Transcription factor choice in the Hippo signaling pathway: *homothorax* and *yorkie* regulation of the microRNA *bantam* in the progenitor domain of the *Drosophila* eye imaginal disc

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The accurate control of cell proliferation and survival is critical for animal development. The Hippo tumor suppressor pathway regulates both of these parameters by controlling the nuclear availability of the transcriptional coactivator Yorkie (Yki), which regulates downstream target genes together with Scalloped (Sd), a DNA-binding protein. Here we provide evidence that Yki can also regulate target genes in conjunction with Homothorax (Hth) and Teashirt (Tsh), two DNA-binding transcription factors expressed in the uncommitted progenitor cells of the *Drosophila* eye imaginal disc. Clonal analyses demonstrate that Hth and Tsh promote cell proliferation and protect eye progenitor cells from apoptosis. Genetic epistasis experiments suggest that Hth and Tsh execute these functions with Yki, in part by up-regulating the microRNA *bantam*. A physical interaction between Hth and Yki can be detected in cell culture, and we show that Hth and Yki are bound to a DNA sequence ~14 kb upstream of the *bantam* hairpin in eye imaginal disc cells, arguing that this regulation is direct. These data suggest that the Hippo pathway uses different DNA-binding transcription factors depending on the cellular context. In the eye disc, Hth and Tsh provide spatial information to this pathway, promoting cell proliferation and survival in the progenitor domain.

[*Keywords*: *Drosophila* eye development; Hippo signaling pathway; Homothorax; Yorkie; apoptosis; cell proliferation]

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The coordination between cell proliferation and differentiation is critical for animal development and organogenesis. The development of the *Drosophila* compound eye is an excellent model system to address how these two processes are coordinated, in part because the transition from proliferation to differentiation can be visualized in a single eye imaginal disc as the morphogenetic furrow (MF) sweeps across the eye disc epithelium (Ready et al. 1976). Consequently, individual eye discs isolated from the third instar larval stage display the entire range of differentiation states, from uncommitted naïve progenitor cells in the anterior of the disc to fully committed photoreceptors in the posterior of the disc.

While much is known about how the network of retinal differentiation genes, together with secreted signals derived from the MF, choreograph the formation of the many cell types present in the differentiated eye (Desplan 1997; Treisman and Heberlein 1998; Silver and Rebay 2005; Morante et al. 2007), much less is known about the uncommitted progenitor cells prior to differentiation. These cells must accomplish at least two tasks. First, they must proliferate and survive in order to generate sufficient cells before the differentiation program begins. Second, they have to keep the differentiation program in check prior to the arrival of the MF. In the developing eye disc, retinal progenitor cells reside anterior to the MF, where the process of retinal differentiation begins. These naïve progenitor cells express two transcription factors—Homothorax (Hth), a TALE-homeodomain protein (Rieckhof et al. 1997), and Teashirt (Tsh), a zinc (Zn) finger transcription factor (Fasano et al. 1991)—both of which have important functions in other contexts during development. In the eye, *hth* is activated by Wingless (Wg), expressed from the periphery of eye field (Pichaud

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and Casares 2000), and is repressed by Decapentaplegic (Dpp), expressed from the MF (Bessa et al. 2002). In vivo, Hth and Tsh interact physically with each other and function together to repress the later-acting retinal differentiation genes, such as *eyes absent* (*eya*) and *sine oculus* (*so*), thus maintaining anterior eye disc cells in an undifferentiated state (Bessa et al. 2002). However, it has remained an open question whether Hth and Tsh also promote cell proliferation and survival of these progenitor cells and, if they do, what pathways and target genes these two transcription factors regulate to mediate these functions.

Recently, the Hippo tumor suppressor pathway has been shown to be an important cell proliferation control pathway in *Drosophila* and mammals (Dong et al. 2007; Pan 2007; Zhang et al. 2009). Activation of the Hippo kinase leads to the phosphorylation of a second kinase, Warts, which in turn phosphorylates and induces the cytoplasmic retention of Yorkie (Yki), a transcriptional coactivator that does not have its own DNA-binding domain (Huang et al. 2005; Dong et al. 2007; Oh and Irvine 2008; J Zhang et al. 2008). In its unphosphorylated state, Yki is nuclear and participates in the activation of growth- and survival-promoting target genes. One such Yki target gene is the microRNA (miRNA) *bantam*, which in turn represses the translation of the proapoptotic gene *hid* (Hipfner et al. 2002; Brennecke et al. 2003; Nolo et al. 2006; Thompson and Cohen 2006). In the wing disc, where it has been best studied, Yki regulates its target genes by binding to Scalloped (Sd), a TEAD/TEF family transcription factor (Goulev et al. 2008; Wu et al. 2008; L Zhang et al. 2008; Zhao et al. 2008). Although the Hippo signaling pathway controls growth in all known tissues, including the eye, Sd's expression pattern and requirement during development may be more limited (Campbell et al. 1992; Srivastava et al. 2004; L Zhang et al. 2008). For example, an enhancer trap into the *sd* locus, which reports *sd*'s expression pattern, is not active in anterior eye disc cells (Campbell et al. 1992), and *sd*-null clones survive well in the eye imaginal disc but not in the wing pouch (L Zhang et al. 2008). These and other observations suggest that nuclear Yki may promote cell survival and proliferation in other tissues by interacting with transcription factors in addition to Sd.

Here we show that Hth and Tsh work together to promote cell proliferation and survival in the anterior eye disc. Genetic epistasis experiments suggest that Tsh and Hth work via the Hippo signaling pathway to execute these functions. Specifically, we provide evidence that *bantam* expression is up-regulated in anterior eye disc cells, and that this up-regulation is *hth*-dependent. Further, *bantam* and *yki* are both necessary for the proliferation-promoting functions of Hth and Tsh. Finally, we show that Hth and Yki are bound at the *bantam* locus in eye disc cells and that Yki and Hth can be coimmunoprecipitated when coexpressed. Together, these results provide strong evidence that Hth and Tsh, together with Yki, promote cell proliferation and survival of eye progenitor cells by directly up-regulating the *bantam* miRNA. Thus, the transcriptional regulation of *hth* provides spatial specificity to the Hippo pathway, ensuring that anterior eye disc cells, but not cells posterior to the MF, remain in a state of active proliferation.

Results

Hth and Tsh are required for cell survival and wild-type proliferation in the eye progenitor domain

The anterior progenitor domain of the eye imaginal disc expresses Hth and Tsh, with Tsh expression extending closer to the MF than Hth (Fig. 1A–E). As noted previously (Bessa et al. 2002), hth^{P2} mutant clones are rarely recovered anterior to the MF, but can be recovered posterior to the MF (Fig. 1F). In contrast, neutral control clones made in parallel are recovered throughout the eye disc (Fig. 1G). This indicates that the absence of *hth* results in poor survival of progenitor cells. The existence of hth^{P2} mutant clones posterior to the MF suggests that hth^{P2} mutant cells can divide and survive long enough to be "fixed" by the passage of the MF, after which *hth* is no longer required for survival. Loss-of-function *tsh* clones are also at a growth disadvantage in the progenitor domain, although in this case we had to use RNAi knockdown of *tsh* in a genetic background that was null for the highly related and functionally redundant gene *tiptop* (*tio*) to see an effect (Supplemental Fig. 1; Fasano et al. 1991; Laugier et al. 2005).

The absence of hth^{P2} clones anterior to the MF is reminiscent of cell competition, where cells that have a growth disadvantage relative to their neighbors are eliminated (Simpson and Morata 1981; Morata and Martin 2007). At least one mechanism leading to the elimination of cells is apoptosis (de la Cova et al. 2004; Moreno and Basler 2004; Morata and Martin 2007). We carried out two experiments to test if hth^{P2} clones were eliminated by apoptosis in the anterior eye disc. When hth^{P2} clones were generated in a heterozygous *Df(3L)H99*/+ background, which removes one copy of the three proapoptotic genes *hid*, *reaper*, and *grim*, small hth^{P2} mutant clones were recovered anterior to the MF (Fig. 1H), although this rescue is not fully penetrant compared with neutral clones made side-by-side (Fig. 1I). Similar partial rescue was observed when hth^{P2} clones were generated in eye discs that express the baculovirus anti-apoptotic protein p35 (Fig. 1J). These results indicate that the poor survival of hth^{P2} clones in the anterior eye disc is, at least in part, because they are eliminated by apoptosis.

