DIAP1 suppresses ROS-induced apoptosis caused by impairment of the *selD/sps1* homolog in *Drosophila*

Marta Morey, Montserrat Corominas and Florenci Serras*

Departament de Genètica, Facultat de Biologia, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain

*Author for correspondence (e-mail: fserras@ub.edu)

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Summary

The cellular antioxidant defense systems neutralize the cytotoxic by-products referred to as reactive oxygen species (ROS). Among them, selenoproteins have important antioxidant and detoxification functions. The interference in selenoprotein biosynthesis results in accumulation of ROS and consequently in a toxic intracellular environment. The resulting ROS imbalance can trigger apoptosis to eliminate the deleterious cells. In *Drosophila*, a null mutation in the *selD* gene (homologous to the human *selenophosphate synthetase type 1*) causes an impairment of selenoprotein biosynthesis, a ROS burst and lethality. We propose this mutation (known as *selD*^{ptuf}) as a tool to understand the link between ROS accumulation and cell death. To this aim we have analyzed the mechanism by which *selD*^{ptuf} mutant cells become apoptotic in *Drosophila* imaginal discs. The apoptotic effect of *selD*^{ptuf} does not require the activity of the Ras/MAPK-dependent pro-apoptotic gene *hid*, but results in stabilization of the tumor suppressor protein Dmp53 and transcription of the *Drosophila* pro-apoptotic gene *reaper* (*rpr*). We also provide genetic evidence that the initiator caspase DRONC is activated and that the effector caspase DRICE is processed to commit *selD*^{ptuf} mutant cells to death. Moreover, the ectopic expression of the inhibitor of apoptosis DIAP1 rescues the cellular viability of *selD*^{ptuf} mutant cells. These observations indicate that *selD*^{ptuf} ROS-induced apoptosis in *Drosophila* is mainly driven by the caspase-dependent Dmp53/Rpr pathway.

Key words: Reaper, p53, DIAP1, SelD, Selenoprotein, ROS

Introduction

Apoptosis is an essential cellular process during the morphogenesis of multicellular organisms and its genetic control has become one of the main topics of study for developmental and cell biologists. However, it is not yet well understood how certain processes such as oxidative stress can trigger specific pathways of apoptosis. Aerobic metabolism uses molecular oxygen as a terminal electron acceptor for mitochondrial respiratory energy production. Reactive oxygen species (ROS) are generated as by-products of this process. These are a variety of oxygen metabolites that have either unpaired electrons (i.e. O_2^-, OH·) or the ability to abstract electrons from other molecules (i.e. hydrogen peroxide). Transient fluctuations in ROS serve important regulatory functions, but when present at high and/or sustained levels, they can cause severe damage to DNA, proteins and lipids, which may finally lead to apoptosis (Simon et al., 2000; Curtin et al., 2002).

A number of defense systems have evolved to counteract the persistent state of oxidative siege associated with aerobic life conditions and among them enzymatic intracellular scavengers play an essential role. In this category, mammalian selenoproteins, which contain selenium in the form of selenocysteine, are important for the control of unwanted ROS as most of them catalyze oxidation-reduction reactions or act as antioxidants (Stadtman, 1996; Rayman, 2000; Hatfield and Gladyshev, 2002). The machinery of selenocysteine incorporation is conserved in bacteria, archaea and eukarya and depends on reading the inframe UGA stop codon as the amino acid selenocysteine and on the recognition of a downstream stem loop structure (Low and Berry, 1996; Stadtman, 1996). Four genes (*selA-D*) of *Escherichia coli* are essential for selenocysteine synthesis, codon recognition and polypeptide elongation. One of them, the *selD* gene, encodes for selenophosphate synthetase which catalyses a selenide-dependent ATP hydrolysis reaction to generate monoselenophosphate, the selenium intermediate necessary for the synthesis of selenocysteine. In flies and mammals two highly conserved *selD* genes have been identified (Low et al., 1995; Guimaraes et al., 1996; Persson et al., 1997; Alsina et al., 1998), *sps1* (*selD* in flies) and *sps2*. Two effects have been described for a mutation in the *Drosophila selD/sps1* gene, perturbation of selenoprotein biosynthesis and accumulation of ROS (Alsina et al., 1999). This mutation, hereafter called *selD*^{ptuf}, is a recessive null mutation and homozygous individuals have extremely reduced and abnormal imaginal discs and die as third instar larvae (Alsina et al., 1998; Alsina et al., 1999). Heterozygous *selD*^{ptuf} flies are apparently wild type, however, a downregulation of the Ras/MAPK pathway has been observed in transheterozygous combinations of the *selD*^{ptuf} mutation and activated members of this signaling pathway (Morey et al., 2001). This downregulation seems to be a consequence of an increase in ROS levels in heterozygous flies (Morey et al., 2001; Morey et al., 2003). Both loss of survival signals and perturbations in the redox balance, among others, are intracellular stimuli that have been shown to trigger

