

The two cytochrome *c* species, DC3 and DC4, are not required for caspase activation and apoptosis in *Drosophila* cells

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In *Drosophila*, activation of the apical caspase DRONC requires the apoptotic protease-activating factor homologue, DARK. However, unlike caspase activation in mammals, DRONC activation is not accompanied by the release of cytochrome *c* from mitochondria. *Drosophila* encodes two cytochrome *c* proteins, *Cytc-p* (DC4) the predominantly expressed species, and *Cytc-d* (DC3), which is implicated in caspase activation during spermatogenesis. Here, we report that silencing expression of either or both DC3 and DC4 had no effect on apoptosis or

activation of DRONC and DRICE in *Drosophila* cells. We find that loss of function mutations in *dc3* and *dc4*, do not affect caspase activation during *Drosophila* development and that ectopic expression of DC3 or DC4 in *Drosophila* cells does not induce caspase activation. In cell-free studies, recombinant DC3 or DC4 failed to activate caspases in *Drosophila* cell lysates, but remarkably induced caspase activation in extracts from human cells. Overall, our results argue that DARK-mediated DRONC activation occurs independently of cytochrome *c*.

Introduction

Apoptosis is a highly conserved process of cell suicide that is mediated by a family of proteases known as caspases. In mammalian cells, activation of caspases can occur through two pathways (for review see Degterev et al., 2003). The death receptor pathway involves ligation of TNF family of death receptors, which results in recruitment of caspase-8 to a death-inducing signaling complex, which in turn, induces activation of caspase-8. Active caspase-8 then activates caspase-3 and other caspases by proteolytic processing. The intrinsic death pathway is induced by stimuli such as stress, UV, or cytotoxic drugs and leads to permeabilization of the mitochondrial membrane, resulting in the release of various mitochondrial proteins, such as cytochrome *c*, into the cytosol.

In mammals, cytochrome *c* triggers the activation of the apoptotic protease-activating factor (Apaf-1), enabling Apaf-1 to recruit and activate caspase-9 through a mechanism that is not completely understood (Li et al., 1997; Rodriguez and Lazebnik, 1999; Zou et al., 1999). The complex of Apaf-1, cytochrome *c* and active caspase-9, which is referred to as the apoptosome, induces the proteolytically processing and activation of caspase-3, the protease responsible for the majority

of morphological changes associated with apoptosis (Degterev et al., 2003).

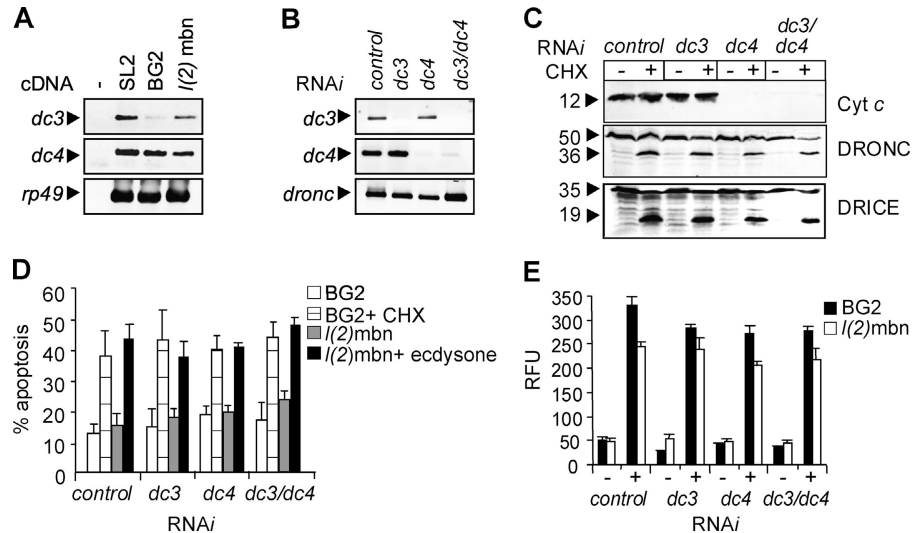
Similar to mammals, the *Drosophila* Apaf-1 homologue DARK, is required for activation of the *Drosophila* caspase-9 homologue DRONC, and apoptosis (for reviews see Kumar and Dumanis, 2000; Kumar, 2004). Like Apaf-1, DARK contains a series of WD repeats, which are the domains in Apaf-1 that interact with cytochrome *c* (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999). One might expect that DARK, like Apaf-1, would require cytochrome *c* for its activation. However, whether this is true is unclear. Addition of mammalian cytochrome *c* to *Drosophila* embryonic extracts, only increased total caspase activity twofold (Kanuka et al., 1999) or had no effect on caspase activation (Varkey et al., 1999). Also, cytochrome *c* is not released into cytosol of *Drosophila* cells during apoptosis, even though DRONC is activated (Varkey et al., 1999; Dorstyn et al., 2002). These findings led to the proposal that cytochrome *c* undergoes a conformational change on the surface of the mitochondrial membrane, and that this change is required and sufficient for the activation of DARK (Varkey et al., 1999). However, silencing the expression of the cytochrome *c* proteins had no effect on apoptosis of *Drosophila* SL2 cells (Zimmermann et al., 2002). Given that apoptosis can proceed in the absence of cytochrome *c* proteins, these results suggest that cytochrome *c* is not important for cell death or caspase activation in *Drosophila* cells.

