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## The *Drosophila* Casein kinase I $\epsilon/\delta$ Discs overgrown promotes cell survival via activation of DIAP1 expression

Ju Guan<sup>a</sup>, Hui Li<sup>a</sup>, Ana Rogulja<sup>a,\*</sup>, Jeff D. Axelrod<sup>b</sup>, and Ken M. Cadigan<sup>a,§</sup>

<sup>a</sup> Department of Molecular, Cellular and Developmental Biology, University of Michigan, Natural Science Building, Ann Arbor MI 48109-1048, USA

<sup>b</sup> Department of Pathology, Stanford University School of Medicine, Stanford CA 94305

### Abstract

The proper number of cells in developing tissues is achieved by coordinating cell division with apoptosis. In *Drosophila*, the adult wing is derived from wing imaginal discs, which undergo a period of growth and proliferation during larval stages without much programmed cell death. In this report, we demonstrate that the *Drosophila* casein kinase I $\epsilon/\delta$ , known as Discs overgrown (Dco), is required for maintaining this low level of apoptosis. Expression of *dco* can suppress the apoptotic activity of Head involution defective (Hid) in the developing eye. Loss of *dco* in the wing disc results in a dramatic reduction in expression of the caspase inhibitor DIAP1 and a concomitant activation of caspases. The regulation of DIAP1 by Dco occurs by a post-transcriptional mechanism that is independent of *hid*. Mutant clones of *dco* are considerably smaller than controls even when apoptosis is inhibited, suggesting that Dco promotes cell division/growth in addition to its role in cell survival. The *dco* phenotype cannot be explained by defects Wingless (Wg) signaling. We propose that Dco coordinates tissue size by stimulating cell division/growth and blocking apoptosis via activation of DIAP1 expression.

### Keywords

*Drosophila*; apoptosis; Dco; Casein Kinase I; DIAP1; Wing disc; Wnt; Wingless

### Introduction

Apoptosis, or programmed cell death (PCD), is a tightly regulated mechanism of cell death that is essential to animal development and homeostasis of adult organs (Jacobson et al., 1997; Meier et al., 2000). In many developmental contexts, PCD is required for the removal of excess cells during morphogenesis. Apoptosis also serves as a quality control mechanism to eliminate abnormal or damaged cells from the organism. In addition, there is a link between cell growth/proliferation and PCD. Cells stimulated to grow and divide often also have an increased propensity for undergoing apoptosis (Abrams, 2002; Green and Evan, 2002). This relationship has been suggested to act as a “fail-safe” system, to prevent unwanted expansion of cell populations. Inhibition of this system is thought to occur during the multi-step process

<sup>§</sup>Corresponding author. Fax: +1 734 647-0884. Email address: cadigan@umich.edu (K. Cadigan).

\*Present address: Institute for Genetics, University of Mainz, Mainz, Germany D-55099

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of oncogenesis (Evan and Vousden, 2001; Green and Evan, 2002; Hanahan and Weinberg, 2000).

PCD is executed by a family of cysteine proteases called caspases. Caspases are synthesized as inactive zymogens and are processed to their active form to cleave cellular substrates, thus promoting the apoptotic pathway. Caspases and many of their regulatory components, such as the Bcl2 family proteins, are evolutionarily conserved from nematodes to mammals (Kaufmann and Hengartner, 2001). However, additional strategies have evolved in insects and higher organisms to provide greater flexibility of regulation. One example is the Inhibitor of Apoptosis (IAP) family of proteins. IAPs can bind to caspases, inhibiting their processing, catalytic activity or the ability of active caspase to bind to substrates (Deveraux and Reed, 1999; Tenev et al. 2005). IAPs can also facilitate caspase degradation by targeting them for ubiquitination via their E3 ubiquitin ligase activity (Wilson et al., 2002; Yang et al., 2000).

Studies of a *Drosophila* member of the IAP family, DIAP1, encoded by the *thread* (*th*) locus, have provided critical insights into the function and regulation of IAPs. DIAP1 can be bound and inhibited by Head involution defective (*Hid*), Grim and Reaper (*Rpr*) (Hay, 2000). Overexpression of these pro-apoptotic factors can down regulate DIAP1 levels by promoting its degradation via the ubiquitin ligase activity of DIAP1 as well as inhibiting DIAP1 translation (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). Once caspases are activated, they can cleave DIAP1, stimulating its ubiquitination and degradation (Ditzel et al., 2003). This multilevel regulation of DIAP1 reflects the critical role it plays in cell survival, as evidenced by the massive activation of caspases and apoptosis observed in *DIAP1* mutants (Ryoo et al., 2004; Yoo et al., 2002).

The connection between cell growth/proliferation and cell death and pattern formation has been extensively studied in the *Drosophila* wing imaginal discs (Edgar, 1999; Serrano and O'Farrell, 1997; Tapon et al., 2001a). During the three larval stages, the wing discs undergo a rapid phase of cell growth and proliferation with very low levels of PCD (Milan et al., 1997). The *Drosophila* homologue of Casein Kinase I $\epsilon/\delta$  (CKI $\epsilon/\delta$ ), known as *discs overgrown/double time* (*dco*), was shown to be essential for imaginal tissue development (Jursnich et al., 1990; Zilian et al., 1999). Weak *dco* mutants exhibit a lag in disc growth, while stronger alleles have small or severely degenerated discs. Mitotic clones of *dco* null mutant cells in the wing disc did not grow or survive when induced during first or second larval stages, though small clones could be observed if induced at later stages (Zilian et al., 1999). Electron micrographs of *dco* mutant discs demonstrated the presence of basally extruded cells with nuclear condensation, suggesting that PCD was occurring. Based on these observations it was concluded that *dco* is required for cell proliferation and/or survival through an undetermined mechanism.

In this report we demonstrate that *Dco* is an essential cell survival factor in the wing imaginal disc. We initially identified *Dco* as a potent suppressor of *Hid* induced cell death in the eye. This *Hid* antagonism requires *Dco* kinase activity. Loss of *dco* in the wing results in massive PCD, which is a major cause of the small disc phenotype seen in *dco* mutants. When apoptosis is inhibited, *dco* mutant wings are normally patterned, suggesting that *dco*'s effect on cell survival is not an indirect consequence of abnormal cell specification. We demonstrate that *dco* mutant cells have a dramatic post-transcriptional reduction of DIAP1 protein levels, which is sufficient to explain the increase in PCD. This regulation of DIAP1 is independent of *hid* and not does appear to require the E3 ligase activity of DIAP1. We also provide results suggesting that *Dco* promotes cell growth/proliferation in the wing disc.

CKI family members, including CKI $\delta$  and CKI $\epsilon$ , have been implicated in several developmentally important signaling pathways. In *Drosophila*, *dco* has been shown to act as a positive regulator of planar cell polarity in the larval eye and pupal wing (Strutt et al.,

2006; Klein et al., 2006). In fly cell culture and the wing imaginal disc, *dco* has been shown to be required for Wnt/Wingless (Wg) signaling (Cong et al., 2004; Klein et al., 2006; Zhang et al., 2006). Wg signaling is known to promote cell survival in the wing imaginal discs (Giraldez and Cohen, 2003; Johnston and Sanders, 2003). However, we find that cells homozygous for a null *dco* allele have normal Wg signaling in the developing wing. Our data support a model where Dco promotes cell survival by activating DIAP1 protein levels independently of Wg signaling.

## Materials and methods

### Fly strains

The following fly strains were used. Oregon R was our wild type stock. The *GMR-hid<sup>10</sup>*, *GMR-hid<sup>1M</sup>*, *GMR-rpr<sup>2</sup>*, *GMR-rpr<sup>5</sup>*, *GMR-grim<sup>2</sup>* and *GMR-grim<sup>3</sup>* (Chen et al., 1996; Rodriguez et al., 1999) stocks were from J. Abrams. *GMR-hid<sup>ala5</sup>* (Bergmann et al., 1998) was from A. Bergmann. UAS-*lacZ* (Brand and Perrimon, 1993), UAS-*p35* (Mergliano and Minden, 2003), *enGal4* (Neufeld et al., 1998) and *ptcGal4* (Johnson et al., 1995) and *th<sup>5c8</sup>* (Hay et al., 1995) were from the Bloomington Stock Center. UAS-*dco* (on the X chromosome; Price and Kalderon, 2002) was from M. Young. A wild-type UAS-*dco* stock and UAS-*dco<sup>D132N</sup>* mutant (Klein et al., 2006) were obtained from M. Mlodzik and a UAS-*dco* and UAS-*dco<sup>K38R</sup>* (Strutt et al., 2006) was provided by D. Strutt. *GMRGal4* (Freeman, 1996) was from M. Freeman. The *hid<sup>05014</sup>* and *hid<sup>X14</sup>* stocks (Grether et al., 1995) were from K. White, while the *Df(3L)H99* and UAS-*DIAP1* stocks (Hay et al., 1995) were from B. Hay. The *dco* mutant alleles *dco<sup>P915</sup>*, *dco<sup>P1396</sup>* and *dco<sup>le88</sup>* (Zilian et al., 1999) were from M. Noll. The *th<sup>6B</sup>* allele (Lisi et al., 2000) was from K. White. *FRT<sup>82B</sup> P[w+, Ubi-GFP<sup>nls</sup>]* and *FRT<sup>82B</sup> P[w+, armlacZ]* (Tapon et al., 2001b) were from I. Hariharan while all other chromosomes for clonal analysis, *yw flp<sup>122</sup>*, *FRT<sup>82B</sup>* (Xu et al., 1993) were from the Bloomington Stock Center. The UAS-*dco<sup>RNAi</sup>* transgene was constructed by cloning an inverted repeat of the C-terminal non-kinase domain coding sequence using the PCR primers 5' CCAGTCTAGACAGCGGCCGACG GAGCGGAC3' and 5' GCTATTTAGATCTAAACCGCAAGGGGTGCGGTGG3' into the pWIZ vector (Lee and Carthew, 2003). Transgenic flies were generated following the standard P element methodology.

### Generation of *dco* clones

To generate *dco* clones with p35 expressed in the posterior domain of the wing disc, the fly strain *yw, flp<sup>122</sup>; enGal4; FRT<sup>82B</sup> P[armlacZ]/TM6* was crossed to *w, UAS-p35; FRT<sup>82B</sup> dco<sup>le88</sup>*. For the *DIAP-lacZ* experiment, the following strain *yw, flp<sup>122</sup>; enGal4; FRT<sup>82B</sup> P[w+, Ubi-GFP<sup>nls</sup>]/TM6* was crossed to UASp35; *th<sup>5c8</sup>, FRT<sup>82B</sup> dco<sup>le88</sup>/TM6*. The progenies were heat shocked during early 2nd instar larval stage for 1 hour at 37°C. The larvae were dissected and fixed with 4% formaldehyde 60 to 72 hours later.