Another way to counteract the elimination of cells as a result of cell competition is to give them a growth advantage relative to their neighbors. In *Drosophila*, this can be accomplished by generating wild-type clones in a *Minute* (*M*)/+ background (Simpson and Morata 1981; Morata and Martin 2007). In the *M(3)95A*/+ background, hth^{P2} $Minute^{+}$ clones were recovered anterior to the MF (Fig. 1K), demonstrating that *hth* is not essential for cells to survive and proliferate in the anterior eye disc. However, the size of these hth^{P2} $Minute^{+}$ clones was dramatically smaller than the size of neutral (hth^{+} $Minute^{+}$) clones generated in parallel in the same *M*/+ background

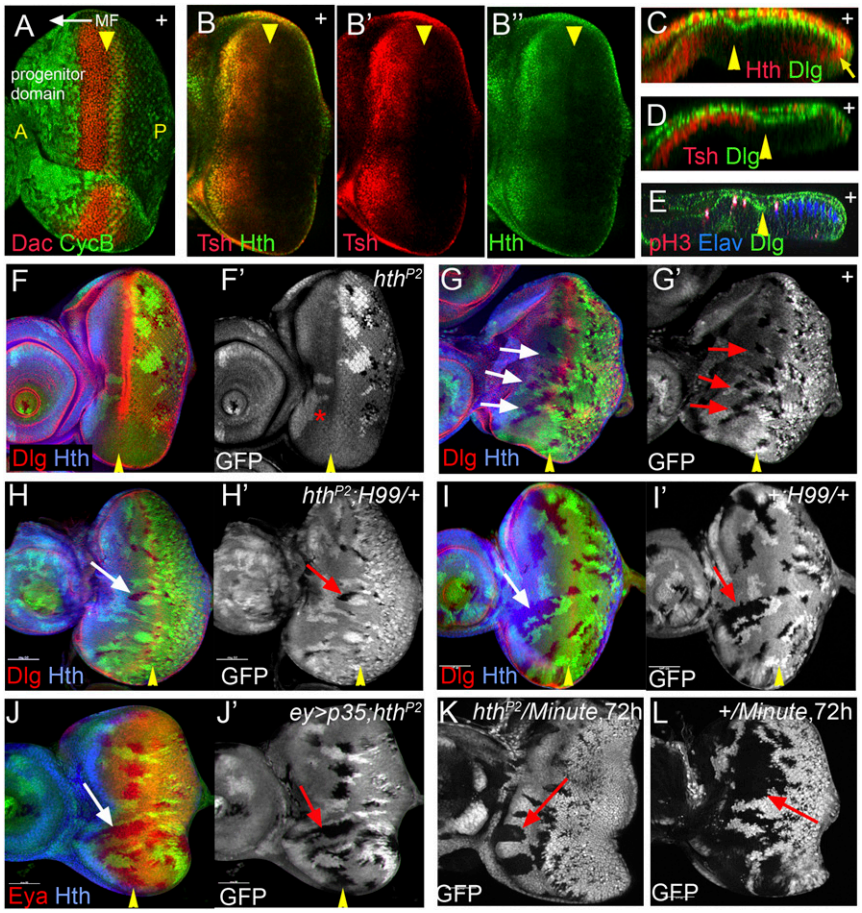

Figure 1. *hth* is required for cell survival in the eye progenitor domain. (*A*) A third instar eye disc stained for CycB (green) and Dac (red, which is expressed anterior to the MF) to highlight the progenitor domain and the initiation of differentiation, respectively. The position and direction of the MF is indicated. (*B*) A third instar eye disc stained for Hth (green) and Tsh (red). (*C–E*) Confocal cross-section of third instar eye discs stained for Dlg (green, all panels), Hth (red, *C*), Tsh (red, *D*), pH3 (red, *E*), and Elav (blue, *E*). The position of MF is indicated (yellow arrowheads). Hth is present in the anterior of the main epithelium, throughout the peripodial epithelium, and in posterior margin cells (arrow). Tsh is expressed only in the main epithelium, and extends closer to the MF compared with Hth. Dlg is used here as a cell membrane marker. (*F,G*) Survival of hth^{P2} (*F*) or neutral clones (*G*) (marked by the absence of GFP) in eye discs stained for Hth (blue) and Dlg (red, which marks the MF, arrowheads). hth^{P2} clones are not recovered anterior to the MF, but twin spots (bright patches in *F'*,*G'*) are recovered. (*H–J*) A few hth^{P2} clones (marked by the absence of GFP, green/white) are recovered anterior to the MF in a *H99*/+ background (*H*) or when the apoptosis inhibitor p35 is expressed (*J*), but not as readily as neutral control clones (*I*). (*K,L*) Comparison of the growth of hth^{P2} $Minute^{+}$ (*K*) and hth^{+} $Minute^{+}$ (*L*) clones made in a $Minute^{+}/Minute^{-}$ background.

(Fig. 1L). Thus, although *hth* is not absolutely required for progenitor eye disc cells to divide, their ability to proliferate is compromised in the absence of *hth*. Together with the partial rescue of hth^{P2} clones observed when apoptosis was blocked, these results suggest that *hth* carries out at least two functions in the eye progenitor domain: It increases cell survival by blocking apoptosis, and it promotes cell proliferation.

Coexpression of Hth and Tsh results in overgrowths

Previous work established that Hth works together with the Zn finger transcription factor Tsh to repress retinal determination genes in *Drosophila* (Bessa et al. 2002). Normally, *hth* is expressed in the anterior progenitor cells of the eye disc as well as in the peripodial cell layer (Fig. 1C,D). Although *hth* is repressed by Dpp derived from the MF, it is also expressed at the very posterior margin of the eye disc (Fig. 1B,C). Unlike *hth*, *tsh* is not expressed in the peripodial cell layer, nor is it expressed in posterior margin cells (Fig. 1B–D). In fact, *tsh*'s restriction to the main epithelium of the eye disc helps to distinguish between these two tissue layers (Bessa and Casares 2005).

If both Tsh and Hth were required to promote proliferation in eye progenitor cells, we would expect that ectopic expression of Tsh would only be able to induce overgrowths in cells that already express *hth*. Consistent with this prediction, when Tsh was expressed ectopically in clones, posterior margin cells and peripodial cells could be induced to overgrow (Fig. 2A). In contrast, Tsh^{+} clones posterior to the MF in the main epithelium did not overgrow and instead differentiated into photoreceptor clusters with apparently normal morphology (Fig. 2A). Thus, there is a strong correlation between Tsh and Hth coexpression and their ability to induce overgrowths. Consistently, when both Hth and Tsh are coexpressed in clones (hereafter referred to as Hth + Tsh clones), they overgrow regardless of where they occur in the eye disc (Fig. 2F).

As an additional experiment to test whether Hth and Tsh are both required to induce overgrowths, we used mosaic analysis with a repressible cell marker (MARCM) to generate hth^{P2} clones that simultaneously express Tsh. These Tsh^{+}; hth^{P2} clones never overgrow, regardless of where they are located in the disc (Fig. 2E [cf. Tsh^{+}; hth^{+} MARCM clones generated in parallel], D). These data strongly support the idea that Hth and Tsh must be coexpressed to induce proliferation.

