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# Systematic *in vivo* RNAi analysis of putative components of the *Drosophila* cell death machinery

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## Abstract

**Despite the identification of numerous key players of the cell death machinery, little is known about their physiological role. Using RNA interference (RNAi) *in vivo,* we have studied the requirement of all *Drosophila* caspases and caspase-adaptors in different paradigms of apoptosis. Of the seven caspases, Dronc, drICE, Strica and Decay are rate limiting for apoptosis. Surprisingly, Hid-mediated apoptosis requires a broader range of caspases than apoptosis initiated by loss of the caspase inhibitor DIAP1, suggesting that Hid causes apoptosis not only by antagonizing DIAP1 but also by activating DIAP1-independent caspase cascades. While Hid killing requires Strica, Decay, Dronc/Dark and drICE, apoptosis triggered by DIAP1 depletion merely relied upon Dronc/Dark and drICE. Furthermore, we found that over-expression of DIAP2 can rescue *diap1*-RNAi-mediated apoptosis, suggesting that DIAP2 regulates caspases directly. Consistently, we show that DIAP2 binds active drICE. Since DIAP2 associates with Hid, we propose a model whereby Hid co-ordinately targets both DIAP1 and DIAP2 to unleash drICE.**
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**Keywords:** apoptosis; IAP; IAP antagonist; caspase; *Drosophila*; RNAi

**Abbreviations:** IAP, inhibitor of apoptosis; BIR, baculovirus IAP repeat; PCD, programmed cell death; dsRNA, double-stranded RNAs; RNAi, RNA interference; IR, inverted repeats; GMR, glass multimer reporter; SEM, scanning electron microscopy; JNK, Jun amino-terminal kinase; TAP, tandem affinity purification; IOC, interommatidial cell; SG, salivary glands; APF, after puparium formation; GFP, green fluorescent protein; GST, glutathione *S*-transferase

## Introduction

Apoptosis requires the action of a set of highly specific cysteine proteases called caspases.[1] Caspases reside in cascades of auto- and transactivation that are typically triggered by activation of initiator caspases. Initiator caspases cleave and activate downstream effector caspases, thereby amplifying the proteolytic activity required for the destruction of the cell.

*Drosophila melanogaster* contains seven caspases, of which Dronc and Dredd/DCP-2 are *bona fide* initiator caspases.[1] Although Dredd is most homologous to mammalian caspase-8, it is not essential for programmed cell death (PCD), and together with its adaptor dFadd, is required for antibacterial immune response.[2,3] In contrast, Dronc, the ortholog of mammalian caspase-9, is essential for many forms of PCD and apoptosis triggered by cytotoxic agents.[4–7] Dronc requires Dark/Hac-1/dApaf1 for activation and, once active, transduces the death signal to downstream effector caspases such as drICE, DCP-1, Decay and Damm/Daydream.[8–12] However, it is currently unclear as to which of these effector caspases are required to execute PCD *in vivo*. While DCP-1 is dispensable for PCD, it is required for stress-induced death in the ovary.[13] drICE seems to be essential for apoptosis of cells in culture.[14–16] The atypical caspase Strica/Dream carries an extensive prodomain typical of initiator caspases. However, it contains a serine-threonine-rich domain instead of a classical caspase recruitment domain or death effector domain.[17] A comprehensive picture of the exact *in vivo* function and epistatic relationship among *Drosophila* caspases remain elusive owing to the limited availability of genetic mutations.

Genetic studies indicate that DIAP1-mediated inhibition of caspases is essential for cell survival. While the baculovirus IAP repeat (BIR)1 region of DIAP1 binds to the effector caspases DCP-1 and drICE, the BIR2 domain directly associates with the initiator caspase Dronc. Mutations that abrogate physical association of DIAP1 with effector or initiator caspases cause unrestrained caspase activation and apoptosis.[18–22] In cells destined to die, cell death is induced by the inhibitor of apoptosis (IAP) antagonists Reaper (Rpr), Grim, Hid, Sickle and Jafrac2.[1] Embryos lacking *rpr*, *grim* and *hid* are virtually devoid of PCD, and die at the end of embryogenesis with the accumulation of supernumeral cells.[23] Hid plays the major role in driving PCD since it is essential for apoptosis in the embryo, developing eye and for histolysis of salivary glands (SGs) during metamorphosis.[24–26] The current dogma dictates that Hid triggers cell death by disrupting DIAP1:caspase association, thereby alleviating DIAP1's inhibition of caspases.[18,21,22]



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Here, we have used an RNA interference (RNAi) approach to systematically dissect the contribution of individual components of the *Drosophila* cell death signal-transduction cascade to apoptosis *in vivo*. Among the seven *Drosophila* caspases, only Dronc, drICE, Strica and Decay seemed to be required for the execution of Hid-dependent apoptosis. Surprisingly, Dronc and drICE were the only caspases that were required for executing cell death initiated by RNAi-mediated DIAP1 depletion. In contrast, Hid killing required Strica and Decay in addition to Dronc and drICE. Thus, Hid-mediated cell death requires a broader range of caspases than the one triggered by the loss of DIAP1, suggesting that Hid not only antagonises DIAP1 to induce apoptosis. Consistently, Hid also efficiently binds to the second *Drosophila* IAP DIAP2, which has been found to interact with the initiator caspase Strica and block Hid-mediated cell death.[17,27] In addition, we find that DIAP2 associates with drICE with the same efficiency as DIAP1, suggesting that DIAP2, like DIAP1, controls cell death by regulating downstream effector caspases such as drICE.

## Results

### Inducible RNAi as a tool to dissect the cell death signal-transduction pathway *in vivo*

Since *Drosophila* encodes seven caspases and two caspase-adaptors, we wished to determine which of these components are required to execute cell death *in vivo*. To this end, we used a RNAi approach to selectively knockdown all *Drosophila* caspases (Dronc, drICE, DCP-1, Dredd/DCP-2, Strica/Dream, Damm and Decay) and caspase-adaptors (Dark/Hac-1/dApaf1 and dFadd) *in vivo*.[28] We generated *target-gene*-specific inverted repeats (IR) separated by a functional intron such that RNA produced by the transgene forms loopless hairpin RNA following splicing.[29] The presence of the intron spacer greatly enhances RNAi efficiency.[29] Independent UAS-*target-gene*-inverted repeat (IR) transgenic *Drosophila* lines were generated and crossed with strains expressing GAL4 under the control of the glass multimer reporter (GMR-GAL4), which drives transgene expression in differentiating photoreceptors and accessory cells of the eye.[30]

To validate the efficiency and selectivity of RNAi towards caspases and caspases-adaptors, we used transgenic fly lines overexpressing Strica (Figure 1a–d), Dronc (Figure 1e–h) and ΔN-DCP-1 (Figure 1i–l). The Strica eye phenotype was only affected by *strica*-RNAi (Figure 1b), but remained unchanged by *dronc*- (Figure 1c) and *dcp-1*-RNAi (Figure 1d) corroborating the specificity of these RNAi constructs. Likewise, the Dronc and ΔN-DCP-1 eye phenotypes were only rescued by their corresponding RNAi transgenes. Moreover, *engrailed*-GAL4-driven expression of *dronc*-RNAi in the wing phenocopied the genetic loss of *dronc* function showing melanized blemishes in the adult wing[4] (data not shown). Ubiquitous expression (tubulin-GAL4) of *dark* double-stranded RNAs (dsRNA) caused developmental defects that are highly reminiscent to the ones of animals that carry the $dark^{CD4}$ hypomorphic mutation.[11] *dark*-RNAi animals showed strong pupal lethality, and rare escapers displayed wing defects (Figure 1m) as well as supernumeral bristles (Figure 1n). Moreover, *dark*-RNAi in the wing phenocopied the genetic loss of *dark* showing melanized blemishes in the adult wing[4] (data not shown). Since drICE overexpression in the eye does not result in any visible phenotypes, we expressed *drice*-dsRNA under the control of the tubulin promoter and examined endogenous drICE protein levels in third instar larvae to assess the efficiency of *drice*-RNAi. Ubiquitous expression of *drice*-dsRNA resulted in significant loss of drICE protein and no detectable levels of drICE were apparent. Likewise, RT-PCR analysis on total RNA extracted from tubulin-driven *decay*- and *damm*-dsRNA expression showed reduced *decay* and *damm* mRNA levels, respectively (data not shown). Moreover, Ubiquitous *dfadd*- and *dredd*-RNAi caused knocks downs of *dfadd* and *dredd* expression and generated immunodeficiencies that were comparable to the ones of *dredd* and *dfadd* null alleles[2,3] (F Leulier and B Lemaitre, unpublished data). Taken together, these results indicate that these RNAi transgenes are highly efficient and selective tools to knock down the function of specific caspases and caspase-adaptors.