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Abbreviations used in this paper: Apaf-1, apoptotic protease-activating factor; dsRNA, double-stranded RNA; RNAi; RNA interference.

Figure 1. **DC3 and DC4 are not required for caspase activation and apoptosis.** (A) Endogenous expression of *dc3* and *dc4* transcripts in *Drosophila* cell lines as detected by RT-PCR.

(B) BG2 cells were treated with dsRNAs for *dc3*, *dc4*, or both *dc3* and *dc4*, and ablation of the target genes assessed by RT-PCR. Amplification of a 405-bp *dronc* region was used as a control. (C) BG2 cells treated with dsRNA to *dc3*, *dc4* or both, were exposed to cycloheximide (CHX) or were left untreated. Absence of DC4 protein was confirmed by immunoblotting with cytochrome *c* antibody. Processing of DRONC and DRICE was detected by immunoblotting. Relative molecular masses of the proteins in kilodaltons are shown. (D) Apoptosis induced in BG2 cells by CHX (10 μ g/ml for 6 h), or *l(2)mbn* cells by ecdysone (10 μ M for 24 h) treatment was estimated by scoring cells with condensed nuclei by DAPI staining. Data (mean \pm SEM) were derived from four independent experiments. (E) Caspase activity, presented as relative fluorescence units (RFU), was determined on DEVD-amc substrate in BG2 or *l(2)mbn* cells treated (+) with CHX (10 μ g/ml for 6 h) or ecdysone (10 μ M for 24 h), respectively, or left untreated (-). Data (mean \pm SEM) were derived from three experiments.



Drosophila encodes two cytochrome *c* proteins, DC3 and DC4 (Limbach and Wu, 1985). DC4 has highest homology to cytochrome *c* from other species, and its expression is abundant but varies in different tissues with stage of development. DC3 is ubiquitously expressed during development but at much lower levels (Limbach and Wu, 1985). DC3 has been implicated in the activation of caspases during spermatid individualization in *Drosophila* (Arama et al., 2003). These observations raise the possibility that DC3 can function as an activator of DARK at some stages of *Drosophila* development. Here, we have investigated whether either DC3 or DC4 proteins are required for activation of caspases in *Drosophila*, and if they can induce caspase activation in a cell-free system. Our data indicate that both cytochrome *c* species are not required for caspase activation in *Drosophila* development and in *Drosophila* cells, even though they can induce activation of Apaf-1.

Results and discussion

DC3 and DC4 are not required for caspase activation in *Drosophila* cells

To compare the expression of *dc3* and *dc4* in *Drosophila* cell lines, we used RT-PCR analysis and found that *dc3* is expressed at lower levels in BG2, SL2 and *l(2)mbn* cells, compared with *dc4* (Fig. 1 A). Expression of *dc3* was the lowest in BG2 cells. To test whether DC3 or DC4 are required for the activation of DRONC or DRICE, we silenced the expression of *dc3* and *dc4* by RNA interference (RNAi) in *Drosophila* BG2 cells, with double-stranded RNAs (dsRNAs) to each gene. The ablation was confirmed by RT-PCR for both *dc3* and *dc4* (Fig. 1 B) and by immunoblotting for DC4 (Fig. 1 C). Silencing both *dc3* and *dc4* in BG2 cell had no effect on the processing of DRONC and DRICE after induction of apoptosis by cycloheximide (Fig. 1 C), the rate of cell death (Fig. 1 D), or caspase activity (Fig. 1 E). We also found that silencing *dc3* and *dc4* did not affect ecdysone-induced apoptosis (Fig. 1 D) or caspase ac-