We next examined the effect of Hth + Tsh expression on cell cycle and differentiation markers. The G2 cyclin Cyclin B (CycB) is normally expressed in proliferating anterior progenitor cells and in a row of cells posterior to the MF that corresponds to the second mitotic wave. In

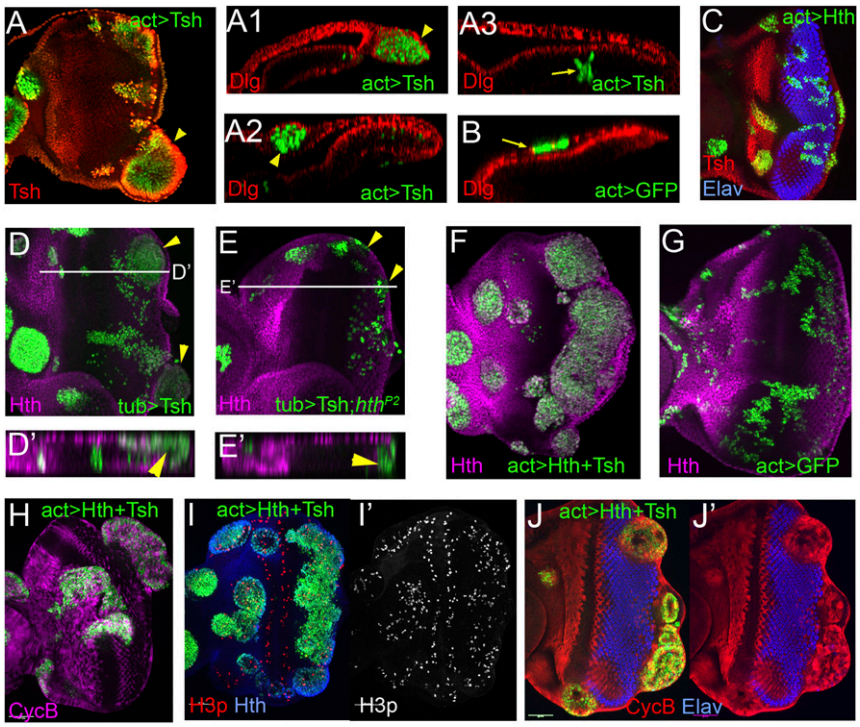

Figure 2. Hth and Tsh function together to induce proliferation. All panels show eye discs with positively marked (GFP$^+$) clones. (*A*,*B*) Eye discs containing clones that ectopically express Tsh (*A*) or GFP (*B*), stained for GFP (green, to mark the clones) and Dlg (red). Compared with neutral clones (*B*), Tsh$^+$ clones result in overgrowths (arrowheads) when they arise in the posterior margin (*A1*) or in the peripodial epithelium (*A2*), but not when they arise in the main epithelium (*A3*). (*C*) Eye disc containing clones that ectopically express Hth (marked with GFP, green), stained for Tsh (red) and Elav (blue). These clones do not result in overgrowths. (*D*,*E*) MARCM clones that ectopically express Tsh (*D*) or that ectopically express Tsh and are also mutant for *hth* (*E*), stained for Hth (purple) and GFP (to mark the clones). Tsh$^+$ clones result in overgrowths (arrowheads in *D*), but Tsh$^+$; *hth*^{P2} clones do not (arrowheads in *E*). *D*′ and *E*′ show confocal cross-sections indicated by the lines in *D* and *E*. (*F*,*G*) Eye discs containing Hth + Tsh clones (*F*) or neutral clones (*G*) stained for Hth (purple) and GFP (to mark the clones). Hth + Tsh clones always overgrow compared with control clones made in parallel. (*H–J*) Eye discs containing Hth + Tsh clones up-regulate CycB (purple, *H*) and pH3 (red/ white in *I*,*I*′), and repress Elav (blue, *J*,*J*′).

Hth + Tsh clones posterior to the MF, CycB expression is up-regulated (Fig. 2H). Similarly, staining for phosphorylated histone 3 (pH3), a marker for cells in mitosis, indicates that the cells in Hth + Tsh clones are actively dividing (Fig. 2I). Finally, we examined Elav, a marker for neural differentiation. In agreement with previous results showing that the retinal determination genes *eya* and *so* are repressed by Hth + Tsh (Bessa et al. 2002), Elav is repressed in Hth + Tsh-expressing clones (Fig. 2J). Together, these results indicate that when Hth and Tsh are coexpressed in the eye disc, they promote proliferation and block differentiation, mimicking the two main properties of anterior progenitor cells, which normally express these transcription factors.

Hth + Tsh function with the Hippo pathway

In order to identify which pathways Hth and Tsh function with to promote proliferation, we carried out several genetic tests using mutations that either activate or inactivate pathways previously implicated in growth control in the eye. We tested the Wg, Notch, and Jak–Stat signaling pathways, all implicated in tissue growth regulation in *Drosophila* (Lee and Treisman 2001; Kenyon et al. 2003; Dominguez et al. 2004; Firth and Baker 2005; Ekas et al. 2006; Tsai et al. 2007). With the exception of Wg, which is required for *hth* expression in the progenitor domain (Pichaud and Casares 2000), manipulation of these pathways had no effect on *hth* or *tsh* expression (data not shown). Moreover, none of these pathways were required for ectopic Hth + Tsh-induced overgrowths (Supplemental Fig. 2). Based on these data, these three pathways are unlikely to mediate the proliferation and survival functions executed by Hth and Tsh in the anterior eye disc.

In contrast to these results, we found that Hth and Tsh require components of the Hippo pathway to carry out their proliferation-inducing functions. First, although *wts*^{P2} clones proliferate well throughout the eye disc and lead to modest overgrowths, *wts*^{P2} *hth*^{P2} double-mutant clones behave like *hth*^{P2} clones: They fail to survive in the anterior of the eye disc (Fig. 3A,B). Similarly, although ectopic expression of Yki results in overgrowths throughout the eye disc, Yki$^+$, *hth*^{P2} clones do not survive anterior to the MF (Fig. 3C,D). These results argue that the inability of *hth* mutant clones to survive anterior to the MF cannot be rescued by activating the Hippo pathway. Conversely, they show that even when the Hippo pathway is in its growth-promoting state (Yki$^+$ or *wts*$^-$), it cannot induce proliferation in the eye progenitor domain in the absence of *hth*.

To provide further genetic support for these conclusions, we tested if the overgrowths produced by Hth + Tsh require *yki*. As described above, Hth + Tsh clones overgrow no matter where they are produced in the eye disc (Fig. 3E,H). In contrast, Hth + Tsh; *yki*^{B5} clones generated in parallel grow much smaller and are rarely recovered anterior to the MF (Fig. 3F,H). Unlike Hth + Tsh clones, Hth + Tsh; *yki*^{B5} clones do not repress Elav, suggesting

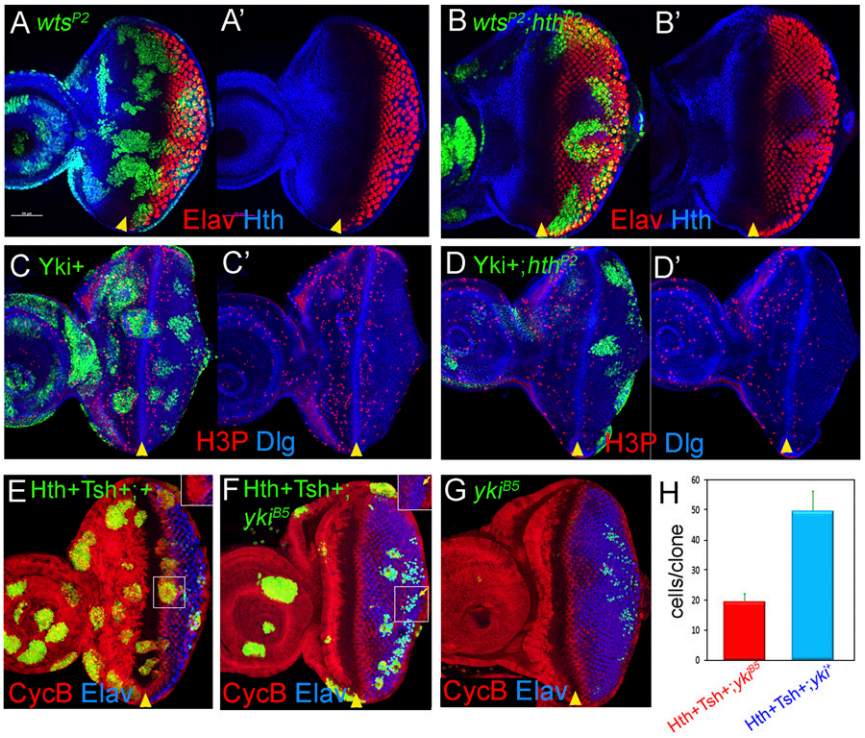

Figure 3. Codependency of the Hippo pathway and Hth + Tsh to induce proliferation in the eye progenitor domain. *A–G* all show eye discs with positively marked (GFP$^+$) MARCM clones. (*A*,*B*) Eye discs with *wts*^{P2} (*A*) and *wts*^{P2} *hth*^{P2} (*B*) clones, stained for GFP (green, to mark the clones), Hth (blue), and Elav (red). *wts*^{P2} clones grow well throughout the eye disc, whereas *wts*^{P2} *hth*^{P2} clones are not recovered in the progenitor domain. (*C*,*D*) Eye discs with Yki$^+$ (*C*) or Yki$^+$; *hth*^{P2} (*D*) clones stained for GFP (green, to mark the clones), pH3 (red), or Dlg (blue). Yki$^+$ clones grow well throughout the eye disc, whereas Yki$^+$; *hth*^{P2} clones are not recovered in the progenitor domain. (*E–G*) Eye discs with Hth + Tsh (*E*), Hth + Tsh; *yki*^{B5} (*F*), or *yki*^{B5} (*G*) clones stained for GFP (green, to mark the clones), CycB (red), and Elav (blue). In *E* and *F*, the *insets* show only the Elav and CycB stains of the boxed regions. Note that Hth + Tsh clones, but not Hth + Tsh; *yki*^{B5} clones, repress the differentiation marker Elav and up-regulate CycB. (*H*) The number of cells present in Hth + Tsh; *yki*^{B5} clones (red bar; $n = 55$) and Hth + Tsh clones (blue bar; $n = 58$).

