Developmental Biology 329 (2009) 25–35

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

The deubiquitinase *emperor's thumb* is a regulator of apoptosis in *Drosophila*

Jeronimo P. Ribaya [a], Madhuka Ranmuthu [a], Jeff Copeland [b], Sergey Boyarskiy [a], Adrienne P. Blair [a], Bruce Hay [b], Frank A. Laski [a,*]

[a] *Department of Molecular Cell and Developmental Biology, and Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90095, USA*
[b] *Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA*

ARTICLE INFO

Article history:
Received for publication 9 May 2008
Revised 14 January 2009
Accepted 3 February 2009
Available online 13 February 2009

Keywords:
Apoptosis
emperor's thumb
Drosophila
Deubiquitinase

ABSTRACT

We have characterized the gene *emperor's thumb* (*et*) and showed that it is required for the regulation of apoptosis in *Drosophila*. Loss-of-function mutations in *et* result in apoptosis associated with a decrease in the concentration of DIAP1. Overexpression of one form of *et* inhibits apoptosis, consistent with *et* having an anti-apoptotic function; however, overexpression of a second form of *et* induces apoptosis, indicating that the two forms of *et* may have competing functions. *et* encodes a protein deubiquitinase, suggesting it regulates apoptosis by controlling the stability of apoptotic regulatory proteins.

Introduction

Apoptosis, a form of programmed cell death, is a highly regulated, evolutionarily conserved process used to dismantle and remove excess, damaged, and deleterious cells with minimal impact on surrounding cells. The core mediators of apoptosis are a family of highly conserved cystein proteases called caspases (*c*ystein *asp*artic acid prote*ases*). Two classes of caspases, "initiator" and "effector," exist within the apoptotic pathway. Upon receiving death stimuli, initiator caspases activate the downstream effector caspases whose protease activity is directed toward the deconstruction of cellular machinery, resulting in a controlled cell death (Thornberry and Lazebnik, 1998). Caspase activation must be strongly regulated in cells not scheduled to die because activated caspases can initiate a cascade of proteolysis. The only known cellular caspase inhibitors are members of the "*i*nhibitor of *a*poptotic *p*roteins" (IAP) family (Vaux and Silke, 2005). IAPs were first identified in baculovirus as cell death inhibitors. These proteins contain several 70-amino acid repeat motifs known as the baculovirus IAP repeats (BIRs) and a C-terminal RING finger domain (Miller, 1999). The BIR domains are important for protein–protein interactions and are required to inhibit caspase function. DIAP1 in flies, for instance, binds and inhibits the caspase Drice (Yan et al., 2004) and Dronc (Wilson et al., 2002) via its BIR domains. These same BIR domains are also required for the deregulation of IAPs in cells fated to die. In flies, the proapoptotic BIR-interacting proteins Reaper, Hid, and Grim (RHG proteins), can bind competitively with caspases (Drice and Dronc) for DIAP1's BIR domains, which displaces the caspases from DIAP1 inhibition (Ditzel and Meier, 2005). The RING finger domain confers E3 ubiquitin ligase activity that allows IAPs to ubiquitinate themselves (autoubiquitination) or bound substrate proteins, such as the caspases and RHG proteins (Ditzel and Meier, 2005; Schreader et al., 2003). A number of proteins that contain one or more BIR repeats have now been identified in vertebrates. Studies on several of these, including *XIAP*, have shown that they act as cell death inhibitors (Miller, 1999; Verhagen et al., 2001).

Many of the proteins involved in the regulation of apoptosis, including DIAP1, the RHG proteins and Dronc, are regulated by ubiquitination. Ubiquitin (Ub) is a 76-amino-acid polypeptide that can be linked covalently to other proteins via an isopeptide bond between the terminal glycine residue of Ub and an internal lysine on the substrate protein (Pickart, 1997). Polyubiquitination marks proteins for degradation by the 26S proteasome, a multisubunit proteolytic complex (Rechsteiner et al., 1993). Once thought to be a mechanism only for disposing of damaged proteins, it is now well established that Ub-mediated proteolysis is widely used to modulate the levels of critical regulatory proteins (Koepp et al., 1999; Maniatis, 1999).

Deubiquitinating enzymes (DUBs) are a large group of proteins that cleave Ub-protein bonds (Soboleva and Baker, 2004) and play an important role in the ubiquitin pathway by reversing the effects of ubiquitination. Where polyubiquitination will mark a protein for degradation, deubiquitination will remove the ubiquitin and stabilize the protein. In this paper we describe *et*, a DUB required for the proper regulation of apoptosis in *Drosophila*. Loss-of-function mutations in *et* result in apoptosis associated with a decrease in the

* Corresponding author. Fax: +1 310 206 7286.
E-mail address: laski@mbi.ucla.edu (F.A. Laski).

0012-1606/$ – see front matter
doi:10.1016/j.ydbio.2009.02.005

concentration of DIAP1, indicating an antiapoptotic function for *et*. Consistent with this notion, overexpression of one form of *et* inhibits apoptosis in the developing retina and apoptosis caused by Reaper and Grim induction; however, overexpression of a second form of *et* induces apoptosis, suggesting that *et* also has a proapoptotic function. Many of the proteins involved in the regulation of apoptosis are ubiquitinated, suggesting a mechanism for the deubiquitinase activity of Et.

Materials and methods

Drosophila stocks

BG2697 and the *et* alleles KG04801 and EY08858 were obtained from Hugo Bellen. (Bellen et al., 2004). EP(3)3076 came from the Szeged *Drosophila* Stock Centre, Szeged, Hungary. Alleles: GS7353, GS13668 and GS13655 came from the *Drosophila* Genetic Resource Center, Kyoto, Japan. Alleles: e00340, d06513, f01742, f01781, Df7586 (Parks et al., 2004; Thibault et al., 2004), and l(3)02331 (Spradling et al., 1999a) came from the Bloomington Stock Center, Bloomington, IN. Df(*et*) was created by deleting the sequences between the FRT sites in et^{d06513} and et^{f01781} using the protocol described in Parks et al., 2004. Df(70)A, Df(3L)exel6107, th^{4} and $th^{EY00710}$ were obtained from the Bloomington Stock Center, Bloomington, IN (Spradling et al., 1999a; Wilson et al., 2002). The UAS-DroncIR and UAS-DriceIR stocks are described in (Ditzel et al., 2003).

et genomic rescue construct

A 7465 bp fragment of Et genomic DNA was amplified by PCR. The DNA was from 3L: nucleotide 5761895 to 5769359 (Berkeley *Drosophila* Genome Project, release 5.3). The fragment was subcloned into the XbaI–EcoRI sites of pCaSper-4 and transformed into flies.

Long and short et constructs

The *et*(D) cDNA AT31021 and the *et*(C) cDNA AT24152 were obtained (Drosophila Genomic Resource Center) and cloned into the Gal4 inducible vector pUAST, the GMR vector pGMR-1N, and the bacterial expression vector pRB.

Antibody staining of Drosophila ovaries

Ovaries from two-day-old Oregon R and et^{roo} adult females were stained with anti-N-fs(2)B rat#1 antibody (Laski, unpublished) followed by a rabbit anti-rat Alexa Fluor secondary antibody (Molecular Probes®, Invitrogen Detection Technologies). These ovaries were further incubated with adducin-related protein (1B1) antibody [Developmental Studies Hybridoma Bank; (Zaccai and Lipshitz, 1996)] followed by a Goat anti-mouse IgG secondary antibody (Molecular Probes®, Invitrogen Detection Technologies).

Flp/Frt mosaic analysis

Genetic crosses to generate P[w^{+}, $et^{EY08858}$] or P[w^{+}, $et^{GS13655}$] mosaic clones were as described in (Chen et al., 2005).

Staining eye discs with DIAP1 and Caspase-3 antibodies

For DIAP1staining, eye discs were incubated with mouse anti-DIAP1 antibody (Yoo et al., 2002) diluted 1:500 at 4 °C overnight, followed by goat anti-mouse Alexa Fluor secondary antibody (Molecular Probes®, Invitrogen Detection Technologies) diluted 1:1000. For Caspase-3 staining, discs were incubated with anti-Active Caspase-3 antibody (BD Biosciences) diluted 1:1000, followed by incubation with goat anti-rabbit Alexa Fluor secondary antibody (Molecular Probes®, Invitrogen Detection Technologies) diluted 1:1000.

Discs large (Dlg) antibody staining

Flies were grown at 18 °C and pupal retinas were dissected 96-hours APF and stained with the Disc large primary antibody [Developmental Studies Hybridoma Bank; (Parnas et al., 2001)] followed by a Goat anti-mouse IgG secondary antibody (Molecular Probes®, Invitrogen Detection Technologies).

RNA in situ analysis of larval eye discs

Probe preparation: a 539 bp region of *et* genomic DNA (from nucleotide 3985 to 4523 of the genomic rescue construct) was cloned into the TOPO® TA vector (Invitrogen); RNA probes were labeled with digoxigenin-UTP by *in vitro* transcription with SP6 and T7 RNA polymerases (Roche Diagnostics). Staining was done by a modification of a previously described protocol (Small et al., 1992)].

qRT-PCR

Total RNA was extracted from pupae of Oregon-R flies using Qiagen RNeasy Mini Kit. RNA concentration was measured with a Nanodrop spectrophotometer, and sample concentrations were normalized. Integrity of isolated RNA was verified on a 2% agarose gel. For qRT-PCR, primers pairs were designed to span intron–exon boundaries and to yield approximately 100 bp amplicon. Actin5C was the reference gene used to normalize amplicon amounts. cDNA synthesis and qRT-PCR were combined into one step using Power SYBR Green RNA-to-CT 1-Step kit (Applied Biosystems) and DNA amount was monitored during the 40-cycle PCR by using an 7300 thermal cycler (Applied Biosystems).

