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Cytochrome c-d regulates developmental apoptosis in the *Drosophila* retina

César S. Mendes[1], Eli Arama[1], Samara Brown[1], Heather Scherr[2], Mayank Srivastava[2], Andreas Bergmann[2], Hermann Steller[1] & Bertrand Mollereau[1†+]

[1]Howard Hughes Medical Institute, Strang Laboratory of Cancer Research, The Rockefeller University, New York, New York, USA, and [2]Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA

The role of cytochrome *c* (Cyt *c*) in caspase activation has largely been established from mammalian cell-culture studies, but much remains to be learned about its physiological relevance *in situ*. The role of Cyt *c* in invertebrates has been subject to considerable controversy. The *Drosophila* genome contains distinct *cyt c* genes: *cyt c-p* and *cyt c-d*. Loss of *cyt c-p* function causes embryonic lethality owing to a requirement of the gene for mitochondrial respiration. By contrast, *cyt c-d* mutants are viable but male sterile. Here, we show that *cyt c-d* regulates developmental apoptosis in the pupal eye. *cyt c-d* mutant retinas show a profound delay in the apoptosis of superfluous interommatidial cells and perimeter ommatidial cells. Furthermore, there is no apoptosis in mutant retinal tissues for the *Drosophila* homologues of apoptotic protease-activating factor 1 (Ark) and caspase 9 (Dronc). In addition, we found that *cyt c-d*—as with *ark* and *dronc*—regulates scutellar bristle number, which is known to depend on caspase activity. Collectively, our results indicate a role of Cyt *c* in caspase regulation of *Drosophila* somatic cells.

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INTRODUCTION

In response to apoptotic stimuli, mammalian cells release cytochrome *c* (Cyt *c*) from the mitochondria into the cytoplasm where it binds to apoptotic protease-activating factor 1 (Apaf 1). This leads to the recruitment of the zymogen form of caspase 9 to a catalytically active multi-protein complex called the apoptosome (Jiang & Wang, 2004). Once activated, the apoptosome, consisting of Cyt *c*, dATP, Apaf 1 and caspase 9, can cleave and activate downstream caspases, including caspase 3. Genetically modified mice have demonstrated the *in vivo* importance of several components of this pathway, including Apaf 1, caspase 9, caspase 3 and Cyt *c*. However, these studies have also shown a surprising degree of complexity and raised questions about how apoptosis is activated in the absence of canonical apoptosome components (Hao *et al*, 2005).

In *Drosophila*, the mechanisms leading to the activation of the Apaf 1 homologue are controversial (Kornbluth & White, 2005). Similar to its mammalian homologue, *Drosophila* Ark (also called Hac 1/Dapaf 1/Dark) contains a series of WD40 repeats, which, *in vitro*, can bind *Drosophila* Cyt *c* and form an apoptosome-like complex and induce caspase activation (Kanuka *et al*, 1999; Rodriguez *et al*, 1999; Zhou *et al*, 1999; Dorstyn *et al*, 2002). RNA interference knock-down experiments, however, failed to support a role for *cyt c* in the apoptosis of S2 culture cells (Zimmermann *et al*, 2002; Dorstyn *et al*, 2004).

The *Drosophila* genome contains two closely related but distinct *cyt c* genes: *cyt c-d* and *cyt c-p* (Limbach & Wu, 1985). *cyt c-p* is involved in mitochondrial respiration and viability, whereas *cyt c-d* is required for caspase activation and sperm differentiation (Arama *et al*, 2003, 2006). In the sperm, caspase activation does not lead to cell death, but to sperm maturation. Here, we report on the role of *cyt c-d* in apoptosis during normal development of the *Drosophila* retina.

RESULTS AND DISCUSSION

cyt c-d regulates apoptosis in the developing eye

In the developing eye, superfluous interommatidial cells (IOCs) and perimeter ommatidial cells (POCs) are eliminated by apoptosis, allowing the precise rearrangement of ommatidia into a honeycomb-like formation (Cagan & Ready, 1989; Wolff & Ready, 1991; Cordero *et al*, 2004; Monserrate & Brachmann, 2006). Antibodies raised against the membrane-bound protein Armadillo (Arm) allow the visualization of each cell in the eye

[1]Howard Hughes Medical Institute, Strang Laboratory of Cancer Research, The Rockefeller University, 1230 York Avenue Box 252, 10021, New York, New York 10021, USA
[2]Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard Box 117, Y4.6024, Houston, Texas 77030, USA
[†]Present address: LBMC, Ecole Normale Supérieure de Lyon, CNRS, UMR 5161, IFR 128, 46 Allée d'Italie, 69364 Lyon, France
[+]Corresponding author. Tel: +1 212 327 8493; Fax: +1 212 327 7076; E-mail: bertrand.mollereau@ens-lyon.fr

