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► **To cite this version:**

S Gaumer, I Guenal, S Brun, L Theodore, Bernard Mignotte. Bcl-2 and Bax mammalian regulators of apoptosis are functional in *Drosophila*. *Cell Death and Differentiation*, 2000, 7, pp.804-814. 10.1038/sj.cdd.4400714 . hal-03034620v2

**HAL Id: hal-03034620**

**<https://hal.science/hal-03034620v2>**

Submitted on 14 Apr 2023

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**Bcl-2 and Bax mammalian regulators of apoptosis are functional in *Drosophila***

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Running title: Expression of Bcl-2 and Bax proteins in *Drosophila*

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## **Abstract**

Studies of apoptosis in *C. elegans* have allowed the identification of three genes, *ced-3*, *ced-4* and *ced-9*. Their products constitute the components of an induction pathway of apoptosis conserved in the nematode and mammals. In *Drosophila*, homologues have been found for CED-3, CED-4 and CED-9. CED-9 belongs to the Bcl-2 family which includes negative (Bcl-2) and positive (Bax) regulators of apoptosis. The recently discovered Bcl-2 family member named Drob-1 acts as a positive regulator of cell death. To address whether a Bcl-2 anti-apoptotic pathway exists in the fly, we studied the effects of expressing the mammalian genes *bcl-2* in *Drosophila*. In embryos, expression of *bcl-2* inhibits developmental and X-ray-induced apoptosis. Expressing *bcl-2* or the pro-apoptotic mammalian *bax* in the developing eye and wing alters these structures, *bcl-2* increasing the number of cells, while *bax* reduces the number of cells. In addition, the functional interaction between Bcl-2 and Bax is conserved. These results indicate that factors necessary for the activity of *bcl-2* and *bax* are present in *Drosophila*. Therefore, a Bcl-2 pathway for inhibition of cell death may exist in the fly.

**Key words :** Programmed cell death; apoptosis; caspase; mitochondria; Bcl-2; Bax; *Drosophila*.

**Abbreviation:** Acridine orange, AO; After egg laying, AEL; Programmed cell death, PCD; Apoptosis inducing factor, AIF; vestigial, vg; daughterless, da.

## Introduction

Programmed cell death (PCD) serves as an important mechanism for the precise regulation of cell number, and as a defense mechanism to remove unwanted and potentially dangerous cells. Genetic studies in *C. elegans* have led to the identification of a dozen of cell death genes (*ced*) that are responsible for different aspects of cell death processes [1]. Two of these genes stand out, *ced-3* and *ced-4*, since they are essential for PCD. A third, *ced-9*, antagonizes the death activities of *ced-3* and *ced-4*. Remarkably, homologues of these genes have been found in various species.

The CED-3 protein is a member of a family of cystein proteases, known as caspases, which are generated as pro-enzymes that are activated upon cleavage by identical or other caspases (for reviews see [2; 3]). They mediate PCD by cleaving selected intracellular proteins, including proteins of the nucleus, nuclear lamina, cytoskeleton, endoplasmic reticulum, and cytosol. In mammals, this family comprises at least fourteen paralogues most of which are definitively implicated in apoptosis. Some of them, called initiator caspases, are activated during the initiation phase of the process while others, called executioner caspases, are activated during the degradation phase. To date, five members of the caspase family have been identified in *Drosophila*. Based on the characteristics of their prodomain, two of these, DREDD/DCP-2 [4] and DRONC [5], are thought to act as initiator caspases while the three others, DCP-1 [6], drICE [7] and DECAPY [8] would be executioner caspases.

More recent studies have identified orthologues of *C. elegans* CED-4. In mammals, Apaf-1 (Apoptotic protease-activating factor-1) has been shown to interact with caspase-9 and to trigger its proteolytic self-activation [9; 10]. Caspase-9 subsequently proteolyzes and thereby activates caspase-3, one of the execution caspases which kills the cell by cleaving key intracellular targets. However, there is a significant difference between the mechanisms for initiation of caspase activation mediated by CED-4 and Apaf-1. In contrast to CED-4, Apaf-1

requires cytochrome c, a mitochondria-derived apoptosis initiation factor which binds to the WD40 repeats at the C terminus of Apaf-1 protein. In *Drosophila*, Dark/Dapaf-1/HAC-1, an homologue of CED-4/Apaf-1 which contains WD40 repeats, also relays death signals to caspases [11-13]. The loss of Dark/Dapaf-1/HAC-1 function result in defective cytochrome c-dependent caspases activities and reduced apoptosis suggesting that an Apaf-1/cytochrome c-dependent apoptosis pathway exist in the fly.

The CED-9 protein belongs to a family of many members termed the Bcl-2 family in reference to the first discovered mammalian cell death regulator (for review see [14]). The Bcl-2 family proteins are involved in positive and negative regulation of apoptotic cell death (review in [15]). Among the anti-apoptotic members, Bcl-2 and Bcl-X<sub>L</sub> are negative regulators of cell death, preventing cells from undergoing apoptosis induced by various stimuli in a wide variety of cell types [16; 17], whereas others, such as Bax and Bid promote or accelerate cell death.

Proteins of the Bcl-2 family dimerize [18], although all possible heterodimers are not formed in the cells [19]. A widely accepted model postulates that homodimers of Bax promote apoptosis [15; 20-23], and that the functional effect of Bcl-2 related proteins is to form competing heterodimers with Bax which are unable to promote apoptosis [24; 25]. However, it seems that Bcl-2 and Bax are both able to regulate apoptosis independently [26; 27].

In both worm and mammalian cells, the anti-apoptotic members of the Bcl-2 family act upstream of the executioner caspases, somehow preventing their proteolytic processing into active killers [28; 29]. Two main mechanisms of action have been proposed to connect Bcl-2 to caspases. In the first one, pro-survival proteins function by directly inhibiting the ability of CED-4 like proteins to activate caspases in a ternary complex. This model arose from the elucidation of the role of CED-4 protein in the nematode [30]. An equivalent ternary

complex was found to be present in mammalian cells involving Apaf-1 [9], caspase-9 and Bcl-X<sub>L</sub>, in which Bcl-X<sub>L</sub> inhibits Apaf-1-mediated maturation of caspase-9 [31]. However, since some Bcl-2 family members inhibit apoptosis in the absence of significant binding to Apaf-1, it has been suggested that, in mammals, the antiapoptotic Bcl-2 homologues do not act by sequestering Apaf-1 [32]. The second way that links Bcl-2 to caspases is more indirect and involves its membrane association. Indeed, Bcl-2 mutants with restricted subcellular location reveal distinct pathways for apoptosis depending on cell type [33]. When bound to the endoplasmic reticulum membrane, Bcl-2 would be involved in the maintenance of calcium homeostasis [34-36], while its nuclear association would modulate protein subcellular trafficking through nuclear pores [37]. However, most of the data available involves mitochondria, which appears today as the central executioner of programmed cell death [28; 38-40]. Indeed, a growing number of data show that mitochondrial membrane-associated Bcl-2 acts by regulating the release of some apoptotic factors usually sequestered in the mitochondrial intermembrane space. These factors include AIF (apoptosis inducing factor), that on its own causes chromatin condensation and large-scale fragmentation of DNA [41], caspases -2 and -9 [42] and cytochrome c which, in combination with Apaf-1 can promote caspase-9 activation [9].

