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Buffy, a *Drosophila* Bcl-2 protein, has anti-apoptotic and cell cycle inhibitory functions

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Bcl-2 family proteins are key regulators of apoptosis. Both pro-apoptotic and anti-apoptotic members of this family are found in mammalian cells, but only the pro-apoptotic protein Debcl has been characterized in *Drosophila*. Here we report that Buffy, the second *Drosophila* Bcl-2-like protein, is a pro-survival protein. Ablation of Buffy by RNA interference leads to ectopic apoptosis, whereas overexpression of *buffy* results in the inhibition of developmental programmed cell death and γ irradiation-induced apoptosis. Buffy interacts genetically and physically with Debcl to suppress Debcl-induced cell death. Genetic interactions suggest that Buffy acts downstream of Rpr, Grim and Hid, and upstream of the apical caspase Dronc. Furthermore, overexpression of *buffy* inhibits ectopic cell death in *diap1* (*th*^{5}) mutants. Taken together these data suggest that Buffy can act downstream of Rpr, Grim and Hid to block caspase-dependent cell death. Overexpression of Buffy in the embryo results in inhibition of the cell cycle, consistent with a G_1/early-S phase arrest. Our data suggest that Buffy is functionally similar to the mammalian pro-survival Bcl-2 family of proteins.

Keywords: apoptosis/cell survival/*Drosophila*/programmed cell death/RNA interference/TUNEL

Introduction

A balance between cell proliferation and apoptosis is essential for development of the multicellular organism. Superfluous or damaged cells must be removed by apoptosis, whilst those cells required for subsequent stages of development are protected by cell survival factors (reviewed in Baehrecke, 2002). In the nematode *Caenorhabditis elegans*, four genes, *egl-1*, *ced-3*, *ced-4* and *ced-9*, are essential for regulating apoptosis (reviewed in Horvitz, 1999). Of these, EGL-1, CED-3 and CED-4 are required for cell death to occur, whereas CED-9 is essential for cell survival. In *C.elegans*, EGL-1 functions upstream of CED-9, while CED-9 interacts with and regulates CED-4-mediated CED-3 activation (Hengartner, 2001).

Not surprisingly, the pathways of cell death are considerably more complex in mammals, where EGL-1, CED-3 and CED-9 are represented by multiple family members (reviewed in Baehrecke, 2002). There are several mammalian homologs of CED-3, some of which have essential functions in apoptosis (reviewed in Cryns and Yuan, 1998). The mammalian homolog of the CED-4 adaptor protein, Apaf-1, is essential for the activation of caspase-9. The mammalian homologs of CED-9, including Bcl-2, Bcl-x_L and Bcl-w, act as inhibitors of caspase activation and function upstream of Apaf-1 (reviewed in Cory and Adams, 2002). The mammalian EGL-1 homologs share a small region of homology (BH3 domain) with CED-9/Bcl-2 proteins and act as pro-apoptotic proteins upstream of Bcl-2 (Conradt and Horvitz, 1998).

Drosophila appears to have cell death machinery of intermediary complexity compared with that of *C.elegans* and mammals. Thus far, *Drosophila* has yielded seven caspases and a single CED-4/Apaf-1 homolog, termed Dark/Dapaf-1/HAC-1, which interacts with the *Drosophila* caspase Dronc and is required for its activation (reviewed in Richardson and Kumar, 2002). EGL-1-related, pro-apoptotic, BH3-only proteins have not yet been identified in *Drosophila*. Like mammals, *Drosophila* contains IAP (inhibitor of apoptosis) pro-survival proteins, which bind to and inhibit caspases (reviewed in Deveraux and Reed, 1999). Two IAP homologs have been reported in *Drosophila*—Diap1/Thread and Diap2 (reviewed in Hay, 2000)—which are antagonized by the IAP inhibitors Reaper (Rpr), Hid (Head involution defective/Wrinkled), Grim (reviewed in McCall and Steller, 1997) and Sickle (Christich *et al*., 2002; Srinivasula *et al*., 2002; Wing *et al*., 2002). The recent analysis of the human genome sequence shows that there are no strongly related homologs of *rpr*, *hid* or *grim* (Aravind *et al*., 2001); however, the mammalian apoptosis inducer Smac/Diablo appears to act as a functional homolog of Rpr, Hid or Grim, as it functions to neutralize caspase inhibitory function of the IAP protein family (Du *et al*., 2000; Verhagen *et al*., 2000).

This paper focuses on the role of the Bcl-2 family of proteins in *Drosophila* programmed cell death. Accumulated evidence suggests that in mammalian cells, mitochondrial Bcl-2 prevents the release of cytochrome *c*, required for the formation of the Apaf-1 apoptosome and therefore caspase activation (Zou *et al*., 1997). Conversely, the pro-apoptotic Bcl-2 proteins promote mitochondrial permeability and cytochrome *c* release. Life or death of the cell is determined by whether the balance is tipped towards the pro-survival or the pro-apoptotic Bcl-2 members (reviewed in Cory and Adams, 2002). In *Drosophila*, there are two homologs of the Bcl-2/Ced-9 family of programmed cell death (PCD) proteins, Debcl/dBorg-1/dRob-1 and Buffy/dBorg-2 (Brachmann *et al*., 2000; Colussi *et al*., 2000; Igaki *et al*., 2000). Although both Debcl and Buffy share the BH1, BH2, BH3 and C-terminal transmembrane domains of the Bcl-2

family of proteins, they appear to lack the N terminal BH4 domain. In mammals, the BH4 domain distinguishes the pro-apoptotic Bcl-2 family members, e.g. Bax and Bok, from the anti-apoptotic members, e.g. Bcl-2, Bcl-xL and Bcl-w (reviewed in Cory and Adams, 2002). Based upon this, both Debcl and Buffy were expected to be pro-apoptotic. Although both Debcl and Buffy are most closely related to the mammalian pro-apoptotic Bok, only Debcl has been shown to have a pro-apoptotic function in *Drosophila*, since ectopic overexpression of *debcl* in transgenic flies results in ectopic PCD and functional knockout of Debcl by RNA interference (RNAi) leads to an inhibition of cell death (Brachmann *et al*., 2000; Colussi *et al*., 2000; Igaki *et al*., 2000).

Here we provide the first evidence that Buffy is a pro-survival relative of Bcl-2/Ced-9. Buffy is required for cell survival and can prevent developmental and irradiation-induced cell death. We also show that Buffy overexpression prevents cell cycle progression and results in the accumulation of cells in G_1, like its mammalian pro-survival counterpart Bcl-2 (O'Reilly *et al*., 1996). Thus, both pro-survival and cell cycle functions of Bcl-2 have been evolutionarily conserved in Buffy, suggesting that Buffy is the *Drosophila* homolog of the pro-survival Bcl-2 proteins.

Results

The buffy expression pattern correlates with debcl expression and apoptotic domains

Buffy encodes a protein that is 19% identical and 56% similar to human Bcl-2 over a 239 amino acid region and shares several conserved motifs with mammalian Bcl-2, including the BH1, BH2 and BH3 domains, and a C-terminal hydrophobic membrane anchor (Figure 1A and B). Although an N-terminal BH4 domain present in pro-survival Bcl-2 proteins is not obvious in the Buffy sequence, there are two α-helical domains in the N-terminal region that might be functionally similar to the BH4 domain.

To examine the expression of *buffy* mRNA during development, we initially carried out northern blot and RT–PCR analysis (Figure 1C; data not shown). Due to the low level of *buffy* mRNA expression, the 1.2-kb *buffy* transcript was scarcely detectable upon northern analysis (data not shown). By RT–PCR, however, *buffy* mRNA was detected at all developmental stages, with the strongest expression detected from the late larval/early pupal stages (Figure 1C).

The spatial distribution of *buffy* mRNA was determined using *in situ* hybridization. The *buffy* transcript was expressed at very low levels and, as with *debcl* mRNA, detection required indirect tyramide-amplification (TSA™), as described previously (Colussi *et al*., 2000). *In situ* hybridization analysis of *Drosophila* embryos revealed *buffy* transcript in non-cellularized, stage 5 embryos. Since zygotic transcription does not occur prior to stage 5, this represents maternally deposited mRNA (Figure 1D). General ubiquitous expression was observed in germ band extended, stage 10 embryos (Figure 1E). Later in embryogenesis the pattern of *buffy* mRNA becomes more restricted, with staining in the midgut, the hindgut and a segmental pattern throughout the epidermal tissue (Figure 1F). *buffy* message becomes more restricted at stage 16 of embryogenesis and is prominent in the epidermis of the gut and regions of the head, including the pharynx and clypeolabrum [compare Figure 1G with I (sense control)]. *buffy* mRNA was detected in the same pattern as the pro-apoptotic *Drosophila* Bcl-2-related gene *debcl* (Colussi *et al*., 2000). The similarity between the expression patterns of *debcl* and *buffy* was particularly striking in stage 16 embryos (compare Figure 1G with H). Such similar expression patterns suggest that coordinated expression may be important for regulating cell death. The patterns of *buffy* and *debcl* expression correlate with regions of cell death in the developing embryo (Colussi *et al*., 2000).

