Yki activation (bi>yki+sgg) not only suppressed Wg (Fig. 8A,A′), but also blocked activation of Hth (Fig. 8B,B′) and Tsh (Fig. 8C,C′) on the DV margins. It suggests that Yki activation triggers Wg signaling, which in turn induces Hth and Tsh to block retinal determination and differentiation in the eye.

**Yki activation does not change eye fate to antenna**

The ectopic expression of Hth or Tsh in yki3SA-expressing cells presented the alternate possibility that the cells overexpressing

---

Fig. 6. Activation of yki suppresses the eye fate by ectopically inducing Wg signaling. (A,A′) Panels show wild-type Wg (green) expression in the third instar eye imaginal disc. Wg (green) is expressed antero-laterally on dorsal and ventral eye margins of the eye imaginal disc. (B,C) Misexpression of yki3SA, under (B-B′) bi-Gal4 (bi>yki3SA) at DV margins or in ‘FLP-out’ random clones (marked by GFP, blue) under (C-C′) hsFLP>yki3SA in the eye disc, results in suppression of eye fate (marked by Elav, red) and ectopic induction of Wg expression (green, marked by white arrows in B,B′,C′). Blocking Wg signaling in bi>yki eye disc by misexpression of antagonists of Wg signaling such as (D,E) dominant-negative dTCFDN (bi>yki+dTCFDN5) and (F,G) Shaggy (Sgg) (bi>yki+SggS9A), resulted in the rescue of eye suppression by yki (bi>yki) in the (D,F) eye disc and (E,G) the adult eye. The orientation and magnification of imaginal discs and adult heads are identical in all panels.

Fig. 7. Activation of yki induces Homothorax (Hth) and Teashirt (Tsh) expression. Eye imaginal discs stained for (A,C,E,G,G′) Hth (green) and (B,D,F,H,H′) Tsh (green) are shown from (A,B) wild-type, (C,D) bi>yki3SA, (E,F) dpp>yki3SA and (G,H′) hsFLP::Act>y+>Gal4 UASyki3SA (hsFLP>yki3SA) larvae. (A) Normally, Hth and (B) Tsh expression is restricted anterior to the MF in the eye. Both (C,E,G′) Hth and (D,F,H,H′) Tsh are upregulated, and (C-H,G′,H′) eye differentiation (Elav, red) is suppressed when yki3SA is overexpressed under (C,D) bi-Gal4, (E,F) dpp-Gal4 or in ‘FLP-out’ clones using (G,H) hsFLP>yki3SA. (G-H′) ‘Flp-out’ clones of yki3SA are marked by GFP (blue).
yki3SA have undergone a change in cell fate from eye disc to antenna or head cuticle. This idea was based on the fact that the larval eye imaginal disc gives rise to not only the compound eye but also to the antenna and the head of the adult fly. In developing eye discs, Cut (Ct) serves as a marker for the antennal specific fate (Kenyon et al., 2003; Duong et al., 2008; Wang and Sun, 2012; Weasner and Kumar, 2013). In the wild-type eye-antennal imaginal discs, Ct expression is found in a circular expression domain in the antenna region, and marks cone cells within the developing eye field (Fig. 9A). In the cells overexpressing yki3SA (bi>yki-GFP, sgg3SAs), expression of Ct is lost (Fig. 9B,D,D‴). Together, the suppression of Cut (Fig. 9B-D) and Elav (Fig. 9D,D‴) in bi>ykiSA and in ‘flp-out’ ykiSA clones suggests that hyperactivation of Yki strongly suppresses differentiation in the developing eye. Furthermore, Ct expression is not induced in the ‘flp-out’ clones, it suggests that yki3SA misexpression does not cause a change in cell fate. Overall, these experiments show that the primary influence of yki3SA misexpression is linked to cell differentiation.