that they are unable to block differentiation (Fig. 3F). Hth + Tsh; *yki*^{B5} clones do, however, grow better than *yki*^{B5} clones (Fig. 3G), suggesting that not all of the growth-promoting functions of Hth + Tsh may require *yki*. These conclusions are consistent with another manipulation of the Hippo pathway that, like removing *yki*, causes cells to proliferate poorly. Clones that overexpress the Hpo kinase grow poorly, especially in the anterior of the eye disc (Supplemental Fig. 3A). Overexpressing Hpo$^+$ can suppress most, but not all, of the growth-promoting effects of ectopic Hth + Tsh expression (Supplemental Fig. 3B,C).

Thus, in the eye progenitor domain, the growth-promoting effects observed when the Hippo pathway is compromised require *hth*. One scenario that could explain these observations is if *hth* or *tsh* were transcriptional targets of the Hippo pathway. This is ruled out, however, because manipulating the activity of the Hippo pathway does not affect the patterns of Hth and Tsh expression in the eye disc (Supplemental Fig. 4A; data not shown). We also tested if Sd, the only previously described transcription factor in the Hippo pathway, was required for proliferation the anterior eye disc. In contrast to *hth*^{P2} clones, *sd*-null clones were recovered in the anterior eye disc, arguing that Sd is not required for eye progenitor cell proliferation or survival (Supplemental Fig. 5A,B). Moreover, we found that Hth + Tsh can induce overproliferation in the eye disc in the absence of *sd* (Supplemental Fig. 5C,D). Together, these data suggest a model in which, like Sd and Yki in the wing pouch, Hth + Tsh and Yki directly regulate Hippo pathway targets in the anterior eye disc. Below, we present biochemical and genetic data that further support this hypothesis.

Hth and Tsh regulate bantam *in eye progenitor cells*

Because the overgrowth-inducing property of Hth + Tsh depends on *yki* and the ability of Yki$^+$ clones to grow in the eye progenitor domain depends on *hth*, we considered the possibility that they work together to regulate common targets in the anterior eye disc. Furthermore, because *hth*^{P2} clones can be partially rescued by blocking apoptosis, we focused on the known Yki target *bantam*, which encodes a miRNA that blocks the translation of the proapoptotic gene *hid* (Brennecke et al. 2003). Based on these findings, we tested whether Hth + Tsh activate *bantam* in the anterior progenitor domain of the eye disc.

To monitor *bantam*, we used a *bantam* sensor in which a green fluorescent protein (GFP) transgene contains two perfect copies of a sequence that is complementary to the *bantam* miRNA in its 3′ untranslated region (UTR) (Brennecke et al. 2003). As this transgene is transcribed ubiquitously via a *tubulin* promoter, GFP levels are inversely proportional to the levels of the *bantam* miRNA. In early third instar eye discs, the *bantam* sensor is expressed at lower levels in the anterior regions compared with the posterior regions (Fig. 4A,B). Double staining with Hth and Tsh demonstrates that where Tsh and Hth levels are high, *bantam* sensor levels are low (indicating high *bantam* levels). At a later stage of eye disc development (mid–late third instar), these relationships remain the same, but now the sensor is expressed at especially high levels in differentiating photoreceptors (Fig. 4C,D). This high level of sensor expression is similar to that observed in *ban*-null clones (data not shown), suggesting that it represents the complete absence of *bantam*. The inverse correlation between Hth + Tsh and

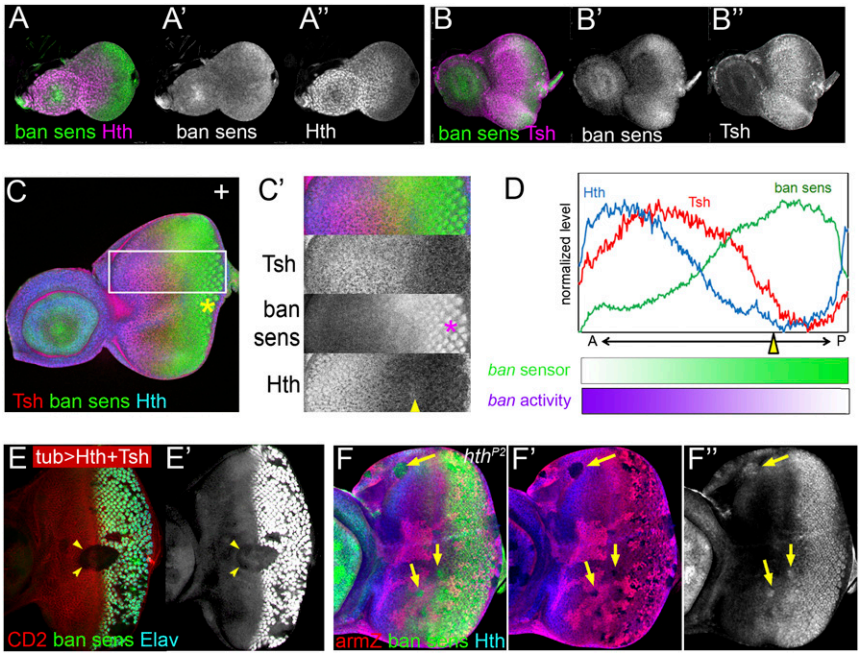

Figure 4. Control of *bantam* expression by Hth + Tsh. (*A*,*B*) Early to mid-third instar eye discs stained for the *bantam sensor* (green) and Hth (purple, *A*) or Tsh (purple, *B*). (*C*) Late third instar eye disc stained for Tsh (red), *ban sensor* (green), and Hth (blue). *C*′ shows a blow-up and individual channels for the region boxed in *C*. (*D*) Quantification of the Hth, Tsh, and *ban sens* stains in the regions boxed in *C*. *bantam* activity (purple gradient) is inferred to be the inverse for what is observed for the *ban sensor* (green gradient). (*E*) Eye disc containing clones expressing Hth + Tsh (absence of red, CD2) stained for the *ban sensor* (green/white in *C*,*C*′) and Elav (blue in *C*). Hth + Tsh clones repress both the very high levels of the *ban sensor* in the Elav$^+$ cells and the intermediate *ban sensor* levels that are present close to the MF (arrowheads). (*F*) Eye disc containing *hth^{P2}* clones made in a *H99*/+ background stained for β-gal (red, absence of *arm-lacZ* expression marks the clones), Hth (blue), and the *ban sensor* (green). An increase in *ban sensor* levels compared with surrounding cells can be seen in three of three *hth^{P2}* clones that survived in the progenitor domain (arrows).

bantam sensor levels suggests that *bantam* is up-regulated by these factors in the anterior of the eye disc.

