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The Bax/Bak ortholog in *Drosophila*, Debcl, exerts limited control over programmed cell death

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Bcl-2 family members are pivotal regulators of programmed cell death (PCD). In mammals, pro-apoptotic Bcl-2 family members initiate early apoptotic signals by causing the release of cytochrome c from the mitochondria, a step necessary for the initiation of the caspase cascade. Worms and flies do not show a requirement for cytochrome c during apoptosis, but both model systems express pro- and anti-apoptotic Bcl-2 family members. *Drosophila* encodes two Bcl-2 family members, Debcl (pro-apoptotic) and Buffy (anti-apoptotic). To understand the role of Debcl in *Drosophila* apoptosis, we produced authentic null alleles at this locus. Although gross development and lifespans were unaffected, we found that Debcl was required for pruning cells in the developing central nervous system. *debcl* genetically interacted with the *ced-4/Apaf1* counterpart *dark*, but was not required for killing by RHG (Reaper, Hid, Grim) proteins. We found that *debcl*^{KO} mutants were unaffected for mitochondrial density or volume but, surprisingly, in a model of caspase-independent cell death, heterologous killing by murine Bax required *debcl* to exert its pro-apoptotic activity. Therefore, although *debcl* functions as a limited effector of PCD during normal *Drosophila* development, it can be effectively recruited for killing by mammalian members of the Bcl-2 gene family.

KEY WORDS: Apoptosis, Bcl-2 genes, Cell death, *Drosophila*

INTRODUCTION

Apoptosis is a form of programmed cell death (PCD) that is required for proper development, for the maintenance of tissue homeostasis during adulthood, and for the elimination of damaged or unwanted cells. Although the core apoptotic machinery is conserved, distinct mechanistic differences in the activation and regulation of this process have evolved. In worms and mammals, both anti- and pro-apoptotic Bcl-2 family members play pivotal roles in regulating cell death early in the apoptotic pathway. In *C. elegans*, the anti-apoptotic Bcl-2 protein CED-9 physically interacts with CED-4 to inhibit cell death. Upon detection of an apoptotic stimulus, the pro-apoptotic BH3-only protein, EGL-1, binds to CED-9, relieving suppression of CED-4 and allowing it to bind to and activate the caspase CED-3. In mammals, the 'BH3-only' members of the Bcl-2 family activate apoptosis either by inhibiting the anti-apoptotic Bcl-2 members, or by directly activating the pro-apoptotic Bcl-2 members, such as Bax and Bak, which are central regulators of apoptotic cell death (Lindsten et al., 2000). Unlike worms, where a direct physical link to the apoptosome is seen, the regulation of apoptosis by the mammalian Bcl-2 gene family occurs indirectly through the regulation of mitochondrial properties. For example, several pro- and anti-apoptotic Bcl-2 proteins impact the mitochondrial outer membrane (MOM) permeability (Kluck et al., 1997; Zou et al., 1997), resulting in the release of cytochrome c and subsequent formation of the apoptosome (Green and Reed, 1998; Gross et al., 1999).

Like worms and mammals, the *Drosophila* genome encodes at least two Bcl-2 family members (Chen and Abrams, 2000). However, unlike mammals, fly cytochrome c is not required for apoptosome formation (Yu et al., 2005), and the roles of *Drosophila* Bcl-2 family members as potential regulators of PCD remain unclear (Arama et al., 2003; Arama et al., 2005; Dorstyn et al., 2004; Dorstyn et al., 2002; Mendes et al., 2006; Zimmermann et al., 2002). To date, *Drosophila* cytochrome c (*cyt-c-d*) has been linked to caspase activation during spermatid differentiation (Arama et al., 2003) and to the proper timing of cell death in the pupal eye (Mendes et al., 2006). Previous studies, based on forced expression and RNAi, reported pro-apoptotic functions for *debcl* (Brachmann et al., 2000; Colussi et al., 2000; Igaki et al., 2000; Senoo-Matsuda et al., 2005; Zhang et al., 2000) and potentially anti-apoptotic functions for *Buffy* (Brachmann et al., 2000; Quinn et al., 2003). Recently, genetic studies found limited roles for either gene in stress-induced apoptosis, but, because partial *debcl* function is likely to occur in these mutants, pivotal questions remain unresolved (Sevrioukov et al., 2007).

To illuminate the functional role of Bcl-2 proteins in PCD, we generated definitive null alleles at *debcl*. We exclude a global role for this gene in developmental PCD, but do find selective roles for *debcl* in regulating cell death and cell numbers in the CNS. *debcl* genetically interacted with the *ced-4/Apaf1* counterpart *dark*, but was not required for killing by RHG proteins. We found no overt role for *debcl* in regulating stress-induced cell death, cell-cycle checkpoint kinetics, genomic instability, or autophagy. In related studies, *debcl*^{KO} mutants were not significantly affected for mitochondrial density or volume. Surprisingly, in a model of caspase-independent cell death, we found that heterologous killing by murine Bax required *debcl* to exert its pro-apoptotic activity. Hence, although it regulates PCD in limited contexts during normal development, the action of *debcl* can be effectively recruited for killing by pro-apoptotic mammalian members of the Bcl-2 gene family.

MATERIALS AND METHODS

Ends-out donor constructs

Genomic DNA from *yw* flies was used as the template for all PCR reactions. Primers 5′-ACA ATC ACA GCG GCC GCG CCT CAC TAA GAG AAA CTT ATG G-3′ and 5′-CGG GGT ACC TAT TGT TGC TGC TGA GGC CTT TGT TGG-3′ were used to PCR amplify the 3.84 kb upstream flanking

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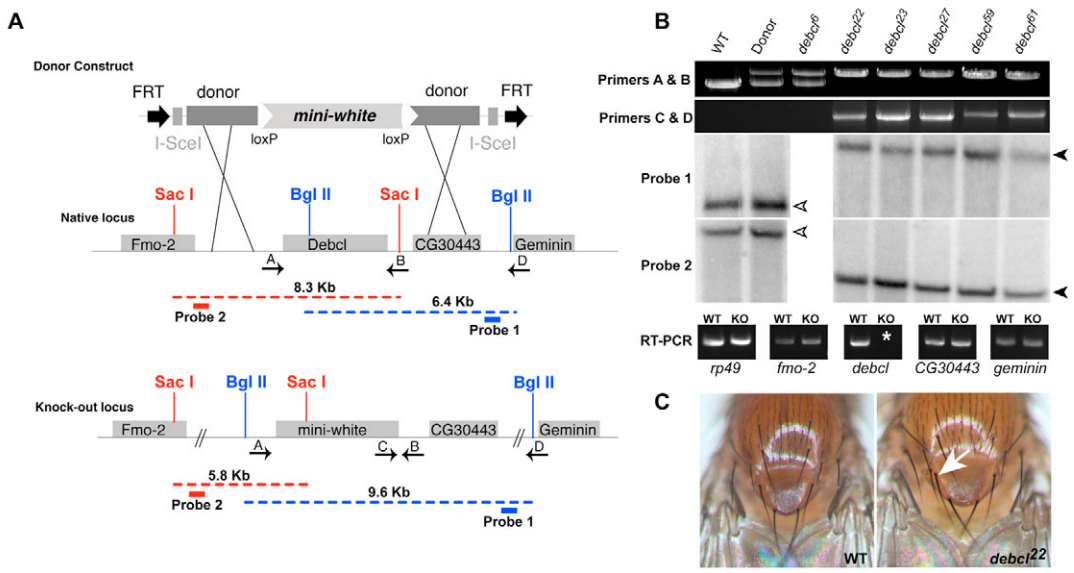

