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Hid arbitrates collective cell death in the *Drosophila* wing

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Abstract

Elimination of cells and tissues by apoptosis is a highly conserved and tightly regulated process. In *Drosophila*, the entire wing epithelium is completely removed shortly after eclosion. The cells that make up this epithelium are collectively eliminated through a highly synchronized form of apoptotic cell death, involving canonical apoptosome genes. Here we present evidence that collective cell death does not require cell-cell contact and show that transcription of the IAP antagonist, *head involution defective* (Abdelwahid, Yokokura et al.), is acutely induced in wing epithelial cells prior to this process. *hid* mRNAs accumulate to levels that exceed a component of the ribosome and likewise, Hid protein becomes highly abundant in these same cells. *hid* function is required for collective cell death, since loss of function mutants show persisting wing epithelial cells and, furthermore, silencing of the hormone *bursicon* in the CNS produced collective cell death defective phenotypes manifested in the wing epithelium. Taken together, our observations suggest that acute induction of Hid primes wing epithelial cells for collective cell death and that Bursicon is a strong candidate to trigger this process, possibly by activating the abundant pool of Hid protein already present.

Keywords

Apoptosis; Collective Cell Death; Eclosion; Development; Hormone

1. INTRODUCTION

Programmed cell death (PCD) is essential for the elimination of tissues and organs during development and aging (Jacobson, Weil et al. 1997, Vaux and Korsmeyer 1999). Apoptosis, one form of PCD, is highly conserved in both vertebrates and invertebrates and involves the condensation of DNA followed immediately by nuclear and cytoplasmic fragmentation. Resulting cellular bodies are engulfed by phagocytes or neighboring cells (Kerr 1991).

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The first observations of wing epidermal cell death were reported for the adult blowfly, *Lucilia cuprina*. Body part ligature experiments were performed and cytoplasmic fragments of dead cells originating from the wings were observed (Seligman, Filshie et al. 1975). In *Drosophila melanogaster*, while the wing is expanding, the epidermal cells detach from the cuticle and all the cellular fragments are removed from the wing blade between 2 to 3 hours after eclosion (Johnson and Milner 1987). Unlike the canonical apoptotic process, death of epithelial cells that form the dorsal and ventral layers of the adult wing blade occurs in a synchronized and coordinated manner rather than at random (Link, Chen et al. 2007). Engulfment does not appear to occur within the wing, but instead apoptotic corpses are washed into the body of the fly (Link, Chen et al. 2007). After elimination of epithelial cells, the dorsal and ventral cuticles fuse together making a functional wing. Although collective cell death in the adult fly wing is uniquely different from the canonical apoptotic process, previous studies have shown that removal of the apoptosome components *dark and dronc*, or the effector caspase *drice* in the wing leads to persisting cells and a distinctive, late-onset blemishing phenotype, characteristic of a PCD defect (Xu, Li et al. 2005) (Link, Chen et al. 2007). Live cell imaging of wings from newly eclosed flies revealed that the apoptotic changes spread rapidly throughout the epithelium, usually starting from the peripheral edges and moving across the wing blade (Link, Chen et al. 2007). These results suggest that execution of wing epithelial PCD requires the tight coordination of death signals, in addition to the canonical apoptotic pathway. The collective nature of these signals and how they engage with apoptotic components are largely unknown.

Hormones play an important role in PCD. In *Drosophila* development, the steroid hormone ecdysone acts as the apical signal to initiate the stage-specific elimination of larval tissues during metamorphosis (Yin and Thummel 2005). Ecdysone-induced expression of reaper (*rpr*) and *hid* is required for destruction of the larval midgut and salivary glands during metamorphosis (Yin and Thummel 2004). While *hid* plays the primary role in the salivary gland PCD, both *rpr* and *hid* act in a redundant manner in the midgut (Yin and Thummel 2004). Another hormone, *bursicon*, is required for the final steps of ecdysis, including cuticle hardening and tanning, wing expansion, and wing epithelial cell death (Peabody, Diao et al. 2008). Furthermore, mutations in *rickets* (*rk*), which encodes the Bursicon receptor, prevents PCD leading to epithelial cells observed 8 hours after eclosion in the wing blade (Kimura, Kodama et al. 2004). Although studies suggest a role for *bursicon* in PCD, the direct link between the *rickets-bursicon* signaling pathway and collective PCD in the wing epithelium is yet to be determined. Here, we present evidence that the pro-apoptotic gene, *hid*, is acutely induced and the corresponding protein becomes highly abundant prior to collective PCD. Furthermore, *bursicon* silencing in the CNS generated characteristic PCD phenotypes in the wing without preventing Hid accumulation. Taken together, our observations suggest that this hormone may be a trigger that elicits collective apoptosis among cells that are already primed for death with an IAP antagonist.

2. RESULTS

2.1 Collective cell death does not require coordination through cell-cell contact

After eclosion, the adult wing expands and the entire epithelium dies within 3 hours. Previously we applied static imaging methods to examine epithelial cell clones mutated for apoptotic components (Link, Chen et al. 2007). To investigate whether cell-cell contact is required for the spreading of apoptotic physiology in the post-eclosion wing, we applied live imaging to visualize epithelia mosaic for cell death defective clones during this process. Figure 1 (and Supplemental Video 1) shows how mosaic patches of wild type and *dark* mutant (*dark*^{82}) tissue were imaged. In this assay, all epithelial cells express nuclear dsRed and nuclear GFP distinguishes small clones of *dark*^{82} mutant cells (GFP−) from wild type clones (GFP+) in the wing. After the two fluorescent signals are overlaid, dual color labeling allows for identification of wild type clones as yellow and *dark*^{82} clone cells as red (Figure 1). Time-lapse images of newly eclosed wings in this assay show that cells lacking the apoptosome (*dark*^{82}) always persist (Figure 1 and Video 1). Importantly, the programmed death of discontinuous wild type clones remained highly coordinated and synchronous, despite the fact that many of these clones were separated over relatively large distances. Additionally, the same *dark*^{82} mosaic wings developed late-onset blemishes, a characteristic phenotype indicating PCD failure (Link, Chen et al. 2007). These observations suggest that collective PCD in the wing epithelium is not coordinated through cell-cell contact and, instead, favor a systemic cell death signal present throughout the wing blade.

