The *myb*-related gene *stonewall* induces both hyperplasia and cell death in *Drosophila*: rescue of fly lethality by coexpression of apoptosis inducers

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Abstract

We carried out gain-of-function mutagenesis screening and identified a mutant in which *GAL4* induction led to both hyperplasia and apoptosis. The gene involved was identified as *stonewall* (*stwl*), a *myb*-related gene involved in germ cell proliferation and differentiation during oogenesis. As observed with *dmyb*, the ectopic expression of *stwl*^{UY823} inhibited endoreplication in salivary glands. We also found that *stwl*^{UY823} overexpression, like overexpression of the wild-type gene, activated G1/S transition and apoptosis. The apoptosis triggered by *stwl*^{UY823} expression is correlated to induction of the proapoptotic gene *reaper*. Finally, the death of flies induced by ectopic *stwl*^{UY823} expression is efficiently prevented *in vivo* by triggering cell death in *stwl*^{UY823}-expressing cells. Our results suggest that *stwl*^{UY823} kills flies by causing inappropriate cell cycle entry, and that triggering the death of these overproliferating cells or slowing their proliferation restores viability.

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Abbreviations: AO, acridine orange; *Brat* gene, brain tumor gene; BrdU, bromodeoxyuridine; CDS, coding DNA sequence; CyO, curly of Oster; *da*, daughterless; *Dpp*, decapentaplegic; *en*, engrailed; *fkf*, fork head; GFP, green fluorescent protein; *gmr*, glass-mediated response; GUS, *gmr* UAS; *hid*, head involution defective; Hs, heat shock; hsp70, heat-shock protein 70; ORF, open reading frame; PBS, phosphate-buffered saline; PCD, programmed cell death; *rpr*, reaper; *Sev*, sevenless; *stwl*, *stonewall*; UAS, upstream activating sequences; *vg*, vestigial; *yw*^c, yellow white C; ZNC, zone of nonproliferating cells

Introduction

The coordination of cell growth, division and death is essential for normal development and adult survival. Cell growth and division are usually coordinated, leading to cell proliferation and the growth of tissues due to increases in cell number. Endoreplicating tissues are a notable exception, growing partly by means of DNA replication without cell division (review: Edgar and Orr-Weaver¹). Data for mammals and *Drosophila* show that mechanisms regulating cell proliferation and apoptosis are intimately linked (review: Hipfner and Cohen and Abrams and White^{2,3}). Neoplasia involves many processes, but the cooperative deregulation of cell proliferation and inhibition of cell death occurs in almost all cases of cancer progression. Indeed, many genes implicated in tumorigenesis act on cell proliferation and/or apoptosis. Some, such as *myc* and *e2f*, activate both processes. Consequently, proliferating cells are more susceptible to death signals. In many cases, the inactivation of proapoptotic tumor suppressors, such as *p53*, is associated with oncogene activation in tumorigenic transformation.

The *Drosophila* genome sequencing project has shown that most mammalian genes involved in proliferation or apoptosis have a counterpart in flies. This is true for genes involved in cell death execution, such as those encoding caspases, *dark* (the homolog of *apaf-1*) and two *bcl-2* homologs, *debcl* and *buffy*. However, although mammalian Bcl-2 and Bax are functional *in vivo* in *Drosophila*,^{4,5} the release of mitochondrial proteins observed in mammalian cell apoptosis is not demonstrated during *Drosophila* cell death. Genetic approaches have also resulted in the discovery of new apoptosis executors in *Drosophila*. The products of these genes (*Rpr*, *Hid*, *Grim*, *Sickle* and *Jafrac2*) act through binding to inhibitor of apoptosis proteins (IAPs), disrupting caspase inhibition.⁶ Their action on IAPs is similar to that of mammalian *Smac*/*Diablo* and *Omi/HtrA2*, but, unlike these proteins, *Rpr*, *Hid*, *Grim* and *Sickle* act upstream from or independently of mitochondria (reviewed in Mignotte *et al.*⁷). *Drosophila* also has a *p53* homolog: *Dmp53*.⁸ *Dmp53*, like mammalian *p53*, is required for apoptosis induced by radiation⁹ or reactive oxygen species,¹⁰ but, unlike mammalian *p53*, is not involved in the cell cycle arrest observed after irradiation.¹¹

Some of the *Drosophila* genes involved in cell proliferation and cell death regulation are homologs of known mammalian oncogenes. Those promoting proliferation may inhibit apoptosis. For example, the *Drosophila* homolog of *ras* stimulates cell growth and proliferation¹² whereas it downregulates head involution defective (*hid*), thereby activating a survival signaling pathway.¹³ Genetic and genomic tools for *Drosophila* recently led to the discovery of micro RNAs regulating apoptosis and cell proliferation (review: Baehrecke¹⁴). One of these micro RNAs, *bantam*, stimulates proliferation by driving G1/S and G2/M transitions and inhibiting apoptosis.

Conversely, as in mammals, many other genes stimulate both proliferation and apoptosis. This is the case for *de2f1*, the homolog of *E2F1*¹⁵ and for the *Drosophila* Src-family kinases,¹⁶ which have been shown to enhance proliferation and apoptosis. *Slik*, which encodes a Ste20 serine/threonine kinase, behaves similarly but also triggers apoptosis when it is downregulated.¹⁷ Similarly, *dmyb*, the single fly homolog of the vertebrate *myb* gene family, not only abolishes endoreplication but also promotes proliferation and apoptosis.¹⁸ C-myc induces both proliferation and apoptosis, and it has been shown that, in *Drosophila*, Dmyc induces cell competition, leading to the death of surrounding normal cells (review: Secombe *et al.*¹⁹).

Genes negatively regulating the cell cycle and controlling apoptosis have also been found in *Drosophila* (review: Sutcliffe *et al.*²⁰). Indeed, a pRB network operates in *Drosophila*. The fly RBF1 – like pRB, which can either inhibit apoptosis through its E2F-inactivating properties (reviewed in Harbour and Dean²¹) or enhance cell death^{22,23} through E2F-independent mechanisms²⁴ – can inhibit G1/S transition by inhibiting E2F factors and either inhibit or enhance apoptosis, depending on the tissue (Rincheval *et al.*, unpublished data).

Screens for *Drosophila* loss-of-function mutations resulting in tissue overgrowth have led to the discovery of three genes promoting both cell cycle exit and cell death. The *hippo*, *warts* and *salvador* genes function together to restrict tissue growth *in vivo* by favoring cell cycle exit and inducing cell death (reviewed in Hay *et al.* and Mignotte *et al.*^{6,7}). This control over programmed cell death (PCD) is achieved by decreases in cell DIAP1 content and the induction of *hid* expression.

We report here the results of the screening of a gain-of-function mutagenesis involving the transposition of a P element containing upstream activating sequence (UAS) sequences, *P{Mae-UAS.6.11}*.²⁵ Genes located downstream from the transposon insertion site should therefore be transcriptionally regulated by the UAS/GAL4 system. We characterized one mutant that behaves as a gain-of-function mutation upon GAL4 induction and as a loss-of-function mutation in the absence of GAL4 induction. Depending on the conditions used, this mutant either induces hyperplasia and apoptosis or leads to the death of flies upon GAL4 induction. We also show that the death of the fly can be prevented by triggering cell death, providing evidence that the lethality of this mutant results from hyperproliferation. Finally, we show that these effects result from the deregulation of *stonewall* (*stwl*). This gene, predominantly expressed in the ovary, has

been shown to encode a nuclear protein required for germline cell proliferation and differentiation.^{26,27} The work presented here provides clues to the mechanism of action of *stwl*.