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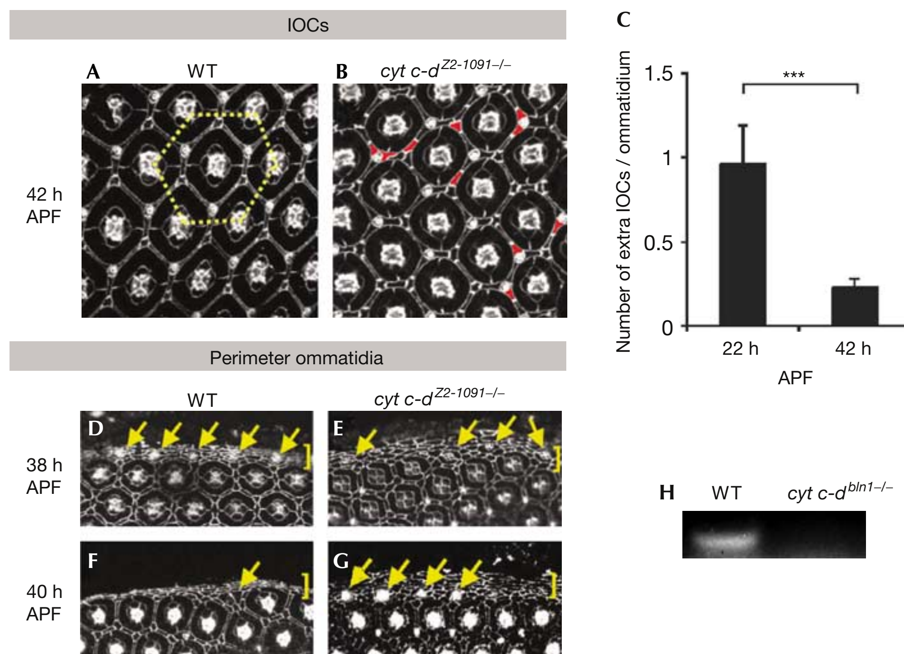

Fig 1 | Interommatidial cell and perimeter ommatidial cell death are delayed in *cyt c-d* mutant retina. (**A,B,D–G**) Pupal retinas were stained with Arm protein to visualize retinal cell membranes. (**A,B**) Extra IOCs are marked red in staged 42 h APF animals. (**A**) Wild-type (WT) ommatidia contain a definite number of IOCs (that is, six secondary and three tertiary cells). The yellow hexagon is a defined area used to count IOCs (see the Interommatidial cell counts section). (**B**) *cyt c-d*$^{Z2\text{-}1091-/-}$ retinas show extra IOCs with the normal shape and position of secondary or tertiary cells (see Table 1 for values). (**C**) Graph showing the difference in the number of IOCs per ommatidium between WT and *cyt c-d*$^{Z2\text{-}1091-/-}$ at 22 and 42 h APF. The number of extra IOCs is significantly higher at 22 h than at 42 h APF (*t*-test, ***$P<0.001$). (**D–G**) Staged 38 and 40 h APF retinas stained against Arm (white). (**D,E**) At 38 h APF, extra ommatidial clusters (yellow arrows) are visualized in (**D**) WT and (**E**) *cyt c-d*$^{Z2\text{-}1091-/-}$ retinas in a thick layer of IOCs (yellow bracket). (**F**) In WT retinas at 40 h APF, POC death is almost complete and only occasional extra ommatidial clusters remain at the edge in a thin layer of IOCs. (**G**) In *cyt c-d*$^{Z2\text{-}1091-/-}$ 40 h APF retinas, many extra ommatidial clusters (yellow arrows) are still visible in a thick layer of IOCs. (**H**) Reverse transcription–PCR analysis of *cyt c-d* expression at 22 h APF. As a negative control, we used *bln*^{1} mutant flies in which no *cyt c-d* transcript is detected (Arama *et al*, 2003). APF, after puparium formation; Arm, Armadillo; IOC, interommatidial cell; POC, perimeter ommatidial cell.

lattice. By 42 h after puparium formation (APF), a fixed number of IOCs form an hexagonal array around each photoreceptor cell cluster, comprising four cone cells, three bristle cells, two primary, six secondary and three tertiary pigment cells (Fig 1A; supplementary Fig S1 online).