Until recently, although caspases had been found in *Drosophila*, neither Apaf-1 homologues nor Bcl-2 family homologues were known. In contrast, a genetic screen in *Drosophila* allowed the identification of three new apoptosis activators yet unknown in mammals: *rpr*, *hid* and *grim*, which are all located in the 75C region of the *Drosophila* genome. Alone or collectively, these genes are involved in cell death in the *Drosophila* embryo [43; 44]. All three genes encode proteins harboring at their N-terminus a 14 amino acid sequence referred to as the RHG motif. *Rpr*, *Hid* and *Grim* are transcriptionally regulated by a variety of death-inducing stimuli, including steroid hormones and DNA damaging

agents. The function of *Hid* is negatively regulated by Ras activation at both the RNA and protein levels suggesting that it is a sensor of Ras-mediated survival signaling. These three genes kill by activating a caspase-dependent apoptosis.

Study of baculovirus inhibitor of apoptosis has led to the discovery of a novel family of apoptosis regulators that interact physically and functionally with Rpr, Grim and Hid: the inhibitors of apoptosis proteins (IAPs) [45]. Proteins of this family have been found in mammals, flies, nematodes and yeast and it has been proposed that *rpr*, *grim*, and/or *hid* promote apoptosis at least in part by disrupting IAP-mediated inhibition of caspase activity [46]. Alternatively, IAPs could function as inhibitors of *rpr*, *grim*, and/or *hid* by preventing their access to yet undiscovered effectors [44].

Since each group of these key inducers and regulators of apoptosis were found in separate taxons, it remained possible until recently that there were distinct mechanisms for the control of apoptosis between flies and mammals. However, *rpr*, *hid* and *grim* were found to induce apoptosis in mammalian cells [47-51], while *ced-3* [52] and *ced-4* [53] can induce cell death in fly tissues, thereby favoring the idea that the core component of the cell death machinery has been highly conserved during evolution. However, mammalian homologues of *rpr*, *hid* and *grim*, as well as *Drosophila bcl-2* anti-apoptotic homologues remain to be identified.

With the aim to test whether a Bcl-2 pathway exists in the fruit fly, we have studied the effects of expressing mammalian *bcl-2* and *bax* in *Drosophila*. In order to drive the production of these proteins, we have used the UAS/GAL4 expression system that allows the specific expression of the transgene in a specific location and at a specific time [54]. The results presented in this paper show that mammalian *bax* and *bcl-2* genes are functional in *Drosophila* during development and upon X-ray irradiation in the embryo, suggesting that a Bcl-2 pathway exists in the fly.

## Results

### **Bcl-2 prevents developmental and X-ray induced cell death during *Drosophila* embryogenesis.**

During normal *Drosophila* embryogenesis, significant cell death first appears during germ band shortening and continues throughout development with a very dynamic pattern [55; 56]. In order to address the ability of mammalian Bcl-2 to participate in apoptosis during *Drosophila* development, we assayed whether expression of mammalian Bcl-2 could counteract cell death patterns in normal or X-ray treated embryos. Therefore, we generated transgenic flies carrying a human *bcl-2* cDNA placed under the control of UAS sequences [54]. Transgenic flies carrying the P[UAS-*bcl-2*] construct were crossed to flies expressing the GAL4 protein under the control of regulatory sequences of the *daughterless* gene (*da-GAL4*). This driver allowed us to induce ubiquitous expression of the transgene during development of the fly. Since the baculovirus caspase inhibitor protein p35 had already been shown to protect most cells from apoptosis in *Drosophila* embryos [57], we constructed a transgenic line carrying the coding sequences of p35 under the control of UAS sequences and used this line as a control. Cell death was analyzed at all stages of embryonic development using acridine orange (AO) staining *in vivo*. Embryos expressing *bcl-2* fail to exhibit most of the cell death pattern normally seen during mid-embryonic development, in a manner similar to that observed with UAS-p35 (Figure 1A-C). Cell death appears particularly reduced in the lateral part of the cephalic region as well as in the ventral portion of the embryo during germ band retraction. The number of dying cells throughout the central nervous system is also reduced as the ventral nerve cord condenses (Figure 1D, E). Some AO-stained cells remain visible, for instance in the nerve cord, when Bcl-2 expression is driven by *da-Gal*, whereas no cell specificity of this driver was detected using a UAS-GFP reporter gene. In addition,



several P[UAS-Bcl-2] lines gave identical results. This result suggests a possible cell-specificity of Bcl-2 in counteracting cell death in the *Drosophila* nervous system.

The reduction of cell death induced by Bcl-2 expression affects the viability of the whole organism in a dose-dependent manner. P[UAS-*bcl-2*] / + heterozygous are sub-viable, for all P[UAS-*bcl-2*] transformant lines tested. This result suggests that a low level of expression from the P[UAS-*bcl-2*] construct is sufficient to affect development. When *bcl-2* expression is induced by *da*-GAL4, the percentage of lethality increases from 25% (47/188) to 37% (66/179) ( $P < 4.10^{-4}$ ). Lethality occurs at earlier stages upon increasing the dose of *da*-GAL4 or P[UAS-*bcl-2*] ( $P < 4.10^{-2}$ ), ranging from mostly larval death to embryonic death.

We tested the ability of mammalian Bcl-2 to inhibit radiation-induced cell death in *Drosophila* embryos. Two hours after egg laying (AEL) at 25°C, embryos were exposed to 4000 rads X-irradiation, then aged for four hours, collected and stained with AO. Using this protocol, Abrams *et al.* obtained 100% embryonic lethality, accompanied by an increase in the number of AO-labeled cells displaying the ultrastructural features of apoptosis [55]. The pattern of cell death in *da*-GAL4 / P[UAS-*bcl-2*] embryos was compared to that of wild-type embryos exposed to the same conditions. Figure 2 shows that ubiquitous expression of *bcl-2* significantly reduces cell death induced by X-rays in all regions of the embryos.

In conclusion, *bcl-2* expression is sufficient to counteract both developmental and X-ray induced cell death in embryos. This result indicates that partners of Bcl-2/CED-9 exist in many embryonic *Drosophila* tissues.

### **Bax induces embryonic cell death and lethality in *Drosophila***

In order to determine whether mammalian Bax is sufficient to kill cells in *Drosophila*, the murine *bax* cDNA was placed under the control of UAS sequences and expressed under the control of various GAL4 drivers. P[UAS-*bax*] / *da*-GAL4 embryos were collected, stained

with AO and compared to wild-type embryos collected at the same time. The expression of *bax* affects the pattern of programmed cell death throughout embryonic development. For instance, from stage 11 to 13, the segmental pattern of dying cells along the antero-posterior axis normally found in wild-type embryos is disorganized (Figure 1F). Intensely stained cell-aggregates can be seen in *bax*-expressing embryos. These clusters likely result from engulfment of the apoptotic bodies by macrophages [55]. The morphology of these embryos is hardly recognizable, and most of them die before hatching. Only rare adults are obtained.

Viability of *bax* expressing embryos was tested using several other drivers. The use of ubiquitous drivers such as *actin-GAL4* or *hs-GAL4* without heat shock (at 18°C) leads to embryonic lethality (data not shown). Other GAL4 drivers with a more restricted pattern of expression like *eyeless-GAL4*, *pannier-GAL4*, *vestigial-GAL4* or *engrailed-GAL4* (*en-GAL4*) also induce lethality (data not shown). The latter result indicates that weak expression of *bax* in vital tissues is sufficient to kill the embryos. In fact, P[UAS-*bax*] / + flies are sub-viable, for all four of the transgenic lines obtained. These data suggest that the UAS-*bax* construct has a low level of expression independent of GAL4 induction. Flies homozygous for UAS-*bax* have a two days lifespan and reduced fertility. Furthermore, these flies exhibit melanotic masses at larval and adult stages (Figure 3). These masses may result from an immune response to the massive programmed cell death induced by *bax*, abnormal tissues being isolated by haemocytes from the organism. Alternatively, *bax*-induced cell death might lead to the aberrant differentiation of some neighboring cells up to a state where they become recognized as "nonself", and therefore eliminated.