Fig. 1. Generation and verification of a targeted *debcl* mutation. (**A**) Targeting scheme for the *debcl* gene. The donor construct was generated by insertional cloning of a 4 kb upstream and a 2.7 kb downstream genomic sequence of the *debcl* gene into the targeting vector pW25. Upon chromosomal targeting, the *debcl* native gene is replaced with a *white*$^{+}$ marker gene. (**B**) Verification of the *debcl*-targeting event by PCR, Southern blot and RT-PCR analysis. Primer pair A and B were used to amplify both the native and the knock-out locus to confirm the replacement of *debcl* (4.2 kb) with the white^{+} marker gene (~5.2 kb). Primer pair C and D were used to verify the right arm of recombination during the screening process for potential recombinants. Primer pair E and F were used to confirm targeted recombination of the left arm of recombination (data not shown). WT refers to the *yw* parental strain, wild type at the *debcl* locus on the second chromosome; Donor refers to the *debcl* donor construct on the third chromosome, wild-type at the *debcl* native locus on the second chromosome; *debcl*^{6,22,23,27,59,61} represent different *debcl* knock-out candidates disrupted at the native locus. *debcl*^{6} was a potential *debcl* targeted deletion, but failed the PCR screen and was therefore eliminated from further characterization analysis. An additional *debcl* disruption allele, *debcl*$^{47/48}$, was also confirmed (not shown). Southern blot analysis using genomic DNA from the indicated fly strains was used to confirm both the right (SBP1) and left (SBP2) arm of recombination. For SBP1 (*Bgl*II digest) and SBP2 (*Sac*I digest), the black arrowheads indicate the expected 6.4 kb and 8.3 kb genomic fragments in the WT and donor strains for SBP1 and SBP2, respectively. The white arrowheads indicate aberrant genomic fragments of 9.6 kb and 5.8 kb, indicative of gene-targeted replacement, for SBP1 and SBP2, respectively. RNA from L3 larvae was used as a template to confirm abolishment of the *debcl* transcript in *debcl*^{KO} flies, and to confirm that transcript levels are unaffected in the neighboring genes *fmo-2*, *CG30443* and *geminin*. *rp49* was used as a control for RT-PCR. The white asterisk indicates the absence of the *debcl* transcript in mutant flies. (**C**) Photograph of extra scutellar bristles in *debcl*^{KO} flies (white arrow).

sequence (from –3876 to –37 bp upstream of the *debcl* start codon) to clone into the respective *Not*I and *Acc*651 sites in the pw25 donor plasmid. Primers 5′-TAT GGC GCG CCT GTT CTA GAT TCG CTT GGG ATC GCG TCG-3′ and 5′-CGC CGT ACG ACA TCA ATG CGG ATG GAT TTC AAT GTG TGG G-3′ were used to PCR amplify the 2.70 kb downstream flanking sequence (including the 3′UTR of *debcl*) to clone into the respective *Asc*I and *Bsi*WI pw25 vector sites. All constructs were transformed into the germ line of *Drosophila melanogaster* by using standard methods (Rubin and Spradling, 1982).

Targeted recombination genetics

Crosses for targeted recombination were performed in standard 25-mm-diameter vials, each carrying three to five females and a corresponding number of the appropriate males. Males carrying the donor construct were crossed to virgin females carrying the heat-inducible (70Flp)(70 I-Sce I)/TM6. The adults were removed a couple days after egg laying and prior to heat shocks, which were performed in a circulating water bath at 38°C for 90 minutes and repeated for three consecutive days. A total of 100 vials were used for heat shock. Mosaic-eyed females were crossed en masse to males carrying the constitutively active P(70Flp). Flies strains were obtained from the Bloomington Stock Center.

Southern blotting

For verification of targeting, genomic DNA was prepared from flies carrying the candidate targeted allele using the Wizard Genomic Purification Kit (Promega). Genomic DNAs were digested as indicated (Fig. 1A), separated by 0.8% agarose gel electrophoresis and transferred to positively charged nylon membranes. The membranes were probed with [^{32}P]dCTP-labeled DNA from PCR product using primers 5′-GCT ACA GTC GAG TGT GCT GGG TTG TTT GCG-3′ and 5′-GAC GGC GGA TTC CAG ACG CTT TCA GAA CGG-3′ for verification of the left recombination arm, and primers 5′-GCG TAC AAT TAG ACC AGC CGT TGT GTT GGC-3′ and 5′-AAG AGG ACA ACA GCG AGG TGG AGG AGG ACG-3′ for verification of the right recombination arm, and hybridized using Express Hyb Hybridization Solution (BD Biosciences).

PCR and RT-PCR

A PCR strategy was used to recognize candidate targeted recombination alleles. The primers 5′-GGT TAT CAT ACC ATT CCT GCT CTT TGG-3′ (Primer C) and 5′-GTC CTG AAG GAG ATC TGC GAA GAG GAC AAC AGC-3′ (Primer D) were used to amplify a PCR fragment unique to targeted recombination. The same strategy using sequence specific primers was used to verify the left recombination arm (data not shown). Additionally, primers flanking the *debcl* ORF, 5′-AAC GAG AAC GGG AAC TCG AAA GAA CCT AGA TCG-3′ (Primer A) and 5′-AAG ACG AAT TGT CGT ACT CAA AAT ATT GGC ACC-3′ (Primer B), were used to distinguish native *debcl* or replacement by the *white*$^{+}$ gene. The genomic sequences from the PCR reactions were fully sequenced, and the sites of recombination at the right and left recombination arms matched endogenous sequence. To access transcript levels, total RNA was prepared from *debcl*^{KO} and wild-type (*yw*, WT) L3 larvae using the High Pure RNA Isolation Kit (Roche). The Superscript One-step RT-PCR System with Platinum Taq (Invitrogen) was used for RT-PCR reactions. The following primers were used to amplify transcript in *debcl* and