To examine the role of the *cyt c* locus in cell death during pupal eye development, we compared the number of IOCs between wild-type and several *cyt c-d* male-sterile and viable, loss-of-function alleles at a stage in which *cyt c-d* is expressed (Fig 1; supplementary Fig S2 online; Table 1; Arama *et al*, 2003, 2006). We focused our analysis on the *cyt c-d*$^{Z2\text{-}1091}$ allele, as it bears a point mutation that creates a stop codon in the *cyt c-d* coding region, which does not affect neighbouring open reading frames. At 42 h APF, a time when IOC death is normally complete, *cyt c-d*$^{Z2\text{-}1091-/-}$ mutant retinas showed extra cells in the secondary or tertiary position (Fig 1B; Table 1). A more pronounced phenotype was observed in *cyt c-d*$^{Z2\text{-}1091}$/Df(2L)H20 retinas, indicating that another neighbouring gene included in Df(2L)H20 contributes to the regulation of IOC death, or that *cyt c-d*$^{Z2\text{-}1091}$ might be a hypomorphic allele. In addition, the extra IOC phenotype observed in *cyt c-d*$^{Z2\text{-}1091-/-}$ was rescued by the ectopic expression of *cyt c-d* in the developing retina. Mutant retinas for the other *cyt c-d* alleles also showed extra IOCs (Table 1).

Table 1 | Comparison of the number of interommatidial cells for different *cyt c-d* mutant allele combinations

Genotype	Cells per hexagon ± s.e.m.	ΔIOCs/ommatidium ± s.e.m. (age matched)
Wild type (CS)	21 ± 0.0	—
cyt c-d$^{Z2\text{-}1091-/-}$	21.80 ± 0.15	0.27 ± 0.05
cyt c-d$^{Z2\text{-}1091}$/*Df(2L)H20*	22.29 ± 0.15	0.43 ± 0.05
cyt c-d$^{Z2\text{-}1091-/-}$;*GMR-cyt c-d*	21.33 ± 0.13	0.11 ± 0.04
cyt c-d$^{bln1-/-}$	23.13 ± 0.27	0.71 ± 0.09
cyt c-d $^{EP2049-/-}$	22.03 ± 0.18	0.34 ± 0.06
cyt c-d $^{EP2305-/-}$	21.70 ± 0.15	0.23 ± 0.05
cyt c-d $^{Ex\text{-}6C-/-}$	22.17 ± 0.18	0.39 ± 0.06

IOCs, interommatidial cells.

To further characterize the role of *cyt c-d*, we counted the number of extra IOCs at different stages of pupal development in the mutant retina (Fig 1C): at 22 h APF, *cyt c-d*$^{Z2\text{-}1091-/-}$ retinas already showed extra IOCs (Fig 1C); at 48 h APF, they still occasionally showed extra IOCs compared with wild type

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(0.16 ± 0.04 extra IOCs/ommatidium). The decrease in the number of extra IOCs between 22 and 48 h APF indicates that IOC death is delayed and not completely suppressed in the *cyt c-d* mutant. Considering that the IOC death process terminates at 36 h APF (Cagan & Ready, 1989), we estimate that IOC apoptosis in *cyt c-d*$^{Z2\text{-}1091-/-}$ retinas can be delayed up to 12 h. We propose that the *cyt c-d* gene is required for the 'on-time' apoptosis of IOCs during pupal development.

Our results show that *cyt c-d* regulates IOC apoptosis in pupal retinas (Fig 1A–C). We then asked whether *cyt c-d* also regulates POC apoptosis. Ommatidia at the edge of the eye (perimeter ommatidia) contain photoreceptor, cone and pigment cells that die by apoptosis (Hay *et al*, 1994; Lin *et al*, 2004). Between 36 and 44 h APF, 80–100 ommatidia are eliminated, allowing the formation of a normal eye edge. POCs were visualized using an anti-Arm antibody in staged *cyt c-d*$^{-/-}$ and wild-type retinas (Fig 1D–G). By 38 h APF, POC elimination has just begun, with numerous small ommatidial clusters along the edge of the retinas (Fig 1D,E). By 40 h APF, many wild-type POCs have been eliminated (Fig 1F). By contrast, *cyt c-d*$^{Z2\text{-}1091-/-}$ retinal edges showed more clusters of malformed ommatidia in a thick layer of IOCs (Fig 1G). The same phenotype was also visible in all the other *cyt c-d* alleles (data not shown). By 54 h APF, POC elimination is complete both in wild-type and mutant retinas (data not shown). These results indicate that *cyt c-d* promotes not only IOC elimination but also POC death.

