

## RESEARCH ARTICLE

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

Erika Wittkorn<sup>1</sup>, Ankita Sarkar<sup>1</sup>, Kristine Garcia<sup>1</sup>, Madhuri Kango-Singh<sup>1,2,3,\*</sup> and Amit Singh<sup>1,2,3,\*</sup>

## 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., 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 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; 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. *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,

<sup>1</sup>Department of Biology, University of Dayton, Dayton, OH 45469, USA. <sup>2</sup>Premedical Program, University of Dayton, Dayton, OH 45469, USA. <sup>3</sup>Center for Tissue Regeneration and Engineering at Dayton (TREND), University of Dayton, Dayton, OH 45469, USA.

\*Authors for correspondence (asingh1@udayton.edu; mkangosingh1@udayton.edu)

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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; 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; Burgy-Roukala et al., 2013). These are *twin of eyeless* (*toy*), *eyeless* (*ey*), *eyegone* (*eyg*), *twin of eyegone* (*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; 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 *atonal* (*ato*)-encoding basic HLH proteins, play important roles in PR differentiation (Jarman et al., 1994; Bertrand et al., 2002; Tanaka-Matakatsu 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  $\beta$ -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 Hth, 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. Hth 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; Napoletano 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 (Yki<sup>S168A</sup> and Yki<sup>S3A</sup>) 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.

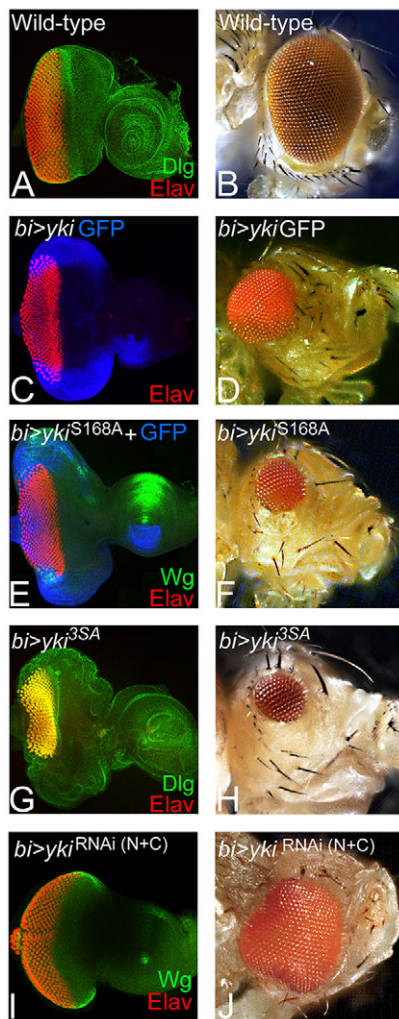
## RESULTS

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-*yki*<sup>S168A</sup> and UAS-*yki*<sup>S3A</sup>) (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