2.2 The pro-apoptotic gene *hid* is acutely induced prior to collective cell death in the wing epithelium

In *Drosophila*, a deletion removing the pro-apoptotic genes *rpr*, *grim*, and *hid*, blocks virtually all PCD in response to different death-inducing signals (Grether, Abrams et al. 1995) (Abrams 1999). Wings mosaic for this deletion (*Df (H99)*) show persisting cells and a late-onset blemishing phenotype, a characteristic indicator of a PCD failure (Link, Chen et al. 2007). Although *rpr*, *grim*, and *hid* may induce apoptosis through similar mechanisms, it is clear that they are not functionally equivalent *in vivo*. Previous studies have shown that these genes are differentially expressed in dying cells and in response to different signals (Peterson, Carney et al. 2002). To determine which of the pro-apoptotic genes encoded in the H99 region might provoke collective PCD, we quantified changes in expression of all genes in the H99 region in pupal- and newly eclosed-wings (Fig. 2A). Using qRT-PCR, we found abundant expression of *hid* in pupal- and newly eclosed-wings while other apoptotic genes in the H99 region, *rpr*, *grim* and *sickle* (Kroemer, Galluzzi et al.), were uniformly low (Fig. 2B). To extend these findings, we quantified *hid*, *rpr* and *skl* expression at different wing developmental stages (wing disc, pupal-, and eclosed- wing) using a droplet digital PCR (ddPCR) system. ddPCR enables an absolute quantification of pro-apoptotic transcripts within a sample (Link, Kurtz et al. 2013) and, as seen in Fig 2C, *hid* is highly expressed and uniquely abundant compared to other genes in the H99 interval (Fig. 2C). In fact, levels of *hid* mRNAs in eclosed wings were about 3 fold higher than levels of a highly abundant transcript, *ribosomal protein 49* (*rp49*) (Fig. 2C). Furthermore, between the pupal stage and eclosion, *hid* transcripts dramatically increased by at least 10 fold, demonstrating that acutely induced expression of this gene anticipates collective PCD (Fig. 2C). Given that

negligible expression was seen for *rpr*, *grim* and *skl*, these results suggest that collective PCD phenotypes associated with the H99 region largely reflect a role for *hid* in this process.

2.3 Hid protein levels increase prior to collective PCD while Diap1 levels remain constant

To investigate whether the dramatic increases seen for *hid* mRNAs also occurred at the protein level, extracts were prepared from wild type pupal and eclosed wings, and blotted using anti-Hid CL1C3 antibody (Haining, Carboy-Newcomb et al. 1999). As shown in Fig. 3A, Hid protein was undetectable in pupal wings but eclosed wings showed high levels of Hid when compared to Tubulin as a loading control. To corroborate that the band we are observing is in fact Hid, we prepared protein extracts from eclosed wings from hid^{A22}/hid^{A329} trans-heterozygotes (Abbott and Lengyel 1991) and blotted with the same anti-Hid CL1C3 antibody. As expected, full-length Hid was absent while a truncated form of Hid, consistent with hid^{A329} allele, appeared in transheterozygous wings (Fig. 3B). These observations establish that, like *hid* mRNAs, Hid protein levels become acutely induced and highly abundant prior to collective cell death in the wing epithelium.

Diap1 is a pivotal regulator of PCD that binds directly to, and inhibits, apical (Dronc) and effector caspases (Dcp1, Drice) (Vaux and Silke 2005). IAP antagonists, such as Hid, promote apoptosis by destabilizing Diap1 and releasing Dronc to initiate cascades of caspase activity. Therefore, collective PCD in the wing could also involve changes that alter levels of Diap1 (Ryoo, Bergmann et al. 2002) and, to test this possibility, we blotted protein lysates from wild type pupal and eclosed wings with an anti-Diap1 antibody (Ryoo, Bergmann et al. 2002) As seen in Fig. 3, despite the dramatic accumulation of Hid protein that anticipates collective PCD, Diap1 levels were unchanged.

2.4 *hid* function is required for collective PCD

To functionally test the role of *hid* during collective PCD, we examined transheterozygotes from the previously reported *hid* mutant alleles, hid^{A22} and hid^{A329} (Abbott and Lengyel 1991). These trans-heterozygote mutants are known to have wing defects, including an opaque appearance and trapped fluid at a lower penetrance (Abbott and Lengyel 1991). We examined trans-heterozygote mutant wings at eclosion and found they were morphologically wild type (Fig. 4A). However, over 4–8 days, melanized blemishes appeared throughout the wing blade and became more severe as animals aged (Fig. 4B). This progressive, late-onset blemishing phenotype was previously observed for mutants defective in *dark*, *dronc* and *drice* and is characteristic for cell death genes (Link, Chen et al. 2007).

To further characterize the defective PCD phenotype observed in *hid* trans-heterozygote wings, we used a ubiquitous nuclear RFP reporter to visualize wing epithelial cells immediately after eclosion. As seen in Fig. 4C, all epithelial cells are cleared from wild type wing blades within 3 hours after eclosion and any remaining RFP expression is restricted to the wing veins. By contrast, in *hid* mutant wings, RFP positive tissue remains in the blade even 1 day after eclosion, demonstrating that these cells were unable to complete PCD (Fig. 4D). These observations are consistent with previous studies using the H99 deletion (Link, Chen et al. 2007). Moreover, since the wings of *hid* mutants phenocopied wings mosaic for

the H99 deletion, *hid* function likely accounts for all collective PCD activity encoded by the Reaper region (Link, Chen et al. 2007).