The detection of *cyt c-d* expression is challenging, as none of the available antibodies allows us to distinguish between the two *cyt* c species or visualize the release of Cyt *c* during apoptosis of ommatidial cells. The analysis of *cyt c* RNA transcripts showed that *cyt c-p* is the prevailing form expressed throughout development and adulthood (Arama *et al*, 2003; data not shown). The *cyt c-d* transcript, however, seems to be mainly restricted to the testis (Arama *et al*, 2006). Here, we found that both *cyt c* transcripts are present at the time of IOC elimination in the retina (Fig 1H; data not shown). This supports the possibility that the two Cyt *c* proteins can function in the elimination of superfluous retinal cells during pupation. The fact that physiological amounts of *cyt c-p* cannot substitute for the loss of *cyt c-d* suggests that the full apoptogenic function of *cyt c* requires the expression of both *cyt c* genes.

Elimination of both *cyt c* genes in the retina might lead to a more pronounced phenotype than *cyt c-d* mutation alone. Unfortunately, this hypothesis is extremely difficult to test, given the general requirement of *cyt c-p* for cell survival (data not shown). In addition, we do not favour the possibility that the loss of both *cyt c* genes would lead to a phenotype as pronounced as the complete inhibition of death observed in retina expressing p35 (Fig 3G) because in *cyt c-d*$^{Z2\text{-}1091}$/Df(2L)H20 flies, in which *cyt c-d* is lost and only one copy of *cyt c-p* is functional, IOC death is delayed to a level comparable with that in *cyt c-d* mutants (Table 1).

cyt c-d does not regulate respiration or cell differentiation

We showed that apoptosis is delayed in the *cyt c-d*$^{-/-}$ retina (Fig 1). This could be due to a direct role of *cyt c-d* in the apoptotic process or an indirect consequence of an impaired respiratory function in the mutant retina. To address the latter possibility, we measured ATP levels in several wild-type strains and *cyt c-d* mutants (Fig 2A). We found no significant difference between wild-type and *cyt c-d* mutants, ruling out an effect in the bioenergetics levels as the cause of extra cells in *cyt c-d*$^{-/-}$ retina. To eliminate any consequence in retinal development, *cyt c-d*$^{Z2\text{-}1091-/-}$ larval and pupal eyes were stained with antibodies against several specific differentiation markers (Fig 2B–G). *cyt c-d*$^{Z2\text{-}1091-/-}$ larval eye discs stained against Elav (neuronal marker), Boss (R8-specific marker) and Spalt-major (R3, R4, R7, R8 and cone cell marker) appeared as in the wild-type control (Fig 2B,E; data not shown). Moreover, tangential plastic sections of *cyt c-d*$^{Z2\text{-}1091-/-}$ adult eyes presented the normal number and arrangement of photoreceptor cells (data not shown).

Other retinal cell types, including primary pigment, cone and bristle cells, visualized at pupal stages in *cyt c-d* mutant, appeared normal in shape and number (Fig 1; supplementary Figs S3,S4 online). We also stained *cyt c-d*$^{Z2\text{-}1091-/-}$ retinas at different stages of pupal development (24, 27, 30 and 42 h APF) with an anti-Homothorax (Hth) antibody, which stains secondary and tertiary pigment cell nuclei (Wildonger *et al*, 2005). All secondary and tertiary cells expressed *hth*, suggesting that the extra IOCs differentiate normally (Fig 2G; supplementary Fig S3B′,D′,F′ online). Thus, the only phenotype associated with *cyt c-d* mutations is the appearance of extra secondary and tertiary cells in the eye lattice, with no disruption of early retinal development. For this reason, the *cyt c-d* mutation can be classified as lattice-specific according to the nomenclature described by Cagan and colleagues (Tanenbaum *et al*, 2000). To determine whether *cyt c-d* is required for development progression, we examined the dynamic IOC rearrangement and maturation in staged *cyt c-d*$^{Z2\text{-}1091-/-}$ and wild-type retinas (supplementary Fig S4 online). Despite the presence of extra IOCs in *cyt c-d*$^{Z2\text{-}1091-/-}$, the process of cell sorting and IOC maturation occurs similarly to wild-type retinas (20–27 h APF). Thus, the dynamic rearrangement and maturation of IOCs are not delayed in *cyt c-d*$^{Z2\text{-}1091-/-}$ retinas, eliminating any significant effect of *cyt c-d* mutations on the progression of retinal cell differentiation.

Together, these results demonstrate that *cyt c-d* is not required for respiration, differentiation or developmental progression in the pupal eye, providing the first genetic evidence for a physiological role of *Drosophila cyt c* in the regulation of developmental apoptosis.

ark and *dronc* are required for apoptosis in the pupal eye

We have shown that *cyt c-d* is required for apoptosis progression during pupal eye development in *Drosophila* (Fig 1). We then asked whether the other homologues of the apoptosome components, Ark and Dronc, are also required for apoptosis in this model. We used *ark* and *dronc* loss-of-function mutant alleles (Xu *et al*, 2005; Srivastava *et al*, 2006); both *ark* and *dronc* alleles are strong loss-of-function or null alleles leading to apoptosis defects at early stages in development and lethality.

