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The *Drosophila* caspase DRONC is regulated by DIAP1

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We have isolated the recently identified *Drosophila* caspase DRONC through its interaction with the effector caspase drICE. Ectopic expression of DRONC induces cell death in *Schizosaccharomyces pombe*, mammalian fibroblasts and the developing *Drosophila* eye. The caspase inhibitor p35 fails to rescue DRONC-induced cell death *in vivo* and is not cleaved by DRONC *in vitro*, making DRONC the first identified p35-resistant caspase. The DRONC pro-domain interacts with *Drosphila* inhibitor of apoptosis protein 1 (DIAP1), and co-expression of DIAP1 in the developing *Drosophila* eye completely reverts the eye ablation phenotype induced by pro-DRONC expression. In contrast, DIAP1 fails to rescue eye ablation induced by DRONC lacking the pro-domain, indicating that interaction of DIAP1 with the pro-domain of DRONC is required for suppression of DRONC-mediated cell death. Heterozygosity at the *diap1* locus enhances the pro-DRONC eye phenotype, consistent with a role for endogenous DIAP1 in suppression of DRONC activation. Both heterozygosity at the *dronc* locus and expression of dominant-negative DRONC mutants suppress the eye phenotype caused by reaper (RPR) and head involution defective (HID), consistent with the idea that DRONC functions in the RPR and HID pathway.

Keywords: apoptosis/caspase/DIAP1/*Drosophila melanogaster*

Introduction

In multicellular organisms, homeostasis is established and maintained by a dynamic balance between cell proliferation and cell death. Programmed cell death (PCD) is used as a means to eliminate damaged or supernumerary cells and to sculpt and whittle structures during development (Evan and Littlewood, 1998; Tschopp *et al*., 1998; Vaux and Korsmeyer, 1999). In addition, PCD provides an important defence against viral infection and the emergence of cancer (Thompson, 1995).

PCD, usually called apoptosis in complex metazoans, is an active process implemented by a machinery that is evolutionarily conserved amongst nematodes, insects and vertebrates. Apoptosis involves execution of a complex and co-ordinated series of events culminating in activation of a family of cysteine proteases called caspases (cysteinyl aspartate-specific proteases) (Thornberry and Lazebnik, 1998). Caspases are expressed as pro-enzymes with little or no intrinsic catalytic activity that comprise three nascent domains: an N-terminal pro-domain, a large subunit containing the catalytically active cysteine (~20 kDa) and a C-terminal small subunit (~10 kDa). They are activated by proteolytic cleavages at sites located between these domains that abscize the pro-domain and release the large and small subunits, which then form the active $(p20/p10)_2$ caspase hetero-tetramer. The inter-domain sites for proteolytic activation of caspases are themselves caspase consensus cleavage sites, indicating that caspases reside in cascades of auto- and trans-activation that are typically initiated by activation of initiating or 'apical' caspases (Alnemri, 1997). Once activated, caspases cleave various cellular substrates, such as lamins, kinases, DNA repair enzymes and proteins involved in mRNA splicing and DNA replication, and this is presumed to trigger many of the morphological processes of cell death defined as apoptosis (Thornberry and Lazebnik, 1998).

Genetic studies in the nematode *Caenorhabditis elegans* provided the first direct evidence for the importance of caspases in PCD. Inactivating mutations in the nematode caspase CED-3 block all of the 131 developmental cell deaths that occur during *C.elegans* development (Ellis and Horvitz, 1986). Later studies indicated analogous requirements for caspases in PCD in *Drosophila* and in mammals. In *Drosophila*, RPR (reaper), GRIM and HID (head involution defective) proteins have been identified as key activators of the apoptotic machinery (White *et al*., 1994; Grether *et al*., 1995; Chen *et al*., 1996). Embryos with a chromosomal deletion that includes the *rpr*, *grim* and *hid* loci show essentially no PCD during ontogeny (White *et al*., 1994). Ectopic expression of RPR, GRIM or HID in the developing *Drosophila* eye results in a highly efficient and dose-dependent ablation of eye structures. This occurs through activation of a caspase-dependent apoptotic machinery, since PCD induced by each of these pro-apoptotic proteins is blocked by expression of the baculovirus protein p35, a promiscuous caspase inhibitor (Grether *et al*., 1995; Chen *et al*., 1996; White *et al*., 1996).

In *Drosophila*, four caspases have been identified thus far: drICE, DCP-1, DCP-2/DREDD and, most recently, DRONC (Fraser and Evan, 1997; Inohara *et al*., 1997; Song *et al*., 1997; Chen *et al*., 1998; Dorstyn *et al*., 1999). Both drICE and DCP-1 possess short pro-domains typical of 'downstream' or 'effector' caspases, such as mammalian caspases-3, -6 and -7, which are activated via proteolytic

cleavage by 'upstream' caspases. In contrast, DCP-2/DREDD and DRONC contain extensive pro-domains, characteristic of 'upstream' or 'apical' caspases. The DRONC pro-domain contains a caspase recruiting domain (CARD), whereas the pro-domain of DREDD shares no significant homology, as judged by Pfam analysis (Bateman *et al*., 1999), with either the CARD or death effector domains (DEDs) found in other caspases. Ectopic expression of RPR, GRIM or HID leads to proteolytic cleavage and activation of drICE, DCP-1 and DCP-2/DREDD. However, nothing is known about the hierarchy of caspase activation, nor how RPR, GRIM and HID engage the apoptotic machinery. Intriguingly, expression of RPR, GRIM or HID leads to proteolytic cleavage of DREDD even in the presence of the caspase inhibitor p35 (Chen *et al*., 1998). Since drICE, DCP-1 and DREDD are each inhibited by p35, this suggests that DREDD is activated by a p35-resistant protease.

The role of the recently reported caspase DRONC in PCD of *Drosophila* has not been established. During development, DRONC is ubiquitously expressed during embryogenesis as well as in the developing eye, brain and adult egg chambers, all places where PCD naturally occurs. Interestingly, in late third instar larvae, DRONC is dramatically up-regulated in salivary glands and midgut before histolysis of these tissues occurs during metamorphosis. Exposure of these tissues to ecdysone leads to a significant increase in *dronc* mRNA levels, suggesting that DRONC may be an ecdysone-inducible caspase (Dorstyn *et al*., 1999).

The inhibitor of apoptosis protein (IAP) family comprises proteins conserved amongst a wide range of eukaryotic species that suppress apoptosis induced by a variety of stimuli (Uren *et al*., 1998; Deveraux and Reed, 1999). In *Drosophila*, ectopic expression in the developing eye of the cellular IAPs, DIAP1 or DIAP2, suppresses cell death induced by RPR or HID (Hay *et al*., 1995). Furthermore, genetic studies of DIAP1 in the eye and ovary suggest that DIAP1 is essential for 'normal' survival of these cell types. However, the mechanisms by which IAPs suppress cell death are poorly understood. In lepidopteran cells, DIAP1 and DIAP2 interact physically with, and block, the pro-apoptotic activity of RPR, GRIM and HID (Vucic *et al*., 1997, 1998a). In addition, DIAP1 inhibits the proteolytic activity of active drICE and DCP-1 *in vitro* (Kaiser *et al*., 1998; Hawkins *et al*., 1999).

At present, however, it is unclear how effector caspases become activated in *Drosophila*, or how the pro-apoptotic proteins RPR, HID and GRIM promote caspase activation, and the DIAP proteins suppress it. To address these issues, we have searched for proteins that interact with the 'effector' caspase drICE and have identified DRONC. We show that DRONC has proteolytic activity that, unlike other caspases, is not blocked by p35. In addition, we show that DIAP1 interacts with the pro-domain of DRONC and appears to be a critical regulator of activation of this 'apical' caspase *in vivo*. Furthermore, we provide evidence that supports the idea that DRONC is a rate-limiting caspase in the RPR and HID pathway.

Results

DRONC interacts with the effector caspase drICE

In *Drosophila melanogaster*, the pro-apoptotic proteins RPR, GRIM and HID induce cell death via activation of caspases. However, thus far, little is known concerning how RPR, GRIM or HID trigger caspase activation. To study the mechanisms underlying caspase activation, we sought to identify molecules that interact with pro-caspases and, hence, might be involved in their regulation and activation. As a target pro-caspase we chose the *Drosophila* caspase drICE, which has been shown to be required for execution of apoptosis in certain fly cells *in vitro* (Fraser *et al*., 1997).

A catalytically inactive mutant of drICE, in which the active site cysteine has been changed to alanine (drICE C→A), was used as bait in a yeast two-hybrid assay to screen a 0–24 h *Drosophila* embryonic cDNA library. From 2×10^6 yeast transformants, we isolated 34 clones encoding potential drICE interactors. Three of these clones were *drICE*-derived; one encoded full-length drICE (1–339) whereas the other two encoded N-terminal truncations of drICE (40–339 and 43–339, respectively). Interaction with these latter two suggests that pro-drICE can dimerize via its core region (the protein region without the pro-domain), even in its inactive pro-form.

We further assessed the physical interaction between DRONC and drICE by testing for the ability of the two proteins to co-immunoprecipitate from cell extracts. FLAG-tagged, full-length, catalytically inactive DRONC (pro-DRONC C→A, 1–451) was co-expressed in 293T cells together with Myc-tagged catalytically inactive pro-drICE C→A (1–339), ΔN drICE C→A (29–339) or Bcl-10 (Figure 1C). The mammalian protein Bcl-10 that contains an N-terminal CARD was used as the control in the co-immunoprecipitation experiments. Pro-DRONC specifically co-immunoprecipitated both pro-drICE and ΔN drICE, but not Bcl-10, indicating that DRONC and drICE form a stable complex in cell extracts.