### Dronc, dark and drICE are indispensable for apoptosis induced by DIAP1 depletion

Loss of DIAP1 function triggers unrestrained caspase-mediated cell death.[18–22] Consistently, eye-specific *diap1*-RNAi resulted in massive ectopic apoptosis in third larval instar eye discs causing severely deformed eyes with highly abnormal external and internal morphologies (Figure 2). *diap1*-RNAi also resulted in the loss of bristles (Figure 2g and h). This phenotype was a direct consequence of RNAi-mediated knock down of DIAP1 since control dsRNAs exerted no apparent effects on eye development or DIAP1 protein levels (data not shown). Moreover, *diap1*-RNAi was highly selective in knocking down DIAP1 and no cross-interference effect was detected since only DIAP1 protein levels were depleted, while levels of the closely related DIAP2 remained unaffected (Figure 2p). Moreover, the RNAi phenotype was fully rescued when UAS-*diap1*-IR flies were crossed to flies overexpressing DIAP1 (UAS-*diap1*) using GMR-GAL4 (Figure 2k).

To further define how loss of DIAP1 triggers cell death, we examined the possibility that DIAP1 depletion triggered apoptosis by activating upstream proapoptotic signals, thereby triggering caspases activation indirectly. As previously shown in the embryo,[18,21] *diap1*-RNAi-mediated cell death was independent of upstream proapoptotic signals such as Rpr, Grim, Hid or the stress-activated protein kinase Jun amino-terminal kinase (JNK) signal-transduction pathway. Inhibition of the JNK pathway through eye-specific expression of the JNK phosphatase Puckered failed to significantly ameliorate the eye phenotype caused by DIAP1 depletion (Figure 2l). Likewise, genetic removal of Hid function, through combining the $hid^{05014}$ null allele with the H99 deletion that removes *hid* as well as *rpr* and *grim* ($hid^{05014}/Df(3L)H99$), also did not suppress the *diap1*-RNAi eye phenotype (Figure 2m). Moreover, the survival of eye pigment cells in *diap1*-RNAi eyes was not suppressed in mosaic flies with eyes that are





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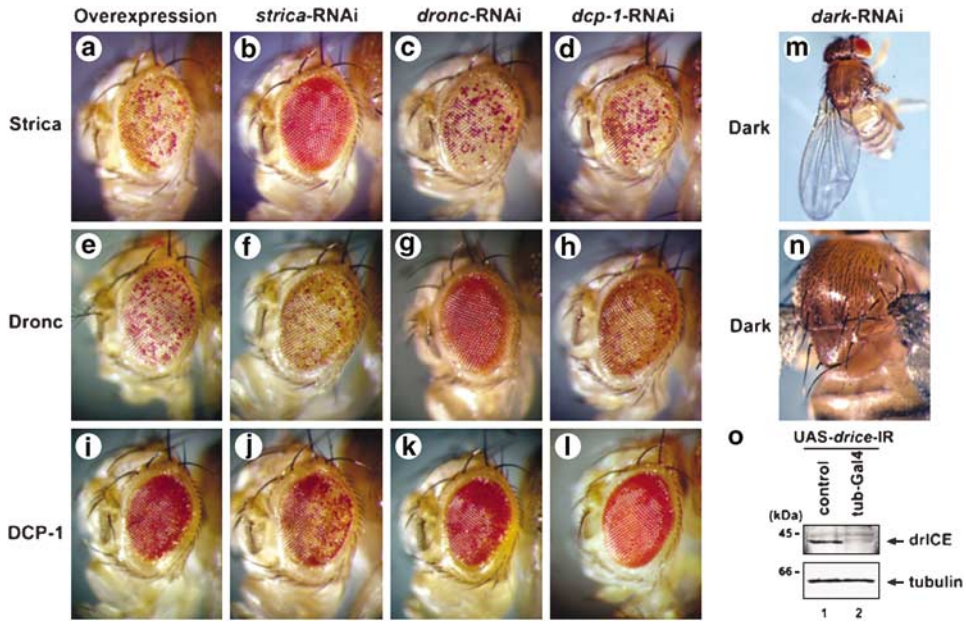


**Figure 1** Inducible *in vivo* RNAi targeting *Drosophila* caspases and caspase-adaptors. To validate the efficiency and selectivity of *in vivo* RNAi towards caspases and caspase–adaptors, we used transgenic fly lines overexpressing Strica (**a**–**d**), Dronc (**e**–**h**) and ΔN-DCP-1 (**i**–**l**). The *strica* overexpression eye phenotype (**a**) was only affected by *strica*-RNAi (**b**) but remained unchanged by *dronc*- (**c**) and *dcp-1*-RNAi (**d**) corroborating the specificity of this RNAi construct. Likewise, the *dronc* overexpression eye phenotype (**e**) was only rescued by *dronc*-RNAi (**g**) but not by *strica*- (**f**) or *dcp*-1-RNAi (**h**). The effects of ΔN-DCP-1 overexpression (**i**) were only suppressed by *dcp-1*-RNAi (**l**) but not by *strica*- (**j**) or *dronc*-RNAi (**k**). (**m**, **n**) Ubiquitous expression of *dark* dsRNA caused developmental defects that are highly reminiscent to the ones of animals that carry the $dark^{CD4}$ hypomorphic mutation.[11] *dark*-RNAi animals showed strong pupal lethality, and rare escapers displayed wing defects (**m**) as well as supernumeral bristles (**n**). (**o**) *drice*-RNAi caused a significant loss of drICE protein. Protein extracts from L3 larvae ubiquitously expressing *drice*-dsRNA (lane 2, *drice*-RNAi (UAS-*drice*-IR/ + ;α*Tubulin84B*-GAL4/ + ) were used to monitor the RNAi efficiency. Extracts from UAS-*drice*-IR/ + ;TM3Ser,*Actin*-GFP/ + animals served as control (lane 1). The presence of drICE was detected by immunoblot analysis using anti-drICE antibodies. The genotypes of the shown phenotypes are: (**a**) GMR-GAL4,GMR-*strica*/ + ; (**b**) GMR-GAL4,GMR-*strica*/ + ;UAS-*strica*-IR/ + ; (**c**) GMR-GAL4,GMR-*strica*/UAS-*dronc*-IR; (**d**) GMR-GAL4,GMR-*strica*/UAS-*dcp-1*-IR; (**e**) GMR-GAL4/ + ;UAS-*prodronc*/ + ; (**f**) GMR-GAL4/ + ;UAS-*prodronc*/UAS-*strica*-IR; (**g**) GMR-GAL4/UAS-*dronc*-IR;UAS-*prodronc*/ + ; (**h**) GMR-GAL4/UAS-*dcp-1*-IR;UAS-*prodronc*/ + ; (**i**) GMR-N-*dcp-1*/ + ; (**j**) GMR-N-*dcp-1*/ + ;GMR-GAL4,UAS-*strica*-IR/ + ; (**k**) GMR-N-*dcp-1*/GMR-GAL4,UAS-*dronc*-IR; (**l**) GMR-N-*dcp-1*/ + ;GMR-GAL4,UAS-*dcp-1*-IR/ + ; (**m** and **n**) α*Tubulin84B*-GAL4/UAS-*dark*-IR

homozygous for H99 and hence completely lack *rpr*, *grim* and *hid* (Figure 2o) (see Materials and Methods for details). Surprisingly, loss of *rpr*, *grim* and *hid* actually enhanced the death of pigment cells, suggesting that DIAP1 ensures survival of H99-mutant pigment cells. Since the overall eye size (external morphology) appears to be slightly improved by the loss of *rpr*, *grim* and *hid* (Figure 2, compare o with f), our data suggest that the *diap1*-RNAi-mediated death of pigment cells occurs relatively late in pupal development and after determination of eye size. Taken together, these results corroborate the notion that cell death triggered by loss of DIAP1 in the developing fly eye does not rely on upstream proapoptotic signals such as Hid, Rpr, Grim or JNK activity, but instead is the direct result of unguarded caspases. Thus, loss of DIAP1 on its own, without any further cell death trigger, appears to be sufficient to cause cell death in the developing eye.

To identify the caspases and caspase-adaptors that are required to execute apoptosis induced by DIAP1 depletion, we crossed *diap1*-RNAi flies to flies lacking specific components of the cell death machinery in the eye. RNAi-mediated knockdown of Dronc and Dark almost completely rescued the eye phenotype caused by DIAP1 depletion (Figure 3a–c). Likewise, *drice*-RNAi also efficiently suppressed the *diap1*-RNAi phenotype (Figure 3d). In complete contrast, however, RNAi-mediated knockdown of DCP-1, which is dispensable for developmental cell death,[13] did not suppress these phenotypes (Figure 3f), even though *dcp-1*-RNAi was highly efficient in rescuing cell death caused by ectopic expression of ΔN-DCP-1 (Figure 3l). Similarly, depletion of the effector caspases Damm and Decay failed to rescue apoptosis induced by loss of DIAP1 (data not shown). Moreover, combined RNAi-mediated depletion of all effector caspases (drICE, DCP-1, Decay and Damm) was only slightly more effective in suppressing the *diap1*-RNAi eye phenotype than *drice*-RNAi on its own (Figure 3f), suggesting a minor contribution by DCP-1, Decay and Damm. RNAi-mediated knockdown of the initiator caspases Strica and Dredd or the adaptor dFadd did not suppress the *diap1*-RNAi eye phenotype (data not shown). Surprisingly, loss of Strica or Decay actually significantly enhanced the *diap1*-RNAi eye phenotype (F Leulier and P Meier, unpublished data). In summary, these results show that loss of DIAP1 engages the canonical cell death signalling cascade comprising of Dronc, Dark and drICE. Since no upstream proapoptotic signals are required for *diap1*-RNAi-mediated apoptosis, these results further suggest that Dark is constitutively active and triggers Dronc activation as soon as 'free' Dronc (non-DIAP1-bound) becomes available. Moreover, of the four effector caspases, drICE seems to be the only rate-limiting caspase executing cell death upon DIAP1 inactivation *in vivo*.