apoptosis in mammals (Kroemer and Reed, 2000; Adrain and Martin, 2001). In *Drosophila*, the cell death genes *reaper* (*rpr*), *head involution defective* (*hid*) and *grim* are potent activators of caspase-dependent apoptosis (reviewed by Richardson and Kumar, 2002). Extensive research has been carried out to uncover how distinct death-inducing stimuli converge to activate a common apoptotic program. Both *rpr* and *grim* are expressed in cells doomed to die (White et al., 1994; Chen et al., 1996). In contrast, *hid* is expressed not only in cells that die, but also in living cells (Grether et al., 1995). Thus, these different expression patterns imply that they integrate different signals regulating apoptosis. Survival signals regulate *Drosophila* apoptosis and several studies have shown the need for Ras/MAPK activity for cell survival in flies (Simon et al., 1991; Diaz-Benjumea and Hafen, 1994; Freeman, 1996; Miller and Cagan, 1998; Sawamoto et al., 1998; Halfar et al., 2001). In the subset of *hid*-expressing cells prone to die, downregulation of the Ras/MAPK pathway increases *hid* expression and activity (Bergmann et al., 1998; Kurada and White, 1998). In the case of *rpr*, besides inducing developmentally programmed cell death, it also triggers apoptosis in response to other stimuli such as aberrant development, steroid hormone signaling and X-irradiation (Asano et al., 1996; Nordstrom et al., 1996; Robinow et al., 1997). *rpr* is also a transcriptional target of the *Drosophila* p53 protein, making its expression responsive to genotoxic stress caused by X-irradiation (Brodsky et al., 2000). Because X-irradiation, in addition to direct DNA damage, generates ROS in the aqueous cytoplasm that can also damage DNA, the Dmp53/Rpr pathway is a candidate pathway to be activated in a situation of increased ROS levels such as in the *selD*^{ptuf} mutant.

We have taken a genetic approach to study how an increase in ROS levels due to impairment of the *selD/sps1* function triggers apoptosis in *Drosophila* imaginal discs. Our results indicate that *hid*-induced apoptosis may not be the major contributor to the apoptosis observed in *selD*^{ptuf} mutant cells and that ROS increase plays an important role in *selD*^{ptuf} apoptosis through the activation of the Dmp53/Rpr pathway. We clearly show that this apoptotic pathway is mediated by DRONC and DRICE caspases and that the inhibitor of apoptosis DIAP1 is able to rescue the viability of *selD*^{ptuf} cells. This work supports the importance of *selD/sps1* in the maintenance of cellular viability and demonstrates that ROS-induced apoptosis triggered by the loss-of-function of *selD/sps1* is caspase dependent and activated by Dmp53/Rpr function.

Materials and Methods

Drosophila stocks

The *selD*^{ptuf} line (*yw; l(2)k11320/CyO*) was obtained from a collection of lethal mutants resulting from *PlacW* insertions on the second chromosome of *Drosophila melanogaster* (Torok et al., 1993; Alsina et al., 1998). The following stocks were used: *yw; Cat*^{n1}*/TM3* (Mackay and Bewley, 1989; Griswold et al., 1993); *e ftz ry/TM3 P(sev-rasV12)* (Fortini et al., 1992); *Sco/SM1 P(GMRhid)* (Kurada and White, 1998); *GMRyan*^{Act} on the second chromosome (Rebay and Rubin, 1995); *rprlacZ* on the third chromosome (Nordstrom et al., 1996); *UAS-DIAP1/TM2* (Lisi et al., 2000); *GMR-GAL4 UAS-dronc #80* (*GMR-GAL4 UAS-dronc/CyO; UAS-dronc)* (Quinn et al., 2000); *UAS-Sem* on the third chromosome (Martin-Blanco, 1998); *+; gl-Dmp53/SM6aTM6B* (Ollman et al., 2000) (Exelixis Inc.); *hh-GAL4/TM2* (Tanimoto et al., 2000); *2xarm-GAL4/TM3Sb*, *69B-GAL4* on the third chromosome (Bloomington Stock Center).

Scanning electron microscopy

To prepare scanning electron microscopy (SEM) samples, flies were dehydrated in 25, 50, 75 and 100% ethanol for 24 hours each. Flies were critical point dried and coated with gold to be examined in a Leica-360 scanning electron microscope.

Generation of mitotic clones

selD^{ptuf} clones in the adult eye were generated using the *ey*FLP/FRT technique coupled to a cell lethal mutation (*cl2R11*) (Newsome et al., 2000), which kills the twin clone allowing the growth of more mutant tissue. Adults of the genotype *yw eyFLP; FRT42D w+ cl2R11/FRT42D selD*^{ptuf} were examined for mutant clones (two copies of the *mini-w* marker from the *PlacW* insertion in the *selD* locus in a *mini-w/ w+* background) in the eye. Histological sections of the eyes were prepared as described previously (Basler and Hafen, 1988).

selD^{ptuf} mitotic clones in imaginal discs were generated using the *hs*FLP/FRT technique (Xu and Rubin, 1993) in combination with the *Minute* (*M*) technique (Morata and Ripoll, 1975), which gives proliferative advantage to the mutant tissue minimizing perdurance of *selD* product. Larvae of the *yw hsFLP; FRT42D arm-lacZ M/FRT42D selD*^{ptuf} genotype were heat shocked at 60 hours after egg laying (AEL) for 30 minutes at 34°C. For *rpr* transcription in *selD*^{ptuf} clones, 60 hours AEL larvae of *yw hsFLP; FRT42D* π*Myc M/FRT42D selD*^{ptuf}*; rpr-lacZ/+* genotype were heat shocked for 1 hour at 37°C and πMyc was induced as described previously (Xu and Rubin, 1993). In these experiments the homozygous mutant tissue was marked by the absence of β-galactosidase or πMyc staining.

To determine the clonal size in wing discs, clones were induced by a 10-minute heat shock pulse at 34°C in *yw hsFLP; FRT42D arm-lacZ/FRT42D selD*^{ptuf}*; hh-GAL4/UAS-DIAP1* larvae (4-hourly egg collections) at 55 hours AEL and analyzed at 120 hours AEL.