2.5 Developmental induction of *hid* in the wing epithelium is transcriptionally regulated

To investigate how *hid* expression is regulated in the wing epithelium, we used a *hid*>GFP line that has been previously generated (Tanaka-Matakatsu, Xu et al. 2009). This GFP reporter was made by fusing the 2.2 kilobase sequence upstream of *hid* to GFP, and it has been shown that this enhancer contains regulatory elements that control *hid* transcription in response to developmental signals (Tanaka-Matakatsu, Xu et al. 2009). We measured GFP expression using quantitative RT-PCR between pupal and eclosed wings. As seen in Fig. 5A, this reporter drives GFP expression in a pattern almost identical to endogenous *hid* expression in the wing epithelium. These observations suggest that this 2.2 Kb fragment regulates *hid* expression in response to wing development signals.

To further investigate whether known regulatory elements within the 2.2 Kb fragment control *hid* expression during communal cell death, we took advantage of a second GFP reporter. This reporter is identical to the *hid*>GFP reporter but has a mutated E2F binding site (*hidmutE2F*>GFP) (Tanaka-Matakatsu, Xu et al. 2009). E2F is a family of transcription factors that regulate a variety of processes such as cell proliferation, differentiation, and apoptosis. In *Drosophila*, there are only two E2F family proteins: dE2F1, which functions as a transcriptional activator and dE2F2, which mediates transcriptional repression (Frolov, Huen et al. 2001). To determine whether the E2F binding site specifies *hid* expression levels in eclosed wings, we quantified GFP expression in eclosed wings from *hid*>GFP and *hidmutE2F*>GFP flies. As shown in Fig. 5B, equivalent GFP expression was seen with both constructs, indicating that E2F does not specify the dramatic induction of *hid* that anticipates collective PCD.

A p53 binding site is also present within the 2.2 Kb fragment upstream of *hid* (Zhang, Mehrotra et al. 2014) but, since $p53^{-}$ animals do not develop late-onset blemishes (Sogame, Kim et al. 2003) (Rong, Titen et al. 2002), this site cannot account for collective PCD.

2.6 Depleting the hormone Bursicon generates collective cell death phenotypes without affecting Hid accumulation

At eclosion, wing epithelial cells express high levels of *hid* but, despite extraordinary accumulation of this IAP antagonist, collective apoptosis is held in check and only occurs once expansion is complete (Kimura, Kodama et al. 2004). These observations, combined with indications that cell-cell contact is not required for collective PCD (Fig 1) suggests the involvement of a hormonal factor as a potential trigger for this process (Link, Chen et al. 2007). It has been shown that the peptide hormone, Bursicon, is released before wing expansion and helps complete the tanning reaction and the hardening of the newly expanded cuticle (Kimura, Kodama et al. 2004). Furthermore, it has been reported that mutations in a putative Bursicon receptor, *rickets*, inhibit cell death in the wing epithelium (Kimura, Kodama et al. 2004) (Baker and Truman 2002). Therefore, we used the Gal4-UAS system (Brand and Perrimon 1993), to investigate whether Bursicon plays a role in collective PCD. Since Bursicon is a peptide hormone made by neurosecretory cells in the nervous system,

transgenic flies expressing *synaptobrevin-gal4 (nsyb-gal4)*, a pan-neuronal driver, were crossed to a *bursicon* RNAi transgenic line. To quantify the efficiency of our *bursicon* knock-down, we prepared protein lysates from wild type eclosed wings and *bursicon* knock-down eclosed wings and blotted using a previously reported anti-Bursicon-alpha antibody, which specifically detects the Bursicon alpha subunit (Luan, Lemon et al. 2006). As shown in Fig. 6B (inset), Bursicon protein levels were considerably reduced when compared to wild type levels. Importantly, we also found that flies expressing *bursicon* RNAi (*nsyb-Gal4*>UAS-*burs RNAi*) appeared wild type at eclosion, but as they aged, melanized blemishes developed throughout the wing blades (Figs. 6A and 6B). These late onset blemishes were indistinguishable from those seen with other cell death mutants (Fig. 4B) and clearly indicative of defective PCD (Link, Chen et al. 2007). To extend these findings and validate the specificity of the RNAi transgenic line, we took advantage of a second driver line that is expressed only in Bursicon neurons, *burs-gal4* (Peabody, Diao et al. 2008). Wings from *bursicon* knock-down flies using the *burs-gal4* driver are indistinguishable from wild type at eclosion (Fig. 6C), and they also developed lateonset blemishes throughout the wing blades (Fig. 6D).

These observations, combined with previous reports linking Bursicon to post-eclosion events in the wing (Kimura, Kodama et al. 2004), raised the possibility that Bursicon could impact the accumulation of Hid that anticipates collective PCD. Therefore, we quantified *hid* transcripts and Hid protein in *bursicon* knock-down pupal and eclosed wings. As seen in Fig 6, induction of *hid* RNA (Fig. 6E) and accumulation of Hid protein were both unaffected by silencing of Bursicon hormone (Fig. 6F). Taken together, these results suggest that Bursicon might trigger collective PCD among cells that are poised for Hid-mediated apoptosis.

3. DISCUSSION

Wing maturation in *Drosophila* requires the complete removal of the epithelial cells by PCD a few hours after eclosion from the pupal case. Using a novel, dual color live cell imaging platform, we found that discontinuous clones of wild type cells died in a coordinated and synchronous manner, indicating that this form of collective PCD does not require cell-cell contact to occur. Since mosaic wings for mutants in the canonical apoptotic pathways share a late-onset blemishing phenotype, we investigated likely components involved in the coordination and synchronization of PCD and established that the pro-apoptotic gene, *hid*, is a major effector of this process. Prior to collective PCD, *hid* transcription is acutely induced and Hid protein levels become highly abundant, without impacting Diap1. Furthermore, *hid* mutants exhibit characteristic late-onset blemishing and persisting cell phenotypes (Link, Chen et al. 2007), providing direct functional evidence that *hid* gene action mediates collective PCD. Taken together, it seems plausible that Hid becomes highly abundant in an inactive form throughout the wing epithelium, thereby priming these cells for collective PCD. Furthermore, given the cell death defective phenotypes caused by Bursicon silencing (Figs. 6B and 6D), exposure to this hormone could define a systemic trigger for collective PCD among Hid-primed cells. Intriguingly, mammalian counterparts of the Bursicon receptor, DLGR2 (Baker and Truman 2002) encode receptors for ligands such as Follicle Stimulating Hormone and Luteinizing Hormone/Choriogonadotropin (dos Santos, Schroeder

et al. 2014), which function during apoptotic removal of uterine tissue during menstruation (Gosden and Spears 1997).