Results

GAL4-induced UY823 expression leads to adult phenotypes reminiscent of cell death and cell proliferation

We carried out gain-of-function mutagenesis screening, with the aim of identifying new regulators of apoptosis. We used *P{Mae-UAS.6.11}* (abbreviated in UY), a P element vector containing UAS sequences. Genes downstream from the transposon insertion site should be transcriptionally regulated by the UAS/GAL4 system. The UY823 transgenic line displayed an interesting phenotype. Specific GAL4 induction in the ventral lateral neurons of UY823 adults, driven by *GAL1118*,²⁸ led to cell death (E Blanchardon and F Rouyer, personal communication). We studied the effects of UY823 induction in various tissues during development, using several drivers (glass-mediated response (*gmr*)-GAL4, daughterless (*da*)-GAL4, engrailed (*en*)-GAL4, heat-shock (*hs*)-GAL4 and vestigial (*vg*)-GAL4). Transcriptional regulation of the gene next to the UY P element in the UY823 line by GAL4 throughout development impaired viability (Table 1). This effect increased with the temperature, consistent with the increase in efficiency of the GAL4 system. *Drosophila* eyes consist of ommatidia precisely positioned within a highly ordered crystalline lattice; they have been widely used to study the effects of apoptosis regulators. We found that specific UY823 induction in the eye during development by the *gmr*-GAL4 driver at 21°C (Figure 1a and b) led to the development of small, rough, less pigmented eyes. Moreover, GAL4 induction of UY823 by the *vg*-GAL4 driver at a sublethal temperature (21°C) induced the formation of notches at the wing margin (Figure 1d). All of these phenotypes may result from excess cell death. However, the *vg*-GAL4 induction of UY823 also led to prominent hyperplastic buds on the wing hinge (Figure 1d). Furthermore, UY823 induction by the *en*-GAL4 driver in the posterior compartment of the wing disc induced hyperplastic tissues in the posterior part of the wing (data not shown). The induction of UY823 in the wing therefore demonstrates that the gene located downstream from the UAS sequences can induce both cell death and proliferation.

Table 1 Percentage viability and phenotypes induced by GAL4 expression in UY823

	18°C		21°C		25°C	
	Viability	Adult phenotype	Viability	Adult phenotype	Viability	Adult phenotype
<i>en</i> > UY823	25.6% ($N_{TM3}=69$)	Notches wing hairs	<0.6% ($N_{TM3}=132$)	Lethality	<0.7% ($N_{TM3}=106$)	Lethality
<i>vg</i> > UY823	76.4% ($N_{TM3}=146$)	Notches	30.5% ($N_{TM3}=116$)	Notches hyperplasia	<0.1% ($N_{TM3}=531$)	Lethality
<i>gmr</i> > UY823	81.4% ($N_{TM3}=34$)	Rough eye	7.5% ($N_{TM3}=51$)	Glass eye	<1.4% ($N_{TM3}=53$)	Lethality
<i>da</i> > UY823	82.1% ($N_{TM3}=59$)	No	50.3% ($N_{TM3}=130$)	No	<0.6% ($N_{TM3}=122$)	Lethality
<i>hs</i> > UY823	ND	ND	ND	ND	8.1% ($N_{TM3}=448$)	No

Percentage viability = $(N_{UY823+}/(1.3 \times N_{TM3})) \times 100$; when no UY823/+ flies are born in the progeny, the percentage viability $< (1/(1.3 \times N_{TM3})) \times 100$. ND: not determined. no: no phenotype observed

Figure 1 Induction of *UY823* triggers adult phenotypes reminiscent of cell death and cell proliferation. (a, b) Photographs of *Drosophila* eye and (c, d) wings mounted in Mowiol. The figure compares the phenotype associated with *GAL4* induction in *UY823* *Drosophila* eye (b, *gmr* > *UY823*) and wing (d, *vg* > *UY823*), with that of control eye (a, *gmr-GAL4/+*) and wing (c, *vg-GAL4/+*). Less pigmented glassy eyes are observed in (b). Arrows indicate hyperplastic buds and stars indicate wing notches. Flies were raised at 21°C

Figure 2 Induction of *UY823* triggers hyperproliferation and apoptosis in third instar larval wing discs. The first and third columns correspond to control discs for *en-GAL4* and *vg-GAL4* induction, respectively. The second and last columns correspond to wing discs with *en-GAL4* and *vg-GAL4* induction, respectively, in *UY823* lines. All discs are shown with the anterior at the top and the posterior at the bottom. The A–P boundary is outlined by a red dotted line. Fluorescence micrographs of fixed discs expressing *UAS-GFP* under *en-GAL4* induction (a, b) and under *vg-GAL4* induction (c, d) are shown. (e–h) Corresponding Nomarski micrographs of discs (a–d). (i, j) AO staining of unfixed discs and their corresponding Nomarski images (k, l). X-gal staining of control *en-GAL4/+ ; rpr-11-lacZ/+* fixed discs (m) and of *en-GAL4/+ ; rpr-11-lacZ, UY823/+* fixed discs (n). BrdU incorporation in *en-GAL4/+* control fixed discs at low and high magnifications (o, q) and in fixed discs *en-GAL4/+ ; UY823/+* at low and high magnifications (p, r). Arrows indicate the ZNC, and an arrowhead indicates the absence of ZNC in the posterior region of the *en-GAL4/+ ; UY823/+* wing disc

Induction of *UY823* in the wing discs triggers hyperproliferation by activating entry into S phase

We studied the effect of *UY823* insertion on proliferation during development, using the *en-GAL4* and *vg-GAL4* drivers to induce *UY823* in wing discs. The pattern of expression of these two drivers in the wing disc is clearly illustrated by green fluorescent protein (*GFP*) expression in Figure 2a and c.

The *en>UY823* and *vg>UY823* discs showed massive hyperplasia (Figure 2). Indeed, the posterior part of the *en>UY823* discs was much larger than the posterior part of the control discs (compare Figure 2f–e and l–k). Similarly, the hinge and wing pouch of *vg>UY823* fixed discs were formed with prominent, multiple folds, resulting from an excess of cells (Figure 2h–g). In the wing discs, cell proliferation occurred throughout larval development, in the entire disc. At the third instar stage, margin cells (in the wing pouch) were blocked in G1, generating a zone of nonproliferating cells (ZNC – see arrows in Figure 2q and r). As observed in control discs (Figure 2o and q), S-phase cells were labeled with bromodeoxyuridine (BrdU) throughout the disc, with the exception of the ZNC. BrdU labeling in *en>UY823* discs was clearly stronger in the hypertrophied posterior part than in the anterior part, demonstrating that *UY823* expression induced an increase in the number of cells in S phase (Figure 2p and r). Moreover, unlike ZNC cells in the anterior part of the *en>UY823* wing pouch, the cells located in the posterior part of the wing disc pouch were labeled with BrdU. This suggests that cells of the ZNC subjected to *UY823* induction overcome the normal G1/S block. Thus, deregulation of the gene next to *UY823* insertion triggers cell proliferation, at least by activating the entry of cells into S phase.