Inmunohistochemistry

Imaginal discs from third instar larvae were dissected in PBS and fixed for 20 minutes in 4% paraformaldehyde (PFA) at room temperature (for β-galactosidase staining) or PLP (2% PFA, 75 mM lysine; 10 mM sodium periodate, 37 mM sodium phosphate) at 4°C (for πMyc staining). Following permeabilization, discs were incubated overnight with primary antibodies: rabbit anti-β-galactosidase 1:1000 (Cappel), mouse anti-β-galactosidase 1:250 (Promega) or mouse anti-πMyc 1:1000 (Babco) to mark clones, and mouse anti-Boss (Cagan et al., 1992) 1:2000 (a gift from S. L. Zipursky), mouse anti-Elav (Robinow and White, 1988) 1:100 (from DSHB), mouse anti-Dmp53 (Ollmann et al., 2000) 1:250 (Exelisis Inc.), rabbit anti-active DRICE (Dorstyn et al., 2002) 1:1000 (a gift from B. Hay) and mouse anti-FASIII (Brower and Jaffe, 1989) 1:1000 (a gift from D. Brower). Rhodamine Red- and FITC-conjugated secondary antibodies 1:200 (Jackson ImmunoResearch) were used. YOYO® nuclear marker (1:5000, Molecular Probes, Inc.) was added together with the secondary antibody. Discs were mounted in Slowfade® Light antifade (Molecular Probes, Inc.) and images collected using a Leica TCS 4D confocal laser scanning microscope. Negative controls for these antibodies in *selD*^{ptuf} discs and clones were performed in parallel and scanned under the same conditions and showed no staining. All images were processed with Adobe PhotoShop 6.0. and ImageJ 1.29v (National Institute of Health, USA).

TUNEL assay in *selD*^{ptuf} clones

After fixation of *yw hsFLP; FRT42D arm-lacZ M/FRT42D selD*^{ptuf}

discs, apoptotic cells were detected by labeling the 3′-OH ends of DNA with Chromatide BODIPY® Texas Red-14-dUTP (Molecular Probes, Inc.) for 1 hour 30 minutes at 37°C using terminal deoxynucleotidyl transferase (Roche). Primary and secondary antibodies were used to detect clones as described above.

In vivo detection of ROS in *selD^{ptuf}* clones

Clones to test for accumulation of ROS were generated in *yw hsFLP; FRT42D GFP/FRT42D selD^{ptuf}* larvae after heat shock at 48 hours AEL for 30 minutes at 34°C. Third instar larvae discs were dissected in Schneider's medium. Staining was performed in medium containing 20 μM dihydroethidium (DHE, Molecular Probes, Inc.) for 5 minutes, and a series of washes in Schneider's medium were performed to protect the sample from light before mounting in antifade solution. The samples were examined by confocal microscopy just after mounting.

Results and Discussion

Drosophila eye development is a paradigm to understand the cellular mechanisms that coordinately promote cell differentiation and survival. During larval development, cell-to-cell interactions in the eye imaginal disc shape the ommatidia, the units that form the insect compound eye. The Ras/MAPK pathway controls differentiation and survival of ommatidial cells (Freeman, 1996; Halfar et al., 2001), and a wide variety of molecular markers are available to verify these processes. In addition, the role of apoptosis in shaping the eye has been extensively described (Brachmann and Cagan, 2003). We have used the eye imaginal disc as a model to explore how apoptosis is triggered in oxidative stress conditions. We first tested whether *selD^{ptuf}* homozygous condition perturbs cell survival and/or normal differentiation in the developing eye. Because the homozygous *selD^{ptuf}* individuals are not viable (Alsina et al., 1998; Roch et al., 1998), we have analyzed the mutant condition in genetic mosaics. Clones of *selD^{ptuf}* mutant cells were generated in the eye disc and recovered to adulthood. These clones resulted in scarred tissue with poor ommatidial differentiation (Fig. 1A). Tangential sections revealed that no or very few photoreceptors were present in the mutant area, suggesting that extensive cell death had occurred during the development of the mutant sector (Fig. 1B). We also analyzed whether recruitment and differentiation of photoreceptor cells was taking place. Although the R8 photoreceptor-specific marker Boss revealed that R8 differentiation and spacing occurred properly (data not shown), the recruitment of the subsequent photoreceptors was disrupted, as revealed by the pattern of the neuronal marker Elav (Fig. 1C). Mutant ommatidia contained less cells than their neighboring wild-type heterozygous ones. Besides aberrant ommatidial organization, the epithelium of *selD^{ptuf}* was severely disrupted. As it has been described that disorganization and loss of polarity in epithelial tissues precedes cell death (Tepass et al., 1990), we checked for apoptosis by TUNEL staining and indeed detected a significant increase in apoptotic cells within *selD^{ptuf}* clones. Interestingly, ectopic apoptotic figures were also observed in the neighboring wild-type cells, suggesting a non autonomous effect of *selD^{ptuf}* mutation (Fig. 1D).

We next examined whether the impairment of cell differentiation and survival in clones of *selD^{ptuf}* homozygous mutant cells was associated with a detectable burst of ROS. We used the ROS-specific nuclear probe dihydroethidium (DHE).