Both loss-of-function and gain-of-function experiments support the idea that *bantam* is regulated by Hth + Tsh in eye progenitor cells. In clones that coexpress Hth + Tsh, the levels of the *bantam* sensor are reduced, suggesting that *bantam* is up-regulated (Fig. 4E). Conversely, *hth^{P2}* clones anterior to the MF [made in a *Df(3L)H99*/+ background to allow their survival] show increased levels of the *bantam* sensor (Fig. 4F). The levels observed in *hth^{P2}* clones are not as high as those in differentiated photoreceptors, suggesting that some *bantam* expression remains in the absence of *hth*. Thus, *hth* boosts the levels of *bantam* above a *hth*-independent baseline. In contrast to these effects on *bantam*, Hth + Tsh clones in eye discs had only a weak effect or no effect on other previously described Hippo pathway targets, *cycE*, *diap1*, and *expanded* (Supplemental Fig. 6). Taken together, these results suggest Hth + Tsh are required for high *bantam* levels that are normally present in the eye progenitor domain.

bantam *is required for ectopic Hth + Tsh-induced overgrowths*

Based on the observations that Hth + Tsh regulate *bantam*, we carried out two additional genetic tests to determine if this regulation was relevant to eye progenitor cells. First, we reasoned that because *bantam* carries out its functions in part by repressing the translation of the proapoptotic gene *hid*, and thereby protects cells from apoptosis, *hth^{P2}* clones might survive in the anterior eye disc if *bantam* expression was provided independently of *hth*. Consistent with this idea, *hth^{P2}* clones could be recovered in the progenitor domain when *bantam* was expressed throughout the eye disc (Fig. 5A,B). This rescue was incomplete, similar to that observed when p35 was used to rescue *hth^{P2}* clones.

Second, we tested whether *bantam* was necessary for Hth + Tsh-induced overgrowths. As shown above, Hth + Tsh clones overgrow, up-regulate CycB, and repress Elav, regardless of where they originate in the eye disc. In contrast, Hth + Tsh; *ban$^{\Delta 1}$* clones generated in parallel are much smaller, fail to survive in the anterior eye disc, and do not alter Elav or CycB expression levels (Fig. 5C-F). *ban$^{\Delta 1}$* clones survive even more poorly than Hth + Tsh; *ban$^{\Delta 1}$* clones (Fig. 5E). From these data, we conclude that, like *yki*, *bantam* is required for Hth + Tsh-induced overgrowths. In addition, these data suggest that Hth + Tsh have functions in addition to up-regulating *bantam*.

Hth and Yki can be copurified from S2 cells

Previous results demonstrated that Hth and Tsh can directly interact with each other in eye discs (Bessa et al. 2002). Here, we tested the idea that Hth and Yki may interact physically with each other to regulate target genes. We tested if Hth and Yki can bind to each other by transfecting S2 cells with HA-tagged Yki and Hth. In cotransfected cells, Hth was coimmunoprecipitated with HA-Yki (Fig. 6A,B). These results suggest that Hth and Yki can interact physically with each other when coexpressed, consistent with the idea that they function together in vivo.

Hth and Yki bind to the bantam *locus*

We next used chromatin immunoprecipitation (ChIP) to ask if Hth and Yki bind to the *bantam* locus in

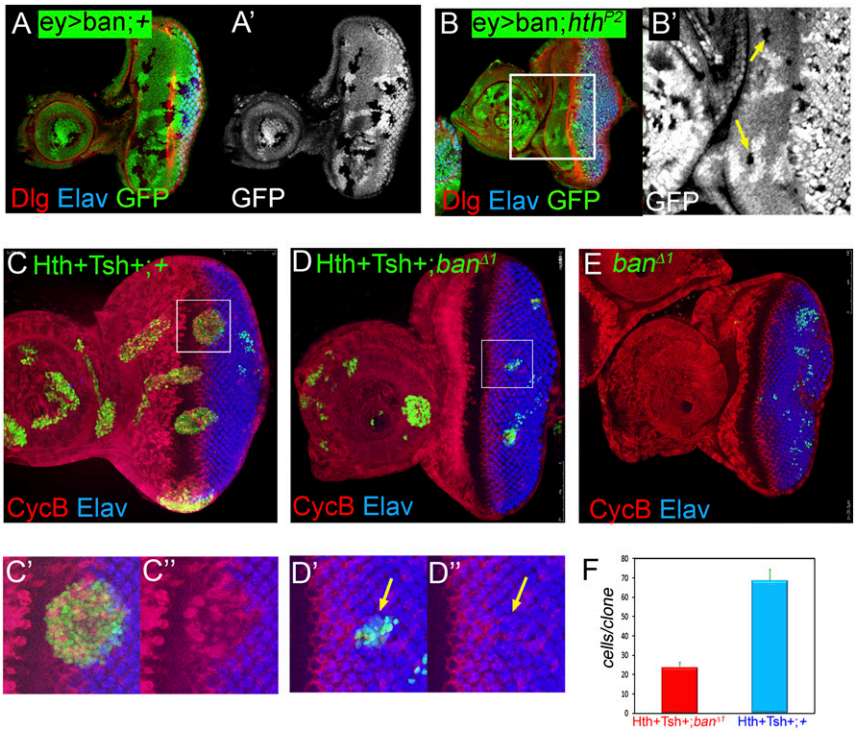

Figure 5. Mutual dependence of *bantam* and Hth + Tsh for cell proliferation and survival. (*A*,*B*) Eye discs expressing *bantam* via *eyeless-Gal4* (*ey* > *ban*) containing neutral clones (*A*) or hth^{P2} clones (*B*) stained for GFP (a clone marker), Elav (blue), and Dlg (red). A few hth^{P2} clones surviving in the progenitor domain are observed in *B* (arrows). (*C*–*E*) Eye discs containing Hth + Tsh clones (*C*), Hth + Tsh; $ban^{\Delta 1}$ clones (*D*), or $ban^{\Delta 1}$ clones (*E*) stained for CycB (red) and Elav (blue). *C*′, *C*″, *D*′, and *D*″ show enlargements of the boxed regions in *C* and *D*, respectively. Hth + Tsh; $ban^{\Delta 1}$ clones (*D*) grow more poorly compared with Hth + Tsh clones (*C*) made in parallel. Note also that Hth + Tsh; $ban^{\Delta 1}$ clones fail to repress Elav and do not activate CycB. (*F*) The number of cells present in Hth + Tsh; $ban^{\Delta 1}$ clones (red bar, $n = 44$) and Hth + Tsh clones (blue bar, $n = 69$).

eye–antenna imaginal discs. Using this assay, we identified a region ~14 kb upstream of the *bantam* hairpin that was immunoprecipitated by both anti-Hth and anti-Yki (Fig. 6D). Based on modENCODE data (http://modencode.oicr.on.ca/cgi-bin/gbrowse/fly), this site appears to be close to a start site for a primary *bantam* transcript (Fig. 6C). Although binding was stronger in the eye–antenna disc, the same region was immunoprecipitated from chromatin isolated from wing and leg imaginal discs (Fig. 6D). Two other regions at the *bantam* locus failed to be immunoprecipitated from either imaginal disc tissue, nor did a negative control region from *pyruvate dehydrogenase* (PDH). These data argue that Hth and Yki regulation of *bantam* is direct. Although the available antibodies against Tsh failed to work in ChIP experiments (data not shown), the observation that Hth and Tsh bind to each other in vivo (Bessa et al. 2002) suggests that Tsh is also a direct regulator of *bantam*.

Discussion

Coordination of cell proliferation, survival, and differentiation in eye development

We provide evidence that Hth and Tsh promote cell proliferation and survival in the undifferentiated progenitor cells of the eye imaginal disc. Further, our experiments suggest that these transcription factors carry out these functions with the coactivator Yki, a downstream component of the Hippo signaling pathway. Sd, which acts with Yki to mediate the regulation of Hippo targets elsewhere in the fly, does not seem to be required in this context. Finally, our data suggest that at least one direct Hth–Yki target in the eye disc is the miRNA *bantam*, which has been shown previously to both promote proliferation and prevent apoptosis in other tissues (Brennecke et al. 2003; Nolo et al. 2006; Thompson and Cohen 2006). Together, these data suggest a model in which a Hth–Tsh–Yki complex functions in eye progenitor cells to promote proliferation and block apoptosis, at least in part by up-regulating the levels of the miRNA *bantam* (Fig. 7). Below we discuss the roles of both Hth + Tsh and the Hippo pathway in tissue growth control.