### **Mammalian Bax and Bcl-2 regulate cell death during imaginal development**

In order to assay for Bax and Bcl-2 effect on cell viability during development in *Drosophila*, the eye and wing imaginal discs provide an appropriate model since their

alteration may lead to viable phenotypes where loss or gain of cells are visible. Targeted expression of *bax* and *bcl-2* in the eye was achieved by crossing the P[UAS-*bax*] or P[UAS-*bcl-2*] lines to *GMR-GAL4* flies. The GMR promoter sequences fused to the GAL4 gene drives expression of the GAL4 protein in the eye imaginal disc during larval development [58]. In order to express *bcl-2* and *bax* specifically in the developing wings, transgenic flies carrying P[UAS-*bcl-2*] or P[UAS-*bax*] were crossed to a line in which GAL4 is expressed under the control of part of the *vestigial* (*vg*) promoter. The expression pattern of this driver is revealed by crossing flies carrying *vg-GAL4* to flies carrying UAS-GFP. This part of the *vg* promoter drives the expression of GAL4 during development in a limited portion of the wing imaginal disc corresponding to the margin of the adult wing (Figure 4A).

Eye specific expression of *bax* alters the ordered array of ommatidia and bristles, normally found in the wild type (Figure 5A, C). This phenotype is observed in 45% of heterozygous adult flies of the P[UAS-*bax*] / +; *GMR-GAL4* / + genotype. At a higher magnification, smaller ommatidia can be observed (Figure 5B, D). This defect in ommatidial organization is reminiscent of that observed in transgenic flies mis-expressing *rpr*, *hid*, *grim* or *ced-4* [53; 59-61]. The presence of small ommatidia may result either from the defective proliferation of photoreceptor precursor cells, or from the loss of cells during morphogenesis. Semi-thin sections through the adult eye of P[UAS-*bax*] / +; *GMR-GAL4* / + flies were made in order to address this question. Figure 6A, B shows that many ommatidia are missing in these flies, the remaining losing some of the pigments cells, photoreceptors and/or cone cells. Holes and vacuoles are also observed indicating a massive cell death. These results show that *bax* induces cell death in the eye without any detectable cell-type specificity.

Using all P[UAS-*bax*] transgenic lines, we showed that induction of the expression of *bax* by *vg-GAL4* reduces the size of the wings, in a temperature-dependent manner (Figure 7). At 18°C (Figure 7B, C), most of the flies have notched wings, while at 21°C (Figure 7D), the

wing phenotype ranges from notched to absent wing. In some cases, the wing appears as a tubular structure, probably because the ventral and dorsal layers which make up the wing did not merge. At 25°C, most of the flies exhibit a gnarled or wrinkled wing phenotype (Figure 7E). Since the GAL4 system is more active at higher temperature, the temperature effect on the strength of the phenotype is most likely due to the increase in the dose of Bax protein, thereby indicating that *bax* expression induces cell loss in the wing during development in a dose-dependent manner.

In the third instar larval imaginal wing disc, *bax* expression under the control of *vg-GAL4* at 18 °C induces a modification of the size and the shape of the disc (Figure 4A-B). At 21 °C, the wing part of the disc is reduced in size and highly disorganized (Figure 4C-D). *bax*-expressing discs are altered specifically in the same area as that labeled by a UAS-GFP marker when driven by *vg-GAL4* (arrowheads Figure 4A, D), thereby indicating that the absence of parts of the wing in adults is likely due to the loss of cells in *bax*-expressing imaginal cells. Taken together, our results in the eye and wing discs suggests that mammalian Bax functions as a pro-apoptotic gene in *Drosophila* when expressed during imaginal development.

The expression of *bcl-2* in the eye disc leads to a rough eye phenotype in 45% of heterozygous flies of the P[UAS-*bcl-2*]/+; *GMR-GAL4*/+ genotype. This phenotype is enhanced and affects 100% of the flies expressing two doses of the UAS-*bcl-2* transgene under the control of the *GMR-GAL4* driver. Induction of the UAS-*bcl-2* transgene results in a type of eye alteration that differs from that observed with the UAS-*bax* transgene (Figure 5). Flies carrying two copies of *bcl-2* under the control of the *GMR-GAL4* driver have supernumerary ommatidia as well as larger ommatidia when compared to wild-type flies (Figure 5E, F). These defects might result from alterations in cell differentiation, or from the inhibition of cell death induced by mammalian Bcl-2 expression. In order to address this

question, cross-sections through the eye were performed (Figure 6C), revealing a disorganized morphology of the retinal cell layer when compared to the regular pattern of the wild type. An increased spacing corresponding to additional cellular material between ommatidia is observed next to R1-R6 for each ommatidial unit. The position of this extra material correlates with the position where some of the pigment cells normally die by apoptosis in the pupal retina (Figure 6D-E, [62]). This result was confirmed by examination of phalloidin staining of pupal eye discs (data not shown), revealing the presence of extra pigment cells at 70 hours after pupariation at 21°C. A normal complement of photoreceptor cells is always observed, ruling out an effect for *bcl-2* in the photoreceptor differentiation pathway. This phenotype is also very similar to that observed in loss of function *hid* mutant flies [60], thereby indicating that Bcl-2 alters the organization of the eye by inhibiting apoptosis of pigment cells in the developing eye.

The expression of *bcl-2* in the wing disc under the control of *vg-GAL4* driver had no detectable effect. According to the study of developmental cell death in the wing imaginal disc by Milán *et al.* [63], except at larval molt, only rare cells die and in the future wing margin, where the *vg-GAL4* driver is active, less than 2% of the cells die. Therefore, this result indicates that expression of mammalian Bcl-2 by itself does not alter cell differentiation in the developing wing.

### **Bcl-2 can inhibit Bax-induced cell death**

We assayed whether Bcl-2 and Bax retain functional interactions in the fly as they do in mammalian cells [64]. When *bcl-2* and *bax* are ubiquitously co-expressed in embryos using *da-GAL4* (Figure 1 G), the pattern of cell death usually observed in *bax*-expressing embryos is altered. The pattern of cell death in these embryos is very close to that observed in *bcl-2* expressing embryos, thereby indicating that *bcl-2* expression can counteract the cell death

induced by expression of *bax* in *Drosophila* embryos. The antagonistic activity of Bcl-2 was also tested by assaying the viability of *vg-GAL4 / P[UAS-*bax*]* flies in the presence or in the absence of *P[UAS-*bcl-2*]* (Figure 8A). Since the efficiency of the UAS/GAL4 system depends on temperature, we raised the flies at 18, 21 and 25°C. No significant effect of Bcl-2 was detected in this study at 18°C ( $P > 3 \cdot 10^{-1}$ ). To the contrary, Bcl-2 was able to rescue the lethality of *bax*-expressing flies at 21°C (146% of *vg-GAL4 / P[UAS-*bax*]* viability,  $P < 2 \cdot 10^{-4}$ ) as well as at 25°C (217% of *vg-GAL4 / P[UAS-*bax*]* viability,  $P < 5 \cdot 10^{-5}$ ).