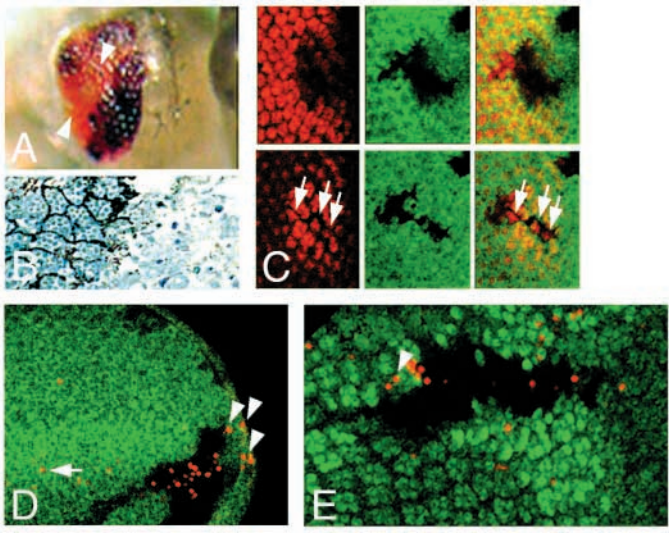

Fig. 1. Genetic mosaics of *selD^{ptuf}* in the eye. (A) *selD^{ptuf}* clones were recovered to adulthood and resulted in aberrant eyes. The mutant area (light red sectors; arrowheads) was scarred and almost no ommatidia differentiated. (B) Tangential sections of *selD^{ptuf}* clones revealed no ommatidial cells present in the mutant area. Right half: mutant sector; left half: normal tissue. (C) *selD^{ptuf}* homozygous clones in the eye imaginal disc stained with the anti-Elav neuronal cell marker (red; left) indicated that differentiation of photoreceptors is altered. In the middle panels mutant clones (black) lack β-galactosidase staining (green). Right panels: merged images. A confocal section at the normal level of Elav pattern (upper panels) showed no labeling in the *selD^{ptuf}* clone; however, deeper in the mutant tissue (lower panels) Elav staining occurs, showing abnormal ommatidia (arrows). (D) TUNEL staining (red) in a *selD^{ptuf}* clone of an eye imaginal disc. It shows that cells in the *selD^{ptuf}* clone (dark area lacking β-galactosidase labeling) entered apoptosis. In addition to wild-type apoptosis in the vicinity of the morphogenetic furrow (arrow), ectopic apoptotic figures were observed in cells adjacent to the clone (arrowhead). (E) In vivo detection of ROS with DHE (red) in *selD^{ptuf}* clones (dark area lacking GFP labeling) DHE labeled cells were present in the clone and also in cells adjacent to it (arrowhead).

This probe specifically detects the superoxide anion (O_2^-), a primary oxygen free radical produced by mitochondria rapidly removed by conversion to hydrogen peroxide. Upon an in vivo burst of O_2^-, DHE oxidizes to ethidium and incorporates into the nuclear DNA emitting fluorescence (Molecular Probes, Inc.). Certainly, some cells in *selD^{ptuf}* clones accumulated ROS, as shown by nuclear DHE staining. In addition to that, DHE-positive cells were detected in wild-type cells adjacent to the clone (Fig. 1E). This could explain the presence of apoptotic cells bordering the clone. ROS are very small molecules that can freely diffuse through cell membranes and after an accumulation in the clone the surrounding heterozygous tissue acts as a sink diluting this accumulation. To confirm that ROS accumulation was the cause and not a consequence of apoptosis, we performed the DHE assay on discs in which apoptosis was ectopically induced by constitutive expression of an inductor of apoptosis (*GMRhid*-expressing discs, see below). In these discs no DHE labeling was observed (data not shown) reinforcing the role of ROS in inducing apoptosis.

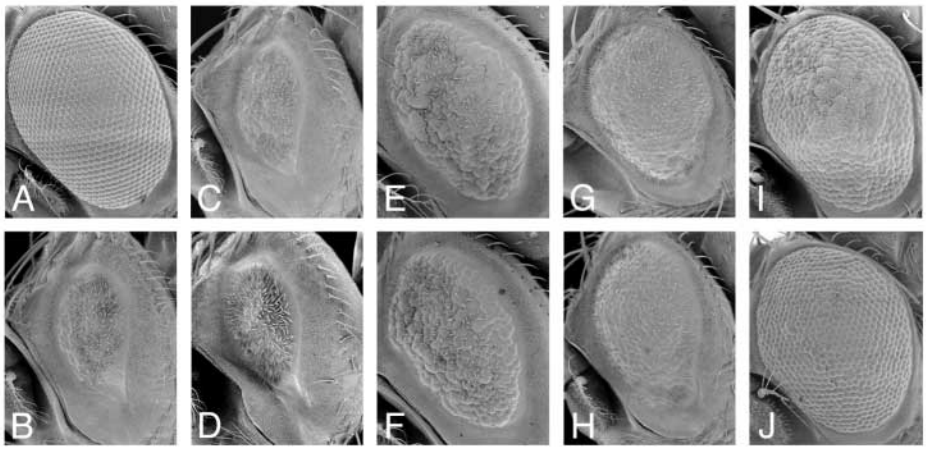

Fig. 2. Phenotypes resulting from genetic interactions of the Hid and the Ras pathway. (A) Wild type eye. (B) GMR*hid*. (C) *GMRhid*/*selD*^{ptuf}. (D) *GMRhid/selD*^{ptuf}; *Cat*^{n1}. (E) *GMRhid; sev-rasV12*. (F) *GMRhid/selD*^{ptuf}; *sev-rasV12*. (G) *GMRyan*^{Act}. (H) *GMRyan*^{Act}/ *selD*^{ptuf}. (I) *sev-rasV12*. (J) *selD*^{ptuf}; *sev-rasV12/ Cat*^{n1}. Anterior part of the eye is to the left.