During oogenesis, the nurse cells dump their cytoplasm into the oocyte, a process coordinated with nurse cell apoptosis, and are regulated by apoptotic stimuli (reviewed in Buszczak and Cooley, 2000). *buffy* mRNA was abundant in the nurse cell chambers from stage 10a ovaries [(compare Figure 1J with K (sense control)], which undergo apoptosis at stage 10b. During early pupal stages, most larval tissues are histolysed, an extensive apoptotic process regulated by pulses of the steroid hormone ecdysone (reviewed in Baehrecke, 2000). During third instar, *buffy* mRNA was strongest in larval midgut (Figure 1L) and salivary glands (Figure 1N), tissues destined for histolysis in pupariation (Jiang *et al*., 1997). *buffy* mRNA was also detected (albeit at lower levels) in larval tissues that are remodeled into the adult tissue during pupal development—a process requiring a balance between apoptosis and cell survival—including the brain lobes (Truman *et al*., 1994) (Figure 1P) and eye imaginal discs (Miller and Cagan, 1998) (Figure 1R). *buffy*, therefore, is expressed throughout development, in the same pattern as the pro-apoptotic gene *debcl* and in tissues susceptible to apoptosis.

Buffy interacts with the pro-apoptotic Drosophila Bcl-2 homolog, Debcl

The mammalian pro-apoptotic Bcl-2 proteins function by binding and sequestering pro-survival Bcl-2 members (reviewed in Cory and Adams, 2002). Debcl binds most mammalian pro-survival Bcl-2 proteins, including Bcl-2 and Bcl-X_L, but not their pro-apoptotic counterparts (Colussi *et al*., 2000). In order to determine whether Debcl heterodimerizes with Buffy, we carried out co-immunoprecipitation experiments (Figure 2). FLAG-tagged Buffy was coexpressed with HA-tagged Debcl in 293T cells. Immunoprecipitation was performed with anti-FLAG or anti-HA antibodies. The control immunoblot with anti-FLAG showed that FLAG-Buffy (33 kDa) was precipitated (Figure 2, middle panel). Immunoblotting of the FLAG immunoprecipitates with anti-HA revealed the HA-Debcl protein (Figure 2, bottom panel), suggesting that the two proteins can co-immunoprecipitate. Therefore, like the pro- and anti-apoptotic members of the mammalian Bcl-2 family, Debcl and Buffy can physically interact.

Buffy protein colocalizes with mitotracker to the mitochondria

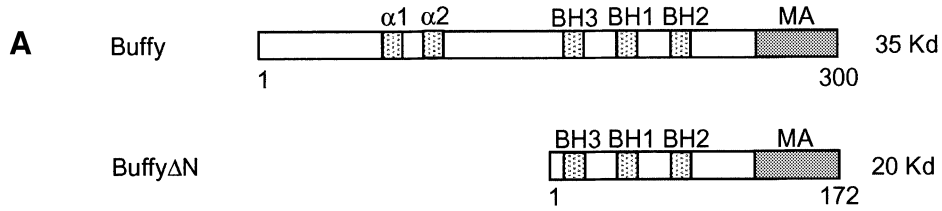
The subcellular distribution of Buffy protein was determined using a rat polyclonal Buffy antibody. We tested the specificity of this antibody using the *en-GAL4* driver to

ectopically express the upstream activator sequence (*UAS*)-*buffy* transgene and a *UAS-GFP* transgene to mark cells expressing *engrailed* (Figure 3A–C). Significantly, increased levels of anti-Buffy antibody staining were observed in the Engrailed (En) stripes compared with the levels of protein in adjacent cells (Figure 3B). Leaky expression of the *UAS-buffy* transgene was suggested by the finding that Buffy antibody staining was consistently higher across the entire embryo when compared with the level of endogenous protein from wild-type embryos (Figure 3D). Further evidence for leaky expression was the greater general protection from irradiation-induced cell death (see below). Although mutants were not available to verify the specificity of our antibodies further, we found a clear reduction in the level of staining for *buffy* double-stranded (ds) RNA ablation embryos (see below). In addition, the pattern of Buffy antibody staining in stage-16 wild-type embryos was similar to that observed for *buffy* mRNA expression (compare Figure 3E with F).

The pro-survival Bcl-2 proteins are localized to intracellular membranes, including mitochondria, endoplasmic reticulum and nuclear envelope (Janiak *et al*., 1994; reviewed in Cory and Adams, 2002). The Buffy C-terminus contains a putative hydrophobic membrane anchor, similar to the sequence found in many Bcl-2 family proteins (Brachmann *et al*., 2000; Colussi *et al*., 2000). To determine whether Buffy localized to mitochondria, we co-stained *Drosophila* tissues with the mitochondrial marker mitotracker and anti-Buffy antibody. Mitotracker has been used previously to show that the mitochondria of *Drosophila* larval brain are scattered throughout the entire cytoplasm and surround the nucleus (Iyengar *et al*., 2002). We reproduced this pattern of mitotracker staining in larval neuroblast cells (Figure 3H), and found co-localization with Buffy protein predominantly in mitotracker-positive regions (Figure 3I). Ionizing radiation has been used previously to induce apoptosis in *Drosophila* tissues (Ollmann *et al*., 2000). Similar Buffy staining was seen in γ-irradiated tissues compared with untreated ones, including eye discs, wing discs, salivary glands (data not shown) and midgut (Figure 3J–O, and high power in Figure 3P–R). Therefore, like Bcl-2, Buffy localizes to mitochondria in both normal and irradiated cells, unlike the pro-apoptotic Bax proteins that only become localized to the mitochondrial membrane following stress signals (reviewed in Cory and Adams, 2002).

Buffy is required for cell survival during embryogenesis

As no specific *buffy* mutants are currently available, we used RNAi to knock down Buffy expression, which has been used extensively in *Drosophila* to knock down gene function (reviewed in Sharp, 2001). *buffy* dsRNA was injected into pre-blastoderm embryos, which were allowed to develop for 6–7 h before TUNEL and staining with anti-Buffy antibody, to measure the efficiency of Buffy protein ablation. On average, a 7-fold increase in TUNEL cells was observed in *buffy* dsRNA-injected embryos (Figure 4). Wild-type, stage-11 embryos have small populations of apoptotic cells in the amnioserosa, brain lobes and



B

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                                                BH4
Buffy      MPGTSYPTNNDNFSNGFPMATTQSERLLQAQNRRKFSFPATLHSASLLEVGGGPKETTRR 60
hBcl-2     --------MAHAGRTGYDNREIVMKYIHYKLSQRGYEWDAGDVGAAPPGAAPAPGIFSSQ 52
                     .   .*:       : :    .:* :.: *   .*:   .. .*   : :

                                                                        
Buffy      RLSNVSDAVTRKLSYTIGWKAAQIPAQDIISQGRCLCGHYIKRRLRRSGLFNKKLGLQRI 120
hBcl-2     PGHTPHTAASRDP------VARTSPLQTPAAPG--------------------------- 79
              .   *.:*.        *   * *   : *

                                        BH3
Buffy      RSILGSTSMGIVRDVFPAVQVLGDELERMHPRIYNGVARQICRNPGGEFHTPDAVSLLLG 180
hBcl-2     -AAAGPALSPVPPVVHLTLRQAGDDFSRRYRRDFAEMSRQLH-------LTPFTARGRFA 131
            :  *.:   :   *. :::  **:.* : * :  ::**:         ** :.   :.