UY823 induction in wing discs causes apoptosis and is correlated with induction of the proapoptotic gene *rpr*

We studied the capacity of the gene next to *UY823* insertion to trigger apoptosis, using a specific marker, acridine orange (AO), to visualize apoptosis *in vivo* in induced *UY823* tissues. In control wing discs, only a few cells died by apoptosis, as shown by AO staining (Figure 2i). By contrast, massive AO staining was observed in the posterior part of *en>UY823* third instar larval wing discs (Figure 2j), corresponding to the area of *en-GAL4* driver expression. Slightly stronger AO staining was also observed in the anterior region of the disc, suggesting that some compensation occurs in this area.

In *Drosophila*, the proapoptotic gene reaper (*rpr*) is transcriptionally induced to trigger apoptosis during embryonic development, metamorphosis and after X-ray irradiation.²⁹ We investigated whether *UY823* induction activated *rpr* transcription, by comparing the staining pattern of *en-GAL4/+;rpr-11-lacZ,UY823/+* discs with that of *en-GAL4/+;rpr-11-lacZ/+* control discs, using the *rpr-11-lacZ* construct.³⁰ In control discs, *in situ* X-gal staining showed that *rpr* expression was prominent in the wing hinge and wing pouch, where it occurred in the margin (dorso-ventral limit) and perpendicularly, at the antero-posterior limit (Figure 2m). Patches of *rpr* expression were also observed in the wing attach and at the tip of the notum. In *en>UY823* discs, *UY823* induction in the

Figure 3 Coexpression of the *p35* partially suppresses the wing phenotype of *vg>UY823* flies. (a, b) Wings of *vg-GAL4, UAS-GFP/+; UY823/+* and *UAS-P35/+; vg-GAL4, UAS-GFP/+; UY823/+* flies, respectively. Asterisks indicate notches in the anterior part of the wing and an arrowhead shows notch in the posterior part of the wing. An arrows indicates the presence of extra tissue folds in the hinge region of the wing. Flies were raised at 21°C

posterior part of the disc was associated with an increase in *rpr* expression (Figure 2n). In addition, consistent with AO staining, *rpr* expression also increased in the anterior part of the disc, but to a lesser extent. These results clearly show that deregulation of the gene next to *UY823* insertion directly or indirectly activated *rpr* transcription. *Rpr*-induced apoptosis has been shown to involve IAP inhibition and caspase activation. We investigated whether the wing notch phenotypes observed in *vg>UY823 Drosophila* were due to ectopic apoptosis. We did this by abolishing these phenotypes, by coexpressing the caspase inhibitor *p35* gene. Flies expressing *p35* alone with the *vg-GAL4* driver at 21°C have normal wing sizes and do not display notch phenotypes. At 21°C, *vg>UY823* flies coexpressing the *p35* gene carried fewer notches than *vg>UY823* flies (Figure 3). Indeed, 21 of the 58 *vg>UY823* flies coexpressing the *p35* gene had more than two notches, versus 48 of 67 for *vg>UY823* flies (χ^2 test, $P=7 \times 10^{-5}$). We also found that *vg>UY823* flies had notches in the posterior part of the wing, but also often in the anterior part of the wing (Figure 3a). This may be due to weaker expression of the *vg-GAL4* driver in the anterior region of the *vg>UY823* wing disc (Figure 2d). Notches in the anterior part of the wing were rarely observed in the presence of *p35* (χ^2 test, $P=1.38 \times 10^{-7}$). These results suggest that the notch phenotype is due to caspase-dependent apoptosis. Furthermore, coexpression of the *p35* gene often results in an expansion of the dorsal compartment in the hinge of the wing, consistent with strong *vg-GAL4* expression in this region (Figure 2d). This expansion is associated with the presence of

extra tissue folds (Figure 3b), possibly arising from excessive proliferation.

Characterization of the *stwl*^{UY823} allele

Homozygous females of the *UY823* transgenic line are sterile and do not lay eggs. We made use of this property for genetic complementation screening. These mutant females have rudimentary ovaries (depleted of post-vitellogenic eggs), but the germaria are hypertrophied, containing abnormal egg chambers (data not shown). As the P element *UY823* is located on the third chromosome, we screened for deletions of this chromosome that do not complement *UY823* female sterility. We identified three such deletions: *Df(3L)fz-D21* (70D1-2;70E7), *Df(3L)fz-GS1a* (70D2;70E4-5) and *Df(3L)Brd6* (70E;71F). We also tested 46 mutations or P insertions located in the genomic region defined by these deletions. Only *P{lacW}stwl^{6C3}* (stock BL-12087), an insertion in the *stwl* gene, failed to complement female sterility, suggesting that *stwl* is mutated in the *UY823* line. We have named this new allele *stwl*^{UY823}. Interestingly, *stwl* loss of function induced female sterility with phenotypes strikingly similar to those observed in sterile *stwl*^{UY823} females.²⁶ We also found that *stwl*¹⁹⁵, a null allele of *stwl*, did not complement female sterility. Finally, we confirmed that the recessive

stwl^{UY823} mutation was a loss-of-function mutation of *stwl* by showing that female sterility is rescued by introducing the *stwl* 'minigene' into the *stwl*^{UY823} genetic background. As expected, *y,w/y,w; P{w⁺, minigene stwl}/+; stwl^{UY823}/stwl^{UY823}* females were fertile, laying viable eggs.

Molecular characterization and regulation of *stwl*^{UY823} mRNA

We used a forward primer specific to the P element (P1^{ISA}) and a reverse primer complementary to the minus strand of *stwl* (P2^{stwlR3}), for classical PCR amplification of genomic DNA to recover the flanking sequences transcribed upon *GAL4* induction. A 2900-base pair (bp) DNA fragment (not shown) was amplified, indicating that the *UY823* P element was inserted in the first exon of *stwl* and that its orientation was consistent with transcription of the coding strand of *stwl* (+ strand). Analysis of the sequences of two independent PCR products indicated that the *UY823* P element was inserted at position +111 in the first exon of *stwl* and that the ATG in -3 of the P element was in frame with the coding sequence of *stwl* (Figure 4a). Thus, whereas the open reading frames (ORFs) starting from the two more internal ATGs of the P element encode very short polypeptides

Figure 4 Molecular localization of the *UY823* mutation and analysis of *stwl*^{UY823} expression. (a) Schematic representation of the 5' end of the UYP element inserted into the coding sequence of *stwl*. The transcription start site -366 bp from the end of the UYP transposon and the ATG start codons are indicated by arrows. The primers used for PCR are indicated by arrows. The sole intron of *stwl*, located from +148 to +2925 bp, is shown in gray. (b) Schematic diagram of Myb family proteins. The N-terminus to the left and the C-terminus is shown to the right. The R1, R2 and R3 repeats of the Myb DNA-binding domain are shown, together with the acidic transactivation domain (TA). The Stwl protein consists of one Myb DNA-binding domain, the R2 and R3 repeats, and a putative acidic transactivation domain. (c) Northern blot analysis of poly(A)⁺ mRNA from adult females. The *stwl*-specific probe demonstrated *stwl* expression in control flies, whereas no *stwl* mRNA was detected in homozygous *stwl*^{UY823}/*stwl*^{UY823} flies. (d) RT-PCR analysis of total RNA from *en>stwl*^{UY823} and *en-GAL4/+* third instar larval wing discs. The *stwl*^{UY823} mRNA was amplified using P1^{ISA} and P2^{stwlR3} primers. The mRNA of *stwl*^{UY823} was detected in *en>stwl*^{UY823} discs but not in control discs. (e) Northern blot analysis of adult male total RNA. The *stwl*-specific probe demonstrated basal constitutive expression of *stwl*^{UY823} in *hs>stwl*^{UY823} males, whereas *stwl* mRNA was not detected in *hs-GAL4/+* control males