Figure 7 illustrates a proposed model for collective PCD in the wing (Fig. 7). Epithelial cells in the newly eclosed wing are loaded with *hid* transcript and abundant Hid protein but the death signal is hypothetically "dormant", perhaps because Hid is modified (Bergmann, Agapite et al. 1998) or because Hid is localized to a specific subcellular compartment (Haining, Carboy-Newcomb et al. 1999) (Abdelwahid, Yokokura et al. 2007). After eclosion, Bursicon is released, initiating a signaling cascade, which alters Hid such that it is permissive for initiating cell death. According to this model, collective PCD occurs through the coincident action of a hormonal trigger together with apoptogenic priming of effector cells that are loaded with abundant levels of a dormant IAP antagonist.

4. MATERIALS AND METHODS

Dual color whole wing clones

Dual color clonal analysis was generated by crossing *ms1096-Gal4, UAS-FLP; FRT42D-GFP* animals to *dark*^{82} *FRT42D; UAS-DsRed*. Newly eclosed adult flies were isolated and immobilized with super glue on glass slides and imaged with a stereomicroscope (SteREO Discovery V.12; Carl Zeiss MicroImaging, Inc.) with PentafluarS using a 1.5× PlanApoS lens, an MRm or MRc5 digital camera (Axiocam), a dual color filter and Axiovision Release 4.6 software.

RT-PCR

Between 20 and 30 pupal and newly eclosed (unexpanded) wings were dissected and frozen in Trizol until the RNA extraction process. Total RNA was isolated using Trizol Reagent and cDNA was generated using Bio-Rad's iScript cDNA synthesis kit.

qPCR analysis was done using iQ™ SYBR Green Supermix by Bio-Rad. The reaction included the mix, cDNA template, and primers at a final concentration of 500mM. The reaction was carried out on the CFX96™ Real-Time PCR Detection system by Bio-Rad. Digital RT-PCR reactions included primers and fluorescent probes specific for each transcript together with Bio-Rad's ddPCR Supermix for Probes. Droplets were generated using the Droplet Generator (Bio-Rad) with 20ul PCR sample and 70ul Droplet Generator Oil (Bio-Rad). Droplets were transferred to 96 well PCR plates (Eppendorf), heat sealed, and run on an Eppendorf Mastercycler Pro to saturation (45 cycles). Droplets were read on the Droplet Reader (Bio-Rad). Quantasoft software (Bio-rad) assesses the number of positive and negative droplets and applies Poisson statistics to generate an absolute measurement of starting DNA molecules. Rp49 transcript was used as RNA quality control.

Immunoblotting and Antibodies

200 pupal and eclosed wings were dissected and protein lysates were prepared and separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore) and immunodetected with respective antibodies. Blots were further developed by incubation with corresponding secondary antibody and visualized using enhanced chemiluminescence (GE Healthcare).

Monoclonal mouse anti-Hid CL1C3 (gift from Hermann Steller) was used at 1:1000, rabbit anti-Diap1 (gift from Hyung Don Ryoo) was used at 1:500, rabbit anti-Bursicon-alpha (gift from Benjamin White) at 1:1000, mouse anti-Tubulin E7 (Hybridoma Bank) at 1:5000, goat anti-mouse HRP (Jackson ImmunoResearch) at 1:2000, and goat anti-rabbit HRP (Jackson ImmunoResearch) at 1:2000.

Microscopy

Adult wing images were acquired using a dissecting microscope (Olympus SZX10) at a 1.25× magnification. CellSens standard software was used to acquire adult wing images. Fluorescent wing images were acquired with a stereomicroscope (Axioplan 2E; Carl Zeiss MicroImaging, Inc.) at a 20× magnification. OpenLab software was used to acquire florescent images. (Seligman, Filshie et al. 1975)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Formation of the *Drosophila* wing is a model for synchronized collective cell death.

Clonal analyses show that collective cell does not require cell-cell contact.

Transcriptional loading of hid defines a pivotal switch anticipating collective PCD.

The Bursicon hormone-receptor axis elicits collective cell death.

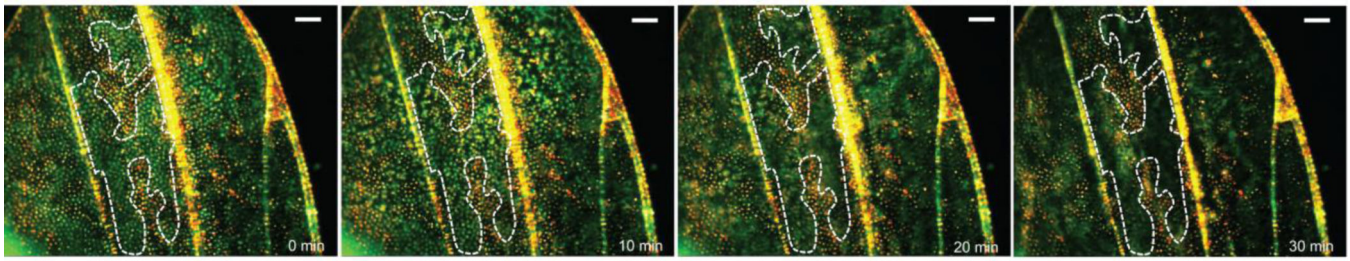

Fig. 1. Collective cell death is not coordinated through cell-cell contact

Timelapse images of a *dark*^{82} mosaic wing from a newly eclosed fly (see Materials and Methods). Images shown here are excerpted frames from supplemental Video 1. All wing cells are labeled with nuclear DsRed while wild type cells are labeled with nuclear GFP. *dark*^{82} cells are GFP^{-}. Two discontinuous patches of wild type cells are outlined (dotted white lines). At 0 minutes, the genetically mosaic epithelium is intact. At 10 minutes, patches of wild type cells that are discontinuous initiate synchronous PCD. Within 30 minutes, virtually all the wild type apoptotic corpses are cleared from the outlined clones while cell death defective *dark*^{82} patches remain unchanged. Scale bar = 50 um. (See Supplemental Video 1).