Results

A male and female sterile mutation causes apoptosis in germ cells

BG2697 is a *Drosophila* line that contains a P-element insertion in the gene *aip1* (*actin interacting protein 1*). A P element excision screen of BG2697 generated 27 P-element excision mutations that were balanced and characterized. Of most interest to this study were four excision lines that had identical male- and female-sterile mutant phenotypes. As described below, these male- and female-sterile mutations were not in *aip1*, but rather in a gene we have named *et*. Males and females homozygous or transheterozygous for these four *et* alleles were viable, appeared healthy, but were sterile. Dissections showed identical phenotypes, the mutant ovaries and testes were small in size and using brightfield microscopy appeared agametic; however, germline markers showed that a small number of germ cells were present in both ovaries (Fig. 1) and testes (data not shown). The ovarian germ cells contain spectrosomes, indicating they were either germline stem cells (GSCs) or 1-cell cystoblasts (Deng and Lin, 1997). Two-cell cysts were also observed. Germaria in *et* mutant ovaries were heavily stained by acridine orange (data not shown), a dye that is readily taken-up by cells undergoing apoptosis. These data suggest that the GSCs in *et* mutant ovaries are dividing, giving rise to daughter GSCs and cystoblasts, but soon after a cystoblast leaves the germarium's stem cell niche it undergoes apoptosis.

The *et* mutations complemented *aip1* mutations and also complemented the deletion Df(70A), which uncovers *aip1* and surrounding genes, indicating that *et* is not an allele of *aip1* and does not map near *aip1*. Deletion mapping of the 2nd chromosome showed that Df(3L)exel6107, an approximately 100 kb deletion covering polytene bands 64E5–64F5, uncovered the *et* mutation. Df(3L)exel6107 deletes 17 genes, some of which had mutant alleles available, including P[PZ]l

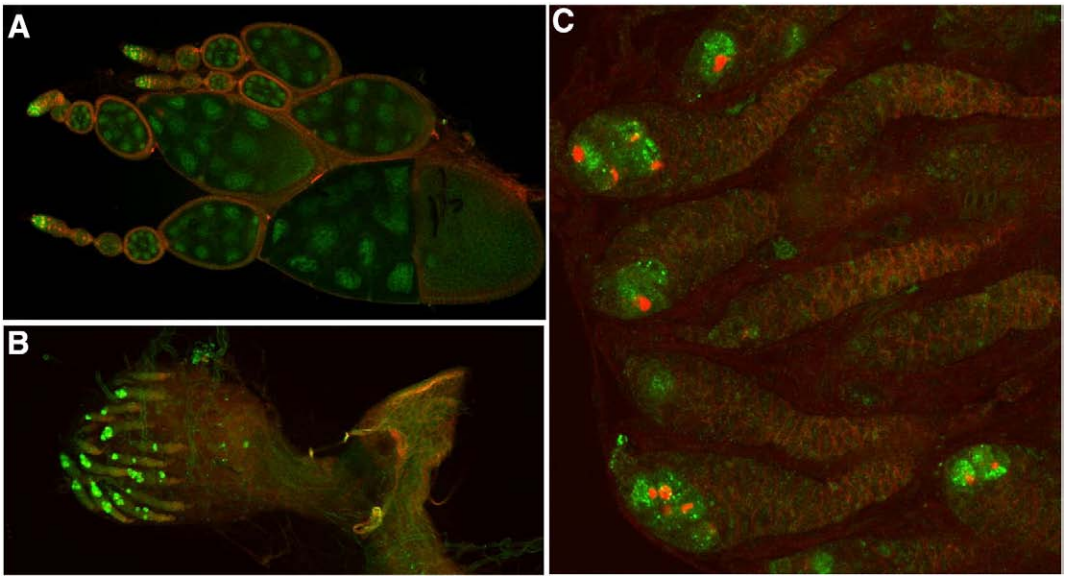

Fig. 1. *et*^{roo} has a germ line specific mutant phenotype. Confocal images of (A) wild type and (B, C) *et*^{roo} mutant ovaries stained with early germ cell markers (anti-N-fs(2)B, green; and anti-spectrin, red). Germline stem cells, cystoblasts and two cell cystoblasts are present in *et*^{roo} mutant ovaries, older germ cells are not detected. A and B were taken with a 10× objective, C with a 60× objective.

(3)02331 (Spradling et al., 1999b). Flies transheterozygous for P[PZ]l (3)02331 and *et* were male- and female-sterile with tiny gonads, a phenotype identical to the *et* homozyogotes. P[PZ]l(3)02331 is inserted into an intron of the gene CG5505 (Fig. 2), indicating the male and female sterile phenotype of *et* is due to a mutation in CG5505. We have renamed the CG5505 gene *et*.

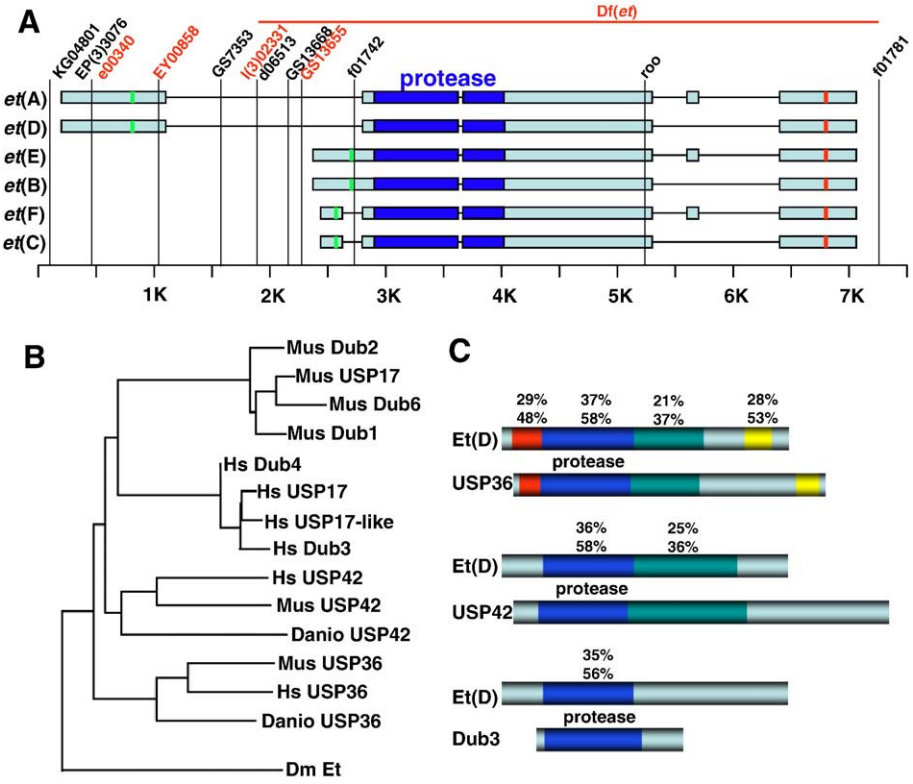

Fig. 2. Gene and protein structure of *et*. (A) *et* has six predicted transcripts, each encoding a similar but different protein. The predicted translational start (green) and stop (red) sites for the transcripts are shown. The conserved ubiquitin protease domain (blue) is present in all six proteins. The A and D transcripts utilize an upstream start site and make larger proteins than the shorter transcripts. The location of the different transposon insertion mutations are shown. The four recessive lethal P-element insertions (e00340, EY00858, l(3)02331 and GS13655) are labeled in red. Df(*et*) is a null mutation that deletes the sequences between the d06513 and f01781. The insert GS13668 has a UAS inducible promoter oriented to the right towards the short Et proteins. (B) A dendogram showing the phylogenic relationships of Et and its human, mouse and zebrafish homologues (Hs—*Homo sapien*, Mus—mouse, Danio—zebrafish) was created by using the ClustalX program (Thompson et al., 1997). (C) The Et proteins have varying regions of homology with USP42, USP36 and Dub3. Homologous regions are shown between Et(D) and Hs USP36, Hs USP42 and Hs DUB3. The conserved ubiquitin protease domain is labeled. The percentage of amino acid similarity (top) and identity (bottom) for each homologous region is shown.

Structure and function of et

et is located on chromosome 3L at position 64E7 and has six identified transcripts encoding six similar but different proteins (Fig. 2A) (Stapleton et al., 2002). Sequence comparisons show that *et* is a member of the deubiquitinase family of proteins. The conserved ubiquitin protease domain is present in all six forms of the Et protein (Fig. 2A). Using the pUB-Arg-β-Gal assay (Baker et al., 1992), we found that both short and long forms of *et* have deubiquitinase activity. Whereas bacterial colonies with the pUB-Arg-β-gal construct were blue in an Xgal assay, those that also expressed short [pRB1-*et*(C)] or long [pRB1-*et*(D)] *et* were white (data not shown), the result of βgal degradation after the cleavage of Ub from βgal by the deubiquitinase activity of Et.

Although there are no direct studies of *et* in the literature, a microarray analysis provides insight into its function. In *Drosophila*, the steroid hormone ecdysone is the main activator of the stage specific destruction of larval tissues during morphogenesis (Jiang et al., 1997; Lee and Baehrecke, 2001). Approximately 10 h after puparium formation (APF) a pulse of ecdysone triggers the rapid destruction of the larval salivary glands. The microarray analysis identified genes that are induced or repressed during salivary gland programmed cell death (Lee et al., 2003). The transcription patterns of over 13,000 genes were analyzed and it was determined that the concentration of *et* transcript increased 71-fold after ecdysone induction; the concentration of only one other gene increased more. It was also shown that *et* expression is not induced during apoptosis caused by radiation exposure. Thus, *et* is not required for apoptosis, but rather appears to have a role in the regulation of apoptosis and is a likely player in the cell death pathway in dying salivary glands.