(<30 amino acids), translation from the ATG in frame with the *stwl* coding DNA sequence (CDS) would lead to the production of a Stwl protein with the first 37 amino acids deleted. Stwl belongs to the Myb protein family. All the proteins of this family have at least one Myb DNA-binding domain (Figure 4b). This domain corresponds to a sequence of approximately 50 amino acids, containing a series of highly conserved residues, which folds into a helix–turn–helix (HTH) structure. Unlike dMyb, which bears three repeats of the Myb DNA-binding domain, Stwl has a single DNA-binding motif related to the Myb domain. Stwl also bears a putative acid-rich transactivator domain, suggesting its possible involvement of transcriptional regulation. The putative N-terminally truncated Stwl protein induced by GAL4 in *stwl^{UY823}* flies contained a truncation of the Myb/HTH DNA-binding motif. However, helices 1 and 2 remained intact in this truncated protein (Figure 4b).

Northern blot analysis on poly (A) + mRNA indicated that *stwl* expression was impaired in *stwl^{UY823}/stwl^{UY823}* females. We detected *stwl* mRNA in control females, but not in sterile *stwl^{UY823}/stwl^{UY823}* females (Figure 4c). We investigated whether *stwl^{UY823}* transcription was activated upon GAL4 induction by carrying out semiquantitative RT-PCR on total RNA extracted from *en>stwl^{UY823}* wing discs. We detected *stwl^{UY823}* mRNA in *en>stwl^{UY823}* larvae, but not in *en-GAL4/+* controls (Figure 4d). We also determined the size of the *stwl^{UY823}* mRNA by Northern blot analysis of total RNA extracted from adult *hs>stwl^{UY823}* flies. The *stwl* cDNA is 3636 bp long (Genbank Acc Num:U41367) and the ATG start codon is located +161 bases from the beginning of the cDNA. The transcription start site within the UYP element is located –366 from the end of the transposon, giving a predicted size of 3761 bases (3636–110–161+366) for *stwl^{UY823}* spliced mRNA. The expression of *stwl^{UY823}* was weak and constitutive in *hs>stwl^{UY823}* adult flies (Figure 4e). The apparent size of *stwl^{UY823}* mRNA was about 3700 bases, consistent with the production of a spliced mRNA starting from the transposon transcription start site.

Effects of *stwl^{UY823}* and full-length *stwl* expression on apoptosis and cell proliferation are similar

As *stwl* deregulation in the *UY823* line is driven by UAS sequences upstream from *stwl*, it is theoretically possible that these UAS drive a neighboring gene other than *stwl*, possibly accounting for the observed phenotypes. Furthermore, as the putative Stwl^{UY823} protein lacks the first 37 amino acids of Stwl, it may exert a dominant-negative effect after ectopic expression. We tried to determine whether one of these possibilities applied, by studying phenotypes associated with the overexpression of full-length *stwl* in another genetic background. We used a heat-shock protein 70 (*hsp70*)-*stwl* line²⁶ in which *stwl* was overexpressed in response to heat-shock. We performed AO staining on wing discs from control or *hsp70-stwl* heat-shocked larvae. Wing discs overexpressing *stwl* showed stronger AO staining than control discs (Figure 5c and d), indicating that *stwl* overexpression causes apoptosis. In BrdU labeling experiments, slightly more stained cells were detected in the ZNC in heat-shocked wild-type

Figure 5 Induction of full-length *stwl* expression triggers hyperproliferation and apoptosis in third instar larval wing discs. All discs were obtained from heat-shocked larvae. The first column corresponds to wild-type discs and the second to *hsp70-stwl*-expressing discs. (a–d) Discs are shown with the anterior at the top and the posterior at the bottom. (e–f) show a magnification of the wing pouch observed with a confocal microscope. (c, d) AO staining of unfixed discs and their corresponding Nomarski micrographs (a, b). (e, f) BrdU incorporation in fixed discs. An arrow indicates the ZNC and arrowheads show the presence of stained cells in the ZNC. The scale bar represents 36 μ m

larval discs (Figure 5e) than in non-shocked wild-type larval discs (data not shown), suggesting that heat-shock may have a slight effect on larval cell proliferation. Nevertheless, following the induction of *stwl* expression by heat shock, BrdU staining increased in the ZNC of the wing pouch (compare Figure 5e and f). This indicates an increase in the number of cells in S phase in the ZNC. Thus, *stwl* expression induced cell proliferation by activating the entry of cells into S phase. As the *hsp70-stwl* transgene is located at a site other than the *stwl* locus, these results show that the data obtained for *stwl^{UY823}* were the consequence of the overexpression of *stwl*, rather than of another, neighboring gene.

Heat-shocked *hsp70-stwl* larvae died soon after induction. We were therefore unable to study the corresponding adult phenotypes. We used the transgenic line *stwl^{GS3129}* to compare the ectopic expression of full-length *stwl* with that of *stwl^{UY823}*. This transgenic line carries a UAS P element inserted upstream from *stwl* CDS,³¹ making it possible to express the full-length *stwl*/CDS. Using the same drivers as for *stwl^{UY823}* expression, with various breeding temperatures, we obtained viable adults, making it possible to compare phenotypes induced by both forms of Stwl. The expression of *stwl^{GS3129}* in the eye and in the wing during development induces effects reminiscent of cell death, similar to those

Table 2 Percentage lethality triggered by *UY823* induction in embryos, larvae and pupae raised at 25°C

% Lethality	<i>w¹¹¹⁸/y,w^c</i> N = 250 (%)	<i>w¹¹¹⁸/y,w^c; UY823/+</i> N = 256 (%)	<i>w¹¹¹⁸/y,w^c; vg-GAL4/+</i> N = 267 (%)	<i>w¹¹¹⁸/y,w^c; vg-GAL4/+; UY823/+</i> N = 272 (%)
Embryonic	3	4	3	1
Larval	16	10	15	16
Pupal	1	2	4	83

N = number of fertilized embryos counted

induced by *stwI^{UY823}* expression. We also demonstrated that *stwI^{GS3129}* induced apoptosis and proliferation at the cellular level. These results are presented in supplementary data.

These results show that *stwI* activates cellular proliferation and apoptosis and suggest that the N-terminal portion of *stwI* is not required for the activation of cellular proliferation and apoptosis.

