Scalloped and Yorkie are required for cell cycle re-entry of quiescent cells after tissue damage

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Summary

Regeneration of damaged tissues typically requires a population of active stem cells. How damaged tissue is regenerated in quiescent tissues lacking a stem cell population is less well understood. We used a genetic screen in the developing *Drosophila melanogaster* eye to investigate the mechanisms that trigger quiescent cells to re-enter the cell cycle and proliferate in response to tissue damage. We discovered that Hippo signaling regulates compensatory proliferation after extensive cell death in the developing eye. Scalloped and Yorkie, transcriptional effectors of the Hippo pathway, drive Cyclin E expression to induce cell cycle re-entry in cells that normally remain quiescent in the absence of damage. Ajuba, an upstream regulator of Hippo signaling that functions as a sensor of epithelial integrity, is also required for cell cycle re-entry. Thus, in addition to its well-established role in modulating proliferation during periods of tissue growth, Hippo signaling maintains homeostasis by regulating quiescent cell populations affected by tissue damage.

Introduction

Tissue regeneration has fascinated biologists for many years, but the molecular mechanisms underlying this process have only recently begun to be understood. Pioneering experiments on regenerating hydra by the Abbe Trembley in the 1700’s and on other organisms in the 1800’s (Morgan, 1901) established that regeneration functions as a reparative process to replace tissues damaged by injury or disease and occurs as a restorative process to repair old and damaged tissues throughout an organism’s life. While early experiments on highly regenerative animals like hydra are fundamental for our understanding of regeneration, the lack of facile genetic manipulation in these organisms made identifying molecular mechanisms difficult.
In the 1970’s, *Drosophila melanogaster* emerged as a powerful and genetically tractable experimental system to study regeneration. Larval imaginal discs, which are epithelial tissues that proliferate during larval life and differentiate during pupation into adult structures, are able to regrow after substantial tissue loss due to irradiation or removal of fragments by surgery (Bryant, 1971; Haynie and Bryant, 1977; Worley et al., 2012). In these imaginal discs, a process called “compensatory proliferation (CP)” replaces cells lost by tissue damage. Research from several labs in the last decade has elucidated some of the mechanisms controlling CP. One important mechanism is the induction of proliferation by apoptotic cells. Caspases are required for robust regeneration in many organisms even though caspase activity and cell death contributes to initial tissue loss following damage. When apoptosis is blocked by the baculovirus effector-caspase inhibitor p35 in damaged *Drosophila* wing discs, “undead cells” that have initiated but not completed apoptosis induce hyperproliferation (Huh et al., 2004; Perez-Garijo et al., 2004). This hyperproliferation is dependent on the initiator caspase Dronc, suggesting this caspase has a role in inducing proliferation independently of apoptosis (Huh et al., 2004). Proliferation induced by undead cells or genuine apoptotic cells may act in various contexts through multiple pathways, including the Wingless/Wnt, Dpp/BMP, Jun N-terminal kinase (JNK), and Hedgehog signaling pathways (Ryoo et al., 2004; Perez-Garijo et al., 2005; Fan and Bergmann, 2008). Apoptosis-induced proliferation involving mitogenic signaling is likely one part of a larger pathway controlling CP (Mollereau et al., 2013).

The participation of apoptotic cells in regeneration is not unique to *Drosophila*. Studies in other organisms have revealed caspase activity is required for regeneration in the *Xenopus laevis* tadpole tail (Tseng et al., 2007) and in the mammalian liver and skin (Li et al., 2010). Additionally, many of the pathways identified as CP regulators in
*Drosophila* are also involved in vertebrate tissue repair. For example, both the Hedgehog (Cai et al., 2011) and JNK (Wuestefeld et al., 2013) pathways are required for mammalian liver regeneration, and Wnt signaling is required for limb regeneration in *Xenopus*, axolotl, and zebrafish (Kawakami et al., 2006). Thus, there are conserved mechanisms for regeneration between invertebrates and vertebrates.

How the cell cycle is regulated during regeneration to ensure proper regrowth remains unclear. Tissue regrowth can be accomplished by increasing proliferation to restore cell number, as in *Drosophila* wing discs, or by cellular growth without cell division, such as during endoreplication (Fox and Duronio, 2013). A strong proliferative response to damage occurs during planaria body regeneration (Wenemoser and Reddien, 2010), zebrafish heart regeneration (Poss et al., 2002), and *Xenopus* tail regeneration (Tseng et al., 2007), while endoreplication contributes to tissue repair in the mammalian liver (Sigal et al., 1999) and the *Drosophila* ovary (Tamori and Deng, 2013). In these tissues, a moderate increase in proliferating or endoreplicating cells quickly replaces lost tissue.

In contrast, how cell cycle exit in quiescent tissues is overcome to allow proliferation following damage is unclear. Robust inhibition of cell cycle re-entry in quiescent tissues is necessary to maintain tissue homeostasis and prevent neoplasia and cancer. Cell cycle inhibition thus presents a high hurdle to overcome before regeneration can take place. We investigated this issue using the *Drosophila* eye imaginal disc. The eye disc contains a population of cells that are normally quiescent but will undergo cell cycle re-entry after induction of massive cell death (Fan and Bergmann, 2008). We used the developing eye as a model for CP and performed a genetic screen to identify regulators of this process. With this approach, we identified the transcription factor Scalloped (Sd) as a novel CP regulator. We show Sd and the
Sd binding partner Yorkie (Yki) are required for cell cycle re-entry following damage in the eye imaginal disc. Yki is a transcriptional effector of the Hippo pathway and was previously identified as a CP regulator in wing discs (Sun and Irvine, 2011; Grusche et al., 2011). We also found that CP in the eye disc requires the Hippo pathway regulator Ajuba (Jub), similar to recent results in the wing disc (Sun and Irvine, 2013). However, Jub activation during CP is likely differentially regulated in these two tissues. Our study demonstrates that Hippo signaling is required for quiescent cells to re-enter the cell cycle following tissue damage and is likely to provide insight into a variety of regenerative systems, particularly those within non-proliferative tissues.

**Results**

**Quiescent cells re-enter the cell cycle after tissue damage in the developing eye**

The developing *Drosophila* eye is ideal for studying regeneration in a quiescent cell population. Many genetic tools are available for manipulating the eye imaginal disc, and subtle defects in eye development are readily apparent in the highly organized adult eye (Domínguez and Casares, 2005; Gutierrez-Avino et al., 2009). The neurocrystalline lattice of the *Drosophila* eye takes shape by precise control of the cell cycle and differentiation during development (Fig. 1A,L). In early larval development, the eye disc grows as undifferentiated cells proliferate asynchronously. During the third and last larval stage, a wave of differentiation associated with an apical constriction of the epithelial sheet called the morphogenetic furrow (MF) moves from posterior to anterior across the disc. Cells within the MF arrest in G1, and a subset begin to differentiate into photoreceptors. After the MF has passed, the remaining undifferentiated cells synchronously enter S-phase in what is termed the second mitotic wave (SMW) (Fig. 1B). After the SMW, these cells become quiescent and await
cues to differentiate (Fig. 1L) (Firth and Baker, 2005). This population is considered quiescent as very few cells posterior to the SMW undergo S-phase (Fig. 1B’) or mitosis (Baker and Yu, 2001). Cells remain quiescent by mechanisms that prevent cell cycle re-entry, including CDK inhibition by Dacapo and E2f1 repression by Retinoblastoma/Rbf1 (Buttitta et al., 2007; Ruggiero et al., 2012), as well as destruction of cell cycle regulators by the anaphase promoting complex (Buttitta et al., 2010; Bandura et al., 2013).