A second screen also provided insight into the function of *et*. *Listeria monocytogenes* is an intracellular Gram-positive bacterial pathogen that lives in the cytosol of host cells. Genome-wide RNAi screens were performed in *Drosophila* S2 cells to dissect the contribution of host genes to *L. monocytogenes* intracellular growth (Cheng et al., 2005). Of 7216 genes examined, only four were identified in which RNAi knockdowns resulted in a large increase in the number of intracellular bacteria. These four genes were *et*, *string*, *MESR4* and *CG5451*. What function *et* has in inhibiting bacterial growth is unknown.

Vertebrate homologues of et

An evolutionary tree showing the relationship between *et* and its human, mouse, and zebrafish homologues is shown in Fig. 2B. The human homologues of Et protein are USP36, USP42 and the DUB/USP17 family. Et has highest homology to USP36, followed by USP42, but all three are homologues because Et is closer in sequence identity to USP36, USP42 and DUB/USP17 than to any other fly protein. Also, the three human homologues are closer to Et in sequence identity than they are to any other human protein. All three human proteins have homologues in mice; only USP36 and USP42 have homologues in zebrafish. The DUB/USP17 family is interesting because in both humans and mice it is located on a micro-satellite repeated sequence, with the exact number of copies varying among different individuals, only 4 copies are shown in Fig. 2B (Okada et al., 2002).

All three of the Et human homologues are highly conserved within the protease domain, with USP36 showing the highest homology (37% identity and 58% similarity) (Fig. 2C). USP36 and USP42 also share homology with Et outside of the protease domain, with USP36 having significant homology to regions both upstream and downstream of the protease domain, whereas USP42 sharing only downstream homology. We were unable to detect any known "motifs" in these regions of homology, providing no clue as to their function. The DUB/USP17 family of proteins only shares homology within the protease domain of Et.

Comparison of the regions of homology between the Et proteins and its homologues provides insight into the function of the Et proteins. The region of homology between Et and USP36 begins at amino acid 42 of the Et protein expressed from either the *et*(A) or *et*(D) transcripts. A 55-amino acid region of homology is found in the upstream exon of *et*(A) and *et*(D); no similar region of homology is observed in proteins made from the other transcripts. This region of homology suggests that the proteins made from *et*(A) and *et*(D) may have a different and conserved function than those made from *et*(B), *et*(C), *et*(E) and *et*(F). Therefore, we refer to the transcripts and proteins made from *et*(A) and *et*(D) as the "long" forms of Et, and the shorter transcripts and proteins as the "short" forms. This suggests the possibility that after the *et* duplication event that formed both USP36 and USP42; USP36 evolved and took over the function of the long Et protein, whereas USP42 evolved and took over the function of the short Et protein.

et has a first instar lethal recessive phenotype

PCR analysis of DNA isolated from a male- and female-sterile *et* allele identified a 9 kb insertion into the gene (data not shown). Sequence analysis revealed a roo reterotransposon insertion at nucleotide 5243 of the *et* genomic sequence shown in Fig. 2A, which is 3′ of the conserved protease domain. We call this allele et^{roo}. Identical roo retrotransposon insertions were found in both of the *et* alleles analyzed, suggesting the mutations were caused by a spontaneous roo insertion in the BG2697 stock before the P-element excision experiment was done.

Eleven different P element insertions at the *et* locus were obtained from stock centers. The location of these P-element insertions are shown in Fig. 2A. Of the 11 inserts, four had a recessive lethal phenotype (et^{e00340}, $et^{EY00858}$, $et^{l(3)02331}$ and $et^{GS13655}$; shown in red in Fig. 2A) and seven were homozygous viable. Thus, a strong mutation in *et* has a recessive lethal phenotype and not the male- and female-sterile phenotype of et^{roo}. Of the seven viable insertions, five were homozygous fertile and two (et^{d06513} and et^{f01742}) were sterile; however, neither of these sterile mutations had a phenotype similar to et^{roo}, having developmental defects at later stages of oogenesis and spermatogenesis (data not shown). All four lethal *et* alleles failed to complement each other. Also, flies trans-heterozygous for et^{roo} and any of the four lethal mutations had the et^{roo} male and female sterile phenotype, verifying that all these alleles were mutations in the same gene. That the mutations are in *CG5505* was confirmed by showing that two lethal alleles, $et^{EY08858}$ and $et^{GS13655}$, could be completely rescued by a P element transformant containing the 7.5 kb of *et* genomic sequences shown in Fig. 2A (data not shown).

Both $et^{EY08858}$ and $et^{GS13655}$ homozygotes die sometime between the 1st and 2nd instar larval stages of development. Many of the dead *et* larvae had two sets of mouth hooks, indicating that lethality primarily occurred at the molt from 1st to 2nd instar. The recessive lethal *et* alleles are likely loss-of-function mutations; however, it was not clear whether they were null for *et* function. To generate a null allele, FLP recombinase was used to delete the sequences between et^{d06513} and et^{f01781}, two FRT-bearing P element insertions (Fig. 2A). The resulting deletion removes a majority of the coding region for *et* creating an assumed null allele. Flies homozygous for this deletion [Df(*et*)] are lethal. Similar to the mutants already tested, viable 1st larval instar animals, but no 2nd larval instar mutants, were detected. However, the dead 1st larval instar animals homozygous for Df(*et*) lack the double mouth hooks seen with the other lethal alleles, suggesting lethality occurs slightly earlier and that this null deletion has a slightly stronger mutant phenotype than the two P-element lethal alleles tested.

Loss of et activity results in apoptosis during eye development

The *et* loss-of-function phenotype was examined in the *Drosophila* eye using FLP/FRT mosaic analysis (Xu and Rubin, 1993).

Mosaic clones of $et^{GS13655}$ resulted in a severely disrupted eye (Figs. 3A, B). A similar result was seen in mosaic clones of $et^{EY08858}$ (data not shown). The $et^{GS13655}$ mutant allele is marked with a mini-white gene, giving the eye its red color. The lack of white mosaic patches indicates that the mutation has a recessive cell lethal phenotype. The mutant eyes were rough and smaller than wild-type eyes; most had a notch at the anterior portion of the eye. Histological analysis revealed degeneration of ommatidial structures within a mutant clone (Fig. 3C), likely the result of cell death. This was confirmed by staining the mosaic clones with an antibody against active-Caspase-3, which is found at increased concentrations in apoptotic cells. To distinguish the mutant clones (et^{-}/et^{-}) from wild-type cells ($et^{-/+}$), we used a GFP marker on the wild-type chromosome transheterozygous from et^{-}. Cells that expressed GFP were heterozygous for *et*, whereas cells that did not express GFP were homozygous for the *et* mutation. Mosaic patches were easily detected in 3rd instar larval eye discs (Fig. 3D) and elevated active-Caspase-3 staining was found almost exclusively in *et* mutant cells (Figs. 3E, F). Not all *et* mutant cells in the mosaic patches had high levels of active-Caspase-3, but since the mosaic adult eyes lacked white cells, we assume all of the mutant cells will eventually undergo apoptosis. However, because all of the *et* mutant cells do not die immediately once formed, it remains to be determined whether the loss of *et* activity by itself is the direct cause of cell death.

et is uniformly expressed in the eye disc

RNA *in situ* hybridizations were done on 3rd larval instar eye discs to determine the RNA expression pattern of *et*. RNA sense and anti-sense probes came from a region of the *et* gene that is present in all six of the *et* transcripts. The anti-sense staining revealed that *et* is uniformly distributed throughout the eye disc (Supplementary Fig. 1).

Expression of long et inhibits apoptosis

The Gal4/UAS system (Brand and Perrimon, 1993) was used to express *et* during the development of the eye. P[UAS-*et*(D)] is a construct that can express the long *et*(D) transcript (Fig. 2A) from a Gal4 inducible promoter. UAS-*et*(D) was expressed with GMR–Gal4, which is a strong eye specific Gal4 driver that is turned on in late 3rd instar larval cells behind the morphogenetic furrow, and remains on throughout eye development (Moses and Rubin, 1991). For these experiments, a GMR–Gal4 insert on the 3rd chromosome (P[GMR–Gal4]3) was used. The eyes of the P[GMR–Gal4]3, UAS-*et*(D) flies looked wild type when grown at 18 °C. At 22 °C (Gal4 is more active at higher temperature) these eyes were moderately rough, and rougher still when grown at 25 °C (Supplementary Fig. 2D–F). During wild type larval development, more interommatidial cells are made than required, these excess cells are removed by apoptosis in pupal development (Brachmann and Cagan, 2003). Since loss of *et* function