We assayed for the strength of the wing phenotype of *vg-GAL4 / P[UAS-*bax*]* flies in the presence or in the absence of *P[UAS-*bcl-2*]* (Figure 8B). No significant effect of Bcl-2 was observed at 21 and 25°C (data not shown). To the contrary, *bcl-2* expression at 18°C induced a decrease of the ratio of strong wing phenotypes as opposed to (intermediate + weak) phenotypes ( $\chi^2 = 14.8$ ,  $P < 2 \cdot 10^{-4}$ ) and an increase of the ratio of weak wing phenotypes as opposed to (intermediate + strong) phenotypes ( $\chi^2 = 22.1$ ,  $P < 3 \cdot 10^{-6}$ ).

Whereas *bax*-expression leads to structural modifications of the wing discs, co-expression of *bax* and *bcl-2* restores partially the structure of wing discs at 21°C (Arrowheads, Figure 4D-E). Therefore, the lack of effect of Bcl-2 over Bax-induced wing phenotype in adults at higher temperature is certainly due to a high level of expression of Bax at 21°C and higher temperatures leading to massive cell death. The organization of the disc might be too affected for the rescued cells to build a structure with an aspect closer to that of the normal wing.

## Discussion

In this paper, we show that mammalian Bcl-2 and Bax are functional *in vivo* in *Drosophila*. Bcl-2 reduces cell death during embryogenesis and in the developing eye, whereas Bax increases the number of dying cells and deregulates the normal cell death pattern both in embryos and in the developing eye and wing discs. Increasing the number of copies of the transgenes results in more severe lethality and phenotypes. In addition, Bcl-2 antagonizes Bax function in *Drosophila* embryos and in the imaginal wing disc. It appears thus that the effectors of the Bcl-2 and Bax pathways are present in the flies. The recently identified DROB-1 [65] is pro-apoptotic. *Drosophila* anti-apoptotic homologues of Bcl-2 as well as upstream activators of Bcl-2 homologues in *Drosophila* remain to be characterized.

Among potential partners of Bcl-2 and Bax in *Drosophila* are the products of *rpr*, *hid*, and *grim*. Although no Bcl-2 homologue with anti-apoptotic function has been isolated yet in *Drosophila*, and no homologue of *rpr*, *hid*, and *grim* has been found in mammals, the use of heterologous systems allows to study possible interactions between the two pathways. GRIM-induced apoptosis of mammalian cultured cells is antagonized by Bcl-2 in a dose-dependent manner [49], suggesting that the two pathways interact. Furthermore, Bcl-2 has been shown to rescue RPR-induced caspase activation in an *in vitro* amphibian model, thereby suggesting that a *rpr* homologue might act upstream of Bcl-2 in vertebrates [66]. However, since in a reciprocal experiment, mammalian and nematode Bcl-2 proteins poorly counteract RPR-induced cell death in *Drosophila* and lepidopteran cells [50; 51], it was suggested that at least part of the downstream apoptotic events induced by RPR in vertebrates are different from those occurring in insects.

Our finding that the *Drosophila* apoptotic machinery recognizes and responds to mammalian Bcl-2 protein suggests that the molecular components that function *via* the Bcl-2 protective cascade are conserved in *Drosophila*. The simplest hypothesis is that a *Drosophila*

homologue of Bcl-2 controls cell death during embryogenesis and that mammalian Bcl-2 can mimic its effect.

Since most of the embryonic cell death in *Drosophila* is under the control of *rpr*, *grim* and *hid* [67], it is likely that these regulators are implicated in the same cell death pathway as *bcl-2*. We have shown that Bcl-2 is also able to prevent ectopic cell death in embryos submitted to X-rays. Since X-ray-induced apoptosis is probably mediated by *rpr* [68], it appears, based on our results, that Bcl-2 is able to prevent *rpr*-induced apoptosis, *in vivo*, in *Drosophila* embryos. The involvement of both Bcl-2 and RPR in ectopic and developmental cell death makes it unlikely that Bcl-2 acts in an alternative cell death pathway. At least two possible explanations can reconcile our *in vivo* observations on Bcl-2 inhibition of apoptosis with experiments showing that Bcl-2 weakly prevents apoptosis triggered by *rpr* over-expression. One explanation would be that Bcl-2 acts upstream of *rpr* or that they are mutually antagonistic, controlling the same target. In this case, a competition could exist in favor of the over-expressed *rpr*. An alternative scenario, which is more consistent with the results obtained in mammals, is that Bcl-2 controls a step essential for *rpr*-induced apoptosis, acting downstream of RPR.

The Bcl-2 protein is localized in the outer mitochondrial membrane. Several reports suggest that Bcl-2 may block apoptosis by preventing the release of cytochrome c from mitochondria [69; 70]. In mammalian cells, the *Drosophila* GRIM protein localizes initially in the cytoplasm but accumulates progressively in the mitochondria during apoptotic cell death. The expression of Bcl-2 in this system blocks GRIM-induced apoptosis in a dose dependent manner [49], which suggests that the balance of their mutually antagonistic functions either promotes or inhibits caspase activation. Although it is not known whether Bcl-2 can modify GRIM localization, this result indicates that, in mammalian cells, GRIM and Bcl-2 can act at the same level in the apoptotic cascade. It has also been shown in insect cells that while the



wild type Bcl-2 protein protects cells from apoptosis induced by a baculovirus infection, Bcl-2 deleted from its C-terminal domain is unable to do so, and no longer localizes to mitochondria [71]. These results show that Bcl-2 inhibits baculovirus-induced apoptosis of insect cells and underlines the importance of its mitochondrial localization for its functionality. A specific alteration of cytochrome c configuration was detected in cultured *Drosophila* cells expressing *rpr* or *grim*. Furthermore, it has been shown that altered cytochrome c display precedes apoptotic cell death in *Drosophila* in ovaries and that this alteration is provoked in a caspase-dependent manner by the death activators *rpr* or *grim* [72]. By analogy with results from studies in mammals, one can hypothesize that mitochondrial events induced by *rpr* and *grim* propagate an apoptotic signal by functioning with a caspase in a Bcl-2 inhibitable positive feedback amplification loop.

Cell-specific inhibition of apoptosis by Bcl-2 in the nerve cord may reflect the conservation of the mammalian activity of the protein since it has already been shown *in cellulo* and *in vivo* that Bcl-2 is unable to inhibit cell death in several types of neurons [73-76]. In addition, since Bcl-2 irradiated embryos are very comparable to non-irradiated Bcl-2 embryos, it appears that Bcl-2 activity is greater in irradiated cells than in non-irradiated cells. Lastly, no head involution defect was detected in embryos ubiquitously expressing two doses of Bcl-2 (data not shown), which contrasts with what is observed in *rpr*, *hid* and *grim* mutant embryos. Together, these results suggest a possible specificity in the pathway in which Bcl-2 is active. Distinct cell killing mechanisms were also proposed to explain cell specificity and specificity of upstream and downstream events displayed with *hid*, *grim* and *rpr* over-expression during embryogenesis [77].

In contrast to the ubiquitous induction of *bcl-2*, ubiquitous induction of *bax* is lethal in most embryos. *bax*-expressing embryos show a significant amount of ectopic cell death. Cells that are abnormally eliminated cannot be replaced, for example by hyper-proliferation of

neighboring cells due to early determination and cell division arrest during embryogenesis. This make irreversible the lethality induced by Bax.