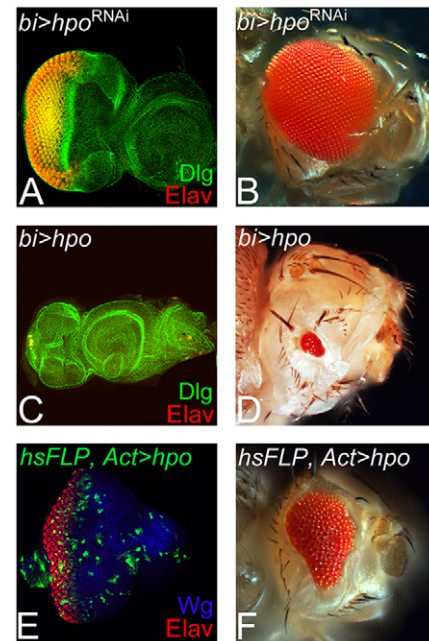




**Fig. 1. Hippo signaling is required for eye development.** (A,B) Wild-type eye imaginal disc and compound eye of adult fly are shown. (A) Eye imaginal disc is stained for the membrane-specific marker Discs large (Dlg, green) and the pan-neural marker Elav (red), which marks the retinal neuron-specific fate. (C,E,G) Eye discs in which *bi*-Gal4 drives misexpression of (C) full-length *yki* (*bi>yki*-GFP), and its hyperactivated forms (E) *yki*<sup>S168A</sup> (*bi>yki*<sup>S168A</sup>) and (G) *yki*<sup>3SA</sup> (*bi>yki*<sup>3SA</sup>). 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 *yki*<sup>RNAi</sup> on DV margins (*bi>dicer+yki*<sup>RNAi(N+C)</sup>) 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 *yki*<sup>S168A</sup> (*bi>yki*<sup>S168A</sup>) or UAS-*yki*<sup>3SA</sup> (*bi>yki*<sup>3SA</sup>) 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-*yki*<sup>RNAi(N+C)</sup> (*bi>yki*<sup>RNAi(N+C)</sup>) resulted in the enlargement of eye field at DV margins (Fig. 1I,J). 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 *hpo*<sup>RNAi</sup> using



**Fig. 2. Modulating Hippo levels affects cell survival in the developing eye.** (A,B) Blocking Hippo (Hpo) signaling by misexpression of *hpo*<sup>RNAi</sup> (*bi>hpo*<sup>RNAi</sup>) using a *bi*-Gal4 driver, resulted in dorsoventral (DV) enlargement of (A) the eye imaginal disc and (B) the adult eye. (C,D) Misexpression of *hpo* at the DV margin (*bi>hpo*) resulted in dramatic reduction of the eye fate, as evidenced from Elav expression in (C) the eye imaginal disc and in (D) the adult eye. (E,F) Gain-of-function ‘flip 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.

*bi*-Gal4 (*bi>hpo*<sup>RNAi</sup>) 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>hpo*<sup>RNAi</sup> 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 ‘flip-out’ clones of *hpo* in the eye that were generated during the late second instar stage. We could not detect *hpo* gain-of-function clones 24 h after clone induction, as these clones were eliminated (supplementary material Table S1). Therefore, we analyzed them 12 h after induction, and found that the GFP-positive *hpo* gain-of-function clones fail to grow (two or three cells in size), did not affect the photoreceptor neurons and resulted in a highly reduced adult eye (Fig. 2F). These data further validate other studies demonstrating that gain of function of *hpo* primarily induces cell death (Verghese et al., 2012). However, our data also show that Yki affects eye differentiation; therefore, we tested the expression of core RD genes when Yki is activated.

#### 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 *yki*<sup>3SA</sup> (*bi>yki*<sup>3SA</sup>) 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*<sup>3SA</sup> using *dpp*-Gal4 (*dpp>yki*<sup>3SA</sup>) and generated ‘flp-out’ clones (*Act>yki*<sup>3SA</sup>+GFP) in the eye imaginal disc. Misexpression of *yki*<sup>3SA</sup> using *dpp*-Gal4 (*dpp>yki*<sup>3SA</sup>), 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*<sup>3SA</sup> eye imaginal disc, Ey (Fig. 3G), Eya (Fig. 3H) and Dac are downregulated (Fig. 3I). Gain-of-function ‘flp-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’,K”,L,L”). Anterior to the MF, Ey was downregulated in the clones (Fig. 3J,J’). The gain-of-function clones of *yki*<sup>3SA</sup> strongly suppressed Eya (Fig. 3K,K”) and Dac (Fig. 3L,L”) expression. We observed that stochastic overexpression of *yki*<sup>3SA</sup> 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*<sup>3SA</sup> later in the early to mid-second instar in ‘flp-out’ clones, resulted in the downregulation of Ey and in