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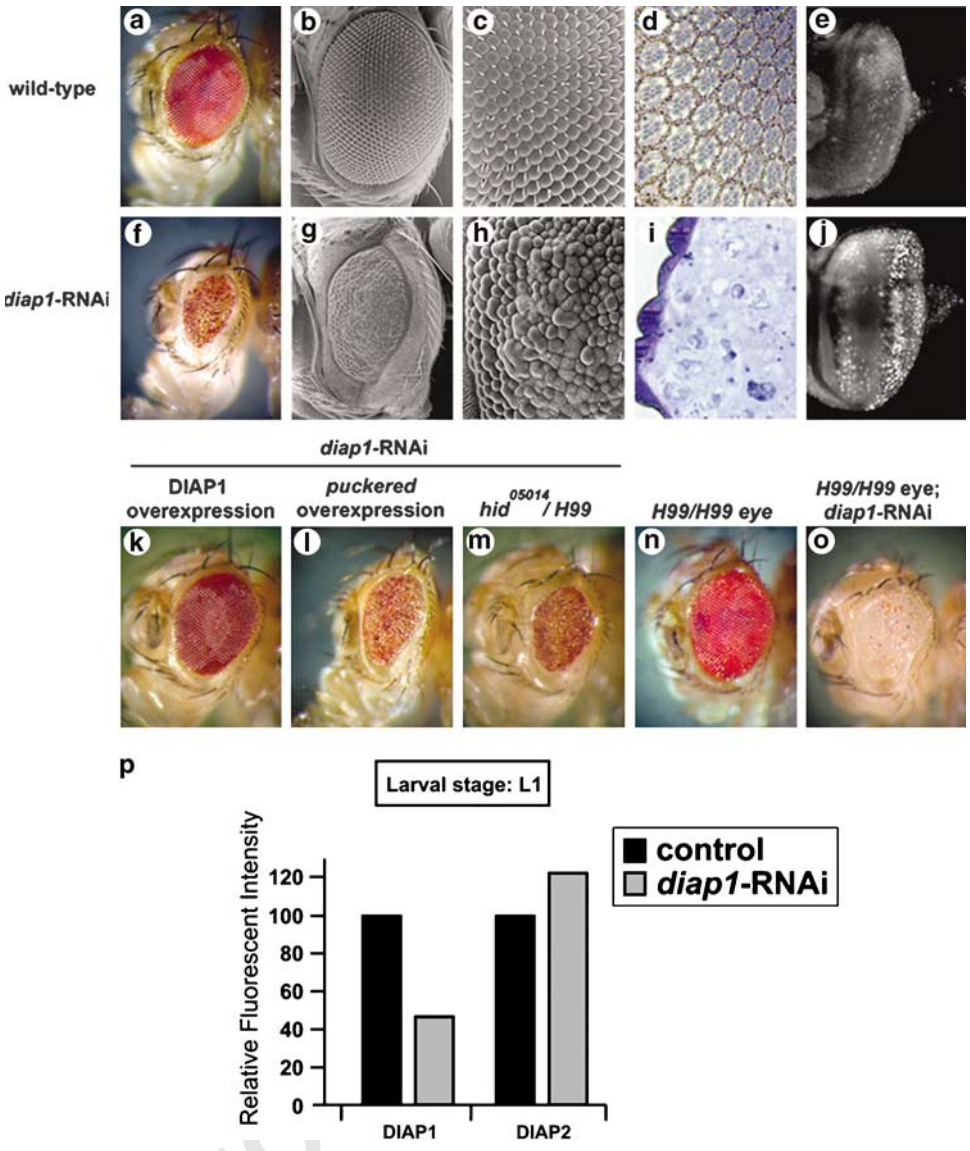


**Figure 2** RNAi-mediated depletion of DIAP1 triggers cell death in the developing eye that is independent of upstream proapoptotic signals. (**a–j**) Expression of *diap1*-dsRNA in the eye causes excessive cell death (**j**) generating a spotted eye phenotype (**f**) with a distorted outer eye morphology (**g** and **h**) and no defined interior structures (**i**). Eye phenotypes were analyzed by light microscopy of whole mounts (**a** and **f**), SEM (**b** and **g** at $\times$ 100; **c** and **h** at $\times$ 250), tangential semithin sections of adult eyes (**d** and **i**) and acridine orange staining of eye discs of third instar larvae (**e** and **j**). (**a–e**) Control flies (GMR-GAL4/ $+$), and (**f–j**) GMR-GAL4/UAS-*diap1*-IR flies. In this and the following figures, anterior is to the left and posterior to the right. (**k**) The *diap1*-RNAi eye phenotype is fully rescued by overexpression of *diap1* (compare **f** with **k**). (**l–o**) Cell death induced by RNAi-mediated depletion of DIAP1 is independent of JNK, *rpr*, *grim* or *hid* activity. *diap1* RNAi eye phenotype remains unchanged by Puckered overexpression (compare **f** with **l**). Likewise, loss of Hid (*hid*$^{05014}$*/H99*, viable allelic combination) fails to suppress the phenotype caused by DIAP1 depletion (compare **f** with **m**). *diap1*-RNAi eye phenotype is not rescued in mosaic flies with eyes that are homozygous mutant for the *H99* deficiency (**o**) and hence completely lack *rpr*, *grim* and *hid*. (**n**) Mosaic control animals with *H99* mutant eye. (**p**) Shown are the relative amounts of endogenous DIAP1 and DIAP2 protein levels. RNAi-mediated knockdown was quantified by Odyssey® Technology (Licor Biosciences) using young first instar larvae expressing *diap1*-dsRNA under the control of the tubulin promoter (tubulin-GAL4). *diap1*-RNAi selectively depletes DIAP1 protein levels by approximately 55%, while DIAP2 levels are not reduced. Note that young L1 larvae carry substantial amounts of maternally derived DIAP1 protein, which is refractory to RNAi-mediated knockdown. Young L1 larvae were used since *diap1*-RNAi is lethal at this developmental stage. Protein extracts from young L1 control larvae (lane 1, UAS-*diap1*-IR/ $+$ ;TM3Ser,*Actin*-GFP/ $+$) and ubiquitously expressing *diap1*-dsRNA (lane 2, UAS-*diap1*-IR/ $+$ ;$\alpha$*Tubulin84B*-GAL4/ $+$) were probed with anti-DIAP1, anti-DIAP2 and anti-tubulin antibodies. Genotypes: (**a–e**) GMR-GAL4/ $+$, (**f–j**) GMR-GAL4/UAS-*diap1*-IR, (**k**) GMR-GAL4,UAS-*diap1*-IR/UAS-*diap1*, (**l**) GMR-GAL4,UAS-*diap1*-IR/ $+$ ;UAS-*puckered*/ $+$, (**m**) GMR-GAL4,UAS-*diap1*-IR/ $+$ ; *hid*$^{05014}$*/H99*, (**n**) *yw,ey*-FLP/$w^+$;CyO;FRT80B,*Rps17*,*arm*-lacZ/FRT80B,*Df(3L)H99* and (**o**) *yw,ey*-FLP/$w^+$;GMR-GAL4,UAS-*diap1*-IR;FRT80B,*Rps17*,*arm*-lacZ/FRT80B,*Df(3L)H99*

## DIAP2 can functionally substitute for loss of DIAP1 *in vivo*

DIAP1's ability to bind and inhibit caspases enables it to act as one of the last lines of defence against caspase-mediated damage. Previous work has suggested that the second *Drosophila* IAP DIAP2 inhibits cell death not by inhibiting caspases, but instead by acting as a decoy-IAP for IAP antagonists.[31] Accordingly, DIAP2 would serve as a sink for IAP antagonists, thereby preventing the liberation of cas-