**Activated Yki regulates retinal differentiation through Ft-Wts branch**

Our studies suggested that loss of Hippo signaling by downregulation of wts or by misexpression of activated forms of Yki affects growth and differentiation likely by separable independent mechanisms. Therefore, we investigated other Hippo pathway components, to test whether all or only specific upstream regulators of the pathway affect differentiation and growth. One of the upstream regulators is the Fat branch of the pathway that regulates Wts stability via the atypical myosin Dachs (D) (Cho et al., 2006). In addition, loss of function of the Fat branch components also shows broad DV and PD defects, and defects in MF progression (Cho and Irvine, 2004; Mao et al., 2006; Matakatsu and Blair, 2012). Misexpression of UAS-ftRNAi (bi>ftRNAi) at the DV margin shows mild suppression of the retinal differentiation and MF progression, marked by the loss of Elav-positive cells (Fig. 10A,A‴). These defects coincide with ectopic Wg expression at the DV margins (Fig. 10B,B‴), suggesting a similar mechanism of suppression of differentiation and MF progression as yki3SA (Fig. 10G,H). Interestingly, overexpression of D, which is negatively regulated by Ft, showed weak overgrowth and extension of MF progression (Fig. 10C,C‴), and downregulation of Wg (Fig. 10D,D‴). Within the Ft branch, D negatively regulates Wts, and overexpression of Wts throughout the eye using ey-Gal4 (data not shown) or GMR-Gal4 (Tapon et al., 2002; Wu et al., 2003; Lai et al., 2005) is shown to cause mild cell death. However, misexpression of Wts using bi-Gal4 resulted in an unexpected phenotype in which, although the overall eye size was not overly overgrown, the MF progression appeared extended at both the dorsal and ventral margins in the bi-Gal4 domain (Fig. 10E,E‴). Wg expression was downregulated in these discs (Fig. 10F,F‴). Overall, these data revealed a shared defect in MF progression in several components of the Ft branch of the pathway that is not shared by other upstream regulators like Hpo.

**DISCUSSION**

During development, patterning and growth are tightly controlled by highly conserved signaling pathways to determine the size and shape of an animal. In *Drosophila* eye, an initial proliferative phase (growth spurt) occurs during early larval development (under the control of several growth regulatory pathways), which is followed by retinal determination and differentiation (Tare et al., 2013a). Together, these processes direct the formation of the fly retina and other associated cells (Wolff and Ready, 1993). Interestingly, the expression of growth regulatory pathway genes continues when cells are undergoing differentiation, suggesting that these genes may play an important role during retinal differentiation.

**The Hippo pathway regulates both growth and retinal differentiation in the developing eye**

In *Drosophila*, the Hippo signaling pathway members regulate the activity of the transcriptional co-activator Yki to control tissue growth. Activation of Yki by downregulation of the Hippo pathway, leads to its nuclear localization, where it activates target genes that trigger cell proliferation and inhibit apoptosis (Huang et al., 2005; Edgar, 2006; Pan, 2010; Halder and Johnson, 2011; Verghe et al., 2013; Yu and Guan, 2013). The Yki homolog YAP/TAZ function as
cells where tested expression of three RD network genes (1995, 1998; Chen et al., 1997, 1999; Shen and Mardon, 1997). We et al., 1994; Mardon et al., 1994; Quiring et al., 1994; Halder et al., epithelial cells into retinal neurons (Bonini et al., 1993; Cheyette specification (Zhang et al., 2011).

Previously it has been suggested that Yki and Sd regulate tissue separable mechanisms. These phenotypes of Yki misexpression the growth of eye field and eye fate specification/differentiation by Taken together, these data suggest that the Hippo pathway regulates

oc oncogenes in the context of many cancers. Recently, other functions of the Hippo signaling pathway have been described, including stem cell renewal and maintenance, regeneration, wound healing and axial patterning (Halder and Camargo, 2013; Yu and Guan, 2013). Earlier data have shown that the prominent effect of downregulation of the Hippo pathway is excess growth due to increased proliferation and reduced cell death (Fig. 2; supplementary material Fig. S1). By contrast, hyperactivation of the pathway results in the reduction of developing eye field (Fig. 2) by hpo-mediated upregulation of the pro-apoptotic gene head involution defective (hid) (Udan et al., 2003). Interestingly, misexpression of yki (bi>yki), which also results in downregulation of the pathway, showed overgrowth of the eye disc but suppression of photoreceptor differentiation in the eye (Fig. 1). Taken together, these data suggest that the Hippo pathway regulates the growth of eye field and eye fate specification/differentiation by separable mechanisms. These phenotypes of Yki misexpression suggested that Yki may regulate the expression of the retinal differentiation genes or other negative regulators of eye development. Previously it has been suggested that Yki and Sd regulate tissue specification (Zhang et al., 2011).