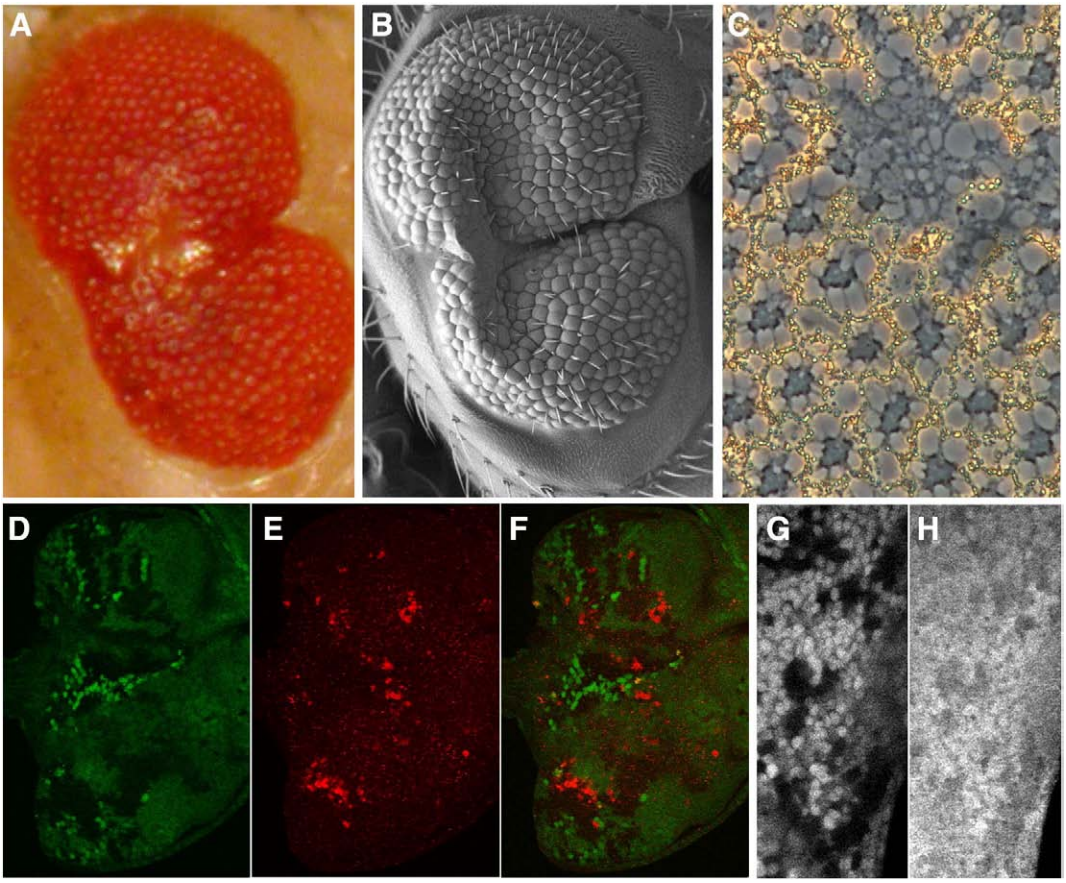

Fig. 3. Loss of *et* function causes a reduction in DIAP1 and induces apoptosis. Somatic recombination was induced during eye development in *w ey*-FLP, $et^{GS13655}$ FRT80B/*w* P[ubi-GFP] Minute FRT80B flies to make mosaic clones mutant for *et* function. (A) Brightfield and (B) scanning electron microscopy shows a small malformed eye that lacks white mosaic patches of cells, indicating the *et* mutation is cell lethal. (C) A histological section through a mutant mosaic patch shows a scar containing disorganized retinal cells. (D–F) Confocal images of 3rd instar larval eye discs showing (D) mosaic pattern of GFP-stained wild-type cells and non-fluorescing *et* loss-of-function clones and (E) the same eye disc stained for active-Caspase-3. (F) A merged image shows that almost all of the cells undergoing apoptosis are mutant for *et*. (G,H) Confocal images of 3rd larval instar eye discs showing (G) mosaic pattern of GFP stained wild-type cells and non-fluorescing *et* loss-of-function clones and (H) the same eye disc stained with anti-DIAP1 antibody. Cells mutant for *et* have lower levels of DIAP1 expression.

induces apoptosis, we thought it was possible that expression of *et* might inhibit apoptosis, which would result in the presence of excess interommatidial cells. To determine whether more cells are present in the interommatidial space of P[GMR–Gal4]3, UAS-*et*(D) eyes, pupal retinas of these flies were stained with anti-Discs large antibody, which stains the periphery of cells. Staining was done after the extra interommatidial cells have undergone apoptosis (which occurs between 24- and 36-hours APF at 25 °C), after which time each ommatidia should be surrounded by 12 cells, including the three bristle cells (Brachmann and Cagan, 2003). At 25 °C and 22 °C we observed an increase in the number of interommatidial cells in the P[GMR–Gal4]3, UAS-*et*(D) pupal retinas (data not shown); however, the ommatida in these eyes were not arranged in a hexagonal array, but instead had a square pattern. It is possible that the increase in the number of interommatidial cells was due to the change in the shape of the ommatidia, rather than a direct effect on apoptosis. We therefore focused on 96-hour APF pupae grown at 18 °C (the equivalent of 48-hours APF at 25 °C), which maintained hexagonally-arranged ommatidia, but still had an increase in interommatidial cells (Fig. 4B). The P[GMR–Gal4]3 control had the normal number of cells (Fig. 4A). This shows that expression of *et*(D) has an anti-apoptotic effect and inhibits the normal apoptosis of interommatidial cells.

To further test the anti-apoptotic activity of long Et, a construct was made that directly expressed long Et from the GMR promoter. Consistent with the above experiment, GMR-*et*(D) was capable of inhibiting the normal apoptosis of interommatidial cells (data not shown). We then asked whether GMR-*et*(D) can inhibit the apoptosis induced by the expression of proapoptotic proteins. Figs. 4C–F shows that overexpression of Grim, Hid, Reaper, and miDIAP1 (a micro-RNA directed against DIAP1) causes a rough eye in the fly because of the induction of apoptosis. GMR-*et*(D) was able to partially suppress the apoptosis induced by Grim and Reaper (Figs. 4G, H), but was unable to suppress the apoptosis caused by Hid and miDIAP1 (Figs. 4I, J). These results confirmed the anti-apoptotic activity of long Et, but suggests a specificity to the types of apoptosis it can inhibit.

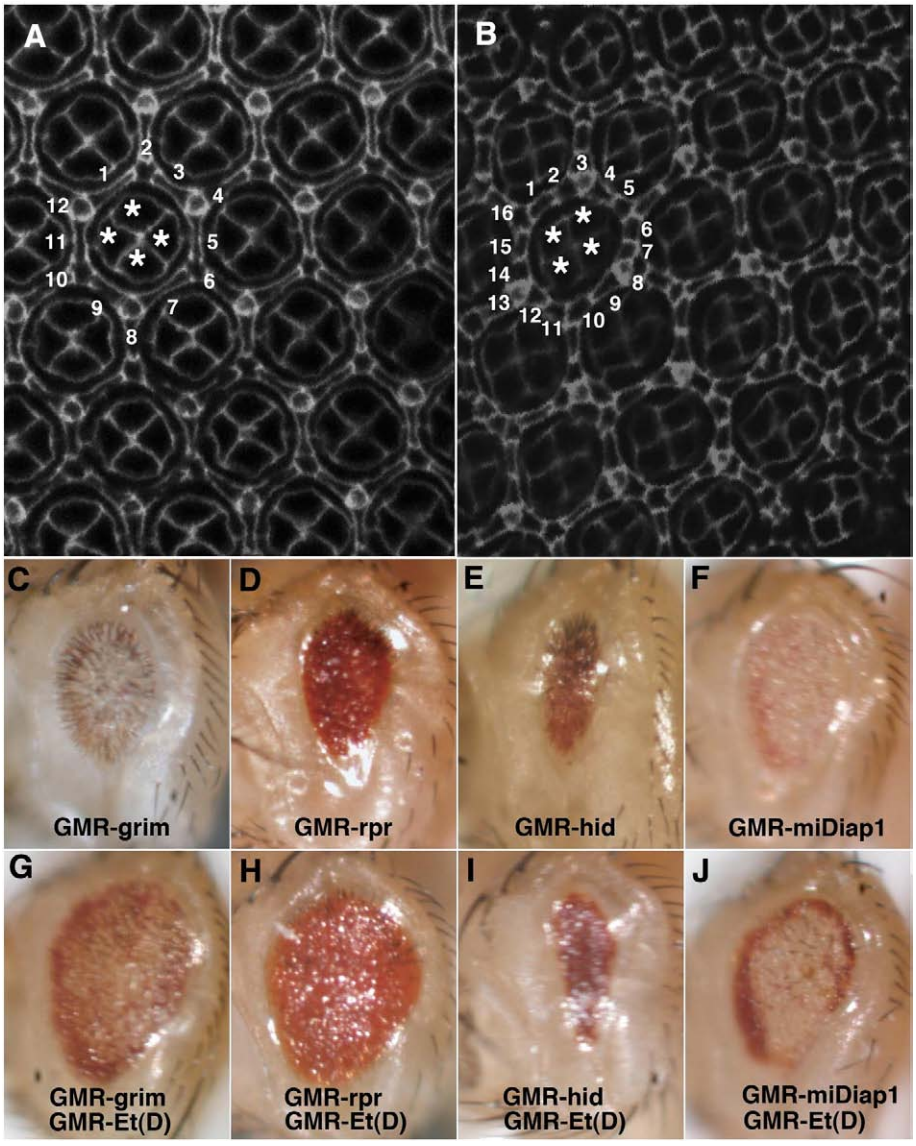

Fig. 4. Overexpression of long *et* inhibits apoptosis. (A) Control GMR–Gal4/+ and B) UAS-*et*(D)/+; GMR–Gal4/+ flies were grown at 18 °C and the pupal retinas dissected and stained with anti-Discs large (Dlg) antibody 96-hours APF. (A) The GMR–Gal4/+ pupal retina has a wild-type appearance with 4 cone cells in the center of each ommatidia (marked by an asterisk) surrounded by 12 interommatidial cells. (B) The UAS-*et*(D)/+; GMR–Gal4/+ pupal retinas have extra interommatidial cells, with 16 surrounding the labeled ommatidia. These extra cells demonstrate that long *et*(D) can inhibit the normal apoptosis of the pupal interommatidial cells. (C–F) Adult eyes are smaller due the induction of apoptosis caused by the overexpression of (C) Grim, (D) Reaper, (E) Hid and (F) miDIAP1. (G–J) The concurrent overexpression of *et*(D) partially suppresses the apoptosis induced by (G) Grim and (H) Reaper, but not (I) Hid or (J) miDIAP1.