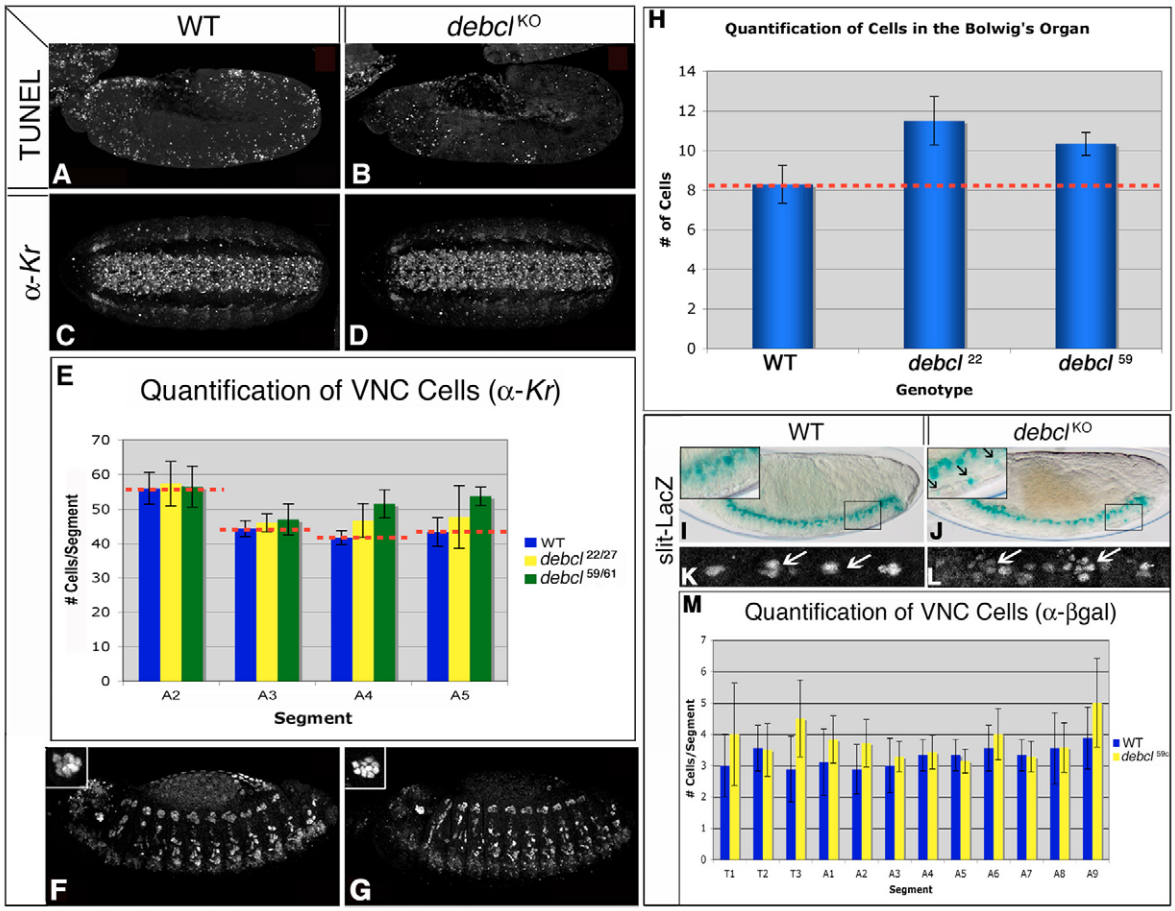

Fig. 2. *debcl* regulates developmental cell death in the nervous system. (**A**,**B**) TUNEL staining is moderately reduced in *debcl* mutant embryos (B) compared with WT (*yw*) embryos (A; stage 11). (**C-H**) The presence of extra cells during embryonic development was examined by α-*Kr* Ab staining (C-G) and *slit-lacZ* staining (I,J *slit-lacZ* reporter; K,L, α-βGal Ab staining). Arrows in J-L indicate extra *slit-lacZ*+ cells. Dashed lines in E and H represent the WT cell count threshold for each segment surveyed. (E) Quantification of extra cells in the VNC by α-*Kr* Ab. Error bars show ±s.d. (*n*=6). (H) Quantification of cells in Bolwig's Organ. Error bars show ±s.d. (*n*=8). (M) Quantification of VNC cells by α-*βGal* Ab staining. Error bars show ±s.d. (*n*=8).

neighboring genes: *rp49* (RT-PCR control) 5′-ATG ACC ATC CGC CCA GCA TAC A-3′ and 5′-ACA AAT GTG TAT TCC GAC CAG G-3′; *fmo-2* 5′-ATC AAA ACT TCA GTG GAC AAG CGT CGT GTT TGC-3′ and 5′-ATC GTA TAC TTG TTG CTC CTG TAC GTG TCC-3′; *debcl* 5′-CCA AGT TCA AGT CCT CGT CGC TGG ACC-3′ and 5′-GCG AAT CTA GAA CAG CAG CGA ATA CAG TTG ACC-3′; *CG30443* 5′-GAG CTG GAC CAG TTC TAC TGC GAA ATA TGC-3′ and 5′-AGC CAA TCT GTA ATA ACT TCC TCG CTG TGG-3′; and *geminin* 5′-CCA GGG TCT ACA TCC AAG TCG AGA CAG AGG-3′ and 5′-TTG ACC TTG TCC TCG TCA CCC GTA GTG TCG-3′.

Cell death assays

Embryo TUNEL labeling was performed as described by White et al. (White et al., 1994), using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Chemicon International). Acridine Orange staining was performed as described by Sogame et al. (Sogame et al., 2003).

Fly strains

dark^{CD4} was meiotically recombined with *debcl*^{KO} to generate double knock-out flies for genetic interaction studies. The following stocks were also used for various analyses: *UAS-mito-GFP* (Cox and Spradling, 2003), *pGMR-Gal4*, *da-GAL4*, pGMR-*rpr*, -*hid* and -*grim4* (Bloomington Stock Center). *UAS-Bax* flies were kindly provided by B. Mignotte (Université de Versailles/Sain-Quentin). Recognizing that these strains tended to accumulate modifiers, we mobilized the *UAS-Bax* transgene by conventional methods. Briefly, we crossed *UAS-Bax* flies to flies carrying Δ2-3 transposase, recovered candidate alleles for transgene mobilization, and mapped the new insertion sites.

Immunohistochemistry

Embryos or L3 tissues were collected and treated as described by Chew et al. (Chew et al., 2004). Primary antibody was incubated overnight at 4°C [1:600 guinea pig α-*Kr* (Kosman et al., 1998); 1:800 α-*βGal* Ab (Promega); 1:500 α-*dHb9*, 1:1 α-*LBe* and 1:500 α-*Eg* (Rogulja-Ortmann et al., 2007); 1:200 rabbit α-phosphohistone H3 (Upstate); 1:50 α-cleaved Caspase 3 (Cell Signaling)]. Secondary antibodies from Molecular Probes were used at a 1:500 dilution.