There are several signaling pathways involved in eye development but the RD genes represent the core transcription factor cascade, which is required to specify and differentiate epithelial cells into retinal neurons (Bonini et al., 1993; Cheyette et al., 1994; Mardon et al., 1994; Quiring et al., 1994; Halder et al., 1995, 1998; Chen et al., 1997, 1999; Shen and Mardon, 1997). We tested expression of three RD network genes (ey, eva and dac) in cells where yki was hyperactivated. Misexpression of yki (bi>ykiSSA) blocked the eye fate by suppressing RD gene expression (Fig. 3). These data suggest that Yki levels and activity need to be regulated during eye growth and differentiation, as inappropriate upregulation of Yki activity leads to suppression of retinal differentiation.

**Yki is required for retinal differentiation and MF progression**

Retinal differentiation is marked by onset of expression of Ato, which marks the R8 photoreceptor fate and initiates the process of retinal differentiation behind the MF (Baker et al., 1990; Mlodzik et al., 1990; Jarman et al., 1994). Misexpression of yki on the posterior margin of the eye disc by the dpp-Gal4 (dpp>ykiSSA) driver resulted in a ‘no-eye’ phenotype (Fig. 3J-L and Fig. 4G,H). Lack of retinal photoreceptor neurons raised an interesting possibility that retinal differentiation process may not have initiated at all in dpp>ykiSSA eye imaginal disc (Fig. 3J-L and Fig. 4G,H). Initiation and progression of MF depends on the function of Dpp and Hh signaling (Dominguez and Hafen, 1997; Borod and Heberlein, 1998; Kumar, 2013). Activation of Yki in a subset of cells at the DV margin can prevent initiation of the MF, as expression of dpp-lacZ (a key gene in maintaining MF progression) and downstream RD genes is suppressed (Figs 4 and 5). Furthermore, MF initiation and R8 specification was completely blocked when Yki was activated at the posterior margin of the developing eye imaginal disc (dpp>ykiSSA). Thus, activated Yki suppressed retinal differentiation. Taken together, these studies suggest that Yki is required for the MF progression.

**Yki regulates Wg signaling to promote MF progression**

The highly conserved Wg signaling pathway is involved in various developmental functions, including eye development and retinal differentiation. Wg, a signaling molecule, is expressed at the antero-lateral margin of the developing eye disc and its expression recedes as the MF progresses anteriorly from the posterior margin. Wg is known to be a negative regulator of retinal development, and blocks MF progression in the developing eye (Ma and Moses, 1995; Treisman and Rubin, 1995). Interestingly, Wg is one of the downstream targets of the Hippo pathway effector Yki (Cho et al., 2006). Misexpression of yki caused ectopic expression of Wg, suggesting that Wg signaling is activated by Yki on the DV margins to block MF progression, as well as to prevent retinal differentiation in the developing eye (Fig. 6). Furthermore, downregulation of Wg signaling caused ectopic differentiation to occur when yki was misexpressed (Fig. 6). Thus, Wg acts downstream of Yki to suppress MF progression and retinal differentiation (Fig. 7). When Wg signaling was blocked by co-expressing antagonists of Wg signaling such as SggSSA or dTCF and the loss of retinal differentiation due to misexpression of Yki was restored to near wild type (Fig. 6). These results further substantiate our earlier conclusions that the Hippo pathway affects multiple steps during differentiation, e.g. MF progression, regulation of expression of RD genes and activation of negative regulators of eye development.