### Immunostainings, TUNEL and in situ hybridization

Immunostainings were performed as previously described (Parker *et al.*, 2002). Mouse anti-DIAP1 (1:200) and rabbit anti-active Drice (1:2,000) (Yoo et al., 2002) were both provided by B. Hay. Guinea pig anti-Sen (1:5000) was made by immunization with GST-Sen as previously described (Nolo et al., 2000). Rabbit anti-Dll (1:200) was from J. Kohtz and rabbit anti-Spalt (1:100) (Kuhnlein et al., 1994) was from R. Schuh and B. Mollereau. Rabbit anti-β-galactosidase (Cappel, ICN, Costa Mesa, CA) and mouse anti-β-galactosidase (Sigma, St. Louis, MO) were both used at 1:500. Rabbit anti-phospho-Histone 3 (1:150) was from Upstate (Lake Placid, NY). Monoclonal anti-En (1:20) and anti-Wg (1:100) were from the Developmental Studies Hybridoma Bank (Iowa City, IA). Cy3- and Alexa Fluor 488-conjugated secondary antibodies were from Jackson Immunochemicals (West Grove, PA) and

Molecular Probes (Eugene, OR), respectively. The TUNEL assay was performed using the Cell Death Detection kit (Roche Diagnostics, Indianapolis, IN) as previously described (Lin et al., 2004). In situ hybridization detection of *hid*, *grim* and *rpr* transcripts was performed as previously described (Lin et al., 2004). Pupal wings were stained as described (Axelrod, 2001) with Alexa568 phalloidin (Molecular Probes) at a concentration of 6.25U/ml.

### Microscopy and image analysis

The adult eyes were photographed on a Leica M10 microscope as previously described (Parker et al., 2002). All fluorescent pictures were obtained with a Zeiss Axiophot coupled to a Zeiss LSM510 confocal apparatus. The pixel values of the clone and twin areas in Fig. 8 were calculated using the histogram tool in Photoshop 6.

## Results

### Dco preferentially suppresses Hid induced cell death in the eye

*dco* was identified in an EP misexpression screen (Rorth et al., 1998) for genes that interact with the Wg signaling pathway. When placed under the control of the eye-specific *GMR* promoter, Wg causes a severe reduction in eye size (Parker et al., 2002). This is due in part to an increase in apoptosis in the late larval/early pupal eye (H. V. Lin, A. Rogulja and K. Cadigan, in preparation). We expected that some EP lines that could suppress the *GMR/wg* phenotype would be inhibitors of PCD. One such example was *EP(3)3280*, where the P-element is inserted into the first intron of *dco* (data not shown). Similar suppression of *GMR/wg* was obtained with a UAS-*dco* line, confirming that *dco* was responsible for the effect. Misexpression of *dco* in several contexts outside the eye revealed no effect on Wg signaling (data not shown). However, expression of *dco* in the eye strongly suppressed the activity of the pro-apoptotic factor Hid (Fig. 1B). *dco* had more subtle effects on the small eye phenotype caused by *GMR-rpr* (Figs. 1D, F) and moderately suppressed *GMR-grim* (Fig. 1H). Because *hid*, *grim* and *rpr* are expressed by the heterologous *GMR* promoter, the suppression is not due to transcriptional inhibition of these pro-apoptotic genes.

It has been previously shown that ectopic expression of activated Ras preferentially suppresses Hid induced cell death in the eye (Bergmann et al., 1998; Kurada and White, 1998). However, the ability of activated Ras to suppress the *GMR-hid* small eye phenotype was blocked when five potential MAP kinase phosphorylation sites were mutated to alanine (*GMR-hid<sup>ala5</sup>*) (Bergmann et al., 1998). Both *EP(3)3280* and UAS-*dco* were able to suppress *GMR-hid<sup>ala5</sup>* to the same extent as wild type *GMR-hid* (Fig. 2B and data not shown), suggesting that *dco* overexpression is not working through a Ras/MAP kinase pathway.

To determine whether endogenous *dco* regulates apoptosis in the eye, we reduced *dco* expression using RNAi via a *dco* hairpin construct (UAS-*dco<sup>RNAi</sup>*). Expression of this hairpin with the *GMR-Gal4* driver resulted in a roughened eye phenotype but did not reduce overall eye size (Fig. 3B). However, *dco<sup>RNAi</sup>* enhanced the small eye phenotype of an intermediate *GMR-hid<sup>IM</sup>* construct (Figs. 3C, D). *dco<sup>RNAi</sup>* had much smaller effects on *GMR-rpr* and *GMR-grim* constructs (data not shown). These data indicate that endogenous *dco* influences the level of apoptosis caused by misexpression of *hid*.

To examine whether the kinase activity of Dco is required for Hid antagonism, two mutant forms of *dco* were co-expressed with *hid* in the eye. *dco<sup>D132N</sup>* is predicted to compromise ATP binding (Peters et al., 1999; Zhu et al., 1998) while *dco<sup>K38R</sup>* contains a mutation within the catalytic site (Peters et al., 1999; DeMaggio et al., 1992; Fish et al., 1995). Both mutants can act as dominant negatives for CKI function (Zhu et al., 1998; Peters et al., 1999; McKay et al., 2001; Klein et al., 2006; Strutt et al., 2006). However, Dco<sup>D132N</sup> was clearly able to suppress

the *GMR-hid* small eye phenotype (Fig. 4B), while Dco<sup>K38R</sup> had the opposite effect. The K38R form of Dco enhanced both a moderate/strong *GMR-hid* (Fig. 4D) and a weaker *GMR-hid* transgene (Fig. 4F). When expressed by *GMRGal4* in an otherwise wild-type eye, Dco<sup>K38R</sup> caused a roughening of the eye, but did not effect eye size (data not shown). Because the D132N mutation has been reported to reduce, but not eliminate CKI kinase activity (Zhu et al., 1998), the data suggest that a partially active Dco can still suppress Hid-mediated apoptosis but some Dco kinase activity is required for antagonizing Hid activity.

### **dco mutant imaginal discs have elevated apoptosis**

Although *dco* overexpression and RNAi knockdown effect the ability of Hid to promote apoptosis, mitotic clones homozygous for a *dco* null allele (*dco*<sup>le88</sup>) are readily detectable in late larval eye discs and mid-pupal eyes (data not shown). *dco* mutant clones are not recovered in the adult eye, although some scarring near the twin spots was seen (data not shown). We have not been able to detect increased apoptosis in the larval or pupal clones. Apparently, cells lacking *dco* are eliminated sometime in late pupal development.

To further explore whether *dco* is required for suppressing PCD, we examined several other imaginal discs. Using the TUNEL DNA fragmentation assay, we compared the level of apoptosis in imaginal discs from wild type and hypomorphic *dco* mutants (*dco*<sup>P915/P1395</sup>). Throughout third instar larval development, TUNEL positive cells in the wing were detected at low levels in wild type, as shown in Figs. 5A & D. In contrast, we found a dramatic increase in TUNEL signal in the *dco* mutant wing discs (Figs. 5B, E). Note that the *dco* hypomorphs are developmentally delayed, and the discs were normalized for developmental stage by examining the pattern of Wg expression. Elevated TUNEL signal was also observed in the leg and haltere discs from *dco* mutants, but no increase was observed in eye imaginal discs (data not shown).

To determine whether the elevated PCD we observe is related to *hid*, *grim* and/or *rpr* function, we lowered the dose of these genes in a *dco* mutant background. Removal of one copy of *hid*, *grim* and *rpr* (using the *Df(3L)H99* deficiency) caused a significant reduction of TUNEL signal in *dco* mutants (Figs. 5E, F). Removal of one copy of *hid* alone did not significantly reduce the level of apoptosis, but a dramatic reduction was achieved by removing both copies of *hid* (data not shown). These data suggest that in *dco* mutant discs endogenous *hid*, *grim* and/or *rpr* are required for the elevated cell death.

As reported previously, *dco*<sup>P915/P1395</sup> mutant animals have a prolonged larval phase with slow imaginal disc growth (Zilian et al., 1999). These animals die in late larval or early pupal stages. We found that the decrease in cell death in *H99*, *dco*<sup>P915/+</sup>, *dco*<sup>P1395</sup> discs was correlated with marked acceleration in disc growth and larval development (from 8–10 days to 5–6 days). These *H99/+*, *dco*<sup>P915/P1395</sup> animals have increased survival into late pupal stages, and the veination pattern of the majority of these animals are similar to wildtype (Figs. 5G, H). Occasional escapers survive to adulthood displaying normal legs and wrinkled or blistered wings (data not shown). These results suggests that the developmental delay in disc growth and larval development seen in *dco* mutants is largely caused by elevated apoptosis. In addition, the data in Fig. 5H indicate that patterning of the wing is normal in *dco* mutants when PCD is reduced.

### **Dco activates DIAP1 expression in the wing imaginal disc**

Both overexpression and loss of function studies with *dco* suggest that it encodes an anti-apoptotic factor in *Drosophila* imaginal tissues. Our initial observation that overexpression of *dco* differentially suppresses *GMR-hid* and *GMR-rpr* is reminiscent of *DIAP1* alleles containing mutations in its RING domain (Lisi et al., 2000; Wilson et al., 2002). It has been

shown that these DIAP1 mutants lack ubiquitin ligase activity and may be present at higher levels due to stabilization of DIAP1. These findings prompted us to look at DIAP1 protein levels when *dco* expression is elevated (with UAS-*dco*) or reduced (UAS-*dco*<sup>RNAi</sup>). Both UAS transgenes were driven by *patched* (*ptc*)-Gal4, which is active in a stripe on the anterior side of the anterior/posterior boundary of the wing imaginal disc (Johnson et al., 1995).

Overexpression of *dco* led to a moderate increase of DIAP1 protein levels in the *ptc* expression domain (Fig. 6B). Conversely, DIAP1 expression was decreased upon RNAi-mediated knockdown of *dco* (Fig. 6C). Using an antibody specific for the activated form of the effector caspase Drice (Yoo et al., 2002), we found that *dco* knockdown also caused a marked increase in Drice activation (Fig. 6E). Z-axis projection of *ptc*-Gal4/UAS-*dco*<sup>RNAi</sup> wing discs reveals that the Drice-positive cells are found basally in the epithelial sheet (Fig. 6G) and that DIAP1 levels are low both apically and basally (Fig. 6H). Loss of DIAP1 is known to cause apoptosis (Ryoo et al. 2004; Wang et al. 1999; Yoo et al. 2002), and the data suggest that Dco promotes cell survival by maintaining DIAP1 expression.

To determine whether *hid* was required for Dco regulation of DIAP1, the *ptc*-Gal4/UAS-*dco*<sup>RNAi</sup> experiments were repeated in a *hid* mutant background. An obvious decrease in DIAP1 levels was still observed (Fig. 6D) indicating that Dco can activate DIAP1 independently of *hid*. The reduction in DIAP1 was also seen in a *H99*/+ background (data not shown). In contrast, the activation of Drice by *dco*<sup>RNAi</sup> was greatly suppressed in *hid* mutants (Fig. 6F) or *H99* heterozygotes (data not shown). This is consistent with the results from *dco* hypomorphic mutants (Figs. 5C, F).