Although cells posterior to the SMW are normally quiescent, cell cycle re-entry occurs after tissue damage (Fan and Bergmann, 2008). Expression of the pro-apoptotic gene hid with the GMR promoter, which is expressed posterior to the MF (Fig. S1A), induces extensive cell death (Fig. 1E,F). Dying, caspase-positive cells with pyknotic nuclei are extruded from the basal surface of the eye disc (Fig. S1B-C), as in other discs (Gibson and Perrimon, 2005; Shen and Dahmann, 2005). Not all cells posterior to the SMW die, and some overcome cell cycle inhibition and re-enter S-phase in a wave of proliferation (Fig. 1D,D’, Fan and Bergmann, 2008). GMR-hid flies have nearly absent adult eyes (Fig. 1C), indicating increased proliferation cannot fully compensate for tissue loss, likely because GMR-hid expression during pupal stages induces extensive apoptosis after the potential to re-enter the cell cycle is lost. Thus, GMR-hid eye discs behave somewhat differently than previous CP models in the wing where tissue regrowth is more complete (Mollereau et al., 2013). Nonetheless, tissue damage-induced cell cycle re-entry in the eye disc provides a valuable model for studying CP in a quiescent cell population.

Because apoptotic cells play a crucial role during CP, we further characterized the relationship between dying and proliferating cells in the eye disc. Rather than uniform apoptosis across GMR-hid discs, two distinct waves of cleaved Caspase-3
(CC3) positive cells occur on either side of the CP wave (Fig. 1E,F, S1D-E). Because CP overlaps substantially with the apoptosis-free zone between the two waves of CC3 staining, it was previously suggested proliferating cells might inhibit apoptosis (Fan and Bergmann, 2008). However, these two waves of apoptosis persist when CP is blocked (see below and Fig. 3B,C), suggesting CP does not inhibit apoptosis. Rather, variations in Hid activity across the disc may account for the observed pattern of apoptosis. Hid accumulation and activity varies posterior to the MF (Fan and Bergmann, 2014). In addition, hid mRNA is not uniformly expressed across GMR-hid discs, with high levels immediately posterior to the MF and lower levels more posteriorly (Fig. 1G,H). hid mRNA can be regulated by the miRNA bantam (ban) (Brennecke et al., 2003), which protects cells from apoptosis in certain damaged tissues after being up-regulated by a Tie-like receptor tyrosine kinase (Tie)-dependent mechanism (Bilak et al., 2014). We did not detect ban induction in GMR-hid discs (Fig. S5C), and tie RNAi does not affect the pattern of apoptosis (Fig. S2A). These results suggest Tie-dependent ban induction is not responsible for decreased hid transcripts in the posterior eye disc.

Because hid transcripts are low in the posterior of the disc, we considered the possibility that dying cells in the first apoptotic wave promote apoptosis-induced apoptosis (AiA), resulting in the second apoptotic wave. AiA is mediated by JNK signaling (Perez-Garijo et al., 2013). However, expression of a dominant-negative version of the Drosophila JNK homolog Basket (Bsk) does not affect the pattern of apoptosis in GMR-hid discs (Fig. S2B). Interestingly, while JNK signaling is required for CP in the wing disc (Ryoo et al., 2004), expression of Bsk^DN or Puckered (Puc), a negative regulator and downstream target of the JNK pathway, does not suppress CP (Fig. S2C,D,G). Additionally, while puc is induced after damage in the wing
(Bergantinos et al., 2010), puc-lacZ is not induced in GMR-hid eye discs (Fig. S2E,F). We conclude from these data that regulation of Hid expression and activity, rather than S-phase entry, ban induction, or AiA, determines the pattern of apoptosis in GMR-hid discs and JNK signaling is not a major CP regulator posterior to the MF.

We next explored contributions to the pattern of CP in GMR-hid discs. CP occurs in a well-defined wave that does not typically extend to the posterior edge of the disc (Fig. 1D,D’). Undifferentiated cells, which are the only cells that re-enter S-phase in GMR-hid discs (Fan and Bergmann, 2008), are present at the posterior edge of the eye disc. We hypothesized cells lose the competency to re-enter S-phase prior to differentiation, perhaps due to prolonged quiescence. To address this hypothesis, we shifted the wave of apoptosis towards the posterior edge of the disc by expressing hid under the control of longGMR (LGMR), a version of the GMR promoter that contains a repressor element and is only expressed in a subset of photoreceptors (Wernet et al., 2003; Fig. S1F). Although we were unable to obtain clear hid mRNA signal in this genotype, likely due to lower levels of accumulation than in GMR-hid discs, we did observe a single wave of apoptosis in the posterior region of LGMR-hid discs (Fig. 1K). In addition, LGMR flies have a reduced adult eye phenotype similar to, but less severe than, GMR-hid flies (Fig. 1I). Unlike in GMR-hid discs, however, CP is not induced in LGMR-hid discs (Fig. 1J,J’,M). This result is consistent with the idea that undifferentiated cells at the posterior of the disc are refractory to cell cycle re-entry in response to tissue damage. Alternatively, lower levels of apoptosis in LGMR-hid discs compared to GMR-hid discs (Fig. 1N) may not be sufficient to induce CP.
An RNAi screen identifies genes required for compensatory proliferation

How do undifferentiated cells overcome quiescence to re-enter the cell cycle in response to tissue damage? The mechanisms that control CP in quiescent eye discs are distinct from those in proliferating wing discs (Fan and Bergmann, 2008). For example, JNK signaling is required for CP in the wing disc (Ryoo et al., 2004) while our data suggest it is not in the eye disc (Fig. S2). To identify pathways necessary for cell cycle re-entry in the regenerating eye, we designed a genetic screen based on adult eye phenotypes. We expressed hairpin RNAs targeting individual genes in GMR-hid eye discs using the GAL4/UAS system and assessed the effect on CP by scoring the adult eye phenotype. To drive UAS-transgene expression, we constructed a GMR-hid, GMR-Gal4/+ (GMR>hid, Gal4) line (unless otherwise noted, genotypes written as GMR>hid, transgene are heterozygous for GMR-hid, GMR-Gal4, and a UAS-transgene). Eyes in GMR>hid, Gal4 flies are drastically reduced compared to wild type but larger than GMR-hid eyes (compare Figs. 1A,C and 2A). GMR>hid, Gal4 larval eye discs exhibit strong CP (Fig. 2A') with similar numbers of cells re-entering S-phase compared to GMR-hid (p-value=0.39). We tested whether inhibiting CP would result in a detectable adult eye phenotype by expressing the mammalian CDK inhibitor p21 or dsRNA targeting Cyclin E in GMR>hid, Gal4 eye discs. In these genotypes, CP is blocked and a reproducible adult eye phenotype results in which pigment is reduced (Fig. 2B-C'). Because pigment cells are one of the last cell types to differentiate, this result suggests the undifferentiated, precursor pool is reduced when CP is blocked and cells that undergo CP contribute to the adult eye. Importantly, the SMW does not appear to be affected in GMR-Gal4, GMR-hid/UAS-p21 discs (Fig. S3A,B), in contrast to GMR-p21 flies in which the SMW is ablated (Fig. S3C, (de Nooij and Hariharan, 1995)). These data suggest the accumulation of Gal4 required to drive high UAS-
transgene expression does not occur until posterior to the SMW, allowing us to assess phenotypes during CP without confounding defects in the SMW. In addition, these data indicate we can detect loss of CP by monitoring adult eye phenotypes.