Role of Hth and Tsh in eye progenitor cells

Our evidence, together with previous findings (Bessa et al. 2002; Bessa and Casares 2005), suggests that Hth and Tsh function as partners to carry out two main functions in anterior eye disc cells: They repress the expression of the later-acting retinal determination factors (Bessa et al. 2002), and they promote cell proliferation (this study). That these functions require *hth* is supported by both loss-of-function studies as well as gain-of-function studies. For example, hth^{P2} clones fail to survive anterior to the MF, and Tsh's ability to induce overgrowths when ectopically expressed is abolished in the absence of *hth*. The involvement of Tsh is supported by gain-of-function experiments and our previous finding that Hth and Tsh directly interact with each other in vivo (Bessa et al. 2002). Carrying out loss-of-function genetics for *tsh* is difficult because this gene is located proximal to the standard Flp recombination targets (FRTs) used to generate mitotic recombination. In addition, the highly related gene *tio*, which is closely linked to *tsh*, functions redundantly with *tsh* in several instances, including some aspects of eye development (Fasano et al. 1991; Laugier et al. 2005). Nevertheless, we found that knocking down *tsh* using

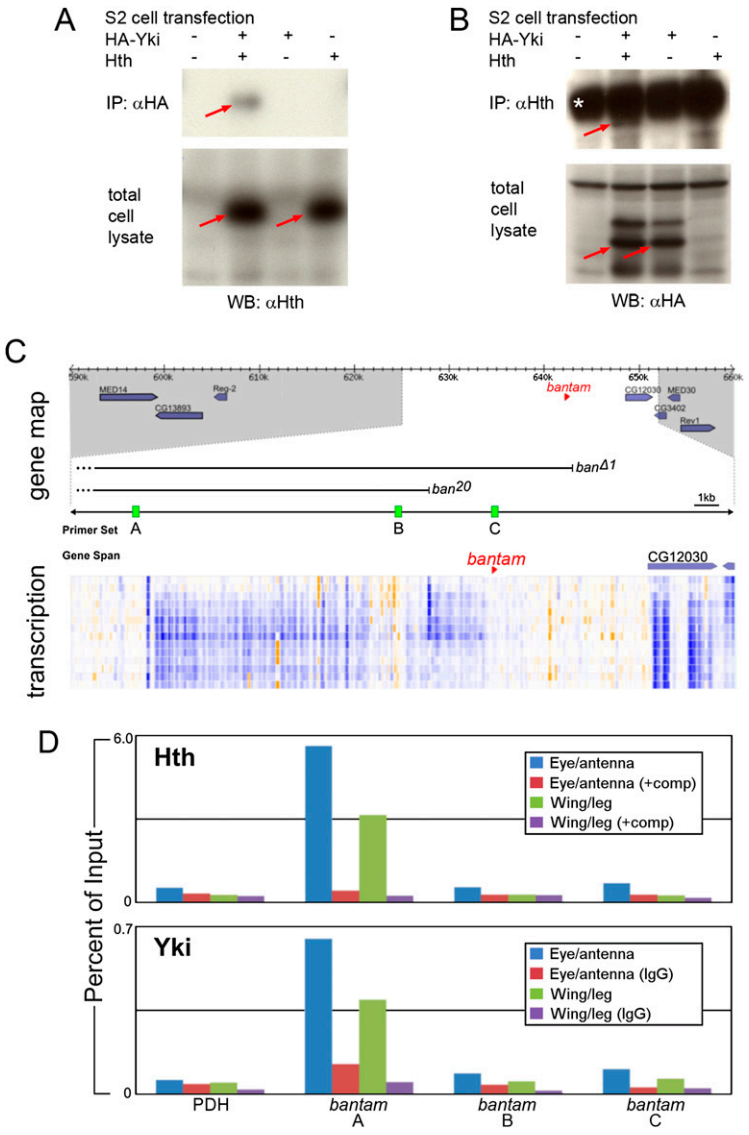

Figure 6. Molecular interactions between Hth, Yki, and the *bantam* locus. (*A*,*B*) Western blots of extracts from S2 cells that were transfected with the indicated expression plasmids. Blots were probed with anti-Hth (*A*) or anti-HA (*B*). The *top* panels show blots of immunoprecipitations with anti-HA (*A*) or anti-Hth (*B*), and the *bottom* panels show blots of total cell lysates. In *A*, Hth is coimmunoprecipitated with HA-Yki. In *B*, HA-Yki is coimmunoprecipitated with Hth. In *A*, the red arrows point to Hth detected in total cell lysates and anti-HA immunoprecipitate. In *B*, the red arrows point to HA-Yki detected in total cell lysates and anti-Hth immunoprecipitate. The asterisk in *B* indicates cross-reaction from the IgG heavy chain in the anti-Hth antiserum. (*C*) The *top* section shows a gene map in the vicinity of *bantam*; the regions deleted in two *ban* deletion mutants ($ban^{\Delta 1}$ and ban^{20}) are indicated (Hipfner et al. 2002; Yang et al. 2009). The *middle* section reproduces data from the modENCODE project measuring the amount of transcription around the *bantam* locus; each row represents a different developmental time point (from embryogenesis to second instar) and provides a measure of amount of mRNA encoded by that genomic position; blue indicates high expression, and orange indicates low expression (see http://www.modencode.org for details). Regions A, B, and C indicate the positions of three of 11 primers used for ChIP experiments; based on the modENCODE transcription map, region A is close to an apparent start of *bantam* transcription and was the only amplicon that gave a positive ChIP signal. (*D*) The results of ChIP experiments using anti-Hth (*top*) and anti-Yki (*bottom*) antibodies to precipitate chromatin prepared from the indicated tissues. In the anti-Hth experiment, "comp" refers to the addition of a competitor peptide that specifically blocks binding to Hth (see the Materials and Methods for details). In the anti-Yki experiments, the control was unprogrammed IgG. Immunoprecipitated chromatin was assayed for the presence of regions A, B, or C or for a negative control locus, PDH. Data are presented as a percentage of the signal obtained relative to input chromatin. For both anti-Hth and anti-Yki, a significant signal was obtained only for region A.

RNAi in a *tio*-null background results in poor survival in the progenitor domain. Taken together, these data provide a compelling argument for Hth + Tsh functioning together to promote cell survival in the anterior eye disc.

A functional relationship between Hth and Tsh also exists in other tissues in *Drosophila*, most notably in both wing and leg imaginal discs, where they are coexpressed in cells that will give rise to the proximal domains of these appendages. In both wings and legs, Tsh has the capacity to regulate *hth* when expressed in clones, and both *tsh* and *hth* have the ability to suppress distal appendage development when misexpressed (Rieckhof et al. 1997; Azpiazu and Morata 2000; Casares and Mann 2000; Wu and Cohen 2000, 2002; Zirin and Mann 2004). However, in these tissues, and unlike the eye disc, Hth + Tsh expression is not correlated with proliferation, which occurs uniformly throughout these discs. Consistently, the expression pattern exhibited by the *bantam* sensor does not correlate with Hth or Tsh in the leg or wing (Brennecke et al. 2003; Nolo et al. 2006; Thompson and Cohen 2006). The special relationship between proliferation and Hth + Tsh in the eye may be due in part to the *Drosophila* Pax6 homolog Eyeless (Ey), which is critical for eye identity (Gehring 1996). Moreover, Ey is found in a complex with Hth in vivo and participates with Hth and Tsh in the repression of retinal determination genes (Bessa et al. 2002). Thus, it may also be the case that Ey directly participates in the regulation of *bantam* together with Hth and Yki.

hth *promotes, but is not essential for, cell proliferation and survival*

Although hth^{P2} clones fail to survive in the eye progenitor domain, our data demonstrate that *hth* is not absolutely required for cells in this domain to proliferate.

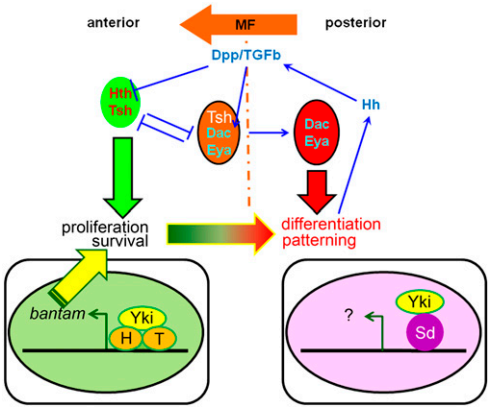

Figure 7. Model for the switch from proliferation to differentiation in the eye disc. Hth and Tsh are expressed in the progenitor domain where they repress retinal differentiation genes such as *dac* and *eya* and also up-regulate *bantam* expression in conjunction with Yki. Signals such as Dpp from the MF repress *hth* and *tsh*, allowing *dac* and *eya* to execute the differentiation program. In this region of the eye disc, Yki may work with other transcription factors such as Sd to regulate a different subset of Hippo pathway target genes.