tivity (Fig. 1 E) in *Drosophila l(2)mbn* cells. These observations are consistent with previous studies (Zimmermann et al., 2002), which demonstrated that silencing expression of cytochrome *c* proteins in SL2 cells had no effect on apoptosis induced by UV, actinomycin D, or ectopic expression of Reaper or Grim. These results clearly indicate that DC3 and DC4, are not essential for caspase activation and apoptosis in *Drosophila* cells.

DRONC processing is not enhanced by overexpression of DC3 or DC4

Although *Drosophila* cytochrome *c* proteins are not required for caspase activation and apoptosis, it is possible that they can induce caspase activation but can be substituted by some redundant activity. We tested this possibility in cultured cells and in cell-free systems. One approach was to test whether overexpression of the cytochromes in BG2 cells had any effect on cell death. We generated BG2 cell lines that expressed DC3 or DC4 protein fused with the HA epitope tag and confirmed expression of proteins by immunoblotting (Fig. 2 A). Ectopic expression of DC3 or DC4 did not induce processing of DRONC or DRICE. When these cells were treated with cycloheximide, we found that cells expressing exogenous DC3 or DC4 underwent apoptosis at the same rate as the parental cells (Fig. 2 B) and showed caspase activity comparable to controls (Fig. 2 C).

We had demonstrated previously that cytochrome *c* is not released from mitochondria in *Drosophila* cells (Dorstyn et al., 2002). The failure to observe cytochrome *c* release could be interpreted as a failure of cytochrome *c* to leave mitochondria or a failure to detect the released protein. We decided to take advantage of the cell lines that ectopically expressed DC3 or DC4 to test whether these proteins, particularly DC3 which is not normally abundant in BG2 cells, are released during apoptosis. We treated cells with cycloheximide and fractionated them by differential centrifugation at various times after treatment. We found

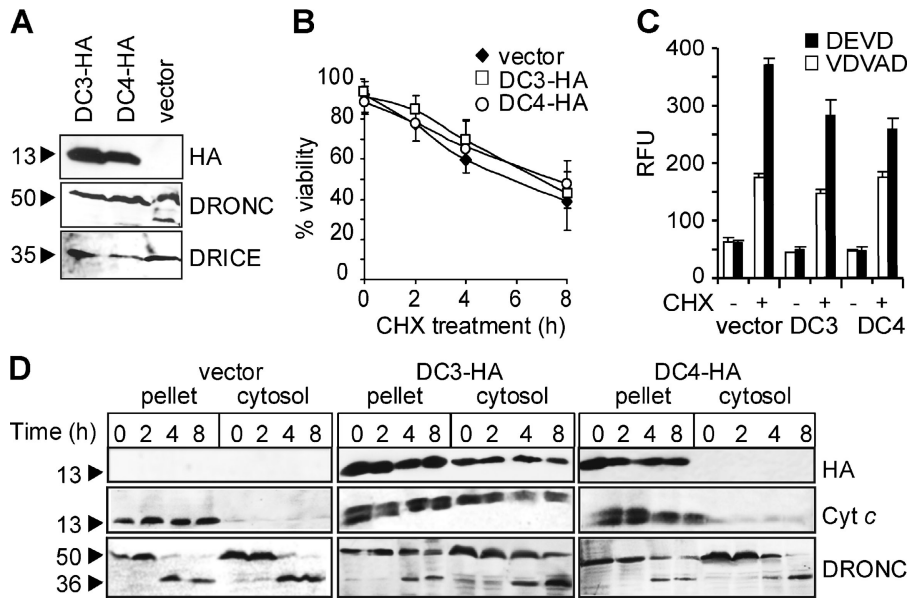


Figure 2. DC3 or DC4 expression does not affect cell death or DRONC processing. (A) BG2 cells were transfected with DC3-HA or DC4-HA and protein expression confirmed by immunoblotting with HA antibody. Immunoblotting for DRONC and DRICE showed a lack of processing of caspases in cells overexpressing DC3 or DC4. (B) DC3-HA or DC4-HA transfected BG2 cells were treated with cycloheximide for the indicated times and cell death was determined by Trypan blue staining and is represented as mean \pm SEM from three independent experiments. (C) Caspase activity in untreated (-) or CHX-treated (+) BG2 cells overexpressing DC3 or DC4. Data (mean \pm SEM) derived from three experiments. (D) Cells were fractionated by differential centrifugation to separate heavy membrane (pellet, containing mitochondria) and cytosol. Fractions were immunoblotted for HA, cytochrome c, or DRONC, as indicated. Cytochrome c antibody detects both transfected and endogenous protein as seen by a doublet. Relative molecular masses of the proteins in kilodaltons are shown.