To rule out the possibility that Dco regulates the transcript levels of *hid*, *grim* or *rpr*, in situ hybridization was performed on *ptc*-Gal4/UAS-*dco*<sup>RNAi</sup> wing imaginal discs. *hid* expression was not detectably effected (Fig. 7A), but a small reduction in *grim* expression was sometimes observed within the Ptc domain (Fig. 7B) while a small increase in *rpr* transcripts was also consistently seen (Fig. 7C). These minor differences were not observed in wild-type discs (data not shown). We consider it highly unlikely that these minor and contradictory differences can explain the dramatic effect observed on DIAP1 protein levels.

We also examined the phenotype of *dco* null (*dco*<sup>le88</sup>) mutant cells induced by mitotic recombination. As reported previously (Zilian et al., 1999), clones of this allele were not obtained when recombination was induced before third larval instar (data not shown). This experiment was repeated in discs where the baculovirus caspase inhibitor p35 was constantly expressed in the posterior compartment via *engrailed* (*en*)-Gal4. As shown in Fig. 8A, *dco* mutant clones were not detected in the anterior compartment (lacking p35), but were found in the posterior compartment expressing p35. Twin spots were visible in both compartments, indicating that mitotic recombination had occurred. These data indicate that caspase-dependent PCD contributes significantly to the loss of cells in *dco* mutant clones.

Consistent with a role of Dco in promoting cell survival by maintaining DIAP1 levels, we found elevated active Drice and decreased DIAP1 in *dco* clones (Figs. 8C–H). The activation of Drice is largely cell autonomous, though we occasionally see some additional signal adjacent to the clones (arrows in Figs. 8C–E). We speculate that this might represent dying cells engulfed by surrounding cells or macrophages. It has been reported in fly embryos that phagocytosis of cells containing activated caspases is not blocked by p35 expression (Mergliano and Minden, 2003). The *dco* null clones have decreased DIAP1 levels regardless of the area in the wing discs (hinge, pouch, notum) where the clones are found, indicating that Dco regulates DIAP1 throughout the wing discs.

When *dco* clones were generated in a Minute/+ background, a strong decrease in DIAP1 levels were observed, but only a small increase in activated Drice was seen, most prominently in the

basal area of the clone (Supplementary Fig. 1). This suggests that cell competition is involved in promoting PCD in *dco* mutant cells, though not in the primary effect on DIAP1 protein expression.

To examine whether this activation of DIAP1 by *dco* is at the transcriptional level, we looked at the expression of a *lacZ* reporter under the control of the *DIAP1* control region (*DIAP1-lacZ*) in *dco* clones and found that it was not altered (Figs. 8I–K). This suggests that Dco regulates DIAP1 by a post-transcriptional mechanism.

### **dco mutant clones are significantly smaller than their twins**

A role for Dco in cell proliferation was suggested by the size of *dco* clones in the presence of the caspase inhibitor p35, which were consistently smaller than the twin spots (e.g., Fig. 8A). When this was quantitated, we found that the *dco* clones were on average 4 times smaller than the twin spots in the posterior compartment (Fig. 8B). Because the twins and clones originate from the two daughter cells of a single cell that underwent mitotic recombination, they grow for the same period of time in a similar location. Therefore the approximate 1:4 ratio of clone/twin size in *dco* mutants suggests a defect in the rate of growth/proliferation. To examine this further, we performed bromo deoxyuridine incorporation and immunostaining for phospho-Histone 3, which label cells that are undergoing DNA replication or mitosis, respectively. Neither analysis revealed any obvious differences between the clones and surrounding tissues (data not shown). These techniques are probably not sensitive enough to reveal subtle differences in cell proliferation that could contribute to the reduced clone size after several generations of cell division. The cells and nuclei in the clones also appear to be of similar size and shape as those in the surrounding tissue, suggesting that reduced cell size cannot account for the small size of *dco* clones.

### **The E3 ubiquitin ligase activity of DIAP1 is not required for its regulation by Dco**

The C-terminus of DIAP1 contains a RING domain that has intrinsic E3 ubiquitin ligase activity which can promote its own degradation via auto-ubiquitination. Mutations that abolish this activity suppress Hid induced cell death but do not affect or enhance Rpr killing (Lisi et al., 2000; Wilson et al., 2002; Yokokura et al., 2004). This is consistent with the differential suppression of Hid and Rpr by ectopic expression of Dco (Figs. 1A–F). It is conceivable that Dco regulates DIAP1 level through modulating the auto-ubiquitination process.

To explore the relationship between Dco and DIAP1 E3 ligase activity, we examined whether a point mutation in one of the essential cysteine residues in the RING domain (*th<sup>6B</sup>*) disrupted the decrease in DIAP1 protein levels upon RNAi depletion of *dco*. One complication of monitoring DIAP1 levels in the RING domain mutant is the possibility that the mutant DIAP1 proteins are unusually stable compared to wild-type, due to lack of degradation by Hid or perhaps Rpr/Grim, since there are reports that these factors can also effect DIAP1 stability (Hays et al. 2002; Ryoo et al. 2002). However, mitotic clones of *Df(3L)H99* did not contain higher levels of DIAP1 immunostaining in the wing imaginal disc (Figs. 9A–C). This was true of clones located throughout the wing pouch and notum (data not shown). While much of the tissue surrounding these clones are heterozygous for the *hid*, *grim* and *rpr* deficiency, the *+/+* twin clones also had similar levels of DIAP1 staining compared to the *H99* clones (data not shown). These data indicate that under normal conditions, endogenous Hid, Grim and Rpr do not significantly regulate the expression of DIAP1 in the wing.

As previously described, expression of *dco<sup>RNAi</sup>* in the *ptc* domain of the wing disc results in a significant decrease in DIAP1 expression (Fig. 6C) and an increase in Drice activation (Fig. 6E). In a *th<sup>6B</sup>/+* background, similar results were obtained (Figs. 9D–E), suggesting that the effect of Dco on DIAP1 protein levels does not require DIAP1's E3 ligase activity.

## **dco mutant clones have normal Wg and Dpp signaling**

CKIε has been proposed to act both as a positive and negative regulator of Wnt signaling (Price, 2006). A positive role for Dco in Wg signaling has recently been demonstrated in cultured *Drosophila* S2 cells (Cong et al., 2004) and wing imaginal discs (Klein et al., 2006; Zhang et al., 2006). Wg signaling regulates cell proliferation and promotes cell survival in the wing imaginal discs (Giraldez and Cohen, 2003; Johnston and Sanders, 2003). Therefore, the phenotypes we have observed in *dco* mutant clones could be explained by a reduction in Wg signaling. However, we find that two targets of Wg signaling in wing imaginal discs, Senseless (Sen) and Distal-less (Dll) (Parker et al., 2002) are unaffected in cells (expressing *p35*) that are homozygous for the *dco* null mutation (Figs. 10A–F). We conclude that the cell survival function of Dco in wing imaginal discs is not linked to the Wg pathway and that Dco is dispensable for Wg signaling under the conditions used in our study.

Like Wg, Dpp signaling has also been shown to promote cell proliferation and cell survival in the wing imaginal disc (Martin-Castellanos and Edgar, 2002; Moreno et al., 2002). CKIε has been shown to influence TGF-β signaling in mammalian cell culture, both negatively and positively, depending on the assay (Waddell et al. 2004). However, Dpp signaling appears to be normal in cells lacking *dco*, as judged by expression of the Dpp target Spalt (Figs. 10G–I).

## **Discussion**

### **Dco promotes cell survival by activating DIAP1 expression**

Dco is required for suppression of PCD in several developing tissues. Hypomorphic *dco* mutants have elevated apoptosis in the wing, leg and haltere discs (Fig. 5 and data not shown). Reduction of *dco* with either RNAi or with mitotic clones of a null allele also caused activation of the effector caspase Drice in wing discs (Figs. 6E and 8D). In the eye disc and early pupal eye, clones of the null *dco* allele did not result in obvious activation of Drice or PCD (data not shown). However, reduction of endogenous *dco* with RNAi did enhance the ability of Hid to promote cell death in the eye (Figs. 3C, D), indicating that Dco promotes cell survival in this tissue as well. It is possible that more subtle phenotype in the eye is caused by other CKI family members acting redundantly with Dco in this tissue.

In the wing disc, the loss of *dco* resulted in a dramatic reduction in DIAP1 expression (Figs. 6, 8, 9). The loss of *DIAP1* in either fly embryos or the wing imaginal discs leads to massive activation of caspases and cell death (Ryoo et al., 2004; Wang et al., 1999; Yoo et al., 2002). Therefore, the reduction in DIAP1 levels observed in *dco* mutants is sufficient to explain the elevated caspase activation and apoptosis in these cells. The reduction in DIAP1 expression is not a secondary consequence of activating the apoptotic machinery, since a strong reduction in DIAP1 levels was observed in the presence of a caspase inhibitor (Fig. 8G), or in mutant backgrounds where active caspases are barely detectable (Figs. 6D, F). These results argue that reduction in *dco* leads to a loss of DIAP1 expression, which results in caspase activation and PCD.

Though our data indicate that Dco activation of DIAP1 is important to suppress apoptosis, the possibility existed that this regulation was due to non-specific effects of mispatterning in *dco* mutants. However, the phenotype of *H99, dco<sup>P915/+</sup>, dco<sup>P1395</sup>* mutants argues strongly against this scenario. Heterozygosity of *H99* suppressed the elevated PCD in the wing (Figs. 5C, F), shortened the developmental lag observed in *dco* hypomorphic mutants and increased the lethal phase of these mutants from early pupation to late pupal stages, with some escapers eclosing. The flies that emerged had normally patterned legs, but the pattern of the wings was difficult to analyze due to lack of unfolding and blistering. However, the venation pattern of the pupal wings (prior to folding) appeared normal (Fig. 5H). This data, combined with the fact that Dpp



and Wg signaling appear normal in *dco* null clones (Fig. 10) strongly suggests that Dco does not have a significant role in patterning, but rather is a direct repressor of apoptosis.

### The role of the IAP antagonists Hid, Grim and Rpr

*dco* interacts genetically with *hid*, *grim* and *rpr* in the eye (Figs. 1–4) and the wing (Fig. 5 and 6), suggesting a link between Dco and these pro-apoptotic factors. Dco appears to regulate DIAP1 at a post-transcriptional level (Fig. 8J) and it has been reported that DIAP1 is inhibited by Hid, Grim and Rpr at both the translational level and by increased protein turnover (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). This raises the possibility that Dco could activate DIAP1 expression through repression of one or more of these IAP inhibitors. Alternatively, Dco could activate DIAP1 through a pathway that acts in parallel to Hid, Grim and Rpr.

If Dco regulates DIAP1 expression by repressing IAP inhibitor activity, it cannot work solely through Hid. The elevated caspase activation and PCD observed upon reduction of *dco* gene activity is greatly suppressed in a *hid* mutant background (Fig. 6F and data not shown). However, DIAP1 levels are still reduced with *dco* RNAi in *hid* mutants (Fig. 6D). The apoptotic phenotype in *dco* mutant wing discs is suppressed by removal of one copy of *hid*, *grim* and *rpr* (Figs. 5C,F), but not by removal of one copy of *hid* (data not shown). Therefore Dco would have to act by inhibiting Hid and Grim and/or Rpr. While Dco overexpression most effectively suppresses Hid activity (Fig. 1B), it also moderately suppresses Grim (Fig. 1H) and has a small effect on Rpr-induced PCD (Fig. 1F).