The effects we observed on the *bantam* sensor are consistent with the idea that *hth* promotes, but is not essential for, cells to proliferate in the eye progenitor domain. In hth^{P2} clones, *bantam* sensor levels increased above those normally observed in progenitor cells, but not as high as the levels observed in differentiated photoreceptors. Thus, if the level in photoreceptors represents the complete absence of *bantam*, these data imply that *hth* only up-regulates *bantam* over a basal *hth*-independent level. Moreover, the levels of the *bantam* sensor in other tissues, such as the wing disc, rarely approach those observed in photoreceptors, suggesting that most cells have some *bantam* expression, and that *bantam* regulators, such as *hth*, only serve to modulate *bantam* levels.

If eye progenitor cells have the capacity to proliferate in the absence of *hth*, how important is the proliferation-promoting function of *hth*? Although normal eyes can develop in animals in which hth^{P2} clones are generated, this is likely due to the ability of neighboring wild-type cells to compensate in this mosaic situation. In contrast, when wild-type and heterozygous cells are killed (using the EGUF method [*ey-Gal4/UAS-flp/GMF-hid*]) (Stowers and Schwarz 1999), we find that the remaining hth^{P2} tissue is unable to produce normal-sized eyes (Supplemental Fig. 7). This experiment suggests that the proliferation-promoting functions of *hth* in the eye progenitor domain are critical for normal eye development, likely by providing a sufficient pool of progenitor cells prior to differentiation.

The role of Yki partners in Hippo pathway specificity

The Hippo pathway has emerged recently as an important regulator of cell proliferation and survival in both vertebrates and invertebrates (Pan 2007; Zhang et al. 2009). In *Drosophila*, this pathway appears to regulate proliferation in nearly all tissues. For example, wts^{-} clones or Yki^{+} clones have the capacity to induce overgrowths throughout the body (Harvey et al. 2003; Udan et al. 2003; Wu et al. 2003; Huang et al. 2005). As Yki and its mammalian ortholog Yap are transcriptional coactivators that do not have their own DNA-binding domain, they are thought to partner with DNA-binding transcription factors to regulate gene expression. Prior to this work, the only transcription factor proposed to work directly with Yki was Sd, a member of the TEAD/TEF family of DNA-binding proteins (Goulev et al. 2008; Wu et al. 2008; L Zhang et al. 2008; Zhao et al. 2008). However, unlike other components of the Hippo pathway, the available data suggest that Sd plays a more limited role in cell proliferation and survival in *Drosophila* (Campbell et al. 1992; Goulev et al. 2008; Wu et al. 2008; L Zhang et al. 2008; Zhao et al. 2008). In contrast to its essential role in the wing pouch, sd^{-} clones survive well in other tissues, including the region of the wing disc that will give rise to the notum of the fly (Supplemental Fig. 5; Wu et al. 2008; L Zhang et al. 2008). Similarly, *sd*-null clones grow well in the eye progenitor domain. Thus, unlike in the wing pouch, *sd* is not required for cell survival and proliferation in the eye progenitor domain.

In contrast to the survival of *sd* clones in this domain, hth^{P2} clones fail to survive in the eye progenitor domain. Thus, analogous to *sd* in the wing pouch, *hth* is required for cells to survive and proliferate in the anterior eye imaginal disc. This observation suggests that *hth* could play an analogous role to *sd* in this progenitor domain, a view that is supported by our results. This evidence includes (1) Hth can interact with Yki when coexpressed in S2 cells, (2) Hth + Tsh regulate the Yki target *bantam*, and (3) Hth and Yki are both bound to the same region of the *bantam* locus in eye discs. Genetically, we show that the Hippo pathway is unable to induce overgrowths in the eye progenitor domain without *hth*, and that Hth + Tsh cannot induce overgrowths in the absence of Yki. These results suggest that Hth + Tsh comprise the DNA-binding transcription factors that function with Yki to regulate proliferation and survival genes, such as *bantam*. Thus, analogous to Sd in the wing pouch, Hth + Tsh are transcription factors used by the Hippo signaling pathway in eye progenitor cells.

The finding that Hth + Tsh play an analogous role in the eye progenitor domain as Sd does in the wing pouch has several implications for how the Hippo pathway is regulated in vivo. For one, the use of different DNA-binding transcription factors to regulate Hippo target genes suggests a previously unknown degree of specificity available to this pathway. Hth, a TALE family homeodomain protein, and Tsh, a Zn finger protein, are likely to bind very different target DNA sequences than Sd, a TEAD/TEF domain DNA-binding factor. Accordingly, we find that ectopic Hth + Tsh clones in the eye disc do not consistently up-regulate *diap1* or *expanded*, known Sd–Yki targets in the wing disc (Supplemental Fig. 6; Harvey et al. 2003; Jia et al. 2003; Udan et al. 2003; Wu et al. 2003).

These results also imply that the transcriptional regulation of *hth*, *tsh*, and *sd* has the potential to change the

output of the Hippo pathway. Because *hth* and *tsh* are transcriptionally repressed by signals coming from the MF, these factors are not available to work with the Hippo pathway posterior to the MF. However, loss of Hippo kinase activity can lead to proliferation of differentiated cells posterior to the MF. In these cells, *sd* is expressed (Campbell et al. 1992), suggesting that Yki may use this transcription factor in this context. Analogously, loss of Hippo kinase activity can cause overgrowths in the notum as well as in the wing pouch. As sd^- clones grow well in the notum, but not in the wing pouch, these data suggest that the notum overgrowths may be mediated by a transcription factor other than Sd. *hth* clones also survive well in the notum, implying that yet another transcription factor or factors may work with Yki in this tissue. In sum, we suggest that Yki, and thus the Hippo pathway, may be able to work with multiple transcription factors to regulate target genes. In principle, the use of several transcription factors that are themselves developmentally regulated allows the Hippo pathway to be interpreted in different ways in different contexts.

Although our data suggest that the Hippo pathway uses Hth + Tsh to up-regulate *bantam*, they also suggest that both Hth + Tsh and Yki have additional, independent targets. For example, the loss of Hippo kinase activity leads to the up-regulation of *diap1* throughout the eye disc (Wu et al. 2003). Because *diap1* is not affected when Hth + Tsh are coexpressed, the Hippo pathway has the capacity to regulate some genes independently of Hth + Tsh, even in the eye progenitor domain. Moreover, at least when Yki is ectopically expressed, *sd* appears to be required in all regions of the eye disc for *diap1* activation (Wu et al. 2008). Thus, although it has not been shown that *sd* is required for endogenous *diap1* expression in this tissue, these data, together with those presented here, suggest that Yki may use both Sd and Hth + Tsh to regulate gene expression in the eye disc. In fact, L Zhang et al. (2008) suggest that *sd* is also a modifier of *bantam* expression in the eye disc and that *sd* is required for normal-sized eyes. However, these clones, which used RNAi to knock down Sd, grew well in the eye progenitor domain. In addition, the smaller eyes observed by L Zhang et al. (2008) when *sd* was knocked down could be due to the earlier embryonic expression of the Gal4 driver used in these experiments. In contrast, when generated during larval stages, hth^- clones, but not sd^- clones, fail to survive in the eye progenitor domain, arguing that, at least post-embryonically, gene regulation by Hth + Tsh, not Sd, is critical for cell survival in this tissue. This conclusion is also supported by our finding that Hth + Tsh can induce proliferation in the absence of *sd* (Supplemental Fig. 5).

As shown previously (Bessa et al. 2002), Hth + Tsh play a key role in blocking eye differentiation by repressing the retinal determination genes *eya* and *so*. The available data do not yet resolve whether this repression works independently of the Hippo pathway. On the one hand, the loss of Hippo kinase activity leads to overgrowths without blocking differentiation, arguing that nuclear Yki promotes proliferation without changing cell fate (Harvey et al. 2003; Udan et al. 2003; Wu et al. 2003). Consistently, we find that wts^- or Yki^+ clones do not alter Elav expression in differentiated photoreceptors. Curiously, however, ectopic expression of Hth + Tsh did not block differentiation in the absence of Yki. Although these data could be interpreted to suggest that Yki is directly required for repressing differentiation, they could alternatively suggest that repression requires cell proliferation. Consistently, Hth + Tsh were also unable to block differentiation in the absence of *bantam*. These observations raise the possibility that the absence of *bantam* or *yki* indirectly inhibits Hth + Tsh's ability to repress differentiation by compromising the proliferation of these cells, although other indirect affects are also possible.