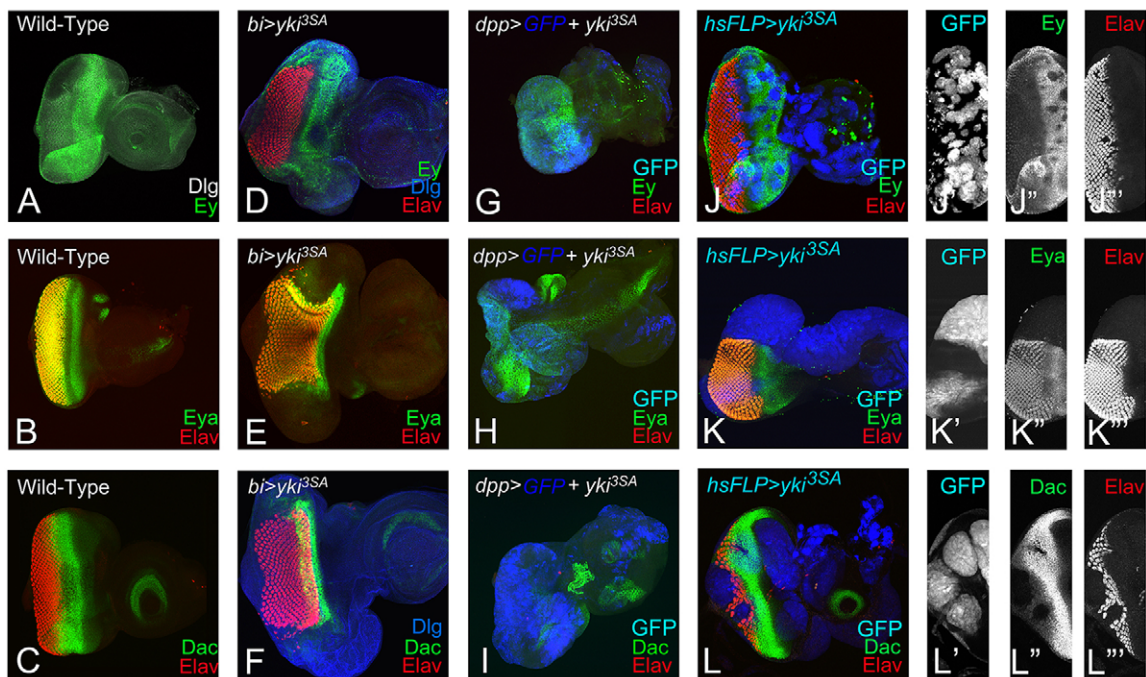
suppression of differentiation. Furthermore, the clone sizes ranged from large to small, reflecting whether the clone was induced during the late first or late second instar of larval development. Thus, suppression of differentiation by downregulation of RD genes (*eya*, *dac*) is a consistent phenotype of *yki*<sup>3SA</sup> 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 *yki*<sup>3SA</sup> using *bi*-Gal4 driver (*bi>yki*<sup>3SA</sup>) 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*<sup>3SA</sup>; Fig. 4E,F) and in random ‘flp-out’ gain-of-function clones of *yki*<sup>3SA</sup> (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*<sup>3SA</sup> in MF progression was tested.

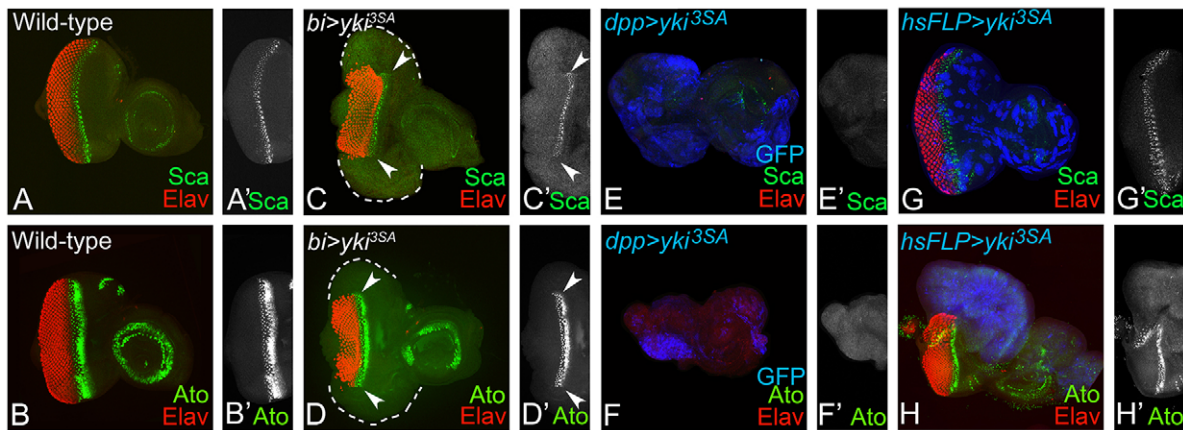
### Yki is required for MF progression

In the developing eye, Dpp and Hh signaling is required for normal development and MF progression (Ready et al., 1976; Wolff and