Coexpression of proapoptotic genes prevents the pupal death triggered by *stwI^{UY823}* expression

The *vg-GAL4*-driven expression of *stwI^{UY823}* leads to pupal death at 25°C (Table 2). Death may result from excessive cell death or from hyperproliferation. We investigated the possible role of cell death triggered by *stwI^{UY823}* induction in pupal death by coexpressing antiapoptotic genes, such as *UAS-qbcd-2*, *UAS-p35* and *UAS-diap1*. Surprisingly, none of these genes abolished pupal death (data not shown). We studied the contribution of hyperproliferation to pupal death, by investigating whether the coexpression of proapoptotic genes (*UAS-bax*, *UAS-rpr*, *UAS-hid*, *UAS-grim*, *UAS-sickle*, *UAS-dronc*, *UAS-dreddδ* and *gmr UAS (GUS)-p53*) or antiproliferative genes (*UAS-dacapo/p21*, *UAS-rbf* and brain tumor gene (*brat^{UY113}*) could prevent pupal death (Table 3). Coexpression of all the proapoptotic genes studied here prevented pupal death. The surviving flies presented a strong wing phenotype (Figure 6), with thoracic defects sometimes observed in the survivors coexpressing *bax*. Furthermore, with the exception of *dacapo/p21*, the coexpression of antiproliferative genes decreased pupal lethality and the few escapers display also a strong wing phenotype (data not shown). Thus, the abolition of pupal death was not gene-specific. Instead, it depended on the elimination of cells decreasing fly viability from tissues. Thus, the *vg-GAL4*-driven induction of *stwI^{UY823}* probably triggers a high level of cell proliferation, interfering with vital functions during pupariation, and blocking cell proliferation in the tissue or triggering the death of these supernumerary cells may therefore restore viability.

Expression of *stwI^{UY823}* is associated with endoreplication defects in salivary glands

Studies on dMyb have shown that the ectopic expression of *dmyb* inhibits endoreplication¹⁸ in salivary glands. As *StwI* belongs to the dMyb family, we investigated the effects of *stwI^{UY823}* expression on endoreplication. We carried out Hoechst staining on the DNA of salivary gland cells from control or induced *stwI^{UY823}* third-instar larvae. We used fork head (*fkh*)-*GAL4* to drive *stwI^{UY823}* expression in salivary

Table 3 Percentage viability of flies expressing different transgenes under *vg-GAL4* control in the *UY823* line at 25°C

Co-expressed genes	N _{UY823} /N _{TM3}	Survival (%)
Control	0/531	<0.1
UAS-GFP	0/78	<1.0
UAS-bax	10/7	120.9
UAS-hid	12/11	83.9
UAS-rpr	20/28	71.4
GUS-p53	33/37	68.6
UAS-grim	6/13	35.5
UAS-sickle	22/48	35.3
UAS-prodronc	7/75	7.2
UAS-dreddδ	3/49	4.7
UAS-rbf	5/151	2.5
UY1131 (<i>brat</i>)	2/102	1.5
UAS-dacapo	0/81	<0.9

Percentage viability = $(N_{UY823}/(1.3 \times N_{TM3})) \times 100$; when no *UY823/+* are born in the progeny, the percentage of viability $< (1/(1.3 \times N_{TM3})) \times 100$

Figure 6 Phenotype of a viable adult *vg> rpr; UY823* fly raised at 25°C. The image shows a viable *UAS-rpr/Y; vg-GAL4, UAS-GFP/+; stwI^{UY823}/+* adult obtained at 25°C. This survivor had a strong wing phenotype

glands. Cell size is generally proportional to the amount of nuclear DNA and endoreplication is an efficient growth strategy in differentiated tissues. Salivary glands with induced *stwI^{UY823}* were only about one-fifth the size of control glands (Figure 7a and f). These salivary glands are composed of much smaller cells than control glands, as shown by Nomarski imaging (Figure 7b and g). DNA staining of control (Figure 7c–e) and induced *stwI^{UY823}* (Figure 7h–j) salivary gland cells is shown. Mutant salivary gland cell nuclei (Figure 7h and j) were less strongly stained than control salivary gland cell nuclei (Figure 7c and e), whereas the nuclei of fat body cells were stained equally strongly in the control and the mutant (Figure 7d and i). The nuclei of induced *stwI^{UY823}* salivary glands were also smaller than control nuclei (Figure 7j and e). Thus, mutant salivary gland cell nuclei contain less DNA than control

salivary gland or fat body cell nuclei, consistent with the lower level of endoreplication in induced *stw^{UY823}* flies.

Some nuclei also display changes to chromatin morphology, suggesting that the cells may be dying. However, Hoechst labeling of these nuclei was weaker than that in apoptotic cells and no AO staining can be observed in *stw^{UY823}*-expressing salivary glands (data not shown). Furthermore, the size of *stw^{UY823}*-expressing cells (Figure 7g and h) was sufficient to account for the smaller volume of the gland. Thus, the main effect of *stw* in salivary glands is the inhibition of endoreplication.

Discussion

Stwl is a nuclear protein required for female germline cell development. Its expression is essentially restricted to germline stem cells, nurse cells and the oocyte nucleus until stage 7 of oogenesis. Analyses of loss-of-function phenotypes have suggested that Stwl is required for maintenance of the stem cell pool, or for oocyte differentiation.^{26,27} However, the precise function of Stwl remains unclear. We generated a new allele of *stw*, *stw^{UY823}* by gain-of-function mutagenesis. We show that this allele behaves as a loss-of-function mutation of *stw* and that its induction by the *UAS/GAL4* system is equivalent to a gain-of-function mutation of *stw*. Our results indicate that ectopic expression of the 5'-truncated -*stw* (*stw^{UY823}*) or the full-length *stw* (*hsp70-stw*) induces apoptosis and cell proliferation. On the one hand, *stw^{UY823}*

expression in the wing disc increases the number of cells in S phase, probably by accelerating G1/S transition. On the other hand, *stw^{UY823}* expression induces transcriptional activation of the proapoptotic gene *rpr*. Nevertheless, it has been shown that *stw* loss of function induces germ cell apoptosis.²⁶ In this case, nurse cells with differentiation defects are eliminated, whereas in the case of *stw* ectopic expression overproliferating cells are eliminated. This is consistent with the observation that ectopic *stw^{UY823}* expression in the ventral lateral neurons of *Drosophila* brain induces the death of these cells (E Blanchardon and F Rouyer, personal communication). We show here that the coexpression of proapoptotic genes (and, to a lesser extent, the coexpression of antiproliferative genes) prevents the pupal death induced by *stw^{UY823}* expression at 25°C. The wing phenotype of survivors was stronger than - that observed at 21°C for *stw^{UY823}*, and stronger than the phenotype observed when proapoptotic genes are overexpressed alone. This rescue provides evidence that the pupal death induced by *stw^{UY823}* expression results from inappropriate cell proliferation and that triggering the death of overproliferating cells or slowing their proliferation restores viability.

The similarity between the changes induced by ectopic expression of *stw^{UY823}*, *stw^{GS3129}* and *hsp70-stw* suggests that these alleles act in a similar manner, in this context. Stwl belongs to the evolutionarily conserved Myb family, and previous studies have shown that *dmyb* overexpression also induces cell proliferation and apoptosis. As reported here for

Figure 7 Ectopic expression of *stw^{UY823}* inhibits endoreplication in salivary glands. Hoechst DNA staining of salivary glands dissected from third instar larvae, carried out on control glands and glands expressing *stw^{UY823}* driven by *fkh-GAL4*. The endocycling salivary glands are indicated by (sg) and fat bodies by (fb). (a–e) show control glands *fkh-GAL4/+*. (f–j) show glands with induced *stw^{UY823}* *fkh-GAL4/stw^{UY823}*. (a, b, f, g) are Nomarski images. (b) and (g) are high magnifications of (a) and (f), respectively, and (c, h) are fluorescence micrographs corresponding to (b, g). (d, e) and (i, j) show high magnifications of the nuclei presented in (c) and (h) respectively. (d) and (i) are nuclei from fat bodies and (e) and (j) are nuclei from salivary glands. The scale bar represents 100 μ m in (a, f) and 20 μ m (across) in (b–e) and (g–j). An asterisk indicates mandibles and imaginal discs

stwl, *dmyb* expression activates G1/S transition.¹⁸ Furthermore, *stwl*^{UY823} expression, like *dmyb* expression, inhibits endoreplication in salivary glands. It was recently suggested that Stwl may be involved in nuclear inhibition of the cell cycle following DNA damage.³² Thus, the expression of *dmyb* and *stwl* probably results in the activation of common pathways involved in regulation of the cell cycle and apoptosis.