Ectopic expression of DRONC is toxic to S.pombe and induces apoptosis in Rat-1 cells

The fission yeast *Schizosaccharomyces pombe* is devoid of caspase homologues or caspase-like activities. However, because many active caspases have been demonstrated to be toxic when expressed in yeast, *S.pombe* has emerged as a useful and facile model system in which to assess caspase functionality (Ekert *et al*., 1999). We inserted sequences encoding pro-DRONC and ΔN DRONC into the *S.pombe* expression vector pNeu under the control of a thiamine-repressible promoter. In the presence of thiamine, yeast transformed with either of the two constructs grew normally. However, both pro-DRONC and ΔN DRONC expression proved toxic and resulted in a time-dependent inhibition of yeast growth (Figure 2A). This toxicity is dependent on DRONC enzymatic activity since catalytically inactive DRONC mutants (pro-DRONC C→A or ΔN DRONC C→A) had no effect on yeast growth. Both pro-DRONC and ΔN DRONC underwent catalytic autoprocessing to a similar extent in *S.pombe*, as judged by immunoblotting of DRONC from cell extracts. However, when expressed at approximately similar levels, pro-DRONC appeared somewhat more toxic than ΔN DRONC.

Many caspases induce apoptosis when expressed in mammalian cells. We therefore asked whether pro-DRONC, ΔN DRONC or the catalytically inactive mutant of ΔN DRONC (ΔN DRONC C→A) killed Rat-1 fibro-

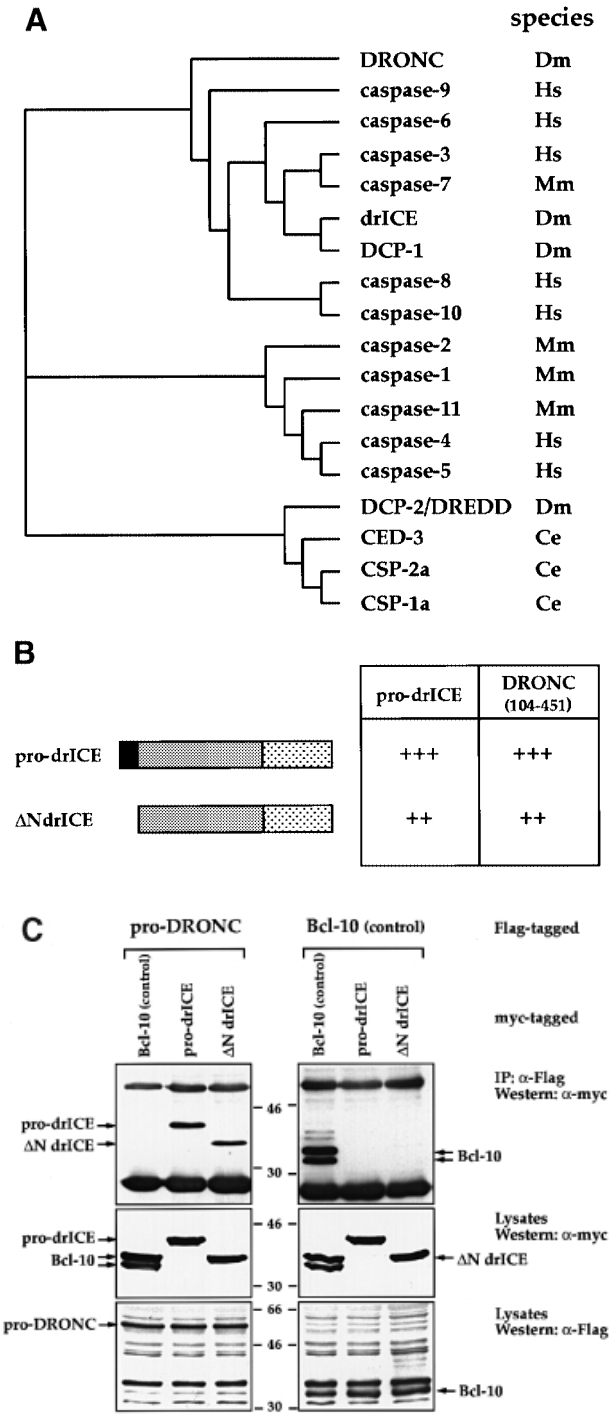


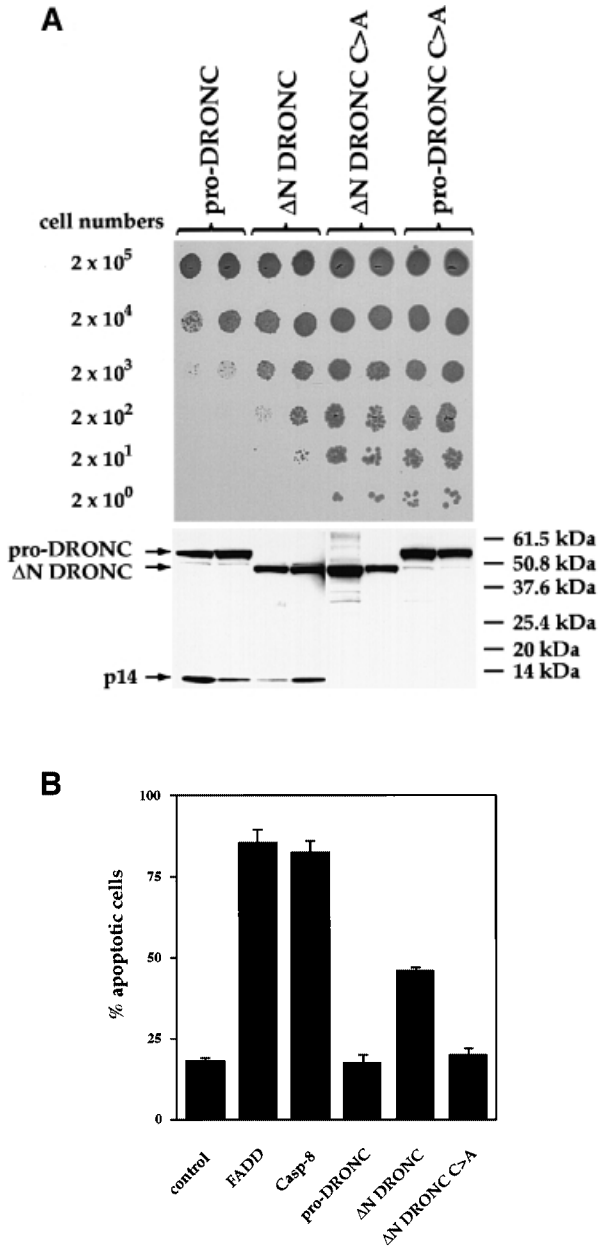
Fig. 1. DRONC is a drICE-interacting caspase. (**A**) The dendrogram shows the phylogenetic relationships of the core region of caspase family members (i.e. the protein sequence without the pro-domain). ClustalX was used for the sequence analysis. (**B**) Yeast two-hybrid analysis showing that DRONC and drICE interact with each other through their core regions. The extent of the β-galactosidase staining, as detected in filter tests, is indicated: +++, intense blue staining of large colonies; ++, light blue staining of medium size colonies. (**C**) Co-immunoprecipitation from 293T cell extracts. FLAG-tagged full-length DRONC (pro-DRONC C→A) and Bcl-10 (control) were co-expressed together with either Myc-tagged pro-drICE C→A, ΔN drICE C→A or Bcl-10 (control). Cell lysates were incubated with M2 anti-FLAG monoclonal antibody resin, washed, and the co-immunoprecipitated Myc epitope-tagged proteins were detected by immunoblot analysis using anti-Myc monoclonal antibody (9E10). Expression of FLAG-tagged and Myc-tagged proteins was confirmed. Molecular mass markers in kDa are shown.

blasts (Figure 2B). Expression of ΔN DRONC, which lacks its pro-domain, was very effective at inducing cell death, as was expression of either of the positive controls, caspase-8 and the Fas pathway adaptor FADD. However, in complete contrast, expression of full-length DRONC exerted no lethal effect. DRONC therefore resembles caspases-4 and -5 (Munday *et al*., 1995), both of which kill mammalian cells only when expressed without their respective pro-domains. As in *S.pombe*, the catalytically inactive ΔN DRONC C→A mutant had no effect on Rat-1 cell viability, consistent with a requirement for the caspase activity of DRONC to induce Rat-1 cell death. The lack of toxicity of full-length DRONC in Rat-1 cells is in stark contrast to the situation in *S.pombe* in which both pro-DRONC and ΔN DRONC are toxic and undergo autocatalytic activation. One possible explanation for this discrepancy is that mammalian cells contain cellular factors that suppress pro-DRONC activation by binding its pro-domain. If true, deletion of the pro-domain in ΔN DRONC would then render the caspase no longer inhibitable by such putative factors, resulting in the spontaneous activation of ΔN DRONC and consequent cell death. Cell line-specific variations in levels of such putative inhibitory factors might explain why the efficacy with which pro-DRONC induces cell death is variable amongst different cell types. In this context, it is noteworthy that although pro-DRONC does not induce cell death in Rat-1 cells, it is lethal to NIH 3T3 cells (Dorstyn *et al*., 1999).

As DRONC interacts with drICE, we next assayed the ability of active DRONC to cleave drICE C→A, lamin Dm_O (Gruenbaum *et al*., 1988), the DNA fragmentation factor DREP-1 (Inohara *et al*., 1998) and the baculovirus caspase inhibitor p35 (Figure 2C). Both DRONC and drICE cleaved drICE C→A, lamin Dm_O and DREP-1. The cleavage products generated by DRONC and drICE were clearly different, indicating that DRONC and drICE each cleave lamin Dm_O and DREP-1 at different sites. Unlike drICE, however, DRONC was unable to cleave p35. Together, these results indicate that *dronc* encodes a catalytically active protease and that its unique active site PFCRG pentapeptide confers upon it a different substrate specificity from classical caspases such as drICE that share the QAC(R/Q/G)(G/E) active site pentapeptide consensus.