Microscopy and imaging

All embryos and imaginal disc imaging was done on a Zeiss LSM 510 META laser scanning confocal microscope, a Leica TCS SP5 spectral confocal microscope, or a Zeiss Axioplan 2E digital light microscope. Images were processed using ImageJ and Adobe Photoshop software. Photographs of fly eyes were taken on a Zeiss SteREO Discovery.V12 microscope.

Notched wing assays

Wandering L3 larvae were treated with 0, 1500 or 2500 Rads of ionizing radiation. Adults with notched wings were scored as a percentage of the total number of adults counted.

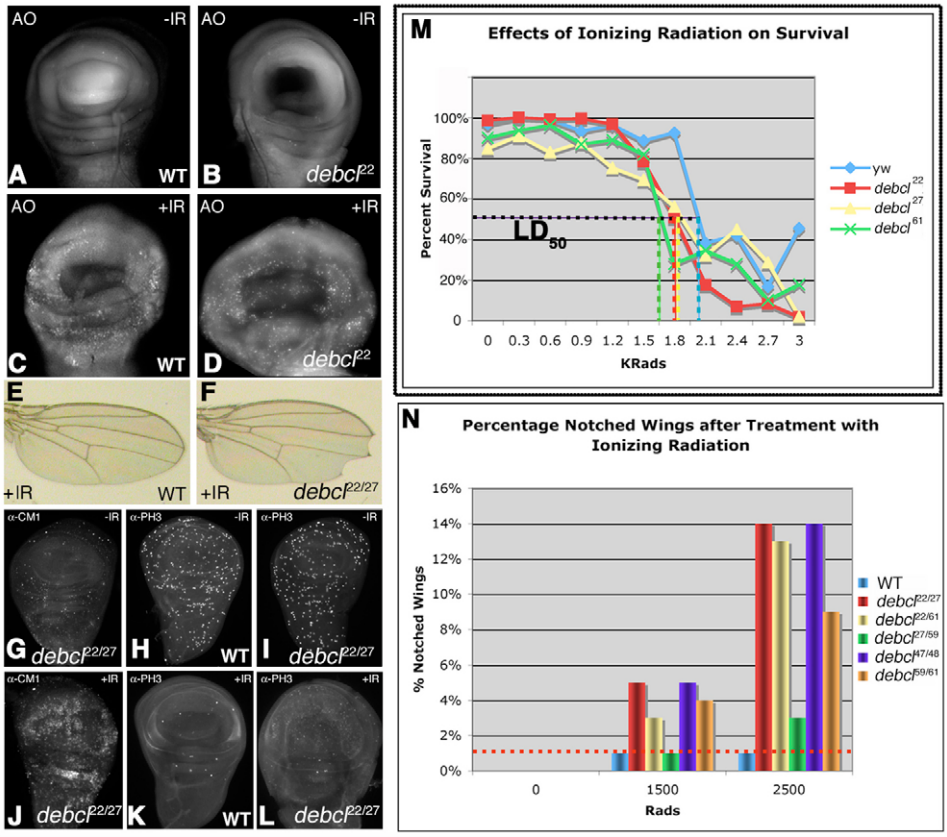

Fig. 3. *debcl* and genotoxic stress. (**A-N**) After irradiation, *debcl* mutants show an elevated incidence of visible defects, but are unaffected for stress-induced apoptosis and cell-cycle arrest. (A,B) Basal levels of cell death are observed in the larval wing imaginal disc. (C,D) Four hours after exposure to 4000 Rads of ionizing radiation (IR), robust cell death is detected by Acridine Orange staining (AO). (M) Adult survival curve after treatment with IR. The corresponding LD_{50} is shown for each sample (dashed line). Effector caspase levels labeled by α-proCaspase3 Ab staining (G) drastically increased after IR challenge in WT and $debcl^{22/27}$ tissue (J, WT not shown). Cell proliferation in the larval L3 wing imaginal disc was examined before (H,I) and after (K,L) treatment with 4000 Rads of IR, by α-phosphohistone H3 Ab staining. Wing from a WT (*yw*, E) and $debcl^{22/27}$ (F) adult after IR challenge, showing a notched wing phenotype in the *debcl* mutant. (N) Histogram quantifying the percentage of notched wings in WT (*yw*) and transheterozygous *debcl* mutant alleles at 0, 1500 and 2500 Rads of IR. Dashed line represents WT percentage of notched wings.

Autophagy assays

Lysotracker dye was used as a marker for autophagy, and assays were performed as described by Rusten et al. (Rusten et al., 2004). The following modifications were made: fat bodies were stained with LysoTracker Red DND-99 (Molecular Probes) diluted 1:4000 in PBS with 1 μM Hoechst for 5-10 minutes. Fat bodies were washed several times in PBS and mounted in 70% glycerol/PBS and visualized immediately. Images were captured using a 40× dry lens. For quantification analysis, three fat body lobes from five independent animals of each genotype were obtained and imaged. The number of lysotracker-positive structures was quantified from *z*-stacked images taken on a Zeiss Axioplan 2E digital light microscope and processed using ImageJ and Adobe Photoshop. A total area of 800 square pixels was quantified for each image. These results are expressed as mean values ±s.d.

Mitochondrial imaging and analyses

Wing imaginal discs and salivary glands from L3 larvae or pupae carrying the transgenes for *UAS-mito-GFP* and *Da-Gal4* were dissected in PBS and fixed for 15 minutes in 4% formaldehyde (EM Grade). The salivary glands were mounted in 70% glycerol/PBS, visualized directly by confocal microscopy using a 63× oil objective lens, and imaged with a zoom factor of 5. Two cells per salivary gland were captured for thorough sampling. Quantification of mitochondrial volume and density was accomplished using Imaris software (Bitplane, Zurich).