the majority of both DC3 and DC4 were in the heavy membrane fraction that contained mitochondria (Fig. 2 D). Interestingly, DC3, but not DC4 was also detectable in the cytosolic fraction of both normal and apoptotic cells. Both DC3 and DC4 were detectable in the light membrane fractions comprising Golgi and ER, but there was no significant change in protein levels after treatment (unpublished data). It is unclear why DC3 is present in

cytosol in healthy cells. This may simply be an artifact of overexpression, or likely represents a portion of apocytochrome c that is unable to enter the mitochondria. However, it is clear that the presence of DC3 in cytosol does not effect DRONC activation or apoptosis. DRICE activation was also unaffected in these cells (unpublished data). We conclude that although DC3 and DC4 are not released into the cytoplasm during apoptosis, DC3

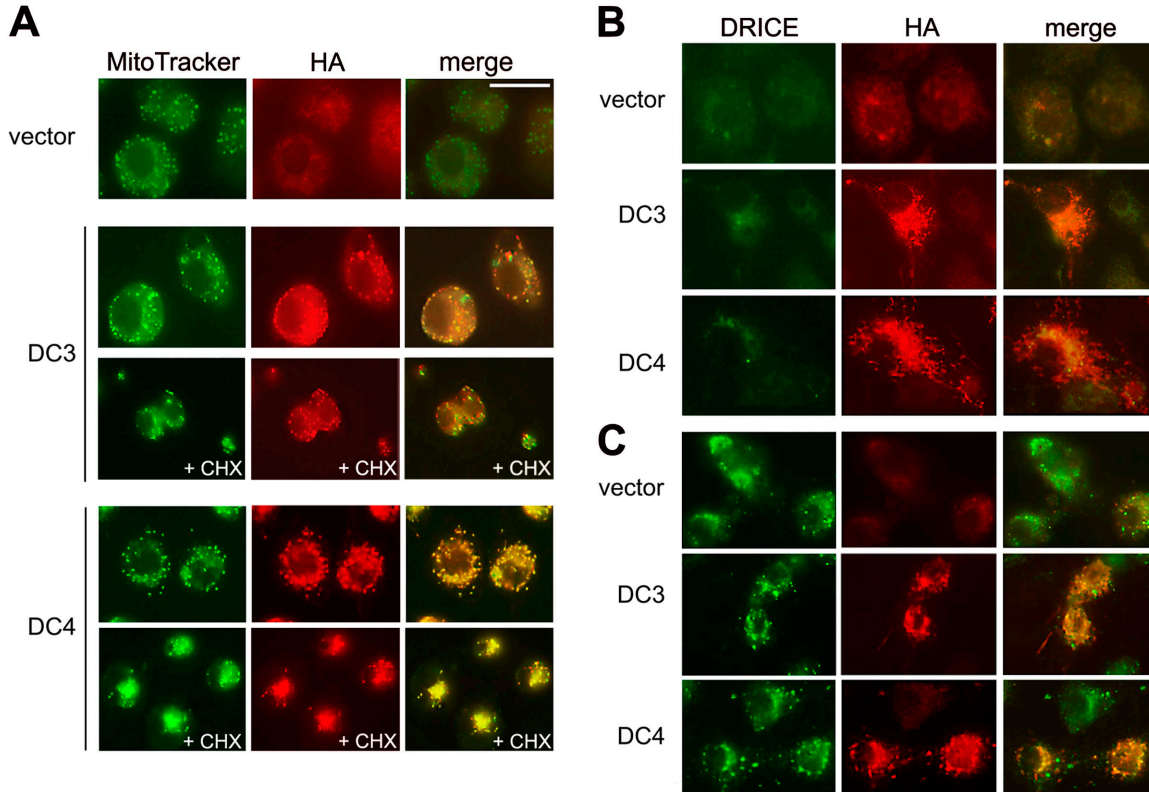


Figure 3. Localization of DC3 and DC4 in healthy and apoptotic cells. (A) Vector, *dc3-HA*, or *dc4-HA*-transfected BG2 cells were treated with CHX for 4 h where indicated and costained with MitoTracker green and anti-HA antibody. (B) Untreated or (C) CHX treated BG2 cells transfected with vector alone (top), *dc3-HA* (middle), or *dc4-HA* (bottom) were costained with anti-DRICE and anti-HA antibodies. The last column displays merged images showing costaining of DC3 and DC4 with mitochondria (A) and partial colocalization with DRICE (C). Bar, 5 μ m.

possibly resides in both cytoplasm and mitochondria of *Drosophila* cells but cannot induce caspase activation and apoptosis.

To verify the results obtained by cell fractionation, we assessed the localization of DC3 and DC4 in these BG2 cell lines. We found that the majority of DC3 and DC4 protein colocalize with MitoTracker, in both live and apoptotic cells (Fig. 3 A), indicating that these proteins remain in the mitochondria during apoptosis. A portion of DC3 was detectable outside of mitochondria in live and apoptotic cells (Fig. 3 A) consistent with its detection in light membrane and cytosol fractions (Fig. 2 D). We used an antibody that detects processed DRICE protein and confirmed that ectopic expression of DC3 or DC4 did not cause DRICE activation. Consistent with our previous observations (Dorstyn et al., 2002) we also found that in cells treated with cycloheximide, active DRICE partially colocalized with mitochondria (Fig. 3 C). However, considering that neither of the cytochromes are required for DRICE activation, the observed association may be unrelated to caspase activation.