Ectopic expression of DRONC driven by an eye-specific promoter induces an eye ablation phenotype in Drosophila

To determine whether ectopic expression of DRONC can induce cell death in *D.melanogaster*, we used the GAL4/UAS system to express various forms of DRONC in the developing *Drosophila* compound eye. Independent transgenic *Drosophila* lines were generated carrying *pro-dronc*, ΔN *dronc*, pro-*dronc C→A*, ΔN *dronc C→A* or *dronc-card* (the pro-domain of DRONC on its own) under the control of GAL4-upstream activating sequences (UAS). These flies were then crossed with *Drosophila* strains expressing GAL4 under the control of the *glass* multimer reporter (GMR-*gal4*; Hay *et al*., 1994) in differentiating photoreceptors and pigment cells posterior to the morphogenetic furrow in the eye imaginal disc (Ellis *et al*., 1993). The DRONC-induced phenotypes that we observed were of variable severity, depending on the insertion line



used, presumably because of insertion site-specific effects on the transgene expression level (Spradling and Rubin, 1983). Accordingly, one representative weak UAS-*pro-dronc* (*pro-dronc*^{W}) and one representative strong UAS-*pro-dronc* line (*pro-dronc*^{S}) were selected for further characterization, along with one UAS-Δ*N dronc* line.

Pro-dronc^{W} flies carrying one copy of the transgene exhibited a 'spotted eye' phenotype when crossed with GMR-*gal4* flies: although *pro-dronc*^{W} flies are *white*$^{+}$, and should therefore have red eyes, their eyes appeared white with occasional red spots (Figure 3B). Such eyes have an essentially normal external morphology and size [compare Figure 3A, F and K (control) with B, G and L], in contrast to eyes expressing RPR under the control of GMR, which are severely reduced in size (Figure 3E, J and O). By comparison, *pro-dronc*^{S} and Δ*N dronc* transgenic flies exhibited dramatically 'roughened eyes' that were severely reduced in size (Figure 3C and D). Scanning electron microscopy (SEM) analysis of *pro-dronc*^{S} and Δ*N dronc* eyes confirmed that surface morphology was severely distorted, erupted and rough (Figure 3H and M, and I and N). As with *pro-dronc*^{W} flies, eyes from *pro-dronc*^{S} and Δ*N dronc* flies were white, not red. This consequence of DRONC expression in eyes is particularly intriguing given that expression of RPR dramatically reduces eye size yet has no effect on eye colour (compare Figure 3B–D with E, and L–N with O). The phenotypes induced by DRONC expression are a consequence of DRONC caspase activity since overexpression of catalytically inactive C→A mutants of DRONC exerted no detectable effect on eye development (data not shown).

To investigate in detail the consequences of DRONC expression on the survival of photoreceptor and pigment cells underlying the eye surface, we examined transverse sections of adult transgenic eyes. Surprisingly, even in the *pro-dronc*^{W} flies, no normal cellular structures of either pigment or photoreceptor cells were visible: only remnants of pigment cells and vacuole-like structures remained (compare Figure 3P with Q–S). These remnant pigment cells, containing the red pigment pteridine, were responsible for the red 'spots' observed in the *pro-dronc*^{W} fly eyes (Figure 3B). We therefore conclude that GMR-driven DRONC expression kills both pigment and photoreceptor cells.

Fig. 2. Ectopic expression of DRONC induces cell death in yeast and in mammalian Rat-1 cells. (**A**) Expression of DRONC is toxic to *S.pombe*. For cytotoxicity assays, yeast from two independent colonies were grown to log phase, the OD_{595} of the culture determined and the yeast then plated in serial 10-fold dilutions on selective, inducing media. Western blot analysis with anti-FLAG M2 antibody was used to confirm expression and autoproteolytic cleavage of C-terminally tagged DRONC. (**B**) Transient transfection of *dronc* leads to induction of apoptosis in mammalian Rat-1 fibroblast cells. Various expression constructs were co-transfected with a CMV-*lacZ* reporter plasmid in a ratio of 10:1. At 24 h post-transfection, cells were fixed and examined for β-galactosidase activity. Shown are the percentage of β-galactosidase-positive cells with apoptotic morphology from three independent experiments (mean ± SD). (**C**) DRONC is a cysteine protease that cleaves drICE C→A, lamin Dm_O and DREP-1 but not p35 *in vitro*. *In vitro* translated substrates were incubated with (1) control (no protease added); (2) pro-DRONC C→A purified from yeast; (3) pro-DRONC purified from yeast; and (4) purified bacterially expressed drICE. The unprocessed substrate is indicated by an arrow and the cleavage product is denoted by an asterisk.

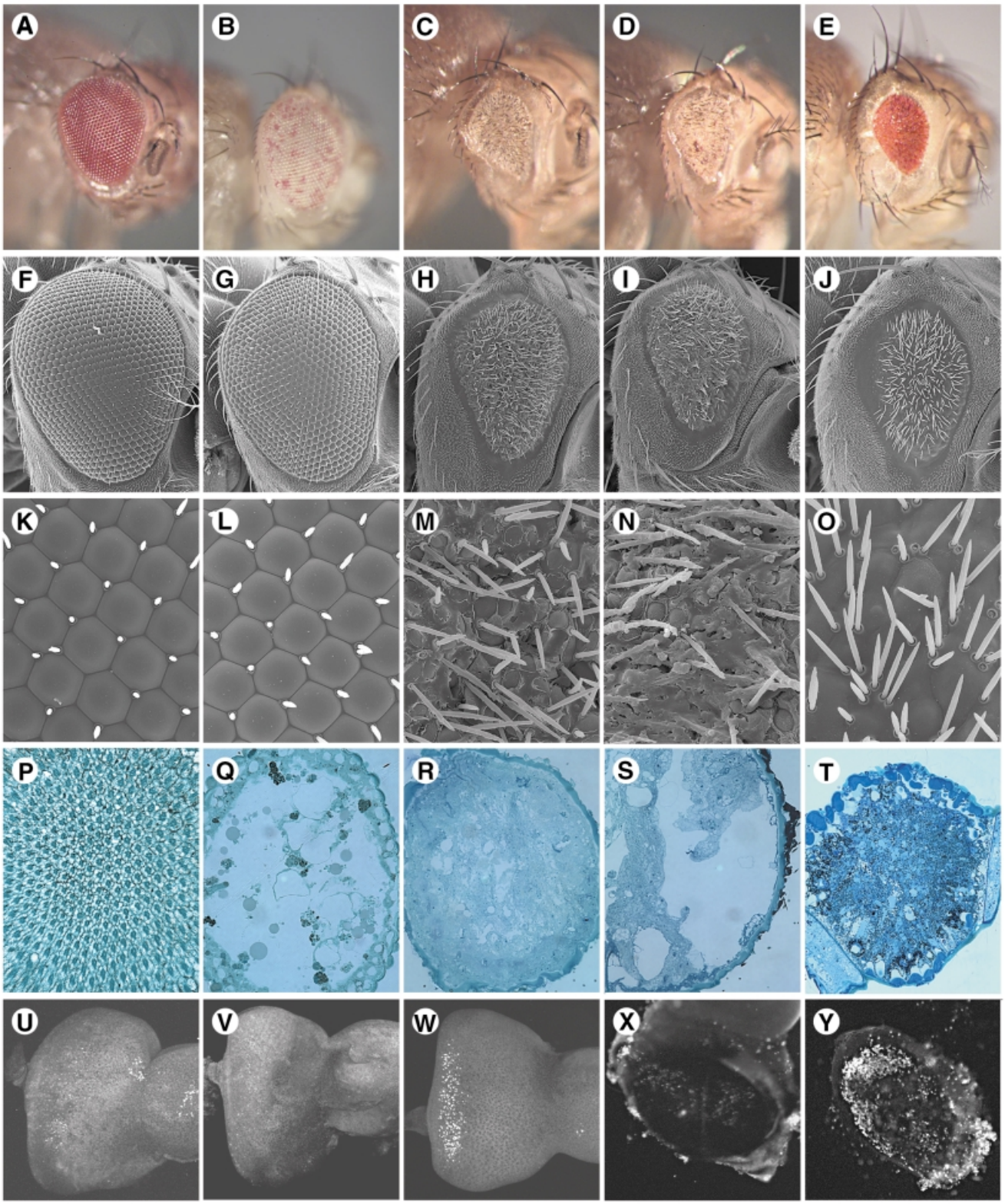


Fig. 3. Ectopic expression of DRONC in the developing eye causes ablation of all retinal structures resulting in a hollow eye. Phenotypes were analysed by light microscopy of whole mounts (A–E), tangential thin sections of adult eyes (P–T), scanning electron microscopy (F–O) and acridine orange staining of eye discs of third instar larvae (U–W) and 60 h after puparium formation (X and Y). (**A**, **F**, **K**, **P**, **U** and **X**) Control flies (+/*GMR-gal4*). (**B**, **G**, **L**, **Q**, **V** and **Y**) The weak *pro-dronc*^{W} transgenic line (GMR-*gal4*/UAS-*pro-dronc*^{W}) displays a spotted eye phenotype (B) with an essentially normal eye morphology on the outside (G and L) but a severely malformed cell arrangement in the interior (Q). (**C**, **H**, **M** and **R**) *Pro-dronc*^{S} transgenic flies that show reduced eye size (C and H) with no defined interior eye structure (R) (GMR-*gal4*/UAS-*pro-dronc*^{S}). (**D**, **I**, **N**, **S** and **W**) Ectopic expression of ΔN DRONC (GMR-*gal4*/UAS-Δ*N dronc*) causes excessive cell death in the eye disc of third instar larvae (W) resulting in a small eye phenotype (D and I). (**E**, **J**, **O** and **T**) GMR-*rpr* flies display eyes of a reduced size (E and J) but unlike *dronc* transgenic fly eyes they are red instead of white (E) (GMR-*rpr*/+). (C, H, M and R) and (D, I, N and S) represent pictures from animals that were crossed with GMR-*gal4* (815, weak) and raised at 18°C. All other images were obtained from animals crossed with GMR-*gal4* (816, strong) and raised at 25°C. In this and the following figures, anterior is to the right and posterior to the left.