Ras/MAPK downregulation as a result of ROS increase has been observed in *selD*^{ptuf} heterozygotes (Morey et al., 2001), and so it is possible that apoptosis through *hid* could account for cell death in *selD*^{ptuf} homozygous mutants. To address this question, we checked for the enhancement of ectopic *hid* phenotype under the control of the eye-specific *glass* multimer reporter [pGMR (Hay et al., 1994)] after removing one copy of *selD*. Flies carrying one copy of *GMRhid* have severely reduced eyes devoid of most normal ommatidial morphology because of massive apoptosis in the eye disc (Fig. 2B) (Grether et al., 1995). The removal of one dose of *selD*, or of *catalase* (as a *selD/sps1*-independent source of ROS) did not enhance the *GMRhid* phenotype (Fig. 2C). Even the combination of *selD*^{ptuf} and *Cat*^{n1} mutations in transheterozygotes could not enhance the *GMRhid* phenotype (Fig. 2D), whereas that combination is able to suppress a strong constitutively activated *rasV12* construct (Fig. 2I,J) (Fortini et al., 1992). As it is likely that the *GMRhid* phenotype is too strong to detect an enhancement, we generated *GMRhid sev-rasV12* flies to get a weaker apoptotic phenotype (Fig. 2E) (Bergmann et al., 1998; Kurada and White, 1998). Again, neither reduction of one dose of *selD*, nor of *catalase*, enhanced the rough eye phenotype of the *GMRhid sev-rasV12* background (Fig. 2F). In addition, we performed a genetic interaction with an activated form of *yan* (*GMRyan*^{Act}), a negative regulator of the Ras/MAPK pathway. Overexpression of *yan* in the developing eye induces apoptosis (Fig. 2G) (Rebay and Rubin, 1995), and increases *hid* mRNA levels (Kurada and White, 1998). *GMRyan*^{Act} eyes display a milder phenotype than the *GMRhid* ones, but no enhancement was observed by reduction of one dose of *selD* (Fig. 2H). In addition to that, ectopic expression of a gain-of-function allele of the MAPK *rolled* locus (*rl*^{sem}) under different drivers (*69B-GAL4* and *arm-GAL4*) does not rescue imaginal disc morphology in homozygous mutants at any time during larval development (data not shown). Together these results indicate that the impairment of the Ras/MAPK pathway may not be the main cause of *selD*^{ptuf} apoptosis and suggested that other apoptotic inducers could be involved.

Several signaling pathways converge to activate a cell-death program triggered by the pro-apoptotic gene *reaper* (White et al., 1994; Asano et al., 1996; Nordstrom et al., 1996; Brodsky et al., 2000). It is known that in mammals ROS can induce apoptosis (Buttke and Sandstrom, 1994; Simon et al., 2000) thus we tested if the increase in ROS caused by *selD*^{ptuf} mutation would trigger apoptosis through *rpr*. Indeed, we found *rpr* transcription in *selD*^{ptuf} homozygous discs using a *rpr-lacZ* reporter. Widespread β-galactosidase staining was observed throughout the mutant disc (Fig. 3A). Moreover, *rpr* expression was observed in clones of *selD*^{ptuf} homozygous mutant cells in both the wing and eye imaginal discs (Fig. 3B,C). In cell cultures ROS can activate the tumor suppressor protein p53, a sensor of genotoxic stress (Yin et al., 1998; Kitamura et al., 1999; Buschmann et al., 2000). As a transcription factor, one of the critical roles of p53 is to regulate the expression of genes involved in eliminating damaged cells via apoptosis. Activation of *p53* occurs largely through posttranslational mechanisms that enhance its stability and DNA binding activity, making possible the transcription of its targets (Burns and El-Deiry, 1999; Sionov and Haupt, 1999). *Drosophila melanogaster* p53 protein (Dmp53) (Brodsky et al., 2000; Jin et al., 2000; Ollman et al., 2000) targets a radiation responsive enhancer at the *rpr* locus (Brodsky et al., 2000). It has been shown that targeted transcription of *rpr* rapidly causes widespread ectopic apoptosis (White et al., 1996). In fact, overexpression of *Dmp53* in the eye gives rise to viable adults that have small rough eyes as a result of massive apoptosis (Brodsky et al., 2000; Jin et al., 2000; Ollman et al., 2000). Nuclear stabilization of Dmp53, in cells of the eye disc overexpressing *Dmp53* under the *glass* (*gl*)-responsive enhancer elements (Moses and Rubin, 1991), can be detected with anti-Dmp53 antibody (Fig. 4A,B) (Ollman et al., 2000). Accordingly, we detected *rpr* transcription in *gl-Dmp53*-expressing cells, as shown by double staining with anti-Dmp53 and *rprlacZ* (Fig. 3D). We wondered whether *rpr* transcription observed in *selD*^{ptuf} mutant cells was associated with Dmp53. In *selD*^{ptuf} homozygous discs, widespread Dmp53 stabilization was detected with anti-Dmp53. This accumulation is characterized by a punctate pattern throughout the whole imaginal disc (Fig. 4C) and localized in the nuclei (Fig. 4D), which is in agreement with the function of p53 as a transcription factor.

