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Multiple mechanisms modulate distinct cellular susceptibilities towards apoptosis in the developing *Drosophila* eye

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Abstract

Although apoptosis is mechanistically well understood, a comprehensive understanding of how cells modulate their susceptibility towards apoptosis in a developing tissue is lacking. Here, we reveal striking dynamics in the apoptotic susceptibilities of different cell types in the *Drosophila* retina over a period of only 24 hours. Mitotic cells are extremely susceptible to apoptotic signals, while post-mitotic cells have developed several strategies to promote survival. For example, photoreceptor neurons accumulate the inhibitor of apoptosis, Diap1. In unspecified cells, Cullin-3-mediated degradation keeps Diap1 levels low. These cells depend on EGFR signaling for survival. As development proceeds, developmentally older photoreceptors degrade Diap1 resulting in increased apoptosis susceptibility. Finally, R8 photoreceptors have very efficient survival mechanisms independently of EGFR or Diap1. These examples illustrate how complex cellular susceptibility towards apoptosis is regulated in a developing organ. Similar complexities may regulate apoptosis susceptibilities in mammalian development and tumor cells may take advantage of it.

INTRODUCTION

Apoptosis is a major form of programmed cell death. Its biochemical mechanisms are evolutionarily conserved (reviewed in (Fuchs and Steller, 2011; Xu et al., 2009)). Essential for apoptosis are caspases, highly specific cell death proteases. They are produced as inactive zymogens and need proteolytic cleavage for activation (Kumar, 2007). Active caspases can be detected using the cleaved Caspase-3 antibody (Cas3*) which cross-reacts with cleaved *Drosophila* caspases (Fan and Bergmann, 2010; Srinivasan et al., 1998; Yu et al., 2002). Once caspases are activated, they cleave a large number of cellular proteins, which triggers the death of the cell. In surviving cells, caspases are inhibited by inhibitor of

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SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures (Figures S1–S6).

apoptosis proteins (IAPs), the most important one in *Drosophila* being *Drosophila* IAP1 (Diap1) (Goyal, 2001; Hay et al., 1994; Wang et al., 1999). Many IAPs including Diap1 carry a C-terminal RING E3 ligase domain capable of auto-ubiquitylation and degradation of the IAP (Yang et al., 2000). The IAP-antagonists Reaper (Rpr), Hid, and Grim induce cell death by stimulating the RING activity and degradation of Diap1 (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Wing et al., 2002; Yoo et al., 2002). Caspases are released from Diap1 inhibition and can now induce apoptosis. Overexpression of the IAP antagonists *rpr*, *hid* or *grim* induces a strong apoptotic response. For example, expression of *hid* or *rpr* under the control of the eye-specific promoter *GMR* causes a strong eye-ablation phenotype (Grether et al., 1995; White et al., 1996) (Figure 1A – C). Hid is unique among the IAP antagonist, because it is negatively regulated by EGFR/Ras/MAPK signaling through inhibitory MAPK phosphorylation and transcriptional downregulation (Bergmann et al., 1998; Kurada and White, 1998).

Despite our detailed knowledge of the biochemical pathways of apoptosis, we know very little about the regulatory mechanisms that control the susceptibility of cells towards apoptosis in the context of a developing organism. It has been widely observed that cells at different developmental stages and of different types can differ in their sensitivity to apoptotic stimuli. As an example, human intestinal cells exhibit segment-specific sensitivities to apoptosis probably due to differential levels of Bcl-2 family of proteins, key regulators of apoptosis in mammals (Gauthier et al., 2001). Furthermore, evading apoptosis is a hallmark of cancer (Hanahan and Weinberg, 2000). Intriguingly, even among different tumor types and depending on their cell cycle status, the susceptibility of tumor cells to apoptosis-inducing anticancer therapies varies (reviewed in (Smith et al., 2000)). However, most of these studies were done in isolated cell lines and only analyzed one or two particular cell types. Thus, a detailed understanding of the survival requirements of all cells in a given tissue at different time points is still lacking.

The developing *Drosophila* eye tissue, the eye imaginal disc, is an ideal system to study distinct cellular apoptotic responses as the timing of specification of every cell type is known, and many cellular markers exist to follow them. In early larval stages, the cells in the eye imaginal disc proliferate continuously to produce the cell mass required for the production of the eye. During mid-third instar larval stage, an indentation known as the morphogenetic furrow (MF) forms at the posterior edge of the eye disc. All cells in the MF are post-mitotic. The MF moves across the eye disc from posterior to the anterior. In and posterior to the MF, cells are assembled into ommatidia, the functional units of the eye. This begins in the MF with the specification of the first photoreceptor neuron, the R8 cell. By definition, this occurs in ommatidial column 0 (Wolff and Ready, 1993). Still in the MF, the R8 cell induces specification of two pairs of photoreceptor neurons, R2, R5 and R3, R4, forming the five-cell precluster. This specification step requires EGFR signaling (Freeman, 1996; Yang and Baker, 2001). While the MF moves on to the anterior, the remaining unspecified cells re-enter the cell cycle and synchronously divide one more time in the second mitotic wave (SMW) to generate sufficient cells for further specification and differentiation. The SMW occurs in columns 2–4. After the SMW, all cells arrest in G1 and are post-mitotic from now on. The next photoreceptor neurons to be specified are R1 and R6

(column 5), followed by R7 (column 6). The specification of these cell types requires EGFR signaling. Due to their position in the photoreceptor cluster, R7 and R8 are referred to as inner photoreceptors, while R1-R6 are outer photoreceptors. The last cell types specified during larval stages are lens-secreting cone cells (columns 11–15). Thus, the portion of the larval eye disc located posterior to the MF represents a developing field with all developmental states. It is composed of mitotic cells in the SMW, post-mitotic yet unspecified cells, and differentiating cells including photoreceptor neurons and cone cells. Therefore, cells in the MF are developmentally the youngest, while cells towards the posterior edge of the disc are becoming increasingly older. During pupal stages, pigment and bristle cells are added. Finally, a wave of apoptosis removes all interommatidial cells that have not been incorporated into the ommatidia. In the end, each ommatidium is composed of 19 different cells (Brachmann and Cagan, 2003).

Here, we conduct a comprehensive analysis of the apoptosis susceptibilities of the different cell types in the posterior half of the eye imaginal disc. We report that these cell types respond differently to expression of the IAP antagonist *hid*. Dividing cells in the SMW are extremely sensitive to *hid*-induced apoptosis. Post-mitotic cells increase their apoptosis resistance in different ways dependent on the status of the specification/differentiation process. Interestingly, as soon as they are specified, photoreceptor neurons accumulate Diap1 and become apoptosis-resistant, while unspecified cells keep Diap1 levels low by Cullin-3-mediated degradation. These cells require EGFR signaling for suppression of *hid*-induced apoptosis. Finally, at later stages of photoreceptor differentiation, outer photoreceptors are re-sensitized to apoptotic signals by down-regulation of Diap1, potentially in a RING-dependent manner. Surprisingly, the developmentally oldest cell, R8, has the highest apoptotic resistance independently of Diap1. In summary, these data give an impression of how mechanistically complex cellular susceptibilities towards apoptosis are regulated in a developing tissue.

RESULTS

Photoreceptor neurons survive and differentiate in *GMR-hid*

To test how the different cell types in the posterior eye disc respond to apoptotic signals, we expressed the IAP-antagonist *hid* in all cells posterior to the MF using the *GMR* promoter (*GMR-hid*), resulting in a strong eye ablation phenotype (Figure 1B) (Grether et al., 1995). *GMR*-driven expression of *hid* causes massive apoptosis in the posterior half of 3rd instar larval eye imaginal discs as observed by cleaved Caspase3-(Cas3*-) labeling and TUNEL (Figure 1D – F) (Fan and Bergmann, 2008, 2010; Srivastava et al., 2007; Udan et al., 2003). However, although *hid* is expressed in all cells posterior to the MF (Figure 4B,D), *GMR-hid*-induced cell death is not uniform, but occurs in two distinct waves separated by an apoptosis-free zone (Figure 1E,F) suggesting that not all cells respond to *hid* in the same manner. The first apoptotic wave is present between ommatidial columns 3–6, the apoptosis-free zone is between columns 7–15, and the second apoptotic wave begins at column 16. Surprisingly, despite the strong apoptotic phenotype and eye ablation of *GMR-hid*, photoreceptor differentiation as judged by the neuronal marker ELAV appears normal in larval *GMR-hid* eye discs (Figure 1G,G'). *hid*-expressing photoreceptor neurons are even

able to project axons into the optic lobe (Figure 1H,H',I,I'), although strong Cas3*-labeling is detectable in the optic stalk (arrows, Figure 1I''). Cleaved caspases are not present in wild-type axons (Figure 1H'') suggesting that they do not fulfill a non-apoptotic function in axons. Thus, although photoreceptor neurons express *hid* and contain cleaved caspases, they are initially surviving and are able to initiate neuronal differentiation.