If Dco regulates DIAP1 in parallel to the IAP inhibitors, then how to explain the suppression of caspase activation and PCD in *dco* mutants when *hid* is mutated (Fig. 6F) or in *Df(3L)H99* heterozygotes (Figs. 5C, F)? These data can be explained by a balance between negative regulators of apoptosis (Dco) and positive inputs (Hid/Grim/Rpr). Reduction of *dco* lowers DIAP1 levels, promoting caspase activation, however, the IAP inhibitors are known to bind to DIAP1 and sequester it from its normal function of caspase inhibition (Hay, 2000). So reduction of these DIAP1 inhibitors allows the lower levels of DIAP1 in the *dco* mutants to bind to caspases and prevent their activation.

In addition to binding to DIAP1 and inhibiting its ability to repress caspases, Hid, Grim and Rpr have been shown to down regulate DIAP1 expression (Hays et al., 2002; Holley et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). Interestingly, we found that clones lacking *hid*, *grim* and *rpr* had no effect on DIAP1 protein levels in the wing disc (Figs. 9A–C). These results suggest that under normal conditions, these IAP inhibitors are not major regulators of DIAP1 expression in the wing imaginal disc. A more informative experiment would be to examine DIAP1 expression in clones lacking *hid*, *grim*, *rpr* and *dco*, to determine whether the decrease in DIAP1 requires the three pro-apoptotic factors. Attempts to perform this experiment have thus far not been possible, due to technical difficulties.

Hid, Grim and Rpr have been suggested to downregulate DIAP1 through its intrinsic E3 ubiquitin ligase activity (Hays et al., 2002; Ryoo et al., 2002; Yoo et al., 2002). However, a mutation that disrupts enzymatic activity (*th<sup>6B</sup>*) is still downregulated in *dco*<sup>RNAi</sup> depleted cells (Figs. 9D–F). This argues against DIAP1 auto-ubiquitination playing an important role in Dco regulation of DIAP1. This experiment could only be performed in a *th<sup>6B</sup>* heterozygous background, because *th<sup>6B</sup>* clones do not survive/grow.

### Dco may promote cell growth/proliferation

In addition to its role in promoting cell survival, our clonal analysis indicates that *dco* may promote cell growth/proliferation. In the presence of p35 caspase inhibitor, *dco* mutant clones

are about four times smaller than their twins (Fig. 8B). One explanation for this is that p35 does not completely suppress the elevated apoptosis in the *dco* clone. However, the UAS-*p35* transgene used is able to fully suppress a strong GMR-*hid* phenotype in the eye, and no TUNEL positive cells are observed in our *dco* clones in the presence of p35 (data not shown).

The conclusion that *dco* mutant cells grow/divide slower than controls is tempered by several caveats. Recent findings show that apoptotic cells in the wing disc can stimulate their neighbors to proliferate (de la Cova et al., 2004; Huh et al., 2004; Moreno and Basler, 2004; Ryoo et al., 2004). This phenomenon has been observed in cells expressing p35, suggesting that caspase activation but not PCD itself generates the proliferative signal (Huh et al., 2004; Ryoo et al., 2004; Perez-Garijo et al. 2005). Induced expression of Wg in the dying cells has been proposed to be part of the mitogenic signal for this compensatory proliferation (Ryoo et al., 2004). While we observed substantial ectopic Wg staining in *dco* mutant wing discs (Supplementary information Fig. 2), ectopic Wg was not observed in the p35 expressing *dco* mutant clones (n=30, data not shown). Despite this, our current data cannot rule out that the low clone/twin ratio we observe for *dco* clones in Fig. 8B could at least be partially due to increased proliferation of the twins.

### Relation of Dco to Wg and other pathways affecting PCD/proliferation

In our study, no defect in Wg signaling was observed in mitotic clones for a *dco* null allele in wing imaginal discs (Fig. 10A–F). A similar result has been recently reported, though a hypomorphic *dco* allele was used (Strutt et al., 2006). In contrast, another recent study generated large clones with a strong *dco* allele (*dco<sup>dbt-P</sup>*) in a *Minute/+* background, and reported no activation of caspases and a loss of Sens and adult wing margin, two readouts of Wg signaling (Klein et al., 2006). We have made *dco<sup>le88</sup>* clones with the Minute technique, and find that they are infrequent and similar in size to the ones reported in Fig. 8. The few at the dorsal/ventral boundary expressed Sens normally (data not shown), while clones throughout the wing pouch had a dramatic reduction in DIAP1 expression and only a small increase in activated Drice (Supplementary information Fig. 1). Another report generating very large *dco<sup>le88</sup>* clones using *MS1096-Gal4/UAS-FLP*, Minute and p35 expression (Zhang et al. 2006) observed a strong decrease in Sens expression. This report also found evidence that other CKI family members could promote Wg signaling (Zhang et al., 2006). Clone size and redundancy/compensation may explain the differences between our results and those of Klein et al. (2006) and Zhang et al. (2006). However, the primary phenotype observed when *dco* is removed under the conditions used in this study is a decrease in DIAP1 levels.

In our misexpression studies in the eye, Dco was able to suppress the ability of a Ras/MAPK resistant version of Hid (Bergmann et al., 1998) to induce apoptosis (Fig. 2). At least in the eye, Dco appears to control cell survival independently from Ras/MAPK signaling.

The phenotype of *dco* is similar to that of *yorkie*, a nuclear protein that is required for DIAP1 expression and proliferation (Huang et al., 2005). Yorkie is inhibited by a complex containing the Lats and Hippo kinases, and the adaptor protein Salvador (Sav). Clones mutant for *lats*, *sav* or *hippo* display an opposite phenotype to *dco*, i.e., dramatic increases in proliferation and a block in PCD (see Edgar, 2006 for a review). Double mutant clones for *sav* and *dco* do not survive/grow (data not shown), suggesting that Dco may act downstream of the Wts/Sav/Hippo complex. However, the expression of *DIAP1-lacZ* is upregulated in *hippo* clones (Wu et al., 2003) and decreased in *yorkie* clones (Huang et al., 2005) but we observe no effect on this transgene in *dco* clones (Figs. 8I–K). In addition, Wts/Sav/Hippo represses Cyclin E expression, but Dco does not (data not shown). Therefore, we consider it unlikely that Dco acts with Wts/Sav/Hippo in a simple linear pathway. Hippo has also been reported to phosphorylate DIAP1, leading to instability (Harvey et al., 2003; Pantalacci et al., 2003), so the possibility remains that Dco may interact with Hippo in this regard.

## CKI $\delta/\epsilon$ in other systems

Our data supports a model where Dco promotes cell survival in fly imaginal discs by maintaining expression of DIAP1 through a novel pathway. Do casein kinase I $\delta/\epsilon$  family members have similar functions in other organisms? The yeast CKI gene Hrr25 is involved in DNA repair, cell growth and viability (Hoekstra et al., 1991). In mammalian cell lines, CKI $\epsilon$  plays a protective role against apoptosis induced by extrinsic death signals (Desagher et al., 2001; Izeradjene et al. 2004). This cell survival activity was correlated with CKI $\epsilon$  phosphorylation of the pro-apoptotic factor Bid (Desagher et al., 2001). Inhibition of CKI $\delta$  induces apoptosis in trophoblast cells (Stoter et al. 2005). Further studies will be required to determine whether there are commonalities between these different systems. The ability of Dco to inhibit apoptosis and possibly promote cell proliferation makes it an attractive candidate for functioning as an oncogene in tumorigenesis.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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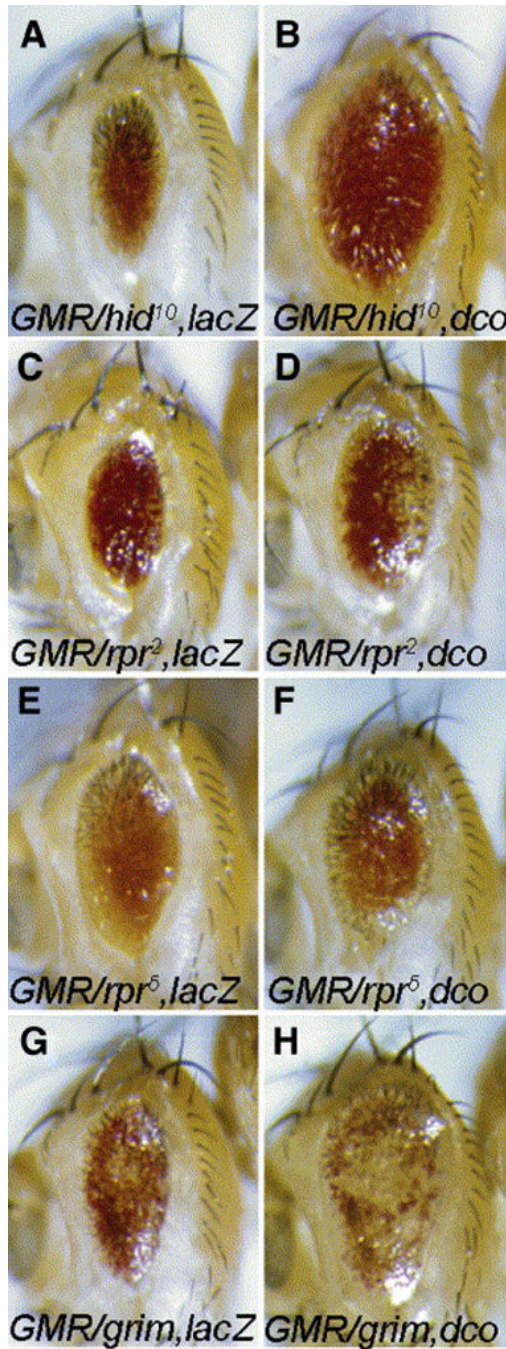
## References

- Abrams JM. Competition and compensation: Coupled to death in development and cancer. *Cell* 2002;110:403–406. [PubMed: 12202030]
- Axelrod JD. Unipolar membrane association of Dishevelled mediates Frizzled planar cell polarity signaling. *Genes Dev* 2001;15:1182–1187. [PubMed: 11358862]
- Bergmann A, Agapite J, McCall K, Steller H. The *Drosophila* gene *hid* is a direct molecular target of Ras-dependent survival signaling. *Cell* 1998;95:331–341. [PubMed: 9814704]
- Brand A, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 1994;118:401–415. [PubMed: 8223268]
- Chen P, Nordstrom W, Gish B, Abrams JM. *grim*, a novel cell death gene in *Drosophila*. *Genes Dev* 1996;10:1773–1782. [PubMed: 8698237]
- Cong F, Schweizer L, Varmus H. Casein kinase Iepsilon modulates the signaling specificities of dishevelled. *Mol Cell Biol* 2004;24:2000–2011.
- de la Cova C, Abril M, Bellosta P, Gallant P, Johnston LA. *Drosophila* Myc regulates organ size by inducing cell competition. *Cell* 2004;117:107–116. [PubMed: 15066286]
- DeMaggio AJ, Lindberg RA, Hunter T, Hoekstra MF. The budding yeast HRR25 gene product is a casein kinase I isoform. *Proc Natl Acad Sci USA* 1992;89:7008–7012. [PubMed: 1495994]
- Desagher S, Osen-Sand A, Montessuit S, Magnenat E, Vilbois F, Hochmann A, Journot L, Antonsson B, Martinou JC. Phosphorylation of bid by casein kinases I and II regulates its cleavage by caspase 8. *Mol Cell* 2001;8:601–611. [PubMed: 11583622]
- Deveraux QL, Reed JC. IAP family proteins--suppressors of apoptosis. *Genes Dev* 1999;13:239–252. [PubMed: 9990849]
- Ditzel M, Wilson R, Tenev T, Zachariou A, Paul A, Deas E, Meier P. Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. *Nat Cell Biol* 2003;5:467–473. [PubMed: 12692559]