In addition to its role in MF progression, Wg is also essential for delimiting the border between the compound eye and the adult head cuticle (Royet and Finkelstein, 1996, 1997). Similarly, expression of the head-specific fate markers such as Hth and Tsh also becomes
restricted to anterior to the MF in the presumptive head cuticle region (Bessa et al., 2002; Singh et al., 2002). Tsh is responsible for Wg-mediated induction of Hth in the ventral eye to define the boundary between the head capsule and the eye field (Bessa et al., 2002; Singh et al., 2002; Tare et al., 2013a). Our studies showed that Yki-mediated suppression of retinal differentiation causes ectopic induction of Wg, Hth and Tsh (Fig. 6). Both tsh and hth have been shown in previous studies to be targets of yki and wg (Rieckhof et al., 1997; Gallet et al., 1998; Peng et al., 2009). Our studies demonstrate that Yki uses its downstream target, Wg, to regulate expression of the negative regulator Hth in order to regulate retinal fate (Figs. 8 and 11). It is possible that cells misexpressing yki undergo transformation from eye to antennal fate. However, the antennal fate marker Ct was not induced in the cells where retinal fate was suppressed by Yki misexpression (Fig. 9). Thus, the Yki misexpressing cells do not undergo transformation from eye to antenna-specific cells.

Mechanism of Hippo-mediated regulation of retinal differentiation

Our results uncovered a role for the Hippo pathway in the regulation of expression of RD genes, by controlling MF progression. These findings also raised the issue of whether all or only some components of the Hippo pathway were involved in the regulation of retinal differentiation. As Hippo gain of function resulted in reduced eye due to cell death (Fig. 2), we tested the effects of upstream components of the Fat branch specifically because prior reports suggested that ft mutant cells show marked delay in MF progression. Indeed, all major components of the Fat branch, e.g. ft, D and wts, showed defects in MF progression that were separate from the defects in growth regulation. Other upstream regulators (e.g. Sav, Mats, Kibra) did not cause an obvious change in MF progression. However, as previously reported, Ex, which also acts (e.g. Sav, Mats, Kibra) did not cause an obvious change in MF progression. These results suggest that this novel employment of the pathway constitutes a new, and potentially general, mechanism for regulating tissue and organ size.

MATERIALS AND METHODS

The stocks used in this study are listed in FlyBase. The fly stocks used were UAS-fa-RNAi (Dietzl et al., 2007), UAS-wt-RNAi (Fernandez et al., 2011; Rauskolb et al., 2011), UAS-yki-RNAi(NC) (Zhang et al., 2008), UAS-D (Mao et al., 2006), UAS-hpo (Udan et al., 2003), UAS-wts (Kwon et al., 2015), y w hsFLP; UAS-hpoRNAi; y w; FRT122/TM6B, Tb (Pantalacci et al., 2003), dpp-Gal4 (Staehling-Hampton et al., 1994), b-Gal4 (Calieja et al., 1996), UAS-Dicer (Missiquita et al., 2008), dpp-i22 (Blackman et al., 1991), UAS-Sgg (Hazelett et al., 1998), UAS-JTCEP (van de Wetering et al., 1991), w P(Act>y+>Gal4)25 P(UAS-GFP)55S/55T/CyO (Ito et al., 1997), y w hsFLP122 (Struhl and Basler, 1993), w FRT82B/TM6B, Tb (Xu et al., 1995) and Canton-S. For activation of Yki, we used the following transgenes: yki-GFP (full length) (Oh and Irvine, 2008), UAS-ykiS188A (Oh and Irvine, 2008) and UAS-ykiS38A (Oh and Irvine, 2008).

We used the Gal4/UAS system for targeted misexpression studies (Brand and Perrimon, 1993). All Gal4/UAS crosses were maintained at 18°C, 25°C and 29°C to sample different induction levels. Genetic epistasis experiments were carried out at 25°C in order to determine the genetic hierarchy.

