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

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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 crosswalk 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., 2002) 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 (Grusche et al., 2010; Boggiano and Feihon, 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; Goulev 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. cylcin 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 (fj) 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,
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; Roignant 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; Roignant and Treisman, 2009; Kumar, 2011; Singh et al., 2012; Burgu-Roukala et al., 2013). These are twin of eyeless (toy), eyeless (ey), eyeopen (eog), twin of eyopen (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 Ey is required for eye field specification, whereas downstream genes eya, so and dac are required for retinal determination and differentiation (Kumar, 2011; Burgu-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; Burgu-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 atonal (ato)-encoding basic HLH proteins, play important roles in PR differentiation (Jarman et al., 1994; Bertrand et al., 2002; Tanaka-Matakatu 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 (Chanat 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, Wg 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 ey causes reduction in the eye size and duplication of antennae (Boedighheimer 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; Napoliello 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 later 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), 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-neural 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...
Misexpression of ey. (C,E) GFP (blue) marks the both dorsal and ventral margins of (C,E,G) the eye disc and (D,F,H) the adult respectively. Hyperactivated Yki resulted in suppression of the eye fate on yki\textsuperscript{S168A} compared with the wild-type eye (Fig. 1B). Misexpression of ey was highly reduced along the DV margins (Fig. 1D) when

\begin{itemize}
    \item bi\textgreater{}yki\textsuperscript{S168A} or UAS-\textit{yki}\textsuperscript{S168A} (bi\textgreater{}yki\textsuperscript{S168A})
    \item bi\textgreater{}yki\textsuperscript{3SA} or UAS-\textit{yki}\textsuperscript{3SA} (bi\textgreater{}yki\textsuperscript{3SA})
\end{itemize}

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) the eye 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 \textit{yki}\textsuperscript{RNAi} on DV margins (bi\textgreater{}dicer\textsuperscript{+}\textit{yki}\textsuperscript{RNAi(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.

\textbf{Yki activity is required for RD gene expression}

The human Pax6 homolog, Eys, 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, Eys is downregulated to allow eye differentiation to proceed; Eys 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 yki^{3SA} (bi>yki^{3SA}) 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 yki^{3SA} using dpp-Gal4 (dpp>yki^{3SA}) and generated ‘flip-out’ clones (Act>yki^{3SA}+GFP) in the eye imaginal disc. Misexpression of yki^{3SA} using dpp-Gal4 (dpp>yki^{3SA}), 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+yki^{3SA} 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′,L″). Anterior to the MF, Ey was downregulated in the clones (Fig. 3J,J′). The gain-of-function clones of yki^{3SA} strongly suppressed Eya (Fig. 3K,K′) and Dac (Fig. 3L,L″) expression. We observed that stochastic overexpression of yki^{3SA} 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 yki^{3SA} 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 yki^{3SA} 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. 3A-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 yki^{3SA} using bi-Gal4 driver (bi>yki^{3SA}) 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>yki^{3SA}; Fig. 4E,F) and in random ‘flip-out’ gain-of-function clones of yki^{3SA} (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 yki^{3SA} 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