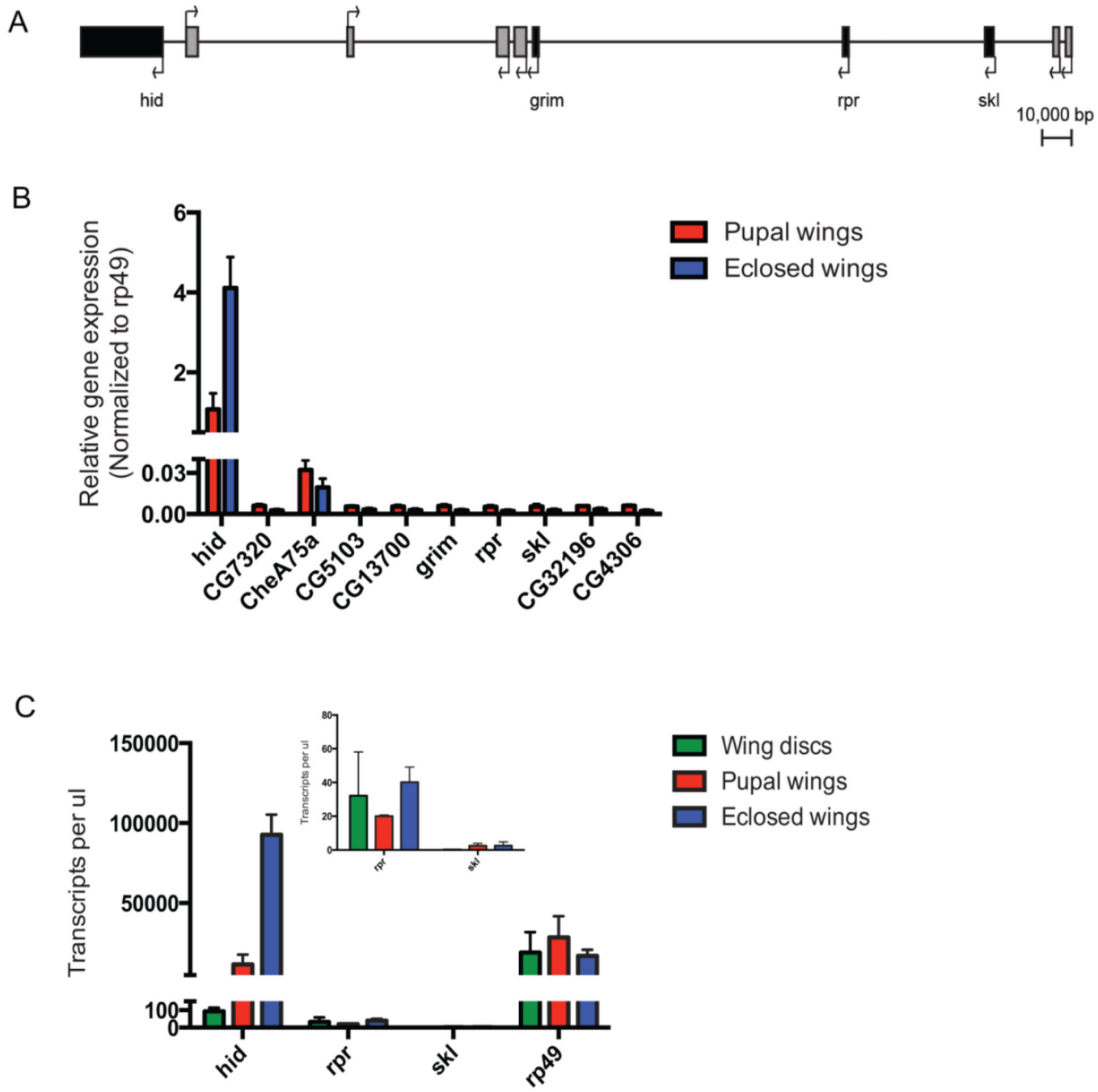

Fig. 2. Induction of *hid* transcript anticipates collective PCD in the wing epithelium

(A) Schematic representation of the H99 genomic interval on chromosome 3L. Black boxes represent pro-apoptotic genes and gray boxes represent unrelated genes. **(B)** Semi-quantitative RT-PCR showing relative transcript levels of the H99 region genes in pupal (red) and eclosed (blue) wings. **(C)** Quantitative Droplet Digital PCR (ddPCR) assay showing the absolute number of transcripts per ul for the pro-apoptotic genes *hid*, *reaper* (*rpr*), *sickle* (Kroemer, Galluzzi et al.), and *ribosomal protein 49* (*rp49*) in wing discs (green), pupal (red) and eclosed (blue) wings.