Using the flipase (FLP)/FLP recombinase target (FRT) technique, we generated *ark* and *dronc* mutant clones in the eye. In these clones, visualized by the absence of green fluorescent protein (GFP), we counted an excess of 5.20 and 4.36 IOCs/ommatidium, respectively (Fig 3A,B,G). These values are comparable with those observed in pupal retinas in which the caspase inhibitor, p35, is ectopically expressed under control of the ubiquitous eye promoter, GMR, showing 5.06 extra IOCs/ommatidium (Fig 3G). These values are higher than the total estimated number of

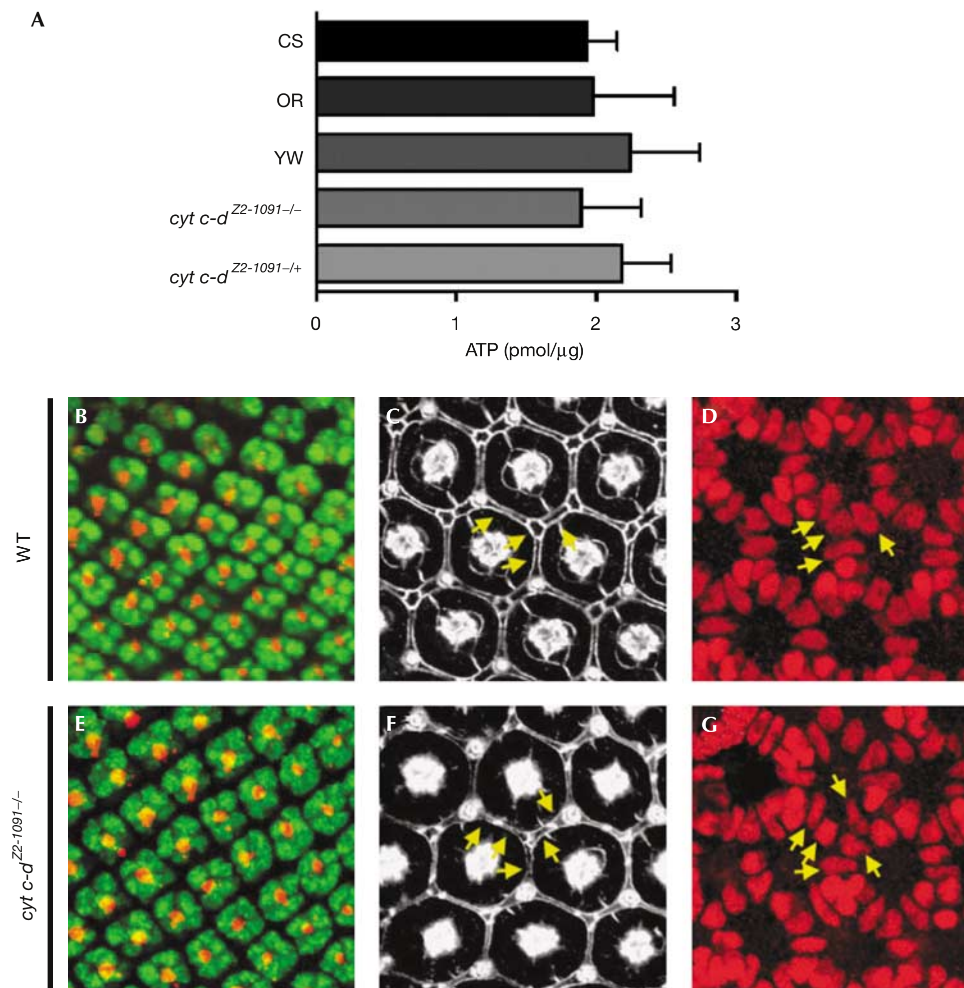

Fig 2 | Loss of *cyt c-d* has no effect on ATP levels and retinal development. **(A)** ATP levels in wild-type (WT; CS, OR and *yw*), *cyt c-d*$^{Z2\text{-}1091-/+}$ and *cyt c-d*$^{Z2\text{-}1091-/-}$ adult fly heads (1 day after eclosion). Each value shows the mean ± s.e.m. of three independent experiments. **(B–D)** WT and **(E–G)** *cyt c-d*$^{Z2\text{-}1091-/-}$ mutant eye discs were stained with various differentiation markers. **(B,E)** Posterior is to the right. Third-instar larval eye discs stained with antibodies against the photoreceptor cell marker, Elav (green), and R8 cell marker, Boss (red), show similar pattern in **(B)** WT and **(E)** *cyt c-d*$^{Z2\text{-}1091-/-}$. 42 h APF **(C,D)** WT and **(F,G)** *cyt c-d*$^{Z2\text{-}1091-/-}$ retinas stained with Arm (apical view; **C,F**) and the secondary and tertiary pigment marker, Hth (basal view; **D,G**), in two distinct focal planes of the retina. In *cyt c-d*$^{Z2\text{-}1091-/-}$, extra secondary or tertiary cells are visualized with Arm staining and their nuclei express Hth (an extra yellow arrow compared with WT retina). APF, after puparium formation; Arm, Armadillo; Hth, Homothorax.