- Edgar BA. From small flies come big discoveries about size control. *Nat Cell Biol* 1999;1:E191–E193. [PubMed: 10587651]
- Edgar BA. From cell structure to transcription: Hippo forges a new path. *Cell* 2006;124:267–273. [PubMed: 16439203]
- Evan GI, Vousden KH. Proliferation, cell cycle and apoptosis in cancer. *Nature* 2001;411:342–348. [PubMed: 11357141]
- Fish KJ, Cegielska A, Getman ME, Landes GM, Virshup DM. Isolation and Characterization of Human Casein Kinase I $\epsilon$  (CKI), a Novel Member of the CKI Gene Family. *J Biol Chem* 1995;270:14875–14883. [PubMed: 7797465]
- Freeman M. Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell* 1996;87:651–660. [PubMed: 8929534]
- Gerether ME, Abrams JM, Agapite J, White K, Steller H. The *head involution defective* gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev* 1995;9:1694–1708. [PubMed: 7622034]
- Giraldez AJ, Cohen SM. Wingless and Notch signaling provide cell survival cues and control cell proliferation during wing development. *Development* 2003;130:6533–6543. [PubMed: 14660542]
- Green DR, Evan GI. A matter of life and death. *Cancer Cell* 2002;1:19–30. [PubMed: 12086884]
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57–70. [PubMed: 10647931]
- Harvey KF, Pflieger CM, Hariharan IK. The *Drosophila* Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. *Cell* 2003;114:457–467. [PubMed: 12941274]
- Haung J, Wu S, Barrera J, Matthews K, Pan D. The Hippo Signaling Pathway Coordinately Regulates Cell Proliferation and Apoptosis by Inactivating Yorkie, the *Drosophila* Homolog of YAP. *Cell* 2005;122:421–434. [PubMed: 16096061]
- Hay BA, Wassarman DA, Rubin GM. *Drosophila* homologs of baculovirus inhibitor and apoptosis proteins function to block cell death. *Cell* 1995;83:1253–1262. [PubMed: 8548811]
- Hay BA. Understanding IAP function and regulation: a view from *Drosophila*. *Cell Death Differ* 2000;7:1045–1056. [PubMed: 11139277]
- Hays R, Wickline L, Cagan R. Morgue mediates apoptosis in the *Drosophila melanogaster* retina by promoting degradation of DIAP1. *Nat Cell Biol* 2002;4:425–431. [PubMed: 12021768]
- Hoekstra MF, Liskay RM, Ou AC, Demaggio AJ, Burbee DG, Heffron F. Hrr25, a putative protein-kinase from budding yeast - association with repair of damaged DNA. *Science* 1991;253:1031–1034. [PubMed: 1887218]
- Holley CL, Olson MR, Colon-Ramos DA, Kornbluth S. Reaper eliminates IAP proteins through stimulated IAP degradation and generalized translational inhibition. *Nat Cell Biol* 2002;4:439–444. [PubMed: 12021770]
- Huh JR, Guo M, Hay BA. Compensatory proliferation induced by cell death in the *Drosophila* wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. *Curr Biol* 2004;14:1262–1266. [PubMed: 15268856]
- Izeradjene K, Douglas L, Delaney AB, Houghton JA. Casein Kinase I attenuates Tumor Necrosis Factor-related apoptosis-inducing ligand-induced apoptosis by regulating the recruitment of Fas-associated death domain and procaspase-8 to the death-inducing signal complex. *Cancer Res* 2004;64:8036–8044. [PubMed: 15520213]
- Jacobson MD, Weil M, Raff MC. Programmed cell death in animal development. *Cell* 1997;88:347–354. [PubMed: 9039261]
- Johnson RL, Grenier JK, Scott MP. *patched* overexpression alters wing disc size and pattern: transcriptional and post-transcriptional effects on hedgehog targets. *Development* 1995;121:4161–4170. [PubMed: 8575316]
- Johnston LA, Sanders AL. Wingless promotes cell survival but constrains growth during *Drosophila* wing development. *Nat Cell Biol* 2003;5:827–833. [PubMed: 12942089]
- Jurnich VA, Fraser DE, Held LIJ, Ryerse J, Bryant PJ. Defective gap-junctional communication associated with imaginal disc overgrowth and degeneration caused by mutations of the *dco* gene in *Drosophila*. *Dev Biol* 1990;140:413–429. [PubMed: 2373260]

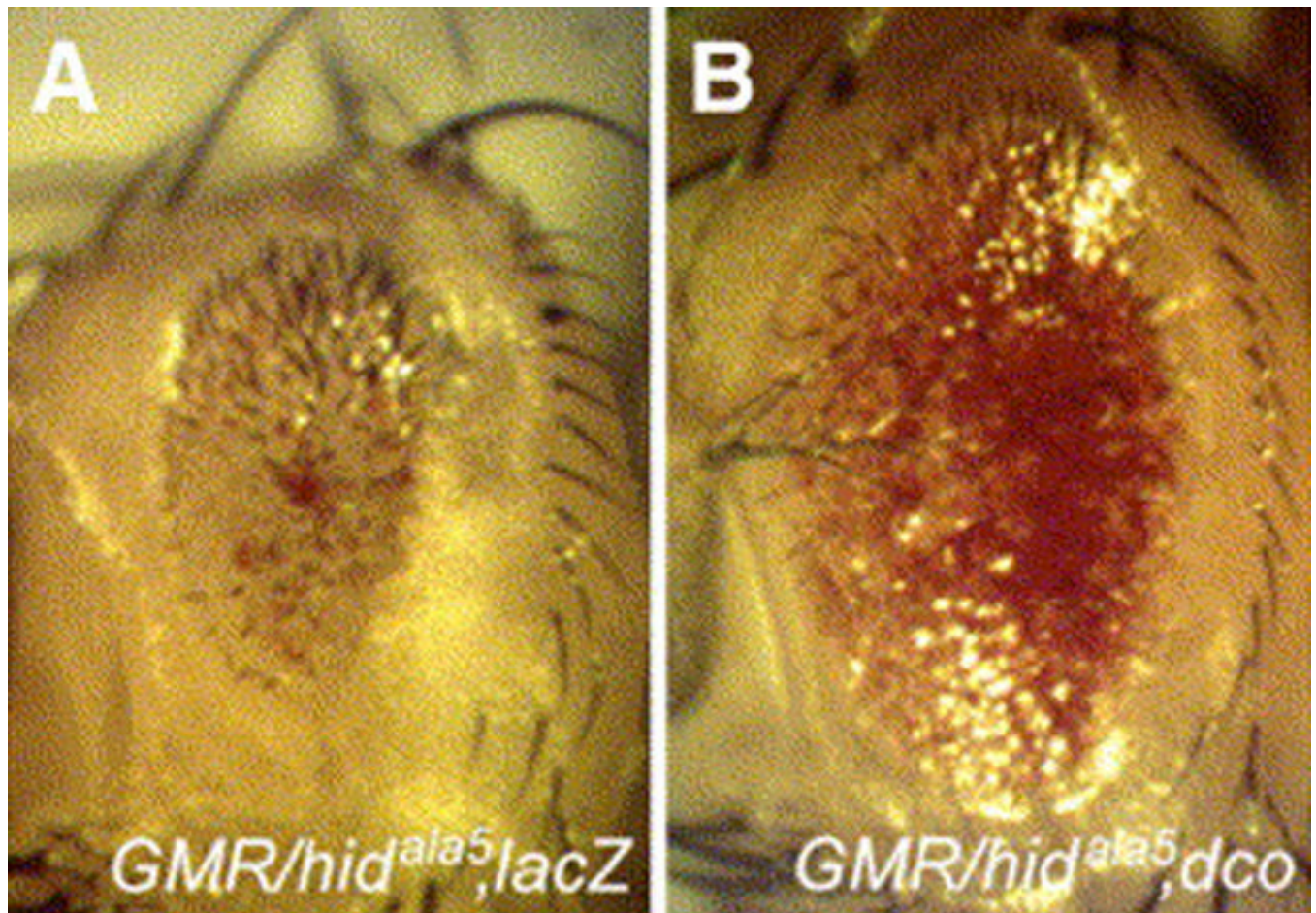
- Kaufmann SH, Hengartner MO. Programmed cell death: alive and well in the new millennium. *Trends Cell Biol* 2001;11:526–534. [PubMed: 11719060]
- Klein TJ, Jenny A, Djiane A, Mlodzik M. *CKI/discs overgrown* promotes both Wnt/b-catenin and Fz/PCP signaling in *Drosophila*. *Curr Biol* 2006;16:1337–1343. [PubMed: 16824922]
- Kuhnlein RP, Frommer G, Friedrich M, Gonzalez-Gaitan M, Weber A, Wagner-Bernholz JF, Gehring WJ, Jackle H, Schuh R. *splat* encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. *EMBO J* 1994;13:168–179. [PubMed: 7905822]
- Kurada P, White K. Ras promotes cell survival in *Drosophila* by downregulating hid expression. *Cell* 1998;95:319–329. [PubMed: 9814703]
- Lee YS, Carthew RW. Making a better RANi vector for *Drosophila*: use of intron spacers. *Methods* 2003;30:322–329. [PubMed: 12828946]
- Lin HV, Rogulja A, Cadigan KM. Wingless eliminates ommatidia from the edge of the developing eye through activation of apoptosis. *Development* 2004;131:2409–2418. [PubMed: 15128670]
- Lisi S, Mazzon I, White K. Diverse domains of THREAD/DIAP1 are required to inhibit apoptosis induced by REAPER and HID in *Drosophila*. *Genetics* 2000;154:669–678. [PubMed: 10655220]
- Martin-Castellanos C, Edgar BA. A characterization of the effects of Dpp signaling on cell growth and proliferation in the *Drosophila* wing. *Development* 2002;129:1003–1013. [PubMed: 11861483]
- McKay RM, Peters JM, Graff J. The Casein Kinase I Family: Roles in Morphogenesis. *Dev Biol* 2001;235:378–387. [PubMed: 11437444]
- Meier P, Finch A, Evan G. Apoptosis in development. *Nature* 2000;407:796–801. [PubMed: 11048731]
- Mergliano J, Minden JS. Caspase-independent cell engulfment mirrors cell death pattern in *Drosophila* embryos. *Development* 2003;130:5779–5789. [PubMed: 14534140]
- Milan M, Campuzano S, Garcia-Bellido A. Developmental parameters of cell death in the wing disc of *Drosophila*. *Proc Natl Acad Sci USA* 1997;94:5691–5696. [PubMed: 9159134]
- Moreno E, Basler K, Morata G. Cells compete for Decapentaplegic survival factor to prevent apoptosis in *Drosophila* wing development. *Nature* 2002;416:755–759. [PubMed: 11961558]
- Moreno E, Basler K. dMyc transforms cells into super-competitors. *Cell* 2004;117:117–129. [PubMed: 15066287]
- Neufeld TP, de la Cruz AF, Johnston LA, Edgar BA. Coordination of growth and cell division in the *Drosophila* wing. *Cell* 1998;93:1183–1193. [PubMed: 9657151]
- Nolo R, Abbott LA, Bellen HJ. Senseless, a Zn finger transcription factor, is necessary and sufficient for sensory organ development in *Drosophila*. *Cell* 2000;102:349–362. [PubMed: 10975525]
- Pantalacci S, Tapon N, Leopold P. The Salvador partner Hippo promotes apoptosis and cell-cycle exit in *Drosophila*. *Nat Cell Biol* 2003;5:921–927. [PubMed: 14502295]
- Parker DS, Jemison J, Cadigan KM. Pygopus, a nuclear PHD-finger protein required for Wingless signaling in *Drosophila*. *Development* 2002;129:2565–2576. [PubMed: 12015286]
- Perez-Garijo A, Martin FA, Struhl G, Morata G. Dpp signaling and the induction of neoplastic tumors by caspase-inhibited apoptotic cells in *Drosophila*. *Proc Natl Acad Sci USA* 2005;102:17664–17669. [PubMed: 16314564]
- Peters JM, McKay RM, McKay JP, Graff JM. Casein kinase I transduces Wnt signals. *Nature* 1999;401:345–350. [PubMed: 10517632]
- Price MA, Kalderon D. Proteolysis of the Hedgehog signaling effector Cubitus interruptus requires phosphorylation by Glycogen Synthase Kinase 3 and Casein Kinase I. *Cell* 2002;108:823–835. [PubMed: 11955435]
- Price MA. CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. *Genes Dev* 2006;20:399–410. [PubMed: 16481469]
- Rodriguez A, Oliver H, Zou H, Chen P, Abrams J. Dark is a *Drosophila* homologue of Apaf-1/CED-4 and functions in an evolutionarily conserved death pathway. *Nat Cell Biol* 1999;1:272–279. [PubMed: 10559939]
- Rorth P, Szabo K, Bailey A, Laverty T, Rehm J, Rubin GM, Weigmann K, Milan M, Benes V, Ansong W, Cohen SM. Systematic gain-of-function genetics in *Drosophila*. *Development* 1998;125:1049–57. [PubMed: 9463351]