For the moment, it is not clear, in vertebrates as well as in *Drosophila*, whether Bax counteracts the anti-apoptotic activity of Bcl-2 family protein or if *bax* expression activates programmed cell death on its own. In our study, all the tested tissues seem sensitive to *bax* expression. Therefore, unlike Bcl-2, RPR and GRIM, Bax does not seem to have a tissue-specificity, suggesting that Bax is active by itself or that all the cells of the fly contain downstream partners of Bax. The effect observed when *bax* is expressed in the eye is weak when compared to that described upon ectopic expression of *rpr*, *hid* or *grim* [59; 61; 78]. However in these latter cases the genes were directly under the control of the GMR promoter, which could result in a higher expression level. Indeed we observed that *hid* and *rpr* expression under the control of *vg*-GAL4 leads to weaker phenotypes than *bax* does (data not shown). Therefore, Bax, RPR, and HID appear to have comparable effect as apoptotic inductors.

In conclusion, data presented here show that *bcl-2* and *bax* mammalian genes are functional *in vivo* in *Drosophila* flies. Therefore, both pro- and anti-apoptotic functions of Bcl-2 family proteins may be evolutionarily conserved in Metazoans, establishing *Drosophila* as an appropriate animal model for studying the control of apoptosis by proteins of the Bcl-2 family.

## Materials and methods

### Fly stocks

Flies were raised on standard *Drosophila* medium at 21°C. Flies carrying *da*-GAL4, *vg*-GAL4 and *GMR*-GAL4 transgenes are described in [79], [80] and [58], respectively. *panier*-Gal4, *actin*-Gal4, *hs*-Gal4, and *en*-Gal4 were generously provided to us by Dario Coen, and *eyeless*-GAL4 by Sébastien Szuplewski. A  $w^{1118}$  stock was used as our reference strain.

### Production of transgenic flies

Flies carrying UAS-*bcl-2*, UAS-*bax* or UAS-*p35* were generated by *P*-element transformation. The *EcoRI*-*EcoRI* fragment of the human *bcl-2* cDNA (subcloned from plasmid SFFV-Bcl-2 nl, provided to us by Dr. SJ Korsmeyer), the *EcoRI*-*XbaI* fragment of the murine *bax* cDNA (GenBank L22472) and the *EcoRI*-*EcoRI* fragment of the baculovirus *p35* cDNA (GenBank M16821) containing the full-length coding region were ligated into the corresponding restriction sites in the UAS vector PUASt [54]. The resulting plasmids were purified with Qiagen tips and injected into  $w^{1118}$  embryos using standard procedures [81; 82]. Several independent transformant lines showing similar phenotypes were obtained. Ten transformant strains were obtained for *bcl-2*, and nine for *p35*. One transformant strain was obtained for *bax*. Three new insertions were subsequently obtained *via* mobilization using  $\Delta 2-3$  as a source of transposase [83].

### Staining of embryos with acridine orange

Acridine orange staining to detect apoptotic cells was performed by methods previously described [55], except that embryos were dechorionated five minutes in 1X bleach, rinsed with water and shacked five minutes in an equal volume of heptane and 0.25  $\mu\text{g/ml}$

acridine orange in 1X PBS. Embryos removed from the interface were mounted in mineral oil (SIGMA). Samples were viewed with a conventional fluorescence research microscope DMRHC Leica using I3 filter to detect green fluorescence or with a confocal microscope (Biorad MP 1024).

### **Irradiation of embryos**

Two hours after egg laying (at 25°C), *w*<sup>1118</sup> and *da-GAL4 / P[UAS-*bcl-2*]* embryos were simultaneously exposed to 4000 rads of X-irradiation. This protocol reduces hatching frequency to 0%. Embryos were then aged for 3-4 hours at 25°C and observed as described above.

### **Viability experiment**

Twelve hours after egg laying, embryos were collected and counted. After 36 hours of development at 25°C the number of dead embryos was counted. Six and eleven days after egg laying, pupae and adult flies were counted. Statistical analysis was performed using a  $\chi^2$  test.

### **Electron microscopy and eye sections**

For electron microscopy analyses, adult *Drosophila* were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 hours at room temperature. Adult flies were then dehydrated through a graded ethanol series with 24 h incubations at each step, and critical point dried. Samples were sputter-coated with platinum and examined and photographed by Dr. Jeril Degrolard at the INRA, Unité de Biologie du Développement, Jouy-en-Josas, France. The preparation of semi-thin sections of adult heads were performed as described [84]. Images were collected on a Nikon Eclipse E800 microscope.

## **Acknowledgements**

We thank Anne-Marie Pret for her critical reading of the manuscript. We are indebted to Jeryl Degrolard (INRA, Jouy-en-Josas) who performed electron microscopy studies, Jacqueline Mesnard for expert technical assistance with eye sections and Michel Laurent (UPS, Orsay) for confocal microscopy analysis. This work was supported in part by grants from the Association pour la Recherche contre le Cancer (#9517). SG was a recipient of a fellowship from the Ligue Nationale Contre le Cancer. We are grateful to the Conseil régional d'Ile-de-France, the Association pour la Recherche contre le Cancer, the Ligue Nationale Contre le Cancer, and the Fondation pour la Recherche Médicale which all contributed financial support towards equipment used in our laboratories.

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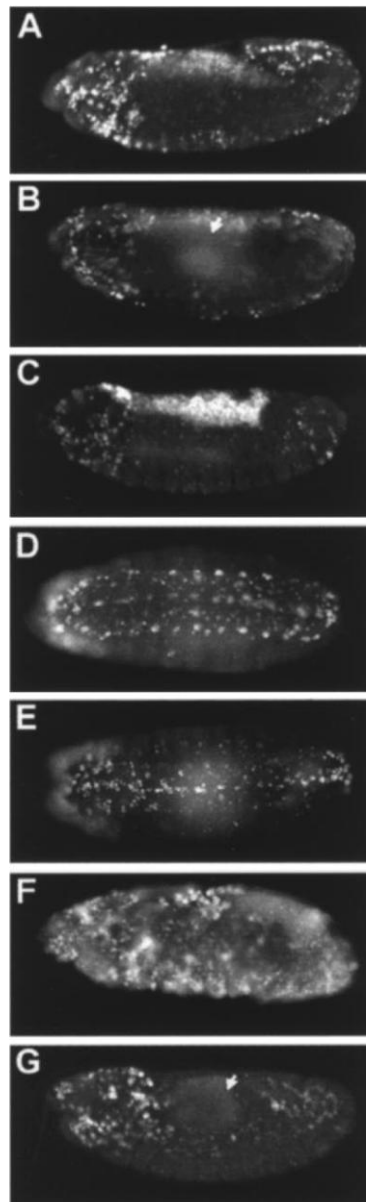
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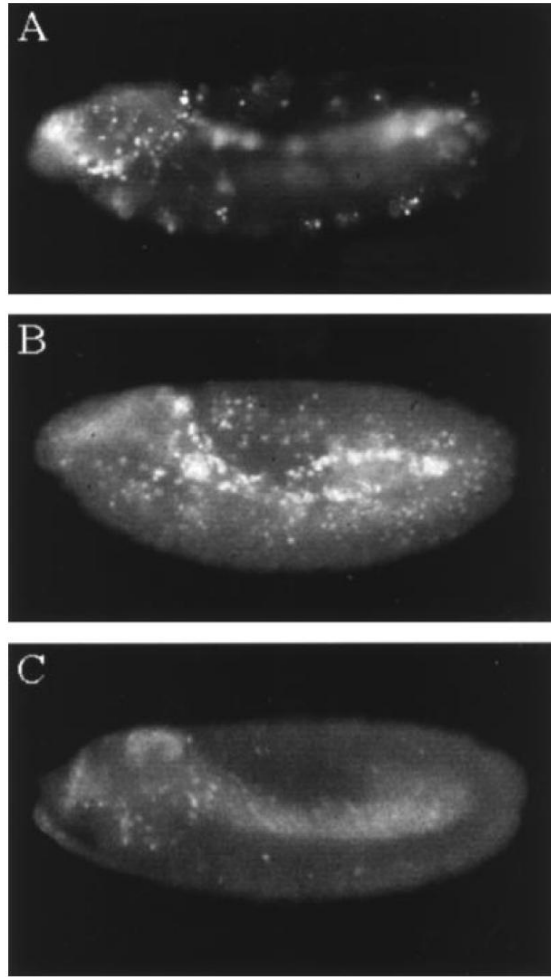
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## Figures