                                  BH1
Buffy      AVGRELFRVEITWSKVISLFAIAGGLSVDCVRQGHPEYLPKLMESVSEVIEDELVPWINE 240
hBcl-2     TVVEELFRDGVNWGRIVAFFEFGGVMCVESVNREMSPLVDNIALWMTEYLNRHLHTWIQD 191
           :* .****  :.*.::::* :.* :.*:.*.:  .  : ::   ::* :: .* .**::

                                                                        
           BH2                          MA
Buffy      NGGWSGINTHVLPTTNSLNPLEWTTLVIGVVFGLILVFMILRFIFNLIVPKIYQRFTNS 299
hBcl-2     NGGWDAFVELYGPSMRPLFDFSWLSLKTLLSLALVGACITLGAYLGHK----------- 239
           ****..:     *: ..*  :.* :*   : :.*: . : *   :.
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developing central nervous system (CNS) (Abrams *et al*., 1993) [Figure 4A (TUNEL = 90 ± 12, n = 4)], and ubiquitous staining for Buffy protein (Figure 4B). Control embryos injected with *GFP* dsRNA and aged to stage 11 have a similar low level of apoptosis [Figure 4C (TUNEL = 93 ± 23, n = 4)] and ubiquitous staining for Buffy protein (Figure 4D). Reduction of Buffy protein, shown using the Buffy antibody (Figure 4F), correlated with increased levels of ectopic apoptosis [Figure 4E (TUNEL = 802 ± 115, n = 4)]. In a separate experiment, embryos were aged to between stages 14 and 16, following injection with either *buffy* dsRNA or buffer only. Antibody staining revealed that Buffy protein was barely detectable by stage 14–16 in embryos injected with *buffy* dsRNA (Figure 4L), compared with control embryos at stage 14, where epidermal and neural staining is observed (Figure 4H), and at stage 16 (Figure 4J). The older *buffy* RNAi embryos were fragile, with many disintegrating

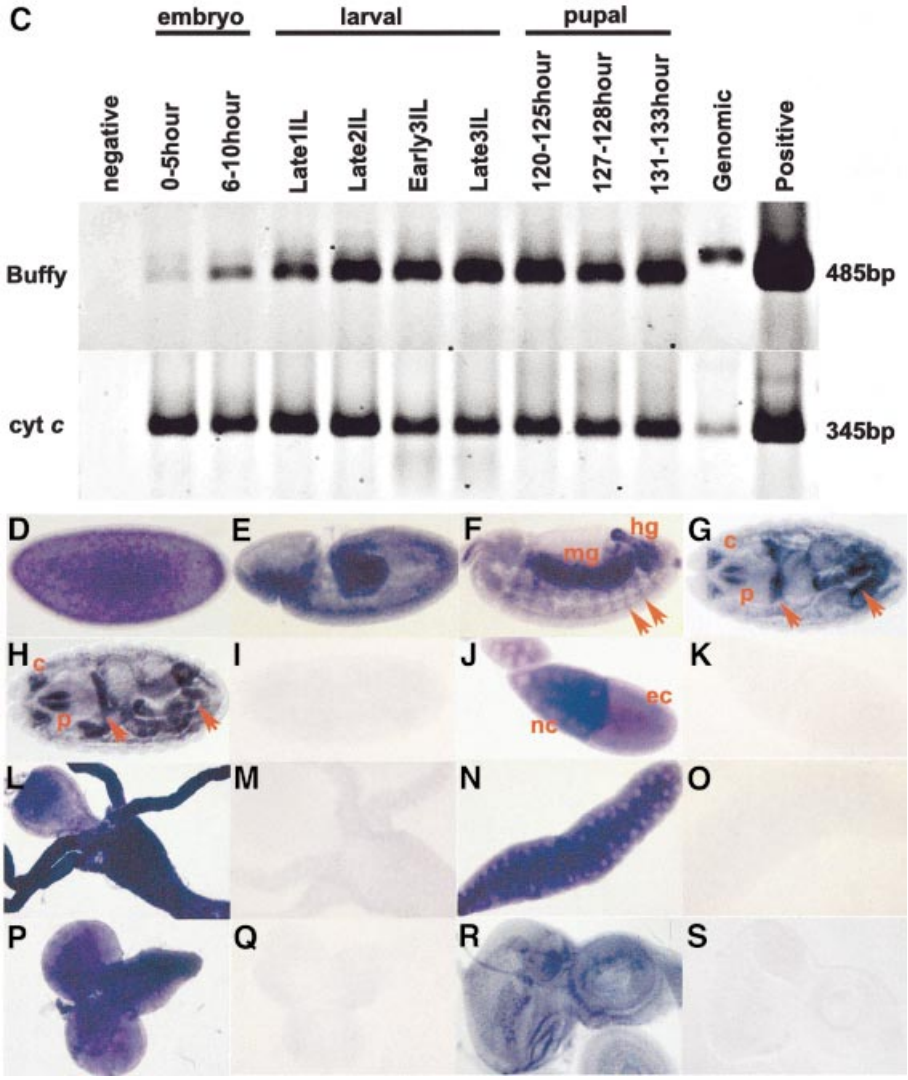

Fig. 1. (**A**) Protein structure of Buffy. The positions of the BH1, BH2 and BH3 domains and a C-terminal hydrophobic membrane anchor are shown. Two upstream α-helical domains are also indicated. The deletion mutant BuffyΔN, used to generate *UAS-buffyΔN* transgenic flies, lacks the first 128 amino acids. (**B**) Alignment of the predicted protein sequences of Buffy and human Bcl-2 [DDBJ/EMBL/GenBank accession no. AAA35591; performed using the program Clustal_W (http://www.ebi.ac.uk/clustalw/)]. Identical residues are indicated by an asterisk (*), conserved residues by a colon (:), and similar residues by a full point (.). Buffy and Bcl-2 share 19% identity and 56% similarity over a 239 amino acid region. The positions of the BH1, BH2, BH3 and BH4 domains of Bcl-2, based on BH domain consensus sequences (http://www.expasy.org/prosite/), are indicated by lines above the sequence. Buffy contains conserved BH1, BH2 and BH3 domains, but lacks a conserved BH4 domain. The putative membrane anchor (MA) of Buffy is shown. (**C**) RT–PCR analysis of *buffy* expression. After reverse transcription, PCR was performed using *buffy* specific primers spanning an intron to amplify a 465-bp fragment. Cytochrome *c* (cyt *c*) was used as a control from the same cDNA samples. *In situ* hybridization analysis using DIG-labeled probes (D–S). *Drosophila* embryos with an antisense *buffy* probe: (**D**) stage 5; (**E**) germ band extended/stage 10; (**F**) germ band retracted stage 13 (red arrows show segmental pattern; mg = midgut, hg = hindgut); (**G**) stage 16 (red arrows show epidermis of the gut; p = pharynx, c = clypeolabrum); (**H**) stage 16 with an antisense *debcl* (**I**) stage 16 embryo hybridized with sense *buffy* (**J**) stage 10a ovaries with antisense *buffy* (nc = nurse cells, ec = egg chamber); (**K**) stage 10a ovaries with sense probe; (**L**) third instar larval midgut with an antisense *buffy* probe; (**M**) third instar larval midgut with the sense probe; (**N**) third instar salivary glands with antisense *buffy*; (**O**) third instar salivary glands with the sense control probe; (**P**) third instar larval brain lobes with an antisense *buffy* probe; (**Q**) third instar larval brain lobes with a sense buffy probe; (**R**) third instar larval eye discs with an antisense *buffy* probe; and (**S**) third instar larval eye discs with a sense buffy probe.

during collection; those remaining had three times the number of TUNEL-positive cells [Figure 4K (TUNEL = 1015 ± 149, n = 4)] compared with control embryos at stages 14 or 16 [Figure 4G and Figure 4I, respectively (TUNEL = 287 ± 79, n = 4)]. Reduced numbers of cells and very few surviving neural cells were also observed in similarly injected and aged embryos (data not shown).

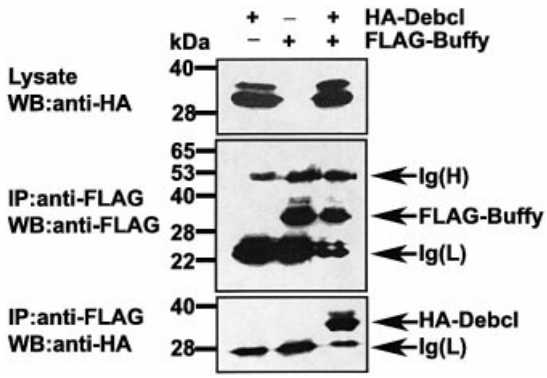

Fig. 2. Buffy interacts with the pro-apoptotic *Drosophila* Bcl-2 homolog, Debcl. Flag-tagged Buffy was coexpressed with HA-tagged Debcl in 293T cells. (Top panel) Lysates blotted with HA antibody. (Middle panel) Lysates immunoprecipitated with an anti-FLAG antibody and blotted with anti-FLAG. (Bottom panel) Lysates immunoprecipitated with anti-FLAG and blotted with anti-HA.