- Ryoo HD, Bergmann A, Gonen H, Ciechanover A, Steller H. Regulation of *Drosophila* IAP1 degradation and apoptosis by reaper and *ubcD1*. *Nat Cell Biol* 2002;4:432–438. [PubMed: 12021769]
- Ryoo HD, Gorenc T, Steller H. Apoptotic cells can induce compensatory cell proliferation through the JNK and the wingless signaling pathways. *Dev Cell* 2004;7:491–501. [PubMed: 15469838]
- Serrano N, O'Farrell PH. Limb morphogenesis: connections between patterning and growth. *Curr Biol* 1997;7:R186–R195. [PubMed: 9162486]
- Stoter M, Bamberger AM, Aslan B, Kurth M, Speidel D, Loning T, Frank HG, Kaufmann P, Lohler J, Henne-Bruns D, Deppert W, Knippschild U. Inhibition of casein kinase I delta alters mitotic spindle formation and induces apoptosis in trophoblast cells. *Oncogene* 2005;24:7964–7975. [PubMed: 16027726]
- Strutt H, Price MA, Strutt D. Planar Polarity is Positively Regulated by Casein Kinase Iε in *Drosophila*. *Curr Biol* 2006;16:1329–1336. [PubMed: 16824921]
- Tapon N, Moberg KH, Hariharan IK. The coupling of cell growth to the cell cycle. *Curr Opin Cell Biol* 2001a;13:731–737. [PubMed: 11698190]
- Tapon N, Ito N, Dickson BJ, Treisman JE, Hariharan IK. The *Drosophila* tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. *Cell* 2001b;105:345–355. [PubMed: 11348591]
- Tenev T, Zachariou A, Wilson R, Ditzel M, Meier P. IAPs are functionally non-equivalent and regulate effector caspases through distinct mechanisms. *Nat Cell Biol* 2005;7:70–77. [PubMed: 15580265]
- Waddell DS, Liberati NT, Guo X, Frederick JP, Wang XF. Casein Kinase Iε plays a functional role in the Transforming Growth Factor-β signaling pathway. *J Biol Chem* 2004;279:29236–29246. [PubMed: 15133026]
- Wang SL, Hawkins CJ, Yoo SJ, Muller HAJ, Hay BA. The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* 1999;98:453–463. [PubMed: 10481910]
- Wilson R, Goyal L, Ditzel M, Zachariou A, Baker DA, Agapite J, Steller H, Meier P. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nat Cell Biol* 2002;4:445–450. [PubMed: 12021771]
- Wu S, Huang JB, Dong JX, Pan DJ. *hippo* encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with *salvador* and *warts*. *Cell* 2003;114:445–456. [PubMed: 12941273]
- Xu T, Rubin GM. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* 1993;117:1223–1237. [PubMed: 8404527]
- Yang Y, Fang S, Jensen JP, Weissman AM, Ashwell JD. Ubiquitin protein ligase activity of IAPs and their degradation in proteasomes in response to apoptotic stimuli. *Science* 2000;288:874–877. [PubMed: 10797013]
- Yokokura T, Dresnek D, Huseinovic N, Lisi S, Abdelwahid E, Bangs P, White K. Dissection of DIAP1 functional domains via a mutant replacement strategy. *J Biol Chem* 2004;279:52603–52612. [PubMed: 15371434]
- Yoo SJ, Huh JR, Muro I, Yu H, Wang LJ, Wang SL, Feldman RMR, Clem RJ, Muller HAJ, Hay BA. Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. *Nat Cell Biol* 2002;4:416–424. [PubMed: 12021767]
- Zhang L, Jia J, Wang B, Amanai K, Wharton KA, Jiang J. Regulation of wingless signaling by the CKI family in *Drosophila* limb development. *Dev Biol* 2006;299:221–237. [PubMed: 16987508]
- Zhu J, Shibasaki F, Price R, Guillenot JC, Yano T, Dotsch V, Wagner G, Ferrara P, McKeon F. Intramolecular masking of nuclear import signal on NF-AT4 by Casein Kinase I and MEKK1. *Cell* 1998;93:851–861. [PubMed: 9630228]
- Zilian O, Frei E, Burke R, Brentrup D, Gutjahr T, Bryant PJ, Noll M. *double-time* is identical to *discs overgrown*, which is required for cell survival, proliferation and growth arrest in *Drosophila* imaginal discs. *Development* 1999;126:5409–5420. [PubMed: 10556065]



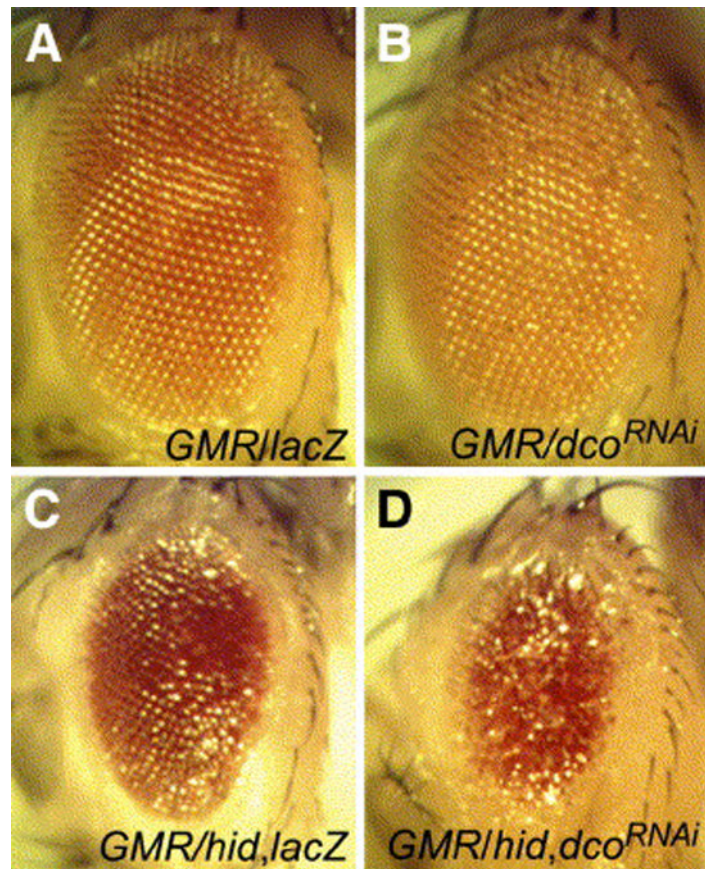
**Fig. 1.**

Ectopic expression of *dco* suppresses the small eye phenotype induced by overexpression of the proapoptotic genes *hid*, *rpr* and *grim*. Micrographs are of adult female eyes. All flies shown contain *GMR*-Gal4 and either UAS-*lacZ* (A, C, E, J) or UAS-*dco* (B, D, F, H). They contain two copies of *GMR-hid<sup>1M</sup>* (A, B), one copy of *GMR-rpr<sup>2</sup>* (C, D), *GMR-rpr<sup>5</sup>* (E, F) or a copy each of *GMR-grim<sup>2</sup>* and *GMR-grim<sup>3</sup>* (G, H). Overexpression of *dco* strongly suppresses *GMR-hid<sup>1M</sup>* and other *GMR-hid* strains (data not shown), while suppression of *GMR-rpr* and *GMR-grim* is weaker and variable.