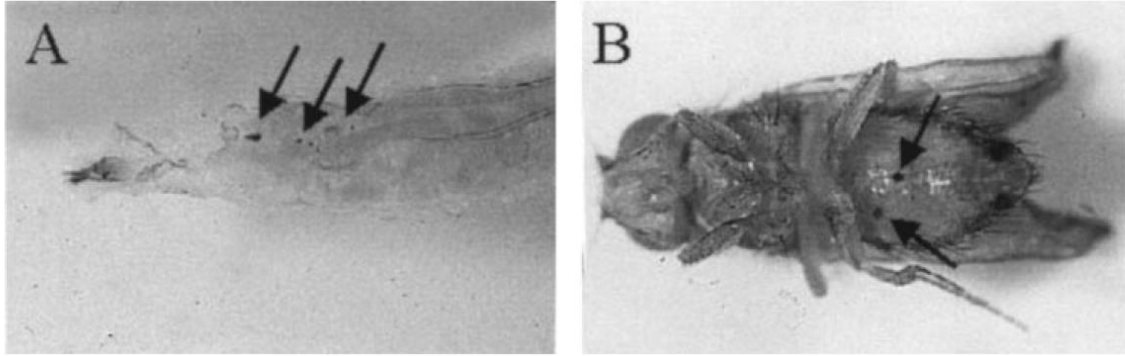


**Figure 1** *bcl-2* and *p35* expression inhibit developmental cell death, while *bax* expression disorganizes the normal cell death pattern in embryos. Apoptosis is visualized by acridine orange staining. Embryos were collected 9-11 hours AEL (A, B, C, F, G) or 12-15 (D, E) hours AEL at 25°C. All views are sagittal with anterior to the left and dorsal towards the top except D and E views which are ventral views. (A, B, C, G) Images generated by confocal microscopy. (D, E, F) Images generated using a fluorescence microscope. (A, D) wild type. (B) P[UAS-*p35*]/+; *da-GAL4*/+. (C, E) P[UAS-*bcl-2*]/+; *da-GAL4*/+. (F) P[UAS-*bax*]/+; *da-GAL4*/+. (G) P[UAS-*bcl-2*]/+; *da-GAL4*/P[UAS-*bax*]. Regions of diffuse staining correspond to yolk autofluorescence (arrows). Despite their differences in morphology, all embryos are age-matched with controls. Note the suppression of naturally occurring cell death in the *bcl-2* expressing embryo (C, E) as in the control embryo expressing *p35* (B). Expression of *bax* disorganizes the pattern of cell death and increases the number of AO-stained cells (F). In addition, these embryos often show abnormal morphology.

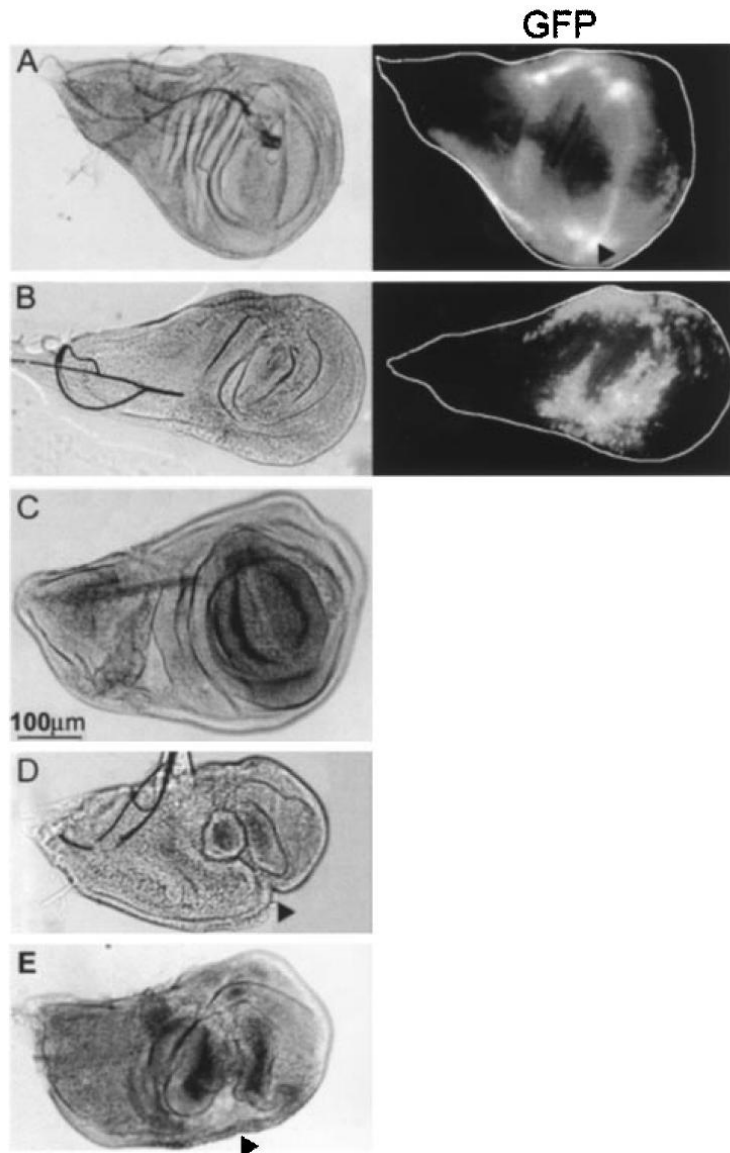


**Figure 2** Bcl-2 inhibits ectopic cell death in irradiated embryos. Embryos were stained with AO. (A, B) wild type. (C) P[UAS-*bcl-2*] / *da-GAL4*. Embryos in (B) and (C) were exposed to 4000 rads of X-rays 2 hours AEL to induce ectopic cell death, and then aged before staining. AO-staining reveals extensive induction of apoptosis in wild type embryos after X-irradiation as shown in panel B. *bcl-2* expression strongly suppresses cell death throughout the entire embryo. Note that cell death is also less prevalent in irradiated *bcl-2* expressing embryos than in wild type non-irradiated embryos and comparable to that observed in *bcl-2* non-irradiated embryos. All views are sagittal with anterior to the left and dorsal towards the top.