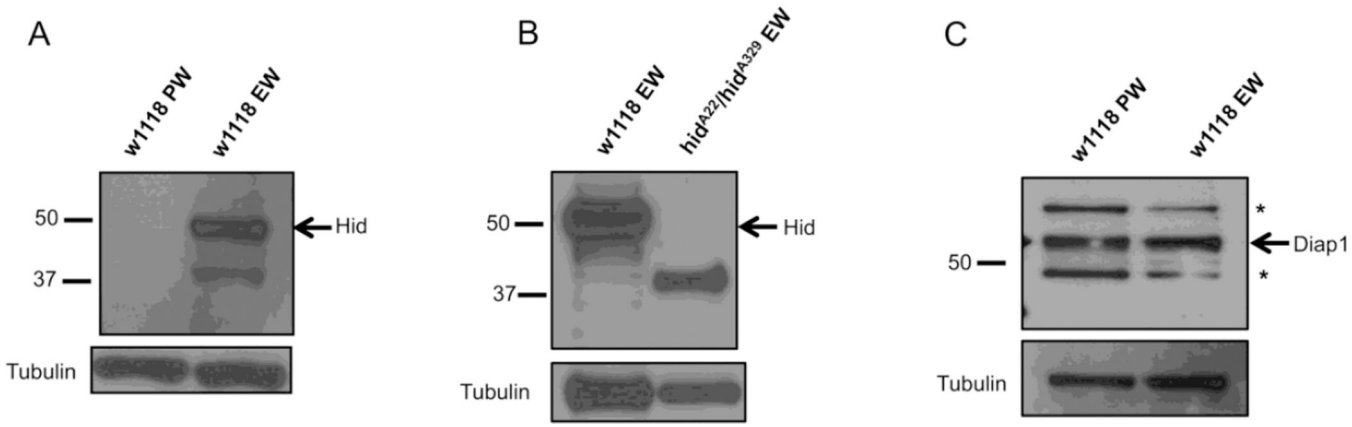

Fig. 3. Accumulation of Hid protein precedes collective PCD but levels of Diap1, remain unchanged

(A) Western blot with anti-Hid CL1C3 shows that abundant Hid protein accumulates in newly eclosed wings from undetectable levels 24 hrs earlier, in pupal (P13) wings. **(B)** Western blot with anti-Hid CL1C3 shows absence of full-length Hid protein in *hid*^{A22}/*hid*^{A329} trans-heterozygous wings. *hid*^{A329} encodes a premature stop that generates a ~37 kDa protein (band in *hid*^{A22}/*hid*^{A329} lane) whereas *hid*^{A22} likely encodes an unstable protein. **(C)** Western blot with anti-Diap1 shows comparable Diap1 levels between wild type pupal and eclosed wings. Asterisks denote non-specific bands. Tubulin is shown as loading control. PW= Pupal Wings, EW= Eclosed Wings

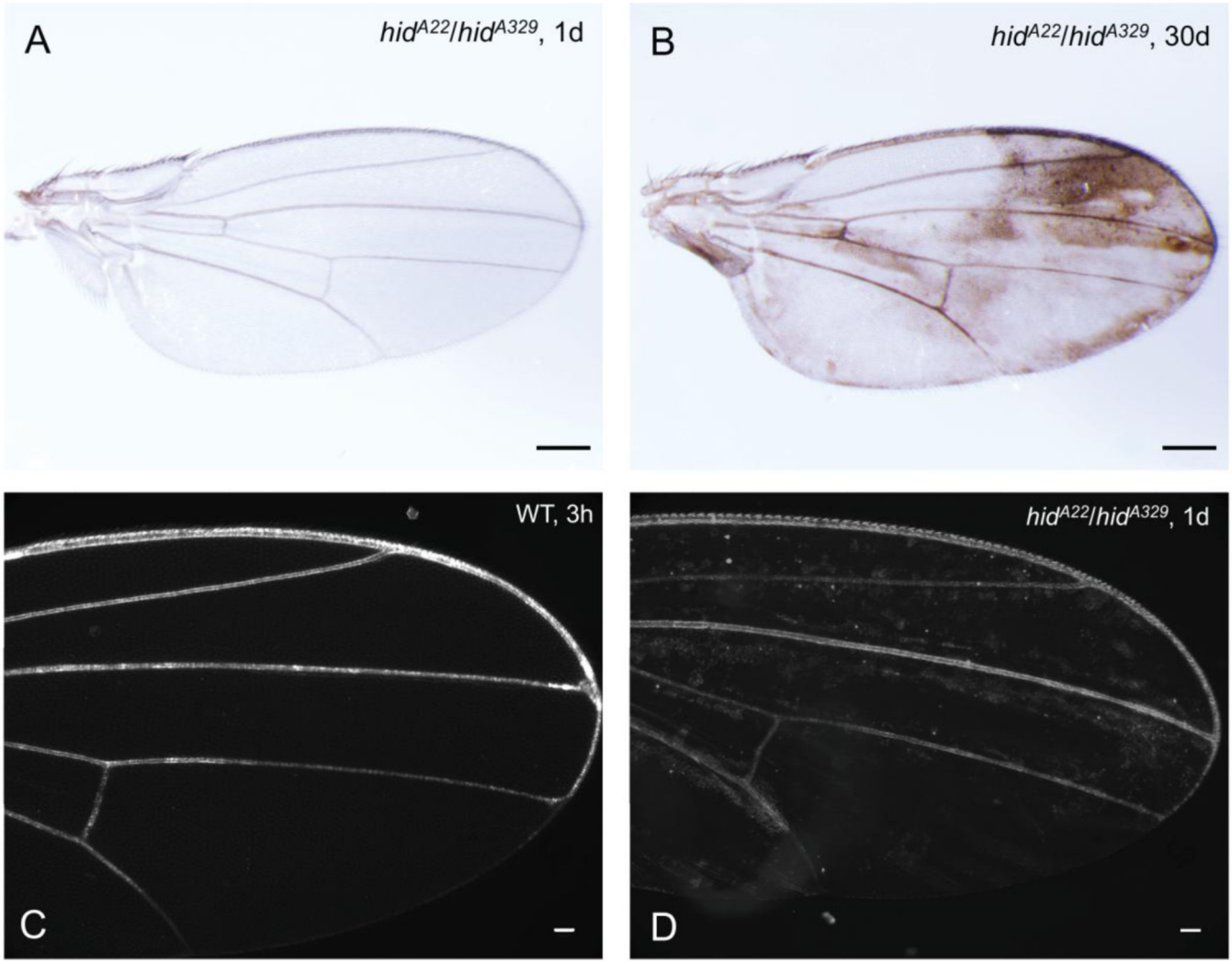

Fig. 4. *hid* function is required for collective PCD in the wing epithelium

(A) Wings of hid^{A22}/hid^{A329} trans-heterozygote flies have wild type morphology 1 day after eclosion but days later progressive, late-onset blemishes occur throughout the wing blade, shown here **(B)** 30 days after eclosion. **(C)** Wild type wing is visualized with a ubiquitous nuclear RFP transgene 3 h post-eclosion. RFP signal is restricted to the wing veins, since all epithelial cells have been eliminated. **(D)** Wings from hid^{A22}/hid^{A329} transheterozygotes retain persisting, RFP positive epithelial cells, shown here 1 d after eclosion. Scale bar in (A) and (B) = 200 um, in (C) and (D) = 50 um.