IOCs that are dying between 18 and 36 h APF (about 3.5 IOCs/ ommatidium; Cordero *et al*, 2004; data not shown). This is probably due to the fact that, in those mutant situations, unwanted IOCs are also rescued during larval development and early pupal development (<18 h APF).

The number of extra IOCs obtained in *dronc* mutant clones is similar to the value observed in the retinas of *dronc* mutant escapers (Xu *et al*, 2005). In addition, clonal analysis showed that only the mutant tissue for *ark* or *dronc*, exhibit extra IOCs, not the surrounding non-mutant tissue. This indicates that *ark* and *dronc* are required cell-autonomously for IOC apoptosis. In addition, we found that the combination of *cyt c-d* mutations and the expression of Dronc dominant negative in the retina induces synergistic reduction of IOC death, suggesting the proximity of these genes in the same pathway (supplementary Fig S5 online).

We also examined the role of *ark* and *dronc* in POC apoptosis. In *ark* and *dronc* mutant clones, POCs are rescued (Fig 3C,D) and TdT-mediated dUTP nick-end labelling (TUNEL) is blocked (Fig 3E,F). Moreover, the mutant retinas present extra POCs that are never eliminated, as seen in GMR-p35 (Hay *et al*, 1994; data not shown). Thus, Dronc has a pivotal role as an initiator caspase in the pupal retina—which differs from embryonic tissues—in which Dronc is required for most, but not all, cell death (Xu *et al*, 2005).

To rule out the possibility that developmental defects in *ark* or *dronc* mutant retinas indirectly affect cell death, we examined retinal cell differentiation in *ark* and *dronc* mutant clones using several larval and pupal eye differentiation markers (supplementary Fig S6 online). We found that retinal cell differentiation is normal in *ark* and *dronc* mutant clones.

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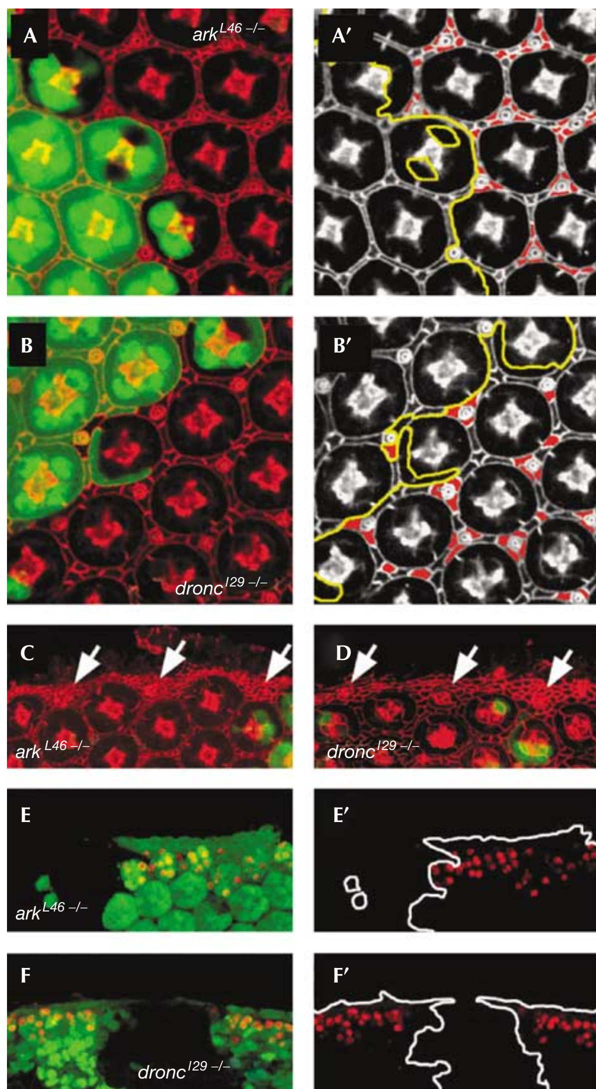