Table 1

A Genetic Cross GMR–Gal4 *etGS13668*/TM6 X OreR	25 °C	22 °C	18 °C
GMR–Gal4 *etGS13668*/OreR	2	94	229
OreR/TM6	215	207	220
B Genetic Cross GMR–Gal4 *etGS13668*/TM6 X P[UAS–Diap1.H]3	25 °C		
GMR–Gal4 *etGS13668*/ P[UAS-Diap1.H]3	528		
P[UAS-Diap1.H]3 /TM6	467		
C Genetic Cross GMR–Gal4 *etGS13668*/TM6 X *th4*/TM3	25 °C	22 °C	18 °C
GMR–Gal4 *etGS13668*/*th4*	0	0	133
GMR–Gal4 *etGS13668*/TM3	0	98	172
th4/TM6	131	130	127
TM3/TM6	131	143	169

A) P[GMR–Gal4]3 $et^{GS13668}$/TM6 was crossed to Oregon R at 25 °C, 22 °C and 18 °C and the progeny counted.
B) P[GMR–Gal4]3 $et^{GS13668}$/TM6 was crossed to P[UAS-Diap1.H]3 at 25 °C and the progeny counted.
C) P[GMR–Gal4]3 $et^{GS13668}$/TM6 was crossed to th4/TM3 at 25 °C, 22 °C and 18 °C and the progeny counted.

Overexpression of short et promotes apoptosis

The P element mutation $et^{GS13668}$ is located within the first intron of *et* (Fig. 2A). This construct has a UAS Gal4 inducible promoter oriented towards the right, thus it should specifically express the short Et proteins. Activation of the UAS promoter in $et^{GS13668}$ with the constitutively active Actin-Gal4 at both 18 °C and 25 °C resulted in embryonic lethality. Activation during eye development with GMR–Gal4 produced a phenotypic series at 18 °C, 22 °C, and 25 °C (Supplementary Fig. 2G–I). At 18 °C, P[GMR–Gal4]3/ $et^{GS13668}$ flies have eyes that are wild type in size and shape, but are slightly rough and exhibit smoothness at the anterior portion of the eye (Supplementary Fig. 2G). At 22 °C, approximately half of the flies fail to eclose (Table 1) and die trapped in their pupal cases. The flies that survive have abnormal eyes that are small and exhibit a smooth or glossy phenotype (Supplementary Fig. 2H). The majority of these eyes also have a scab of black tissue at the anterior region of the eye. At 25 °C, less than 1% of P[GMR–Gal4]3/ $et^{GS13668}$ flies survive (Table 1). Adult escapers had eye defects more severe than those grown at 22 °C (Supplementary Fig. 2I).

Overexpression of the long *et*(D) protein inhibits apoptosis. It was therefore surprising to find that overexpression of short Et with $et^{GS13668}$ causes cell death (that the small eye phenotype is at least partially caused by cell death is shown by the ability of Diap1 overexpression to suppress the small eye phenotype, see below). To verify that the $et^{GS13668}$ overexpression phenotype was caused by the overexpression of a short Et protein, an *et*(C) cDNA (Fig. 2A) was cloned into a Gal4 inducible vector and transformed into flies. When UAS-*et*(C) was overexpressed with P[GMR–Gal4]3 at 25 °C, the resulting progeny died during the 1st and 2nd larval instar. The mutant larvae were smaller than wild type and had blackened tracheae (data not shown). Considering that the GMR promoter is not

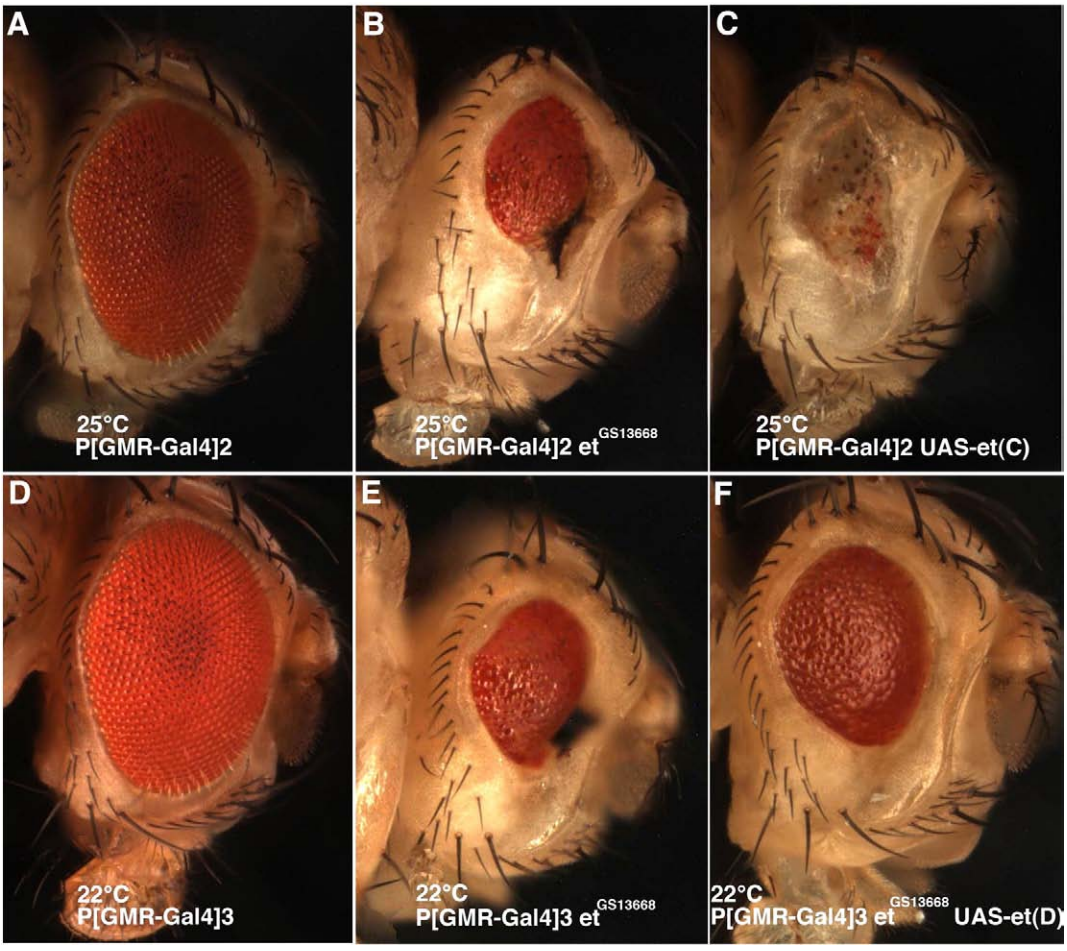

Fig. 5. Overexpression of short *et* induces apoptosis. (A) A P[GMR–Gal4]2/+ control grown at 25 °C is wild type in appearance. Expression of short *et* in (B) P[GMR–Gal4]2, $et^{GS13668}$ or (C) P[GMR–Gal4]2/ UAS-*et*(C) results in apoptosis and a small and rough eye. (D) P[GMR–Gal4]3/+ grown at 22 °C has a normal appearance, whereas the overexpression of short *et* in (E) P[GMR–Gal4]3 $et^{GS13668}$ results in a small and rough eye. (F) This phenotype can be suppressed by the overexpression of *et*(D), as seen in P[GMR–Gal4]3 $et^{GS13668}$/UAS-*et*(D). Flies in A–C were grown at 25 °C, D–F at 22 °C.

active at this time, it is clear that low levels of *et*(C) expression is harmful. Two independent inserts of UAS-*et*(C) gave the same phenotype, so the larval lethality was not due to a position effect. If the flies were grown at 18 °C, some larvae survived to the late pupal stage. These pupae had many defects, including a rough eye. The simplest explanation for the differences in phenotypes in over-expressing $et^{GS13668}$ versus UAS-*et*(C) is that *et*(C) had more activity. To verify this, we screened through a number of GMR–Gal4 lines to find one that had less background expression and would not result in dead progeny when crossed to UAS-*et*(C). A line with an insert on the second chromosome met these criteria. When P[GMR–Gal4]2 was crossed to $et^{GS13668}$, it produced viable progeny with an eye phenotype similar to, but more severe than $et^{GS13668}$ (Figs. 5B, C). This confirms that the severe phenotype is caused by the overexpression of a short form of *et*.

Overexpression of a long form of *et* inhibits apoptosis, whereas overexpression of the short form has a proapoptotic effect. *et* was named for this ability to regulate both life (thumbs up) and death (thumbs down). We asked whether overexpressing long *et* could suppress the severe phenotype caused by overexpressing short *et*. At 22 °C, flies expressing both long and short *et* (Fig. 5F) showed eyes that are larger and less rough than those expressing only short *et* (Fig. 5E). These results indicate that long and short Et proteins have opposite or competing functions.

In addition to being small and rough, most of the P[GMR–Gal4]3/$et^{GS13668}$ flies at 22 °C and 25 °C have a scab of dead tissue at the anterior side of each eye (Fig. 6B). This scab comes from a mass of tissue on the eye observed in GMR–Gal4/$et^{GS13668}$ pupae (arrow in Fig. 6A). It appears that cells within the eye are "leaking" out of the anterior portion of the developing eye, but it is unclear whether these cells are hemorrhaging from the eye or growing on top of the eye. It has been shown that cells undergoing apoptosis can induce proliferation in adjacent cells, and these cells may take on a new cellular identity (Huh et al., 2004; Ryoo et al., 2004). Since cells in the eye are undergoing a high rate of apoptosis, it is possible that they are giving a signal to other cells to grow and that this is the origin of this extraneous tissue.

et genetically interacts with DIAP1

The E3 ubiquitin ligase DIAP1 is the primary inhibitor of apoptosis in the fly. We asked whether overexpression of DIAP1 could suppress the apoptosis caused by overexpression of short *et*. At 25 °C, DIAP1 overexpression completely suppressed the lethality caused by short *et* overexpression and partially suppressed the mutant eye phenotype (Table 1B and Fig. 6C). We then asked whether a reduction in DIAP1 would enhance the mutant phenotype. At 22 °C, many of the flies expressing short *et* are viable; however, all flies that are also heterozygous for a mutation in DIAP1 (th^4) are lethal (Table 1C). Thus, a 50% reduction in DIAP1 activity enhanced the lethal phenotype caused by overexpressing short *et*. This enhancement is also seen at 18 °C, where P[GMR–Gal4]3 $et^{GS13668}$ /TM3 flies have a mild rough eye phenotype (Fig. 6E), which becomes more severe if a fly is also heterozygous for th^4 (Fig. 6F).