Thus, ablation of Buffy function results in cell death, indicating that Buffy is necessary for embryonic cell survival.

Buffy inhibits developmental cell death and apoptosis induced by ionizing radiation

Apoptosis commences during stage 11 of *Drosophila* embryogenesis, and as development proceeds, TUNEL-labeled cells are observed throughout the embryo, particularly in cells of the nervous system (Abrams *et al.*, 1993). In order to determine whether *buffy* overexpression inhibits developmental PCD, we overexpressed *buffy* in *Drosophila* embryos using the *UAS-GAL4* system (Brand and Perrimon, 1993). Buffy protein was expressed using the *En-GAL4* driver, which drives expression in the pair-rule striped pattern of the embryo (Kornberg *et al.*, 1985) (Figure 5A and D). En stripes from *En-GAL4*,*UAS-GFP*,*UAS-Buffy* embryos [Figure 5E (2.3 ± 0.8, n = 14)] contained approximately half the number of TUNEL-positive cells when compared with control embryos [Figure 5B (4.86 ± 1.2, n = 14)]. Therefore, ectopic expression of Buffy can inhibit developmentally regulated PCD during *Drosophila* embryogenesis. Furthermore, ubiquitous expression of *buffy* using the *Armadillo-GAL4* driver can result in additional neural cells, suggesting that Buffy overexpression can block the normal pattern of PCD in the developing peripheral nervous system (data not shown).

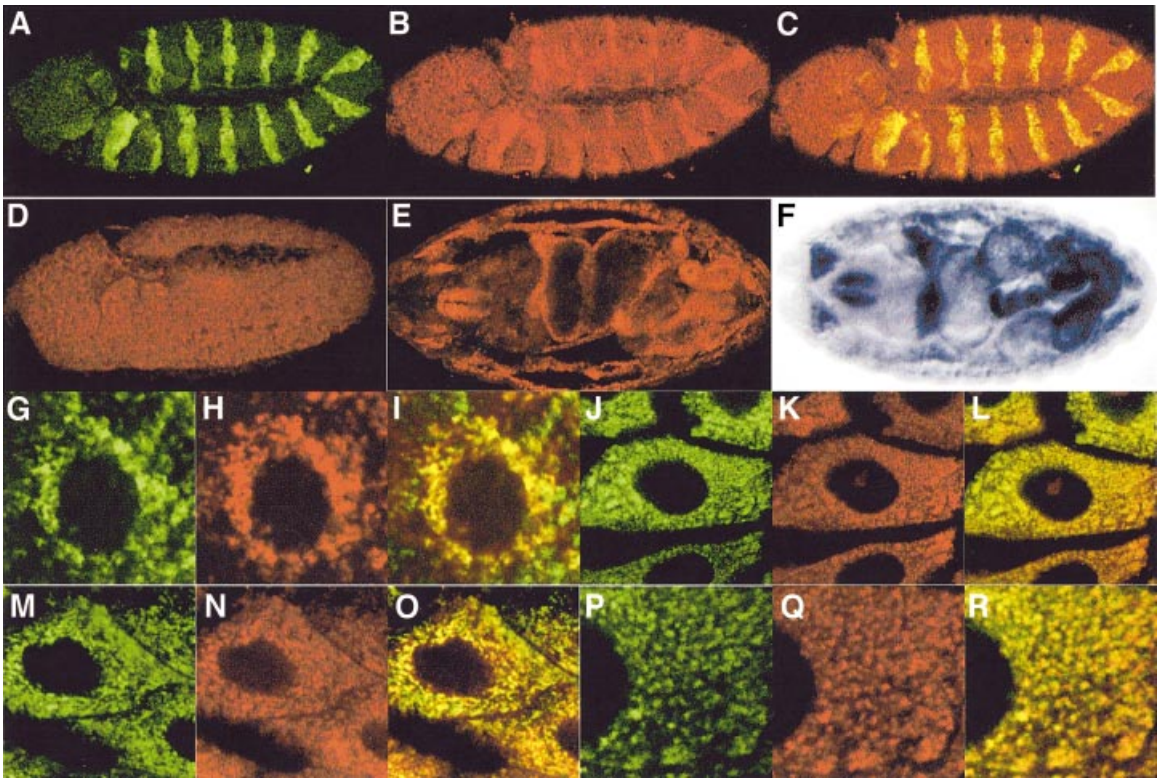

Fig. 3. Analysis of subcellular distribution of Buffy protein. (A–C) Stage 11 *en-GAL4*,*UAS-Buffy*,*UAS-GFP*/+ embryos with (**A**) anti-GFP antibody in green, (**B**) anti-Buffy antibody in red, and (**C**) merge. (**D**) *en-GAL4*,*UAS-GFP*/+ embryos with anti-Buffy antibody in red. Intensity of the confocal laser was equivalent in (B) and (D). Wild-type stage 16 embryos with (**E**) anti-Buffy antibody in red and (**F**) mRNA *in situ* for *buffy*. (G–I) Wild-type third instar brain lobe cells: (**G**) anti-Buffy antibody (green), (**H**) mitotracker (red), and (**I**) merge. (J–L) Wild-type larval midgut cells: (**J**) anti-Buffy antibody, (**K**) mitotracker, and (**L**) merge. (M–O) Wild-type larval midgut cells 2 h after treatment with 8 Gy ionizing radiation: (**M**) anti-Buffy antibody, (**N**) mitotracker, and (**O**) merge. (P–R) High power wild-type, unirradiated larval midgut cells: (**P**) anti-Buffy antibody, (**Q**) mitotracker, and (**R**) merge.

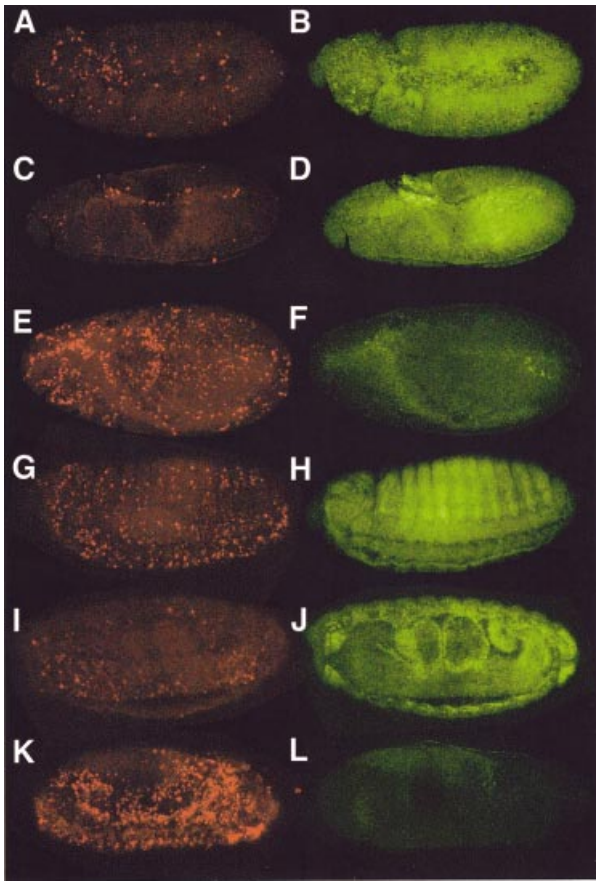

Fig. 4. RNAi using *buffy* dsRNA fragments. (A, C, E, G, I, K) Embryos stained for TUNEL. (B, D, F, H, J, L) Embryos stained for Buffy protein. (**A** and **B**) Wild-type stage 11 embryos. (**C** and **D**) Control embryos, injected with *GFP* dsRNA and aged to stage 11. (**E** and **F**) Embryos injected with *buffy* dsRNA fragments and aged to stage 11. (G–J) Embryos injected with buffer and aged at stage 14 (**G** and **H**) or stage 16 (**I** and **J**). (**K** and **L**) Embryos injected with *buffy* dsRNA fragments and aged between stages 14 and 16 (note that these embryos are difficult to stage due to impaired development).