Using this approach, we screened a collection of UAS-RNAi lines targeting Drosophila transcription factors. Our rationale for this strategy is that many signaling pathways affecting cell cycle entry and CP have transcriptional output. In addition, multiple inputs can result in activation of the same transcription factor, and we reasoned that knocking down the transcription factor itself would result in a stronger phenotype than knocking down an upstream component. Of the 544 transcription factors included in both the Fly Transcription Factor Database (Pfreundt et al., 2010) and the Animal Transcription Factor Database (Zhang et al., 2012), we tested the 373 genes for which there was an available RNAi line (Table S1). In our primary screen, candidate genes were identified based on suppression or enhancement of the GMR>hid, Gal4 eye adult phenotype (Fig. 2D). luciferase (luc) RNAi was used as a negative control. Twelve UAS-RNAi lines caused lethality with both GMR>hid, Gal4 and GMR-Gal4 alone and were not examined further (Table S1).

Fifty two UAS-RNAi lines modified the GMR>hid, Gal4 adult eye phenotype: three acted as suppressors, including glass, which binds to and activates GMR (Fig. 2E,S4); twelve displayed a small eye (Enhancer Category I, Fig. 2F,S4); seven displayed moderate pigment loss (Enhancer Category II, Fig. 2G,S4); twenty one fell displayed mild pigment loss (Enhancer Category III, Fig. 2H,S4); and two fell into a category we termed “Other” (Fig. S4). These 52 lines were also crossed to GMR-Gal4 alone to determine whether they disrupted eye development independently of GMR-hid (Fig. S4). These 52 candidates include genes required for cell proliferation, such
as *E2f1* and *Dp*, and genes required for eye development, such as *cut* and *prospero*, confirming the validity of our approach.

Since many UAS-RNAi lines caused a rough eye phenotype with *GMR-Gal4* alone and thus may disrupt eye development in a process distinct from CP, we performed a secondary screen to assess CP within the larval eye discs (Fig. 2D). In the 52 RNAi lines tested, we observed normal or slightly disrupted CP (Fig. S4). Four RNAi lines caused a striking loss of CP: *Dp, scalloped (sd), fork head*, and *knirps*. *Dp* is known to be required for entry into S-phase (Frolov et al., 2005) and thus was not characterized further. Of the remaining three genes, we focused our studies on *Sd*, a TEAD/TEF transcription factor that regulates growth and apoptosis in many developing tissues (Simmonds et al., 1998; Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008).

### Scalloped is required for compensatory proliferation

CP is substantially reduced in *GMR-hid* discs by *sd* knockdown with either of two independently derived RNAi lines (Fig. 3A,B,G). To verify this result, we tested whether a *sd* mutation would also block CP. Since *sd* null mutants are embryonic lethal (Deshpande et al., 1997), we generated *sd* mutant clones in *GMR-hid* eye discs using the *ey>Fpl/FRT* system (Fig. 3C–C”). While wild type clones contain EdU positive cells in the position of the CP wave, *sd* mutant clones do not (Fig. 3C”,C”). Although apoptosis is slightly decreased in *GMR>hid, sd RNAi* discs compared to *GMR>hid, luc RNAi* (Fig. 3A,B,H), the decrease in CP is not due to the decrease in apoptosis because many *GMR>hid, sd RNAi* discs have the same extent of apoptosis as controls, while none have the same degree of CP as controls (Fig. 3H). Taken together these results indicate Sd is a CP regulator.
Recent results suggest in certain contexts Sd can act as a suppressor of genes controlling apoptosis and growth. This suppressor activity is dependent on the Tondu-domain-containing Growth Inhibitor (Tgi) cofactor (Koontz et al., 2013). We reasoned the slight decrease in apoptosis in GMR>hid, sd RNAi discs could reflect Sd suppressor activity. However, the amount of apoptosis is unchanged in GMR>hid, tgi RNAi discs relative to control (Fig. 3D,H). Interestingly, CP increases in GMR>hid, tgi RNAi discs (Fig. 3D,G), possibly as a result of increased growth gene expression (Fig. 3D). The opposing phenotypes of sd and tgi RNAi in the GMR-hid background make it unlikely Sd is acting as a suppressor to induce CP.

_Yorkie is required for compensatory proliferation_

Sd activates expression of target genes in the eye disc as part of a complex with Yorkie (Yki), the transcriptional effector of the Hippo pathway (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008). The Hippo pathway controls tissue growth by regulation of Yki, whose targets include cell cycle regulators like Cyclin E (Huang et al., 2005) and E2f1 (Goulev et al., 2008) and anti-apoptotic genes such as diap-1 (Huang et al., 2005) and ban (Thompson and Cohen, 2006). Flux through the Hippo pathway is governed by Hippo (Hpo) phosphorylation of Warts (Wts), which in turn phosphorylates Yki. Phosphorylated Yki cannot translocate to the nucleus (Oh and Irvine, 2008). Thus, increased Hippo signaling reduces transcription of Yki target genes. Unphosphorylated, nuclear Yki acts as a co-activator for transcription factors like Sd and the Homothorax (Hth)/Teashirt (Tsh) complex (Peng et al., 2009). Since Yki itself does not bind DNA, it was not included in our initial screen. Targeting yki with two independently derived UAS-RNAi transgenes reduced CP and resulted in an adult eye phenotype similar to sd RNAi (Fig. 3E,G). Levels of apoptosis in GMR>hid, yki RNAi
discs were not significantly different from controls (Fig. 3H). We further probed the contribution of Yki to CP by overexpressing the Hpo kinase domain (dMSTn (Zhang et al., 2008)), which blocks Yki activity. The adult eye phenotypes were similar to sd or yki RNAi (Fig. 3F), and CP in the eye disc was significantly reduced compared to controls (Fig. 3F,G). We conclude Yki is necessary to induce CP in eye discs, suggesting a shared role for Hippo signaling in regulating CP in eye and wing discs.

_Cyclin E is induced by Sd/Yki during compensatory proliferation_

Sd/Yki targets include _Cyclin E, diap1, expanded (ex), and ban_ (Wu et al., 2008; Zhang et al., 2008). Since Cyclin E is required for CP (Fig. 2C), we compared Cyclin E levels in wild type and _GMR-hid_ discs. In proliferating cells, like those anterior to the MF or within the SMW, Cyclin E levels rise during G1 to induce entry into S-phase (Fig. 4A, (Knoblich et al., 1994)), then fall during S-phase and mitosis due to transcriptional (Duronio and O’Farrell, 1995) and post-translational (Moberg et al., 2001) regulation. In wild type eye discs, Cyclin E levels are low in quiescent cells posterior to the SMW (Fig. 4B, (Richardson et al., 1995)). In contrast, Cyclin E expression is high posterior to the SMW in _GMR-hid_ and _GMR>hid, luc RNAi_ discs (Fig. 4C,D). This increase in Cyclin E accumulation occurred primarily in Yan+ undifferentiated cells rather than in photoreceptors (Fig. 4C,D). _sd_ or _yki_ RNAi prevents Cyclin E accumulation (Fig. 4E,F,G). These data suggest Sd/Yki induce Cyclin E expression in regenerating eye discs, driving S-phase entry during CP.

We next examined expression of the Yki targets _ex_ and _ban_ in _GMR-hid_ tissues. While we observe CP (Fig. S5A) and apoptosis (Fig. S5B) in _GMR-hid clones_, we do not observe differences in expression of a transgene that detects _ban_ activity (Brennecke et al., 2003) (Fig. S5C) or _ex-lacZ_ expression (Fig. S5D) between wild
type and GMR-hid clones. Additionally, we do not observe an increase in ban sensor expression or ex-lacZ in GMR-hid discs compared to wild type discs (Fig. S5E,F).