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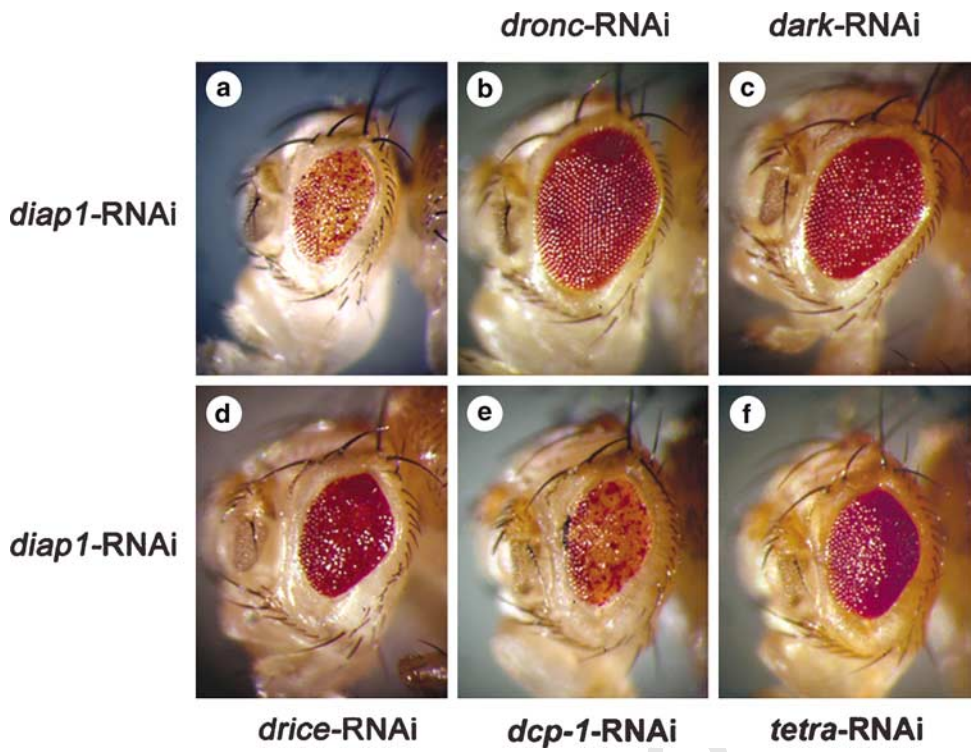


**Figure 3** Dronc, Dark and drICE are indispensable for apoptosis induced by DIAP1 depletion in the developing eye: the *diap1*-RNAi eye phenotype (**a**) was rescued by coexpression of dsRNA of *dronc* (**b**), *dark* (**c**) and *drice* (**d**), but not by coexpression of *dcp-1* dsRNA (**e**). Simulatneous knock down of all four *Drosophila* effector caspases (drICE, DCP-1, Decay and Damm – referred to as *tetra*-RNAi) rescues the *diap1*-RNAi phenotype only slightly better than *drice*-RNAi alone (compare **d** and **f**). Genotypes: (**a**) GMR-GAL4,UAS-*diap1*-IR/+, (**b**) GMR-GAL4,UAS-*diap1*-IR/UAS-*dronc*-IR, (**c**) GMR-GAL4,UAS-*diap1*-IR/UAS-*dark*-IR, (**d**) GMR-GAL4,UAS-*diap1*-IR/UAS-*drice*-IR, (**e**) GMR-GAL4,UAS-*diap1*-IR/+;UAS-*dcp-1*-IR/+ and (**f**) GMR-GAL4,UAS-*diap1*-IR/UAS-*drICE*-IR,UAS-*decay*-IR;UAS-*dcp-1*-IR,UAS-*damm*-IR

pases from DIAP1. To assess whether DIAP2 could functionally replace DIAP1's role in neutralizing caspases *in vivo*, we examined its ability to suppress *diap1*-RNAi-mediated cell death. While loss of DIAP1 triggers spontaneous and unrestrained caspase activation and cell death,[18–21] overexpression of DIAP2, like p35, efficiently rescued this death (Figure 4). Intriguingly, DIAP2 behaved like p35, both of which rescued the *diap1*-RNAi eye size and pigmentation to an apparent normal morphology (Figure 4b and c), but failed to restore the formation of bristles (Figure 4g, l and h, m). The notion that DIAP2 phenocopies p35, in the absence of any IAP-antagonist activation, suggests that DIAP2 can act as a direct caspase inhibitor for a p35-sensitive caspase, such as drICE. Given that drICE is the predominant effector caspase that executes *diap1*-RNAi-mediated cell death (see Figure 3d–f), these results suggest that DIAP2 overexpression may regulate drICE *in vivo*.

## DIAP2 physically interacts with drICE but not DCP-1

To test whether DIAP2 physically interacts with p35-sensitive effector caspases such as drICE and DCP-1, we used wild-type DIAP2-TAP (tandem affinity purification tag) fusion protein as an affinity reagent to purify drICE or DCP-1 from cellular extracts. DIAP1- and DIAP2-TAP were expressed in 293T cells and purified using the TAP system.[32] Resin-bound DIAP1- and DIAP2-TAP were subsequently incubated with 293T cellular extracts containing active drICE-V5 or DCP-1-V5. The active form of drICE efficiently copurified with DIAP2 and DIAP1 (Figure 4 and Zachariou *et al*.[22] and Tenev *et al*.[33]), while it failed to interact with a control protein fused to TAP (data not shown). Interestingly, the efficiency of DIAP2 binding to drICE was similar to the one of DIAP1 (compare lane 3 with lane 5). Surprisingly, DCP-1 completely failed to copurify with DIAP2, while it readily bound to DIAP1 (Figure 4q, compare lane 3 with lane 5). The observation that drICE physically interacts with DIAP1 and DIAP2 strongly implies that DIAP1 and DIAP2 both regulate drICE.

## Loss of bristles in *diap1*-RNAi eyes is Dronc-dependent but independent of p35-sensitive caspases

While overexpression of p35 and DIAP2 suppressed the *diap1*-RNAi eye phenotype, they failed to restore the formation of eye bristles, suggesting that their loss is either caspase independent or, alternatively, is triggered by a p35-insensitive caspase such as Dronc. Intriguingly, the bristle phenotype was strictly Dronc-dependent because mosaic flies with eyes that were homozygous for the null allele $dronc^{I29}$,[7] or expressing *dronc*-dsRNA fully restored these



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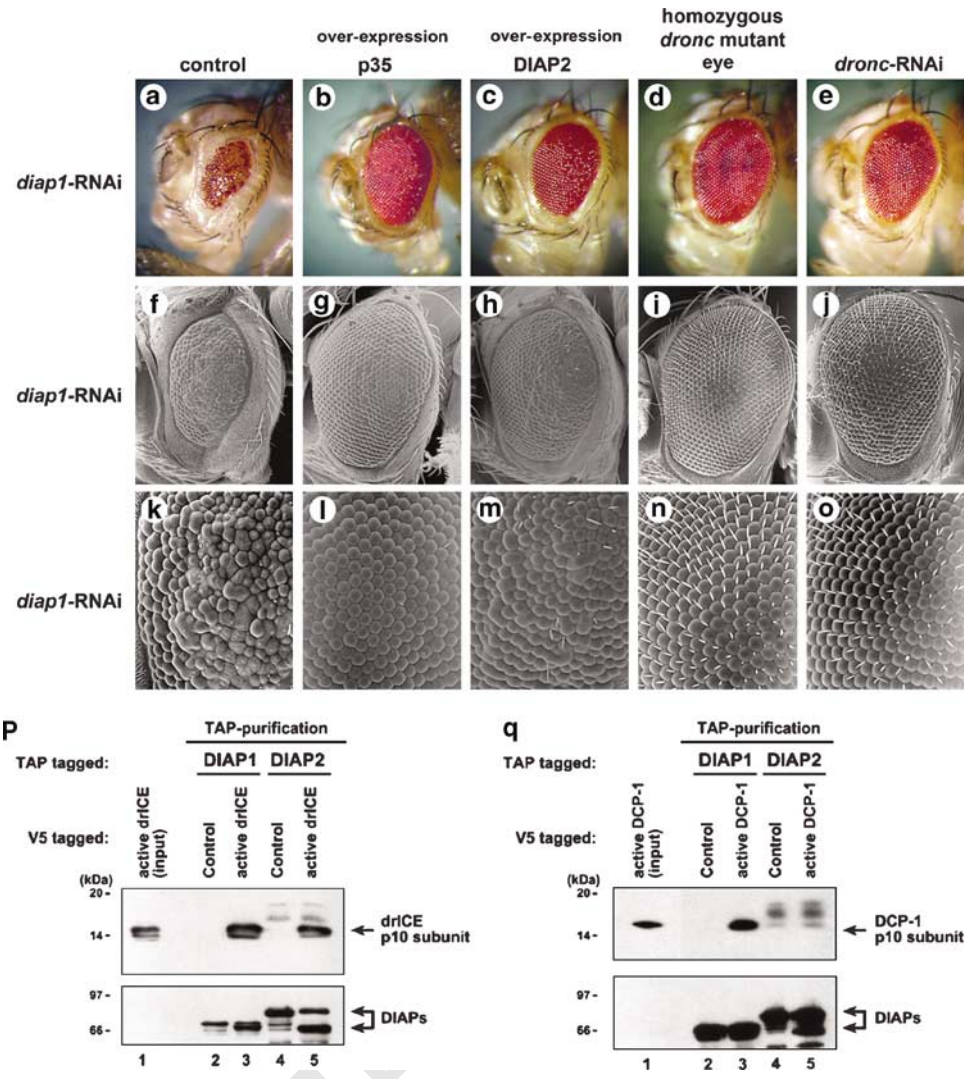