Due to the initial resistance of photoreceptor neurons towards *hid*-induced apoptosis (Figure 1G), *GMR-hid* imaginal discs are only slightly reduced in overall size at the end of third larval instar stage compared to wild-type eye discs. We found that ~21 ommatidial columns form in *GMR-hid* eye discs compared to ~24 columns in wild-type eye discs (Cagan and Ready, 1989; Wolff and Ready, 1993). Yet, the resulting adult *GMR-hid* eye is almost completely ablated (Figure 1B) (Grether et al., 1995). Therefore, we analyzed the size of the eye disc after puparium formation (APF). 2 hours APF, the size of the pupal *GMR-hid* eye disc is slightly reduced compared to wild-type (Figure S1A,B). The density of the photoreceptor clusters at the posterior end is reduced. 4 hours APF, the second apoptotic wave covers half of the eye disc and the density of the photoreceptor clusters is further declining (Figure S1C,D). The disc is now clearly reduced in size. Interestingly, although the size of the eye disc is decreasing, *GMR-hid* eye-antennal discs start eversion normally. We were unable to recover any *GMR-hid* eye discs after eversion. Thus, in early pupal stages most cells are eliminated in *GMR-hid*. These observations indicate that although photoreceptor neurons are initially resistant to *hid*-induced apoptosis, they appear to become sensitive to apoptosis in later stages.

These observations prompt several important questions: Which cells are dying in the first and second apoptotic waves? Why is cell death blocked in the apoptosis-free zone? How do photoreceptors survive in the presence of cleaved caspases? How are they becoming sensitive to apoptotic signals in later stages?

Proliferating SMW cells die in the first apoptotic wave of *GMR-hid*

The first apoptotic wave and the second mitotic wave (SMW) are spatially very close (Figure 2A,A'; Figure S2). In the SMW, all cells except the specified photoreceptors R8, R2/R5, and R3/R4 are proliferating (Baker and Yu, 2001). The photoreceptor neurons are not affected in the first apoptotic wave (see below and Figure 3). Instead, although the proliferation and apoptosis markers BrdU and Cas3* do not overlap (Figure 2A'; Figure S2) because apoptotic cells do not enter S phase, it appears that proliferating SMW cells are subject to cell death in the first apoptotic wave. This is inferred from the reduced number of cells that label positively for the mitotic marker phosphorylated histone 3 (PH3) in *GMR-hid* imaginal discs compared to wild-type eye discs (arrows, Figure 2B,C). On average, *GMR-hid* eye imaginal discs contain 29±5 PH3-positive cells derived from the SMW compared to 66±4 in wild-type discs (20 discs counted for each phenotype). This reduction of mitotic cells is rescued by overexpression of the caspase inhibitor P35 in *GMR-hid* eye discs (Figure 2D,E). Thus, in the first apoptotic wave of *GMR-hid*, proliferating cells in the SMW are dying suggesting that proliferating cells are very susceptible to *hid*-induced cell death.

Outer photoreceptor neurons R1-R6 and unspecified cells die in the second apoptotic wave of *GMR-hid*

Next, using several cell-type-specific markers, we determined the cell types that undergo cell death in the second apoptotic wave in *GMR-hid* imaginal discs. In this part of the larval eye disc, there are unspecified (interommatidial) cells, photoreceptor neurons and cone cells. Using Yan as a marker for unspecified cells (Figure 3A,B) (Rebay and Rubin, 1995), we found that some unspecified cells are missing in the second apoptotic wave (arrows, Figure 3B'') suggesting that they are dying. Labeling with ELAV as neuronal marker revealed that the density of photoreceptor clusters and the number of photoreceptors per cluster is progressively reduced towards the posterior end (compare Figure 3C with Figure 3D'). Cas3* labeling overlapped with photoreceptor clusters (Figure 3D,D''; arrows) suggesting that photoreceptor neurons are also eliminated in the second apoptotic wave.

To obtain a better understanding of photoreceptor cell death in the second apoptotic wave we analyzed individual or subgroups of photoreceptor neurons. Markers for outer photoreceptor neurons, Rough (R2,5,3,4-specific) and Seven-up (Svp, R3,4,1,6-specific), are initially induced in *GMR-hid* eye discs (Figure 3F,G), but are subsequently eliminated in the second apoptotic wave (Figure 3F–H; see also Figure S3A,B for expression of Rough and Svp in wild type discs). Senseless (Sens) is a marker for R8 photoreceptor neurons, the first photoreceptor to be specified (Nolo et al., 2000). Interestingly, while in many cases almost the entire photoreceptor cluster labels positive for Cas3*, this does not include the R8 cell, and only a few of them are eliminated in the second apoptotic wave during larval stages (Figure 3E–E''').

The last photoreceptor neuron to be specified is R7. Labeling with the R7 marker Prospero (Pros) demonstrates that the pattern of R7 cells in *GMR-hid* is normal (Figure S3C,E). We did not find any Pros-positive cells that are also labeled with Cas3* (Figure S3E,F). Therefore, the majority of R7 neurons, if not all, survive at this stage. Similarly, labeling with Cut, a marker for cone cells, shows that most of them also survive in the second apoptotic wave in *GMR-hid* (Figure S3D,G,H). Thus, these examples demonstrate that *hid*-induced cell death affects differentiating cells differently depending on position and developmental age. The outer photoreceptor neurons R1-R6 are largely eliminated by *GMR-hid* in the second apoptotic wave. R7 and cone cells are the developmentally youngest cells and most of them are still alive at the end of larval stages. Most remarkable, however, is R8, the developmentally oldest cell, which still survives *hid*-induced apoptosis by the end of larval stages. This suggests that R8 contains a very strong intrinsic program that protects it from apoptosis.

EGFR signaling protects unspecified cells in the apoptosis-free zone

To explain the apoptosis-free zone in *GMR-hid* (Figure 1G, Figure 3B), we first examined the distribution of Hid protein. Although Hid is strongly induced posterior to the MF in *GMR-hid* eye discs compared to wild type (Figure 4A,B), a clear reduction of Hid protein is present in the apoptosis-free zone in *GMR-hid* (Figure 4B, arrow). Because EGFR signaling is known to negatively regulate Hid activity (Bergmann et al., 1998; Kurada and White, 1998), we considered that the reduction of Hid protein in the apoptosis-free zone is the result

of EGFR activity. Consistently, the reduction of Hid levels is lost when a dominant negative allele of *EGFR* (*EGFR*^{DN}) is expressed in *GMR-hid* eye discs (Figure 4C). Furthermore, EGFR signaling stimulates MAPK to phosphorylate Hid. Therefore, we tested if MAPK phosphorylation destabilizes Hid protein in the apoptosis free zone. A MAPK-unresponsive mutant of Hid (Hid^{Ala5}) is indeed stable in the apoptosis-free zone (Figure 4D). These data imply that EGFR/MAPK-dependent regulation of Hid protein levels contributes to the apoptosis-free zone.

The apoptosis-free zone in *GMR-hid* contains photoreceptor neurons at the apical side and unspecified cells at the basal side of the disc. Although it is known that EGFR signaling protects cells between columns 7 and 15 (Baker and Yu, 2001), it is unclear which of these cell types require EGFR signaling for survival, because Baker and Yu (2001) analyzed *egfr* mutant clones (Baker and Yu, 2001) in which photoreceptor differentiation is blocked. To reinvestigate this question, we examined eye discs expressing the EGFR/MAPK-unresponsive *GMR-rpr* and *GMR-hid*^{Ala5} transgenes (Bergmann et al., 1998; Kurada and White, 1998). In these discs, Cas3*-labeling now covers the entire posterior half of the disc including the area corresponding to the apoptosis-free zone in *GMR-hid* (Figure 4E',F'). The majority of photoreceptor neurons as indicated by ELAV still survives in EGFR/MAPK-unresponsive *GMR-rpr* and *GMR-hid*^{Ala5} eye discs (Figure 4E'',F''). Consistently, sections through the areas which correspond to the apoptosis-free zone show that ELAV labeling is largely excluded from Cas3* labeling which occurs mainly at the basal side of the discs (Figure 4E,F and sections therein). Moreover, expression of *EGFR*^{DN} results in loss of the apoptosis-free zone in *GMR-hid* eye discs (Figure 4G'), while expression of *EGFR*^{DN} in otherwise wild-type background induces cell death specifically in the area which corresponds to the apoptosis-free zone in *GMR-hid* (Figue 4H'). Taken together, these observations indicate that photoreceptor neurons still survive under the conditions of reduced EGFR signaling, while unspecified cells die. Therefore, EGFR signalling is required to protect unspecified cells in the apoptosis-free zone in *GMR-hid* eye discs.

Intriguingly, while EGFR signalling is required for photoreceptor differentiation (Freeman, 1996; Yang and Baker, 2001), these cells do not rely completely on EGFR signalling for survival and appear to have developed other mechanisms to protect themselves from apoptosis (see below). The opposite statement can be made for unspecified cells. While EGFR signalling is not sufficient to induce photoreceptor differentiation of unspecified cells, it does protect them from *hid*-induced apoptosis.

A transient increase of Diap1 reduces apoptosis susceptibility of photoreceptor neurons

As shown in Figure 4D, *GMR-hid* is expressed in all cells posterior to the MF. However, photoreceptor neurons are protected from apoptosis in the first apoptotic wave and apoptosis-free zone independently of EGFR although they contain cleaved caspases (Figure 1G', I''). We therefore investigated how photoreceptor neurons survive despite the presence of cleaved caspases. Because Diap1 is known to inhibit cleaved effector caspases (Ditzel et al., 2008; Li et al., 2011), we examined its protein level in 3rd instar eye discs using Diap1 antibodies. Interestingly, as soon as photoreceptor neurons are specified as judged by ELAV labeling, we detect a strong increase of Diap1 levels in photoreceptor neurons (Figure

5A,B). Diap1 levels remain low in the unspecified interommatidial cells at levels comparable to the tissue anterior to the MF (Figure 5A',B'). A detailed high-resolution analysis suggests that all eight photoreceptor neurons accumulate Diap1 protein while Diap1 levels in interommatidial cells remain low (Figure S4A–C). In each ommatidium, the photoreceptor axons form a bundle projecting towards the optic lobes of the brain. The brightest signals of Diap1 expression appear to be in axon bundles (Figure 5A'',B,B') implying that they inhibit cleaved caspases which are observed here at high levels (Figure 1I''). Consistent with a protective function of Diap1, loss of one copy of *diap1* enhances the *GMR-hid*-induced eye ablation phenotype (Hay et al., 1995) (Figure 5I,J) through increased cell death in both apoptotic waves and a reduction in the size of the apoptosis free zone (Figure S4D,E). The specificity of the Diap1 antibody used was confirmed in *diap1* mosaics (Figure S4F).