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**Fig. 3. Activation of yki suppresses eye fate by downregulating the retinal determination genes.** (A–C) Wild-type expression of (A) Eyeless (Ey, green), (B) Eyes absent (Eya, green) and (C) Dachshund (Dac, green) in the third instar eye imaginal disc. (A) Ey expression is restricted anterior to the morphogenetic furrow (MF). (B) Eya is expressed both posterior as well as anterior to the MF. (C) Dac is expressed in a wide band anterior to the MF. (D–F) Misexpression of yki^{3SA} (bi>yki^{3SA}) at the dorsoventral (DV) margin of the developing eye disc resulted in (D) Ey (green) expression and suppression of Elav (red). (E) Eya (green) and (F) Dac (green) at the DV margin. (D,F) Dlg (blue) marks the outline of the eye disc. (G–I) Misexpression of yki^{3SA} at the posterior margin of the developing eye disc using dpp-Gal4 driver (dpp>yki^{3SA}) resulted in complete loss of eye field, as evidenced by the absence of Elav (red), (H) mild downregulation of Ey (green), and (I) loss of Dac (green). (J–L) Elav expression (red) is downregulated in yki^{3SA} clones. Orientation and magnification of all imaginal discs are identical.
of hyperactivated Wg is known to block MF progression (Ma and Moses, 1995; Kumar, 2011). Therefore, we tested the involvement of Hippo signaling pathway in MF progression, dpp-lacZ reporter expression was examined. In the wild-type eye imaginal discs, dpp-lacZ, a transcriptional reporter of dpp gene (Blackman et al., 1991) is expressed in a thin stripe that overlays the apical constrictions caused by the MF cells, and marks the anterior extent of Elav positive differentiated cells (Fig. 5A,A′). Misexpression of hpoRNAi (bi>hpoRNAi) resulted in the extension of dpp-lacZ expression anteriorly, along the DV margins due to extension of MF, leading to generation of enlarged eyes caused by ectopic Elav expression (Fig. 5B,B′). However, misexpression of yki3SA led to the suppression of eye fate and MF progression causing the furrow to bend posteriorly (Fig. 5C,C′). Large overgrowths are observed in the bi-Gal4 domains in bi>yki3SA discs despite suppression of retinal differentiation and suppression of MF progression (Fig. 5C,C′). It suggests that Yki is involved in regulating MF progression. A comparison of effects of misexpression of hpoRNAi and yki3SA revealed that unlike Yki, loss of hpo does not affect MF progression, suggesting that the Hippo pathway regulates growth and differentiation via separable mechanisms.

**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-dTCFDN5) (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 dTCFDN5 (bi>yki+ dTCFDN5) (Fig. 6D,E) or with Sgg (bi>yki+ sggsa) (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+sggsa 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 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 and Ready, 1993; Schlichting and Dahmann, 2008; Kumar, 2013). To test this, we evaluated the role of Hth and Tsh in regulating MF progression.

**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 (hsFLP>yki3SA) marked by GFP (blue) suppressed the (C,C′,E,E′,G,G′) Sca (green) and (D,D′,F,F′,H,H′) Ato (green) expression in the eye disc. (C,D) White dotted line marks the outline of the eye imaginal disc. The orientation and magnification are identical for all imaginal discs. Arrowheads in C-D′ indicate the region of R8 specification in eye imaginal disc to show that R8 specification does not extend into the bi domain.

**Fig. 5. Activation of yki blocks morphogenetic furrow (MF) progression.** A comparison of dpp-lacZ expression (green), a MF marker, is shown in the (A) wild-type, (B) bi>hpoRNAi and (C) bi>yki3SA eye imaginal discs. (A-C) Differentiated photoreceptor cells are marked by Elav (red). (C,C′) Misexpression of yki3SA resulted in 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.
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 bi>yki3SA 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 ‘flip-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’). 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
yki\textsuperscript{3SA} 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 antennal region, and marks cone cells within the developing eye field (Fig. 9A). In the cells overexpressing yki\textsuperscript{3SA} (bi>yki\textsuperscript{3SA}), the cone cell-specific Ct expression was lost (Fig. 9B,D,D′). Together, the suppression of Cut (Fig. 9B-D) and Elav (Fig. 9D,D′) in bi>yki\textsuperscript{3SA} and in ‘flp-out’ yki\textsuperscript{3SA} clones suggests that hyperactivation of Yki strongly suppresses differentiation in the developing eye. Furthermore, as Ct expression is not induced in the ‘flp-out’ clones, it suggests that yki\textsuperscript{3SA} misexpression does not cause a change in cell fate. Overall, these experiments show that the primary influence of yki\textsuperscript{3SA} 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 or all 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-ft\textsuperscript{RNAi} (bi>yki\textsuperscript{3SA}) 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 yki\textsuperscript{3SA} (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 cy-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′). 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; Verghese et al., 2013; Yu and Guan, 2013). The Yki homolog YAP/TAZ function as
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 (eya, eya 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; Modzik 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 coexpressing antagonists of Wg signaling such as SggSSA or dTCFΔN3, 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 downstream of Ft, shows reduced eye size, and defects in MF progression and differentiation (data not shown) (Boedigheimer and Laughon, 1993; Tyler and Baker, 2007). Taken together, our studies show that Ft-wts branch of the Hippo signaling pathway affect MF progression via Yki-mediated regulation of its downstream target Wg (Fig. 11). Wg, in turn, then induces Hth and Tsh to suppress the eye fate and MF progression (Fig. 11). These functions of the Hippo pathway are separable from its role in the regulation of overall tissue size.