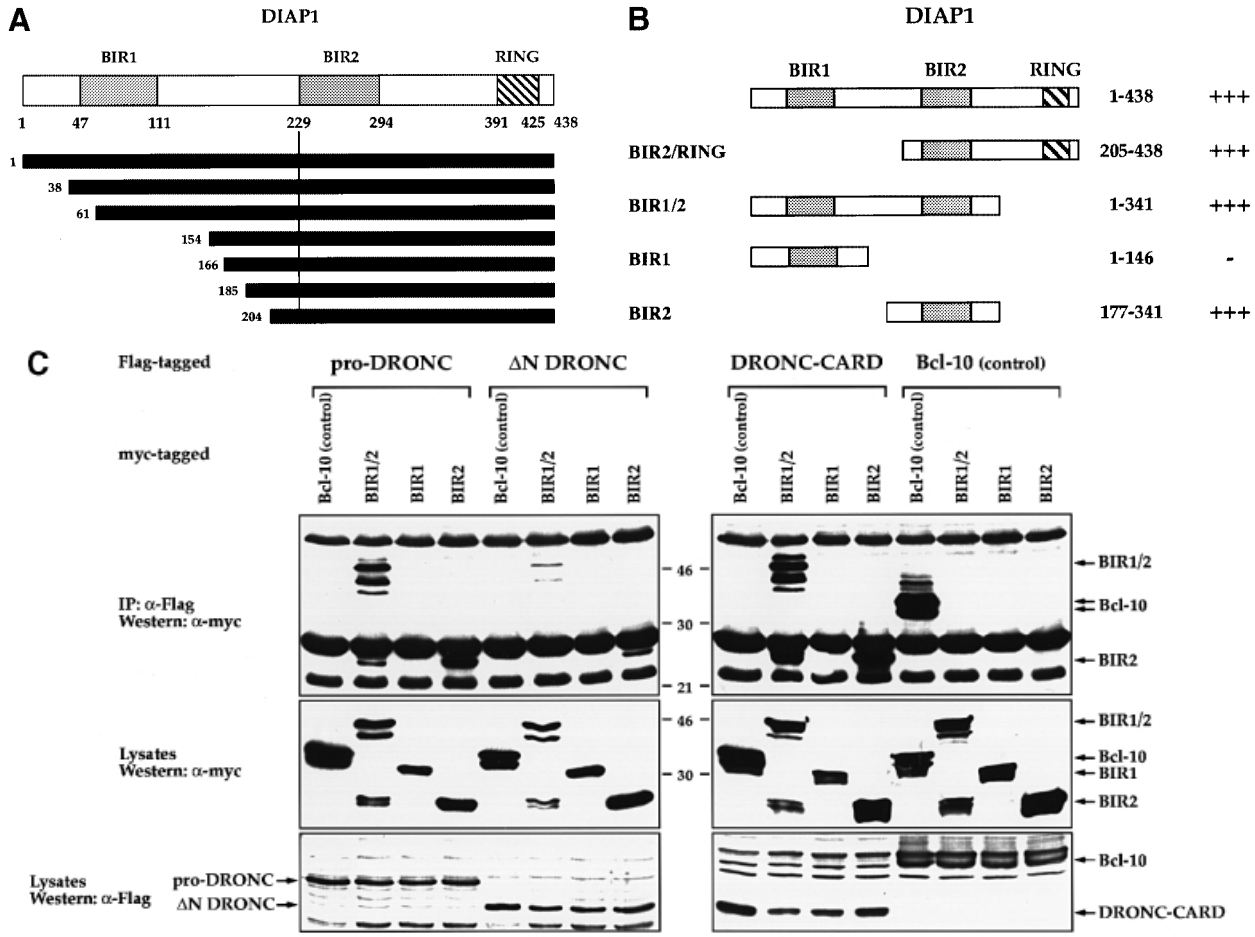
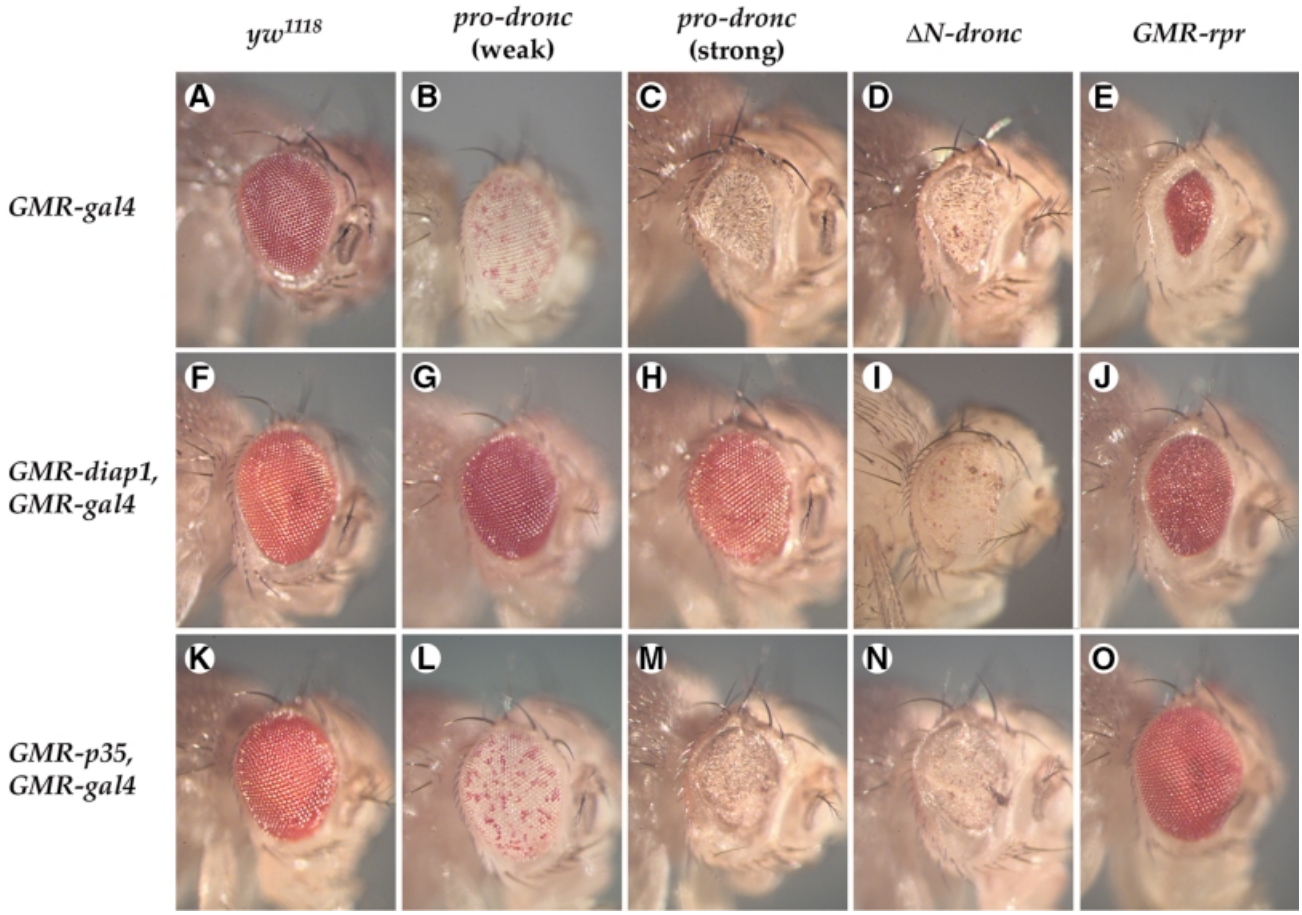


Fig. 4. DIAP1 physically interacts with DRONC. (**A**) Seventeen DRONC-interacting clones encoded full-length and N-terminal truncations of DIAP1 of which seven representative DIAP1 clones are indicated. The positions of the first amino acid of the clones relative to full-length DIAP1 are denoted on the left. (**B**) Various DIAP1 deletion mutants were used in a yeast two-hybrid assay to map the interaction domain between DIAP1 and the pro-domain of DRONC. The BIR2 region of DIAP1 was sufficient for the interaction with the pro-domain of DRONC (**C**) Co-immunoprecipitation of DRONC and DIAP1 from cellular extracts. 293T cells were transiently transfected with plasmids expressing FLAG-tagged DRONC, ΔN DRONC, DRONC-CARD or Bcl-10 (control) and Myc-tagged BIR1/2, BIR1, BIR2 or Bcl-10 in the indicated combinations. Cell lysates were immunoprecipitated with anti-FLAG and immunoblotted with anti-Myc as in Figure 1C. Expression of FLAG-tagged and Myc-tagged proteins was confirmed. Molecular mass markers in kDa are shown.