In conclusion, our results, showing that *stwl* expression has effects similar to *dmyb* expression, provide evidence that these genes have conserved functions in the control of cell cycle progression and of apoptosis in *Drosophila*. Our results also show that *stwl* activates *rpr* transcription, consistent with the putative role of Stwl in the response to DNA damage.³² Comparisons between *dmyb* and *stwl* may make it possible to improve our understanding of the functions of Stwl in germline cells. Our results suggest that Stwl stimulates cell cycle progression, consistent with the requirement to maintain the stem cell pool. Furthermore, Stwl could be involved in inhibiting the endocycling of DNA in the oocyte, which must enter meiosis. By contrast, nurse cells become polyploid. Finally, it would be of interest to screen for suppressors of pupal death due to the *vg-GAL4*-driven expression of *stwl*^{UY823} as a means of identifying new genes potentially interacting with *stwl*.

Materials and Methods

Fly stocks

Flies were raised on standard medium. The *UAS-GFP*, *UAS-bax*, *UAS-bcl-2*, *da-GAL4*, *vg-GAL4*, *gmr-GAL4*/curly of Oster (*CyO*) and *en-GAL4* lines have been described elsewhere.^{4,5} The *UAS-rpr* and *UAS-hid* transgenic lines were generated by recombination of the X-linked *UAS-rpr*, *UAS-hid* transgenic line described previously.³³ The *rpr-11-lacZ* construct was generated by Nordstrom *et al.*³⁰ The *stwl*^{UY823} and *brat*^{UY1131} lines were generated by mobilizing *p[Mae-UAS.6.11]*.²⁵ *brat*^{UY1131} is an inducible allele of the antiproliferative gene *brain tumor suppressor* (personal communication from M Simonelig). The *UAS-grim* line was generated by Wing *et al.*³⁴ The *UAS-sickle* line was generated by Srinivasula *et al.*³⁵ The *UAS-pro-dronc* line has been described by Quinn *et al.*³⁶ *UAS-dredd* has been described by Vidal *et al.*³⁷ *UAS-rbf* was generated by Du *et al.*³⁸ *UAS-dacapo* was generated by Lane *et al.*³⁹ The *w;P(w⁺, minigene stwl)* and *w;P[hsp70-stwl]* lines were generously provided by D McKearin and were generated by Clark *et al.*²⁶ The GS3129 line was generously provided by T Aigaki, and was generated by gain-of-function mutagenesis, by Toba *et al.*³¹ The *fkh-GAL4* was generously provided by Dr. S Beckendorf.⁴⁰ *UAS-p35* (*P[w(+ mC) = UAS-p35.H]BH3, w[*]*), *P[lacW]stwl*^{6c3} and *P[UAS-DIAP1.H]3*, and all the lines required for genetic localization of the *UY823* transgene originated from the Bloomington *Drosophila* Stock Center. The *y,w^c* and *w¹¹¹⁸* lines were used as control strains.

Northern blot analyses

For total RNA analysis, 25 *hs-GAL4/+;stwl*^{UY823/+} males and 25 *hs-GAL4/+* males raised at 25°C were ground in TRIzol (Invitrogen). RNA was extracted according to the manufacturer's instructions. We used 20 µg of total RNA for the Northern blot.

For poly (A)⁺ RNA analysis, 250 *y,w^c* control females and 250 *stwl*^{UY823} homozygous females (1-week-old) raised at 21°C on standard

medium supplemented with fresh yeast were ground in TRIzol. Total RNA was extracted, and poly (A)⁺ RNA was isolated using the Micro-FastTrack™ 2.0 Kit (Invitrogen). We used 20 µg of poly (A)⁺ RNA for the Northern blot.

The *stwl* DNA probe (1085 bp) was obtained by PCR from the cDNA of *stwl* cloned in pBS Σ c3.5 (#1) (generously provided by D McKearin), using primers P3^{stwlS2} (GCCGAGTGAGAACGCCTGGAA) and P4^{stwlR4} (CTGGCCCGATGGCGGCTTGT). The *rp49* DNA probe (431 bp) was generated by PCR from the cDNA of *rp49* (inserted into pGEM-5Zf(+)) using forward (GTGTATTCCGACCAGGTTACA) and reverse (ATACAGGCCCAAGATCGTGA) primers. The single-stranded DNA probes for *stwl* and *rp49* were radiolabeled with α -³²P-dCTP by random priming, using the Megaprime RPN1606 Kit (Amersham).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

For total RNA analysis, 200 *en-GAL4/+;stwl*^{UY823/+} imaginal wing discs and 200 *en-GAL4/+* imaginal wing discs of larvae raised at 25°C were used. RNA was extracted with the 'SV Total RNA Isolation System' following the manufacturer's instructions (Promega; reference Z3100).

A semiquantitative RT-PCR method was used to determine the levels of *stwl*^{UY823} mRNA in *en>stwl*^{UY823} imaginal wing discs. This technique is based on evaluation of the amount of *stwl*^{UY823} cDNA fragment amplified during the exponential phase of PCR. As a control, *rp49* cDNA was amplified in the same PCR tube. Approximately 500 ng of total RNA from *en>stwl*^{UY823} imaginal wing discs was denatured by heating before reverse transcription (RT). RT was performed in the presence of random hexamers, in a total volume of 25 µl, according to the recommendations of the kit manufacturer (M-MLV, 28025-013, Invitrogen). A 224-bp fragment was amplified from the *stwl*^{UY823} cDNA sequence, using primer P1^{ISA} (CTCTCAACAAGCAAACGTGC), which binds at -87 bp, in the *UY* P element sequence, and primer P2^{stwlR3} (GTCCATCGGCCAGCGGATAA), which binds at +3025 bp. A 430-bp fragment was amplified from the *rp49* cDNA using the primers (GTGTATTCCGACCAGGTTACA) and (ATACAGGCCCAAGATCGTGA). PCR was carried out over 27 cycles (30 s each at 92, 64 and 72°C).

Genomic localization of the *UY823* transposon

Genomic DNA was extracted from *stwl*^{UY823} flies with a standard fly 'miniprep' method, by E Jay Rehm (BDGP). PCR on genomic DNA, using the P1^{ISA} (CTCTCAACAAGCAAACGTGC) and P2^{stwlR3} (GTCCATCGGCCAGCGGATAA) primers, generated a fragment of about 2900 bp. The forward primer used, P1^{ISA} is specific to the 5' end of the *UY* transposon and can be used to amplify the DNA strand transcribed upon *UAS/GAL4* induction (+ strand). The reverse primer, P2^{stwlR3} allows amplification of the noncoding strand of *stwl* from position +3006 of the coding sequence. Two independent PCR products were then sequenced, using P1^{ISA} (MWG Biotech Germany).