Recombinant DC3 and DC4 do not promote caspase activation in cell extracts

We set out to test whether DC3 or DC4 can induce caspase activation in a cell-free system. Recombinant DC3 and DC4 were generated by coexpressing these proteins with haem lyase in *E. coli*. The absorbance spectrum of the purified recombinant DC3 and DC4 proteins showed peaks at 419, 520, and 549 nm, similar to the spectrum shared by human cytochrome *c* (Fig. 4 A). The identity of these purified proteins was confirmed by immunoblotting with cytochrome *c* antibody (Fig. 4 B). We added recombinant DC3 or DC4 to S100 extracts from *Drosophila* BG2 or mammalian 293T cells that do not contain endogenous cytochrome *c* (Fig. 4 C). We found that the addition of DC3 and DC4, in the presence of dATP, did not significantly increase caspase activity in *Drosophila* BG2 cells (Fig. 4 D). Interestingly, if we used apoptotic extracts from BG2 cells we detected a small but significant increase in caspase activity after the addition of either *Drosophila* or human cytochrome *c* (unpublished data). When apoptotic extracts from cells subjected to *dc3* and *dc4* RNAi were used, the increase in caspase activity was still evident upon addition of recombinant cytochrome *c* proteins (unpublished data). These results indicate that additional apoptotic factors may augment caspase activation in the presence of cytochrome *c*. In contrast, we could clearly see that both DC3 and DC4 induced caspase activity in mammalian 293T extracts (Fig. 4 D). This activity is comparable to the increase in caspase activity seen when purified human cytochrome *c* is added. Interestingly, human cytochrome *c* was unable to induce caspase activity in BG2 cell extracts. These data indicate that whereas cytochrome *c* is necessary for caspase activation in mammalian cells, it does not directly activate caspases in *Drosophila* cells.

DC3 and DC4 are not essential for caspase activation in vivo

To examine whether DC3 or DC4 is required for cell death in *Drosophila* development, we assessed whether lysates from