**Yki overexpression rescues GMR-hid phenotypes**

Since Yki overexpression induces Cyclin E and proliferation (Huang et al., 2005), we tested whether increased Yki expression rescues GMR-hid phenotypes. We utilized both wild type Yki and a version of Yki (YkiS168A) that is hyperactive due to reduced phosphorylation by Wts (Oh and Irvine, 2008). GMR-Gal4 driven expression of either Yki or YkiS168A induces Cyclin E and ectopic S phase entry but not apoptosis posterior to the MF (Fig. S6). YkiS168A expression induces a considerable number of cells to enter S phase, resulting in extensive overgrowth in both the larval disc (Fig. S6B’’) and the adult eye (Fig. S6A’’). Similar results were obtained in the GMR>hid, Gal4 background (Fig. 5). Both Yki and YkiS168A expression results in high Cyclin E accumulation throughout the posterior of GMR>hid, Gal4 discs (Fig. 5C-C’’). While the adult eye morphology, CP, and apoptosis appear similar in control and GMR>hid, yki discs (Fig. 5A’,B’,D’), there is an increase of cells in S-phase in GMR>hid, ykiS168A discs (Fig. 5B’’), and overgrowth occurs in both the disc and adult eye (Fig. 5A’’,B’’). Therefore, overexpression of YkiS168A rescues the small eye phenotype caused by GMR-hid, indicating Yki activation is sufficient to overcome cell loss after Hid induction.

Although suppression of the GMR>hid, Gal4 eye phenotype by YkiS168A expression is likely due primarily to extensive over-proliferation, we also measured apoptosis in this genotype. Apoptosis is significantly decreased in GMR>hid, ykiS168A discs, particularly in the second apoptotic wave (Fig. 5D’’,G). Since diap1 is a Yki target and suppressor of apoptosis, we measured diap-lacZ levels after Hid and YkiS168A expression. diap-lacZ levels posterior to the furrow increase after Hid expression and increase further with Hid and YkiS168A expression (Fig. 5E,F). Thus,
Yki-dependent diap1 expression may play a role in limiting cell death after hid induction. Although apoptosis is not increased in GMR>hid, yki RNAi discs, apoptosis is increased in GMR>hid, dMSTn discs (Fig. 3F,H). GMR>hpo discs also display moderate apoptosis, independent of Hid expression (Verghese et al., 2012). These data suggest that in addition to its role in CP, Yki activity can regulate apoptosis during tissue damage.

Our data suggest Yki activation is a key step in CP initiation. We therefore hypothesized Yki activity is limiting for entry into S phase during CP. If true, an increase in Yki posterior to the MF would sensitize cells to CP signals, resulting in earlier S phase entry that would manifest in a shift of the CP wave towards the anterior. Indeed, the distance between the anterior border of the SMW and the anterior border of the compensatory wave in GMR>hid, yki discs is decreased when compared to GMR>hid, luc RNAi (Fig. 5B,H). Because distance along the A/P axis of the eye disc is a proxy for time, these data indicate cells re-enter S-phase sooner in GMR-hid discs expressing additional Yki compared to those without. These data suggest levels of Yki are important for controlling CP timing and active Yki may be the limiting factor for inducing cell cycle re-entry.

Ajuba, an inhibitor of Hippo signaling, is required for compensatory proliferation

How is Hippo signaling inhibited to allow Yki activation during tissue regeneration? Flux through the Hippo pathway is modulated by events at the cell cortex that monitor epithelial integrity and cell-cell interaction (Yu and Guan, 2013). One mechanism for inhibiting Hippo signaling is through the LIM domain protein Ajuba (Jub). Jub antagonizes Hippo signaling and is essential for eye development (Das Thakur et al., 2010). Activated Jub is thought to inhibit Hippo signaling by binding Wts, preventing
Wts from phosphorylating Yki (Rauskolb et al., 2014). *jub* RNAi enhances the GMR>hid, Gal4 adult eye phenotype and reduces CP (Fig. 6A,B,L). This observation is consistent with results in the wing disc where reduced Jub inhibits regeneration (Sun and Irvine, 2013). Jub localizes with DE-cadherin at apical adherens junctions in larval eye discs (Fig. 6C), as in pupal eye discs (Das Thakur et al., 2010) and wing discs (Sun and Irvine, 2013). This localization is most apparent at the apical surface of photoreceptors (Fig. 6D). We did not observe obvious accumulation or re-localization of Jub in GMR-hid clones (Fig. 6E), suggesting increased activation rather than re-localization of Jub may be required to inhibit Hippo signaling during CP.

Jub can be activated in wing discs by JNK signaling (Sun and Irvine, 2013) or by an increase in cellular tension (Rauskolb et al., 2014). Since our data suggest JNK signaling does not play a significant role in CP in the eye disc (Fig. S2B-G), we investigated whether increased cellular tension modulates CP. We induced an increase in cellular tension by expressing the catalytic domain of Rho-kinase (RokCAT), which phosphorylates myosin and leads to increases in myosin contractility (Winter et al., 2001). In GMR>hid, rokCAT discs, CP increases by two-fold, while the adult eye phenotype is not noticeably altered (Fig. 6F,L). Conversely, rok RNAi results in a mild but statistically significant decrease in CP (Fig. 6L). Rok is activated by the Rho1 GTPase (Warner et al. 2010), which is inhibited in the pupal retina by Cdc42 (Warner and Longmore, 2009). Cdc42 also inhibits CP in irradiated wing discs (Warner et al., 2010). We observed an increase in CP in GMR-hid discs with expression of dominant-negative Cdc42 (Cdc42<sup>DN</sup>) (Fig. 6I,L). These results suggest regulation of cellular tension through Cdc42/Rho/Rok plays a role in CP, presumably through Jub regulation.
We predicted increases in cellular tension in the absence of Hid expression would also activate Jub and induce cell cycle re-entry. Indeed, when RokCAT was expressed with \textit{GMR-Gal4} alone, we observed ectopic S phases in the posterior of the disc (Fig. 6G,L). We also observed a low level of apoptosis in \textit{GMR>rokCAT} eye discs (Fig. 6G), similar to previous results in wing discs (Warner et al., 2010). We investigated whether the cell cycle entry in the posterior of \textit{GMR>rokCAT} discs depends on the presence of these apoptotic cells by labeling \textit{GMR>rokCAT}, \textit{p35} eye discs with EdU and anti-CC3 antibodies. p35 blocks RokCAT-induced apoptosis, which we confirmed by the lack of basally extruded cells and pyknotic nuclei, but does not block CC3 staining due to the presence of non-cleaved-caspase epitopes of the anti-CC3 antibodies in undead cells (Fan and Bergmann, 2010). Suppression of apoptosis in \textit{GMR>rokCAT}, \textit{p35} discs did not prevent the appearance of ectopic EdU-positive cells posterior to the SMW, indicating that RokCAT directly induces S phase entry (Fig. 6H,L). We also observed cell cycle re-entry in \textit{GMR>cdc42\textsuperscript{DN}} discs, even when p35 was co-expressed (Fig. 6J-L). In wing discs, co-expression of RokCAT and p35 induces hyperproliferation (Warner et al., 2010), which could be attributed to the formation of undead cells rather than increases in tension directly inducing proliferation. However, as p35 expression blocks CP in post-furrow eye discs and does not induce mitogenic undead cells (Fan and Bergmann, 2008), our results suggest modulation of cellular tension directly induces cell cycle re-entry posterior to the SMW.

**Discussion**

To maintain tissue homeostasis and prevent inappropriate cell divisions, the threshold for S-phase entry is higher in quiescent cells than in cycling cells. Consequently, to efficiently undergo regeneration, quiescent tissues must overcome robust controls that
restrain cell cycle entry. The mechanisms that drive cell cycle re-entry and regeneration in a quiescent cell population are largely unknown, in part because many studies of regeneration have been performed in proliferative tissues. Here we report the results from a genetic screen identifying regulators of tissue damage-induced cell cycle re-entry of a quiescent cell population in *Drosophila*.