Interestingly, we also observed that protein levels of Diap1 are gradually reduced in developmentally older photoreceptor neurons towards the posterior end of eye discs (Figure 5A'; arrow). We hypothesized that this reduction of Diap1 protein re-sensitizes older photoreceptors to *hid*-induced apoptosis such that they die in the second apoptotic wave. To test this hypothesis, we overexpressed Diap1 using *GMR* (*GMR-diap1*) (Hay et al., 1995). However, while *GMR-diap1* is sufficient to suppress the first apoptotic wave, it did not suppress the second apoptotic wave (Figure 5E) causing only a partial suppression of *GMR-hid* (Hay et al., 1995) (Figure 5G). Nevertheless, we noted that even overexpressed Diap1 protein is subject to the same mechanism that induces down-regulation of Diap1 in older photoreceptor neurons (Figure 5C, arrow). Because RING mutants of Diap1 can suppress *GMR-hid*-induced apoptosis (Goyal et al., 2000), we tested expression of a RING domain-deleted form of Diap1 (*GMR-BIR*) (Hay et al., 1995) in *GMR-hid* background. Consistently, loss of the RING domain resulted in a stabilization and accumulation of Diap1 in developmentally older photoreceptor neurons (Figure 5D, arrow). As a consequence, *GMR-BIR* completely suppressed *GMR-hid*-induced apoptosis (Figure 5F) and its adult eye ablation phenotype (Figure 5H). These data suggest that the protein levels of Diap1 determine the apoptosis susceptibility of photoreceptor neurons. While young photoreceptor neurons at the MF increase Diap1 levels, older photoreceptors down-regulate these levels, likely by a RING-dependent mechanism. Therefore, reduced Diap1 levels may account for increased susceptibility to apoptotic signals in older photoreceptor neurons, explaining the formation of the second apoptotic wave in *GMR-hid* eye discs (Figure 3).

Control of Diap1 protein levels by Cullin-3 E3 ligase

Next, we asked how Diap1 levels are transiently increased in differentiating photoreceptor neurons. We first examined whether the increased levels of Diap1 are due to transcriptional regulation. In situ hybridization of *diap1* suggested that, unlike the protein level, transcription of *diap1* does not show a neuron-specific pattern (Figure S5) consistent with previous reports (Hays et al., 2002; Udan et al., 2003)). To further investigate this at the single cell resolution, we used *diap1-lacZ*, an enhancer trap indicating transcriptional expression of *diap1* (Ryoo et al., 2002). Both photoreceptor neurons and interommatidial, unspecified cells show comparable expression levels of P-galactosidase (Figure 6A)

suggesting that the high protein levels of Diap1 in photoreceptor neurons are due to post-transcriptional regulation.

To further investigate how Diap1 is regulated post-transcriptionally, we focused our analysis on ubiquitin-dependent protein degradation (reviewed in (McCarthy, 2013)). In this process, ubiquitin is transferred and attached to the substrate protein through E1-activating enzymes, E2-conjugating enzymes, and E3-ubiquitin ligases consecutively. Poly-ubiquitylated proteins are then degraded by the 26S proteasome. As Uba1 is the only E1-activating enzyme in *Drosophila* (Lee et al., 2008; Lee et al., 2011; Pfleger et al., 2007), we analysed Diap1 levels in *uba1* mutant tissues. Diap1 is increased in *uba1* mutant clones located both anterior and posterior to the MF (Figure 6B). Posterior to the MF, this increase of Diap1 is prominent in unspecified, interommatidial cells (Figure 6B”; arrows). Similarly, an increase of Diap1 was observed in *prosβ2* mutant tissues, a key component of the 26S proteasome (Figure 6C). These data suggest that in posterior eye tissue, Diap1 levels are kept low specifically in unspecified interommatidial cells through an ubiquitin-dependent protein degradation mechanism maintaining these cells in an apoptosis-susceptible state if they are not protected otherwise (EGFR). Diap1 protein in young photoreceptor neurons, however, is not subject to this degradation mechanism. These cells accumulate Diap1 and increase their apoptotic resistance.

We sought to identify the E3 ubiquitin ligase that targets Diap1. Cullin (Cul) family proteins are the largest known class of ubiquitin E3 ligases (reviewed in (Petroski and Deshaies, 2005)). Both Cul1 and Cul3 have been implicated in regulation of Ci, a key transcription factor in the Hedgehog signaling pathway, in developing *Drosophila* eye tissues (Ou et al., 2002). We therefore analyzed whether Diap1 can be regulated by Cul1 and Cul3. While *Cul1* mutants do not affect Diap1 protein levels (Christiansen et al., 2013), they are increased in *Cul3* mutant tissues (Figure 7A,A’). As controls, such an increase is not due to increased cell proliferation (Figure 7A’’) or increased numbers of cells (Figure S6A). Consistent with the role of Cul3 in protein degradation, in situ hybridization analysis shows that *diap1* transcripts are not increased in *Cul3* mutant tissues (Figure S6B).

We also asked whether the increase of Diap1 in *Cul3* mutants is functional and can suppress apoptosis. To address this, *Cul3* mutant clones were generated in *GMR-hid* eye discs. *GMR-hid*-induced Cas3* activity is suppressed in *Cul3* clones (Figure 7B). Consequently, *GMR-hid*-induced adult eye ablation phenotype is suppressed by *Cul3* mosaic tissues (Figure 7C,D). We further examined whether Cul3 can regulate developmental apoptosis. We observed that, in mid-pupal eye discs, expression of Diap1 is specifically increased in *Cul3* mutant interommatidial cells (arrows, Figure 7E), but not in photoreceptor neurons. Such an increase does inhibit developmental apoptosis of interommatidial cells at this stage (Figure 7F). As a result, many additional interommatidial cells survive in *Cul3* mutant clones (Figure 7G, arrows). Therefore, Cul3 mediates Diap1 degradation in unspecified cells to render them susceptible towards apoptosis during development of the *Drosophila* eye.

We also note that there are more interommatidial cells in *Cul3* clones than what would be expected if only apoptosis was suppressed (Figure 7G). Because we do not see a strong effect on proliferation in *Cul3* clones (Figure 7A’’), this may not be due to increased

proliferation. Instead, it appears that *Cul3* clones partially affect photoreceptor specification resulting in an increased number of interommatidial cells.

DISCUSSION

In this paper, using the *GMR-hid* apoptotic model in *Drosophila*, we reveal striking differences in the apoptotic susceptibilities of different cell types in the *Drosophila* retina over a developmental period of only 24 hours (Figure 7H,I). Proliferating cells in the SMW are very susceptible to apoptotic stimuli. In contrast, post-mitotic cells have acquired different mechanisms which can confer – at least transiently - resistance to apoptosis. As previously shown, unspecified cells rely on EGFR signaling for survival (Baker and Yu, 2001). The signal for EGFR activation, Spitz, is likely coming from photoreceptor neurons which require EGFR signaling for specification (Baker and Yu, 2001). Interestingly, although photoreceptor cells require EGFR signaling for specification (Tio and Moses, 1997), the survival of photoreceptor neurons does not solely depend on EGFR signaling. Instead, photoreceptor cells dramatically increase the protein levels of Diap1 at the onset of specification. In contrast, unspecified cells at the basal side of the disc degrade Diap1 by a Cul3-dependent mechanism. The survival of inner photoreceptors R8 and possibly R7 appears to be independent of EGFR signaling and increased Diap1 levels.

Dynamic Diap1 protein pattern

The protein level of Diap1 is under very dynamic control in developing eye imaginal discs (Figure 7H,I). Its level is relatively low in proliferating cells anterior to the MF, in SMW cells and in unspecified cells posterior to the MF. Under normal conditions, these low levels are sufficient to ensure survival of the cells, however, they are very susceptible to apoptotic signals. Our genetic analysis indicates that a Cul3-mediated degradation process maintains Diap1 at low levels in unspecified cells posterior to the MF (Figure 7H,I). Loss of *Cul3* results in accumulation of Diap1 causing survival of many additional interommatidial cells in pupal eye discs. Developmentally, it is likely that this Cul3-mediated Diap1 degradation keeps interommatidial cells susceptible to apoptosis so that they can be eliminated by apoptosis if they have not been incorporated into ommatidia at around 28 hours APF (Brachmann and Cagan, 2003).

The control of Diap1 levels by Cul3 adds another level of complexity to Diap1 regulation. For example, Diap1 uses its own RING E3 domain for auto-ubiquitylation and degradation (see below) (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Wing et al., 2002; Yoo et al., 2002). The kinase IKKε phosphorylates Diap1 and promotes its degradation for proper cell fate specification (Kuranaga et al., 2006). Furthermore, Diap1 is transcriptionally and post-translationally controlled by Hippo signaling to prevent apoptosis during tissue growth (Harvey et al., 2003; Udan et al., 2003; Wu et al., 2008; Zhang et al., 2008).