**Fig. 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*<sup>3SA</sup> (*bi>yki*<sup>3SA</sup>) 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*<sup>3SA</sup> at the posterior margin of the developing eye disc using *dpp*-Gal4 driver (*dpp>yki*<sup>3SA</sup>) resulted in complete loss of eye field, as evidenced by (G–I) loss of Elav (red), (G) mild downregulation of Ey (green) near the posterior margin, (H) loss of Eya (green) and (I) loss of Dac (green). *dpp>yki*<sup>3SA</sup> resulted in a highly reduced eye field. (J–L”) Random gain-of-function clones of *yki*<sup>3SA</sup> (*hsFLP>yki*<sup>3SA</sup>) marked by (J’,K’,L’) GFP (blue) reporter resulted in suppression of (J,J”) Ey (green), (K,K”) Eya (green) and (L,L”) Dac (green). (J”,K”,L”) Elav expression (red) is downregulated in *yki*<sup>3SA</sup> clones. Orientation and magnification of all imaginal discs are identical.





**Fig. 4. Activation of *yki* blocks retinal differentiation in the developing eye disc.** Panels show the expression pattern of (A) Scabrous (Sca, green) and (B) Atonal (Ato, green), the two markers for R8 specification in the wild-type developing eye disc. Misexpression of *yki*<sup>3SA</sup> by (C,D) *bi*-Gal4 (*bi>yki*<sup>3SA</sup>), (E,F) *dpp*-Gal4 and (G,H) 'flip out' clone of *yki*<sup>3SA</sup> (*hsFLP>yki*<sup>3SA</sup>) 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.

Ready, 1993; Schlichting and Dahmann, 2008; Kumar, 2013). To test 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 *hpo*<sup>RNAi</sup> (*bi>hpo*<sup>RNAi</sup>) 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 *yki*<sup>3SA</sup> led to the suppression of eye fate and MF progression causing the furrow to bend posteriorly (Fig. 5C,C', arrowheads). Large overgrowths are observed in the *bi*-Gal4 domains in *bi>yki*<sup>3SA</sup> 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 *hpo*<sup>RNAi</sup> and *yki*<sup>3SA</sup> revealed that unlike *Yki*, loss of *hpo* does not affect MF progression, suggesting that the Hippo pathway regulates growth and differentiation via separable mechanisms.

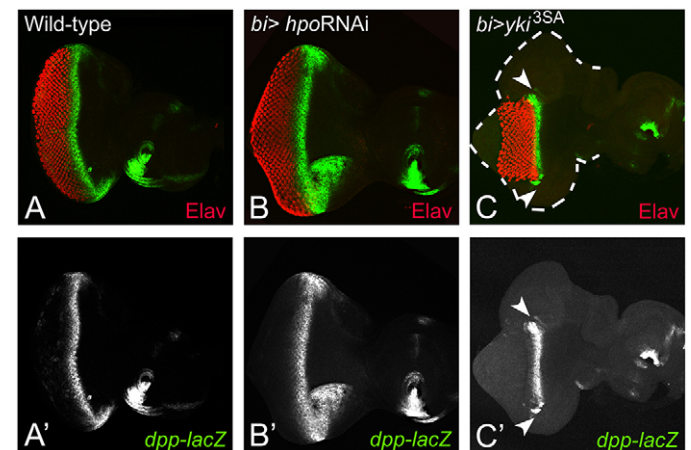
#### ***Yki* activation induces Wntless signaling to regulate MF progression**

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 *yki*<sup>3SA</sup> using *bi*-Gal4 (*bi>yki*<sup>3SA</sup>) (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 *yki*<sup>3SA</sup> suppresses MF progression. We therefore tested effects of modulating levels of *Wg* signaling in the cells misexpressing *yki*<sup>3SA</sup>. 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-*dTCF*<sup>DN5</sup>) (van de Wetering et al., 1997) or the activated form of *shaggy* (UAS-*sgg*<sup>S9A</sup>), the negative regulator of the pathway (Hazelett