**Figure 4** DIAP2 physically interacts with drICE and can functionally substitute for DIAP1 *in vivo*. The eye phenotype caused by DIAP1 depletion (**a**, **f, k**) is rescued by overexpression of p35 (**b**, **g**, **l**), DIAP2 (**c**, **h**, **m**), homozygosity of the loss-of-function mutation *dronc*$^{I29}$ (**d**, **i**, **n**) and *dronc*-RNAi (**e**, **j**, **o**). DIAP1 depletion also results in the loss of bristle cells in the eye (**k**), which is rescued by loss of Dronc (**n–o**) but not by p35 or DIAP2 overexpression (**b**, **c**, **g**, **h**, **l** and **m**). DIAP2 directly interact with active drICE but not DCP-1. DIAP2-TAP and DIAP1-TAP were used as affinity reagents to copurify active drICE (**p**) or DCP-1 (**q**) from cellular extracts. Top panel, caspase expression (lane 1) and their copurification with DIAPs (lanes 2–5) were determined by Western blot analysis using anti-V5 antibody. Bottom panel, TAP-purified DIAP1 and DIAP2 proteins from 293T cell lysates. Genotypes: (**a**, **f**, **k**) GMR-GAL4,UAS-*diap1*-IR/+, (**b**, **g**, **l**) GMR-GAL4,UAS-*diap1*-IR/UAS-p35, (**c**, **h**, **m**) GMR-GAL4,UAS-*diap1*-IR/GMR-*diap2*, (**d**, **i**, **n**) *yw,ey*-FLP/$w^+$;GMR-GAL4,UAS-*diap1*-IR/+;FRT80B,*dronc*$^{I29}$/FRT80B,*Rps17*,*arm*-lacZ and (**e**, **j**, **o**) GMR-GAL4,UAS-*diap1*-IR/UAS-*dronc*-IR

bristles (Figure 4d, i, n and e, j, o). Similarly, *dark*-RNAi also rescued this bristle phenotype (data not show). It is noteworthy to mention that *dronc*-RNAi phenocopies the effect of null allele of *dronc*, validating the potency of the *dronc*-IR transgene. Since Dronc is a p35-resistant caspase,[8,34] these results further indicate that the bristle phenotype is strictly Dronc/Dark-dependent and independent of p35-sensitive caspases, such as drICE. Consistently, *drice*-RNAi failed to rescue the formation of bristles (data not shown), although it suppressed the *diap1*-RNAi eye phenotype (Figure 3d). However, it remains possible that this phenotype is caused by a putative p35-insensitive effector caspase that requires Dronc for its activation.

## Hid targets DIAP1 and DIAP2 equally

Consistent with a model whereby DIAP2 protects from Hid killing,[27,31] we find that Hid strongly binds to endogenous DIAP2 and DIAP1 (Figure 5a). The association of Hid with DIAP1 or DIAP2 was strictly dependent on its IAP-binding motif (IBM) because only AVP-Hid but not VP-Hid bound to endogenous DIAP1 and endogenous DIAP2. Further, Hid bound to DIAP1 and DIAP2 with comparable efficiencies, suggesting that Hid targets both these IAPs equally under physiological conditions (Figure 5b, compare lane 2 with lane 3).[31]



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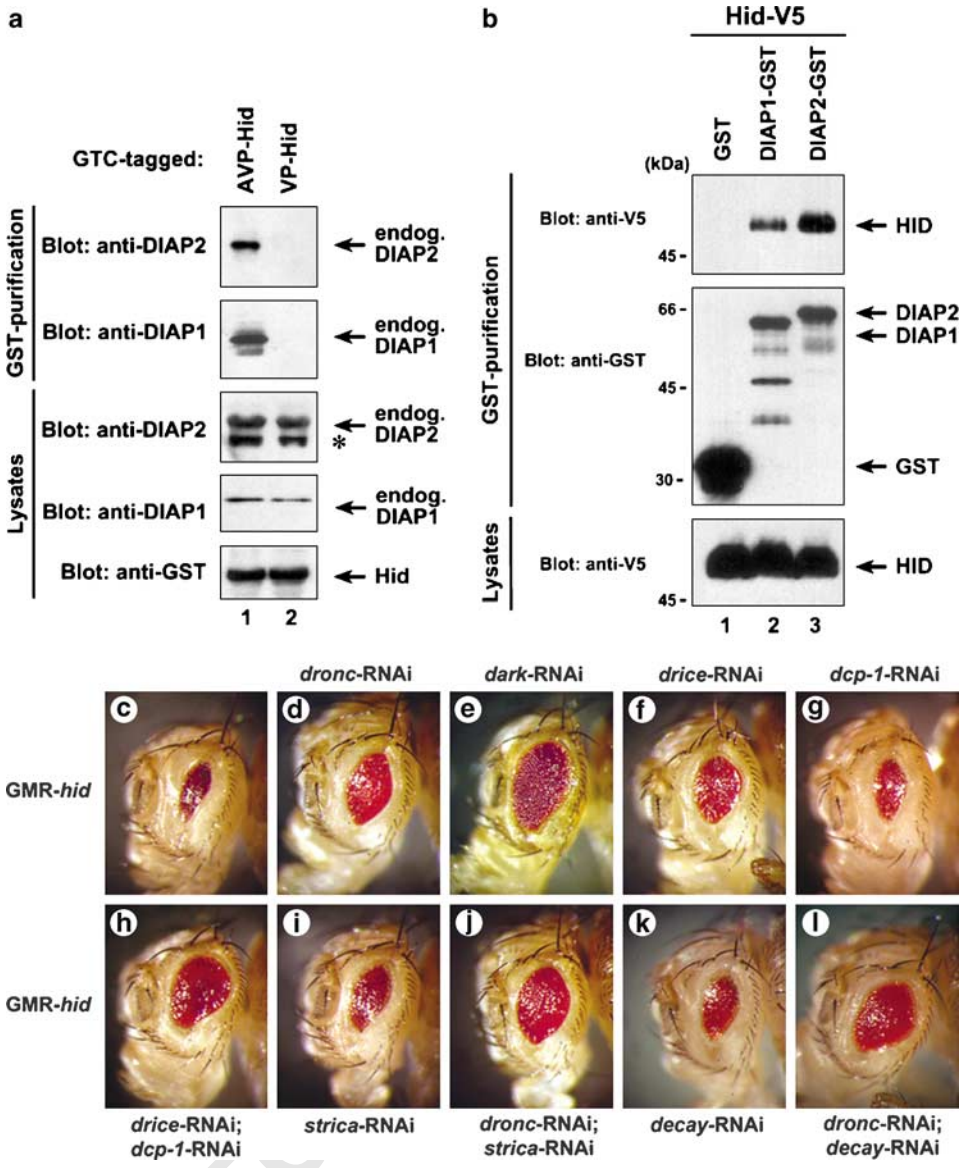


**Figure 5** Hid interacts with DIAP1 and DIAP2 and triggers cell death that is mediated by Strica, Decay, Dark, Dronc and drICE. (**a**) Hid efficiently associates with endogenous DIAP2 and DIAP1. AVP-Hid$^{1-214}$-GTC (lane 1) and VP-Hid$^{2-214}$-GTC (lane 2), which lacks Ala1, were used as affinity reagents to purify endogenous DIAPs from S2 cellular extracts. AVP-Hid, but not VP-Hid, efficiently bound to endogenous DIAP2 (top panel) and DIAP1 (2nd panel). Expression of Hid (bottom panel) and DIAPs (3rd and 4th panel) as well as endogenous DIAPs copurification (1st and 2nd panel) were verified by immunoblot analysis using the indicated antibodies. (**b**) Hid binds equally to DIAP1 and DIAP2. GST on its own (lane 1), DIAP1-GST (lane 2) or DIAP2-GST (lane 3) was coexpressed with Hid-V5 in S2/p35 cells. Cells were lysed and the cellular extracts were subject to GST purification. Expression (bottom panel) and GST-copurification (top and middle panel) were determined by immunoblot analysis using the indicated antibodies. (**c–l**) Hid killing in the eye requires Strica, Decay, Dronc, Dark and drICE, and to a minor extent DCP-1. Knockdown of Dronc (**d**), Dark (**e**), drICE (**f**), Strica (**i**) and Decay (**k**) significantly suppresses Hid-induced eye phenotypes. Double-RNAi of *strica* and *dronc* (**j**) and *decay* and *dronc* (**l**) suppressed Hid-mediated cell death significantly more efficiently than RNAi against each caspase on its own. (**h**) Coexpression of *dcp-1* and *drice* dsRNA shows an improved protection compared to *drice*-RNAi alone (compare **h** with **f**) but *dcp-1* dsRNA expression on its own as no apparent effect on Hid-induced eye phenotypes (**g**). Genotypes: (**c**) GMR-GAL4,GMR-*Hid*/+, (**d**) GMR-GAL4,GMR-*Hid*/UAS-*dronc*-IR, (**e**) GMR-GAL4,GMR-*Hid*/UAS-*dark*-IR, (**f**) GMR-GAL4,GMR-*Hid*/UAS-*drice*-IR, (**g**) GMR-GAL4,GMR-*Hid*/+;UAS-*dcp-1*-IR/+, (**h**) GMR-GAL4,GMR-*Hid*/UAS-*drice*-IR;UAS-*dcp*-1-IR/+, (**i**) GMR-GAL4,GMR-*Hid*/+;UAS-*strica*-IR/+, (**j**) GMR-GAL4,GMR-*Hid*/+;UAS-*dronc*-IR,UAS-*strica*-IR/+, (**k**) GMR-GAL4,GMR-*Hid*/UAS-*decay*-IR and (**l**) GMR-GAL4,GMR-*Hid*/UAS-*dronc*-IR,UAS-*decay*-IR/+.