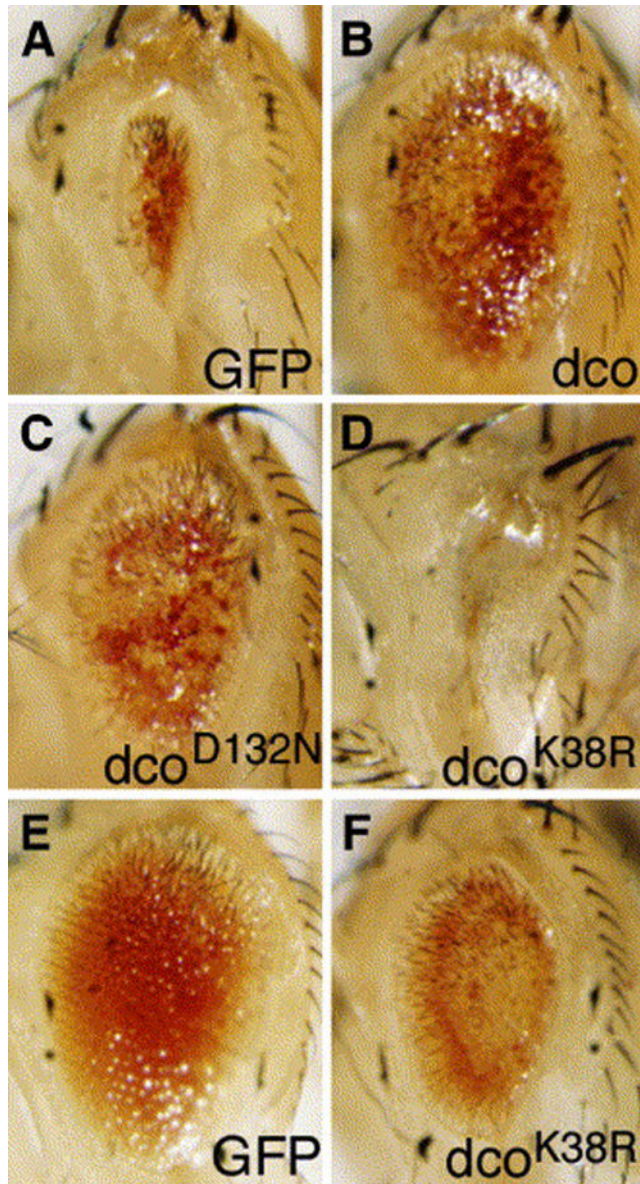


**Fig. 2.** Ectopic expression of *dco* suppresses the small eye phenotype induced by a Ras/MAPK resistant form of Hid. Micrographs are of adult female eyes. Flies contain *GMR*-Gal4, *GMR-hid<sup>ala5</sup>* and either UAS-*lacZ* (A) or UAS-*dco* (B). Overexpression of *dco* strongly suppresses the *GMR-hid<sup>ala5</sup>* phenotype.

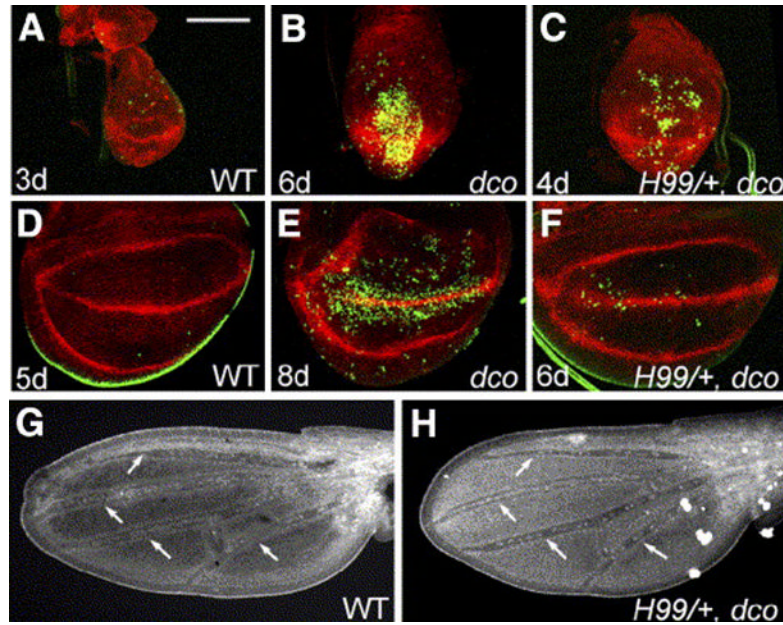




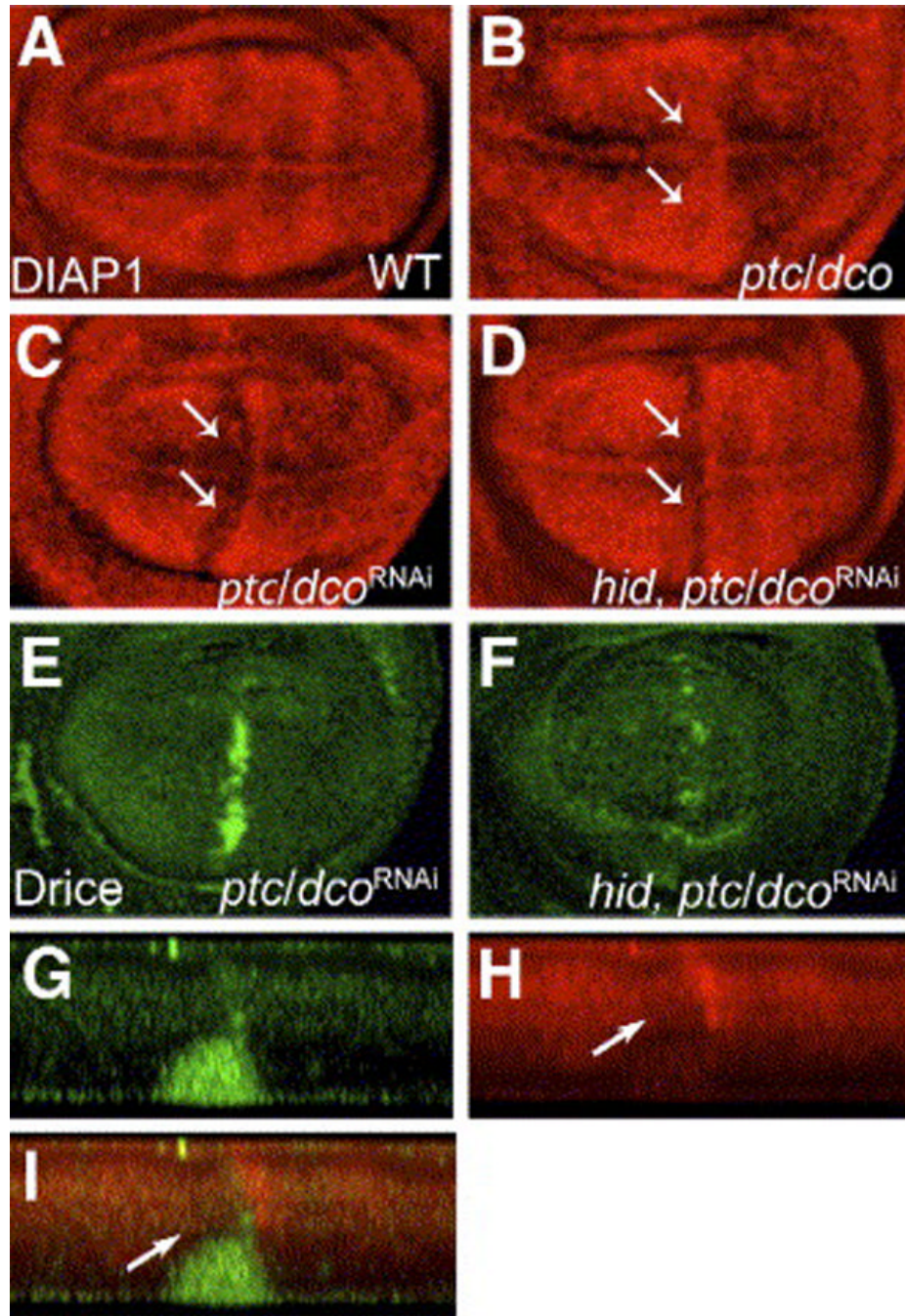
**Fig. 3.** RNAi inhibition of *dco* enhances Hid-mediated apoptosis in the eye. Adult eyes from *GMR-Gal4/UAS-lacZ* (A, C), *GMR-Gal4/UAS-dco<sup>RNAi</sup>* (B, D) without (A, B) or with one copy of *GMR-hid<sup>IM</sup>* (C, D). Depletion of *dco* roughens the eye but does not cause a reduction in size in an otherwise wild-type background (B), but does significantly enhance the *GMR-hid<sup>IM</sup>* phenotype (compare C and D).



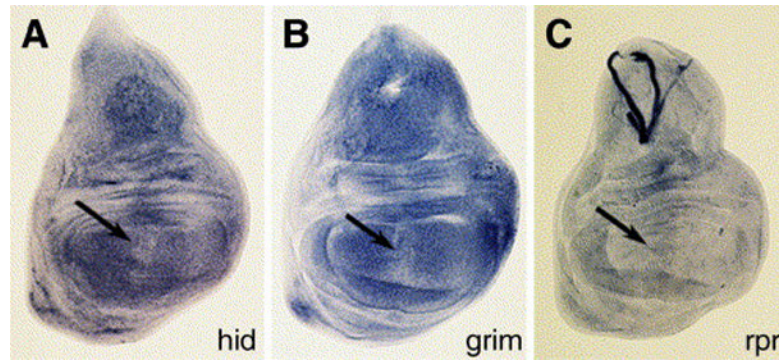
**Fig. 4.** Kinase activity is required for *dco* to suppress Hid-mediated apoptosis in the eye. Adult eyes from *GMR-Gal4*, *GMR-hid<sup>ala5</sup>* and either UAS-*GFP* (A), UAS-*dco* (B), UAS-*dco<sup>D132N</sup>* (C) or UAS-*dco<sup>K38R</sup>* (D). While the ATP-binding mutant *dco<sup>D132N</sup>* suppresses Hid activity as well as wild-type *dco*, the kinase dead mutant *K38R* enhances the small eye phenotype. (E, F) Enhancement was also observed with a weaker *GMR-hid* transgene. *GMR-Gal4*, *GMR-hid<sup>1M</sup>* and either UAS-*GFP* (E) or UAS-*dco<sup>K38R</sup>* (F). Wild type *dco* and *dco<sup>D132N</sup>* strongly suppressed *GMR-hid<sup>1M</sup>* (data not shown).