Hth + Tsh are also likely to regulate genes in addition to *bantam* to promote proliferation and survival in the eye progenitor domain. This is most strongly supported by our observation that ectopic expression of *bantam* only partially rescues the survival of hth^{P2} clones. In addition, we found that the overgrowths generated by ectopic expression of Hth + Tsh are only partially suppressed by the coexpression of Hpo, whose overexpression removes Yki from the nucleus. These data suggest that some of the Hth + Tsh targets that mediate growth and survival in the eye progenitor domain are regulated independently of Yki.

hth *and* tsh *as focal points for the switch from proliferation to differentiation*

In summary, these results suggest that the transcriptional regulation of *hth* and *tsh* along the anterior–posterior axis of the eye disc changes the output of the Hippo pathway. In the eye progenitor domain, where Hth and Tsh are both present, the pathway uses these transcription factors to promote proliferation and cell survival, at least in part by up-regulating *bantam*. Once *hth* and *tsh* are repressed by signals coming from the MF, the Hippo pathway may use other transcription factors, such as Sd, to regulate a different set of target genes. Thus, together with other functions carried out by these transcription factors, their regulation across the anterior–posterior axis coordinates the complex switch from proliferation to differentiation during eye development.

Materials and methods

Drosophila *strains and genetics*

Wild-type flies were Oregon R or *yw*. Standard methods were used to recombine and balance chromosomes bearing mutations, markers, or transgenes. The mutations and transgenes used were hth^{P2} (Rieckhof et al. 1997); tsh^{8}, $tsh^{1,LacZ}$, *UAS-hth::GFP*, *UAS-hth::3′UTR* (Noro et al. 2006); and *UAS-tsh* (Zirin and Mann 2004). For gain-of-function clones, *act* > y^+ > *Gal4* or *tub* > *CD2,*y^+ > *Gal4* flip-out cassettes were used (Struhl and Basler 1993). Heat shocks were given 48 h ± 12 h after egg laying (AEL). For mutant clones, standard FRTs (Xu and Rubin 1993) with dominant visible markers (*ubi* > *GFP* or *arm*>*LacZ*) were used. Heat shocks were given 60 h ± 12 h AEL. For MARCM clones

(Lee and Luo 1999), unless otherwise indicated, *tub* > *Gal4* was used to express *UAS-GFP* and other UAS transgenes. The stocks generally contain *yw*, *hsFLP*, *tub* > *Gal4*. Heat shocks were given 36 h ± 12 h AEL to allow for Gal80 to fade. FRT arms containing *tub* > *Gal80* are from Bloomington Stock Center or from G. Struhl and M. Zecca. *UAS-p35*, *UAS-DIAP1*, and *Df(3L)H99* are from L. Johnston. The *Minute* mutation was *M(3)95A*. For the *sd* experiments, the null allele $sd^{\Delta B}$ was used; for the $sd^{\Delta B}$; Hth + Tsh MARCM experiment, *act-Gal4* was used instead of *tub-Gal4*.

wts^{P2} and $diap1^{lacZ}$ were obtained from L. Johnston. *UAS-Yki* and yki^{B5} were from D.J. Pan (Huang et al. 2005). *UAS-bantam* and $ban^{\Delta 1}$ were from S.M. Cohen (Brennecke et al. 2003). $bantam^{EP3622}$ was from Bloomington Stock Center and gave the same results as *UAS-bantam*. *ey* > *Gal4* and *GMR* > *Gal4* drivers were from Bloomington Stock Center. *UAS-Hpo/dMST1* was from J. Jiang (Jia et al. 2003). *tub* > *EGFP::2xanti-bantam* (*bantam sensor*) lines were constructed by Brennecke et al. (2003) and were obtained from L. Johnston.

Antibodies

Guinea pig anti-Hth (GP52), rabbit anti-Hth, guinea pig anti-Dll, and guinea pig anti-Tsh were described previously (Casares and Mann 2000; Zirin and Mann 2004; Estella and Mann 2008). Rabbit anti-CycB and rabbit anti-CycE were gifts from H. Richardson (Richardson et al. 1995). Rabbit anti-Histone H3 subunit, Ser-10 phosphorylated, anti-Caspase-3, and anti-GFP were from Upstate Biotechnologies. Monoclonal antibody against Dlg, Dacapo, Wg, Elav, Eya, Dac, and CycE were from the Developmental Studies Hybridoma Bank. Phalloidin-AlexaFluor555 conjugate was from Molecular Probes and was used at the recommended concentration with secondary antibodies. Rabbit anti-β-Gal was from Cappel. Mouse anti-rat CD2 was from Invitrogen. Guinea pig anti-Stat92E was from S. Hou (Hou et al. 1996). Anti-Hth (dG-20) polyclonal antibody and Hth competitor peptide (dG-20P) used for ChIP analysis were purchased from Santa Cruz Biotechnologies. Anti-HA (3F10) monoclonal antibody was purchased from Roche.

Immunohistochemistry

Imaginal discs were dissected and immunostained using standard procedures. Secondary antibodies used were AlexaFluor488, AlexaFluor555, and AlexaFluo647 conjugates from Molecular Probes and were used at 1:1000. After staining, discs were washed five times in PBST, 15 min each at room temperature, and were dissected onto glass slides in VectaShield.

Optical section single images or z-series were collected on either Zeiss AxioScope/ApoTome, Bio-Rad MRC1024 confocal microscope, or Leica SP5 LSM confocal system. Z-series were analyzed by ImageJ (http://rsbweb.nih.gov/ij). Other image analysis was done with Photoshop CS3.

S2 cell transfection, immunoprecipitation, and Western blot

S2 cells were from N. Senoo-Matsuda and maintained at room temperature in *Drosophila* Schneider medium with glutamate supplemented with 10% fetal bovine serum, 5mg/mL penicillin–streptomycin, and 2.5 mg/mL Bacto Peptone. pAc-HA-Yki plasmid was from D.J. Pan (Huang et al. 2005). pAc-Hth and pAc-GFP were made by J. Culi (Culi and Mann 2003). For each construct, 15 μg of plasmid DNA were transfected into S2 cells by Effectene (Qiagen). Cells were lysed in Noro's RIPA buffer (20 mM Tris at pH 7.5, 0.5% NP-40, 150 mM NaCl, 10 mM $MgCl_2$). Lysates were passed through a 25-gauge needle five times and cleared by centrifugation. Immunoprecipitation in RIPA buffer was with either 1 μg/mL anti-HA (3F10) or 2 μL/mL GP52 antiserum. Protein A/G-agarose beads captured the immunoprecipitates and were washed five times with RIPA buffer. Immnoprecipitates were denatured in 1× SDS sample buffer.

Immunoprecipitates and control lysates were separated by 10% SDS-PAGE and blotted to PVDF membrane (Millipore). The blot was blocked in TBST (10 mM Tris at pH 7.6, 150 mM NaCl, 0.1% Tween-20) with 5% skim milk for 30 min at room temperature and was incubated with primary antibody (1:2000 for GP52 anti-Hth, 1:400 for anti-HA, 3F10) in TBST + 5% milk at 4°C overnight. After five 15-min washes at room temperature in TBST, blots were incubated for 2 h at room temperature with HRP-conjugated secondary antibody. The blot was washed five times for 15 min at room temperature in TBST, and was detected by ECL Plus (GE Healthcare).

ChIP

Mid-third instar larvae (96 h AEL) were dissected and imaginal discs were collected in PBS on ice. Dissection time was minimized to <1 h to process 80–120 animals. Discs were fixed with 1.8% formaldehyde. Chromatin preparation and immunoprecipitation were performed as described (McKay et al. 2009). Anti-Hth (dG-20) (1.5 μg) was used for each immunoprecipitation. Specificity for the anti-Hth antibody was confirmed by comparing the signal obtained from parallel immunoprecipitations carried out in the presence of the dG-20P competitor peptide, which specifically blocks antigen recognition (Santa Cruz Biotechnologies). Rabbit anti-Yki (D. Pan) was used at a final dilution of 1:300, and specificity was tested by parallel immunoprecipitations carried out with normal rabbit IgG (Santa Cruz Biotechnologies). Eleven real-time PCR amplicons surrounding the *bantam* hairpin (from ~14 kb 5′ to ~3 kb 3′) were used to quantify immunoprecipitated chromatin. Of these, only the primer set labeled A in Figure 6 produced a positive signal. Sequences of the primers are available on request.

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