Overexpression of Bcl-2 impairs the stress-induced apoptotic response of cells (Strasser *et al*., 1994; Cory and Adams, 2002). In order to determine whether *buffy* overexpression inhibits stress-induced apoptosis, we compared TUNEL from γ-irradiated *en-GAL4*,*UAS-GFP*,*UAS-Buffy* embryos with control embryos (Figure 5). Two *UAS-buffy* constructs were generated: a wild-type construct predicted to generate full-length protein and an N-terminal deletion construct (*buffy*Δ*N*). The deletion eliminates 128 amino acids from the N-terminus, removing two putative α-helices that may be ancestral to the amphipathic α-helix from the BH4 domain of pro-survival Bcl-2 proteins (Figure 1A and B). As the BH4 domain is required for anti-apoptotic function of Bcl-2 (Hunter *et al*., 1996), by comparing the properties of *buffy*Δ*N* with full-length *buffy* we sought to determine whether the extended N-terminal region of Buffy was important for cell survival.

Embryos expressing either full-length *buffy* (Figure 5F) or *buffy*Δ*N* (Figure 5K) in the En pattern were protected from γ-irradiation-induced apoptosis (Figure 5B). TUNEL labeling within the En stripe was reduced 7-fold for full-length *buffy* (4.3 ± 1.7, n = 10) and 6-fold for *buffy*Δ*N* (5.6 ± 1.5, n = 10), compared with wild type (32 ± 7, n = 9). There was also a reduced level of TUNEL in the inter-stripe region for full-length *buffy* (34 ± 8, n = 10) and *buffy*Δ*N* (29 ± 10, n = 10) compared with wild type (64 ± 17, n = 9). This general reduction of TUNEL labeling, which was reproducible over three experiments, may be due to leaky expression of the *UAS-buffy* transgene (see above). To determine whether Buffy could inhibit stress-induced apoptosis in other tissues, we expressed Buffy using *en-GAL4*, which is also expressed in the posterior of third instar larval wing discs (Kornberg *et al*., 1985). Expression of two copies of *UAS-buffy* with *en-GAL4* is embryonic lethal (data not shown), therefore wing discs expressing only one copy of full-length *buffy* were examined. Cells in the posterior compartment of the wing disc were protected from γ-irradiation-induced apoptosis, compared with the high level of cell death observed in the anterior compartment (Figure 5I and J), or with the extensive TUNEL observed in irradiated wild-type discs (Figure 5G). The observation that protection from apoptosis does not occur in the anterior compartment of the wing disc suggests that either: (i) leaky expression of the *UAS-buffy* transgene does not occur to the same degree in the wing discs as in the embryo; or (ii) that when only one copy of *UAS-buffy* is present, leaky expression does not occur at a level that provides protection from irradiation-induced cell death.

Increased levels of Buffy are therefore sufficient to inhibit the *Drosophila* apoptotic pathway that normally responds to DNA damaging agents such as ionizing radiation. Furthermore, since both full-length Buffy and BuffyΔN protected embryos from γ-irradiation-induced apoptosis, the region of the protein encompassing the three BH domains, and C-terminal membrane anchor, is sufficient for the anti-apoptotic function of Buffy.

Genetic analysis places Buffy downstream of Rpr, Grim and Hid, and upstream of the the apical caspase Dronc

To examine genetic interactions between Buffy and other apoptotic pathway genes, we used Glass multimer reporter (*GMR*)-*GAL4* to drive the *UAS-buffy* transgene in the posterior region of the third instar eye imaginal disc. Recombinants of the *UAS-buffy* transgene with *GMR-GAL4* on the second chromosome, when heterozygous (*GMR-GAL4:UAS-buffy/+*), produce flies with eyes of wild-type appearance (compare Figure 6B with A). Similarly, ectopic expression of the *Drosophila* inhibitor of apoptosis, *DIAP1*, using the *GMR* driver results in normal-appearing adult eyes (data not shown). However, expression of *diap1* can inhibit apoptotic phenotypes generated by overexpression of caspases (Hawkins *et al*., 2000; Meier *et al*., 2000; Quinn *et al*., 2000), *rpr* and *hid* (Goyal *et al*., 2000; Meier *et al*., 2000). *GMR-diap1* also suppresses the *GMR-GAL4/+;UAS-debcl/+* ablated eye phenotype (data not shown), consistent with the notion that Debcl induces apoptosis by functioning upstream of DIAP1-dependent caspase inhibition (Colussi *et al*., 2000).

The strong ablated eye phenotype from *GMR-GAL4/+;UAS-debcl/+*, shown in Figure 6C, could be partially suppressed by coexpression of *buffy* (Figure 6D). The

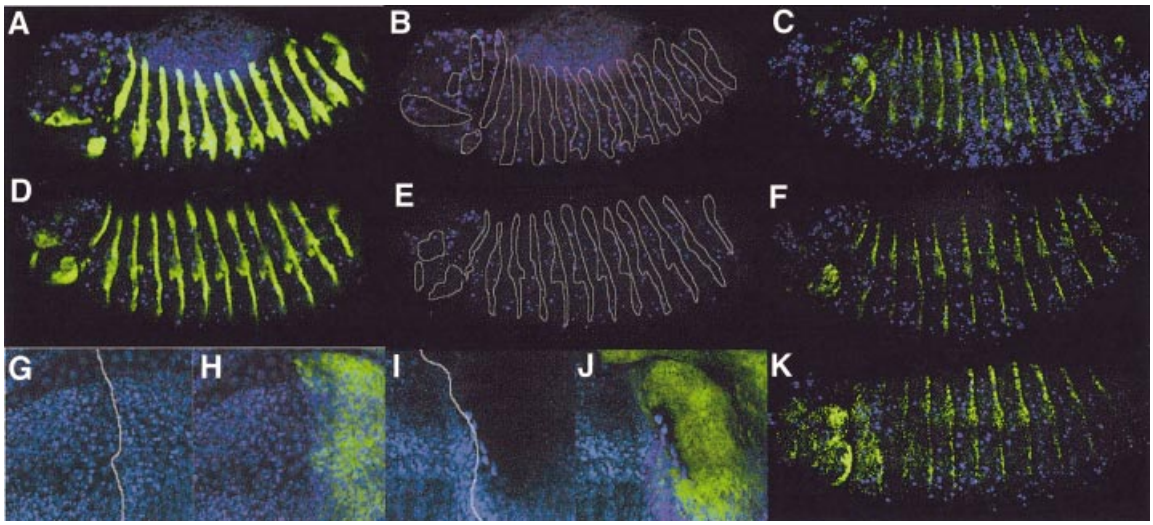

Fig. 5. Overexpression of Buffy with the *engrailed* (*en*)-*GAL4* driver blocks developmental and irradiation-induced cell death in stage 13 embryos, and irradiation-induced cell death in third instar wing discs. (**A**) Control *en-GAL4*/+ embryo stained with TUNEL (blue) and anti-GFP antibody (green). (**B**) The embryo in (A) stained with TUNEL (positions of the En stripes are circled). (**C**) Irradiated control *en-GAL4*/+ embryo stained with anti-En (green) and TUNEL (blue) after 2.5 h recovery. (**D**) *en-GAL4*/+,*UAS-GFP*/+,*UAS-buffy*/+ stained with TUNEL (blue) and anti-GFP antibody (green). (**E**) The embryo in (D) showing the position of the En stripes. (**F**) Irradiated *en-GAL4*/+,*UAS-buffy*/+ embryo after 2.5 h of recovery, stained with anti-En (green) and TUNEL (blue). (G–J) Irradiated third instar larval wing discs after 4 h of recovery, stained with anti-GFP (green) and labeled with TUNEL (blue). (**G** and **H**) *en-GAL4*/+,*UAS-GFP*/+; (**I** and **J**) *en-GAL4*/+,*UAS-buffy*/+,*UAS-GFP*/+. (**K**) Irradiated *en-GAL4*/+,*UAS-buffyΔN*/+ embryo stained with anti-En (green) and TUNEL (blue).

extreme nature of the *GMR-GAL4;UAS-Debcl*/+ phenotype suggests a high level of Debcl protein expression, and thus the slight suppression by Buffy suggests that this *UAS-buffy* line is not expressed at high enough levels to sequester the excess Debcl protein. However, the *en-GAL4-UAS-debcl* ablated wing phenotype (Figure 6O) was suppressed by coexpression of *UAS-buffy* (Figure 6R). TUNEL labeling of third instar wing imaginal discs revealed that this suppression was due to Buffy inhibiting Debcl-induced apoptosis in the posterior compartment (compare Figure 6Q with M).