Fig 3 | Ark and Dronc are required for apoptosis in the pupal retina. **(A,B)** *ark* and *dronc* mutant clones are visualized in 42 h APF retinas by the absence of green fluorescent protein (GFP; green) and the membrane outlined with Arm antibody (red). Numerous extra IOCs are observed in the clonal area of **(A)** (ey-FLP; FRT42D ark^{L46}/FRT42D Ubi-GFP) and **(B)** (ey-FLP;; $dronc^{I29}$ FRT80B/Ubi-GFP FRT80B). **(A′)** and **(B′)** show the corresponding schematic ommatidium, in which the yellow line represents the clonal boundaries and extra IOCs are marked in red. **(C–F)** Retinal edges in *ark* and *dronc* mutant clones at 42 h APF are visualized by the absence of GFP. **(C,D)** POCs stained with Arm antibody (red) in **(C)** ark^{L46} and **(D)** $dronc^{I29}$ mutant clones show extra POCs (white arrows) compared with wild-type retina. **(E,F)** Retinal edges stained with TdT-mediated dUTP nick end labelling (TUNEL; red) in **(E)** ark^{L46} and **(F)** $dronc^{I29}$ mutant clones show the absence of TUNEL staining compared with the non-mutant tissue (GFP positive). **(E′)** and **(F′)** are the corresponding panels without GFP staining, in which a white line marks the boundary of the clone. **(G)** Comparison of the number of IOCs in ark^{L46}, $dronc^{I29}$ mutant clones and retinas expressing GMR-p35 at 42 h APF. APF, after puparium formation; Arm, Armadillo; IOC, interommatidial cell; POC, perimeter ommatidial cell.

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Genotype	Cells per hexagon ±s.e.m.	ΔIOCs ommatidium ±s.e.m. (age matched)
WT	21±0.0	—
ark [L46], FRT 42D	36.60±0.63	5.20±0.21
dronc [I29], FRT 80B	34.07±0.55	4.36±0.19
2×GMR-p35	36.17±0.98	5.06±0.33

Together, these results demonstrate that *ark* and *dronc* are required for the initiation and/or execution of IOC and POC apoptosis, placing these genes hierarchically at the top of the apoptotic cascade during pupal eye development.

cyt c-d regulates scutellar bristle cell number

cyt c-d, *ark* and *dronc* regulate apoptosis during eye development (Figs 1–3). To further explore the role of *cyt c-d* in the regulation of caspase activation, we used the elimination of sensory organs (macrochaetes) as a model. A recent study proposed that caspase activation does not lead to apoptosis but inhibits the Wingless pathway to ensure the correct number of sensory organ precursors (SOPs; Kanuka *et al*, 2005). Consistently, loss-of-function mutations in *ark* or *dronc* lead to the appearance of extra bristles on the *Drosophila* notum (Kanuka *et al*, 1999; Rodriguez *et al*, 1999; Chew *et al*, 2004; Leulier *et al*, 2006). To determine the role of *cyt c-d* during SOP development, we counted the number of posterior scutellar bristles on the thorax of *cyt c-d* mutant flies. In all the *cyt c-d* mutant alleles examined, we found a significant number of flies that had one extra bristle (Fig 4; supplementary Table S1 online). Using a recently characterized allele of *ark* (ark^{N5}; Srivastava *et al*, 2006), we also observed an extra bristle cell phenotype. As for the extra IOCs, *ark* has a more pronounced phenotype than *cyt c-d* mutants (supplementary Table S1 online), suggesting that similar mechanisms lead to caspase activation in the two models. Together, these results provide further support that *cyt c-d* promotes caspase activation required for accurate developmental progression.

We also identified extra bristles in flies mutant for the executioner caspase *dcp 1* ($dcp\text{-}1^{prev1}$; supplementary Table S1 online; Laundrie *et al*, 2003), for which no role in the regulation of bristle cell number has yet been reported. Interestingly, we found no extra bristle phenotype in *drICE* mutant ($drICE^{17}$; Xu *et al*, 2006), suggesting that *dcp 1* could be the main executioner caspase in this model.