One possibility is that the ablation of internal eye structures seen in *dronc* transgenic flies may result from excess cell death in the developing eye disc. We therefore examined third instar larval eye discs for the appearance of apoptotic cells using acridine orange, which stains apoptotic cells (Abrams *et al*., 1993). Compared with controls, third larval instar eye discs expressing ΔN DRONC exhibited dramatic and supernumerary apoptosis posterior to the morphogenetic furrow (compare Figure 3U with W). In contrast, no such sign of excessive apoptosis was evident in eye discs from third instar larvae expressing full-length *pro-dronc*^{W} (Figure 3V). However, during later development (60 h after puparium formation), eye discs of *pro-dronc*^{W} pupae exhibited a dramatic increase in numbers of apoptotic cells (compare Figure 3X with Y). It is presumably this very late activation of apoptosis, essentially after the eye lens structure has formed, which gives the eyes of *pro-dronc*^{W} flies their characteristic morphology wherein the eyes show an essentially normal outer structure with internal ablation. In contrast, the devastating 'small eye' phenotype seen in *pro-dronc*^{S}, *ΔN dronc* or GMR-*rpr* transgenic flies (Figure 3C–E) is consistent with the observed induction of cell death much earlier during larval eye development.

The pro-domain-less ΔN DRONC generates a consistently more severe eye ablation phenotype than does pro-DRONC. Indeed, all *ΔN dronc* transgenic lines die when crossed with GMR-*gal4* (816, strong) and maintained at 25°C, although viability of some of these lines can be sustained by crossing them to a weak GMR-*gal4* driver line (815, weak) and maintaining them at 18°C. The lethality is most likely not to be a trivial result of misexpression of GMR-*gal4* in tissues other than the developing eye but, rather, to be due to the inability of ΔN DRONC flies to open the pupae case with their heads because of extreme head malformation. As a consequence, such flies die trapped in their pupae cases. In confirmation of this, we found that flies with severely deformed and black eyes could indeed be rescued by manually opening the puparium at the end of their development (data not shown).



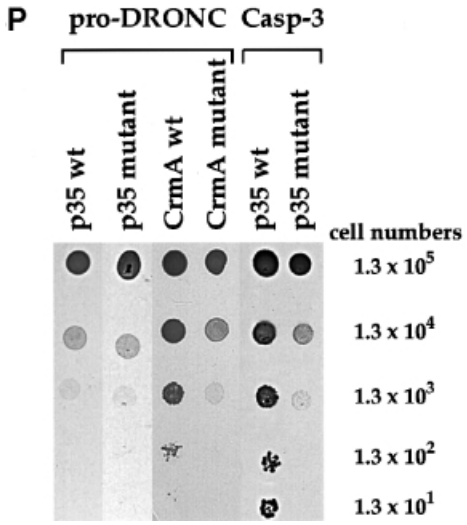


Fig. 5. The eye ablation phenotype caused by ectopic expression of DRONC in the developing eye can be suppressed by co-expression of DIAP1, but not by p35. The effect of overexpressing the different *dronc* constructs or *rpr* alone (**A**–**E**), or in combination with DIAP1 (**F**–**J**) or p35 (**K**–**O**) is shown. (F–J) Ectopic expression of DIAP1 suppresses the eye phenotype caused by pro-DRONC (G and H) but not by ΔN DRONC (I). (K–O) Co-expression of p35 is unable to rescue the eye phenotype caused by pro-DRONC (L and M) or ΔN DRONC (N) overexpression but blocks RPR-induced cell death (O). Flies from *pro-dronc*^{S} and Δ*N dronc* lines were crossed to GMR-*gal4* (815), GMR-*diap1*-GMR-*gal4* (815) or GMR-*p35*-GMR-*gal4* (815) and kept at 18°C. For all other crosses, GMR-*gal4* (816), GMR-*diap1*-GMR-*gal4* (816) or GMR-*p35*-GMR-*gal4* (816) were used and kept at 25°C. (**P**) Expression of p35 fails to suppress DRONC-mediated toxicity in yeast. Vectors to express p35, a non-cleavable p35 mutant (where the caspase recognition motif DQMD has been changed to DQME), CrmA and the CrmA mutant T291R were introduced into *S.pombe* transformed with *pro-dronc* or *caspase-3-lacZ*, respectively. The viability of the resultant transfected yeast cells following induction of DRONC expression was assessed as described above.

The pro-domain of DRONC interacts with DIAP1

The observed difference between the pro-apoptotic activity of pro-DRONC and pro-domain-lacking ΔN DRONC in *Drosophila* and mammalian cells raises the possibility that spontaneous activation of pro-DRONC is suppressed through interaction of its pro-domain with some putative

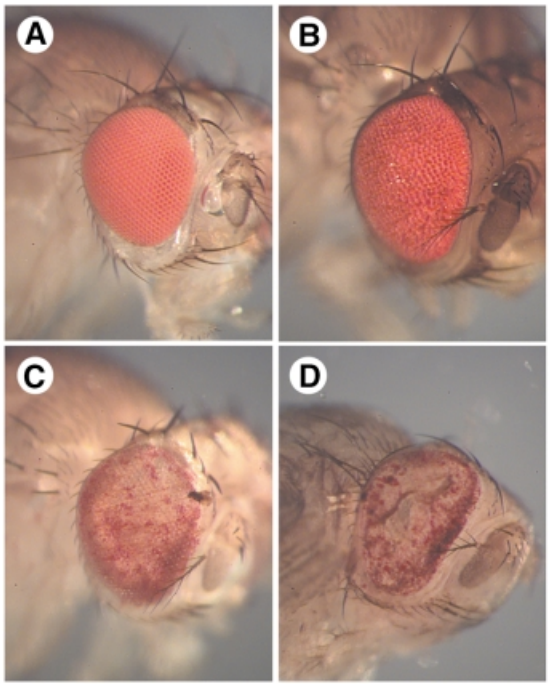


Fig. 6. Heterozygosity at the *diap1* locus bearing the deficiency [Df(3L)th102] enhances the eye phenotype caused by DRONC overexpression. (**A**) Flies with a 50% reduced *diap1* gene dose are viable and show a perfectly normal compound eye [+/SM6; Df(3L)th102/UAS-*pro-dronc*^{W}]. (**B**) Overexpression of GAL4 induces a rough eye phenotype in heterozygous *diap1* flies [+/GMR-*gal4*; Df(3L)th102/TM3]. (**C**) Ectopic expression of *pro-dronc*^{W} induces a spotted eye phenotype (+/GMR-*gal4*; UAS-*pro-dronc*^{W}/TM6c). Note: these flies show a less prominent spotted eye phenotype when compared with the flies shown in Figure 5B due to their different genetic background. (**D**) Flies that express *pro-dronc*^{W} and are heterozygous for *diap1* [Df(3L)th102] display severely deformed eyes and die trapped in their pupae case [+/GMR-*gal4*; Df(3L) th102/UAS-*pro-dronc*^{W}]. All flies were embedded in holocarbon oil and photographed using a stereo-microscope.

cellular inhibitor. To identify such an inhibitor, we searched for *Drosophila* proteins that interact specifically with the DRONC pro-domain in a yeast two-hybrid assay using a 0–24 h *Drosophila* embryonic cDNA library. From 1×10^6 yeast transformants, we recovered 56 DRONC-interacting clones, of which 17 encoded DIAP1 (Figure 4A). The second BIR domain of DIAP1 was necessary and sufficient for the interaction with the pro-domain of DRONC (DRONC-CARD, Figure 4B). This is particularly intriguing since the BIR2 region of DIAP1 is also known to interact physically with, and block the pro-apoptotic activity of, RPR, GRIM and HID (Vucic *et al*., 1997, 1998a,b).

To verify the observed interaction between DIAP1 and DRONC, we performed co-immunoprecipitation experiments on cellular extracts obtained from 293T cells (Figure 4C). FLAG-tagged pro-DRONC C→A, ΔN DRONC C→A and DRONC-CARD (the pro-domain of DRONC on its own) were each tested for interaction with Myc-tagged DIAP1 deletion mutants (BIR1/2, 1–341; BIR1, 1–146; and BIR2, 177–341; see schematic representation in Figure 4B). As expected, full-length DRONC and the isolated pro-domain of DRONC (DRONC-CARD) both co-immunoprecipitated with BIR1/2 and BIR2 but not with BIR1, consistent with our yeast two-hybrid data showing that the BIR2 domain of DIAP1 is required for the interaction with DRONC. Somewhat surprisingly, however, ΔN DRONC lacking the pro-domain also co-immunoprecipitated with DIAP1, although to a far lesser extent than full-length DRONC or DRONC-CARD. The BIR2 region of DIAP1 was required for this interaction between ΔN DRONC and DIAP1 since ΔN DRONC formed stable complexes only with BIR1/2 and BIR2 and not with BIR1. Taken together, these results indicate that DIAP1 physically interacts with unprocessed pro-caspase DRONC and that the BIR2 region of DIAP1 is able to bind both the pro-domain and the core region of DRONC.

Expression of DIAP1 rescues the eye phenotype induced by ectopic expression of pro-DRONC but not ΔN DRONC

To assess the ability of DIAP1 to modulate DRONC activation *in vivo*, we co-expressed DIAP1 with DRONC and ΔN DRONC (Figure 5F–I). Ectopic expression of DIAP1 in the developing eye of *pro-dronc*^{W} transgenic flies completely rescued the phenotype caused by ectopic expression of pro-DRONC (compare Figure 5B with G). Furthermore, GMR-*diap1* also rescued the more severe small eye phenotype of *pro-dronc*^{S} transgenic flies back to a normal eye size (Figure 5H). In contrast, GMR-*diap1* failed to rescue the eye phenotype caused by ΔN DRONC (Figure 5I). However, although GMR-*diap1*/Δ*N dronc* flies still contained a severely distorted eye lacking pigment cells, their eye size was slightly larger than that in Δ*N dronc* transgenic flies. This indicates that DIAP1 does, to some extent, ameliorate the effect of ΔN DRONC (compare Figure 5D with I) and is consistent with our observation that DIAP1 does interact weakly with ΔN DRONC in co-immunoprecipitation analyses (see Figure 4C). Together, these results indicate that the pro-domain and the core region of DRONC are both required for DIAP1 to interact maximally with DRONC and to inhibit DRONC-induced apoptosis. Presumably, DIAP1 binds to unprocessed pro-caspase DRONC and so suppresses its autoproteolytic cleavage and activation.