## Hid killing requires strica and decay in addition to the canonical cell death pathway of Dronc, Dark and drICE

Although it is clear that Hid mediates cell death by inactivating DIAP1, it remains unclear whether this is the sole mechanism through which Hid kills cells. To examine which caspases and caspase-adaptors are required to execute this Hid-mediated cell death, we used the RNAi technique to deplete specific gene products of the caspase cascade. Surprisingly, we found that an overlapping but distinct set of caspases are required for Hid killing, compared to cell death triggered by loss of DIAP1 alone. As for *diap1*-RNAi flies, knock down of Dronc, Dark or drICE showed a significant suppression of the Hid eye



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phenotype (Figure 5d–f). In addition, however, Hid killing also seemed to rely upon Strica and Decay since flies lacking Strica and Decay showed a weak but significant suppression of the Hid eye phenotype (Figure 5i and k). While RNAi against Dronc, Strica and Decay rescued to various extents, double-RNAi of *strica* and *dronc* or *decay* and *dronc* rescued Hid-mediated cell death much more effectively than individual RNAi constructs on their own (compare Figure 5d with j and l). The suppression of the Hid eye phenotype was specific to the respective *target-gene*-RNAi since RNAi-mediated knock-down of dFadd, Damm or Dredd showed no significant rescue of the Hid eye phenotype (data not shown). *dcp-1*-RNAi on its own also failed to appreciably modulate Hid-induced apoptosis (Figure 5, compare c with g). However, coexpression of dsRNA of *dcp-1* and *drice* showed a slightly improved protection (Figure 5, compare h with f) indicating that DCP-1 contributes, to some extent, to Hid killing. Taken together, these results demonstrate that Hid-induced cell death is predominantly mediated by Strica, Decay, Dronc, Dark, drICE and to a minor extent DCP-1. On the other hand, Dredd, dFadd and Damm seem not to contribute significantly to Hid killing. The observation that Strica and Decay are rate-limiting caspases for Hid killing, but not for cell death triggered by DIAP1 depletion, raises the possibility that Hid activates caspases not only by antagonizing DIAP1 but also by engaging one or more alternative pathways. Thus, Hid seems to activate the canonical cell death pathway (Dronc/Dark, drICE) by neutralizing DIAP1. In addition, however, Hid also seems to trigger the activation of Strica and Decay through an unknown mechanism. Similar results were also obtained from Rpr-induced cell death (data not shown).

## Strica, Dronc, Dark and drICE are required for the elimination of extranumeral interommatidial cells (IOC) during pupal retina development

PCD normally occurs during pupal retina development to adjust the number of IOCs. The death of superfluous IOCs is Hid-dependent and occurs between 24 and 36 h after puparium formation (APF). By 42 h APF, all superfluous IOCs are removed and individual ommatidia are positioned within a hexagonal lattice of shared pigment cells and mechanosensory bristles. Each photoreceptor cell cluster consists of four cone cells, three bristle cells, two primary, six secondary and three tertiary pigment cells[26,35] (Figure 6a and e). To determine the physiological role of Dronc, Dark, drICE and Strica for the death of superfluous IOCs, we stained pupal retinae, 68 h APF, of *dronc*- (Figure 6b), *drice*- (Figure 6c), *dark*- (Figure 6d) and *strica*-RNAi flies (Figure 6g) with phalloidin, which detects actin and therefore reveals the outline of cells. At 68 h APF, retinae from *dronc*-, *drice*- and *dark*-RNAi flies contained an aberrant number of IOCs, indicating that Dronc, Dark and drICE are required for the proper adjustment of lattice cells during normal development. *strica* dsRNA expression also affected apoptosis of superfluous IOC: at 42 h APF, *strica*-RNAi retinae (Figure 6f) carried additional IOCs, indicating that Strica contributes to IOC elimination from the early stages of death. However, at 68 h APF, *strica* dsRNA expressing retinae exhibited normal numbers of lattice cells (Figure 6g). The decrease in the number of extra IOCs between 42 and 68 h APF in *strica*-RNAi retinas indicates that IOC death is delayed following knock down of Strica. The observation that depletion of Strica results in delayed death of extra lattice cells suggests that endogenous Strica contributes to the apoptotic signal. However, since

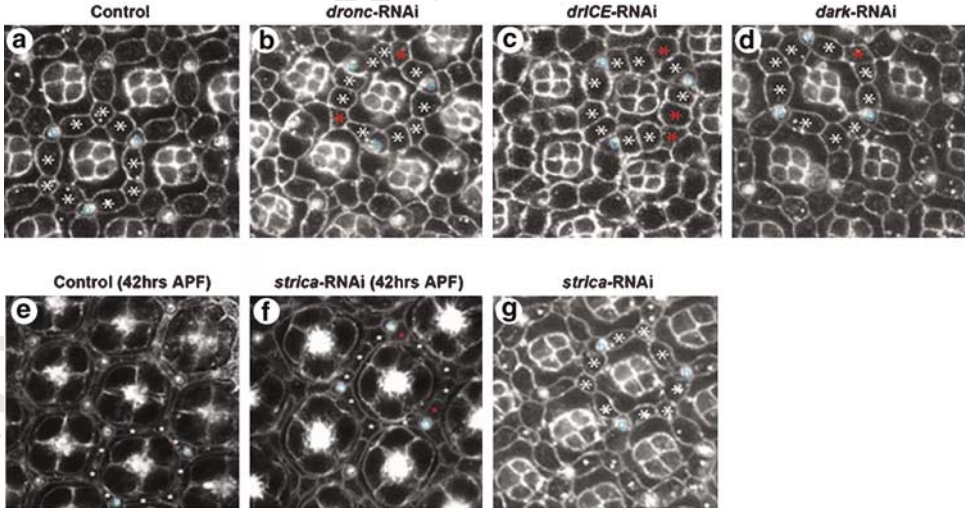


**Figure 6** Strica, Dronc, drICE and Dark are essential for PCD during eye development. To evaluate the effect of *strica*-, *dronc*-, *drice*- and *dark*-RNAi during retina development, pupae were dissected at 68 h (**a**–**d** and **g**) or 42 h APF (**e**–**f**) and actin was stained with phalloidin-488 to reveal cell bodies. Blue 'b' designates bristle complexes, white asterisks indicate normal IOCs and red asterisks indicate additional IOC. At 42 h (**e**) or 68 h APF (**a**) at 25°C, superflous lattice precursor cells have been eliminated by apoptosis to refine a hexagonal interommatidial lattice. Under normal conditions, each hexagonal lattice consists of three bristle complexes with six secondary pigment cells and three tertiary pigment cells. (**b**–**d**) Expression of *dronc*, *drice* and *dark* dsRNA results in an aberrant number of lattice cells (red) at 68 h APF. (**g**) *strica*-RNAi does not result in extra IOCs at 68 h APF but at 42 h APF *strica*-RNAi retinae carried additional IOCs (**f**), indicating that loss of Strica delayed but did not block IOC death. Representative retinae of flies of the following genotypes are shown: (**a** and **e**) GMR-GAL4/+, (**b**) GMR-GAL4/UAS-*dronc*-IR, (**c**) GMR-GAL4/UAS-*drICE*-IR, (**d**) GMR-GAL4/UAS-*dark*-IR and (**e** and **f**) GMR-GAL4/UAS-*strica*-IR





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*strica*-RNAi retinae contain normal number of IOC at later time points, the level of RNAi-mediated knockdown of Strica may either be insufficient or, alternatively, may indicate that Strica is required for the timing, rather than execution, of IOC death.