CONCLUSIONS

In most tissues, with the exception of developing testis, SOP and retina, *cyt c-d* has no apparent role in caspase activation or apoptosis, suggesting that apoptosis can occur in the absence of this protein. The existence of a Cyt-*c*-independent pathway for apoptosis in *Drosophila* was previously proposed on the basis of RNA interference studies in *Drosophila* cell lines or a cell-free system, showing that apoptosis can occur independent of Cyt *c* function but requires Ark (Zimmermann *et al*, 2002; Dorstyn *et al*, 2004; Means *et al*, 2005). Therefore, at least in

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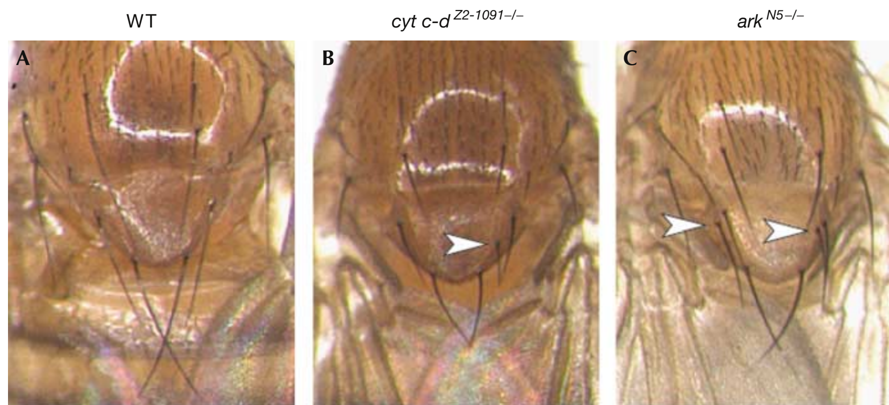

Fig 4 | Adult flies with the mutation *cyt c-d*$^{-/-}$ have an extra bristle in the scutellum. (**A**) Wild-type (WT; CS) flies show four bristles. (**B**) *cyt c-d*$^{Z2\text{-}1091-/-}$ mutant showing an extra bristle (arrowhead). (**C**) *ark*$^{N5-/-}$ fly with two extra bristles (arrowheads). See supplementary Table S1 online for statistical analysis.

some models, Ark-dependent caspase activation might be either constitutive or regulated by other pathways. In support of the latter, ectopic expression of Ark is not sufficient to trigger apoptosis *in vivo*, suggesting that Ark must be activated to function (Akdemir *et al*, 2006). Likewise, analysis of mice devoid of Cyt *c* apoptogenic function (K72A) indicates that caspase activation in thymocytes can occur independently of Cyt *c* (Hao *et al*, 2005). Mammalian Apaf 1 might either have some constitutive activity or might be regulated by factors other than Cyt *c* (Green, 2005).

Conversely, our results indicate that a Cyt-*c*-dependent mechanism for apoptosis in the retina might be necessary for the rapid removal of a precise number of cells during development. An even stricter requirement is observed during sperm or SOP development, in which imbalanced caspase activation or loss of *cyt c-d* function leads to male sterility and extra bristle cells, respectively (Arama *et al*, 2006; Fig 4). Our results indicate that Cyt *c* is able to promote the activation of Ark to form an apoptosome that leads to Dronc activation and cell death. In support of this hypothesis, Dronc is recruited into a >700 kDa complex in *Drosophila* cell extracts supplemented with Cyt *c* and dATP (Dorstyn *et al*, 2002), similar to the mammalian apoptosome. In addition, Ark interacts with Cyt *c*, an interaction dependent on the WD40 domain of Ark (Kanuka *et al*, 1999; Rodriguez *et al*, 1999). However, recent structural data suggest that Ark does not require Cyt *c* to form an apoptosome-like structure (Yu *et al*, 2006). Although the authors used horse and not *Drosophila* Cyt *c* for the apoptosome assembly, *Drosophila* apoptosome formation might not require Cyt *c*. If so, it raises a question on the inhibitory function of the WD40 domain of Ark. The WD40 is conserved between vertebrates and *Drosophila* but not *Caenorhabditis elegans*, in which it is thought to maintain Apaf 1 in an inactive conformation that is relieved on Cyt *c* binding (Hu *et al*, 1998; Srinivasula *et al*, 1998). How Ark activation *in vivo* is dependent on Cyt *c* awaits further analysis.

The identification of *Drosophila* Cyt *c* as part of the apoptotic process opens new and exciting opportunities to elucidate novel mechanisms for apoptosome activation.

METHODS

Genetics, generation of transgenic flies, immunofluorescence, ATP assay and reverse transcription–PCR were performed using standard procedures.

Interommatidial cell counts. At least 30 hexagons from three different animals were scored for each experimental condition, corresponding to 90 complete ommatidia, as described by Wolff & Ready (1991). In Figs 1C,3G and Table 1, the number of extra IOCs per ommatidium (also called ΔIOC/ommatidium) is the difference of the means between wild-type and the mutant conditions divided by 3 (each hexagon contains three ommatidia).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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