Heterozygosity at the diap1 locus enhances the eye phenotype caused by pro-DRONC overexpression

The observation that apoptosis in *pro-dronc*^{W} flies occurs relatively late during eye development might indicate that activation of overexpressed pro-DRONC is suppressed, at least in part, through the interaction of its pro-domain with endogenous DIAP1. To validate this hypothesis, we assessed genetically whether endogenous DIAP1 is responsible for the relatively weak eye phenotype observed in *pro-dronc*^{W} flies. *Pro-dronc*^{W} flies were crossed to heterozygous *diap1* flies that carry a deletion in the *thread* [i.e. *diap1,* Df(3L)th102] locus (Figure 6). Whereas heterozygous *diap1* flies were viable and exhibited a normal compound eye, *pro-dronc*^{W} flies with a 50% reduced dosage of the *diap1* gene died trapped in their pupae cases and displayed severely deformed eyes (compare Figure 6A with D). This indicates that a deletion which removes DIAP1 converts the weak eye phenotype caused by ectopic expression of pro-DRONC into a lethal severe eye phenotype. This is consistent with the notion that endogenous DIAP1 negatively regulates pro-DRONC activation and is responsible, at least in part, for the

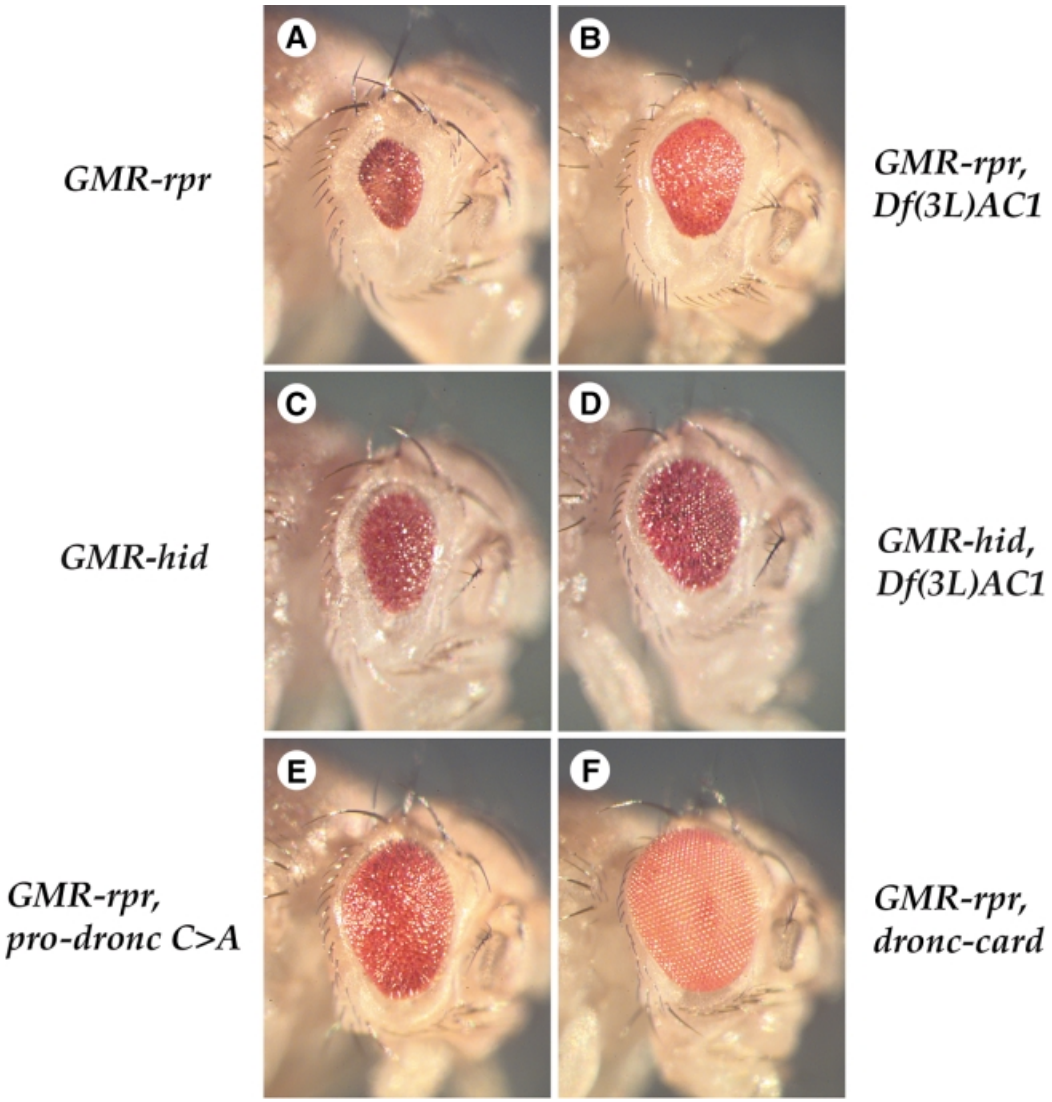


Fig. 7. DRONC is a rate-limiting caspase in the RPR and HID death pathway. (A–D) Cell death induced by RPR and HID is sensitive to *dronc* gene dosage. Flies with a chromosomal deletion that removes the *dronc* locus [Df(3L)AC1] show a suppressed RPR and HID eye phenotype. (**A**) GMR-*rpr*,+; (**B**) GMR-*rpr*,*Df(3L)AC1*; (**C**) GMR-*hid*,+; (**D**) GMR-*hid*,*Df(3L)AC1*. (E and F) The expression of dominant-negative DRONC mutants suppresses the RPR eye phenotype. (**E**) GMR-*rpr*/GMR-*gal4*, UAS-*pro-dronc C→A*; (**F**) GMR-*rpr*/GMR-*gal4*, UAS-*dronc-card*.

relatively late onset of cell death in eyes of *pro-dronc*^{W} transgenic flies.

The baculovirus caspase inhibitor p35 does not rescue the eye phenotype induced by DRONC overexpression

p35 is a promiscuous baculovirus-encoded inhibitor of caspases (Hay *et al*., 1994; Zhou *et al*., 1998). Because the unusual pentapeptide surrounding the active site in DRONC might confer a novel substrate specificity, we were interested in determining whether p35 rescues the eye phenotype of *dronc* transgenic flies. Co-expression of p35 in the developing eye of *pro-dronc*^{W}, *pro-dronc*^{S} or Δ*N dronc* flies failed to rescue the eye phenotype caused by DRONC, although it did, to some extent, ameliorate it (Figure 5K–N). So, for example, p35 slightly increased the eye size of *pro-dronc*^{S} or Δ*N dronc* transgenic flies although all such flies still showed a white and small eye phenotype (Figure 5M and N). In parallel experiments, p35 efficiently blocked RPR-induced cell death and completely rescued the small eye phenotype resulting from RPR overexpression (compare Figure 5E with O). The inability of p35 to rescue the DRONC-mediated phenotype was not due to insufficient levels of p35 expression since the DRONC eye phenotype was not modified by increasing the dosage of p35 in these experiments (data not shown). Furthermore, DRONC-induced cell killing was also not blocked by co-expression of p35 in *S.pombe* (Figure 5P). In contrast to p35, expression of the pox virus caspase inhibitor CrmA, but not the loss-of-function CrmA mutant T291R, did inhibit DRONC-induced cell death in *S.pombe*. We conclude that p35 does not inhibit DRONC appreciably, making DRONC the first identified caspase resistant to inhibition by p35.

DRONC functions in the RPR and HID pathway

The observation that p35 blocks RPR- but not DRONC-induced cell death raises a question over whether DRONC acts in an RPR-dependent or an RPR-independent death pathway. To investigate this question more carefully, we

examined whether RPR-induced cell death is sensitive to *dronc* gene dosage. Because no single gene mutations in *dronc* are currently available, we used mutant flies with a larger chromosomal deletion that includes the *dronc* locus [Df(3L)AC1]. We crossed Df(3L)AC1 to GMR-*rpr* flies and found that flies carrying Df(3L)AC1 show a significant suppression of the RPR eye phenotype (Figure 7A and B). Furthermore, Df(3L)AC1 also suppresses HID-mediated cell killing in the eye (Figure 7C and D). To investigate further whether this observed suppression is due specifically to loss of *dronc*, we assessed whether the expression of dominant-negative DRONC mutants (pro-DRONC C→A and DRONC-CARD) also suppresses the RPR eye phenotype. Pro-DRONC C→A strongly suppressed RPR cell killing, and, surprisingly, the pro-domain of DRONC on its own (DRONC-CARD) completely rescued the RPR eye phenotype (Figure 7E and F). These results, in which DRONC function is ablated either by the Df(3L)AC1 deletion or by the action of dominant-negative DRONC, are consistent with the notion that DRONC is a rate-limiting caspase in the RPR and HID death pathway.

Discussion

Apoptosis is a highly conserved process by which eukaryotic cells commit suicide. In *D.melanogaster*, RPR, GRIM and HID serve as upstream transducers of apoptotic stimuli that induce cell death by triggering caspase activation. Our approach to characterizing the pathways activating one effector caspase, drICE, was to search for proteins that interact with the inactive, unprocessed pro-caspase and that might, therefore, modulate its activation.