Test of phenotype suppression by coexpression of the *p35* gene

We crossed *vg-GAL4, UAS-GFP/CyO; stwl*^{UY823/TM3} *Drosophila* males with *UAS-p35/UAS-p35* or *w¹¹¹⁸* females at 21°C. The progenies of both crosses were counted and classified in different groups according to the number of notches on their wings. Flies with a notch in the anterior part of the wing were also counted. The data were analyzed by means of χ^2 tests.

Viability experiment

At 12 h after egg laying, embryos were collected and counted. After 36 h of development at 25°C, we counted the dead embryos. We counted pupae and adult flies 6 and 12 days after egg laying (Table 2).

Heat-shock protocol

Flies of the *w¹¹¹⁸* and *hsp70-stwl* genotypes were raised at 21°C. Third instar larvae were placed for 20 min at 37°C and then returned to 21°C for a further 20 min. This temperature shift was performed three times, and the larvae were finally placed at 21°C and collected 20 h after the last shock.

Histology

As *stwl^{UY823}* expression driven by *en-GAL4* is lethal at the late larval or pupal stage, at 21°C (data not shown), and *stwl^{UY823}* expression driven by *vg-GAL4* is pupal lethal at 25°C, we dissected and analyzed wing discs of third instar larvae grown at both lethal temperatures, to ensure that the most severe phenotypes were observed.

Detection of *vg-GAL4* and *en-GAL4* expression in wing discs

vg-GAL4,UAS-GFP and *en-GAL4,UAS-GFP* females were crossed with *y,w^c* males and to *stwl^{UY823}* males. Progeny was raised at 21°C for *en-GAL4* induction and at 25°C for *vg-GAL4* induction. Third instar larvae in the progeny were dissected in 1 × PBS, pH 7.6. Larvae were then fixed in PBS/4% formaldehyde, washed three times, for 3 min each in PBS and placed in PBS/glycerol (1 : 1) overnight at 4°C. Discs were then mounted in glycerol and observed with a conventional Leica DMRHC research microscope, using the L5 filter to detect green fluorescence and the Nomarski filter for bright-field micrographs.

AO staining of wing discs

en-GAL4 females were crossed with *stwl^{UY823}* males and *vg-GAL4* females were crossed with *stwl^{UY823}* and *stwl^{GS3129}* males. *en-GAL4/+*; *stwl^{UY823}/+* larvae were raised at 21°C, *vg-GAL4/+*; *stwl^{UY823}/+* and *vg-GAL4/+*; *stwl^{GS3129}/+* larvae were raised at 25°C. Control discs were dissected from *en-GAL4/+* and *vg-GAL4/+* larvae. Discs were dissected in 1 × PBS, pH 7.6, and then stained for 2 min in 100 ng/ml AO (Molecular Probes), for cell death assessment. Discs were then mounted in PBS and observed with a conventional Leica DMRHC research microscope, using the I3 filter to detect AO fluorescence and the Nomarski filter for bright-field micrographs.

X-gal staining of wing discs

en-GAL4,UAS-GFP females were crossed with *rpr-11-lacZ, stwl^{UY823}* males and with *rpr-11-lacZ* males for the control. The progeny were raised at 21°C and larvae were dissected in 1 × PBS, pH 7.6, on ice. Larvae were fixed in PBS/4% formaldehyde/1% glutaraldehyde at 20°C, washed three times, for 3 min each in 1 × PBS, stained for 4 h 20 min at 37°C in 1 × PBS, 4 mM K₄Fe₂, 4 mM K₃Fe₃, 1 mM MgCl₂, 1% Triton X-100, 800 ng/ml X-gal, washed twice for 3 min each in PBS, fixed for 5 min and washed three times, for 3 min each, in 1 × PBS. Discs were then placed in PBS/glycerol (1 : 1) overnight at 4°C, mounted in glycerol and observed with a conventional Leica DMRHC research microscope, using the Nomarski filter.

BrdU labeling of wing discs

en-GAL4 females were crossed with *stwl^{UY823}* males, and with *y,w^c* males for the control. The progeny were raised at 21°C, and larvae were fed for 4 h on medium supplemented with 1 mg/ml BrdU. Larvae were then dissected in 1 × PBS, pH 7.6, and fixed in PBT/formaldehyde (1 × PBS/4% formaldehyde/0.3% Triton X-100) for 20 min at room temperature, washed three times, for 3 min each in PBT, denatured in 2.2 N HCl/0.1% Triton X-100 by two incubations, for 15 min each, neutralized with 100 mM sodium tetraborate (Borax) by two incubations, for 5 min each. For immunohistochemistry, larvae were blocked by incubation in PBT/10% normal goat serum (NGS) for 45 min, incubated with 1 : 20 diluted mouse anti-BrdU monoclonal antibody containing nuclease (Amersham, RPN202) overnight at 4°C and washed three times, for 20 min each, in PBT/NGS. Discs were then incubated with 1 : 1000 diluted Alexa-Fluor-488 goat anti-mouse IgG or with 1 : 50 diluted biotinylated anti-mouse IgG (Amersham) and washed three times, for 20 min each, in PBT. For biotinylated anti-mouse IgG (Figure 2), we used the Vectastain Elite ABC Kit (Vector Laboratories) and the DAB substrate kit for peroxidase (Vector Laboratories) for BrdU detection. Discs were mounted in glycerol and observed with a conventional Leica DMRHC research microscope, using the Nomarski filter. For Alexa-Fluor-488 goat anti-mouse IgG (Figure 6), discs were mounted in glycerol and observed with a Leica SP2 right confocal microscope.

DNA staining of salivary glands

Salivary glands were dissected from third instar larvae, fixed by incubation for 30 min in paraformaldehyde 4% and labeled by incubation with 1 μg/ml Hoechst stain at room temperature for 15 min. Images of salivary glands were captured with a conventional Leica DMRHC research microscope, using the A filter to detect Hoechst fluorescence and the Nomarski filter for bright-field micrographs. The same exposure time was used for all images.

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References

1. Edgar BA and Orr-Weaver TL (2001) Endoreplication cell cycles: more for less. *Cell* 105: 297–306
2. Hipfner DR and Cohen SM (2004) Connecting proliferation and apoptosis in development and disease. *Nat. Rev. Mol. Cell Biol.* 5: 805–815