### Strica, Dronc, dark And drICE are required for the timely removal of larval SGs

PCD of SGs during *Drosophila* metamorphosis is triggered by a pulse of the steroid hormone ecdysone at approximately 12 h APF. Ecdysone induces a signalling cascade that culminates in the upregulation of a number of proapoptotic genes such as *hid*, *rpr*, *dark*, *dronc*, *strica* and *drice*.[36] In addition to the apoptosis machinery, autophagy seems to contribute significantly to SG histolysis.[36] Since the entire cell death machinery is present in the SGs, PCD of this tissue provides an ideal system to study the requirement of each component. We used an SG-specific driver (SG-GAL4) to express our RNAi constructs selectively in SGs and monitored their persistence during metamorphosis using green fluorescent protein (GFP) fluorescence, as described in Ward *et al*.[37] Using GFP fluorescence as an indirect indicator for efficient histolysis of SGs, we found that Strica, Dronc, Dark and drICE seemed to be required for the timely removal of SGs (phenotypes for *dronc*-, *dark*- and *drice*-RNAi were 100% penetrant, $n>50$, while *strica*-RNAi was 56% penetrant, $n=55$). In the absence of Strica, Dronc, Dark and drICE, like expression of p35, removal of GFP-labelled SGs was significantly delayed as indicated by the presence of persisting GFP fluorescence even at 30 h APF (Figure 7). However, RNAi-mediated depletion of Strica, Dronc, Dark and drICE, like expression of p35, merely delayed but did not block SG removal. No GFP fluorescence was detectable in animals that were examined at 40 h APF (data not shown). This observation is consistent with the notion that SGs are removed by autophagy but not apoptosis.[36] The delay in SG removal suggests that caspases and autophagy together ensure efficient clearance of larval tissues during metamorphosis. The RNAi-mediated delay of SG clearance was highly specific to the knock down of Strica, Dronc, Dark and drICE since, under the same conditions, RNAi-mediated depletion of dFadd, Dredd, DCP-1, Damm and Decay had no apparent effect, suggesting that these caspases play no or only a minor role in SG histolysis (data not shown).

## Discussion

In this report, we provide *in vivo* evidence for the physiological importance of *Drosophila* caspases and caspases-adaptors. We have used a systematic genetical approach to reveal the biological role of putative core components of the *Drosophila* cell death machinery. Of the seven caspases and two caspase-adaptors, we find that cell death *in vivo* is predominantly executed by the canonical caspase cascade consisting of Dronc, Dark and drICE. This is evident because RNAi-mediated knockdown of Dronc, Dark and drICE significantly compromised apoptosis induced by Hid overexpression in the developing eye as well as PCD of superfluous IOCs during pupal retina development. In addition to Dronc, Dark and drICE, we find that Strica and Decay also contribute to apoptosis induced by Hid. Knock down of Strica or Decay significantly suppressed cell death triggered by Hid overexpression. Similarly, Rpr-induced cell death was also suppressed by depletion of Strica, Decay, Dronc, Dark and drICE (data not shown). However, while Dronc, Dark and drICE depletion results in aberrant numbers of interommati-

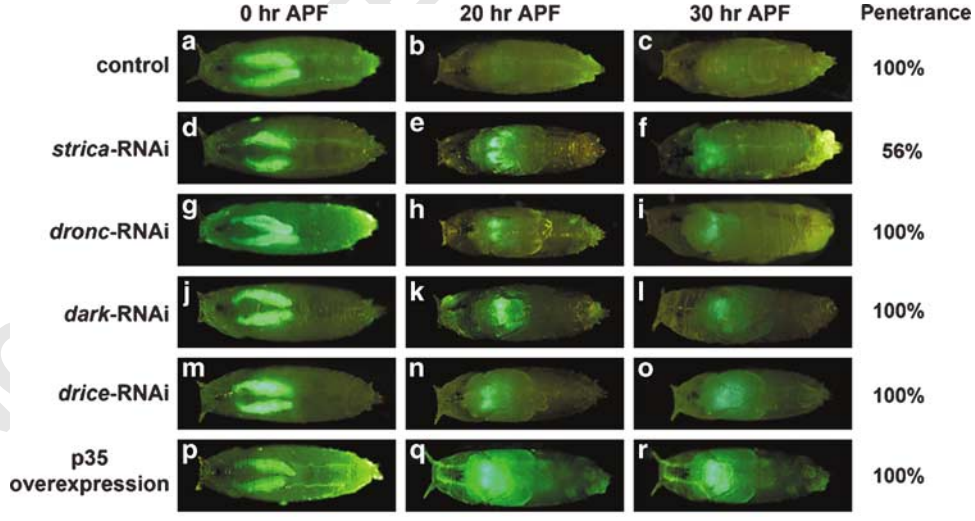


**Figure 7** Strica, Dark, Dronc and drICE are required for the timely removal of larval SGs during *Drosophila* metamorphosis. Programmed cell death of SGs is triggered at approximately 14.5 h APF. By 20 h APF, histolysis is complete and no GFP-marked SGs are detectable in wild-type animals (**a**–**c**). In contrast, RNAi-mediated depletion of Strica- (**d**–**f**), Dronc- (**g**–**i**), Dark- (**j**–**l**) and drICE- (**m**–**o**) like overexpression of p35 (**p**–**r**) severely delayed the removal of GFP-positive SGs. Persistent GFP fluorescence was apparent up to 30 h APF. While the delay in SG removal was fully penetrant in *dronc*-, *dark*- and *drice*-RNAi-treated SG, *strica*-RNAi was only 56% ($n=55$) penetrant. Histolysis of GFP-labelled SGs at different time points is shown in (**a**–**c**) SG-GAL4/+;UAS-*GFP*/+, (**d**–**f**) SG-GAL4/+;UAS-*GFP*/UAS-*strica*-IR, (**g**–**i**) SG-GAL4/UAS-*dronc*-IR;UAS-*GFP*/+, (**j**–**l**) SG-GAL4/UAS-*dark*-IR;UAS-*GFP*/+, (**m**–**o**) SG-GAL4/UAS-*drice*-IR;UAS-*GFP*/+ and (**p**–**r**) SG-GAL4/UAS-*p35*;UAS-*GFP*/+



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dial lattice cells, loss of Strica merely delayed, but not blocked, the removal of superfluous IOCs. This may suggest that Strica is required for the timing, rather than execution, of IOC death. In a second paradigm of PCD, we find that depletion of Strica, Dronc, Dark and drICE delayed, albeit not completely blocked, the removal of larval SGs during metamorphosis. Consistent with the notion that DCP-1 is dispensable for PCD,[13] we find that this effector caspase is not rate limiting for apoptosis *in vivo*. Nevertheless, DCP-1 contributes to apoptosis in the developing eye to a minor extent since simultaneous RNAi of drICE and DCP-1 is slightly more effective in suppressing Hid killing than *drice*-RNAi on its own. In contrast, Dredd, dFadd and Damm appear not to mediate Hid-induced apoptosis. Expression of dsRNA of *dredd*, *dfadd* or *damm*, either alone or in combination, had no effect on the destruction of SGs or Hid killing in the eye, even though these RNAi transgenes were highly effective in phenocopying known genetic mutations of *dredd* and *dfadd,* or knocking down *damm* mRNA levels. Although we did not observe any physiological role for *dredd*, *dfadd* or *damm* in cell death, it remains possible that they are important to execute apoptosis under distinct, tissue- or stage-specific conditions.

Genetic studies show that Hid is indispensable for apoptosis in the developing eye as well as for histolysis of larval tissues, such as the SGs, during metamorphosis.[25,26] The current model suggests that Hid promotes cell death by directly antagonizing DIAP1 and disrupting DIAP1–caspase association, thereby alleviating IAP-mediated inhibition of caspases. If this was the case, then apoptosis initiated by either Hid expression or DIAP1 depletion should require the same set of caspases. Surprisingly, we find that Hid killing is mediated by a broader set of caspases than cell death triggered by loss of DIAP1 alone. Apoptosis triggered by Hid overexpression requires Strica, Decay, Dronc, Dark and drICE. In contrast, cell death initiated by DIAP1 depletion solely requires Dronc, Dark and drICE. This indicates that Strica and Decay are not rate-limiting caspases for apoptosis initiated by DIAP1 depletion. Thus, Strica and Decay mediates cell death exclusively downstream of Hid. The notion that Hid killing requires a broader set of caspases than apoptosis initiated by DIAP1 depletion suggests that Hid causes cell death not only by neutralizing DIAP1 but also by activating DIAP1-independent caspase cascades, consisting of Strica and Decay (Figure 8).