**Scalloped and Yorkie regulate compensatory proliferation in eye imaginal discs**

We found that Sd and Yki are required for quiescent cells in the eye imaginal disc to enter the cell cycle in response to tissue damage. Specifically, Sd and Yki are required for Cyclin E accumulation following damage, presumably through transcriptional control of *Cyclin E*. Initial experiments suggested Yki and Sd act together to drive gene transcription, as Sd is required for overgrowth and target gene induction following Yki over-expression in the eye disc (Wu et al., 2008; Zhang et al., 2008). However, their roles during eye development are clearly distinct as *yki* mutant clones grow poorly in the eye disc (Huang et al., 2005) while *sd* mutant clones grow normally (Zhang et al., 2008). In addition, Sd and Yki have both overlapping and unique binding sites throughout the genome, many of which are tissue-dependent (Slattery et al., 2013). Curiously, mutation of *sd* rescues growth defects in *yki* mutant clones, suggesting Yki may relieve Sd-mediated gene repression (Koontz et al., 2013). Additional factors such as Tgi likely function as co-factors for Sd-suppressor function (Koontz et al., 2013). Conversely, Sd/Yki may act synergistically with E2f1 (Nicolay et al., 2011) and GAGA factor (Bayarmagnai et al., 2012; Oh et al., 2013) to drive gene expression. Although our data suggests Sd activates transcription during CP, it will be important to determine how Sd/Yki co-factors are regulated during development and tissue damage to allow robust Sd/Yki target gene expression.
Cyclin/Cdk regulation is a conserved mechanism for inducing regeneration

Cyclin/Cdk regulation is essential for regeneration in many model organisms. In post-mitotic *Caenorhabditis elegans* muscle cells, ectopic expression of cyclins drives DNA replication and cell division (Korzelius et al., 2011). Mice mutant for the CDK inhibitor p21 robustly regenerate lost skin in the ear, which normally does not regenerate (Bedelbaeva et al., 2010). This suggests regulation of Cyclin/Cdk activity may confer regenerative capabilities on otherwise non-regenerative tissues.

Previous work has shown high levels of cell cycle regulators are required drive cell cycle re-entry in post-mitotic cells (Buttitta et al., 2007). The G1-S transition is driven by a positive feedback loop between E2f1 and Cyclin E where E2f1 induces *Cyclin E* transcription, and Cyclin E/Cdk2 inhibits Rbf1, the E2f1 inhibitor. In post-mitotic photoreceptors, high levels of Rbf1 and Dacapo keep E2f1 and Cyclin E/Cdk2 in check, and activation of both E2f1 and Cyclin E/Cdk2 is necessary to overcome cell cycle exit (Buttitta et al., 2007). In quiescent, undifferentiated cells of the developing eye disc high levels of ectopic Cyclin E are sufficient to overcome cell cycle arrest and drive S-phase entry (Richardson et al., 1995). Undifferentiated eye disc cells may be poised to respond to damage-induced Cyclin E expression.

While our results suggest Sd/Yki induce Cyclin E accumulation during CP, there are likely other inputs that drive cell cycle re-entry. The Hedgehog pathway transcription factor Ci, which is required for CP in the post-furrow eye (Fan and Bergmann, 2008), can directly activate *Cyclin E* transcription in the eye disc (Duman-Scheel et al., 2002). Yki and Ci also have other cell cycle targets: Yki can activate *E2f1* transcription in the wing disc (Goulev et al., 2008) and Ci induces Cyclin D expression in the eye disc (Duman-Scheel et al., 2002). We postulate that a combination of Ci
and Sd/Yki activity drives a *Cyclin E*-containing gene expression program that induces cell cycle entry and CP.

**Apoptotic force may link cell death to Yki activation**

In the wing imaginal disc, Yki activity during CP is driven by Jub-dependent inhibition of Hippo signaling (Sun and Irvine, 2013). Our data indicate Jub is also required for CP in the eye, suggesting a shared mechanism for Yki activation between tissues. However, while Jub-dependent inhibition of Hippo signaling in the wing disc requires JNK signaling, we did not find a requirement for JNK signaling for CP in the eye disc. An alternative mechanism for inhibition of the Hippo pathway is tension-induced activation of Jub (Rauskolb et al., 2014). Mechanical force has also been shown to regulate activity of the mammalian Yorkie homolog Yes-associated protein (YAP) (Aragona et al., 2013) and induce cell proliferation in culture (Streichan et al., 2014). We show that expression of Rok or dominant-negative Cdc42, which can increase cellular tension, increases cell cycle re-entry, and we propose cellular tension in the eye disc epithelium modulates CP.

One possible source of increased cellular tension in *GMR-hid* discs is from “apoptotic force” generated by extrusion of dying cells. The phenomenon of apoptotic force has been described in *Drosophila* embryos, where actin/myosin-driven constriction of dying amnioserosa cells exerts force on overlying epithelial cells to drive dorsal closure (Toyama et al., 2008). Apoptotic force in *GMR-hid* discs may increase cellular tension and drive Jub activation. Several observations are consistent with a model that physical extrusion of apoptotic cells is required to generate the force necessary to induce CP. *LGMR-hid*, which induces less apoptosis than *GMR-hid* and presumably less apoptotic force, does not induce CP. Additionally, the apoptotic-
inhibitor p35, which blocks basal extrusion of apoptotic cells, inhibits CP in the eye disc posterior to the MF (Fan and Bergmann, 2008). Finally, GMR-hid clones exhibit S-phase entry in cells within and immediately bordering the clone (Fig. S5, (Fan and Bergmann, 2008)). This observation suggests CP could result from local changes in tension rather than a long range signal.

Conclusions
The Hippo pathway is a well-conserved regulator of tissue growth and is modulated in processes such as regeneration and tumor growth. YAP is required for intestinal regeneration in mice (Cai et al., 2010) and confers regenerative ability in normally non-regenerative mouse hearts (Xin et al., 2013). Importantly, tight control of the Hippo pathway is critical for tissue homeostasis, as YAP hyperactivity or inhibition of the Hippo pathway promotes cancer in many contexts (Johnson and Halder, 2014). Therefore, knowledge gained from studies of CP in Drosophila imaginal discs will contribute to understanding the role of Hippo signaling in mammalian regeneration and cancer.

Experimental Procedures
Mutants and Transgenes
Fly stocks were obtained from the Bloomington Stock Center or from colleagues (see Supplemental Experimental Procedures for more details), with the exception of LGMR-hid (construction described in the Supplemental Experimental Procedures). All lines used for the RNAi screen are from the TRiP at Harvard Medical School, with details provided in Table S1.
Immunostaining

Discs were incubated with 100 μg/mL EdU or 1 mg/mL BrdU for 60 minutes and fixed for 20 min with 4% formaldehyde. EdU detection was performed with the Click-iT EdU Alexa Fluor 555 kit (Molecular Probes) according to the manufacturer’s protocol. Antibodies used are as follows: 1:200 rabbit anti-cleaved Caspase-3 (Cell Signaling), 1:100 rat anti-Elav (7E8A10, DSHB), 1:1000 mouse anti-βGal (DSHB), 1:200 mouse anti-Cyclin E (8B10), 1:500 mouse anti-Yan (8B12H9, DSHB), 1:1000 rat anti-DE-cadherin (DCAD2, DSHB), 1:1000 mouse anti-22C10 (DSHB), 1:200 mouse anti-BrdU (BD Biosciences), 1:500 goat anti-rabbit 488 (Jackson), 1:500 donkey anti-rat Cy5 (Jackson). For CC3 staining, discs were incubated with primary antibodies for 48 hours at 4°C in PBS and a 2 hour PBS-0.1% Triton, 5% NGS block was performed prior to applying secondary antibodies. Fluorescence in situ hybridizations were performed as previously described (Tomancak et al., 2002) with the following modifications: RNA probe was generated by T7 polymerase in vitro transcription in the presence of digoxigenin-11-UTP (Roche) from linearized hid cDNA (clone AT13267, BDGP). Detection was performed with 1:100 peroxidase conjugated anti-DIG (Roche) and 1:50 Cy5 tyramide reagent (Perkin Elmer). See Supplemental Experimental Procedures for details on image quantification.