Genetic mosaic analysis

Gain-of-function clones of UAS-yki GFP, UAS-ykiS188A and UAS-hpo were generated using ‘flip-out’ technique, by crossing y w hsFLP122; P(Act>y+>Gal4)25 P(UAS-GFP)55S/55T/CyO (Struhl and Basler, 1993) virgins with males from UAS-yki GFP or UAS-ykiS38A or UAS-hpo to generate GFP-positive clones. Eggs were collected at 6 h intervals at 25°C from a synchronous culture, and subjected to a single heat shock (20 min for UAS-hpo and 45 min for UAS-yki) at 37°C at about 24 h after egg laying (AEL) or as indicated. The larvae were transferred to 25°C for recovery and further development. Loss-of-function clones of wts were generated by crossing w; wtsFRT82B/TM6B, Tb males with eFLP, FRT82B Ubi-GFP virgins.

Immunohistochemistry

Eye-antennal discs from wandering third instar larvae were dissected and stained following the standard protocol (Singh et al., 2002). The primary antibodies used were rabbit anti-Dlg (1:200, a gift from K. Cho, KAIST, Korea), mouse anti-Dlg (1:50, DSHB, 4F3), rat anti-Elav (1:100, DSHB, 7E8A100), mouse anti-Wg (1:50, DSHB, 4D4), mouse anti-Ey (1:100, DSHB, EY), mouse anti-Eya (1:100, DSHB, eya1016H), mouse anti-Dac (1:100, DSHB, mAbd2-3), mouse anti-Cut (1:20, DSHB, 2B10), mouse anti-Sca (1:100), rabbit anti-Hth (1:100, a gift from Henry Sun, Institute of Molecular Biology, Taiwan), rabbit anti-Tsh (1:150, a gift from Stephen Cohen, Institute of Molecular and cell Biology, Singapore), rabbit anti-β galactosidase (1:100, Promega, Z3781) and guinea pig anti-Atonal (1:500, a gift from Daniel Marenda, Drewel University, USA). The secondary antibodies were:


RESEARCH ARTICLE


Supplementary Figure 1. Loss-of-function of wts results in suppression of eye fate on the margins. (A, A’) Wild type eye imaginal disc stained for Dlg (Green) and Elav (Blue). (B, B’) Loss-of-function clones of wts marked by absence of GFP reporter (green) results in suppression of eye fate marked by Elav (Blue) generated in the developing eye imaginal disc. The orientation and magnification of imaginal discs and adult heads are identical in all panels.
Supplementary Table 1. Frequency of phenotypes observed in eye imaginal discs in various genetic backgrounds analyzed in this study.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No of flies showing phenotypes</th>
<th>Percentage of discs With phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>bi-Gal4</em> (Control)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>bi-yki</em> (<em>Full length</em>)</td>
<td>14/41</td>
<td>20.93%</td>
</tr>
<tr>
<td>3SA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bi&gt;yki^{S168A}</em></td>
<td>9/39</td>
<td>23.07%</td>
</tr>
<tr>
<td><em>bi&gt;yki^{3SA}</em></td>
<td>19/59</td>
<td>32.20%</td>
</tr>
<tr>
<td>3SA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>dpp&gt;yki^{3SA}</em></td>
<td>6/32</td>
<td>18.75%</td>
</tr>
<tr>
<td><em>hsFLP&gt;yki^{3SA}</em></td>
<td>15/70</td>
<td>21.22%</td>
</tr>
<tr>
<td><em>bi&gt;hpo</em></td>
<td>11/28</td>
<td>42.30%</td>
</tr>
<tr>
<td><em>bi&gt;hpo^{RNAi}</em></td>
<td>5/22</td>
<td>22.72%</td>
</tr>
<tr>
<td><em>hsFLP&gt;hpo</em> Analyze after</td>
<td>6/94</td>
<td>19.35%</td>
</tr>
<tr>
<td>12hr</td>
<td>0/29</td>
<td>0%</td>
</tr>
<tr>
<td>24hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bi&gt;yki^{3SA} + dTCF^{DN5}</em></td>
<td>7/33</td>
<td>21.21%</td>
</tr>
<tr>
<td><em>bi&gt;yki^{3SA} + sgg^{S9A}</em></td>
<td>5/31</td>
<td>16.1%</td>
</tr>
</tbody>
</table>