In most cases, apoptotic cell death culminates in the activation of the caspase family of cysteine proteases, leading to the orderly dismantling and elimination of the cell. Caspases are central components of the apoptotic machinery induced by the pro-apoptotic genes. The *Drosophila* initiator caspase DRONC (Dorstyn et al., 1999) has been shown to be necessary in *rpr*-induced apoptosis (Hawkins et al., 2000; Meier et al.,

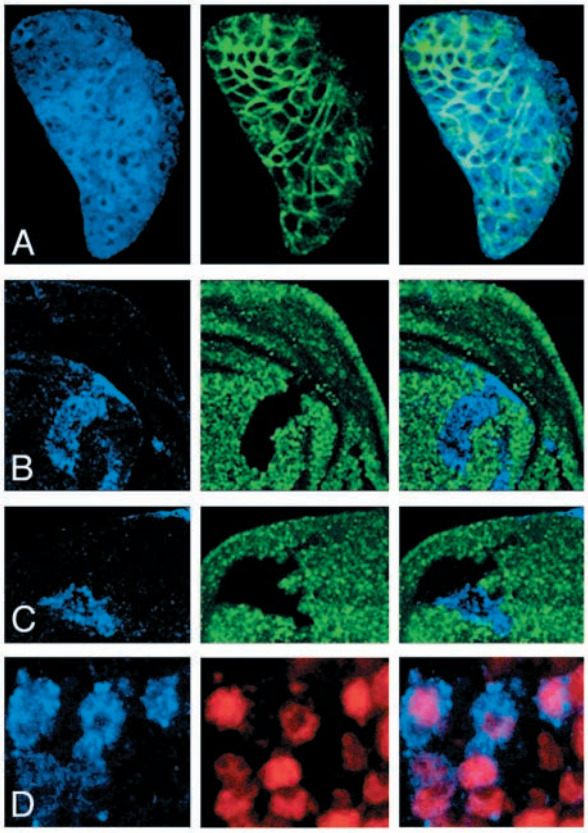

Fig. 3. Transcription pattern of *reaper* in *selD*^{ptuf} cells. (A) Widespread *rpr* transcription (blue) in a *selD*^{ptuf} homozygous mutant imaginal disc where the cell surface was outlined using anti-Fasciclin III antibody (green). (B,C) *rpr* transcription (blue) in *selD*^{ptuf} clones (area lacking the green π-Myc labeling) in the wing (B) and the eye (C) disc, respectively. (D) Detail of some cells of a *gl-Dmp53* eye disc showing Dmp53 stabilization (red) and *rpr* transcription (blue). Panels on the right correspond are merged images.

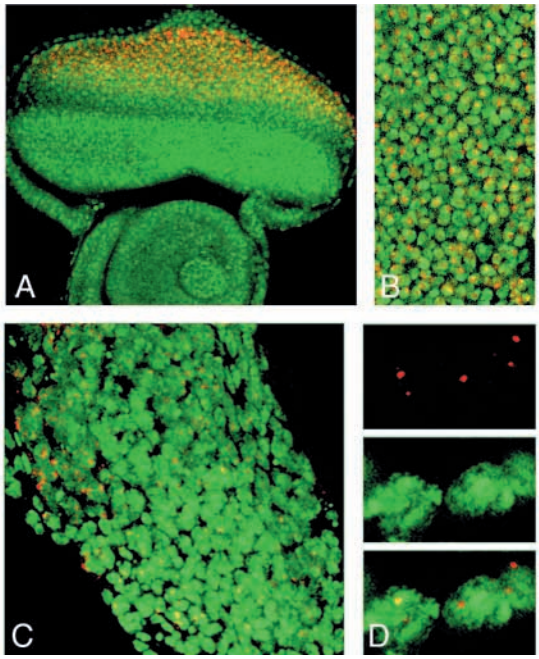

Fig. 4. Dmp53 stabilization in *selD*^{ptuf} cells. (A) Detection of Dmp53 (red) in an eye disc overexpressing *Dmp53* under *glass* enhancer sequences. (B) High magnification of nuclear confocal section counterstained with YOYO nuclear marker (green) showed co-localization of Dmp53 (red) into the nuclei. (C) Dmp53 accumulation (red) in a *selD*^{ptuf} homozygous mutant imaginal disc stained with YOYO. (D) Detail of a high magnification image focusing on two nuclei stained with YOYO nuclear marker (green, middle panel) showed co-localization of Dmp53 (red upper panel) into disrupted *selD*^{ptuf} nuclei. Lower panel shows merged images.

2000; Quinn et al., 2000). Likewise, DRONC induces cell death in a dose-dependent manner when ectopically expressed in the developing eye under the *GMR* promoter (Meier et al., 2000; Hawkins et al., 2000; Quinn et al., 2000). Adult flies have slightly rough and mottled eyes, because of ablation of photoreceptors and pigment cells (Fig. 5A) (Meier et al., 2000). To test the involvement of this caspase in ROS-induced apoptosis, we performed a series of crosses using the *selD*^{ptuf} mutant as well as a mutation in the *catalase* gene (*Cat*^{n1}) as an example of ROS production in a *selD/sps1*-independent mode. Reduction of one dose of *selD* enhanced the mottled-eye phenotype as deduced from the presence of wider areas of *white* tissue (Fig. 5B). The same result was obtained when one dose of *catalase* was removed, reinforcing the idea that an increase in ROS levels might be responsible for *selD*^{ptuf} apoptosis (Fig. 5C). Moreover, when removing one dose of both *catalase* and *selD* this phenotype became even more severe (Fig. 5D).