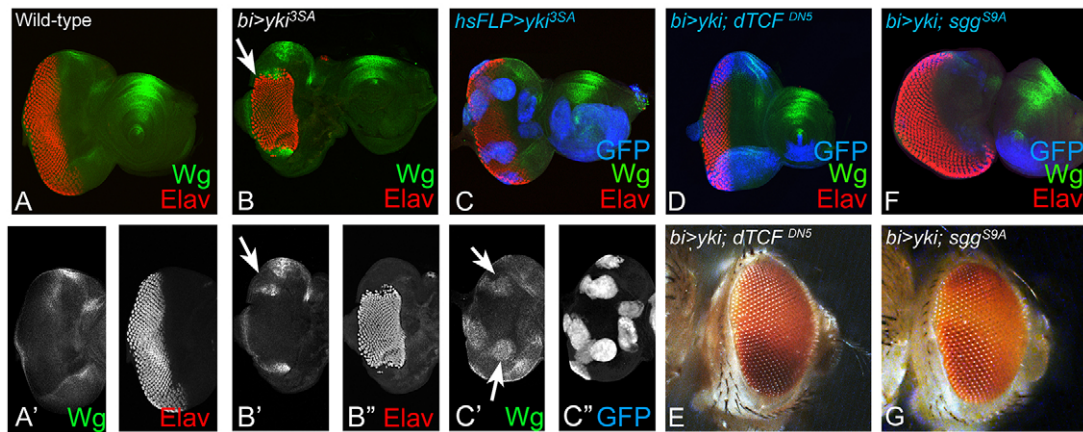
et al., 1998). Co-expression of *yki* with dTCF<sup>DN5</sup> (*bi>yki+dTCF*<sup>DN5</sup>) (Fig. 6D,E) or with *Sgg* (*bi>yki+sgg*<sup>S9A</sup>) (Fig. 6F,G) resulted in a significant rescue of retinal differentiation (marked by Elav) in the *bi* domain of the eye disc (supplementary material Table 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+sgg*<sup>S9A</sup> 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



**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>hpo*<sup>RNAi</sup> and (C) *bi>yki*<sup>3SA</sup> eye imaginal discs. (A-C) Differentiated photoreceptor cells are marked by Elav (red). (C,C') Misexpression of *yki*<sup>3SA</sup> 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.



**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, B') *bi*-Gal4 (*bi>yki<sup>3SA</sup>*) at DV margins or in 'FLP-out' random clones (marked by GFP, blue) under (C-C') *hsFLP>yki<sup>3SA</sup>* 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 dTCF<sup>DN5</sup> (*bi>yki+dTCF<sup>DN5</sup>*) and (F, G) Shaggy (*bi>yki+Sgg<sup>S9A</sup>*), 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.

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 *bi>yki<sup>3SA</sup>* 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>yki<sup>3SA</sup>*) (Fig. 7C, D) or *dpp*-Gal4 (*dpp>yki<sup>3SA</sup>*) (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-*yki<sup>3SA</sup>*, 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

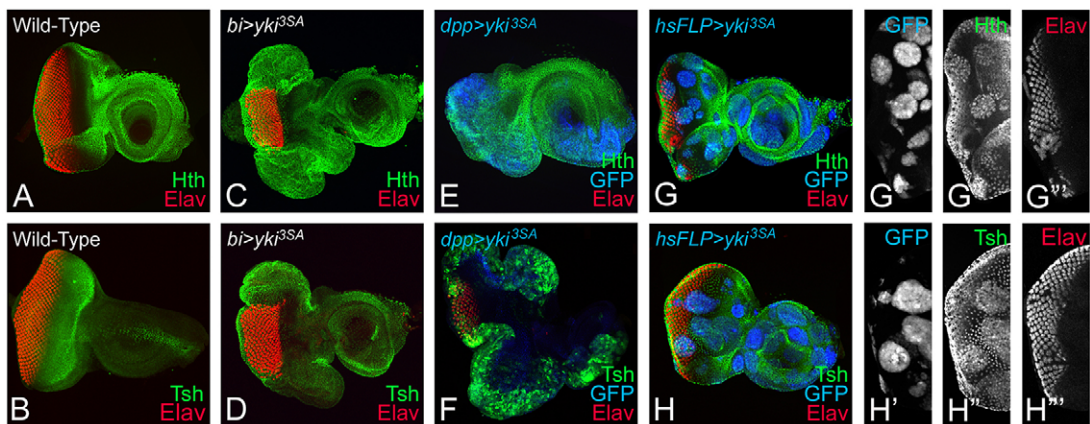
showed that activated Yki induced ectopic expression of Hth and Tsh to suppress retinal differentiation in the eye.