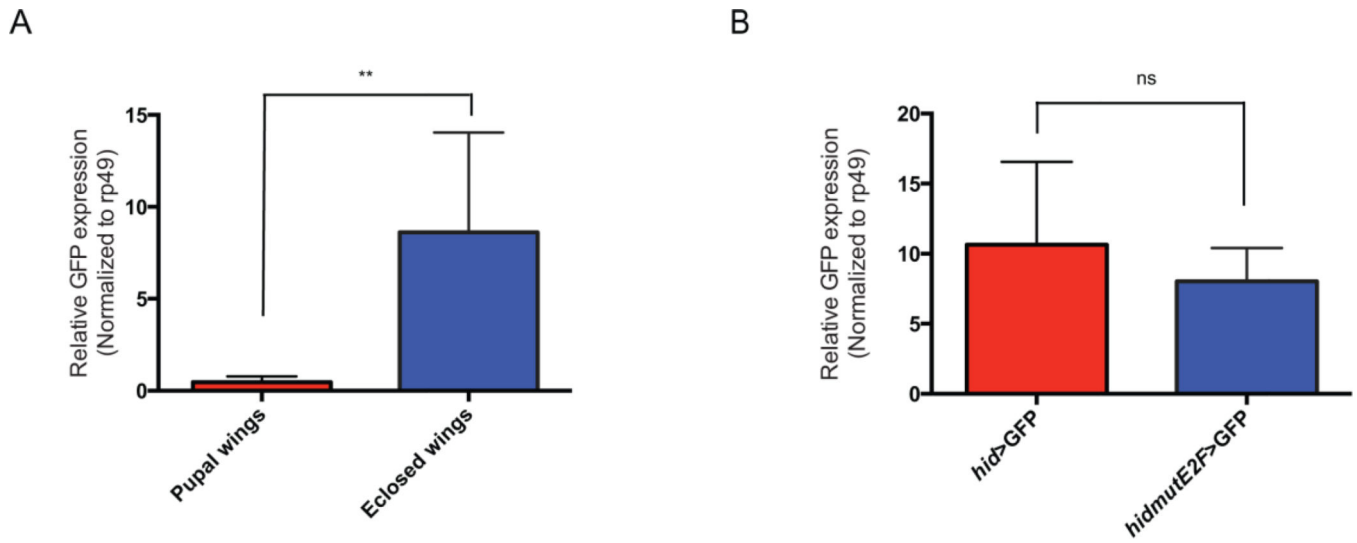

Fig. 5. Developmental induction of *hid* is transcriptionally regulated in the wing and is E2F independent

(A) Quantitative RT-PCR assay showing GFP expression in *hid*>GFP (hid5'F-WT) pupal and eclosed wings. GFP expression pattern resembles endogenous *hid* expression (** indicates p-value = 0.006). **(B)** Quantification of GFP expression in eclosed wings from *hid*>GFP and *hidmutE2F*>GFP (hid5'-E2FMut) flies. No significant difference is observed in GFP levels when the E2F binding site in the 2.2 kilobase promoter region of *hid* is mutated (ns indicates not significant).