DRICE is an effector caspase essential for apoptosis in *Drosophila* cells activated by *rpr* overexpression (Fraser and Evan, 1997; Fraser et al., 1997). It has been shown to physically interact with, and to be processed by, the initiator caspase DRONC, thus being one of its downstream targets (Meier et al., 2000). We assessed the involvement of DRICE caspase in *selD*^{ptuf} apoptosis using the processed DRICE-specific antibody (Dorstyn et al., 2002) as a marker for active DRICE. In *selD*^{ptuf} homozygous mutant imaginal discs we found active DRICE in several cells (Fig. 5E). Accordingly, in *selD*^{ptuf} clones in the wing and eye imaginal discs we found DRICE in some of the mutant cells (Fig. 5F). Interestingly, we also found non autonomous labeling of DRICE, as shown by non-mutant cells adjacent to the mutant clone. This observation is in agreement with the previous observation that ROS and apoptotic cells can also be non autonomous. Importantly, we have also detected active DRICE in *gl-Dmp53* discs (Fig. 5G), which reinforces the idea that *selD*^{ptuf} apoptosis is mediated through a Dmp53/Rpr caspase-dependent pathway.

Since caspases promote and amplify proteolysis cascades, the inhibitor of apoptosis proteins (IAPs) provide a critical barrier to impede apoptosis through direct binding and inhibition of caspases (reviewed by Hay, 2000). Thus, apoptotic stimuli could induce cell death through degradation of IAPs. It has been shown that *rpr* can negatively regulate the levels of the *Drosophila IAP1* (*DIAP1*) to trigger apoptosis (Goyal et al., 2000; Holley et al., 2002; Yoo et al., 2002). The inhibition of DRONC and DRICE caspases by DIAP1 has also been extensively demonstrated (Kaiser et al., 1998; Meier et al., 2000; Hawkins et al., 2000; Quinn et al., 2000). To confirm

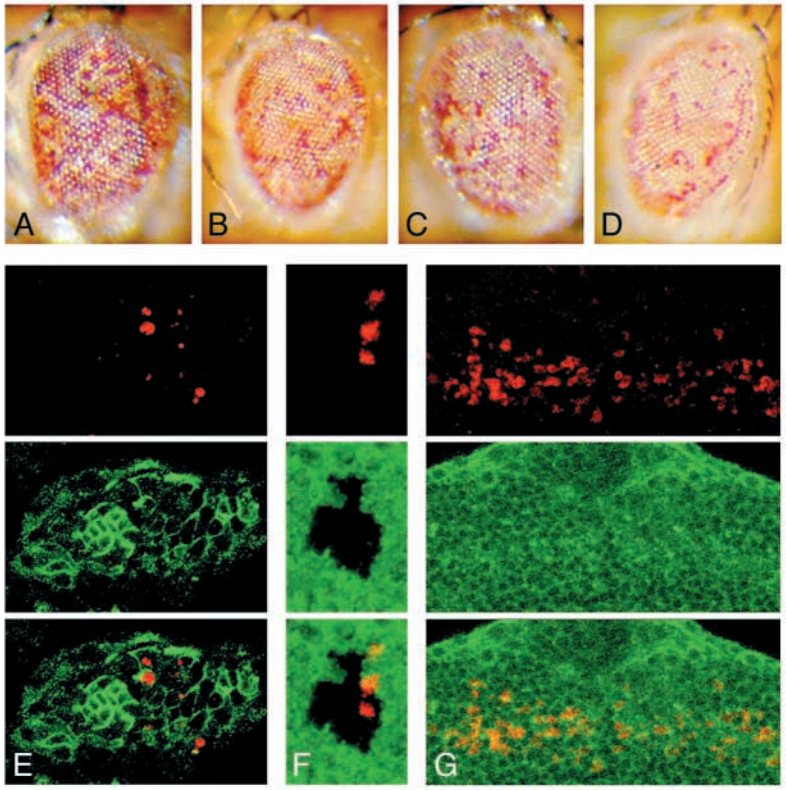

Fig. 5. DRONC and DRICE caspases are involved in *selD*^{ptuf} apoptosis. (A) Overexpression of DRONC caspase under the control of *GMR-GAL4* driver gives rise to slightly rough and mottled eyes as a result of ectopic cell death. *white* patches result from the ablation of pigment cells. (B,C) Reduction of one dose of *selD* or *catalase*, respectively, enhanced the mottled eye phenotype. Wider areas of *white* tissue were observed indicating that an increase in ROS levels enhances DRONC apoptotic activity. (D) Combined reduction of *selD* and *catalase* strongly enhanced DRONC overexpression phenotype and few pigmented cells were observed. (E) Active DRICE (red) in a *selD*^{ptuf} homozygous mutant imaginal disc. The cell surface was outlined using anti-Fasciclin III antibody (green). (F) Active DRICE in *selD*^{ptuf} clones (dark area lacking β-galactosidase labeling, green) in the eye disc. Consistent with the finding of non autonomous apoptosis and ROS, active DRICE was found as well outside the clone. (G) Overexpression of *Dmp53* triggers apoptosis through DRICE caspase as shown by the presence of active DRICE (red) in *gl-Dmp53* discs. In E, F and G lower panels are merged images.

that *selD*^{ptuf} apoptosis is caspase dependent, we misexpressed the *Drosophila IAP1* (*DIAP1*) to block caspase activity and test for viability rescue. The analysis was performed in the wing imaginal disc since divisions occur throughout the whole disc, whereas in the eye disc the pattern of cell divisions changes along the anterior-posterior axis which renders a statistical analysis difficult. By using *hh-Gal4* as a driver, *DIAP1* can be ectopically expressed in the posterior compartment, and the anterior compartment can act as a control in the same imaginal disc. We generated twin clones (i.e. wild-type control *selD*$^{+}$ clone and *selD*^{ptuf} mutant clone produced in the same recombination event) in both the anterior and posterior compartment of these wing discs. We next measured the size of mutant *selD*^{ptuf} clones and defined them as the ratio of their own area relative to the area of their twin control *selD*$^{+}$ clones and also compared the size of mutant clones in the anterior compartment to the size of mutant clones in the posterior compartment. We found that the size of *selD*^{ptuf} mutant clones in the posterior compartment is almost double that in the anterior compartment (Fig. 6). While the mean area of anterior mutant clones is 34% of the area of their wild-type twin clones, the mean area of posterior mutant clones is 58% of the area of their wild-type twin clones. Ectopic expression of DIAP1 robustly rescues viability and doubles the size of *selD*^{ptuf} mutant clones, which strongly supports the idea that *selD*^{ptuf} ROS-induced apoptosis is caspase dependent.