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**Author Contributions**

Experiments were conceived by JHM and RJD. Experiments were performed and data was analyzed by JHM. Figures were generated by JHM, and the manuscript was written by JHM and RJD.

**Figures**

**Fig. 1.** Hid expression induces CP in the eye imaginal disc.

For all figures, eye discs are from third instar larvae, anterior is orientated to the left, and wild type control is w1118 unless stated otherwise.
A,C,I) Adult eyes of the indicated genotypes. Wild type= *Oregon R*.

B,D,J) DAPI (DNA, single confocal slice in grey) and EdU (S-phase, projection in yellow) staining of eye imaginal discs, indicating the MF (arrowhead) and the SMW (double arrowhead), respectively. Note EdU+ cells in the row of cells around the disc (arrows, B) are margin cells and not part of the disc proper. Asterisk denotes CP (D). Boxes indicate areas of magnification in B’D’,J’.

E,F,K) Cleaved Caspase-3 (CC3) staining of apoptotic cells in discs of the indicated genotypes. The first (1) and second (2) apoptotic waves are indicated in (F).

G,H) In situ hybridization for *hid* mRNA in the indicated genotypes.

L) The eye disc, attached to the anterior (A) antennal disc, is comprised of undifferentiated, proliferating cells (blue) anterior to the MF (green) and both undifferentiated and differentiated quiescent cells (yellow) posterior to the SMW (red). Cells furthest from the MF (most posterior) are the most differentiated.

M) Quantification of CP in the indicated genotypes. All post-SMW, EdU+ eye disc cells were counted. For all graphs, each circle represents the number of cells counted for a single disc, and bars represent mean and one standard deviation. For each genotype, n≥22 discs. **p-value=2.6x10^{-17}

N) Quantification of % of total disc area with CC3 staining. **p-value=2.4x10^{-14}

Scale bars=20 μM.
Fig. 2. Genetic screen for regulators of compensatory proliferation.

A-C) Adult eyes and high magnification view of posterior eye imaginal discs (A’-C’; as in Fig. 1B’) expressing the indicated UAS-transgenes in GMR-hid, GMR-Gal4/+ individuals.

D) Schematic of the RNAi screen.

E-H) Representative examples of the four categories of adult eye phenotypes resulting from the RNAi screen. See also Table S1 and Figs. S3,4.

Scale bars=20 μM.
Fig. 3. *sd* and *yki* are required for compensatory proliferation.

A,B,D-F) Adult eyes (left panels), high magnification of posterior eye discs stained with EdU (middle panels), and apoptosis/CC3 (right panels) in *GMR-hid, Gal4* discs expressing the indicated transgenes. *luciferase (luc)* RNAi is used as a control (A).
C) Clones of wild type (RFP+, cyan) and sd mutant (RFP-) cells in the GMR-hid background. Boxed area in (C) indicates area of magnification (C'-C’’'). Double arrowhead indicates SMW; asterisk indicates CP.

G) Quantification of CP in the GMR>hid, Gal4 background for the indicated UAS-transgenes. For each genotype, n>19 discs.

H) Quantification of % of total disc area with CC3 staining in the GMR>hid, Gal4 background for the indicated UAS-transgenes. For each genotype, n≥15 discs.

*p-value≤3x10^{-3}, **p-value≤3x10^{-13}

Scale bars=20 μM.
Fig. 4. Sd and Yki are required for elevated Cyclin E levels during CP.

A) Eye disc labeled with EdU (magenta) and anti-Cyclin E antibodies (green). Cyclin E accumulates prior to and during S phase of the SMW (arrowhead). Box in left panel indicates area of magnification in middle and right panels.
B-F) Eye discs of the indicated genotypes stained with anti-Cyclin E (green) and anti-Yan (marker of undifferentiated cells, magenta) antibodies.

G) Quantification of Cyclin E staining in Yan+ cells of the indicated genotypes. The ratio of post-SMW Cyclin E staining versus SMW Cyclin E staining is displayed (see Supplementary Experimental Procedures for details). *p-value<1.8x10^{-7}. Significance was calculated for wild type (w^{1118}) vs GMR-hid, GMR-hid vs GMR>hid, luc RNAi, GMR>hid, luc RNAi vs GMR>hid, sd RNAi, and GMR>hid, luc RNAi vs GMR>hid, yki RNAi.

Scale bars=20 μM.
Fig. 5. Expression of transgenic *yki* modifies the *GMR-hid* phenotype.

A) *GMR>hid, Gal4* adult eyes (control in A) after expression of *yki* (A’) or *yki^{S168A}* (A’’).
B) EdU staining (yellow) in eye discs of the indicated genotypes. Arrowhead indicates SMW. The white bar (B,B') indicates the distance measured between the SMW and CP (*) in (H).

C) Cyclin E staining of the indicated genotypes.

D) CC3 staining of the indicated genotypes.

E) β-Gal staining of the indicated genotypes detects diap1-lacZ expression.

F) Quantification of diap1-lacZ expression by β-Gal staining (ratio of posterior to anterior eye disc, normalized to w1118 control) in the indicated genotypes. *p-value<6x10^{-4}, n>11 discs.

G) Quantification of % of total disc area with post-furrow CC3 staining in GMR>hid, Gal4 eye discs expressing luc RNAi (n=16) or yki^{S168A} (n=18). **p-value=3.64x10^{-6}.

H) Quantification of the distance between the SMW and CP wave in GMR>hid, Gal4 eye discs expressing luc RNAi (n=25) or yki RNAi (n=22). **p-value=8.47x10^{-11}.

Scale bars=20 μM.
Figure 6: Jub and cellular tension regulate compensatory proliferation.

A,B,F,I) Adult eyes (left panel) and high magnification of post-furrow EdU staining (right panels) of the indicated genotypes.

C,D) Jub-GFP (yellow) co-localizes with DE-cadherin (magenta, C) and 22C10, a marker of neuronal membranes (magenta, D), at the apical surface of post-furrow eye discs.
E) Clones of GMR-hid (no marker) and wild type (RFP+, magenta) cells expressing Jub-GFP (yellow). Boxes in top panels indicate area of magnification in bottom panels. G,H,J,K) High magnification of post-furrow EdU staining (left panels) and CC3 staining (right panels) of the indicated genotypes. Arrowheads mark the MF. GMR>rokCAT (G) and GMR>cdc42DN (J) induce CC3 positive apoptotic cells posterior to the MF. Interestingly, apoptotic cells are also observed anterior to the furrow in these discs. The mechanism triggering this apoptosis is unknown, but its absence in GMR>rokCAT, p35 (H) or GMR>cdc42DN, p35 (K) discs suggests the anterior induction of apoptosis in GMR>rokCAT and GMR>cdc42DN discs is dependent on apoptosis posterior to the furrow. CC3 staining persists posterior to the furrow after p35 expression (H,K) because undead cells express non-cleaved-caspase epitopes of the anti-CC3 antibodies (Fan and Bergmann, 2010).