Using a yeast two-hybrid screen with pro-drICE as bait, we identified DRONC as a drICE-interacting caspase. DRONC shares homology with members of the caspase family and most closely resembles caspase-9. However, DRONC does not contain a typical caspase active site pentapeptide **Q**AC(R/Q/G) (G/E) but instead has the novel sequence ***PF***CRG (Dorstyn *et al*., 1999). Based on the X-ray crystal structure of human caspase-1, the glutamine at position 1 of the pentapeptide forms part of the substrate-binding pocket (Walker *et al*., 1994; Wilson *et al*., 1994). A change at this position may therefore indicate that DRONC has a different substrate specificity from that of classical caspases. Our finding that the promiscuous caspase inhibitor p35 is neither cleaved by DRONC *in vitro* nor blocks the DRONC activity *in vivo* supports the notion that DRONC has a different substrate specificity. Although the physiological cellular substrate(s) for DRONC have yet to be determined, it may be of note that DRONC cleaves three ascribed caspase substrates, drICE, lamin Dm_O and DREP-1, in an *in vitro* assay.

We have shown that ectopic DRONC action is lethal to yeast, insect and mammalian cells. However, expression of full-length pro-DRONC has a more restricted lethality: although it is toxic to yeast cells, it fails to kill Rat-1 cells and, when expressed in the developing *Drosophila* eye, it generates an unusual phenotype in which the eye exhibits apparently normal outer morphology with internal ablation of all photoreceptor and pigment cells, resulting in a 'hollow' eye. Macroscopically, this manifests as white eyes with occasional red spots, even though these flies are genetically *white*$^+$ and would therefore be expected to have red eyes. This 'spotted eye' results from ablation of most of the internal eye structures apart from a few remnant red pigment cells. Why should the outer morphology of *pro-dronc*^{W} eyes be maintained when virtually all retinal cells are severely affected? Specializations that make the eye a functional organ take place relatively late during pupal development, with rhabdomeres, pigment cells and lens structures differentiating only after pattern formation is complete, after the first third of pupal life. Formation of the compound eye with its ~800 ommatidia depends on the correct three-dimensional structure of the underlying cluster of photoreceptor cells. The preservation of external eye structure we see in *pro-dronc*^{W} eyes indicates that massive cell death occurs only very late, after almost the entire eye development has taken place. This is in contrast to the ablation of eye cells early in development, which is evident in flies expressing RPR or ΔN DRONC and generates small and abnormally shaped eyes. The ability of DRONC to kill both pigment and photoreceptor cells is similar to that of CED-4 and differs from RPR, which seems to be selective for photoreceptor cells and generates eyes that, although hypotrophic, remain red (Kanuka *et al*., 1999).

Previous studies have shown that ectopic expression of the promiscuous baculovirus caspase inhibitor p35 in *D.melanogaster* blocks most of the naturally occurring cell death during development, as well as cell death arising from DNA damage or overexpression of either RPR, GRIM or HID (Hay *et al*., 1994; Grether *et al*., 1995; Chen *et al*., 1996; White *et al*., 1996). This is consistent with studies that indicate that p35 is a promiscuous inhibitor of all known mammalian and invertebrate caspases. It is therefore surprising that p35 proved unable to revert the spotted or small eye phenotype induced by DRONC. Three lines of evidence indicate that DRONC is not inhibited by p35. First, in *Drosophila*, p35 does not suppress the DRONC eye phenotype. This is not simply due to insufficient expression levels of p35 because even two copies of GMR-*p35* are still unable to suppress DRONC-induced cell death. Secondly, DRONC-induced cell death in *S.pombe* is not inhibited by p35. In contrast, p35 strongly suppressed caspase-3-induced cell death in yeast in parallel experiments. It is of note that the pox virus caspase inhibitor CrmA does block DRONC-induced cell death and is an effective inhibitor of DRONC. Thirdly, p35 must be proteolytically processed in order to inhibit caspases. However, DRONC does not cleave p35 in an *in vitro* cleavage assay, indicating that p35 is not a substrate for DRONC.

The fact that p35 completely rescues the phenotype caused by ectopic expression of either RPR, GRIM or HID yet only slightly ameliorates DRONC eyes raises the possibility that DRONC is not part of the RPR, GRIM or HID pathway but instead functions in an independent death signalling system. However, several lines of evidence argue that *dronc* is an important effector of the pro-apoptotic proteins RPR and HID. Specifically, heterozygosity at the *dronc* locus significantly suppresses the eye ablation induced by RPR and HID, and expression of dominant-negative DRONC mutants suppresses RPR eyes. Strikingly, expression of the DRONC-CARD alone completely suppresses the RPR eye phenotype, indicating that

CARD-containing proteins are critically involved in the apoptotic signal transduction initiated by RPR. Nonetheless, although we favour the notion that DRONC is an effector of RPR and HID, it is formally possible that DRONC functions in a pathway that is additive to, but independent of, RPR and HID. We currently cannot distinguish between these two alternatives.

If RPR and HID function through DRONC, why should p35 block RPR- and HID-, but not DRONC-induced death? During the apoptotic process, caspases are activated in an amplifying proteolytic cascade, cleaving one another in turn. Thus, it may be that the activation of endogenous DRONC by RPR and HID is insufficient to induce cell death on its own but requires amplification of the apoptotic signal through the activation of other caspases such as drICE, DCP-1 or even DCP-2/DREDD. In contrast, when DRONC is overexpressed, this amplifying proteolytic cascade may not be required to kill the cell. If the downstream caspases were p35 sensitive, this could explain why p35 ameliorates, yet cannot block, DRONC killing. Interestingly, recent studies on DREDD indicate that a p35-resistant caspase (or some other class of protease) is indeed responsible for proteolytic cleavage and activation of DREDD following overexpression of RPR, GRIM or HID (Chen *et al*., 1998). The initial cleavage and activation of DREDD is not blocked by p35 although p35 does block the eventual cell death that would otherwise result. This must mean that a p35-resistant caspase is activated following RPR, GRIM or HID induction but that its activation does not lead to cell death in the presence of p35. Given its resistance to p35, this makes DRONC an intriguing candidate for such a 'DREDD-activating caspase'.

Several lines of evidence suggest that pro-DRONC activation is negatively regulated via its pro-domain. This is best illustrated by the biology underlying the relatively weak eye phenotype in flies expressing full length *pro-dronc*^{W}. First, most UAS-*pro-dronc* lines are viable when crossed to a strong GMR-*gal4* line and kept at 25°C: in contrast, virtually all GMR-driven Δ*N dronc* transgenic lines tested die under such conditions. Secondly, most *pro-dronc* lines exhibit essentially normal outer eye structure, whereas rare surviving Δ*N dronc* transgenic flies never display this 'weak' eye phenotype and have severely deformed eyes. Thirdly, ectopic expression of pro-DRONC induces no significant increase of cell death in the eye discs of third instar larvae, whereas excessive cell death is evident posterior to the morphogenetic furrow in the eye discs of third instar larvae expressing ΔN DRONC. Finally, ectopic expression of DRONC in mammalian Rat-1 cells induces apoptosis only when its pro-domain had been removed, suggesting the existence of an innate inhibitor of DRONC activation acting through the DRONC-CARD domain. All of these observations implicate the DRONC pro-domain in repressing activation of the caspase and suggest that DRONC activation is kept in abeyance in metazoan cells through the action of some CARD-binding innate inhibitor. In contrast, our studies of DRONC in *S.pombe* unambiguously show that isolated pro-DRONC is, by itself, perfectly capable of undergoing catalytic autoprocessing resulting in its activation. Indeed, in yeast, pro-DRONC proved more toxic than ΔN DRONC, suggesting that the presence of the pro-domain may actually enhance DRONC activation in the absence of other modulating influences.

In insect cells, a candidate for such an innate DRONC repressor is the inhibitor of apoptosis, DIAP1, which we have shown to interact with the DRONC pro-domain: co-expression of DIAP1 completely reverts the eye ablation phenotype of *pro-dronc*^{W} flies, whereas the eye ablation phenotype induced by ΔN DRONC is largely unaffected. If endogenous DIAP1, or an analogue, were expressed in the *Drosophila* eye until very late in its development, this would provide the requisite mechanism for holding the activity of DRONC in abeyance until very late, so generating the 'spotted eye' phenotype we observe. Indeed, heterozygosity at the *diap1* locus greatly enhances the eye phenotype induced by pro-DRONC overexpression, indicating that endogenous DIAP1 negatively regulates DRONC activation *in vivo*. This is analogous to the way in which c-IAP1, c-IAP2 and XIAP bind to, and inhibit activation of, the pro-form of the apical caspase-9 in mammalian cells (Deveraux *et al*., 1998). The notion that it is DIAP1, in particular, that most likely fulfils the role of *in vivo* suppressor of DRONC is reinforced by our studies in yeast which show that whilst DIAP1 both interacts with, and protects from the lethal effects of, pro-DRONC, *Drosophila* DIAP2 and the mammalian IAP homologues MIHA, MIHB, MIHC, MIHD and XIAP offer no such protection (data not shown).

Currently, very little is known about how IAPs suppress apoptosis, although the most convincing biological evidence for the ability of IAPs to regulate cell death comes from genetic studies in *D.melanogaster* (Hay *et al*., 1995; Wang *et al*., 1999; Goyal *et al.*, 2000). Deletion of the chromosomal region encoding DIAP1 enhances cell death induced by ectopic expression of RPR, and genetic loss of DIAP1 function leads to early and widespread apoptosis, indicating that DIAP1 is essential for survival of many cell types (Wang *et al*., 1999; Goyal *et al*., 2000). Furthermore, overexpression of DIAP1 suppresses cell death induced by either RPR, GRIM or HID through direct interaction between these various pro-apoptotic proteins and the second BIR domain of DIAP1 (Harvey *et al*., 1997; Vucic *et al*., 1997, 1998a,b), the same BIR domain that is sufficient for its interaction with pro-DRONC. It is noteworthy that it is also the second BIR repeat of the mammalian IAP family members c-IAP1, c-IAP2 and XIAP that appears sufficient for their anti-apoptotic activity (Deveraux *et al*., 1997; Roy *et al*., 1997; Takahashi *et al*., 1998).