RESULTS

Generation of *debcl* mutant flies

To elucidate the biological function of *debcl*, we produced a null mutation at this locus. We applied ends-out homologous recombination (Gong and Golic, 2003) to replace the endogenous *debcl* gene with the *white*$^{+}$ marker gene. The *debcl* gene is located on the right arm of the second chromosome 42C2, 4.0 kb downstream of *fmo-2*, and 2.7 kb upstream of *CG30443* (Fig. 1A). We screened 56,000 flies for recombination events and obtained 10 candidate alleles (Fig. 1B, top panel). Seven of these were confirmed for the predicted event by PCR and Southern blot analysis (Fig. 1B, middle panel). RT-PCR was used to verify normal expression of the flanking genes (*fmo-2*, *CG30443* and *geminin*) and the absence of the *debcl* transcript from these mutant alleles (Fig. 1B, bottom panel, data shown for $debcl^{22}$). For most studies, a combination of transheterozygous individuals were examined. $debcl^{KO}$ animals are viable, fertile and exhibit a normal life-span (data not shown). Hence, *debcl* is not essential for survival or fertility. At variable penetrance (25%-57%), extra scutellar bristles were found on the notum of $debcl^{KO}$ adults (Fig. 1C, white arrow). Notably, this extra bristle phenotype also occurs in *dark*, *dredd*, *dronc* (*Nc* – FlyBase), *Dcp-1*, *cyto-c-d* and *mir-9a* (microRNA-9a) mutant adults (Chen et al., 1998; Laundrie et al., 2003; Li et al., 2006; Rodriguez et al., 1999; Xu et al., 2005). Other phenotypes, observed at lower penetrance, include rotated male genitalia, imperforate vaginas in females, melanotic-like cells in the maxillary palps, and patterning abnormalities of tergites and sternites.

debcl regulates PCD and proper cell number in the developing central nervous system

$debcl^{KO}$ embryos were examined for PCD and, when compared with WT, a moderate decrease in the number of TUNEL-positive cells was consistently observed (Fig. 2A,B). We also examined $debcl^{KO}$ embryos using well-established antibodies that mark cells which normally die in the nervous system, but which fail to die in PCD mutants (Rogulja-Ortmann et al., 2007). For instance, the number of Kr^{+} cells in the ventral nerve cord (VNC; Fig. 2C,D) was determined and quantification of these cells (Fig. 2E) in abdominal segments A2 and A3 showed no significant differences in cell number. However, extra Kr^{+} cells were consistently present in

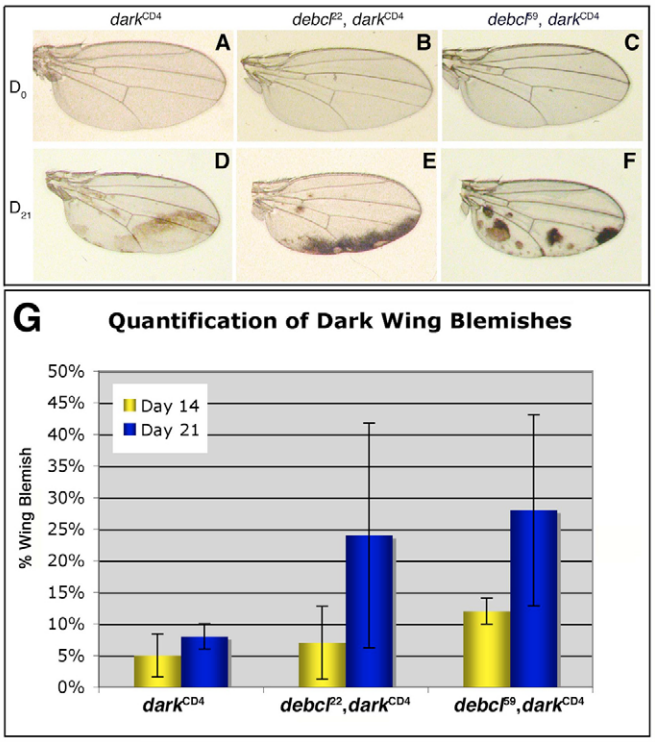

Fig. 4. *debcl* genetically interacts with *dark*. (**A-C**) Adult wings are normal in morphology and appearance at the time of eclosion (D_0). (**D-F**) Wing blemishes are progressive in nature and more severe in *debcl*, $dark^{CD4}$ double mutants (E,F) than in $dark^{CD4}$ single mutants (D), seen here in 14-day-old animals. (**G**) Histogram quantifying the severity of wing blemishing. The incidence of wing blemishing is expressed as a percentage of the wing afflicted with melanized spots over the entire wing region. Error bars show ±s.d. (n=10).

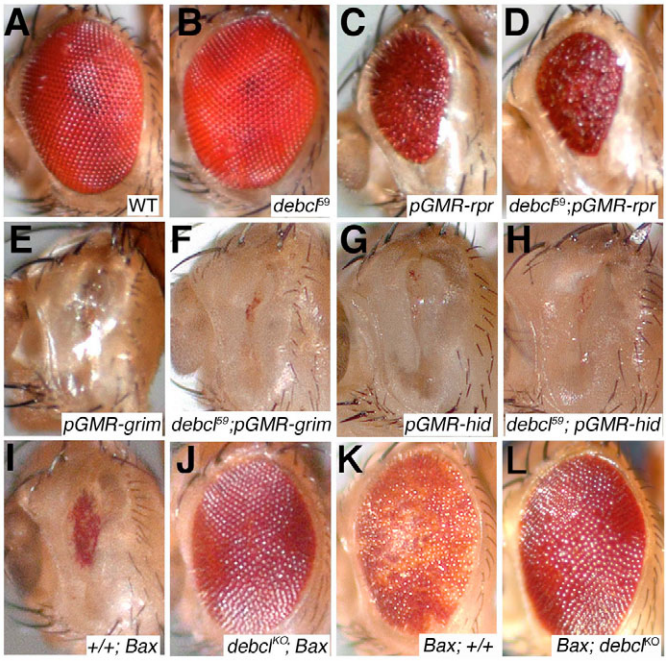

Fig. 5. Ectopic expression of pro-apoptotic genes in *debcl* mutant animals. (**A**,**B**) A morphologically normal eye in WT (A) and $debcl^{59}$ (B). Killing by the RHG proteins is not dependent on *debcl* function. (**C-H**) pGMR-*rpr* (C,D), pGMR-*grim4* (E,F) and pGMR-*hid* (G,H) in the eye showing no effect on the ablated eye phenotype. (**I**,**K**) Two different alleles of *UAS-Bax1*, *GMR-Gal4* showing severe ablation of the eye in the WT background. (**J**,**L**) This severe eye phenotype is partially restored in a *debcl* heterozygous background (not shown) and is nearly completely reversed in homozygous $debcl^{KO}$; *UAS-Bax1, GMR-Gal4*.

segments A4 and A5, and in Bolwig's organ of $debcl^{KO}$ embryos (Fig. 2E-H). We also examined midline glia using two distinct markers for *slit*-expressing cells (α-*βGal* Ab and a *slit-lacZ* reporter), and both detected supernumerary $slit^+$ cells in $debcl^{KO}$ embryos (Fig. 2I-L). Quantification of these cells is shown (Fig. 2M). Not all markers, however, exhibited extra cells in $debcl^{KO}$ animals. For example, antibodies directed against the neuronal transcription factors *dHb9* (*exex* – FlyBase; see Fig. S1A,B in the supplementary material), *lbe* and *eg* (not shown) showed wild-type cell numbers in all segments and normal PCD of Crz^+ neurons in the pupal CNS (see Fig. S1C,D in the supplementary material) (Choi et al., 2006). Likewise, examination of pupal eyes by staining for *discs large* revealed normal loss of interommatidial cells (see Fig. S1G,H in the supplementary material).