Interestingly, Cul3 is produced as soma-specific ($Cul3_S$) and testis-specific ($Cul3_T$) isoforms (Arama et al., 2007). $Cul3_T$ has been shown to regulate caspase activity in a non-apoptotic process called individualization in developing sperm. However, $Cul3_T$ does not target Diap1 for degradation, but another IAP, termed dBruce (Kaplan et al., 2010). Therefore, in the testis, $Cul3_T$ regulates dBruce for a non-apoptotic function of caspases, while in the eye

disc, $Cul3_S$ controls the apoptotic susceptibility of unspecified cells by triggering Diap1 degradation.

While unspecified cells keep Diap1 levels low by the above-described $Cul3_S$-dependent process, photoreceptor neurons accumulate high levels of Diap1 immediately after they are specified and thus survive the first apoptotic wave. A $Cul3_S$-dependent degradation process of Diap1 does not seem to operate in photoreceptor neurons. However, $Cul3_S$ is ubiquitously expressed in eye imaginal discs (Ou et al., 2002), raising the question how $Cul3_S$-activity and thus Diap1 degradation is restricted to unspecified cells. One possibility is the specific expression of the substrate recognition protein in unspecified cells. Cul3 utilizes BTB-domain containing proteins to recruit substrates for ubiquitylation and degradation (reviewed in (Petroski and Deshaies, 2005)). In *Drosophila*, several BTB proteins including Kelch10 (Arama et al., 2007), Roadkill (Baker et al., 2009), Kelch (Hudson and Cooley, 2010), Insomniac (Pfeiffenberger and Allada, 2012; Stavropoulos and Young, 2011), and Diablo (Strutt et al., 2013) have been shown to mediate Cul3-dependent degradation of various protein substrates. However, none of these BTB proteins is required for regulation of Diap1 by Cul3 (data not shown). Another possibility is that an inhibitor of Cul3 is specifically expressed in photoreceptor neurons keeping Cul3 activity low in these cells. An example of such a Cul3 inhibitor is Soti in the testis (Kaplan et al., 2010). Soti competes with dBruce for binding to Kelch10 which regulates activation of caspases during sperm individualization. However, loss of *Soti* in the eye disc does not affect Diap1 levels (data not shown). Therefore, future work is needed to dissect the specificity of $Cul3_S$-mediated degradation of Diap1 in unspecified cells.

While photoreceptor neurons accumulate high levels of Diap1 starting in the MF, later in development towards the posterior edge of the disc, Diap1 levels are down-regulated. As a result, older photoreceptor neurons, especially R1-R6, become sensitive to apoptotic signals and are eliminated in the 2nd apoptotic wave in *GMR-hid*. This down-regulation of Diap1 appears to be the result of RING-dependent self-degradation of Diap1 because a RING-depleted mutant of Diap1 is not subject to this type of down-regulation. That raises the question how the RING E3 ligase activity is stimulated at the posterior edge of the discs. It was previously shown that the IAP antagonists Reaper, Hid and Grim stimulate RING activity (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Wing et al., 2002; Yoo et al., 2002). However, loss of these genes (*H99* mutant) does not block the down-regulation of Diap1 at the posterior edge of the disc (data not shown), suggesting that another, yet unknown mechanism or factor accounts for stimulation of the RING domain of Diap1 at the posterior edge of the eye disc.

Another question addresses the functional significance of the Diap1 accumulation in specifying photoreceptor neurons. There is no definitive answer to this question either, but there is a possible hint. In strong *diap1* mutant clones which are protected from apoptosis by expression of the caspase inhibitor *p35*, photoreceptor neurons fail to differentiate (Figure S4F) suggesting that Diap1 may have a role in early specification of photoreceptor neurons. This function of Diap1 does not seem to require the RING domain as mutant alleles lacking the RING domain allow photoreceptor differentiation (Figure S4G).

R8, R7 and cone cells

R8, R7 and cone cells survive in the second apoptotic wave during larval stages. R7 and cone cells are developmentally the youngest cells and their early specification status may permit their survival during larval stages. However, R8 is exceptional. It is the first photoreceptor neuron to be specified and thus has been exposed to Hid activity for the longest time. Yet, compared to R1–6, R8 is more apoptosis-resistant and survives to the end of larval stages (Figure 3E). Because R8 also does not require EGFR signaling for either specification or survival at any time during development (Baker and Yu, 2001; Yang and Baker, 2001) and it down-regulates Diap1, the survival of R8 appears to require a different mechanism. A possible survival mechanism may be exerted by the R8 specification gene *senseless* (*sens*) (Nolo et al., 2000). *sens* has been shown in a different cellular context (salivary glands in embryos) to have a strong anti-apoptotic activity (Chandrasekaran and Beckendorf, 2003). It is currently unknown if *sens* has a similar protective function in R8.

Relevance for cancer

Cancer cells have developed many strategies to evade apoptosis. One such strategy is the accumulation of IAPs in many tumor types (LaCasse et al., 2008). It is therefore very important to identify the mechanisms that control IAP levels, both transcriptionally and post-translationally. Our work here in *Drosophila* shows that the lack of a Cul3-mediated degradation process can lead to accumulation of Diap1 and survival of interommatidial cells. It is conceivable that similar Cullin-dependent processes control IAP levels in mammals including humans and that cancer cells may inactivate them to up-regulate IAPs.

In conclusion, the cellular steady-state level of Diap1 appears to be a key modulator of cellular apoptosis susceptibility. Multiple mechanisms are employed to regulate Diap1, hence the cellular susceptibility to apoptosis varies from cell to cell depending on the developing context. As several IAP family proteins exist in mammals as caspase inhibitors (reviewed in (Silke and Meier, 2013)), it is likely that cellular sensitivity and susceptibility to apoptosis can also be modulated at the level of IAP proteins in mammals.

EXPERIMENTAL PROCEDURES

Fly Strains and Crosses

Both transgenic lines *GMR-hid*^{10} (Grether et al., 1995) and *GMR-hid*^{326} (Fan and Bergmann, 2008) with insertions on the 2nd and 3rd chromosome, respectively, gave similar waves of cell death in developing eye discs. *4xGMR-rpr* was obtained by combining *CyO; 2xGMR-rpr* (Kurada and White, 1998) and homozygous *GMR-rpr46* (White et al., 1996). *GMR-hid*^{Ala5} (Bergmann et al., 1998), *UAS-EGFRD*^{N} (Freeman, 1996), *UAS-rpr* (Ryoo et al., 2002), *th*$^{33\text{-}1s}$ (Goyal et al., 2000), *GMR-diap1, GMR-BIR* and *th*^{5} (Hay et al., 1995), *GMR-p35* and *UAS-p35* (Hay et al., 1994), *uba-1*^{D6} (Lee et al., 2008), *Cul3*^{gft2} and *Cul1*$^{[EX]}$ (Ou et al., 2002) are as described. *GMR-Gal4, ey-GAL4, elav*^{C155}*-GAL4, UAS-CD8GFP, ey-Flp, UAS-Flp, diap1-lacZ (th*^{j5c8}*)* and *Pros*β*2* ^{EP3067}*were* obtained from the Bloomington Stock Center.

Mosaic Analysis

The following genotypes were generated to analyze *uba-1*, *Pros*β*2*, or *Cul3* mutant clones in mosaic eye discs: (1) *ey-Flp/+; uba-1^{D6} FRT80B/ P[ubiGFP] FRT80B;* (2) *ey-GAL4/UAS-Flp UAS-p35; Pros*β*2^{EP3067} FRT80B/ P[ubiGFP] FRT80B;* (3) *ey-Flp/+; Cul3^{gft2}FRT40A/ P[ubiGFP] FRT40A;* to analyze *Cul3* clones in *GMR-hid* background: *ey-Flp/+; Cul3^{gft2}FRT40A/ P[ubiGFP] FRT40A; GMR-hid/+*. Crosses were raised at 25°C unless otherwise specified. For each genotype, at least 30 discs were analyzed. Representative data are shown.

Immunohistochemistry

Eye-antennal imaginal discs from wandering 3rd instar larvae or pupae at indicated stages were dissected, fixed (with 4% paraformaldehyde for 30min at room temperature), and then labeled with primary and secondary antibodies as described (Fan et al., 2005). Guinea Pig anti-Diap1 (SK14) was kindly provided by P. Meier (Tenev et al., 2002). Guinea Pig anti-Sens was kindly provided by H. Bellen (Nolo et al., 2000). Rabbit anti-Svp is a gift from R. Schulz. Commercial antibodies used are mouse anti-BrdU (BD), rabbit anti-PH3 (Upstate), rabbit anti-cleaved Caspase-3 (Cell Signaling), rat anti-ELAV, mouse anti-Rough, mouse anti-Pros, mouse anti-Cut and mouse anti-Yan (DHSB, U. of Iowa). Secondary antibodies were donkey Fab fragments conjugated to FITC, Cy3 or Cy5 from Jackson ImmunoResearch.

BrdU Labeling, TUNEL and EdU Double Labeling

For BrdU labeling to detect proliferating cells, larval discs were incubated with 0.5 mg/ml BrdU (Sigma) in Schneider's media for 1 hour at room temperature. Discs were then fixed in 1% paraformaldehyde with 0.01% Tween 20 at 4°C overnight followed by RQ1-DNase (1 mg/ml, Promega) digestion for 2h at 37°C and antibody staining thereafter.