#### 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.

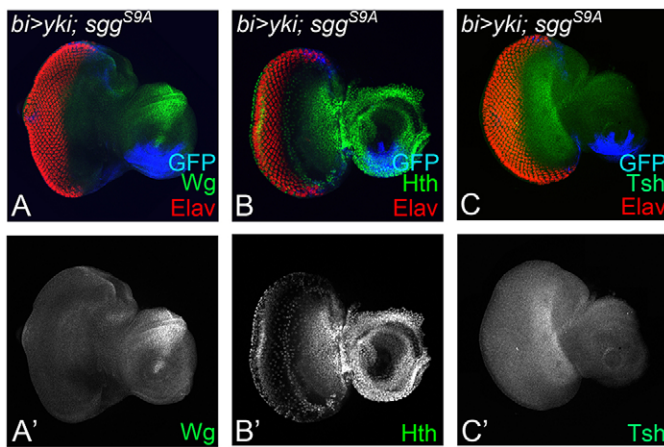
#### Yki activation does not change eye fate to antenna

The ectopic expression of Hth or Tsh in *yki<sup>3SA</sup>*-expressing cells presented the alternate possibility that the cells overexpressing



**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>yki<sup>3SA</sup>*, (E, F) *dpp>yki<sup>3SA</sup>* and (G-H'') *hsFLP>yki<sup>3SA</sup>* (*hsFLP>yki<sup>3SA</sup>*) larvae. (A) Normally, Hth and (B) Tsh expression is restricted anterior to the MF in the eye. Both (C, E, G, G') Hth and (D, F, H, H') Tsh are upregulated, and (C-H, G'', H'') eye differentiation (Elav, red) is suppressed when *yki<sup>3SA</sup>* is overexpressed under (C, D) *bi*-Gal4, (E, F) *dpp*-Gal4 or in 'FLP-out' clones using (G, H) *hsFLP>yki<sup>3SA</sup>*. (G-H') 'Flp-out' clones of *yki<sup>3SA</sup>* are marked by GFP (blue).





**Fig. 8. Activation of Yki suppresses eye fate through Wg signaling-mediated induction of Hth.** Panels show effects of co-expression of activated *yki* and *sgg* with *bi*-Gal4 (*bi*>*yki*-GFP, *sgg*<sup>S9A</sup>) on expression of (A,A') Wg, (B,B') Hth and (C,C') Tsh in the DV margin of eye imaginal disc. The orientation and magnification of imaginal discs are identical in all panels.

*yki*<sup>3SA</sup> 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 *yki*<sup>3SA</sup> (*bi*>*yki*<sup>3SA</sup>), 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*<sup>3SA</sup> and in 'flp-out' *yki*<sup>3SA</sup> 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*<sup>3SA</sup> misexpression does not cause a change in cell fate. Overall, these experiments show that the primary influence of *yki*<sup>3SA</sup> 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

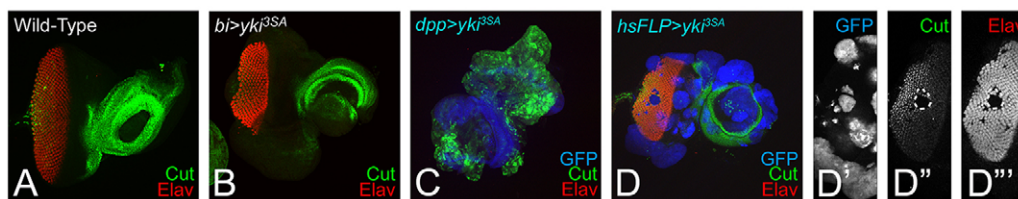
differentiation and growth. One of the upstream regulators is the Fat branch of the pathway that regulates Wts stability via the atypical myosin Dachs (D) (Cho et al., 2006). In addition, loss of function of the Fat branch components also shows broad DV and PD defects, and defects in MF progression (Cho and Irvine, 2004; Mao et al., 2006; Matakatsu and Blair, 2012). Misexpression of UAS-*ft*<sup>RNAi</sup> (*bi*>*ft*<sup>RNAi</sup>) 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*<sup>3SA</sup> (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