debcl is not required for apoptotic cell death in response to genotoxic stress

To test a possible role for *debcl* in stress-induced cell death, wing imaginal discs from larvae exposed to ionizing radiation (IR) were stained for apoptotic cells using Acridine Orange (AO). During larval development, low basal levels of PCD exist in the wing imaginal disc (Fig. 3A,B). Upon treatment with IR, WT and $debcl^{KO}$ showed comparable increases in damage-induced cell death (Fig. 3C,D). Likewise, no discernable difference in effector caspase levels between WT and $debcl^{KO}$ tissue was observed (Fig. 3G,J). To evaluate the effects of IR on viability, larvae were treated with varying doses of IR and scored for survival at the adult stage (Fig. 3M). $debcl^{KO}$ animals were slightly compromised for viability after IR challenge, exhibiting a reduced LD_{50}, as shown in Fig. 3M (dashed line). In these experiments, especially at higher IR doses, *debcl* mutants exhibited a far greater tendency towards some visible defects, scored here in the form of notched wing phenotypes (Fig. 3N).

We also examined irradiated wing imaginal discs for damage-induced cell cycle checkpoint phenotypes using an anti-phosphohistone H3 antibody to label mitotic cells (Fig. 3H,I). After treatment with IR, proliferative arrest (see Fig. 3K,L) and resumption of proliferation (not shown) occurred with similar kinetics in WT and $debcl^{KO}$ discs. Likewise, no genotype-specific differences in wing sizes before and after IR stress were seen and, using *mwh* as a loss of heterozygosity readout, we did not observe evidence that the *debcl* status affected levels of genomic instability (not shown).

Interaction with other pro-apoptotic death genes: Debcl is not required for killing by RHG proteins, but is required for heterologous killing by murine Bax

Adult eyes of $debcl^{KO}$ flies show normal gross patterning (Fig. 5B), and eye ablation phenotypes caused by forced expression of the pro-apoptotic genes *grim*, *rpr* and *hid* were unaffected by $debcl^{KO}$ genotypes (Fig. 5C-H). Hence, killing by forced expression of IAP antagonists does not require *debcl* function. We also tested for

possible genetic interactions between *debcl* and other members of the apoptotic pathway. For example, flies hypomorphic for the apoptosomal gene *dark*, develop progressive melanized blemishes that can be quantified as shown in Fig. 4G and, using this phenotype as an indicator of defective PCD (Link et al., 2007), positive genetic interactions between *dark* and other cell death genes, *dp53* (Sogame et al., 2003) and *dronc* (Chew et al., 2004), have previously been established. Similarly, we found that both the incidence and severity of melanized wing blemishing was clearly exacerbated in *debcl*^{KO}, *dark*^{CD4} double mutants (Fig. 4E,F), compared with single *dark*^{CD4} mutants (Fig. 4D). This observation was quantified in Fig. 4G.

In previous studies, murine Bax provoked cell killing in the *Drosophila* eye (Gaumer et al., 2000) and, as previously shown, Bax provoked eye ablation phenotypes (Fig. 5I,K). We therefore tested whether Bax-induced cell death might be affected by *debcl* status. Surprisingly, these phenotypes were almost completely suppressed in the *debcl*^{KO} background (Fig. 5J,L). Together, these data indicate that a *debcl*-dependent step is required for heterologous killing by Bax.

debcl and autophagy

We examined the possibility that *debcl*, like other Bcl-2 family members (Shimizu et al., 2004), may play a role in autophagy. Using lysotracker as a marker for starvation-induced autophagy (Munafo and Colombo, 2001), we examined larval fat bodies during fed and starved conditions. Autophagy was quantified in larval fat bodies, and in all trials no significant differences between WT and *debcl*^{KO} animals were observed (Fig. 6B). Examples of fat body staining imaged for quantification analysis using lysotracker and Hoechst are shown (Fig. 6C-F). Likewise, WT and *debcl*^{KO} larvae exhibit similar survival curves when transferred from fed to starved conditions (Fig. 6A), with both genotypes displaying an LD_{50} of approximately 6.5 days after challenge.

Mitochondrial properties are independent of Debcl status

We examined the possibility that *debcl*, like other Bcl-2 family members (Karbowski et al., 2006), may function to specify mitochondrial properties. We first measured steady-state ATP levels in larval and adult tissues and found no indication that respiration was perturbed in *debcl*^{KO} mutants (not shown). Next, using the *Gal4-UAS* system together with a GFP reporter (*mito-GFP*), we visualized mitochondria in imaginal wing disc cells and in salivary gland cells before and after metamorphosis. In both tissues, mitochondria in WT (Fig. 7A,C) and *debcl*^{KO} cells (Fig. 7B,D) appeared to have similar morphological distributions. Using Imaris software for unbiased comparisons, we surveyed distributions of mitochondrial volume and found no differences between WT and *debcl*^{KO} with respect to this parameter (Fig. 7G,H). We also determined that, on a per cell basis, mitochondrial densities were comparable in WT and *debcl*^{KO} animals (Fig. 7E,F). We also note here that mitochondrial fragmentation was associated with PCD in pupal salivary glands, but that this change was not evidently impacted on by *debcl* genotypes (not shown).