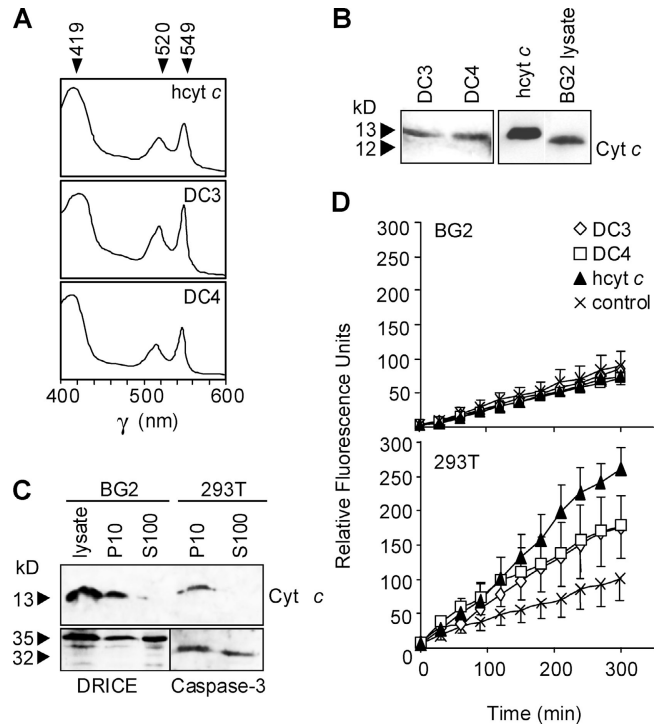


Figure 4. Effect of recombinant DC3 or DC4 on caspase activity in BG2 cell lysates. (A) Absorption spectrum of human cytochrome *c* (hcyt_{c-His6}), DC3_{His6}, and DC4_{His6}. Absorption was measured between 400 and 600 nm at a scanning speed of 1 nm/s. (B) Immunoblot analysis of cytochrome *c* proteins with anti-cytochrome *c* antibody detects recombinant DC3_{His6}, DC4_{His6}, and hcyt_{c-His6}. White line indicates that intervening lanes have been spliced out. (C) BG2 and 293T cytosolic (S100) extracts immunoblotted with anti-cytochrome *c* antibody confirm absence of cytochrome *c* in these fractions. Expression of DRICE and caspase-3 is shown in bottom panel. (D) Purified recombinant DC3_{His6}, DC4_{His6}, or hcyt_{c-His6} (10 μM) were incubated with BG2 S100 (top) or 293T S100 (bottom) lysates together with 1 mM dATP and caspase activity measured on DEVD-amc. Values represent the mean ± SEM from three independent experiments.

dc3 and *dc4* loss of function mutants were deficient in caspase activation. Two independent P-element mutations in each of the cytochrome *c* genes and a deficiency in this region have been reported previously (Arama et al., 2003). A P-element insertion in *dc4* (*l(2)k13095* allele) is homozygous lethal, which is likely due to its important role in respiration. The P-element insertion, which disrupts *dc3* (*bln¹* allele) is homozygous viable but results in male sterility (Castrillon et al., 1993; Arama et al., 2003). Recent studies have shown that the *bln¹* insertion results in significantly reduced levels of activated DRICE in spermatids (Arama et al., 2003). Both P-element insertions, however, disrupt multiple genes of unknown function (Huh et al., 2004). Therefore, it remains unclear whether DC3 is required for DRICE activation. A deficiency in the *dc3* and *dc4* gene region (*Df(2L)H20*) has a homozygous lethal phenotype as expected, but also disrupts the expression of multiple genes located within this region, making it difficult to attribute a function to either *dc3* or *dc4*.

Pre-pupae were collected 4 h after puparium formation, a time when various larval tissues including salivary glands undergo cell death and *dronc* has been shown to be up-regulated (Cakouros et al., 2002, 2004). We prepared lysates from *bln¹*

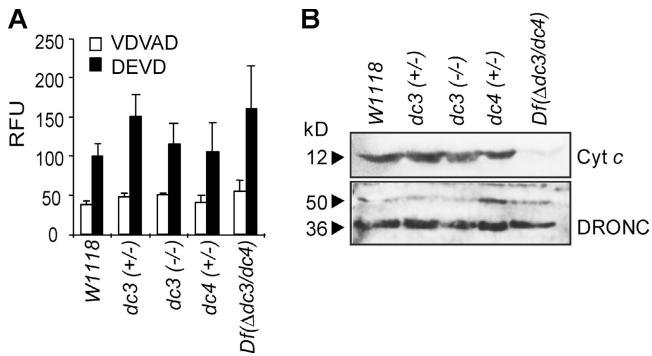


Figure 5. DRONC and DRICE activation can occur in the absence of *dc3* and *dc4* during *Drosophila* development. (A) Lysates were prepared at 4 h after puparium formation, from wild-type (W1118), *bln¹* homozygous (*dc3^{-/-}*), *bln¹* heterozygous (*dc3^{+/-}*), *l(2)k13095* heterozygous (*dc4^{+/-}*), or *Df(2)H20* heterozygote deficiency strains [*Df(Δdc3/dc4)*] and caspase activity was assessed on DEVD-amc or VDVAD-amc. Values are represented as mean ± SEM from four independent experiments. (B) Cytochrome *c* protein levels (top) and DRONC processing (bottom) in each lysate was detected by immunoblotting.