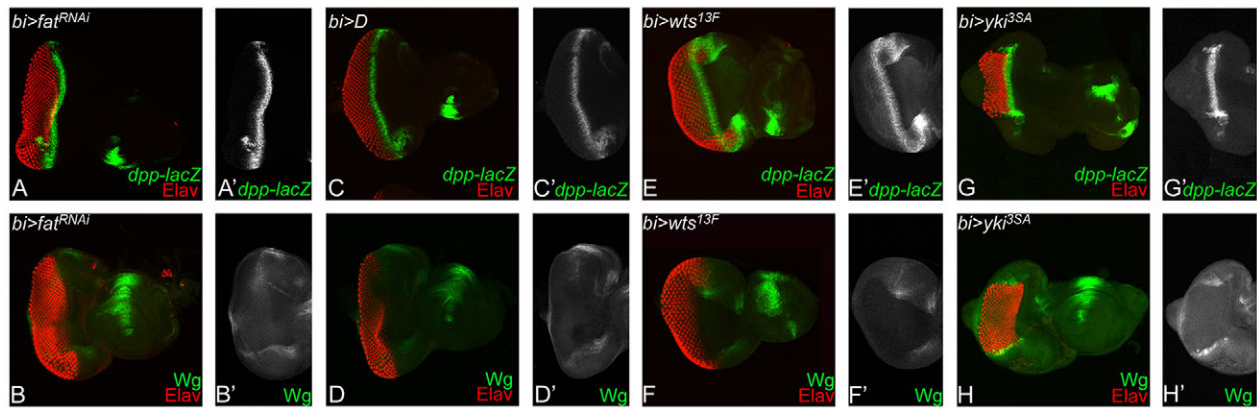
During development, patterning and growth are tightly controlled by highly conserved signaling pathways to determine the size and shape of an animal. In *Drosophila* eye, an initial proliferative phase (growth spurt) occurs during early larval development (under the control of several growth regulatory 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; Verghese et al., 2013; Yu and Guan, 2013). The Yki homolog YAP/TAZ function as



**Fig. 9. Activation of Yki does not change the cell fate from eye to antenna.** (A) The wild-type expression of Cut (Ct, green), a marker for antennal fate, is restricted to a circular domain in the antennal region, as well as in the cone cells of the eye field. (B) Misexpression of *yki*<sup>3SA</sup> using a *bi*-Gal4 driver (*bi*>*yki*<sup>3SA</sup>) at the DV margin suppresses the retinal differentiation, as evident from Elav (red) expression, but does not induce ectopic Cut expression. (C) Misexpression of *yki*<sup>3SA</sup> on the posterior margin of the eye disc using a *dpp*-Gal4 driver (*dpp*>*yki*<sup>3SA</sup>) results in suppression of Elav (red) as well as Ct (green) expression. (D-D'') Random gain-of-function clones of *yki*<sup>3SA</sup> (*hsFLP*>*yki*<sup>3SA</sup>) marked by (D,D') GFP (blue) reporter results in suppression (D,D'') Ct (green). (D,D'') Elav expression (red) is downregulated in *yki*<sup>3SA</sup> clones. The orientation and magnification of imaginal discs and adult heads are identical in all panels.



**Fig. 10. Modulating the Fat branch of the Hippo signaling pathway affects retinal differentiation in the developing eye.** Panels show comparison of *dpp-lacZ* (green) and *Elav* (red) expression in the eye imaginal discs from larvae of the following genotypes: (A-B') *bi-Gal4* driver *UAS-ft<sup>RNAI</sup>* (*bi>fat<sup>RNAI</sup>*), (C-D') *bi-Gal4* driver *UAS-D* (*bi>D*), (E-F') *bi-Gal4* driver *UAS-wts<sup>13F</sup>* (*bi>wts<sup>13F</sup>*) and (G-H') *bi-Gal4* driver *UAS-yki<sup>3SA</sup>* (*bi>yki<sup>3SA</sup>*). The magnification of all images is identical.