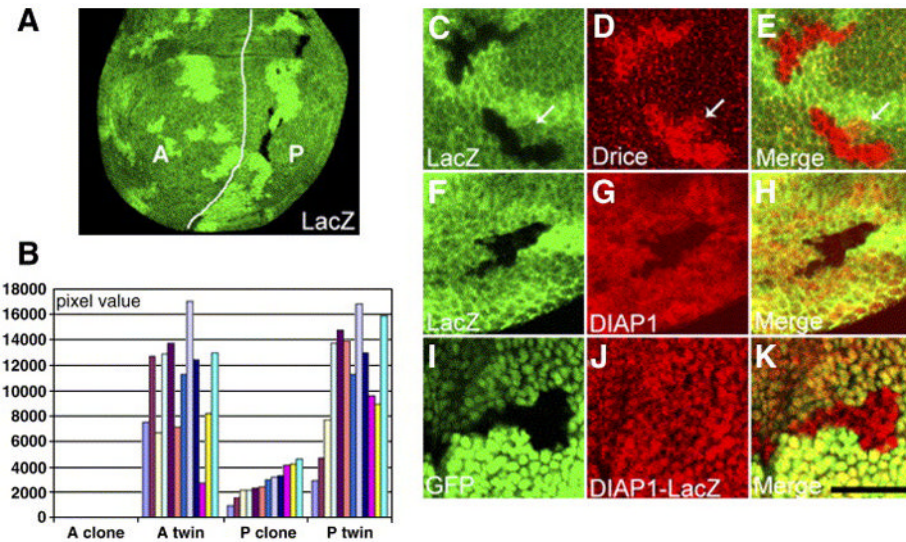
**Fig. 5.** *dco* mutant wing discs have elevated cell death and growth delay that is largely suppressed by removing one copy of *hid*, *rpr* and *grim*. Confocal images of wing discs stained for TUNEL (green) to label apoptotic cells and Wg (red, apical optical section) to identify the developmental age of each disc. The discs in panels A–C are early third instar and those in panels D–F are late third instar. (A, D) Wild type discs 3 and 5 days after egg laying. (B, E) *dco* mutant discs (*dco*<sup>P915</sup>/*dco*<sup>P1396</sup>), 6 and 8 days after egg laying. (C, F) *Df(3L)H99/+*, *dco* (*dco*<sup>P915</sup>/*dco*<sup>P1396</sup>) discs 4 and 6 days after egg laying. *H99* is a deficiency that removes a chromosomal region including *hid*, *rpr* and *grim*. The green signals at the edge of the wing discs in D and F are non-specific staining of nuclei from the peripodal membrane. (G, H) Wild type (G) and *Df(3L)H99/+*, *dco* (H) pupal wings stained with phalloidin. The wings are 33 hr and 38 hr after the white prepupal stage, respectively. The white arrows denote the position of the longitudinal veins. The overall pattern of the *H99/+*; *dco* wings is identical to wild-type. Scale bar: 100µm.



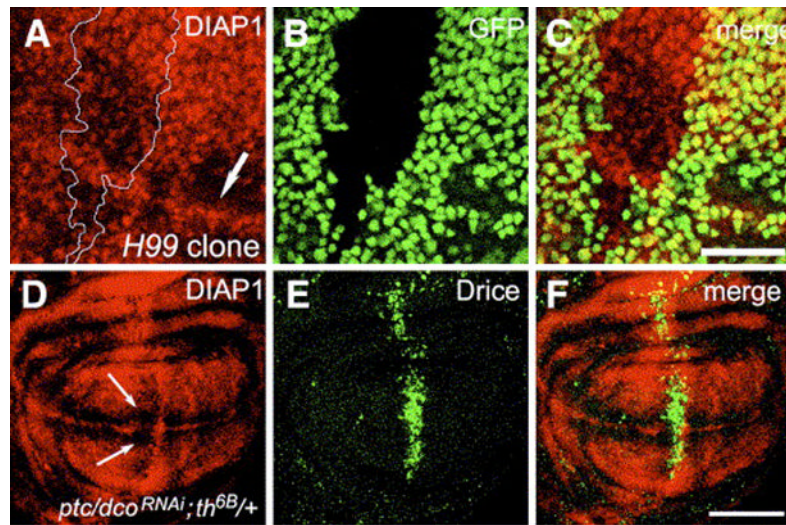
**Fig. 6.** Dco regulates DIAP1 protein levels in the wing imaginal disc. Confocal images of late third instar larval wing discs stained for DIAP1 in red (A–D,H,I) and activated Drice in green (E–G,I). *ptcGal4* is expressed at an anterior strip next to the anterior-posterior boundary (indicated by arrows in B–D). All discs are oriented anterior to the left. (A) Wild type. (B) *ptc-Gal4/UAS-dco*. (C, E) *ptc-Gal4/UAS-dco*<sup>RNAi</sup>. (D, F) *ptc-Gal4/UAS-dco*<sup>RNAi</sup>; *hid*<sup>05014</sup>/*hid*<sup>X14</sup>. (G–I) Z axis projection of *ptc-Gal4/UAS-dco*<sup>RNAi</sup> disc showing basal localization of Drice positive cells. Arrows indicate the reduction in DIAP1 staining in the *ptc* expression domain.



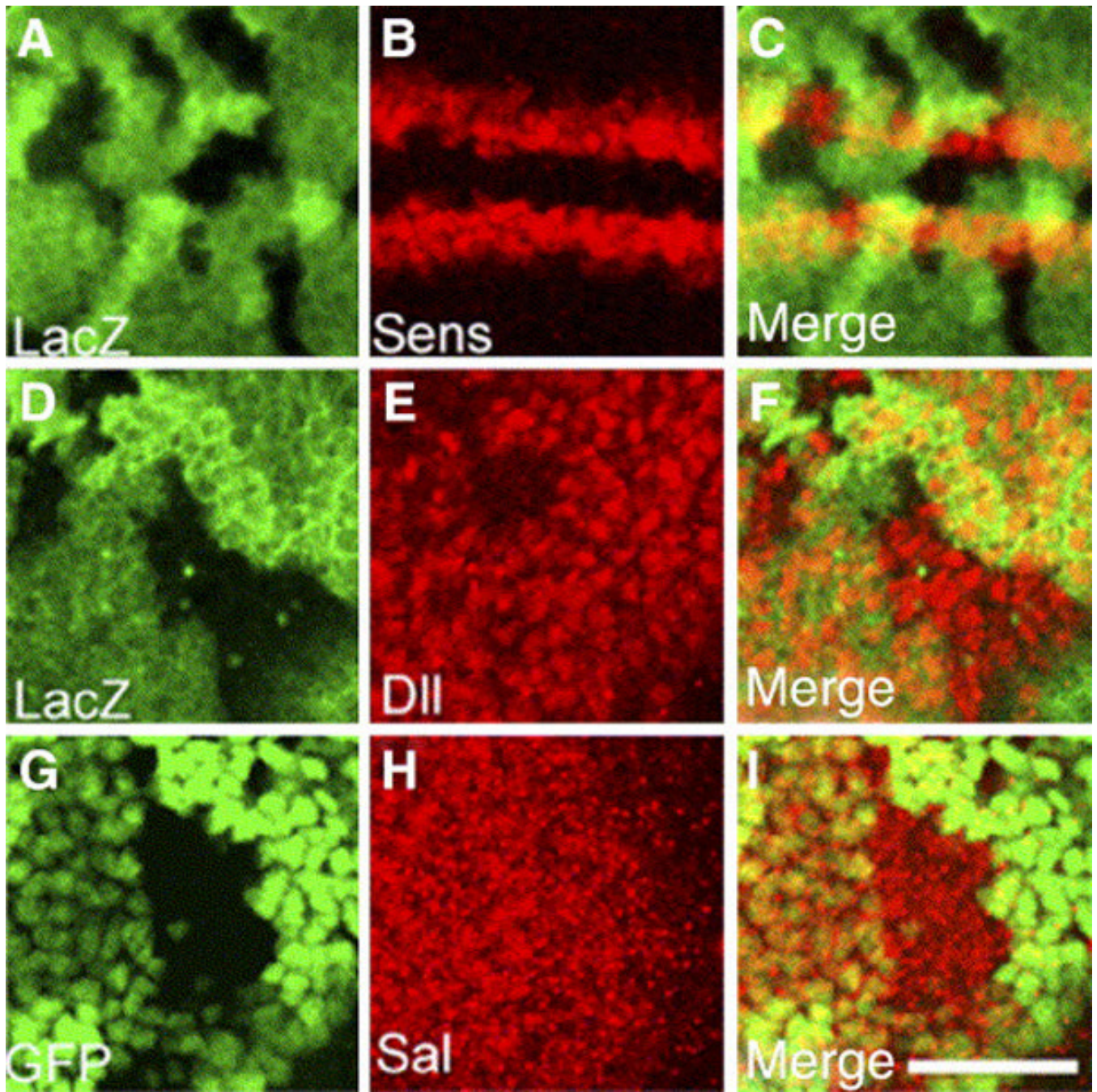
**Fig. 7.** Inhibition of *dco* has little effect on *hid*, *grim* or *rpr* transcript levels. *ptc*-Gal4/UAS-*dco*<sup>RNAi</sup> discs stained for *hid* (A), *grim* (B) and *rpr* (C) transcripts. The location of the *ptc* expression domain is indicated by the arrows. There is no consistent change in *hid* expression by *dco*<sup>RNAi</sup>, but a small decrease in *grim* was sometimes observed, as was a small increase in *rpr* expression.

**Fig. 8.**

*dco* mutant clones are rescued by inhibition of caspases and exhibit increased Drice activation and decreased DIAP1 protein, but not DIAP-lacZ. *dco* null (*dco<sup>le88</sup>*) mutant clones were generated in an *en-Gal4/UAS-p35* background and are marked by the absence of lacZ staining (green; A,C–H) or GFP (green; I–K) as indicated. (A) *dco* clones are found in the posterior (P) compartment where p35 is expressed by *en-Gal4*, but the anterior (A) clones are not observed. The white line marks the AP boundary. The presence of the twins at the anterior indicates that mitotic recombination had been induced. (B) Quantification of the *dco* clone/twin area. The pixel value of each bar represents the total area of clones or twins in A or P in one disc, and 12 discs containing a total of more than 30 individual clones were analyzed. The difference between the areas of A twins (average: 10451 pixels/disc) and P twins (average: 11138 pixels/disc) is not statistically significant ( $p > 0.20$  by the Mann-Whitney test). The average size of the P clones (2855 pixels/disc) is 25.6% of the size of the P twins. (C–E) Active Drice (red) is elevated in *dco* clones. Occasionally active Drice is observed adjacent to the clones (arrows). (F–H) DIAP1 protein (red) is decreased in *dco* clones. (I–K) *dco* clones marked by the absence of GFP were generated in a DIAP-lacZ background (*th<sup>i5c8</sup>*). DIAP-LacZ (red) is not altered in the clones. Scale bar: 20µm.



**Fig. 9.** Hid, Grim and Rpr do not regulate DIAP1 expression in the wing disc and DIAP1 E3 ligase activity is not required for Dco regulation of DIAP1. (A–C) Confocal images of late third wing imaginal discs containing clones of *Df(3L)H99* (A–C), stained for DIAP1 (red). Clones were marked by GFP (green) and the location of the D/V boundary is indicated by the white arrow. The expression pattern of DIAP1 is unaffected in the clones. (D–F) Confocal images of wing imaginal discs stained for DIAP1 (red) and activated Drice (green) from *ptc-Gal4/UAS-dco<sup>RNAi</sup>; th<sup>6B</sup>/+* animals. As in *ptc-Gal4/UAS-dco<sup>RNAi</sup>* controls (see Fig. 4), a strong decrease in DIAP1 levels and increase in activated Drice is observed in the *ptc* stripe. The scale bars are 20  $\mu$ M in C and 100 $\mu$ M in F.



**Fig. 10.**

The *dco* mutant cells have unaffected Wg and Dpp signaling. Confocal images of late third instar wing imaginal discs containing *dco*<sup>le88</sup> null clones, generated as described in Fig. 5. Clones are marked by the lack of lacZ staining (green) and the Wg targets Sens and Dll and Dpp target Spalt are stained in red. Sens (A–C) and Dll (D–F) and Spalt (G–I) expression are normal in *dco* clones.