Ectopic expression of Rpr, Hid and Grim causes the *Drosophila* IAP homolog *Diap1/Thread*(*th*) to be sequestered and inactivated, thus resulting in ectopic cell death (reviewed in Hay, 2000). Overexpression of *buffy* with *GMR-GAL4* partially suppressed the ablated eye phenotypes of *rpr* (Figure 6F), *hid* (Figure 6H) and *grim* (Figure 6J). Furthermore, the ectopic TUNEL observed in homozygous *diap1* (th^5) mutant embryos (Wang *et al*., 1999) (Figure 6S and V) was inhibited by overexpression of the *UAS-buffy* transgene with *en-GAL4* (Figure 6T, U, W and X). Taken together, this suggests that Buffy acts downstream of Rpr, Grim, Hid and DIAP1 to block caspase-dependent cell death.

Expression of the *UAS-dronc* transgene with *GMR-GAL4* results in a small, mottled eye phenotype as a consequence of ectopic cell death, particularly of pigment cells (Figure 6K) (Meier *et al*., 2000; Quinn *et al*., 2000). This phenotype was not modified by coexpression of the *UAS-buffy* transgene (Figure 6L), suggesting that Buffy acts upstream of caspase activation. Similarly, overexpression of the N-terminal deletion construct (*UAS-buffyΔN*) with *GMR-GAL4* suppressed the Rpr, Grim, Hid and Debcl-ablated eye phenotypes, but did not alter the Dronc mottled eye phenotype (data not shown). Therefore, only the C-terminal portion of the Buffy protein is required for suppression.

Buffy induces a G_1 phase delay when overexpressed in embryos

The mammalian Bcl-2 protein can inhibit cell cycle entry, independent of its anti-apoptotic function (Huang *et al*., 1997). Although the overall growth rate of proliferating cell cultures is not affected by ectopic Bcl-2, increased withdrawal from the cell cycle into G_1 phase occurs (Vairo *et al*., 1996, 2000) and cell cycle re-entry is retarded (Linette *et al*., 1996; Mazel *et al*., 1996). Here we show that overexpression of the *UAS-buffy* transgene with the *en-GAL4* driver results in inhibition of rapid embryonic cell cycles and an accumulation of cells in G_1 (Figure 7). Although the cell cycle pattern is dynamic, generally there are comparable numbers of S-phase cells for the same sized region both inside and outside the En stripe in a normal stage-11 embryo (Quinn *et al*., 2001). The number of S-phase cells was clearly reduced, although not eliminated, in cells overexpressing two copies of the *buffy* transgene in the En-stripe (Figure 7C and D) (BrdU in the En stripe = 6.7 ± 1.1, $n = 6$) compared with the interstripe regions (BrdU in the interstripe = 37.7 ± 4.5, $n = 6$; the equivalently sized region to the En stripe was calculated by dividing total interstripe count by 3) and the control embryo (Figure 7A and B) (BrdU in the En stripe = 19.2 ± 1.5; $n = 6$, BrdU in the interstripe =33.3 ± 3.8, $n = 6$). Mitotic cells, visualized using the anti-phosphohistone H3 antibody (PH3), were scattered across the epithelium of stage-11 embryos (Quinn *et al*., 2001) (Figure 7E–H). Mitotic cells were almost eliminated within the *buffy*-expressing En stripe (PH3 in En stripe =

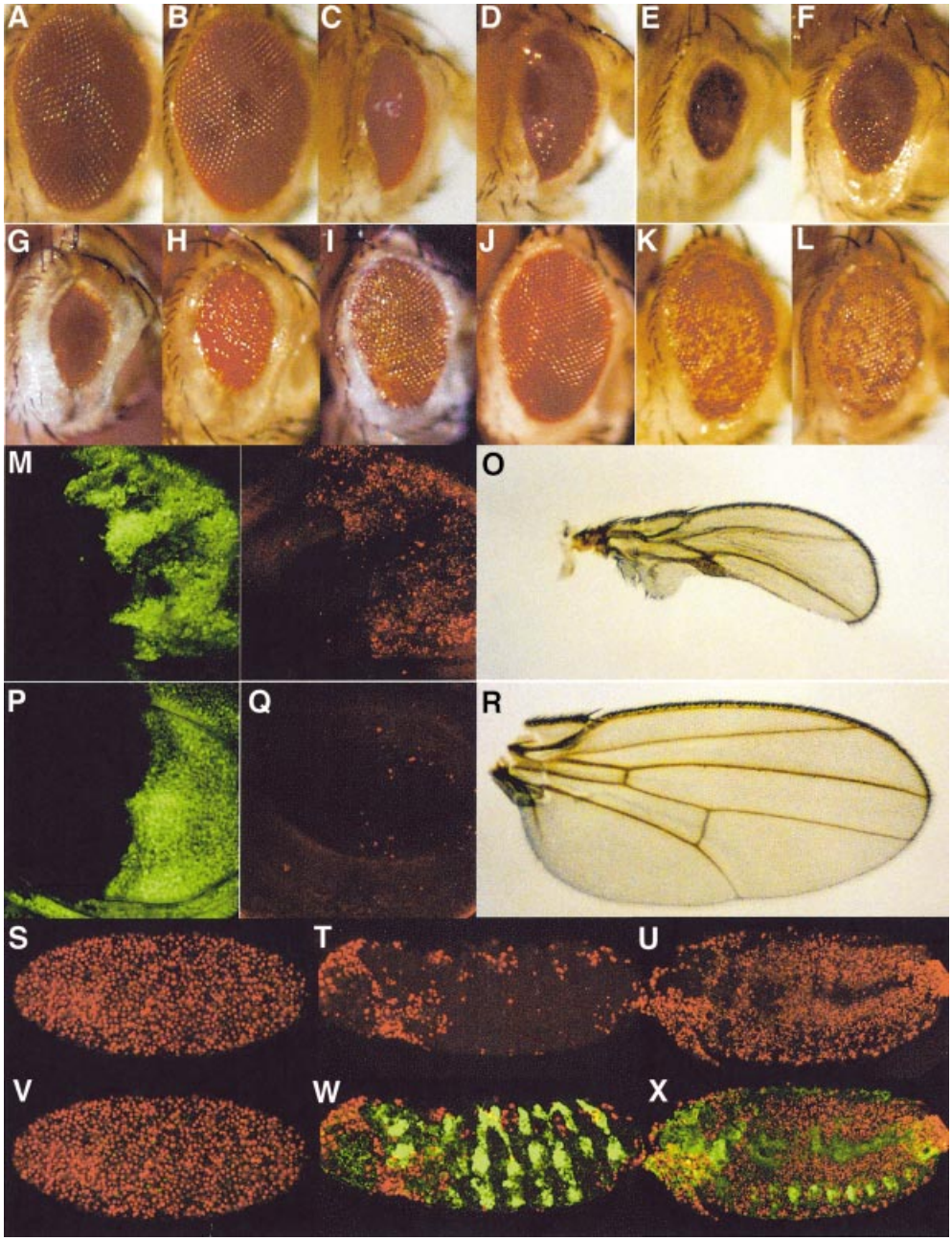

Fig. 6. Genetic interactions between Buffy and cell death genes for Rpr, Grim, Hid, Debcl and Dronc. Adult eyes of the following genotypes: (**A**) *GMR-GAL4*/+;+/+; (**B**) *GMR-GAL4*/+,*UAS-buffy*/+;+; (**C**) *GMR-GAL4*/+;*UAS-debcl*/+; (**D**) *GMR-GAL4*/+,*UAS-buffy*/+;*UAS-debcl*/+; (**E**) *GMR-rpr*/+;+; (**F**) *GMR-rpr*/+,*GMR-GAL4*/+,*UAS-buffy*/+;+/+; (**G**) *GMR-hid*/+;+/+; (**H**) *GMR-hid*/+,*GMR-GAL4*/+,*UAS-buffy*/+;+/+; (**I**) *GMR-grim*/+;+/+; (**J**) *GMR-grim*/+,*GMR-GAL4*/+,*UAS-buffy*/+;+/+; (**K**) *GMR-GAL4*/+,*UAS-dronc*/+;+/+; and (**L**) *GMR-GAL4*/+,*UAS-buffy*/+,*UAS-dronc*/+;+/+. (M–P) Third instar larval wing discs: (**M** and **N**) *en-GAL4*/+,*UAS-GFP*/+;*UAS-debcl*/+; (**O** and **P**) *en-GAL4*/+,*UAS-buffy*/+,*UAS-GFP*/+;*UAS-debcl*/+. En-expressing cells stained using GFP (green) (M and O) and TUNEL (red) (N and P). (Q and R) Wings from adult females: (**Q**) *en-GAL4*/+,*UAS-GFP*/+;*UAS-debcl*/+ and (**R**) *en-GAL4*/+,*UAS-buffy*/+,*UAS-GFP*/+;*UAS-debcl*/+. (S–X) *en-GAL4*/+,*UAS-GFP*/+;th^5/th^5 (**S** and **V**), and *en-GAL4*/+,*UAS-buffy*/+,*UAS-GFP*/+;th^5/th^5 at stage 10 (**T** and **W**) and stage 14 (**U** and **X**). Embryos are labeled with TUNEL (red) (S, T and U) and stained with the anti-GFP antibody (green) (merged images V, W and X). Note, in (V), that cells expressing GFP have been destroyed.