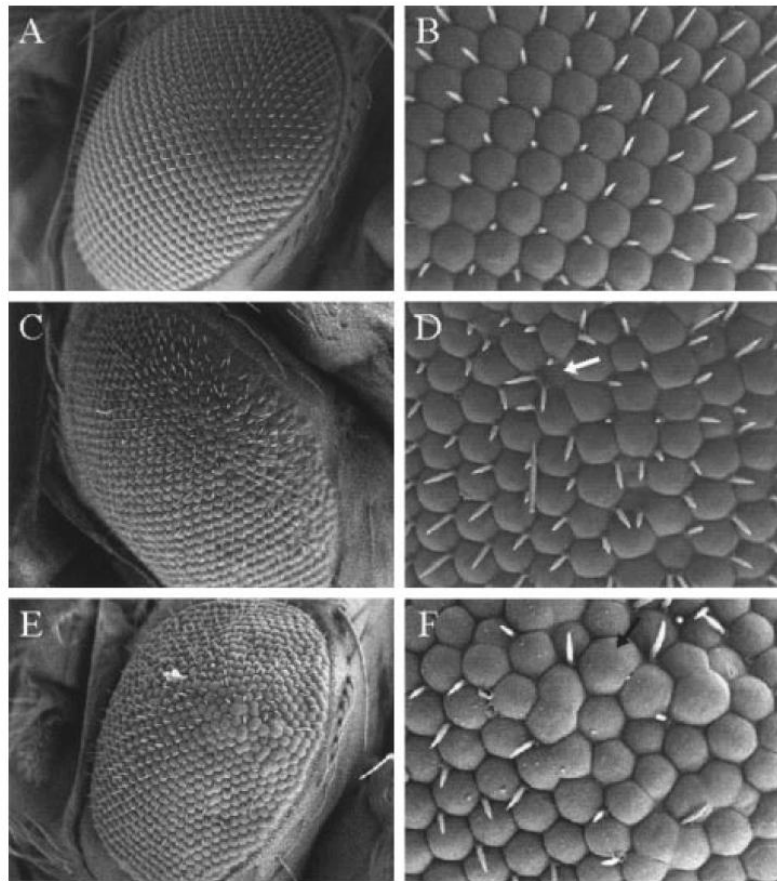




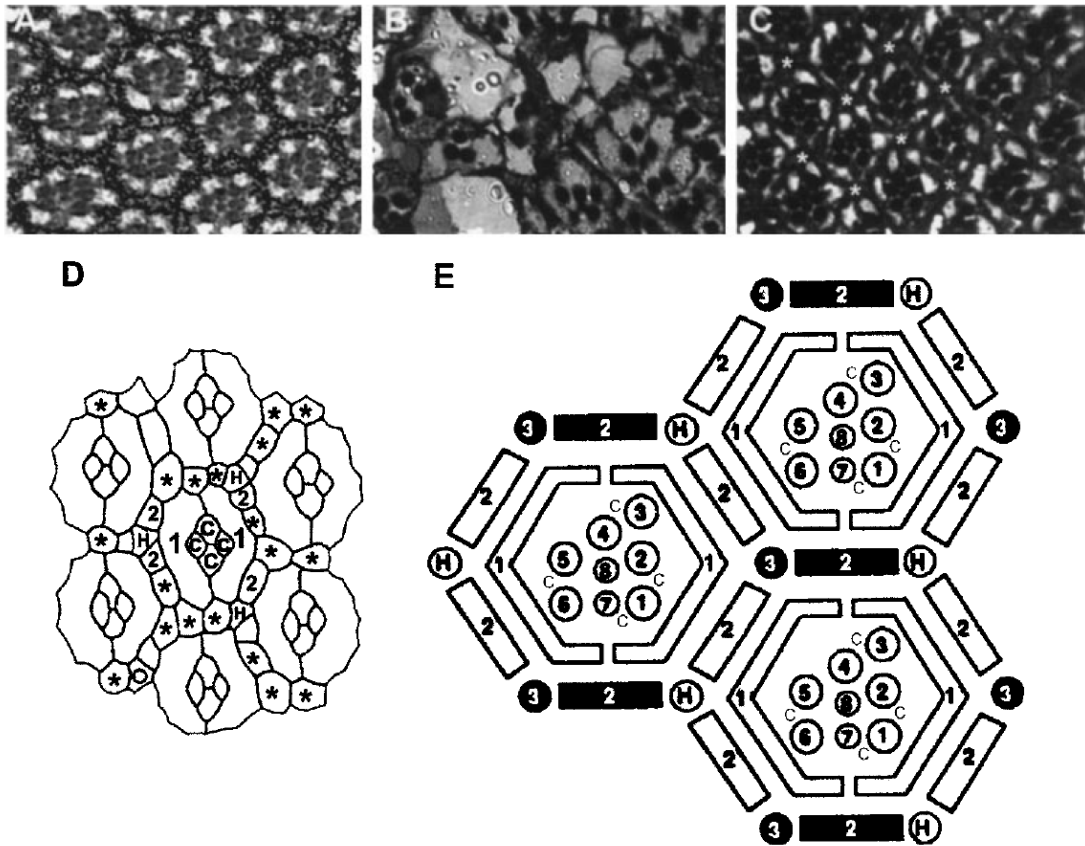
**Figure 3** Low doses of Bax induce melanotic masses in all P[UAS-*bax*] / P[UAS-*bax*] individuals. Homozygous larvae and flies were recognized by using a TM6C,*Tb* (or CyO,*act-GFP*) balancer chromosome. Arrows indicate melanotic masses found in a third instar P[UAS-*bax*] / P[UAS-*bax*] larva (A) and in a two days old adult fly (B).