The main conclusion of the work presented here is that *selD*^{ptuf} cells generate a ROS oxidative stress that triggers a Dmp53/Rpr-mediated apoptosis. The increased levels of ROS, Dmp53 stabilization, *rpr* transcription and activation of caspases that can be inhibited by DIAP1, strongly hint at an important contribution of the Dmp53/Rpr caspase pathway in *selD*^{ptuf} apoptosis. However, when a cell is committed to die, several pro-apoptotic molecules may be activated to irreversibly execute an apoptotic program. For example, the Ras/MAPK down-regulation induced by *selD* (Morey et al., 2001) could contribute, to a lesser extend and beyond our detection levels, to apoptosis (Fig. 7).

Our results could be explained by an activation of the apoptotic machinery resulting from oxidative stress triggered by the absence of selenoproteins in the *selD*^{ptuf} cells. However, there are some observations that tempt us to propose that the fly and mammalian *selD/sps1* could have derived a novel function. There are only three selenoprotein genes described so far in the

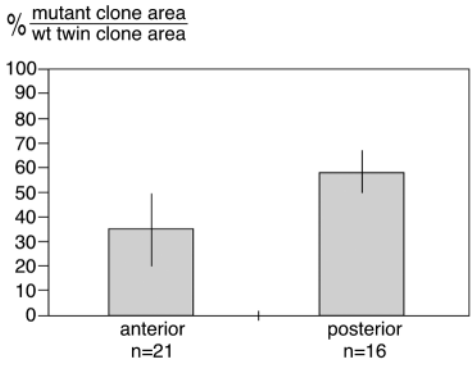

Fig. 6. Rescue of *selD*^{ptuf} clone size by DIAP1 overexpression. *selD*^{ptuf} clones were induced in the posterior DIAP1 expressing compartment and in the anterior compartment of the wing pouch. Bars represent mean values of the area of *selD*^{ptuf} clones as a percentage of the area of their wild-type *selD*$^{+}$ twin clone±s.d., *n*=number of mutant and wild-type twin clone pairs analyzed in the anterior and posterior compartment. Overexpression of DIAP1 in the posterior compartment almost doubled *selD*^{ptuf} clonal size. Mean area of anterior and posterior *selD*^{ptuf} clones, 34% and 58% of their wild-type twin clone respectively, were significantly different (*t*-test: $P<0.001$).

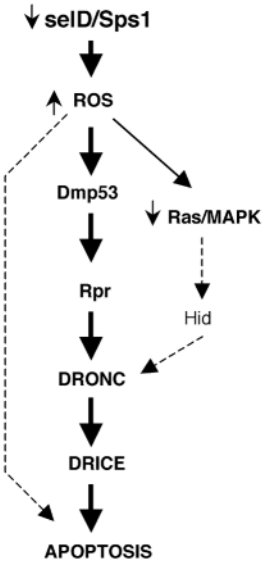

Fig. 7. A model for ROS-induced apoptosis in cells devoid of *selD/sps1*. We propose that the increase in ROS caused by the *selD^{ptuf}* mutation leads to stabilization of Dmp53 and activation of Rpr caspase-dependent apoptosis. We cannot discount the possibility that other pathways such Ras/MAPK downregulation or a ROS-induced caspase-independent pathway may have a minor contribution to *selD^{ptuf}* apoptosis.

fly genome: *selenophosphate synthetase type 2* (*sps2*) (Hirosawa-Takamori et al., 2000), and *dselM* and *dselG*, both of unknown function (Castellano et al., 2001; Martin-Romero et al., 2001). The presence of non-selenoprotein paralogs of *dselM* and *dselG*, makes it hard to account for the lethality of *selD^{ptuf}* by simply removing the selenoproteins. Every known selenoprotein in vertebrates that acts as a detoxifyer enzyme (carrying a UGA-coded selenocysteine in the active center) is not a selenoprotein in the fly (i.e. the selenocysteine is substituted by another amino acid). Taken together, it is unclear whether *Drosophila* selenoproteins are part of the oxidative stress defense system. In addition, the enzymatic activity of the two highly conserved eukarya *selD* gene products differs. The selenocysteine amino acid residue of Sps2 is essential for its selenophosphate synthetase activity in mammals (Kim et al., 1997), whereas the mammalian Sps1 only weakly complements an *E. coli selD* mutation (Low et al., 1995). Similarly, the fly Sps2 contains a selenocysteine in its catalitic domain (Hirosawa-Takamori et al., 2000), whereas the *Drosophila selD/sps1* does not complement a bacterial *selD* mutation (Persson et al., 1997). It is, therefore, possible that *selD/sps1* has a dual function: one mediated by selenoproteins, or involved in their biosynthesis as basal selenophosphate synthetase activity (Guimaraes et al., 1996), and a second ROS-related function, independent of selenoproteins, conserved in flies and mammals.

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