Thus, identification of Ft, D, Wts and Yki as key components involved in retinal differentiation and MF progression provides a striking parallel with the recently discovered involvement of the Wts-Hpo pathway and Yki/YAP in regulating primordial cell populations in vertebrates, notably the segregation of trophoectoderm and inner cell mass in early mammalian embryos (Nishioka et al., 2009; Zecca and Struhl, 2010), patterning and differentiation of airway epithelial progenitors (Mahoney et al., 2014), and patterning of neural and endodermal progenitor cells into spinal cord neurons and gut (Camargo et al., 2007; Cao et al., 2008). Our findings are also supported by recent reports from mammalian model organisms where the Hippo pathway is proposed to play a role in differentiation (Asaoka et al., 2014; Chen et al., 2014). We 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-faRNAi (Dietz et al., 2007), UAS-wtsRNAi (Fernandez et al., 2011; Rauskolb et al., 2011), UAS-ykiRNAi(N;C) (Zhang et al., 2008), UAS-D (Mao et al., 2006), UAS-hpo (Udan et al., 2003), UAS-wts131 (Kwon et al., 2015), y w hsFLP; UAS-hpoRNAi (mp17) SM6-TM6B, Tb (Pantalacci et al., 2003), dpp-Gal4 (Staehling-Hampton et al., 1994), btl-Gal4 (Calieja et al., 1996), UAS-Dicer (Misquitta et al., 2008), dpp-ia2Z (Blackman et al., 1991), UAS-Sgg (Hazelett et al., 1998), UAS-JTCPE35 (van de Wetering et al., 1991), w P(Act>y>Gal4)25 P(UAS-GFP5657)/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: UAS-yki Gfp (full length) (Oh and Irvine, 2008), UAS-ykiS168A (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-ykiS168A and UAS-hpo were generated using ‘flip-out’ technique, by crossing y w hsFLP122; P(Act>y>Gal4)25 P(UAS-GFP5657)/CyO (Struhl and Basler, 1993) virgins with males from UAS-yki Gfp or UAS-ykiS168A 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; wts+/FRT82B/TM6B, Tb males with cyFLP, 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-Ely (1:100, DSHB, 7E8A10), mouse anti-Wg (1:50, DSHB, 4D4), mouse anti-Ey (1:100, DSHB, E), mouse anti-Eya (1:100, DSHB, eya10H6), mouse anti-Dac (1:100, DSHB, mAbd2a3-2), 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-fibroblast growth factor (1:100, Promega, Z3781) and guinea pig anti-Atonal (1:500, a gift from Daniel Marenda, Drexel University, PA, USA). The secondary antibodies were:
donkey anti-rat IgG conjugated with Cy5 (1:250, Jackson, 712-175-153),
donkey anti-mouse IgG conjugated to Cy3 (1:300, Jackson, 715-165-150),
donkey anti-mouse IgG conjugated to FITC (1:200, Jackson, 715-095-150),
donkey anti-rat IgG conjugated to Cy3 (1:300, Jackson, 715-165-150),
goat anti-rabbit IgG conjugated to Cy5 (1:250, Jackson, 111-175-144),
donkey anti-guinea pig IgG conjugated to Cy3 (1:250, Jackson, 706-165-152),
donkey anti-rabbit IgG conjugated to Cy3 (1:250, Jackson, 711-165-152) and

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

E.W. and A. Sarkar performed experiments and data analysis; K.G. performed
experiments; M.K.-S. carried out data analysis and co-wrote the manuscript;
A. Singh developed the concept, carried out data analysis and co-wrote the
manuscript.

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