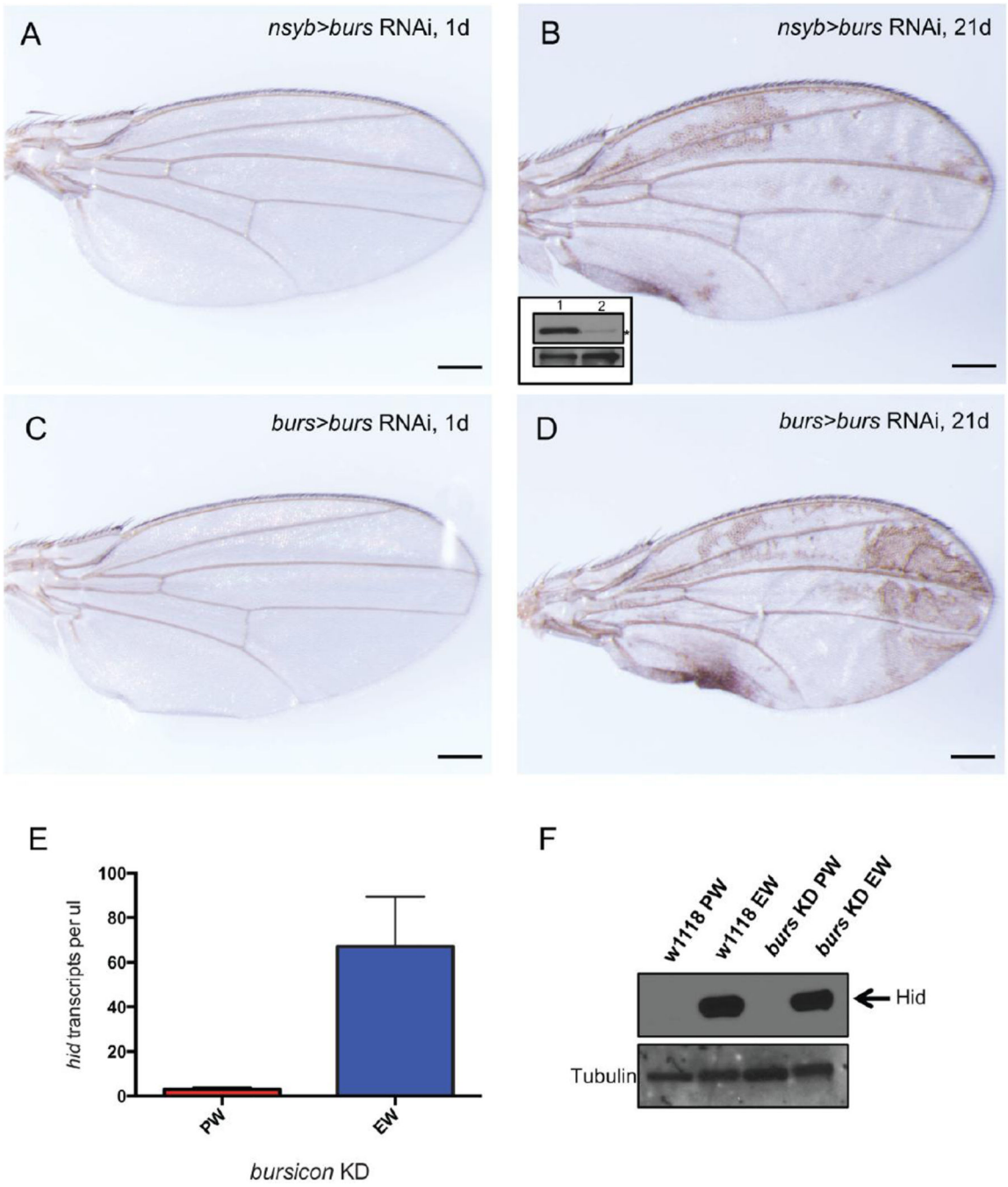

Fig. 6. Depleting the hormone Bursicon generates collective cell death phenotypes without affecting Hid accumulation

(A) Wings from flies with genotype: *nsyb-gal4>burs* RNAi (pan-neuronal *burs* knock-down) are indistinguishable from wild type at 1 day after eclosion. **(B)** Wings from the same *nsyb-gal4>burs* RNAi animals developed late-onset melanized blemishes, shown here 21 days post-eclosion. Inset shows *bursicon* knock-down efficiency. Lane 1: wild type eclosed wings, lane 2: *nsy-bgal4>burs* RNAi eclosed wings. Asterisk indicates Bursicon protein levels. Tubulin was used as loading control. **(C)** Wings from flies with genotype: *burs-*

gal4>burs RNAi, which silence *burs* only in Bursicon expressing neurons, are morphologically identical to wild type at 1 d after eclosion. **(D)** Wings from the same *burs-gal4>burs RNAi* animals also develop late-onset blemishes, shown here 21 days post-eclosion. Scale bar = 200 um. **(E)** Quantitative ddPCR assay showing absolute number of *hid* transcripts in *nsy-bgal4>burs* RNAi pupal and eclosed wings. Note that transcript induction is comparable to that in wild type wings. **(F)** Western blot with anti-Hid CL1C3 shows high levels of Hid in *nsyb-gal4>burs* RNAi eclosed wings while there is no detectable Hid in *nsy-bgal4>burs* RNAi pupal wings, similar to patterns observed in wild type wings. Tubulin is shown as loading control. PW= Pupal Wings, EW= Eclosed Wings.

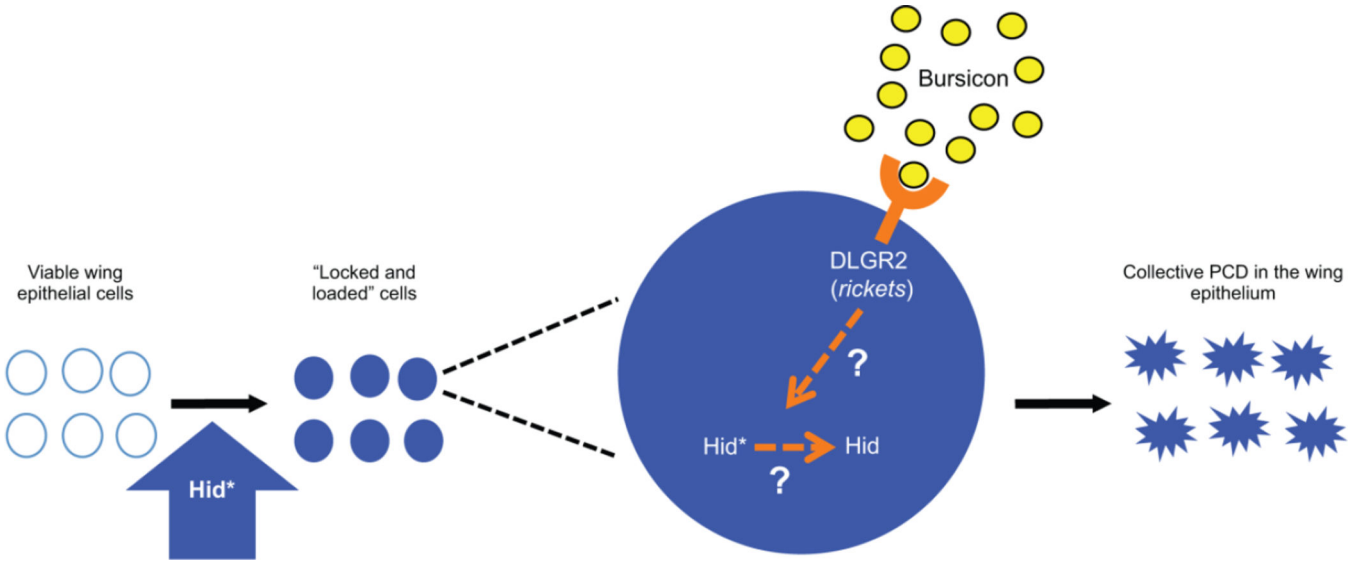

Fig. 7. A "Lock, load and trigger" model for collective cell death

By eclosion, transcriptional regulation has loaded wing epithelial cells with abundant Hid protein, but the action of this IAP antagonist is putatively locked (Abdelwahid, Yokokura et al.) and cells remain alive. Bursicon is released into the hemolymph and binds to DLGR2. The Bursicon/ DLGR2 signaling pathway (dashed arrows) could modify Hid* or alter Hid* localization, enabling active Hid to trigger collective PCD.