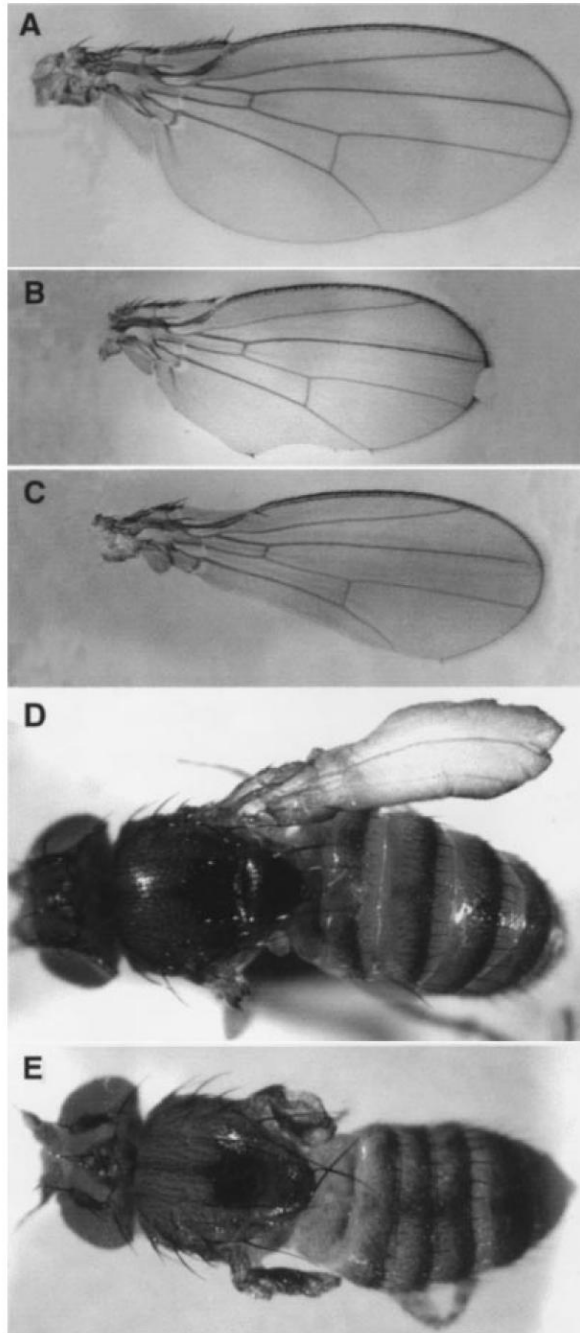
**Figure 4** *bax*-induced cell death in the wing imaginal disc. (A, B) Wing imaginal disc of *vg-GAL4 / UAS-gfp* (A) or *vg-GAL4 / UAS-gfp; UAS-bax / +* (B) fly raised at 18°C. Images visualized under fluorescence on the right panels correspond to the bright field images of the left panels. Arrowhead in (A) indicates the region where UAS-GFP is the most expressed. Note that the structure of the *bax*-expressing disc is weakly affected at this temperature and that the green fluorescence appears punctuated. (C, D, E) Inhibition of *bax*-induced loss of structure by Bcl-2 in wing imaginal discs. Bright field images of wing imaginal disc of wild type (C), *vg-GAL4 / UAS-bax* (D) or *vg-GAL4 / UAS-bax; UAS-bcl-2 / +* (E) fly raised at 21°C. (D) Alterations of the structure of the wing disc is restricted to the GFP expression domain observed in (A). Arrowhead indicates the notched part of the wing disc which corresponds to the region where UAS-GFP is the most expressed in (A). (E) When *bcl-2* and *bax* are co-expressed, the structure of the wing disc is partially restored. An arrowhead indicates the position of a small notch in the wing disc.



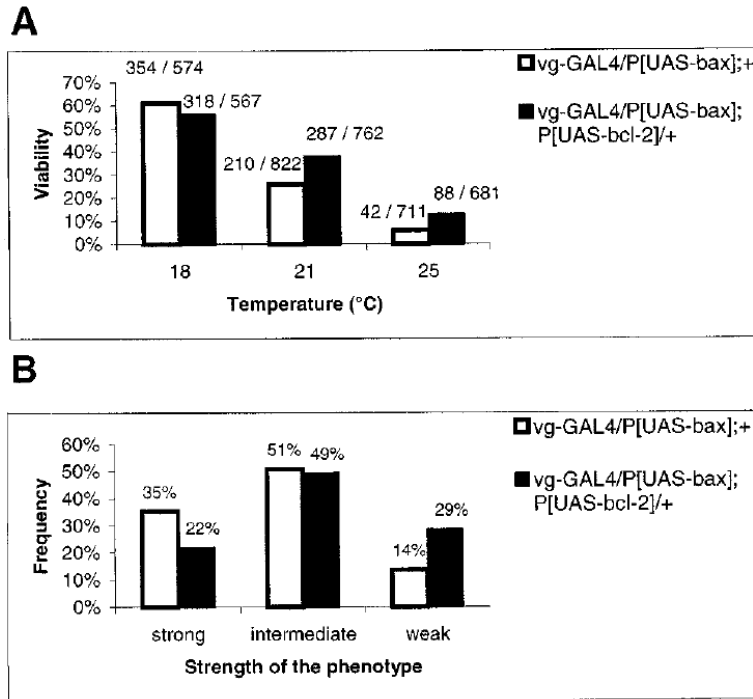
**Figure 5** *bcl-2* and *bax* expression affect the structure of the *Drosophila* compound eye. (A, B) Wild-type Canton-S adult eye. Note the highly regular array of ommatidia and bristles. (C, D) Adult fly eyes of P[UAS-*bax*] / *GMR-GAL4* genotype. Expression of the UAS-*bax* transgene in the eye eliminates some of the ommatidia (white arrow), inducing disorder in the eye. (E, F) Adult fly eyes of P[UAS-*bcl-2*] / P[UAS-*bcl-2*]; *da-GAL4* / *da-GAL4* genotype. Ubiquitous expression of two copies of the UAS-*bcl-2* transgene causes a rough eye phenotype accompanied by the presence of larger ommatidia (black arrow). All panels are scanning electron micrographs at magnifications of (A, C, E) x 150, (B, D, F) x 600.



**Figure 6** Semi-thin sections of *bax* and *bcl-2*-expressing eyes. (A) Eye of a wild type (Canton S) fly. (B) Representative eye of a P[UAS-*bax*] / *GMR-GAL4* fly. Expression of *bax* leads to the loss of ommatidia and to abnormal ommatidia morphology as well as to a reduced number of photoreceptor neurons. Holes and vacuoles resulting from massive cell death are also observed. (C) Representative eye of a P[UAS-*bcl-2*] / *GMR-GAL4* fly. Increased spacing between ommatidia can be seen (\*), suggesting an increase in the number of pigment cells and resulting in disorganized ommatidia. (D) Schematic representation of wild type pupal retina, 40 hrs after pupariation at 21°C (adapted from [62]). Two or three of the pigment cells (per ommatidial unit) marked by \* are eliminated by apoptosis between 40 and 50 hrs after pupariation at 21°C. (E) Structure of wild type adult eye (adapted from [84]) shown in the same orientation as in (A-C) (bottom = equator). Cells marked in black corresponds to those who escaped to normal cell death during the eye development. Their position correlates with the sites where extra material is found between ommatidia in (C). Photoreceptors in the middle of the ommatidia are numbered. Pigment cells: (1) primary, (2) secondary and (3) tertiary. Hair nerve cells: (H). Cone cells (c).



**Figure 7** Expression of the UAS-*bax* transgene in the wing imaginal discs alters the wing. (A) Wild type wing. (B-E) Expression of the UAS-*bax* transgene was driven by *vestigial*-GAL4 in the wing margin of *vg*-GAL4 / +; P[UAS-*bax*] / + flies. At 18°C (B, C), most of the flies have ‘notched’ wings, while at 21°C (D), wing phenotypes range from notched to absent wing. Sometimes, the wing displays a tubular aspect (right wing D). At 25°C (E), all the flies exhibit a gnarled or wrinkled wing phenotype.



**Figure 8** Bcl-2 counteracts Bax-induced cell death. (A) The antagonistic activity of Bcl-2 was tested at 18, 21 and 25°C for viability of *vg-GAL4 / P[UAS-*bax*]* flies issued from a cross between *vg-GAL4* and *P[UAS-*bax*]* / *CyO*, or from a cross between *vg-GAL4*; *P[UAS-*bcl-2*]* and *P[UAS-*bax*]* / *CyO*. Percentages of viability was majored by dividing the number of *P[UAS-*bax*]* flies by the number of *CyO* flies in the progeny. Numbers are indicated above each bar. (B) The strength of the wing reduction was studied at 18°C for 349 *vg-GAL4 / P[UAS-*bax*]* and for 318 *vg-GAL4 / P[UAS-*bax*]; P[UAS-*bcl-2*]* flies. Phenotypes were grouped into three categories. The "weak" category includes flies which have weakly notched wings. The "strong" category includes flies in which both wings are almost or completely absent. The intermediate category corresponds to phenotypes where one wing is notched and the other one is reduced. *Bcl-2*-expressing flies have phenotypes of weaker strength than those which only express *bax*.