heterozygote (*dc3^{+/-}*) and homozygous (*dc3^{-/-}*) mutant prepupae, *l(2)k13095* heterozygotes (*dc4^{+/-}*) and from prepupae heterozygous for the deficiency, *Df(2)H20* [*Df(Δdc3/dc4)*]. As shown in Fig. 5 A, there was a high level of caspase activity in wild-type (W1118) prepupae. There was no reduction in the level of caspase activity in pupae from *dc3* and *dc4* mutants (Fig. 5 A) or in the processing of DRONC (Fig. 5 B). Animals heterozygous for the deficiency *Df(2)H20* also did not show lower caspase activity, even though the expression of cytochrome *c* in these flies is clearly reduced (Fig. 5 B). In addition, TUNEL staining of homozygous mutant embryos did not show any decrease in developmental cell death (unpublished data).

Conclusions and perspective

Our data clearly show that neither of the two cytochrome *c* species in *Drosophila* are required for caspase activation or apoptosis. Previous studies reported that a P-element insertion in the *dc3* gene (*bln¹*) results in loss of DRICE activity in testis (Arama et al., 2003). However, a recent report indicates that the *bln¹* P-element insertion also disrupts a number of other genes (Huh et al., 2004), thus questioning whether DC3 is responsible for DRICE activity. Additionally, DRICE activation during spermatogenesis appears to be independent of DARK and DRONC (Huh et al., 2004). If DC3 is required for caspase activation in *Drosophila*, a loss of function mutation in *dc3* should lead to severe developmental defects and lethality. Furthermore, although a tissue-specific function has been suggested for DC3, it is unlikely that DC3 functions only during spermatogenesis given its ubiquitous expression. Although disruption of the *dc4* gene is embryonic lethal, we have shown that DC4 cannot induce caspase activation and apoptosis in *Drosophila* cells.

The question remains as to how DARK mediates DRONC activation. One possibility is that other factors can substitute for cytochrome *c* function during apoptosis. Alternatively, removal of DIAP1 from DRONC may be sufficient to allow an interaction with DARK and activation. Given that transcription plays a major role in developmental PCD

in *Drosophila*, changes in the concentration of DIAP1, DRONC, and DARK proteins could facilitate caspase activation in the fly (Kumar and Cakouros, 2004). Our studies, combined with published work, demonstrate that *Drosophila* and mammalian cytochrome *c* proteins are functionally similar as they can both mediate respiration and Apaf-1 activation in mammalian cell lysates. Therefore, the requirement for cytochrome *c* in caspase activation in mammals is likely to have evolved late in evolution.

Materials and methods

Constructs

Full-length *dc3* and *dc4* cDNAs were PCR amplified with a 3' HA-tag, from a *Drosophila* larval cDNA library, and cloned into pE1.4 vector (Novagen). For bacterial expression, *dc3* and *dc4* cDNAs were cloned into pQC1.2 backbone (provided by A. Rodriguez, The Rockefeller University, New York, NY, H. Mizuno, Massachusetts Institute of Technology, Cambridge, MA, and A. Matapurkar, Cold Spring Harbor Laboratory) along with cDNA encoding haem lyase.

Cell culture

Drosophila ML-DmBG2c2 (BG2) or *l(2)mbn* cells were maintained at 27°C as described previously (Cakouros et al., 2002; Dorstyn et al., 2002). DC3 and DC4 expressing cell lines were generated by transfecting BG2 cells with pE1.4-DC3_{HA} or pE1.4-DC4_{HA} together with a pHS-Neo expression construct (provided by N. Silverman, University of Massachusetts Medical School, Worcester, MA) as described previously (Cakouros et al., 2004). Transfected cells were selected with 1 mg/ml G418 (GIBCO BRL). Cells were induced to undergo apoptosis as described previously (Cakouros et al., 2002; Dorstyn et al., 2002). Cell viability/death was assessed by Trypan blue exclusion and staining with DAPI.