To further examine the relationship between *et* and DIAP1, we asked whether the concentration of DIAP1 protein is affected by the loss of *et* activity. et^- mosaic clones in 3rd instar larval eye discs were stained with antibodies specific for DIAP1 and although subtle, a DIAP1 pattern very similar to the GFP pattern was seen, with *et* mutant cells having lower concentrations of DIAP1 protein (Figs. 3G, H). This shows that *et*, either directly or indirectly, is required for maintaining the normal concentrations of DIAP1 protein. It is likely that these low levels of DIAP1 result in the apoptosis of these cells.

et induced apoptosis requires Dronc but not Drice activity

The induction of apoptosis requires the activation of the caspase proteins. We asked whether an RNAi mediated reduction of the

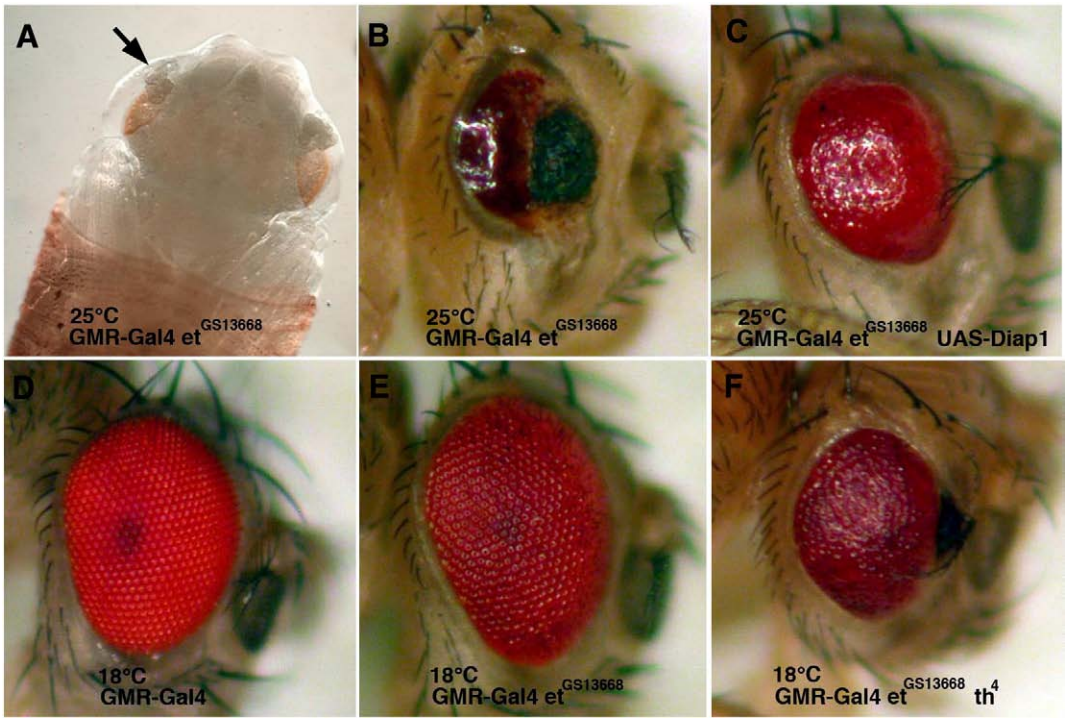

Fig. 6. Genetic interaction between *et* and DIAP1. (A) Flies of the genotype P[GMR–Gal4]3 $et^{GS13668}$ are semi–lethal at 25 °C, with most dying late during pupal development. A mass of tissue is seen in these pupae at the anterior of the eye (arrow). (B) The rare escapers have very small eyes and a crust of black tissue at the anterior of their eye. (C) P[GMR–Gal4]3 $et^{GS13668}$/UAS–DIAP1 flies (grown at 25 °C) are fully viable and have a larger eye, indicating the DIAP1 is inhibiting the apoptosis induced by short *et* overexpression. (D) A P[GMR–Gal4]3/+ control eye is wild type in appearance at 18 °C, (E) while the expression of short *et* in P[GMR–Gal4]3 $et^{GS13668}$ flies causes a mild rough eye phenotype. (F) This phenotype is enhanced if the fly is also heterozygous for *thread* (P[GMR–Gal4]3 $et^{GS13668}/th^4$). Flies in A–C were grown at 25 °C, D–F at 18 °C.

caspases Dronc and Drice can suppress the apoptosis caused by short *et* overexpression. We found that expression of an RNAi construct of Dronc (UAS-*DroncIR*) (Ditzel et al., 2003) could suppress the mutant phenotype. At 25 °C, UAS-*DroncIR* expression completely rescues the lethality caused by short *et* overexpression (Supplementary Table 1). At 22 °C, P[GMR–Gal4]3 $et^{GS13668}$/*UAS-DroncIR* flies have a partially suppressed mutant eye phenotype (Supplementary Fig. 3). These flies have eyes with a larger and bulging phenotype (Supplementary Fig. 3C). These data show that the loss of *Dronc* function partially suppresses the lethality and mutant eye phenotypes caused by short *et* overexpression. It was surprising to find that expression of an RNAi construct for Drice (Ditzel et al., 2003) did not suppress the short *et* overexpression mutant phenotype (Supplementary Table 1). These data suggest that Dronc plays an important role in the manifestation of the short *et* overexpression phenotype. One of the roles of Dronc is to activate Drice, so it is likely that overexpressing short *et* results in the activation of Dronc that in turn activates Drice. It appears the activation of Dronc alone has an important role in the apoptotic phenotype.

Interestingly, the overexpression of the baculovirus-encoded protein p35 did not suppress the short *et* overexpression phenotype (data not shown). This result was unexpected because both DIAP1 and p35 can bind to and inhibit caspases (Deveraux and Reed, 1999), however, whereas DIAP1 can inhibit both Drice and Dronc, p35 can inhibit Drice, but not Dronc. This data is consistent with the above data, that the activation of Dronc by short *et* appears to be an important step in the pathway by which it induces cell death.

Both short and long et transcripts are induced during larval salivary gland programmed cell death

A previous report showed that *et* is induced 71-fold during pupal salivary gland programmed cell death (Lee et al., 2003). Genetic analysis is required to determine the function of *et* during this process, but determining whether the long or short form of *et* is induced will provide a clue. qRT-PCR was used to determine which transcripts are induced. Total RNA was isolated from 5-hour APF (after pupal formation) and 15-hour APF, which is respectively before and after the ecdysone pulse which initiates salivary gland programmed cell death. A primer pair that amplifies a region found in all six transcripts detected a 21-fold increase in total *et* message, confirming the large induction of *et* during this time period (Supplementary Fig. 4). Using primers that only amplify distinct transcripts, qRT-PCR detected a 15-fold induction of the A/D transcripts, a 16-fold induction of the B/E transcripts, and a 49 fold induction of the C/F transcripts (Supplementary Fig. 4). Thus, during salivary gland programmed cell death, both long and short *et* transcripts are induced, with the short RB/RE transcripts induced to a greater extent.

Discussion

Mutations in *et* result in apoptosis coupled with a decrease in the concentration of DIAP1. Overexpression of long *et* inhibits apoptosis, whereas overexpression of short *et* promotes apoptosis. In addition, *et* has been shown to be induced 71-fold during the programmed cell death of larval salivary glands. Together, these data make a compelling argument that *et* is required for the proper regulation of apoptosis in *Drosophila*.

Many *Drosophila* proteins involved in apoptosis are regulated by ubiquitination, including the E3 ubiquitin ligase DIAP1, Dronc and the RHG proteins (Fig. 7). In unstressed cells, DIAP1 and the caspases are kept at low concentrations by ubiquitination and proteosomal degradation. *et* may function by deubiquitinating one or more of these proteins (Fig. 7). A mechanism comparable to this is seen with the mammalian deubiquitinase HAUSP, a regulator of p53 (Brooks and Gu, 2006; Hu et al., 2006). p53 is primarily regulated by the E3 ubiquitin ligase Mdm2, which ubiquitinates p53 and keeps it at low concentrations in unstressed cells. To maintain p53 at low levels, a cell must maintain Mdm2 at optimal levels. It does this through HAUSP, which is capable of deubiquitinating and thereby stabilizing both p53 and Mdm2 by creating a feedback loop between the three proteins that keeps them at the required concentrations. Et belongs to the same deubiquitinase family of proteins as HAUSP and could regulate DIAP1 and the caspases in a manner similar to that of HAUSP.