L) Quantification of CP in the indicated genotypes. GMR>hid, Gal4 genotypes were compared to GMR>hid, luc RNAi, while GMR>Gal4 genotypes were compared to wild type (w^{1118}). For each genotype, n>14 discs. *p-value<0.02, **p-value<1x10^{-7}.

M) Model for induction of CP in a quiescent epithelium. Text in grey are proposed and have not formally been observed in this study.

1. Hid expression/tissue damage induces apoptosis in a subset of cells.
2. Basal extrusion of apoptotic cells induces apoptotic force (AF), which activates Jub, leading to Wts inhibition.
3. Wts inhibition results in Yki to translocae to the nucleus and act as a transcriptional co-activator for Sd
4. Sd/Yki induce high levels of Cyclin E, which induces cell cycle re-entry (5).

Scale bars=20 μM.
References


Supplementary Data

Supplementary Figures
**Supp. Figure 1 (related to Fig. 1). Characterization of GMR-Gal4, longGMR-Gal4, and GMR-hid transgenes.**

A,F) Expression of GFP (green) driven by GMR-Gal4 (A) or longGMR-Gal4 (LGMR-Gal4, (F)). Higher magnification of posterior cells in the right two panels indicates photoreceptors stained with ELAV (purple). GFP is expressed in all photoreceptors with GMR-Gal4 (A) and in a subset of photoreceptors with LGMR-Gal4 (F).

B-C) DAPI staining of nuclei (red) and staining of apoptotic cells with anti-CC3 antibodies (cyan) on the basal surface of a GMR-hid disc. Box in (B) indicates area of magnification in (C). Pyknotic nuclei (arrows) and glial cell nuclei (arrowheads) are present on the basal surface of the disc.

D-E) EdU staining of cells in S-phase (red in D'') in GMR-hid discs marks the SMW (double arrowhead) and CP (*). CC3 staining (cyan in D'') marks the two waves of apoptosis (1 and 2). Box in D'' indicates area of magnification in E.

Anterior is oriented to the left. Scale bars: 20 μM.
Supp. Figure 2 (related to Fig. 1). Inhibition of signaling through JNK or Tie does not modify the GMR-hid phenotype.

A,B) Apoptotic cells stained with anti-CC3 antibodies in the indicated genotypes.
C,D) EdU incorporation in the posterior of eye discs of the indicated genotypes.
E,F) puc-lacZ expression, marked by βGal in yellow, is similar in wild type (E) and GMR-hid (F) tissues.
G) Quantification of compensatory proliferating cells in the GMR>hid,Gal4 background for the indicated UAS-transgenes. All post-SMW, EdU* eye disc cells were counted. Each circle on the graph represents the number of cells counted for a single disc. For each genotype, n≥17 discs. Bars represent mean and one standard deviation. n.s., not significant. While GMR>hid, bskDN CP is significantly increased compared to GMR>hid, luc RNAi (*P=0.047), it is not significantly different from another control (GMR>hid, Gal4/CyO; P=0.43). Therefore, we are wary about drawing conclusions about the biological significance of an increase in CP in GMR>hid, bskDN discs.

Anterior is oriented to the left. Scale bars: 20 μM.
Supp. Figure 3 (related to Fig. 2). Transgenes expressed using *GMR-Gal4* do not affect the second mitotic wave.

A) *GMR>*hid, luc RNAi* eye discs stained with DAPI (grey) and EdU (yellow) to indicate MF (arrowhead) and SMW (double arrowhead), respectively.

B) The SMW appears normal when p21 is expressed with *GMR-Gal4* in the *GMR-hid* background.

C) Expressing p21 via *GMR-p21* results in ablation of the SMW.

Anterior is oriented to the left. Scale bars: 20 μM.
Supp. Figure 4 (related to Fig. 2). 52 RNAi lines caused a change in the *GMR-hid* adult eye phenotype.

Each UAS-RNAi line was crossed to *GMR>hid, Gal4*. For each line, the adult eye phenotype is displayed in the left column, while the EdU staining of the posterior eye disc is displayed in the right column (centered on the compensatory wave; scale bar: 20 μM and magnification is the same throughout; anterior is oriented to the left). Each line was also crossed to *GMR-Gal4* alone (adult eye phenotype in third column). See Table S1 for full gene names and all RNAi lines tested. First row of images represents the “No RNAi” control. Phenotype classes (see text for explanation): Suppressors=green, Enhancer Class I=blue, Enhancer Class II=purple, Enhancer Class III=pink, Other=orange.
Supp. Figure 5 (related to Fig. 4). A *bantam* sensor and *ex-lacZ* are not induced by *hid* expression in the eye disc.

A-D) *GMR-hid* clones (RFP negative, arrowheads) exhibit CP, marked by EdU (cyan, A), and apoptosis, marked by anti-CC3 staining (cyan, B). *bantam*, measured by the *bantam* sensor-GFP (cyan, C), and *ex-lacZ*, marked by β-Gal (cyan, D), are not induced in *GMR-hid* clones.

E) *bantam* sensor-GFP expression in control (E) and *GMR-hid* (E’) eye discs.

F) *ex-lacZ* induction, marked by β-Gal, in control (F) and *GMR-hid* (F’) eye discs.

Anterior is oriented to the left. Scale bars: 20 μM.
Supp. Figure 6 (related to Fig. 5). Expression of Yki or Yki$^{S168A}$ induces larval and adult eye phenotypes.

A) Adult eyes with GMR-Gal4 alone (A), with UAS-Yki (A’), or with UAS-Yki$^{S168A}$ (A”).
B) DAPI staining of nuclei (grey) and EdU staining of S phase cells (yellow) in the indicated genotypes.
C) Staining with anti-Cyclin E antibodies in the indicated genotypes.
D) Staining of apoptotic cells with anti-CC3 antibodies in the indicated genotypes.

Anterior is oriented to the left. Scale bars: 20 μM.
**Supplementary Experimental Procedures**

**Fly stocks**

RNAi lines used in the screen are listed in supplementary material Table S1. Additional fly stocks used are as follows, with full genotype and Bloomington Stock Center number or providing lab listed in parentheses: **GMR-hid** \(P[w^{+mC}=GMR-hid]G1, \#5771\), **Tie RNAi** \(y^1 \text{v}^1; P\{TRiP.HMJ21428\}attP40, \#54005\), **UAS-bsk^{DN}** (made by K. Matsumoto, obtained from J. Poulton), **UAS-puc** (made by A. Martinez Arias, obtained from J. Poulton), **puc-lacZ** \((\text{puc-lacZ}^{E69}, \text{made by A. Martinez Arias, obtained from J. Poulton})\), **Luciferase (luc) RNAi** \((y^1 \text{v}^1; P\{TRiP.JF01355\}attP2, \#31603\), **Cyclin E RNAi** \((y^1 \text{w}^{1118}; P\{GMRP21\}Ex3/TM3, Sb^1 \text{Ser}^1, \#8414\), **sd RNAi-2** \((sd (N+C) \text{RNAi}, J. Jiang)\), **FRT sd** \((\text{FRT19A sd}^{47m}, \text{D. Pan})\), **FRT RFP** \((P\{\text{Ubi-mRFP.nls}\}1, \text{w}^{1118}, P\{\text{neoFRT}\}19A, \#31416\), **FRT GMR-hid; ey>FLP** \((P\{\text{GMR-hid}\}SS1, y^1 \text{w}^* P\{\text{neoFRT}\}19A; P\{\text{UAS-FLP.D}\}JD2, \#5248\), **Tgi RNAi** \((y^1 \text{sc}^* \text{v}^1; P\{TRiP.HMS00981\}attP2, \#34394\), **yki RNAi-1** \((y^1 \text{v}^1; P\{TRiP.HMS00041\}attP2, \#34067\), **yki RNAi-2** \((y^1 \text{v}^1; P\{TRiP.JF03119\}attP2, \#31965\), **UAS-dMSTn** \((J. Jiang)\), **bantam sensor** \((\text{made by S. Cohen, obtained from T.T. Su})\), **ex-lacZ** \((\text{w}^*; \text{ex}^{6T})\), **Diap1-lacZ** \((y^1 \text{w}^*; P\{\text{lacW}\}Diap1^{5C8}/TM3, Sb^1, \#12093\), **UAS-yki** \((y^1 \text{w}^*; P\{\text{UAS-yki.GFP}\}4-12-1, \#28815\), **UAS-yki^{S168A}** \((\text{w}^*; P\{\text{UAS-yki.S168A.V5}\}attP2, \#28818\), **jub RNAi** \((y^1 \text{sc}^* \text{v}^1; P\{TRiP.HMS00714\}attP2, \#32923\), **jub-GFP** \((\text{w}^*; P\{\text{Jubl.T.Avic.GFP}\}18A/TM2, 56806\), **UAS-RokCAT** \((y^1 \text{w}^*; P\{\text{UAS-Rok.CAT}\}3.1, \#6669\), **UAS-Cdc42^{DN}** \((\text{w}^*; P\{\text{UAS-Cdc42.N17}\}3, \#6288\).