For TdT-mediated dUTP nick end labeling (TUNEL) and EdU double labeling, larval discs were first incubated with 10μM EdU (Molecular Probes) similar as the BrdU labeling described above and then fixed in 4% paraformaldehyde for 30min at room temperature. Fixed discs were further incubated in 100mM Na-Citrate with 0.1% TritonX-100 for 30 min at 65°C followed by detection of dying cells using an *in situ* cell death detection kit (Roche). EdU labeling was done after TUNEL using a Click-iT® EdU imaging kit (Molecular Probes).

In Situ Hybridization

For *in situ* hybridization to detect *diap1* transcripts, *Drosophila* cDNA clone GH15248 (BDGP ESTs, *Drosophila* Genomics Resource Center) was used as a template to generate digoxigenin (DIG)-labeled sense and anti-sense RNA probes (Roche). Labeled probes were detected with NBT/BCIP (Roche) or Tyramide Signal Amplification (TSA, PerkinElmer) as previously described (Chotard et al., 2005).

Imaging

Larval or pupal disc images were taken with either a Zeiss LSM700 or an Olympus FV500 confocal microscope. Adult eyes were photographed by a Zeiss AxioImager equipped with a Zeiss AxioCam HR.

Quantification of mitotic cells

PH3-positive cells in the SMW were counted in wild type, *GMR-hid*, and *GMR-hid;GMR-p35* eye discs (mean ± SD). For each genetic background, 20 representative eye discs were counted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Mitotic cells are extremely sensitive to apoptotic signals.
- Post-mitotic, yet undifferentiated, cells require EGFR signaling for survival.
- Photoreceptor neurons accumulate the apoptosis inhibitor Diap1 for survival.
- Cullin-3 degrades Diap1 in undifferentiated cells.

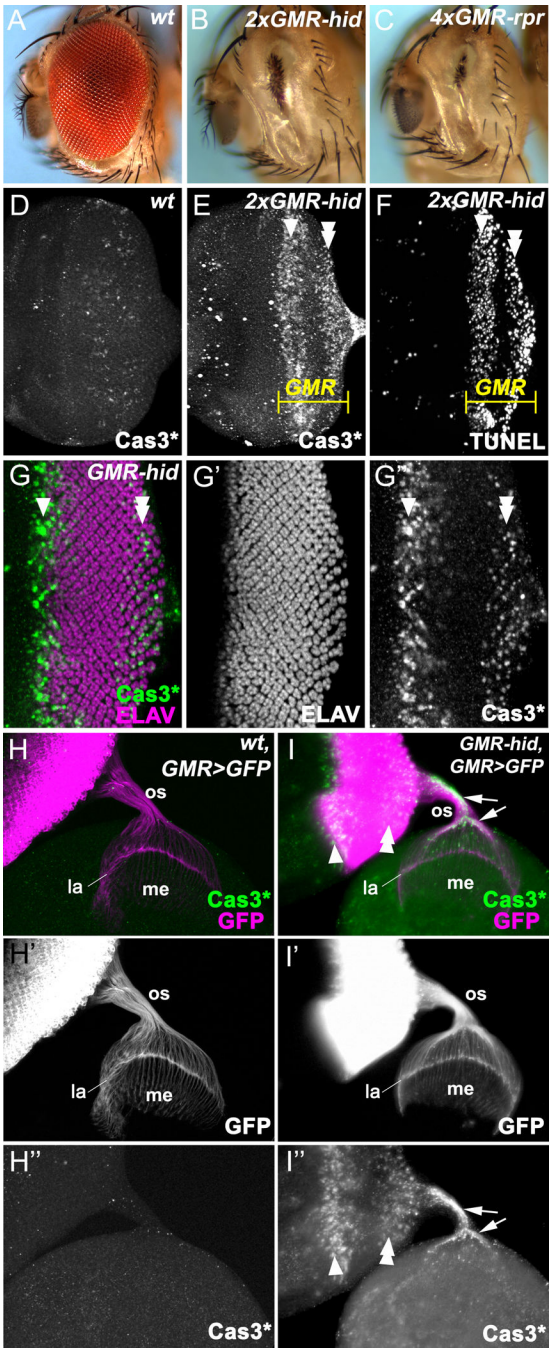

Figure 1. Photoreceptor neurons survive and differentiate in *GMR-hid*

Here, and in the following figures, anterior is to the left. White arrowheads in this and subsequent figures indicate the first apoptotic wave induced by *GMR-hid*, while white double arrowheads indicate the second apoptotic wave.

(**A–C**) Adult eyes of wild type flies (**A**), of flies carrying two copies of *GMR-hid*(**B**), or four copies of *GMR-rpr*(**C**).

(**D–F**) Wild-type (**D**) and *2×GMR-hid* eye discs (**E,F**) labeled with cleaved Caspase-3 (Cas3*) antibodies (**D,E**) and TUNEL assay (**F**). The *GMR* expression domain is indicated

in yellow. In contrast to a few scattered dying cells in wild type (**D**)*GMR-hid* induces two waves of cell death separated by an apoptosis-free zone (**E,F**).
(**G–G’’**)*GMR-hid* eye disc labeled with Cas3* and the neuronal marker ELAV. Despite expression of *hid*, photoreceptor differentiation appears largely normal.
(**H–I**) Eye-brain complexes double labeled with GFP and Cas3* (**H,I**) or GFP (**H’,I’**) or Cas3* (**H’’, I’’**). In wild type (*GMR-GAL4 UAS-CD8GFP*, referred to as *GMR>GFP*), GFP-positive photoreceptor neurons (R1-R8) project their axons through the optic stalk (os) to the optic lobe. R1-R6 axons terminate at the lamina and form the lamina plexus (la) while R7 and R8 axons project to the medulla (me) (**H, H’**). Cleaved caspases are not present in wt optic stalks (**H’’**). The projection pattern of photoreceptor axons in *GMR-hid* eye discs appears normal (**I, I’**). Strikingly, strong Cas3*-labeling was also observed in the optic stalk (**I’’**; arrows) indicating that they contain cleaved caspases. Two apoptotic waves are shown in the *GMR-hid* eye disc in (**I’’**).
See also Figure S1.

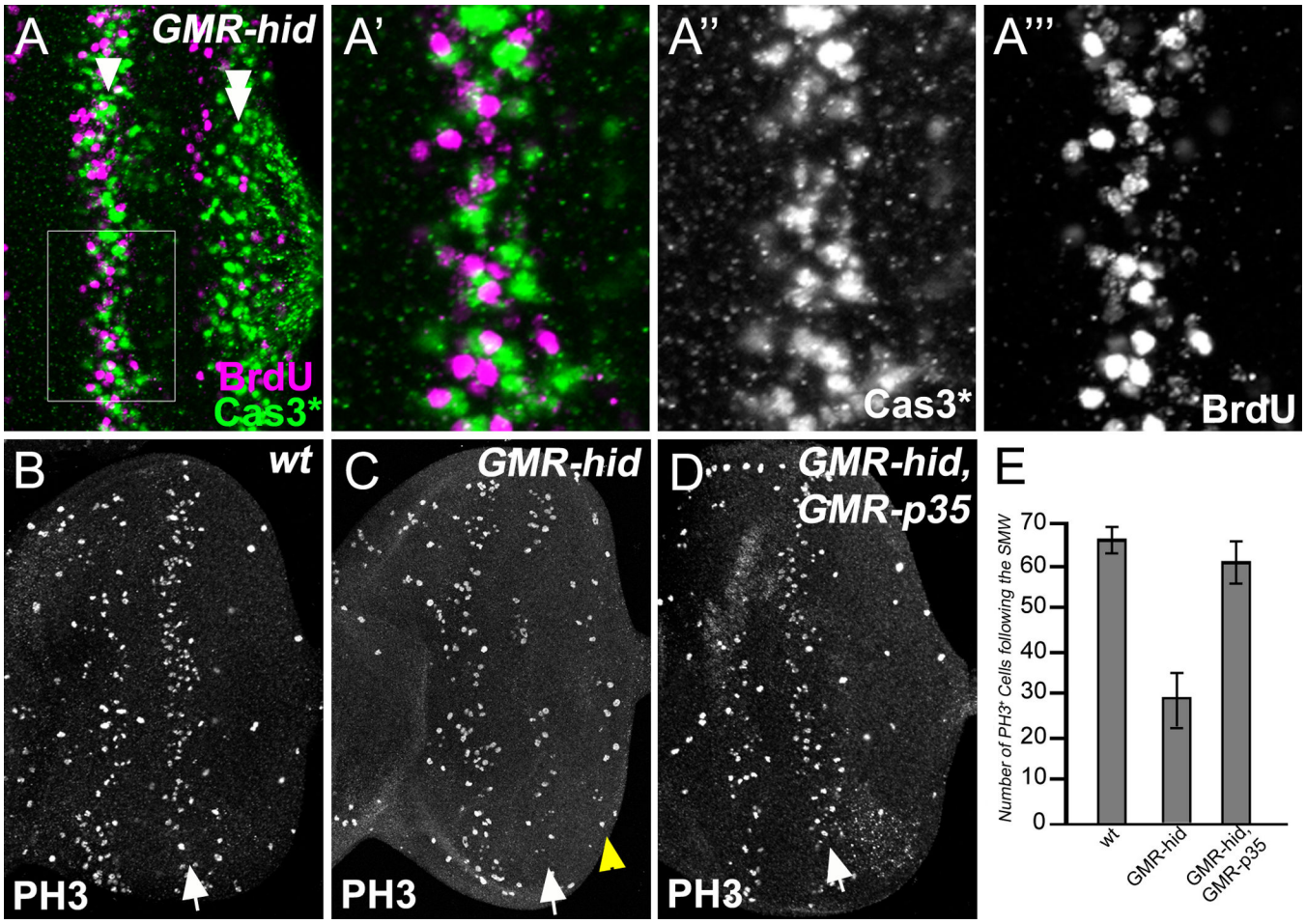

Figure 2. Proliferating SMW cells die in the first apoptotic wave

(**A–A'''**) Late 3rd instar *GMR-hid* eye disc labeled with Cas3* and BrdU. (**A'**) shows an enlarged view of the outlined region in (**A**). The first apoptotic wave (Cas3*-positive, green) and the second mitotic wave (SMW, BrdU-positive, magenta) are spatially very close.