2.8 ± 1.8, $n = 5$), compared with cells between the stripe (Figure 7G and H) (PH3 between stripe = 23 ± 10, $n = 5$) and control embryos (Figure 7E and F) (PH3 in En stripe = 21 ± 3.9, $n = 5$; PH3 between stripe = 39 ± 11, $n = 5$).

That mitotic figures were almost eliminated, whilst some BrdU incorporation was observed, might suggest a G_2 phase arrest, which would result in high levels of the G_2–M cyclin, Cyclin B. However, staining with a Cyclin B antibody showed that Cyclin B was low in *buffy*-overexpressing cells when compared with neighboring regions (Figure 7K and L) and control embryos (Figure 7I and J). Therefore, high levels of Buffy do not appear to cause a G_2 arrest or delay. Since there are decreased numbers of S-, G_2- and M-phase cells, and the nuclei size is smaller (Figure 7O and P), arrest is consistent with a G_1/early S phase arrest. Consistent with a cell cycle arrest

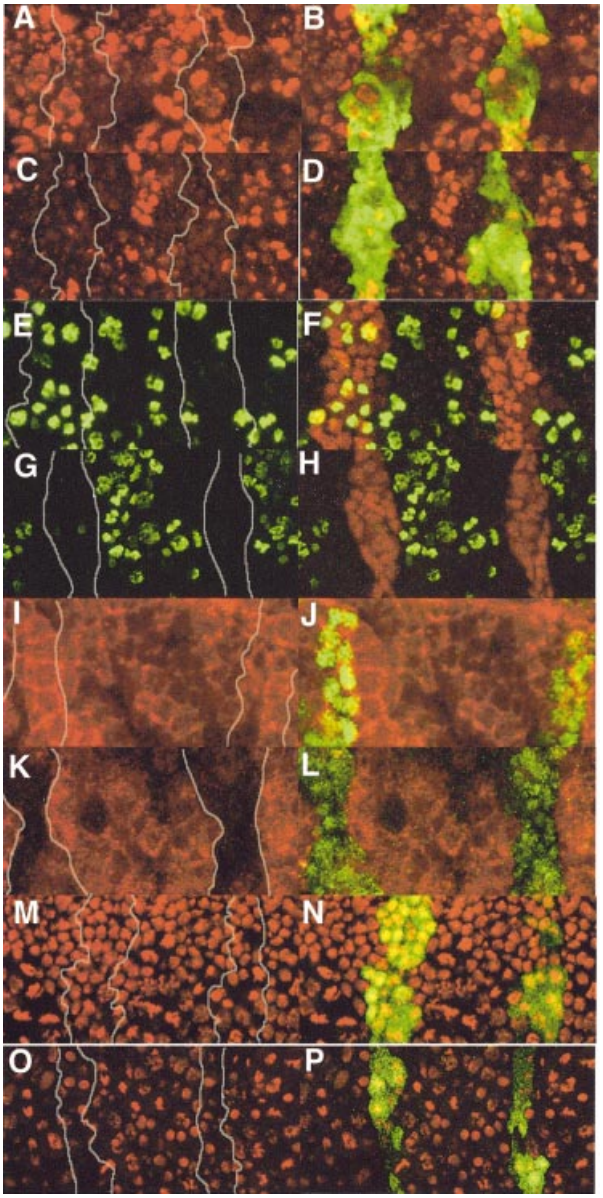

Fig. 7. Cell cycle analysis of *Drosophila* embryos expressing the *UAS-buffy* transgene in En stripes. The En stripes shown are epidermal cells spanning the ventral region of thoracic segments 4 and 5 from stage 11 embryos. (a–d) S-phase cells labeled with BrdU (red) and with En marked with GFP [green in (B) and (D), or white lines in (A) and (C)]. (**A** and **B**) *en-GAL4/en-GAL-4,UAS-GFP/UAS-GFP*; (**C** and **D**) *en-GAL4/enGAL4,UAS-GFP/+,UAS-buffy/+*. (e–h) Mitotic cells of stage-11 embryos visualized using the anti-phosphohistone H3 antibody (PH3) in green, En antibody staining in red (**F** and **H**) or a white line (**E** and **G**). (E and F) *en-GAL4/en-GAL4,UAS-GFP/UAS-GFP*; (G and H) *en-GAL4/enGAL4,UAS-GFP/+,UAS-buffy/+*. (i–l) Cyclin B distribution in stage-11 embryos, Cyclin B (red) and En stripe marked with GFP (green) (**J** and **L**), or by a white line (**I** and **K**). (I and J) *en-GAL4/en-GAL4,UAS-GFP/UAS-GFP*; (K and L) *en-GAL4/enGAL4,UAS-GFP/+,UAS-buffy/+*. (m–p) DNA morphology analysis using propidium iodide (PI) in red and the En stripe marked by GFP in green (**N** and **P**) or by a white line (**M** and **O**). (M and N) *en-GAL4/en-GAL4,UAS-GFP/UAS-GFP*; (O and P) *en-GAL4/en-GAL4,UAS-GFP/+,UAS-buffy/+*.

when Buffy is overexpressed, the En stripe from an equivalent region of the embryo is often thinner and contains fewer cells compared with the control in stage 11/early stage 12 embryos (Figure 7O and P compared with M and N). The variability in the width of the En band is likely to be a consequence of the extremely rapid cycling of stage 11 embryos, combined with the fact the there will be a gradual accumulation of Buffy in the En stripes, because En only starts to be highly expressed at stage 11. Leaky expression of the *UAS-buffy* transgene (see above) does not appear to greatly affect cell cycle progression in the inter-stripe region of *en-GAL4,UAS-buffy* embryos, possibly because the level of Buffy protein required is higher than that needed to prevent apoptosis. Importantly, these results provide the first evidence within a whole animal that a member of the Bcl-2 family has a cell cycle inhibitory role.

Discussion

In this report, we have shown that Buffy functions in a similar manner to the pro-survival mammalian Bcl-2 proteins since it: (i) is required for cell survival; (ii) inhibits developmentally regulated apoptosis; (iii) inhibits γ-irradiation-induced apoptosis; (iv) binds the *Drosophila* pro-apoptotic Bcl-2 homolog Debcl, and can suppress Debcl-induced cell death; and (v) when overexpressed has an inhibitory effect on cell cycle progression.

In mammals there are multiple pro-survival Bcl-2 proteins that play a tissue-specific role in protecting cells from apoptosis. Therefore, targeted knockout of individual pro-survival Bcl-2 members does not result in the death of the entire organism, suggesting that other pro-survival Bcl-2 proteins can compensate. For example, knockout studies show that Bcl-2 is required for survival of stem cells from kidney and melanocytes and adult lymphocytes (Veis *et al*., 1993), whilst Bcl-X_L is required for survival of neuronal and erythroid cells (Motoyama *et al*., 1995). In contrast, RNAi knockdown of *buffy* resulted in general embryonic cell death, suggesting that Buffy is a principle cell survival protein at this stage of *Drosophila* development. Indeed, database analysis shows no other Bcl-2-related proteins, apart from Buffy and Debcl.

Overexpression of Bcl-2 impairs the apoptotic response to DNA damaging agents such as ionizing radiation (Strasser *et al*., 1994). One of the reasons many tumors are resistant to chemotherapy and radiation therapy is that they mis-express Bcl-2 (reviewed in Cory and Adams, 2002). Here we have shown increased levels of Buffy are sufficient to inhibit the *Drosophila* apoptotic pathway that normally responds to DNA damaging agents.