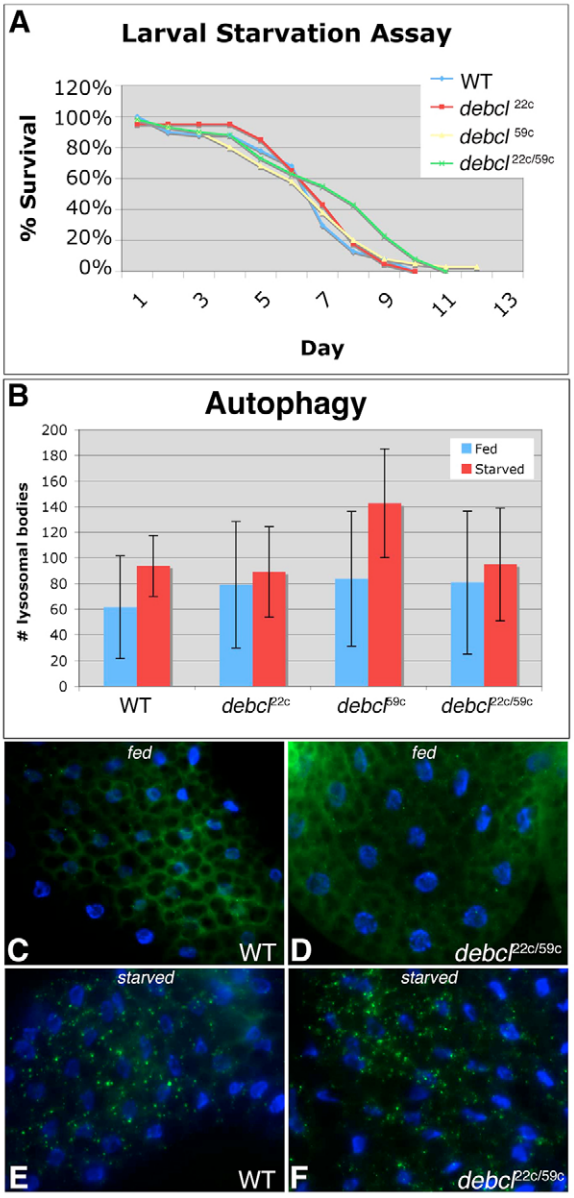

Fig. 6. *debcl* mutants display normal starvation-induced autophagy. (**A**) Histogram illustrating the survival curve of L2 larvae when placed under starved conditions (20% sucrose). (**B**) Fat bodies of young L3 larvae were quantified for autophagic bodies under fed (wet yeast) and starved (20% sucrose) conditions using lysotracker as a marker for autophagy. Error bars show ± s.d. (*n*=5). (**C-F**) Live fat bodies stained with Hoechst (blue) and lysotracker (green) in fed and starved larvae of wild type and mutant.

DISCUSSION

Bcl-2 genes exert pivotal functions that regulate PCD either by direct physical interaction with the apoptosome in worms (Conradt and Horvitz, 1998), or by indirectly promoting apoptosome formation in mammals, a step that requires cytochrome c (Jurgensmeier et al., 1997; Kluck et al., 1999; Li et al., 1998; Wei et al., 2001). The fly genome encodes two well-conserved Bcl-2 family members, yet, in this animal, cytochrome c is dispensable for apoptosome assembly (Yu et al., 2005), raising the possibility that insect Bcl-2 genes could mediate cytochrome c independent activities that may or may not be related to cell death. To clarify the functional roles of this gene family in the *Drosophila* model, we genetically eliminated *debcl*, the fly ortholog of the mammalian pro-apoptotic members Bax, Bak and Bok.

Using a targeted recombination strategy, we recovered seven allelic strains that are definitively amorphic for *debcl*. Our studies exclude a general requirement for *debcl* as a global apoptotic

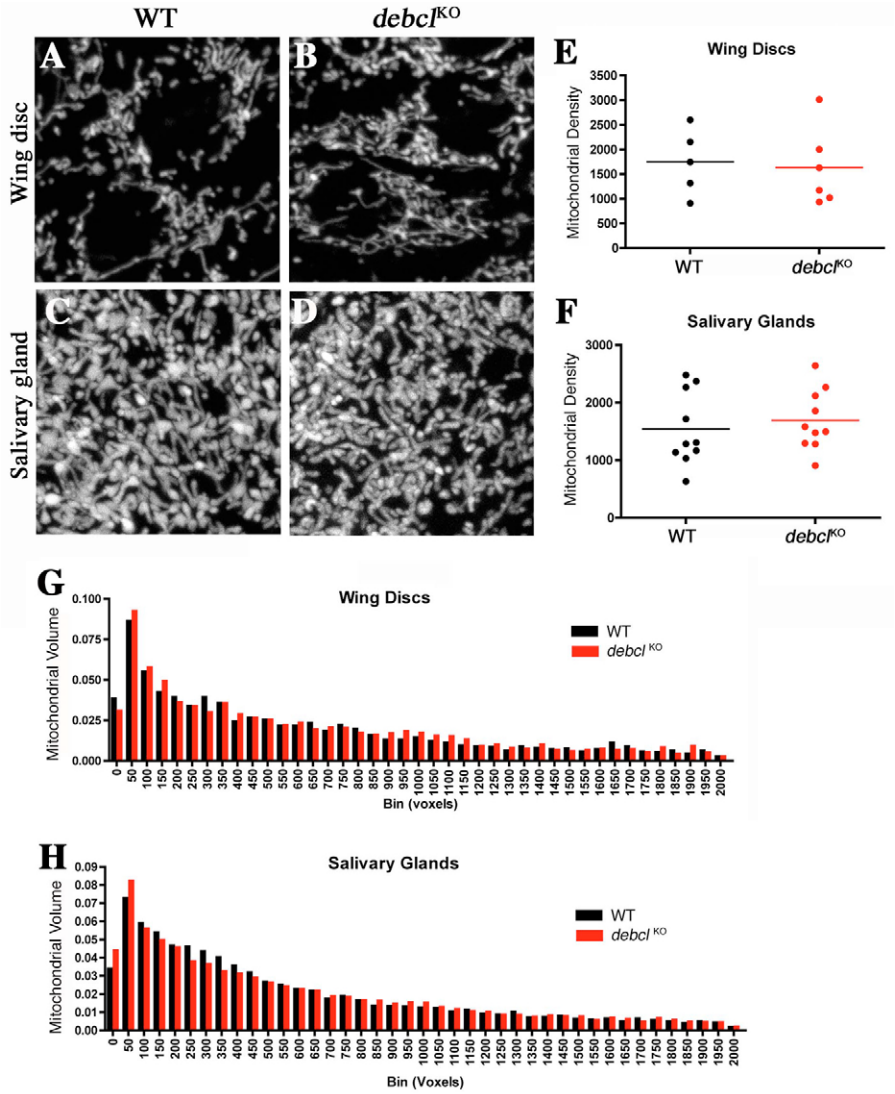

Fig. 7. *debcl* and mitochondrial dynamics. (**A-D**) Images of WT (A,C) and *debcl*^{KO} (B,D) mitochondria (*UAS-mito-GFP; Da-Gal4*) in cells of L3 wing imaginal discs and salivary glands. Mitochondria from pupal salivary glands ~13 hours after puparium formation (APF) are fragmented in response to PCD in WT and *debcl*^{KO} cells (not shown). (**E-H**) Mitochondrial density (E,F) and volume (G,H) in both tissues is comparable.