(**B–D**) Late 3rd instar eye discs labeled with PH3 antibodies to visualize mitotic cells. Compared to wild type (**B**), the number of mitotic cells in the SMW (arrows) in *GMR-hid*(**C**) is strongly reduced. This reduction is rescued in *GMR-hid;GMR-p35* eye discs (**D**). Yellow arrowhead in (C) indicates the wave of compensatory proliferation (Fan and Bergmann, 2008).

(**E**) Quantification of number of PH3-positive cells in the SMW in various genetic backgrounds. PH3-positive cells in the SMW were counted in wild type, *GMR-hid*, and *GMR-hid;GMR-p35* eye discs (mean ± SD).

See also Figure S2.

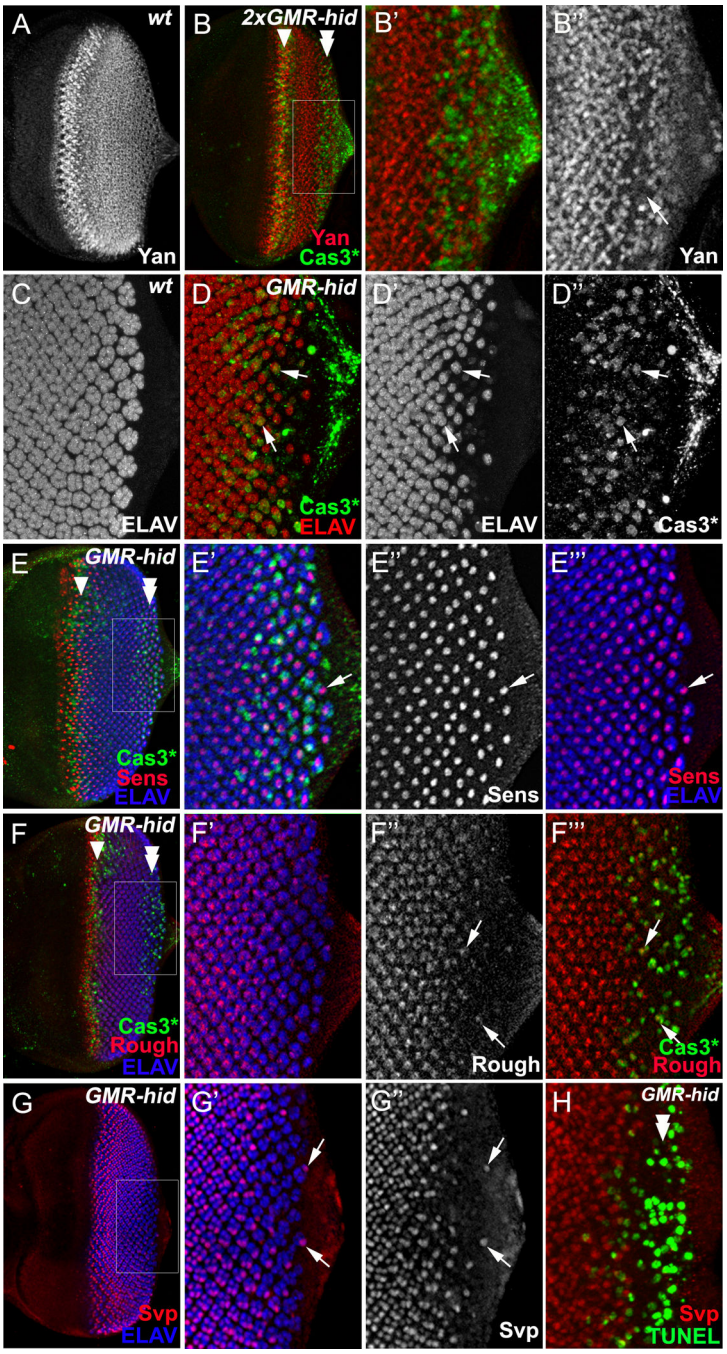

Figure 3. Outer photoreceptor neurons R1-R6 die in the second apoptotic wave

(**A**) Wild-type late 3rd instar eye disc labeled with Yan (a marker for unspecified cells). (**B–B''**) Late 3rd instar *GMR-hid* eye disc labeled with Cas3* and Yan. (**B',B''**) show enlarged views of the outlined region in (**B**). Compared to wild type (A), some unspecified cells are missing in *GMR-hid* as indicated by arrows (B'').

(**C**) Wild-type late 3rd instar eye disc labeled with ELAV (a marker for all photoreceptor neurons R1-R8).

(**D–D’’**) *GMR-hid* late 3rd instar eye disc labeled with ELAV and Cas3*. Arrows highlight examples of ELAV-positive photoreceptor neurons that are also Cas3*-positive.
(**E–E**’’’) Late 3rd instar *GMR-hid* eye disc labeled with Cas3*, ELAV and the R8-specific marker Senseless (Sens). (**E’-E’’’**) show enlarged views of the outlined region in (**E**). Although many photoreceptor clusters are Cas3*-positive in the second apoptotic wave, most R8 cells survive to the end of larval stages. An example with only two photoreceptor neurons left in an ommatidium is indicated by white arrows. One of the two surviving neurons in this ommatidium is R8.
(**F–F’’’**) Late 3rd instar *GMR-hid* eye disc labeled with Cas3*, ELAV and Rough (R3,4,2,5-specific). (**F’-F’’’**) show enlarged views of the outlined region in (**F**). Most Rough-positive cells are excluded from the second apoptotic wave because they are either immediately eliminated by *hid*, or they lose the Rough marker as soon as they become apoptotic. Arrows point to a few Rough-positive cells which also label for Cas3*.
(**G–H**) Late 3rd instar *GMR-hid* eye discs labeled with ELAV and Seven-up (Svp, R2,5,1,6-specific) (**G-G’’**) or with TUNEL and Svp (H). (**G’-G’’**) show enlarged views of the outlined region in (**G**). Most Svp-positive cells are excluded from the second apoptotic wave because they are either immediately eliminated by *hid*, or they lose the Svp marker as soon as they become apoptotic. Arrows point to a few Svp-positive cells left in the area corresponding to the second apoptotic wave.
See also Figure S3.

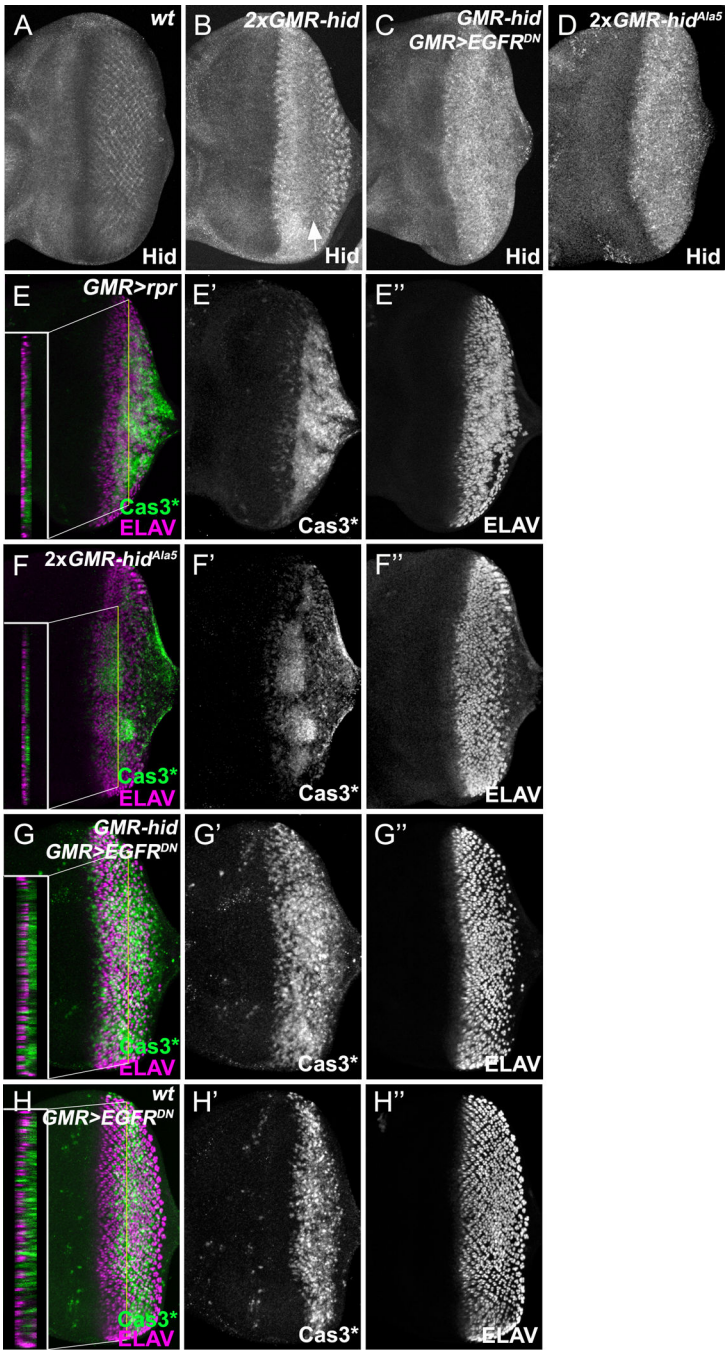

Figure 4. EGFR signaling protects unspecified cells in the apoptosis-free zone

(**A–D**) Late 3rd instar eye discs labeled with anti-Hid antibody. Compared to wild type (**A**), Hid is increased posterior to the MF in *GMR-hid* eye discs with a reduction of protein levels in the apoptosis-free zone (**B**, arrow). Reduction of Hid in the apoptosis free zone does not occur in *GMR-hid;GMR>EGFR*^{DN}(**C**) and *GMR-hid*^{Ala5} eye discs (**D**) suggesting that EGFR/MAPK signaling destabilizes Hid protein.