Fly strains

Drosophila wild-type (W1118), *dc3* "blanks" mutants [(*bln¹/bln¹*), (*bln¹/CyoKr>GFP*)], *dc4* mutants [(*l(2)k13095/CyoKr>GFP*)], and *dc3/dc4* deficiency strains [*Df(2)H20/CyoKr>GFP*] were obtained from Bloomington Stocks Center.

Cytochrome *c* expression and purification

E. coli DH5α cells were transformed with either pQC1.2-DC3, pQC1.2-DC4, or pQC1.2-hcytc expression constructs. 500-ml cultures were induced with 1 mM IPTG overnight at 37°C. Cells were lysed by sonication and cleared lysates were incubated with 300 μl prewashed Ni-NTA agarose beads (QIAGEN) for 1 h at 4°C. Beads were washed with 50 vol lysis buffer and cytochrome *c* proteins eluted with 5 vol elution buffer (300 mM NaCl, 10 mM Tris-HCl, pH 8, 500 mM imidazole).

Caspase assays

Fluorogenic substrate assays were performed as described previously (Dorstyn et al., 1999). Cytosolic S100 protein lysates were isolated as reported previously (Dorstyn et al., 2002). Pre-pupae were snap frozen in liquid nitrogen and lysed by homogenisation. 20–50 μg of lysates or S100 were used in DEVD-amc or VDVAD-amc cleavage reactions. Where indicated, S100 lysates were incubated with 10 μM of purified DC3_{His6}, DC4_{His6}, or human cytochrome *c* (hcytc), and 1 mM dATP, in caspase assay buffer (Dorstyn et al., 1999) at RT and activity was measured over time using a fluorometric plate reader (PerkinElmer) (excitation 385 nm, emission 460 nm).

Cell fractionation

Mitochondria and cytosol from untreated and cycloheximide (15 μg/ml)-treated cells were separated by differential centrifugation as described previously (Dorstyn et al., 2002).

Immunoblotting

Aliquots (50 μg protein) were immunoblotted using the following antibodies: monoclonal cytochrome *c* 7H8.2C12 (PharMingen) used at 0.5 μg/ml; rat HA (Roche) used at 0.1 μg/ml; rabbit DRONC 1.5 μg/ml; and rabbit DRICE (provided by B. Hay, California Institute of Technology, CA) at 1 μg/ml (Dorstyn et al., 2002; Huh et al., 2004).

Immunofluorescence

Cells were stained as described previously (Dorstyn et al., 2002). Where indicated, cells were incubated with 1 $\mu\text{g/ml}$ HA antibody alone or together with DRICE antibody at 4 $\mu\text{g/ml}$ overnight at 4°C followed by incubation with Alexa Fluor 488 (green), Alexa Fluor 568 (red) conjugated anti-rabbit or goat anti-rat Texas red secondary antibodies (Molecular Probes). Fluorescence images were captured using a fluorescence microscope (model BX51; Olympus) and a camera (model UCMAD3/CV-M300; Olympus). Cells were visualized under 100 \times UPLAPO objective lens with NA = 1.3, under oil immersion. All green and red fluorescent images were automatically merged. Image analysis was performed with Metasystems Isis software (Robert Bosch). Image background brightness was adjusted to vector control cells. Images were compiled using Adobe Photoshop version 6.0.

RNAi

dc3 and *dc4* were cloned into pCDNA3 (Invitrogen) and a 700-bp coding region of GFP was cloned into pGEM-T Easy vector (Promega) for use as a control. dsRNA was generated as described previously (Colussi et al., 2000; Cakouros et al., 2002). 40 nM of dsRNA was added to 2 \times 10⁶ cells in 1 ml serum-free medium. In the case of coablation of *dc3* + *dc4*, 40 nM of each dsRNA reactions were mixed and added to cells. Cells were then incubated for 1 h at 27°C after which 2 ml of complete media containing 10% FBS was added. Cells were incubated over 48 h and treated with cycloheximide (10 $\mu\text{g/ml}$) for 6 h, or with ecdysone (10 μM) for 24 h.

RT-PCR

Total RNA was used for RT-PCR. To assess ablation of *dc3* and *dc4*, transcripts were amplified using a 5' primer designed to 55 bases upstream of the ATG, and a 3' primer designed to the terminus of each transcript. Amplification of *dronc* was performed using primers designed from the 5' ATG and a 3' primer designed to a region 400 bp downstream of the start ATG.

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