Our finding that DIAP1 directly binds to and inhibits cell death caused by ectopic expression of DRONC, as well as by RPR, GRIM and HID, underscores the key role played by DIAP1 in the regulation of apoptosis in *D.melanogaster* and raises the possibility that RPR, HID or GRIM may exert some, or all, of their pro-apoptotic action through displacement of DIAP1 from the pro-domain of DRONC, so allowing activation of the caspase and consequent cell death (Figure 8). The isolation of DIAP1 mutants that display greatly reduced binding for RPR, HID and GRIM and significantly suppress RPR, HID and GRIM cell killing strongly supports this idea (Goyal *et al*., 2000). According to this model, IAPs function as 'guardians' of the apoptotic machinery, which act to suppress the chance of spontaneous activation of

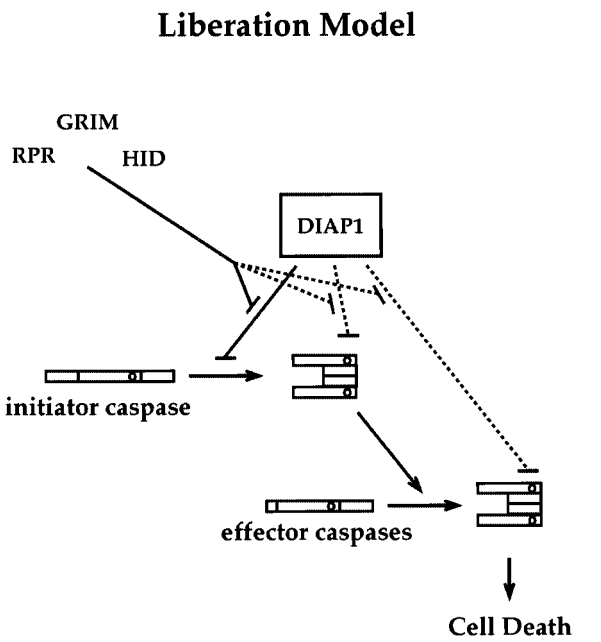


Fig. 8. The observation that DIAP1 interacts with the pro-caspase DRONC as well as with apoptotic inducers such as RPR, GRIM and HID places DIAP1 in a potentially pivotal position to regulate apoptosis. In the proposed model, DIAP1 functions as 'guardian of the caspase machinery' by binding to and suppressing spontaneous pro-caspase activation in the absence of RPR, GRIM and active HID. As indicated by studies using heterologous systems, DIAP1 may also act by directly inhibiting the proteolytic activity of spontaneously activated caspases (dotted lines) (Kaiser *et al*., 1998; Hawkins *et al*., 1999). According to the 'liberation model', RPR, GRIM or HID exert some, or all, of their pro-apoptotic action by liberating initiator caspases, such as DRONC, from the activation-inhibitory effect of DIAP1. This displacement of DIAP1 from DRONC could then result in the activation of DRONC through DARK, the *Drosophila* homologue of Apaf-1/CED-4 (Rodriguez *et al*., 1999).

the intrinsic cell death machinery by neutralizing pro-apoptotic caspases, so establishing a buffered threshold that must be either exceeded or neutralized in order to initiate the destruction of a cell.

Materials and methods

Yeast two-hybrid assay and cloning of dronc

The catalytically inactive mutant of drICE (drICE C→A, described in Fraser and Evan, 1997) was cloned into the pAS2.1 vector (Clontech) and used as bait in a yeast two-hybrid screen against a 0–24 h *D.melanogaster* embryonic cDNA library constructed in pACT2 (kindly provided by S.J.Elledge) following the Matchmaker Two-Hybrid System protocol (Clontech). Three *drICE* cDNA plasmids, encoding amino acids 1–339, 40–339 and 43–339, respectively, and one cDNA encoding amino acids 104–451 of DRONC (pACT2-Δ*N dronc*) were isolated. A BLAST search of the GenBank DNA sequence database identified an expressed sequence tag identical to *dronc*. The corresponding full-length clone (LD28292) was obtained and was used to generate all subsequent *dronc* constructs. pAS2.1-*drICE C→A*, pAS2.1-Δ*N drICE*, *pAS2.1-BIR1/2*, *pAS2.1-BIR1* and *pAS2.1-BIR2* were generated by PCR. All constructs were verified by DNA sequencing.

Cytotoxicity assay in S.pombe

All transformations of *S.pombe* were performed using a standard lithium acetate protocol and cells were then plated on selective medium. All clones were maintained throughout on selective media. Yeast was grown to log phase, the OD_{595} of the culture was determined, and the yeast was then plated in serial 10-fold dilutions on selective and inducing media. DNA fragments containing *pro-dronc*, *ΔN dronc*, *pro-dronc C→A* or *ΔN dronc C→A* were cloned into the *S.pombe* expression vector pNeu. The vector pNeu and the *caspase-3-lacZ* construct have been described (Ekert *et al*., 1999). The mutant p35 was created by changing the caspase cleavage site from DQMD to DQME. All constructs were verified by restriction digest and sequencing.

Transient transfection of Rat-1 cells and apoptosis assay

Transient transfections of Rat-1 cells were performed using the FuGENE transfection reagent according to the manufacturer's instructions (Roche). CMV-*lacZ* reporter DNA was co-transfected with pTracer-CMV2-based constructs (pT, Invitrogen) in a ratio of 1:10. At 24 h post-transfection, cells were assayed for β-galactosidase (β-Gal) activity and cell viability was determined based on the morphology of at least 400 β-Gal-positive cells. pT-*FADD* and pT-*caspase-8* were used as positive controls for apoptosis induction. *Pro-dronc*, ΔN *dronc* and ΔN *dronc C→A* were amplified by PCR and cloned into pTracer plasmids. The catalytically inactive mutants *pro-dronc C→A* and ΔN *dronc C→A* were generated using the Altered Sites II *in vitro* Mutagenesis System according to the manufacturer's instructions (Promega). All constructs were verified by sequencing.

Immunoprecipitation and immunoblot analysis

293T cells were transiently transfected using Superfect according to the manufacturer's instructions (Qiagen). Equal amounts of FLAG- and Myc-based pcDNA3.1 construct were used in the transfection experiments. At 48 h post-transfection, cells were harvested and lysed in ice-cold lysis buffer. Aliquots of cell lysates were incubated for 2 h at 4°C with M2 anti-FLAG monoclonal antibody resin (Sigma). Co-immunoprecipitated proteins were analysed by immunoblot analysis using the anti-Myc monoclonal antibody 9E10.

P-element-mediated germline transformation and transgene analysis

Transgenic flies were generated by microinjecting pUAST-*pro-dronc*, pUAST-Δ*N dronc*, pUAST-*pro-dronc C→A* and pUAST-*CARD-only* (the pro-domain of DRONC-only) DNA according to standard protocols. Several independent lines were established from each transgene, and their integration sites were mapped to individual chromosomes. Sixteen pUAST-*pro-dronc*, seven pUAST-Δ*N dronc*, eleven pUAST- *pro-dronc C→A* and four pUAST-*dronc-card* independent transgenic lines were obtained. For ectopic expression of the various transgenes, *dronc* transgenic flies were crossed with the GAL4 lines: GMR-*gal4* (816, strong) and GMR-*gal4* (815, weak). To assess the effect of DIAP1 or p35 overexpression on the DRONC-mediated phenotype, GMR-*gal4* 815 and 816 were recombined onto the chromosome bearing GMR-*diap1* or GMR-*p35* to generate GMR-*gal4*-GMR-*diap1* and GMR-*gal4*-GMR-*p35*, respectively. GMR-*rpr* and GMR-*hid* were obtained from M.Grether and H.Steller; GMR-*p35* and GMR-*diap1* were obtained from B.Hay and G.Rubin. In addition, the following strains were used: 55100 (Df(3L)th102, h[1] kni[ri-1] e[s]/TM6C, cu[1] Sb[1] e[1] ca[1]), Df(3L)AC1 (with the breakpoints 67A2;67D13), 5248 (P{w+mC= GMR-hid}SS1, y1 w* P{ry+t7.2=neoFRT}19A; P{w+m*=GAL4-ey.H}SS5, P{w+mC=UAS-FLP1.D}JD2).

Histology and scanning electron microscopy

Histological sections were prepared and analysed as described in Basler *et al*. (1991). Third instar larval eye discs were stained with acridine orange as described in Abrams *et al*. (1993). The cytological map position for *dronc* was determined according to standard protocols using a biotinylated *dronc* probe (Ashburner, 1989). Flies were prepared for SEM as described in Kimmel *et al*. (1990).

Purification of processed DRONC and protease assays

FLAG-tagged versions of DRONC and DRONC C→A were expressed using the *S.pombe* system described above. At 15 h post-induction of DRONC expression, DRONC and DRONC C→A were isolated from yeast extracts by immunoprecipitation using anti-FLAG M2 agarose affinity gel (Sigma). Protease assays were performed as described in Chen *et al*. (1998). cDNAs encoding drICE C→A, lamin Dm_O, p35 and DREP-1 were cloned into pBluescript (Stratagene) and *in vitro* transcribed/translated using the reticulocyte lysate TnT system (Promega) in the presence of [^{35}S]methionine (Amersham).

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