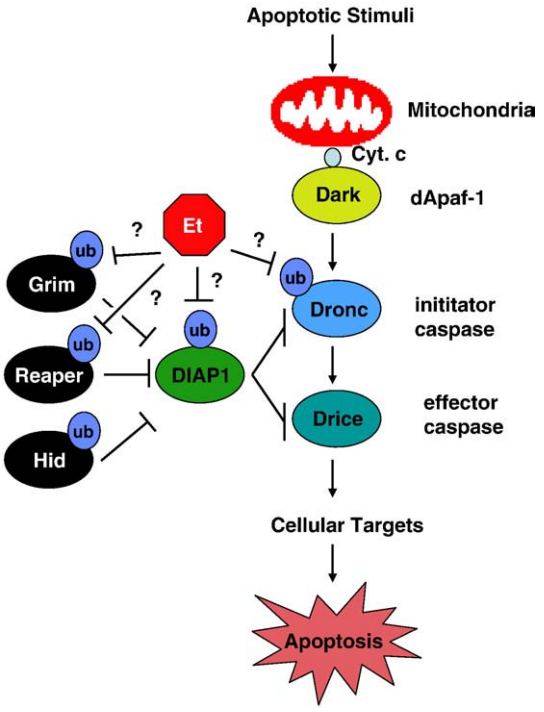

Fig. 7. *Drosophila* apoptotic pathway. The adapter protein Dark (homologue of mammalian Apaf-1) promotes activation of the initiator caspase Dronc, which can initiate a proteolytic cascade via the activation of Drice and other effector caspases. DIAP1 can inhibit apoptosis by binding to and inhibiting Dronc and Drice. The RHG proteins Reaper, Hid, and Grim can bind to and inhibit DIAP1, relieving the repression on the caspases and promoting cell death. The stability of DIAP1, Dronc, and the RHG proteins are regulated by ubiquitination. We propose that Et functions by deubiqutinating and stabilizing proteins required for the proper regulation of apoptosis in *Drosophila*.

Loss-of-function mutations in *et* induced apoptosis in the ovary and developing eye, therefore *et* has an antiapoptotic function in these tissues. Consistent with these results was the finding that overexpression of long *et* also inhibits apoptosis. It was surprising to find that overexpression of short *et* induces cell death. The simplest explanation for this finding is that the long and short Et proteins have competing functions, this is supported by the finding that long *et* overexpression can partially suppress apoptosis induced by short *et*. RT-PCR analysis showed that both short and long *et* transcripts are present in larval eye discs (data not shown). We therefore propose that there is a balance between short and long Et proteins in the eye. A minimal amount of long Et is necessary to prevent apoptosis, but that long Et activity is inhibited by short Et. Loss of both proteins would result in apoptosis, but overexpression of either long or short Et would throw the balance towards life or death. Our results also reveal a strong association between *et* and DIAP1, including a strong genetic interaction and a reduction in DIAP1 concentration in cells mutant for *et*. Also pertinent is the finding that overexpression of long *et* suppresses apoptosis induced by Reaper and Grim, but not Hid. It has been shown in one study that overexpression of Reaper and Grim causes a reduction in the concentration of DIAP1, but overexpression of Hid does not affect DIAP1 levels (Hays et al., 2002). This suggests that Reaper and Grim induce apoptosis by reducing DIAP1 concentrations, possibly by promoting Ub-mediated DIAP1 degradation,

whereas Hid must induce apoptosis by a different mechanism. Our data therefore suggests that long *et* may inhibit the ability of Reaper and Grim to promote the degradation of DIAP1.

Our results put forward the possibility that the human homologues of *et* also function as regulators of apoptosis. The biology of USP36 has not been studied in detail, except for determining that it has deubiquitinase activity and that it is polyubiquitinated in tissue culture cells (Kim et al., 2005). USP42 has also not been studied in detail; however, a manuscript reported that a seven-year-old boy with acute myeloid leukemia (AML) had a novel cryptic translocation in the cancer cells that made a hybrid gene between RUNX1 and USP42, and the hybrid RUNX1/USP42 fusion protein was expressed at high levels in blood cells (Paulsson et al., 2006). It is possible that the overexpression of the USP42 ubiquitin protease activity has a role in the AML phenotype. The DUB/USP17 family of genes are located on the RS447 mega-satellite DNA, which has a 4.7 kb repetitive unit that contains the DUB/USP17 gene, with the number of repeats varying from 20 to 103 per person (Okada et al., 2002). Work on mouse and human DUB/USP17 genes show that their expression is induced by a number of different cytokines and that overexpression in tissue culture cells can block cell proliferation and induce apoptosis (Burrows et al., 2004; Zhu et al., 1997). Further analysis will be required to determine whether Et and its vertebrate homologues have conserved functions.

The germline specificity of et^{roo} is unique as all other *et* alleles affect both germline and soma. RT-PCR shows that et^{roo} makes a hybrid transcript containing both *et* and roo sequences (data not shown). We assume this hybrid transcript makes a truncated, but still active Et protein, which would explain the lack of a somatic phenotype. There are many possible explanations for why this truncated protein functions in the soma but in the not germline. One possibility is that the truncated Et protein is fully functional in the somatic tissue, but the protein coding region downstream of the roo insertion (which is conserved in USP42) has a germline-specific function. A second possibility is that an RNAi mechanism is involved. The expression of retrotransposons has been shown to be inhibited by rasiRNA (repeat associated small interfering RNA), and that rasiRNAs work preferentially in the germline (Klattenhoff et al., 2007; Megosh et al., 2006; Pelisson et al., 2007; Vagin et al., 2006). It is possible that the hybrid *et*-roo transcript is repressed in the germline by rasiRNAs.

Acknowledgments

We thank Elisa Park, Mike Montag, Marina Stavchanskiy, and Jessica Wickland for their help and contributions to this work. We also thank Hugo Bellen, the Harvard Exelixis *Drosophila* stock collection, the Bloomington *Drosophila* Stock Center, the Szeged *Drosophila* Stock Centre, and the *Drosophila* Genetic Resource Center, Kyoto, Japan for providing fly stocks. J.P.R. was supported by the UCLA Genetics Training Grant and a UCLA Dissertation Year Fellowship. This work was funded by grants from the NIH and NSF to F.A.L. and the NIH to B.H.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.02.005.

References

Baker, R.T., Tobias, J.W., Varshavsky, A., 1992. Ubiquitin-specific proteases of Saccharomyces cerevisiae. Cloning of UBP2 and UBP3, and functional analysis of the UBP gene family. J. Biol. Chem. 267, 23364–23375.

Bellen, H.J., Levis, R.W., Liao, G., He, Y., Carlson, J.W., Tsang, G., Evans-Holm, M., Hiesinger, P.R., Schulze, K.L., Rubin, G.M., Hoskins, R.A., Spradling, A.C., 2004. The BDGP gene disruption project: single transposon insertions associated with 40% of *Drosophila* genes. Genetics 167, 761–781.

Brachmann, C.B., Cagan, R.L., 2003. Patterning the fly eye: the role of apoptosis. Trends Genet. 19, 91–96.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Brooks, C.L., Gu, W., 2006. p53 ubiquitination: Mdm2 and beyond. Mol. Cell. 21, 307–315.

Burrows, J.F., McGrattan, M.J., Rascle, A., Humbert, M., Baek, K.H., Johnston, J.A., 2004. DUB-3, a cytokine-inducible deubiquitinating enzyme that blocks proliferation. J. Biol. Chem. 279, 13993–14000.

Chen, J., Call, G.B., Beyer, E., Bui, C., Cespedes, A., Chan, A., Chan, J., Chan, S., Chhabra, A., Dang, P., Deravanesian, A., Hermogeno, B., Jen, J., Kim, E., Lee, E., Lewis, G., Marshall, J., Regalia, K., Shadpour, F., Shemmassian, A., Spivey, K., Wells, M., Wu, J., Yamauchi, Y., Yavari, A., Abrams, A., Abramson, A., Amado, L., Anderson, J., Bashour, K., Bibikova, E., Bookatz, A., Brewer, S., Buu, N., Calvillo, S., Cao, J., Chang, A., Chang, D., Chang, Y., Chen, Y., Choi, J., Chou, J., Datta, S., Davarifar, A., Desai, P., Fabrikant, J., Farnad, S., Fu, K., Garcia, E., Garrone, N., Gasparyan, S., Gayda, P., Goffstein, C., Gonzalez, C., Guirguis, M., Hassid, R., Hong, A., Hong, J., Hovestreydt, L., Hu, C., Jamshidian, F., Kahen, K., Kao, L., Kelley, M., Kho, T., Kim, S., Kim, Y., Kirkpatrick, B., Kohan, E., Kwak, R., Langenbacher, A., Laxamana, S., Lee, C., Lee, J., Lee, S.Y., Lee, T.H., Lee, T., Lezcano, S., Lin, H., Lin, P., Luu, J., Luu, T., Marrs, W., Marsh, E., Min, S., Minasian, T., Misra, A., Morimoto, M., Moshfegh, Y., Murray, J., Nguyen, C., Nguyen, K., Nodado II, E., O'Donahue, A., Onugha, N., Orjiakor, N., Padhiar, B., Pavel-Dinu, M., Pavlenko, A., Paz, E., et al., 2005. Discovery-based science education: functional genomic dissection in *Drosophila* by undergraduate researchers. PLoS Biol. 3, e59.

Cheng, L.W., Viala, J.P., Stuurman, N., Wiedemann, U., Vale, R.D., Portnoy, D.A., 2005. Use of RNA interference in *Drosophila* S2 cells to identify host pathways controlling compartmentalization of an intracellular pathogen. Proc. Natl. Acad. Sci. U. S. A. 102, 13646–13651.

Deng, W., Lin, H., 1997. Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in *Drosophila*. Dev. Biol. 189, 79–94.

Deveraux, Q.L., Reed, J.C., 1999. IAP family proteins—suppressors of apoptosis. Genes Dev. 13, 239–252.

Ditzel, M., Meier, P., 2005. Ubiquitylation in apoptosis: DIAP1's (N-)en(d)igma. Cell. Death Differ. 12, 1208–1212.