(**E–H**) Late 3rd instar eye discs labeled with Cas3* and ELAV. ELAV labels apically located photoreceptor neurons. The apoptosis free zone is missing in EGFR/MAPK-

unresponsive *GMR-GAL4 UAS-rpr*(*GMR>rpr***E**) and *GMR-hid*^{Ala5}(**F**) eye discs, as well as in *GMR-hid;GMR>EGFR*^{DN}(**G**). Sections indicate non-overlap of ELAV and Cas3* labelings suggesting that unspecified cells at the basal side of the discs, but not photoreceptor neurons, require EGFR signaling for survival. In the sections, apical is to the left, basal to the right.

(**H**) Expression of *GMR>EGFR*^{DN} in otherwise wild-type eye discs induces cell death in the region of the disc which corresponds to the apoptosis-free zone.

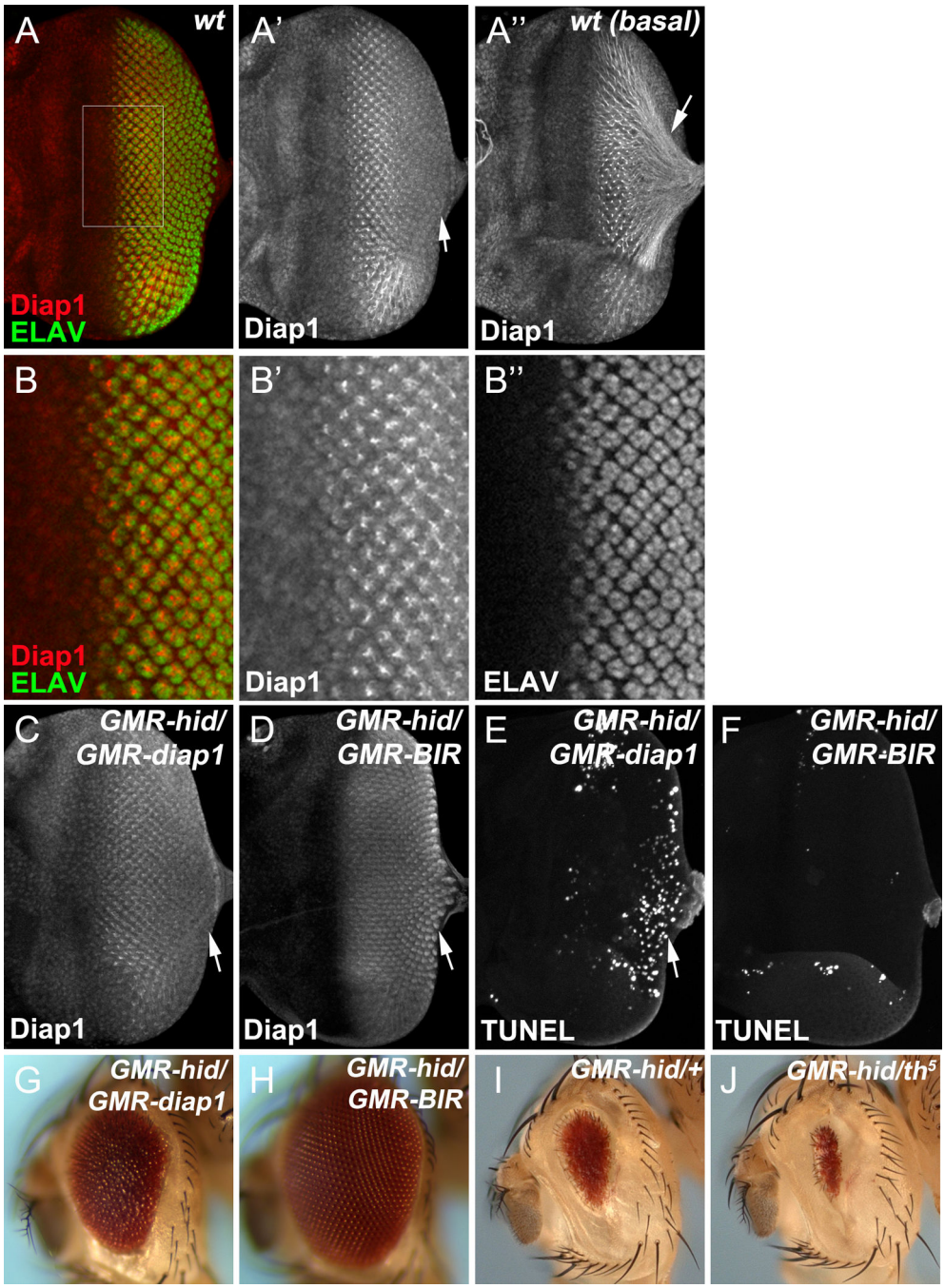

Figure 5. A transient increase of Diap1 protects photoreceptor neurons from apoptosis

(**A, B**) Wild type late 3rd instar eye disc labeled with Diap1 and ELAV. (**B**) shows an enlarged view of the outlined region in (**A**). (**A''**) shows the basal side of the same disc in (**A**). Diap1 strongly accumulates in ELAV-positive photoreceptor neurons (**A, B**) and in basally projecting axonal bundles (arrow, **A''**). In the posterior edge of the disc, levels of Diap1 are gradually reduced (**A'**, arrow).

(**C–F**) Late 3rd instar eye discs labeled by Diap1 (**C, D**) or TUNEL (**E, F**). Overexpression of full length Diap1, *GMR-diap1*, in the *GMR-hid* eye disc shows the same gradual decrease

of Diap1 towards the posterior end of the disc (arrow, **C**) as endogenous Diap1. In contrast, expression of a stabilized form of Diap1, *GMR-BIR*, in the *GMR-hid* eye disc maintains accumulated Diap1 levels at the posterior end of the disc (**D**, arrow). Consequently, *GMR-diap1* suppresses the first apoptotic wave in *GMR-hid*, but not the second (posterior) one indicated by TUNEL-positive cells (**E**)*GMR-BIR* suppresses both apoptotic waves in *GMR-hid*(**F**).

(**G–J**) Adult eyes of *GMRhid/+;GMR-diap1/+* flies (**G**)*GMRhid/+;GMR-BIR/+* flies (**H**)*GMRhid/+* flies (**I**), or *GMRhid/+ ;th^5/+* flies (**J**). Compared to *GMR-diap1, GMR-BIR* shows a stronger suppression of *GMR-hid* (compare H to G). Loss one copy of *diap1* by using a null mutant *(th^5)* enhances eye ablation of *GMR-hid* (compare J to I).

See also Figure S4.