Overexpression of mammalian Bcl-2 in *Drosophila* tissues has been shown to inhibit apoptosis, induced by either irradiation or Rpr overexpression (Gaumer *et al*., 2000; Brun *et al*., 2002). As we have found for Buffy, genetic analysis places the anti-apoptotic activity of Bcl-2 downstream of Rpr when expressed in flies (Brun *et al*., 2002). This is most likely a consequence of Bcl-2 protein binding to and sequestering Debcl, as has been shown previously (Colussi *et al*., 2000). In mammals, Bcl-2-mediated inhibition of apoptosis requires an α-helical domain within the N-terminal BH4 domain (Hunter *et al*., 1996). In contrast, Buffy's N-terminus, and the putative α-helices therein, were not required for either inhibition of irradiation-induced apoptosis or suppression of Rpr-, Grim- and Hid-induced apoptosis. Thus, the C-terminal region containing the BH1, BH2, BH3 and membrane anchor is sufficient for Buffy's cell survival function.

Certain factors controlling cell cycle progression are also sensitive to apoptotic stimuli (reviewed in Evan and Littlewood, 1998). Indeed, cell cycle factors may promote apoptosis under conditions unfavorable for proliferation, thus rendering cycling cells more vulnerable to apoptosis. Evidence that Bcl-2 plays a role in controlling cell cycle progression has been accumulating steadily (Linette *et al*., 1996; Mazel *et al*., 1996; O'Reilly *et al*., 1996; Vairo *et al*., 1996, 2000; Lind *et al*., 1999), and in this study we provide the first *in vivo* evidence that a member of the Bcl-2 family can result in cell cycle inhibition. However, in contrast to the serum-deprived G0 cells that have been used previously (Linette *et al*., 1996; Mazel *et al*., 1996; Vairo *et al*., 1996), *Drosophila* embryonic cells cycle normally prior to overexpression of Buffy.

The cell cycle delay that occurs as a consequence of Buffy overexpression is dose dependent. Expression of two copies of *UAS-buffy* via *en-GAL4* is embryonic lethal, presumably as a consequence of the G_1–S cell cycle arrest, which results in less cells in the En-stripes and insufficient cells to complete embryonic development. Two copies of *en-GAL4*,*UAS-buffy* (i.e. high level of Buffy protein) induce a cell cycle arrest and are embryonic lethal; however, flies expressing one copy (lower level of expression) are viable. Indeed, a low level of ubiquitous Buffy expression does not cause a cell cycle arrest, but results in the production of additional neural cells (L.Quinn and H.Richardson, unpublished data). Thus, the effect of Buffy overexpression is dose dependent; high levels of Buffy overexpression induce cell cycle arrest, which will ultimately result in fewer cells, whilst lower levels can inhibit developmental cell death and are associated with increased cell numbers.

Consistent with cell cycle arrest, Bcl-2 overexpression in mammalian cells correlates with increased levels of the CycE/Cdk2 inhibitor p27 (Brady *et al*., 1996; Linette *et al*., 1996), hyperphosphorylated and inactive retinoblastoma (RB) tumor suppressor (Mazel *et al*., 1996), and increased levels of RB-related protein p130 (Lind *et al*., 1999; Vairo *et al*., 2000). The inhibitory effect of Bcl-2 on the cell cycle is independent of p53, the cdk4/6 inhibitor p16 and RB, but requires p130 and p27 (O'Reilly *et al*., 1996; Vairo *et al*., 1996, 2000). The cell cycle inhibitory function of Bcl-2 can be separated from its cell survival function since the tyrosine residue, Y28, in the N-terminal BH4 domain is important for Bcl-2 inhibition of cell cycle re-entry, but is not required for cell survival (Huang *et al*., 1997). Although Buffy does not have an obvious BH4 domain, the cell cycle inhibitory function has been conserved between Buffy and Bcl-2. Thus, it will now be important to determine whether Buffy uses a similar mechanism to Bcl-2 to inhibit cell cycle progression.

Materials and methods

Cloning of Buffy cDNA

buffy was identified in a TBLASTN search using the Bcl-2 protein sequence (reported as 48A-E in Colussi *et al*., 2000). A 950-bp partial cDNA clone was isolated from a mixed stage *Drosophila* embryo cDNA library in λgt11 using a 450-bp probe derived from *Drosophila* genomic DNA using PCR. Sequencing of this clone confirmed that it encoded a Bcl-2 family member in the genomic region 48A-E and that it contained the predicted coding sequence for *buffy*. Further 5′ non-coding exon sequence was identified using 5′ RACE.

mRNA expression analysis

For RT–PCR, total RNA was prepared using RNAzol B (Tel-Test Inc.) and reverse transcribed using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). PCR was performed using *buffy*-specific primers spanning an intron with forward sequence 5′-ATGCATCCCCG-GATATACAAC-3′ and reverse sequence 5′-GCGGGATCCTTAG-GAATTCGTAAATCGTTGGTA-3′ to yield a product of 465 bp. The cytochrome *c* control was amplified with the forward primer (5′-CCGGAATTCATGGGCGTTCCTGCTGGTGAT-3′) and reverse primer (5′-CCGGAATTCTTACTTGGTCGCCGACTTCAG-3′) to obtain a product of 345 bp. Since *buffy* mRNA is expressed at very low levels, and it is difficult to visualize using the standard immunological staining methods, the TSA™ system (New England Nuclear Life Science Products) was used in to amplify the *in situ* signal according the methods described in Colussi *et al*. (2000).

Buffy/Debcl co-immunoprecipitation experiments

The 900-bp coding region of *buffy* was PCR amplified using Pfu Turbo DNA Polymerase (Stratagene) with an in-frame N-terminal FLAG-tag, and cloned into mammalian expression vector pcDNA3 (Invitrogen). HA-tagged pcDNA3-Debcl has been described previously (Colussi *et al*., 2000). 293T cells were transfected with 2 μg of pcDNA3-HA Debcl or pcDNA3-FLAG Buffy, or co-transfected with 2 μg of each construct using FuGENE 6 Transfection Reagent (Roche). Twenty-four hours after transfection, cell lysates were prepared in lysis buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, 0.1% NP-40) supplemented with Complete protease inhibitors (Roche). Immunoprecipitations were carried out as described previously (Colussi *et al*., 2000) using anti-Flag antibody (Sigma) and HA antibody (Roche).

Generation of Buffy antisera, antibody staining, BrdU, TUNEL and microscopy

To express Buffy in bacteria, the coding region of *Buffy* was PCR amplified from the cDNA clone and cloned into *pGEX*. Bacterially expressed Buffy protein was purified on a SDS polyacrylamide gel and used to inoculate rats. Rats were terminally bled after three boosts. Immunohistochemistry, including TUNEL and BrdU labeling of *Drosophila* tissues, was carried out as described previously (Quinn *et al*., 2000, 2001). Rat anti-Buffy antibody was detected using anti-rat-biotin conjugated secondary antibody followed by either streptavidin-lissamine rhodamine (Jackson) or Alexa-488 (Molecular Probes). TUNEL staining was carried out using the Roche *in situ* cell death kit, TRred. Other antibodies were: anti-BrdU monoclonal antibody (Becton Dickenson), anti-En monoclonal, rabbit anti-phosphohistone H3 (Santa Cruz) and rabbit anti-GFP (Molecular Probes). Staining with MitotrackerRed (Molecular Probes) was performed as described previously (Iyengar *et al*., 2002). All fluorescence-labeled samples were analyzed by confocal microscopy (Bio-Rad MRC1000), while AP colormetrically detected samples were analyzed on the Zeiss Axiophot Photophot using Nomarski optics.

RNA interference

buffy RNAi was pre-formed as described by Quinn *et al*. (2000). RNA transcripts were generated using the Ambion Megascript kit with linearized *pOT2-buffy* templates. Sense and antisense transcripts were purified, annealed and then dissolved in injection buffer (5 mM KCl in 0.1 mM phosphate buffer, pH 7.8) at 0.75 mg/ml. Between 250 and 300 pre-cellularized embryos were injected at 50% egg length with either *buffy* dsRNA or injection buffer alone, and aged until either stage 11–12 or stage 14–16.

Generation of transgenic flies and genetic interactions

PCR-generated, full-length *buffy* and *buffyΔN-Flag* fragments were cloned into *pUAST* (Brand and Perrimon, 1993) and transgenic flies were generated as previously described (Richardson *et al*., 1995). A *UAS-buffy* transgene on the second chromosome was used for all experiments. Recombinants of *GMR-GAL4* and *UAS-buffy* on the second chromosome were used for genetic interactions. All general fly stocks were obtained from the Bloomington Stock Centre, except for *en-GAL4*,*UAS-GFP* (from Laura Johnston). All genetic interaction crosses with *GMR-GAL4*,*UAS-buffy* lines were carried out at 25°C, except those with *GMR-hid* and *GMR-rpr*, which were carried out at 18°C.

Acknowledgements

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