During the apoptotic process, caspases are activated in an amplifying proteolytic cascade, cleaving one another in turn. The initiator caspase Dronc is most homologous to mammalian caspase-9, which, following its dimerization-induced activation, cleaves and activates effector caspases such as caspase-3 and -7.[38] By analogy, following its activation by Dark, Dronc is expected to activate the effector caspase drICE. Consistently, active Dronc cleaves drICE efficiently *in vitro* placing Dronc upstream of drICE[8] (Figure 8). The epistatic position of Strica is less clear as it combines features of both initiator and effector caspases. Reminiscent of initiator caspases, Strica, contains an extensive regulatory prodomain; however, its large and small caspase subunits conform to effector caspases. Our genetic analysis, together with the current available molecular information of Strica, cannot yet reveal Strica's position in the cell death machinery. Since Hid

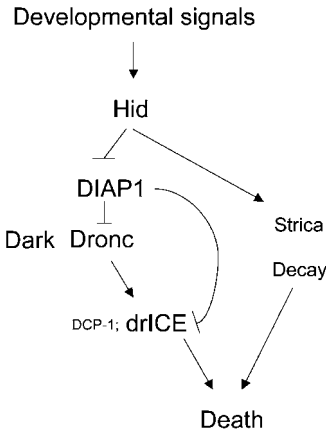


**Figure 8** A putative model for the regulation of cell death in *Drosophila*

overexpression but not *diap1*-RNAi-mediated cell death requires Strica, it appears, however, that Strica acts genetically upstream or in parallel to Dronc, Dark and drICE (Figure 8). Simultaneous knockdown of Dronc and Strica, or Dronc and Decay, has a much more protective effect than each one on its own, indicating that Strica and Decay may cooperate with Dronc to induce cell death. Intriguingly, DIAP2, the *Drosophila* ortholog of the mammalian caspase inhibitor XIAP, has been reported to physically interact with Strica,[17] raising the possibility that the Strica/Decay caspase cascade is under the control of DIAP2. However, DIAP1 has also been reported to associate with Strica.[17] Clearly, additional work is required to clarify the biological significance of these molecular interactions.

In agreement with the notion that Hid promotes cell death by antagonizing both IAPs, we find that Hid physically interacts with DIAP1 and DIAP2 with comparable efficiencies (Figure 5a and b).[31] This suggests that DIAP1 and DIAP2 together set up an apoptotic threshold. Consistently, overexpression of DIAP1 or DIAP2 potently suppresses Hid-induced death. Moreover, overexpression of DIAP2 can also protect against cell death triggered by loss of DIAP1, which clearly indicates that DIAP2 and DIAP1 share common caspase targets. Accordingly, we find that DIAP2 efficiently binds to drICE, indicating that DIAP1 and DIAP2 share the regulation of drICE. However, DIAP2 only partially rescued the *diap1*-RNAi eye phenotype since it failed to restore the loss of bristle cells, which is the result of deregulated Dronc. Thus, it appears that DIAP2 does not control Dronc, which is consistent with our previous observation that DIAP2 offers no protection from the lethal effects of Dronc.[34] DIAP2's ability to rescue the *diap1*-RNAi phenotype is highly reminiscent to the one of p35, which also does not regulate Dronc and failed to suppress the formation of bristles. Since drICE is an important effector caspase to execute apoptosis, it seems likely that DIAP2 and p35 suppress apoptosis by directly inactivating drICE. However, even though DIAP2 can regulate certain components of the cell death machinery when overexpressed, genetic loss-of-function mutations in DIAP2 will be required to identify its physiological role in apoptosis regulation.

While in mammals mitochondrial release of cytochrome *c* is the key determining factor for apoptosome assembly, it





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appears that in *Drosophila* mere liberation of Dronc from DIAP1 is sufficient to trigger Dronc/Dark apoptosome formation and cell death. This is evident because mere depletion of DIAP1 by RNAi instigates spontaneous activation of caspase-mediated cell death. Importantly, this death occurs completely independent of upstream proapoptotic signals. Thus, loss of DIAP1 does not trigger caspase activation indirectly by causing 'cellular stress', which in turn would result in Rpr/Grim/Hid expression or activation of the stress-activated protein kinase JNK signal-transduction pathway. Therefore, depletion of DIAP1 protein levels on its own, and in the absence of any additional death insults, triggers unrestrained cell death in a Dronc/Dark-dependent manner. This must mean that Dark is constitutively active and that loss of DIAP1 function allows Dark-dependent caspase activation. Hence, DIAP1-mediated inhibition of Dronc appears to be the key regulatory step in controlling apoptosome formation and cell death in *Drosophila*.

Our genetic study highlights the biological importance of DIAP1, Strica, Decay, Dronc, Dark and drICE in apoptosis and, therefore, forms the foundation from which to explore the molecular nature of the observed genetic interactions among IAPs, caspases and caspase-adaptors. Future studies will have to address how Hid activates Strica and Decay, how their activity impinges on cell viability and how these caspases are regulated. Intriguingly, although in mammals all caspases activation has been thought to require mitochondrial disruption and consequential apoptosome formation, recent studies indicate that caspase activation may occur before mitochondrial membrane disruption, and independently of the caspase-9/Apaf1 apoptosome, which has been postulated to amplify rather than initiate the caspase cascade.[39] Therefore, it is attractive to speculate that a better understanding of the molecular nature of Strica and Decay will provide fresh insights into the proteolytic signal-transduction cascade that precipitates the destruction of the cell.

## Materials and Methods

### Fly strains and crosses

Inducible RNAi transgenic fly lines were generated by P-element-mediated transformation as described previously.[3] Two independent transgenic fly lines carrying IRs were analyzed for each construct showing undistinguishable phenotypes. The target sequence (500 bp, sequences available upon request) were amplified by PCR and inserted as an IR into pUAST-R57. Following induced expression, the dsRNA is produced as a spliced hairpin structure to maximize interference effects.[29] The following fly strains were used for phenotypical analysis: $\alpha$*Tubulin84B*-GAL4, GMR-GAL4, *Salivary Gland*-GAL4, UAS-*diap1*, UAS-*prodronc*, UAS-*p35*, UAS-*Puckered*, GMR-*Hid*, GMR-*strica*, GMR-N-*dcp-1*, GMR-*diap2*, *hid*$^{05014}$, *Df(3L)H99*, *w*;FRT80B,*dronc*$^{I29}$/TM6b, *w*;FRT80B,*Df(3L)H99*/TM2 and *yw*,*ey*-FLP;FRT80B,*Rps*$^{17}$,*arm*-lacZ/TM6b,Tb. All these stocks are described in Flybase. Eye-specific FRT/Flp-mediated mitotic recombination[40] was used to generate mosaic flies with *Dronc* or *H99* homozygous mutant clones in the eyes. The use of a *Minute* mutation (*Rps*$^{17}$) on the heterologous FRT chromosome allows more than 90% of the cells in the generated eye to be *Dronc* or *H99* mutant.[54] All flies were raised at 25°C. All shown phenotypes are fully penetrant, unless stated otherwise.

### Histology and scanning electron microscopy (SEM)

Histological sections, acridine orange stainings and SEM were performed as described previously,[34] except that serial dilutions of hexametyldisilazone (Agar scientific, UK) were used to dehydrate SEM specimens.

### Tissue culture and immunoblot analysis

S2 and 293 cells were cultured as described in Tenev *et al*.[33] The copurification experiments were performed as in Tenev *et al*.[33] Briefly, for Figure 4p–q, DIAP1- and DIAP2-TAP proteins were affinity purified from 293 cellular extracts and resin-bound DIAP-TAP was then incubated with 293 cellular extracts containing active drICE-V5 or DCP-1-V5. Following 1 h incubation at 4°C, samples were washed, eluted and analyzed by immunoblotting. For Figure 5a, AVP-Hid$^{1-214}$-GTC or VP-Hid$^{2-214}$-GTC was used to copurify endogenous DIAP2 and DIAP1 from S2 cellular extracts as described previously.[22] For Figure 5b, AVP- or VP-Hid-V5 was coexpressed with glutathione *S*-transferase (GST) alone, DIAP1-GST or DIAP2-GST in S2 cells. Cells were lysed and cellular extracts were subject to GST purification as described previously.[22]

### Immunoblot analysis of fly protein extracts

Protein extracts of 50 first instar larvae (*diap1*-RNAi) and five wandering third instar larvae (*drice*-RNAi) were analyzed by immunoblotting with anti-DIAP1, anti-DIAP2, anti-tubulin (Sigma, UK) and anti-drICE as described previously.[22] Anti-DIAP2 was generated in guinea pigs using a purified, recombinant DIAP2 fragment (amino acids 431–496). For immunoblot quantification, Odyssey® technology was used according to the manufacturer's instructions (Licor Biosciences, UK).

### Salivary gland histolysis assay

Embryos were collected for 12 h on apple juice plates. White pre-pupae were collected when spiracles were everted (0 h APF). Larval SG persistence during metamorphosis was monitored by observation of the SG-specific GFP signal as described previously.[37]

### Pupal retina analysis

Pupae were staged at 25°C and the retinae were dissected at 42 or 68 h APF. Briefly, retinae were dissected in PBS and fixed in 4% formaldehyde for 20 min. Phalloidin-488 (Alexa) was used to stain actin and reveal the cell bodies. Corresponding whole-mount retinae were observed though the facet lens.

### Image processing

Pictures of adult eyes and wings were taken under visible light with a Leica MZ APO stereomicroscope using a Nikon D1 digital camera. Pictures of GFP-expressing larval SGs were taken with a Leica MZ APO stereomicroscope using a Leica digital camera. Semithin adult eye sections were analyzed under visible light using a Leica DMRA2 microscope. Acridine orange staining were observed under green fluorescence using a Zeiss Axioplan2 microscope. SEM was performed using a Hitachi S4000 SEM at 15 kV accelerating voltage. Pupal retinae were observed using a Biorad radiance confocal microscope. All pictures were processed using Adobe Photoshop CS2 software.





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