effector, which had been suggested from gene silencing analyses (Colussi et al., 2000; Senoo-Matsuda et al., 2005). Nevertheless, three compelling lines of evidence establish that *debcl* does function to regulate a limited number of developmental cell deaths. First, in every allelic combination tested, TUNEL labeling was consistently and markedly reduced (see Fig. 2). Second, in every allele tested, *debcl* genetically interacted with a hypomorphic allele of the apoptosomal gene *dark*. Third, extra cells in *debcl* embryos were detected using markers that visualize persisting or 'undead' cells in canonical PCD mutants (Chew et al., 2004; Rodriguez et al., 2002; Rogulja-Ortmann et al., 2007; White et al., 1994). Although the impact caused by eliminating *debcl* was modest, we note that reproducible and consistent PCD phenotypes were observed for all alleles tested. Furthermore, it is also worth noting that our data reflect counts of marked cell populations, only a small fraction of which actually die (Rogulja-Ortmann et al., 2007). Hence, if we consider only the cells that are lost and compare these against benchmarks seen in animals completely defective for PCD, the effects caused by eliminating *debcl* are substantial. For example, in *H99* animals where no PCD occurs, a 37% excess of *Kr*+ cells is seen in Bolwig's organ (Link et al., 2007) and, by comparison, an excess of up to 23% *Kr*+ cells was seen in *debcl* mutants (Fig. 2). Graded effects along the anteroposterior axis is another familiar phenotype seen here, and, likewise, was previously reported for persisting motoneurons in *H99* mutants (Rogulja-Ortmann et al., 2007). Specifically, in cases where extra neuronal cells were observed (e.g. α-*Kruppel*, see Fig. 2), these cells tended to appear more commonly among the posterior segments. This trend of extra cells in more posterior segments is consistent with previous studies reporting the prominence of neuronal degeneration in the abdominal ganglion (Kimura and Truman, 1990; Robinow et al., 1993). Segments with supernumerary cells averaged 17% additional cells in *debcl*^{KO} animals (e.g. Fig. 2) and, by comparison, mutations in the apoptosomal genes *dark* and *dronc* produced a range from 33-50% excess cells (Chew et al., 2004; Rodriguez et al., 2002). From these combined results, we speculate that perhaps *debcl* augments apoptotic signaling in certain cell types. Not all tissues were impacted in *debcl*^{KO} animals, however. Notable examples of *Drosophila* PCD that were unaffected by *debcl* status included PCD of interommatidial cells in the eye, PCD of the salivary gland and elimination of *Crz*$^{+}$ neurons during larval to pupal transition (Choi et al., 2006), as well as of markers specific for motoneurons during embryogenesis (see Fig. S1 in the supplementary material). Hence, to the extent represented by the markers studied here, the impact of *debcl* status on *Drosophila* cell death appears to be limited to certain embryonic neurons and glia, but not on the PCD of motoneurons.

Taken together, these studies exclude a universal requirement for *debcl* in PCD, yet establish a limited role for this gene in the death of certain cell types.

Recently, Sevrioukov et al. studied *debcl* mutants and, in contrast to results presented here, they found no evidence linking the action of *debcl* to PCD. At least two important differences explain this discrepancy. First, the main allele studied by Sevrioukov et al., $debcl^{E26}$, probably reflects the hypomorphic condition, as it leaves the entire *debcl* open reading frame intact along with at least half of the 5′ UTR (Sevrioukov et al., 2007). Second, the markers used in the two studies were quite different. In studies presented here, we applied established markers proven to detect extra cells that persist in canonical PCD mutants (*H99*, *dronc* and *dark*) by virtue of specific failures in caspase-dependent cell death (Chew et al., 2004; Rodriguez et al., 2002; Rogulja-Ortmann et al., 2007). By contrast, Sevrioukov et al. applied markers that highlight axonal bundles (Mab^{+} cells) and a glial cell marker ($repo^{+}$ cells), neither of which previously detected supernumerary cells in PCD defective *H99* mutants (Rogulja-Ortmann et al., 2007).

We also carefully examined other cellular processes in addition to PCD. *debcl* mutants showed normal levels of starvation-induced autophagy and, likewise, were unperturbed for radiation stress responses, including cell cycle arrest and loss-of-heterozygosity post-challenge. Interestingly, however, we did find elevated levels of ‘notched’ phenotypes in $debcl^{KO}$ wings after IR challenge. Although the cellular basis for this observation is not clear, we suggest that *debcl* may be recruited to promote effective compensation when development is perturbed (de la Cova et al., 2004; Ryoo et al., 2004).

In numerous models of apoptosis, including in flies, mitochondria are remodeled through fusion and fission (Abdelwahid et al., 2007; Goyal et al., 2007; Green and Reed, 1998; Karbowski et al., 2006; Youle and Karbowski, 2005). In mammals, Bax and Bak are involved in this pathway of mitochondrial remodeling (Gross et al., 1999; Karbowski et al., 2006; Wei et al., 2001; Youle and Karbowski, 2005), but whether this activity plays a role in transducing or amplifying the apoptotic response is hotly debated, in part because it has been difficult to separate these activities. Indications that these activities can, in fact, be uncoupled was shown by Delivani et al., who found that expression of the worm Bcl-2 proteins Egl-1 and Ced-9 in mammalian cells influenced mitochondrial dynamics, but not the translocation of cytochrome c from mitochondria during apoptosis (Delivani et al., 2006). Recently, mitochondrial remodeling was also linked to PCD in flies (Abdelwahid et al., 2007; Goyal et al., 2007). Therefore, we investigated the effect of *debcl* genotypes upon constitutive mitochondrial dynamics in both larval and pupal stages. Using automated imaging analyses, we showed that mitochondrial density and mitochondrial volume in WT and $debcl^{KO}$ cells were similar in both L3 wing imaginal discs and salivary glands (Fig. 7E-H). These results suggest that the ortholog of Bax in flies does not play a crucial role in the regulation of mitochondrial properties, as is seen in mice lacking Bax and Bak (Karbowski et al., 2006). It is also worth noting here that mitochondrial fragmentation was associated with histolysis of the pupal salivary gland but that these changes occurred irrespective of the *debcl* status (not shown).

Despite the absence of mitochondrial phenotypes, our findings highlight a role for *debcl* in regulating a limited number of cell deaths. At the same time, we can exclude a general requirement for this gene in PCD, but the possibility remains that *Buffy* may play redundant roles in the tissue-specific regulation of *Drosophila* PCD. Therefore, during evolution, insects may have de-emphasized ancestral roles for the Bcl-2 proteins in PCD or, alternatively, mammals and worms may have evolved in ways that emphasized roles for this gene family in apoptotic cell death (Cory and Adams, 2002; Danial and Korsmeyer, 2004; Karbowski et al., 2006). Remarkably, *debcl* function was also required for the heterologous killing of fly cells by murine Bax. This observation rules out non-specific toxicity as an explanation for Bax killing in this system. Instead, to kill fly cells, Bax evidently recruits a native activity encoded by *debcl* that is not otherwise essential for all apoptotic cell death in *Drosophila*. Together with known actions of Bax (Karbowski et al., 2006; Wei et al., 2001; Youle and Karbowski, 2005) and with a previously described insensitivity to p35 (Gaumer et al., 2000b), these findings suggest that, in order to kill fly cells, Bax instigates a *debcl*-dependent, but caspase-independent, pathway of cell death. Extending this rationale, it may be possible to use this system to identify effectors of Bax without the potentially confounding effects that are linked to coincident caspase activation in mammalian cells.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/275/DC1

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