Genotypes for clones are as follows:

- **sd clones:** FRT19A sd^{47m}/FRT19A Ubi-mRFP.NLS; GMR-hid/+; ey-Gal4, UAS-FLP/+  
- **GMR-hid clones:** FRT19A GMR-hid/FRT19A Ubi-mRFP.NLS; ey-Gal4, UAS-FLP/+  
- **GMR-hid clones with bantam sensor:** FRT19A GMR-hid/FRT19A Ubi-mRFP.NLS; bantam sensor GFP/+; ey-Gal4, UAS-FLP/+
GMR-hid clones with ex-lacZ: FRT19A GMR-hid/FRT19A Ubi-mRFP.NLS; ex-lacZ/+
ey-Gal4, UAS-FLP/+  
  
jug-GFP clones: FRT19A GMR-hid/FRT19A Ubi-mRFP.NLS; jug-GFP/ey-Gal4, UAS-FLP

**longGMR-hid transgene construction**

To make longGMR-hid transgenic flies, the longGMR (LGMR) enhancer and hid ORF were cloned into pMINTGATE, a kind gift from J. Pearson. LGMR-Gal4 transgene was obtained by PCR amplification from LGMR-Gal4 flies (Bloomington #8121) with primers white 2161 (forward, GTGTCGCTCGTTGCAGAATA) and Gal4R (reverse, GCCTTGATTCCACTTCTGTCA). The longGMR enhancer was then PCR’ed from this fragment with primers LGMRpE F (forward, CACCCAAGCTTTCGCGAGCTCG) and LGMRpE R (reverse, TTTCGCCGGATCTCGACAATAG) and cloned into pENTR/D-TOPO (Invitrogen); pENTR LGMR was then recombined into pMINTGATE using the Gateway LR cloning system (Invitrogen), resulting in pMG LGMR. The hid ORF (sequence from BDGP clone AT13267) was synthesized by GenScript with AgeI and SpeI sites for cloning into pMG LGMR, which replaced the GFP, but retained the plasmid’s SV40 3’UTR. The resulting construct (pMG LGMR hid) was injected by BestGene Inc into the attP40 site to make transgenic flies.

**Image quantification**

ImageJ (NIH) was used for all quantification. For all statistical measurements, p-values were calculated using the T-test function in Microsoft Excel with two-tailed distribution and two-sample unequal variance.

**Measurement of SMW to CP distance**

Z-projections were made in ImageJ of EdU staining in control (GMR>hid, luc RNAi) or experimental (GMR>hid, yki) eye discs. From these images, the physical distance from the anterior edge of the SMW to the anterior edge of the CP wave was measured. To
mitigate confounding effects from preparation artifacts at the dorsal and ventral edges of the disc (e.g. curling over), the distance between the SMW and CP was measured at the approximate midpoint (determined qualitatively) along the D-V axis. In all cases, the farthest distance was measured. Since the CP wave is not entirely synchronous, single EdU⁺ cells considerably anterior to other cells in the wave (>10 µM away) were considered anomalies and were not considered in our determination of the anterior CP edge.

Cleaved-Caspase 3 staining
For cleaved Caspase-3 staining quantification, projections of images with anti-CC3 antibody staining were used to calculate total disc area in ImageJ (Huang thresholding to capture entire disc, followed by measurement of total area of particles >100 pixels, which in all cases was one particle, ie the whole disc) and area of CC3 posterior to the furrow (RenyiEntropy thresholding to capture CC3 staining, followed by measurement of total area of particles >3 pixels, which were all cells with CC3 staining). Thresholding was set manually to account for differences in background and signal between samples. Area was used as a measurement rather than total number of CC3⁺ cells as fragmented cells with pyknotic nuclei could not be unambiguously counted as one or multiple cells. The total area of the disc was used to normalize the area of CC3 staining so that the measurement of CC3 staining is displayed as a percent of total disc area.

Diap-lacZ / β-Gal staining
β-Gal staining from discs with Diap-lacZ expression was quantified by calculating the ratio of average intensity of staining posterior and anterior to the furrow. For posterior measurements, a selection containing at least 50 undifferentiated cells (identified by their location apical to the glial cells) in a single slice was made posterior to the furrow. This selection specifically did not include any glial, peripodial, margin, or pre-furrow nuclei, which could confound our measurements. DAPI staining was used to create a ROI containing nuclei. β-Gal fluorescence intensity was then measured in this nuclear ROI. A similar measurement was made with cells anterior to the furrow to normalize differences
in staining between samples. The ratios of average nuclear β-Gal intensity in posterior versus anterior disc cells were used to compare genotypes.

**Cyclin E staining**

Because staining with anti-Cyclin E antibodies is variable throughout the posterior of eye discs, presumably due to differences in Cyclin E protein accumulation, in each disc we measured the average Cyclin E staining intensity for undifferentiated cells, where differences between genotypes appeared greatest. Since fluorescence from glial, peripodial, or photoreceptor cells could confound our measurements, we gated for undifferentiated cells by applying a mask of Yan staining to Z-stacks of Cyclin E staining. A maximum projection was generated from each gated Cyclin E Z-stack. The resulting image of Cyclin E staining in undifferentiated cells was thresholded using cells with high Cyclin E levels in the SMW as a reference point for Cyclin E positive cells. Since nuclei could not be separated in Z-projected images, area was used as a proxy for cell number. The area of Cyclin E positive cells posterior to the SMW was normalized to the area of Cyclin E positive cells within the SMW. The measurements displayed in Figure 4 and used for quantification are a ratio of post-SMW versus SMW Cyclin E staining area. We considered that the SMW Cyclin E area itself might be different between genotypes, especially considering that Hid expression disrupts the SMW. Therefore, we also measured the area of Cyclin E positive cells posterior to the SMW as a percentage of the total posterior area, based on projections of Yan staining. Statistical comparisons of percent of total posterior disc area with Cyclin E staining for each genotype gave similar significant P-values as our post-SMW versus SMW ratios. We chose to display the post-SMW versus SMW ratios in our results as we feel these measurements better account for differences in staining efficiency than post-SMW area alone.
Table S1.

Click here to Download Table S1