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 proapoptotic 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*, *eya* and *dac*) in cells where *yki* was hyperactivated. Misexpression of *yki* (*bi>yki<sup>3SA</sup>*) 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>yki<sup>3SA</sup>*) 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>yki<sup>3SA</sup>* 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 Hafén, 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>yki<sup>3SA</sup>*). 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 *Sgg<sup>S9A</sup>* or *dTCF<sup>DN5</sup>*, 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.

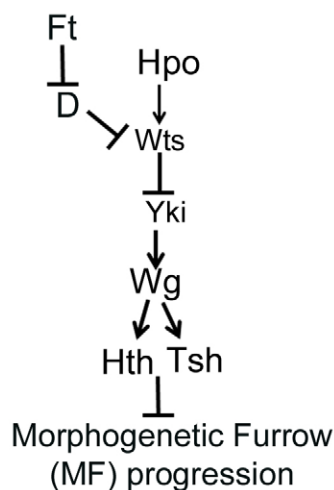
In addition to its role in MF progression, Wg is also essential for delimiting the border between the compound eye and the adult head cuticle (Royet and Finkelstein, 1996, 1997). Similarly, expression of the head-specific fate markers such as *Hth* and *Tsh* also becomes



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



**Fig. 11. Model to demonstrate the role of Hippo signaling in regulating retinal differentiation and MF progression.** The Ft-Wts branch of Hippo signaling downregulates Wts-mediated phosphorylation of Yki, which triggers Wg, a downstream target of Yki, to induce Hth- and Tsh-mediated block of retinal differentiation.

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-*fat*<sup>RNAi</sup> (Dietzl et al., 2007), UAS-*wts*<sup>RNAi</sup> (Fernandez et al., 2011; Rauskolb et al., 2011), UAS-*yki*<sup>RNAi(N+C)</sup> (Zhang et al., 2008), UAS-*D* (Mao et al., 2006), UAS-*hpo* (Udan et al., 2003), UAS-*wts*<sup>13F</sup> (Kwon et al., 2015), *y w; hsFLP*; UAS-*hpo*<sup>RNAi;imp19</sup>/SM6-TM6B, Tb (Pantalacci et al., 2003), *dpp-Gal4* (Staebling-Hampton et al., 1994), *bi-Gal4* (Calleja et al., 1996), UAS-*Dicer* (Misquitta et al., 2008), *dpp-lacZ* (Blackman et al., 1991), UAS-*Sgg* (Hazelett et al., 1998), UAS-*dTCF<sup>DN5</sup>* (van de Wetering et al., 1991), *w; P(Act>y<sup>+</sup>>Gal4)25 P(UAS-GFP<sup>S65T</sup>)/CyO* (Ito et al., 1997), *y w hsFLP<sup>122</sup>* (Struhl and Basler, 1993), *w; wts<sup>x1</sup>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-*yki*<sup>S168A</sup> (Oh and Irvine, 2008) and UAS-*yki*<sup>3SA</sup> (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-*yki*<sup>3SA</sup> and UAS-*hpo* were generated using ‘flp-out’ technique, by crossing *y w hsFLP<sup>122</sup>; P(Act>y<sup>+</sup>>Gal4)25 P(UAS-GFP<sup>S65T</sup>)/CyO* (Struhl and Basler, 1993) virgins with males from UAS-*yki GFP* or UAS-*yki*<sup>3SA</sup> 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<sup>x1</sup>FRT82B/TM6B, Tb* males with *eyFLP*; 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, 7E8A10), mouse anti-Wg (1:50, DSHB, 4D4), mouse anti-Ey (1:100, DSHB, Ey), mouse anti-Eya (1:100, DSHB, eya10H6), mouse anti-Dac (1:100, DSHB, mABdac2-3), mouse anti-Cut (1:20, DSHB, 2B10), mouse anti-Scd (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 Marendia, 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 goat anti-rabbit IgG conjugated to FITC (1:200, Jackson, 711-165-152). Images were captured using the Olympus Fluoview 1000 Laser Scanning Confocal Microscope. The adult eye imaging was carried out using Zeiss Apotome Imager.Z1 microscope. The images were processed and analyzed using the Adobe Photoshop CS4 software.

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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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#### Supplementary material

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