Ditzel, M., Wilson, R., Tenev, T., Zachariou, A., Paul, A., Deas, E., Meier, P., 2003. Degradation of DIAP1 by the N-end rule pathway is essential for regulating apoptosis. Nat. Cell. Biol. 5, 467–473.

Hays, R., Wickline, L., Cagan, R., 2002. Morgue mediates apoptosis in the *Drosophila melanogaster* retina by promoting degradation of DIAP1. Nat. Cell. Biol. 4, 425–431.

Hu, M., Gu, L., Li, M., Jeffrey, P.D., Gu, W., Shi, Y., 2006. Structural basis of competitive recognition of p53 and MDM2 by HAUSP/USP7: implications for the regulation of the p53-MDM2 pathway. PLoS. Biol. 4, e27.

Huh, J.R., Guo, M., Hay, B.A., 2004. Compensatory proliferation induced by cell death in the *Drosophila* wing disc requires activity of the apical cell death caspase Dronc in a nonapoptotic role. Curr. Biol. 14, 1262–1266.

Jiang, C., Baehrecke, E.H., Thummel, C.S., 1997. Steroid regulated programmed cell death during *Drosophila* metamorphosis. Development 124, 4673–4683.

Kim, M.S., Kim, Y.K., Kim, Y.S., Seong, M., Choi, J.K., Baek, K.H., 2005. Deubiquitinating enzyme USP36 contains the PEST motif and is polyubiquitinated. Biochem. Biophys. Res. Commun. 330, 797–804.

Klattenhoff, C., Bratu, D.P., McGinnis-Schultz, N., Koppetsch, B.S., Cook, H.A., Theurkauf, W.E., 2007. *Drosophila* rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. Dev. Cell. 12, 45–55.

Koepp, D.M., Harper, J.W., Elledge, S.J., 1999. How the cyclin became a cyclin: regulated proteolysis in the cell cycle. Cell 97, 431–434.

Lee, C.Y., Baehrecke, E.H., 2001. Steroid regulation of autophagic programmed cell death during development. Development 128, 1443–1455.

Lee, C.Y., Clough, E.A., Yellon, P., Teslovich, T.M., Stephan, D.A., Baehrecke, E.H., 2003. Genome-wide analyses of steroid- and radiation-triggered programmed cell death in *Drosophila*. Curr. Biol. 13, 350–357.

Maniatis, T., 1999. A ubiquitin ligase complex essential for the NF-kappaB, Wnt/Wingless, and Hedgehog signaling pathways. Genes Dev. 13, 505–510.

Megosh, H.B., Cox, D.N., Campbell, C., Lin, H., 2006. The role of PIWI and the miRNA machinery in *Drosophila* germline determination. Curr. Biol. 16, 1884–1894.

Miller, L.K., 1999. An exegesis of IAPs: salvation and surprises from BIR motifs. Trends Cell Biol. 9, 323–328.

Moses, K., Rubin, G.M., 1991. Glass encodes a site-specific DNA-binding protein that is regulated in response to positional signals in the developing *Drosophila* eye. Genes Dev. 5, 583–593.

Okada, T., Gondo, Y., Goto, J., Kanazawa, I., Hadano, S., Ikeda, J.E., 2002. Unstable transmission of the RS447 human megasatellite tandem repetitive sequence that contains the USP17 deubiquitinating enzyme gene. Hum. Genet. 110, 302–313.

Parks, A.L., Cook, K.R., Belvin, M., Dompe, N.A., Fawcett, R., Huppert, K., Tan, L.R., Winter, C.G., Bogart, K.P., Deal, J.E., Deal-Herr, M.E., Grant, D., Marcinko, M., Miyazaki, W.Y., Robertson, S., Shaw, K.J., Tabios, M., Vysotskaia, V., Zhao, L., Andrade, R.S., Edgar, K.A., Howie, E., Killpack, K., Milash, B., Norton, A., Thao, D., Whittaker, K., Winner, M.A., Friedman, L., Margolis, J., Singer, M.A., Kopczynski, C., Curtis, D., Kaufman, T.C., Plowman, G.D., Duyk, G., Francis-Lang, H.L., 2004. Systematic generation of high-resolution deletion coverage of the *Drosophila melanogaster* genome. Nat. Genet. 36, 288–292.

Parnas, D., Haghighi, A.P., Fetter, R.D., Kim, S.W., Goodman, C.S., 2001. Regulation of postsynaptic structure and protein localization by the Rho-type guanine nucleotide exchange factor dPix. Neuron 32, 415–424.

Paulsson, K., Bekassy, A.N., Olofsson, T., Mitelman, F., Johansson, B., Panagopoulos, I., 2006. A novel and cytogenetically cryptic t(7;21)(p22;q22) in acute myeloid

leukemia results in fusion of RUNX1 with the ubiquitin-specific protease gene USP42. Leukemia 20, 224–229.
Pelisson, A., Sarot, E., Payen-Groschene, G., Bucheton, A., 2007. A novel repeat-associated small interfering RNA-mediated silencing pathway downregulates complementary sense gypsy transcripts in somatic cells of the *Drosophila* ovary. J. Virol. 81, 1951–1960.
Pickart, C.M., 1997. Targeting of substrates to the 26S proteasome. FASEB J. 11, 1055–1066.
Rechsteiner, M., Hoffman, L., Dubiel, W., 1993. The multicatalytic and 26 S proteases. J. Biol. Chem. 268, 6065–6068.
Ryoo, H.D., Gorenc, T., Steller, H., 2004. Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. Dev. Cell. 7, 491–501.
Schreader, B.A., Wang, Y., Nambu, J.R., 2003. *Drosophila* morgue and the intersection between protein ubiquitination and programmed cell death. Apoptosis 8, 129–139.
Small, S., Blair, A., Levine, M., 1992. Regulation of even-skipped stripe 2 in the *Drosophila* embryo. EMBO J. 11, 4047–4057.
Soboleva, T.A., Baker, R.T., 2004. Deubiquitinating enzymes: their functions and substrate specificity. Curr. Protein Pept. Sci. 5, 191–200.
Spradling, A.C., Stern, D., Beaton, A., Rhem, E.J., Laverty, T., Mozden, N., Misra, S., Rubin, G.M., 1999a. The Berkeley *Drosophila* Genome Project gene disruption project: single P-element insertions mutating 25% of vital *Drosophila* genes. Genetics 153, 135–177.
Spradling, A.C., Stern, D., Beaton, A., Rhem, E.J., Laverty, T., Mozden, N., Misra, S., Rubin, G.M., 1999b. The Berkeley *Drosophila* Genome Project gene disruption project: single P-element insertions mutating 25% of vital *Drosophila* genes. Genetics 153, 135–177.
Stapleton, M., Carlson, J., Brokstein, P., Yu, C., Champe, M., George, R., Guarin, H., Kronmiller, B., Pacleb, J., Park, S., Wan, K., Rubin, G.M., Celniker, S.E., 2002. A *Drosophila* full-length cDNA resource. Genome Biol. 3 RESEARCH0080.
Thibault, S.T., Singer, M.A., Miyazaki, W.Y., Milash, B., Dompe, N.A., Singh, C.M., Buchholz, R., Demsky, M., Fawcett, R., Francis-Lang, H.L., Ryner, L., Cheung, L.M., Chong, A., Erickson, C., Fisher, W.W., Greer, K., Hartouni, S.R., Howie, E., Jakkula, L., Joo, D., Killpack, K., Laufer, A., Mazzotta, J., Smith, R.D., Stevens, L.M., Stuber, C., Tan, L.R., Ventura, R., Woo, A., Zakrajsek, I., Zhao, L., Chen, F., Swimmer, C., Kopczynski, C., Duyk, G., Winberg, M.L., Margolis, J., 2004. A complementary transposon tool kit for *Drosophila melanogaster* using P and piggyBac. Nat. Genet. 36, 283–287.
Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Thornberry, N.A., Lazebnik, Y., 1998. Caspases: enemies within. Science 281, 1312–1316.
Vagin, V.V., Sigova, A., Li, C., Seitz, H., Gvozdev, V., Zamore, P.D., 2006. A distinct small RNA pathway silences selfish genetic elements in the germline. Science 313, 320–324.
Vaux, D.L., Silke, J., 2005. IAPs, RINGs and ubiquitylation. Nat. Rev. Mol. Cell. Biol. 6, 287–297.
Verhagen, A.M., Coulson, E.J., Vaux, D.L., 2001. Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. Genome. Biol. 2 REVIEWS3009.
Wilson, R., Goyal, L., Ditzel, M., Zachariou, A., Baker, D.A., Agapite, J., Steller, H., Meier, P., 2002. The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. Nat. Cell Biol. 4, 445–450.
Xu, T., Rubin, G.M., 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117, 1223–1237.
Yan, N., Wu, J.W., Chai, J., Li, W., Shi, Y., 2004. Molecular mechanisms of DrICE inhibition by DIAP1 and removal of inhibition by Reaper, Hid and Grim. Nat. Struct. Mol. Biol. 11, 420–428.
Yoo, S.J., Huh, J.R., Muro, I., Yu, H., Wang, L., Wang, S.L., Feldman, R.M., Clem, R.J., Muller, H.A., Hay, B.A., 2002. Hid, Rpr and Grim negatively regulate DIAP1 levels through distinct mechanisms. Nat. Cell Biol. 4, 416–424.
Zaccai, M., Lipshitz, H.D., 1996. Differential distributions of two adducin-like protein isoforms in the *Drosophila* ovary and early embryo. Zygote 4, 159–166.
Zhu, Y., Lambert, K., Corless, C., Copeland, N.G., Gilbert, D.J., Jenkins, N.A., D'Andrea, A.D., 1997. DUB-2 is a member of a novel family of cytokine-inducible deubiquitinating enzymes. J. Biol. Chem. 272, 51–57.