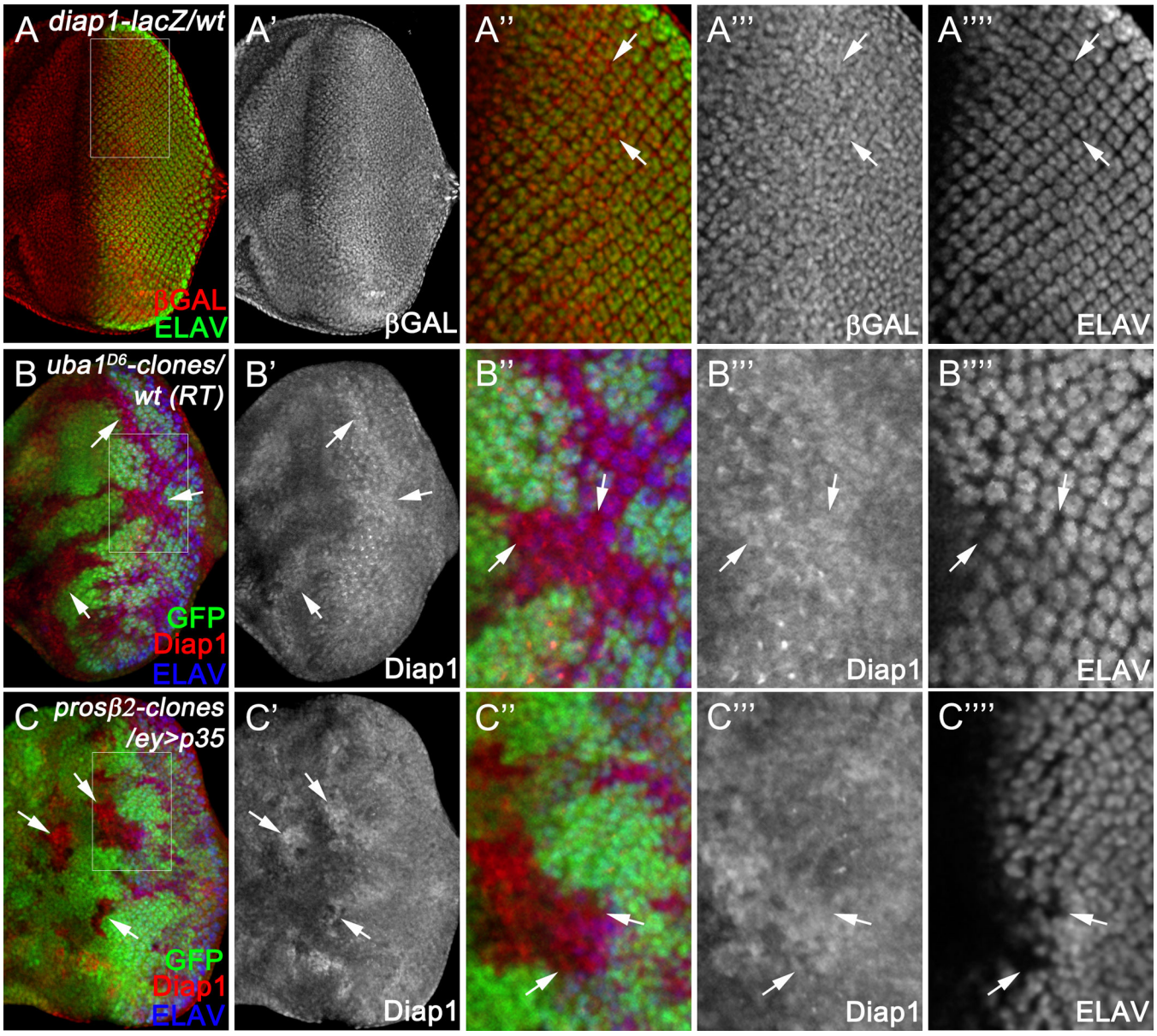

Figure 6. Ubiquitin-dependent degradation of Diap1 in unspecified cells

(**A**) Late 3rd instar eye disc labeled with ELAV. Transcription of *diap1* is indicated by the *diap1-lacZ* reporter (labeled for βGAL). (**A''**) shows an enlarged view of the outlined region in (**A**)*diap1-lacZ* (βGAL) in unspecified, i.e. interommatidial, cells (examples indicated by arrows) is indistinguishable from that in ELAV-positive photoreceptor neurons.

(**B–C**) Late 3rd instar eye discs labeled with Diap1 and ELAV. *uba1*(**B**) or *prosβ2*(**C**) mutant clones are labeled by lack of GFP. (**B'', C''**) show enlarged views of the outlined regions in (**B, C**), respectively. *UAS-p35* was expressed under the control of *ey-GAL4 (ey>p35)* to block apoptosis in *prosβ2* mutant clones (**C**). Expression of Diap1 is increased in *uba1* (B) or *prosβ2* mutant clones (C) specifically in ELAV-negative unspecified, i.e. interommatial, cells (arrows, B'', C'').

See also Figure S5.

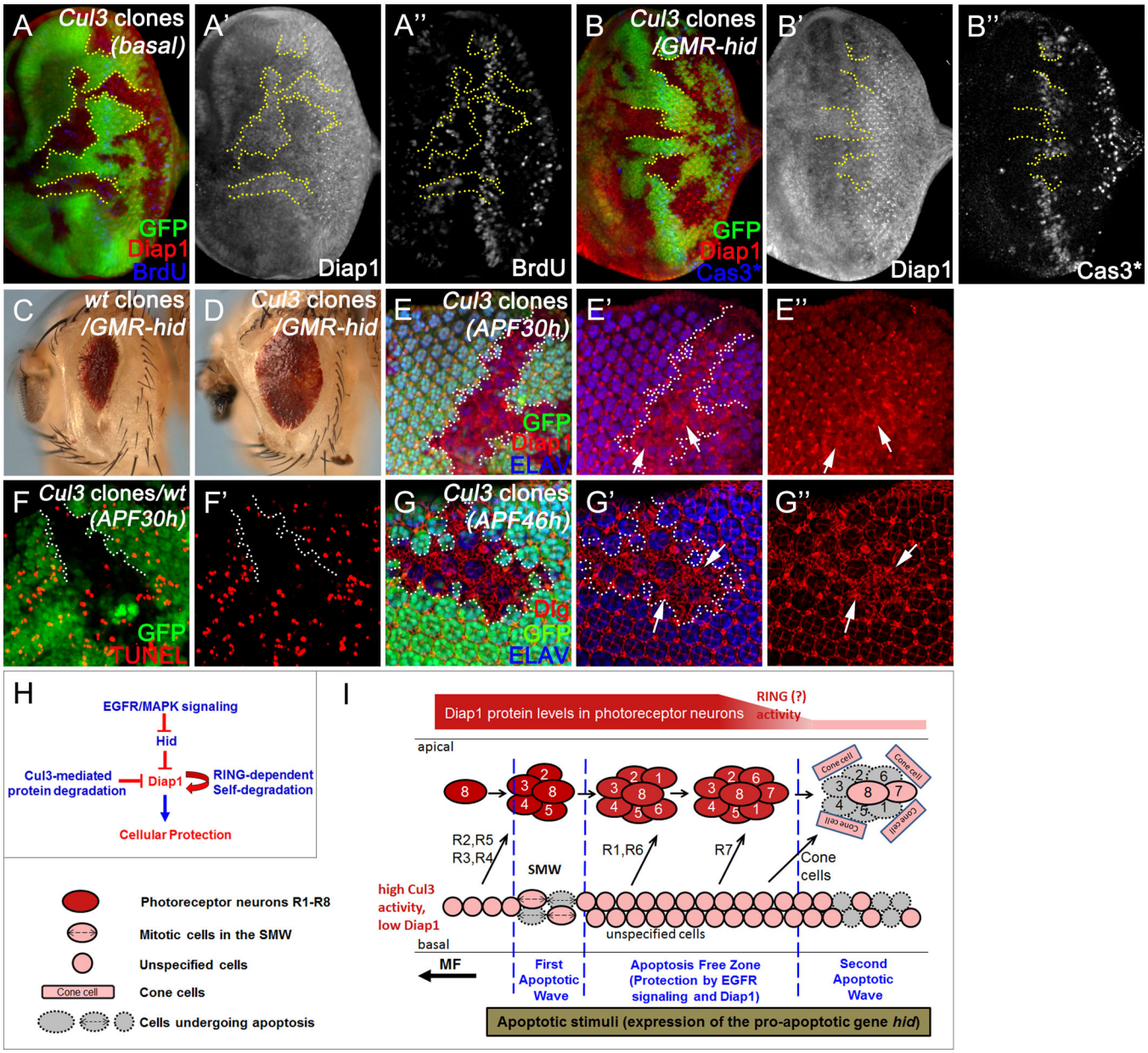

Figure 7. Cullin-3-mediated degradation of Diap1 in unspecified cells

(**A–B**) Late 3rd instar eye discs labeled by Diap1 and BrdU (**A**; wt), or Diap1 and Cas3* (**B;***GMR-hid*)*Cul3* clones are labeled by lack of GFP. Diap1 protein is increased in *Cul3* clones (A', B'). The proliferation pattern does not change in *Cul3* clones (A''). When *Cul3* clones are generated in the *GMR-hid* eye disc (B), *hid*-induced apoptosis is suppressed in *Cul3* mutant clones (B'').

(**C, D**) Adult mosaic eyes of *GMR-hid* flies with wild type control clones (**C**) or with *Cul3* mutant clones (**D**)*hid*-induced eye ablation was partially suppressed by *Cul3* mutant clones.

(**E–G**) Pupal eye discs at 30-hour (**E, F**) or 46-hour (**G**) after pupal formation (APF30h or APF46h) labeled with Diap1 and ELAV (**E**), TUNEL (**F**), or Dlg and ELAV (**G**)*Cul3* clones are labeled by lack of GFP. Diap1 is increased specifically in *Cul3*-mutant interommatidial

cells (arrows, E-E’’), but not in photoreceptor neurons. Consequently, developmental apoptosis of interommatidial cells is blocked in *Cul3* mutant clones (F, F’). This results in additional interommatial cells in *Cul3* clones at APF46h (G-G’’).

(**H**) Several mechanisms modulate the apoptosis susceptibility of various cell types in the eye imaginal disc. First, EGFR/MAPK signaling directly inhibits Hid activity in unspecified cells; Second, Cul3-mediated degradation keeps Diap1 levels low in unspecified cells. Third, *H99*-independent stimulation of the RING-E3 ligase domain contributes to apoptotic sensitivity of older photoreceptor neurons.

(**I**) Schematic outline of distinct cellular apoptosis susceptibilities in the developing *Drosophila* eye tissue. Anterior is to the left. In response to expression of Hid, two apoptotic waves are induced in the SMW (First Apoptotic Wave) and the posterior portion of the developing eye (Second Apoptotic Wave). Unspecified cells located at the basal side of the disc between the two apoptotic waves (the apoptosis-free zone) are protected by EGFR signaling. Moreover, the protein levels of Diap1 are transiently high (indicated by intense red color) in differentiating photoreceptor neurons protecting them from apoptosis at this stage. Later in development, Diap1 levels are down-regulated presumably by RING-dependent self-degradation (indicated by pale red). Therefore, developmentally older photoreceptor neurons become susceptible to apoptosis and die in the second apoptotic wave together with unspecified cells. In addition, R8, R7 and cone cells do not die in the second apoptotic wave suggesting that there are unknown mechanisms that renders these cells resistant to apoptosis.

See also Figure S6.