3. Abrams JM and White MA (2004) Coordination of cell death and the cell cycle: linking proliferation to death through private and communal couplers. *Curr. Opin. Cell Biol.* 16: 634–638
4. Gaumer S, Guénal I, Brun S, Théodore L and Mignotte B (2000) Bcl-2 and Bax mammalian regulators of apoptosis are functional in *Drosophila*. *Cell Death Differ.* 7: 804–814
5. Brun S, Rincheval V, Gaumer S, Mignotte B and Guénal I (2002) *rpr* and *bax* initiate two different apoptotic pathways affecting mitochondria and antagonized by *bcl-2* in *Drosophila*. *Oncogene* 21: 6458–6470
6. Hay BA, Huh JR and Guo M (2004) The genetics of cell death: approaches, insights and opportunities in *Drosophila*. *Nat. Rev. Genet.* 5: 911–922
7. Mignotte B, Colin J, Brun S and Guénal I (2005) Apoptosis: the fly point of view. In *Apoptosis*, Scovassi AI (ed) (Kerala, India: Research Signpost) pp. 169–186 (ISBN: 81-308-0021-7)
8. Brodsky MH, Nordstrom W, Tsang G, Kwan E, Rubin GM and Abrams JM (2000) *Drosophila* p53 binds a damage response element at the *reaper* locus. *Cell* 101: 103–113
9. Sogame N, Kim M and Abrams JM (2003) *Drosophila* p53 preserves genomic stability by regulating cell death. *Proc. Natl. Acad. Sci. USA* 100: 4696–46701
10. Morey M, Corominas M and Serras F (2003) DIAP1 suppresses ROS-induced apoptosis caused by impairment of the selD/sps1 homolog in *Drosophila*. *J. Cell Sci.* 116: 4597–4604
11. Schumacher B, Hofmann K, Boulton S and Gartner A (2001) The *C. elegans* homolog of the p53 tumor suppressor is required for DNA damage-induced apoptosis. *Curr. Biol.* 11: 1722–1727
12. Prober DA and Edgar BA (2000) Ras1 promotes cellular growth in the *Drosophila* wing. *Cell* 100: 435–446
13. Bergmann A, Agapite J, McCall K and Steller H (1998) The *Drosophila* gene *hid* is a direct molecular target of Ras-dependent survival signaling. *Cell* 95: 331–341
14. Baehrecke EH (2003) miRNAs: micro managers of programmed cell death. *Curr. Biol.* 13: R473–R475
15. Du W, Xie JE and Dyson N (1996) Ectopic expression of dE2F and dDP induces cell proliferation and death in the *Drosophila* eye. *EMBO J.* 15: 3684–3692
16. Pedraza LG, Stewart RA, Li DM and Xu T (2004) *Drosophila* Src-family kinases function with Csk to regulate cell proliferation and apoptosis. *Oncogene* 23: 4754–4762
17. Hipfner DR and Cohen SM (2003) The *Drosophila* sterile-20 kinase slik controls cell proliferation and apoptosis during imaginal disc development. *PLoS Biol.* 1: E35. (Epub 2003 Nov 2017)
18. Fitzpatrick CA, Sharkov NV, Ramsay G and Katzen AL (2002) *Drosophila myb* exerts opposing effects on S phase, promoting proliferation and suppressing endoreduplication. *Development* 129: 4497–4507
19. Secombe J, Pierce SB and Eisenman RN (2004) Myc: a weapon of mass destruction. *Cell* 117: 153–156
20. Sutcliffe JE, Korenjak M and Brehm A (2003) Tumour suppressors – a fly's perspective. *Eur. J. Cancer* 39: 1355–1362
21. Harbour JW and Dean DC (2000) Rb function in cell-cycle regulation and apoptosis. *Nat. Cell Biol.* 2: E65–E67
22. Knudsen KE, Weber E, Arden KC, Cavenee WK, Feramisco JR and Knudsen ES (1999) The retinoblastoma tumor suppressor inhibits cellular proliferation through two distinct mechanisms: inhibition of cell cycle progression and induction of cell death. *Oncogene* 18: 5239–5245
23. Lemaire C, Godefroy N, Costina-Parvu I, Rincheval V, Renaud F, Trotot P, Bouleau S, Mignotte B and Vayssière JL (2005) Caspase-9 can antagonize p53-induced apoptosis by generating a p76(Rb) truncated form of Rb. *Oncogene* 24: 3297–3308
24. Hsieh JK, Chan FS, O'Connor DJ, Mitnacht S, Zhong S and Lu X (1999) RB regulates the stability and the apoptotic function of p53 via MDM2. *Mol. Cell.* 3: 181–193
25. Monnier V, Girardot F, Cheret C, Andres O and Tricoire H (2002) Modulation of oxidative stress resistance in *Drosophila melanogaster* by gene overexpression. *Genesis* 34: 76–79
26. Clark KA and McKearin DM (1996) The *Drosophila stonewall* gene encodes a putative transcription factor essential for germ cell development. *Development* 122: 937–950
27. Akiyama T (2002) Mutations of *stonewall* disrupt the maintenance of female germline stem cells in *Drosophila melanogaster*. *Dev. Growth Differ.* 44: 97–102
28. Blanchardon E, Grima B, Klarsfeld A, Chelot E, Hardin PE, Preat T and Rouyer F (2001) Defining the role of *Drosophila* lateral neurons in the control of circadian rhythms in motor activity and eclosion by targeted genetic ablation and PERIOD protein overexpression. *Eur. J. Neurosci.* 13: 871–888
29. Baehrecke EH (2002) How death shapes life during development. *Nat. Rev. Mol. Cell Biol.* 3: 779–787
30. Nordstrom W, Chen P, Steller H and Abrams JM (1996) Activation of the *reaper* gene during ectopic cell killing in *Drosophila*. *Dev. Biol.* 180: 213–226
31. Toba G, Ohsako T, Miyata N, Ohtsuka T, Seong KH and Aigaki T (1999) The gene search system. A method for efficient detection and rapid molecular identification of genes in *Drosophila melanogaster*. *Genetics* 151: 725–737
32. de Vries HI, Brunsting JF, Lemstra W, Kampinga HH and Sibon OCM (2003) Identification of *stonewall* as a novel *Drosophila* gene involved in DNA damage response. In *43rd Annual Drosophila Research Conference* (Chicago: The Genetics Society of America)
33. Zhou L, Schnitzler A, Agapite J, Schwartz LM, Steller H and Nambu JR (1997) Cooperative functions of the *reaper* and *head involution defective* genes in the programmed cell death of *Drosophila* central nervous system midline cells. *Proc. Natl. Acad. Sci. USA* 94: 5131–5136
34. Wing JP, Zhou L, Schwartz LM and Nambu JR (1998) Distinct cell killing properties of the *Drosophila reaper*, *head involution defective*, and *grim* genes. *Cell Death Differ.* 5: 930–939
35. Srinivasula SM, Datta P, Kobayashi M, Wu JW, Fujioka M, Hegde R, Zhang Z, Mukattash R, Fernandes-Alnemri T, Shi Y, Jaynes JB and Alnemri ES (2002) *sickle*, a novel *Drosophila* death gene in the *reaper/hid/grim* region, encodes an IAP-inhibitory protein. *Curr. Biol.* 12: 125–130
36. Quinn LM, Dorstyn L, Mills K, Colussi PA, Chen P, Coombe M, Abrams J, Kumar S and Richardson H (2000) An essential role for the caspase dronc in developmentally programmed cell death in *Drosophila*. *J. Biol. Chem.* 275: 40416–40424
37. Vidal S, Kush RS, Leulier F, Tzou P, Nakamura M and Lemaître B (2001) Mutations in the *Drosophila dTAK1* gene reveal a conserved function for MAPKKs in the control of rel/NF- κ B dependent innate immune responses. *Genes Dev.* 15: 1900–1912
38. Du W, Vidal M, Xie JE and Dyson N (1996) RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila*. *Genes Dev.* 10: 1206–1218
39. Lane ME, Sauer K, Wallace K, Jan YN, Lehner CF and Vaessin H (1996) Dacapo, a cyclin-dependent kinase inhibitor, stops cell proliferation during *Drosophila* development. *Cell* 87: 1225–1235
40. Zhou B, Bagri A and Beckendorf SK (2001) Salivary gland determination in *Drosophila*: a salivary-specific, fork head enhancer integrates spatial pattern and allows fork head autoregulation. *Dev. Biol.* 237: 54–67

Supplementary Information accompanies the paper on the Cell Death and Differentiation website (<http://www.nature.com/cdd>)