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The Drosophila Bcl-2 family protein Debcl is targeted to the proteasome by the β-TrCP homologue Slimb

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Abstract:

The ubiquitin-proteasome system is one of the main proteolytic pathways. It inhibits apoptosis by degrading pro-apoptotic regulators, such as caspases or the tumor suppressor p53. However, it also stimulates cell death by degrading pro-survival regulators, including IAPs. In *Drosophila*, the control of apoptosis by Bcl-2 family members is poorly documented. Using a genetic modifier screen designed to identify regulators of mammalian *bax*-induced apoptosis in *Drosophila,* we identified the ubiquitin activating enzyme Uba1 as a suppressor of *bax*-induced cell death. We then demonstrated that Uba1 also regulates apoptosis induced by Debcl, the only counterpart of Bax in *Drosophila*. Furthermore, we show that these apoptotic processes involve the same multimeric E3 ligase — an SCF complex consisting of three common subunits and a substrate-recognition variable subunit identified in these processes as the Slimb F-box protein. Thus, *Drosophila* Slimb, the homologue of β-TrCP targets Bax and Debcl to the proteasome. These new results shed light on a new aspect of the regulation of apoptosis in fruitfly that identifies the first regulation of a *Drosophila* member of the Bcl-2 family.

Introduction

The ubiquitin-proteasome system (UPS) is one of the main proteolytic pathways in the cell. Ubiquitination requires three specialized enzyme families. The first step of ubiquitination involves a ubiquitin activating enzyme called E1, which covalently binds a ubiquitin monomer. This activated ubiquitin is transferred to a ubiquitin conjugating enzyme, E2. A ubiquitin ligase (E3) then catalyzes the transfer of ubiquitin to a specific target. A broad diversity of E3 ligases exists, to cope with the very large number of substrates of the UPS. E3-dependent polyubiquitination of the substrate can lead to its recognition by the proteasome — a macromolecular complex responsible for the degradation of ubiquitin-tagged proteins into short peptides. The UPS controls many processes such as the cell cycle or apoptosis by targeting some of their main regulators.

In mammals, members of the Bcl-2 family are key elements of the apoptotic machinery (review : (1)). This multigenic family has about 20 members, which may either promote or inhibit apoptosis. Members of the Bcl-2 family control cell death either by inhibiting or promoting mitochondrial outer membrane permeabilization. This permeabilization is a key step in the execution of the mitochondrial apoptotic pathway in mammals, as it allows the release of apoptogenic factors, such as cytochrome c, from the mitochondrion into the cytosol. This cytochrome c release is essential for Apaf-1-mediated caspase-9 activation and cell breakdown.

One of the best described examples of the UPS regulation of Bcl-2 family members-induced cell death in mammals is provided by the degradation of the anti-apoptotic protein Mcl-1. Three E3 ligases have been shown to target Mcl-1 for degradation *via* two independent pathways. The first of these enzymes, Mule (Mcl-1 ubiquitin ligase E3) is a HECT domain-containing E3 ligase (2). It contains a BH3 domain, also found in members of the Bcl-2 family, which mediates the physical interaction between Mule and Mcl-1. Mcl-1 can also be downregulated by phosphorylation on two serine residues by GSK-3β (glycogen synthase kinase-3β). This hyperphosphorylation of Mcl-1 leads to its recognition by the F-box protein β-TrCP (3). β-TrCP acts within a multimeric RING domain-containing E3 ligase, called SCF complex. The F-box protein is a variable subunit of this complex responsible for substrate recognition, leading to ubiquitination by the SCF complex and subsequent degradation by the proteasome. More recenltly, it has been shown that Trim17-mediated ubiquitination and degradation of Mcl-1 initiate apoptosis in neurons (4). Other members of the mammalian Bcl-2 family are regulated in a proteasome-dependent manner. Such regulation has been demonstrated for anti-apoptotic proteins, such as Bcl-2 (5), and pro-apoptotic proteins, such as Bid (6) and Bim (7). However, the precise mechanisms underlying this regulation, including the post-translational modifications targeting them for UPS-mediated degradation, and the identity of the E3 ubiquitin ligases involved remain unclear.

Bax, a multidomain pro-apoptotic member of the Bcl2 family, is a target of the UPS (8, 9). Some evidence suggest that the signal for Bax degradation could be the Akt-mediated phosphorylation of serine 184 (10-13) and that the DNA repair protein Ku70 could be involved in Bax deubiquitination (14). However, the ubiquitin ligase responsible for Bax targeting to the proteasome as well as the regulation of this degradation pathway remain to be characterized.

Only two members of the Bcl-2 family have been identified in Drosophila so far, Buffy and Debcl. Both display a mitochondrial subcellular localization and are apoptotic regulators, suggesting that mitochondria play an important role in apoptosis in Drosophila. Buffy was originally described as an anti-apoptotic Bcl-2 family member (15, 16), but in some cases it can promote cell death (17-20). Debcl (death executioner Bcl 2 homolog), is a multidomain death inducer (20-24) that can be inhibited by direct physical interaction with Buffy (15). When overexpressed in mammalian cells, Debcl induces both cytochrome c release from mitochondria and apoptosis. This protein interacts physically with anti-apoptotic members of the Bcl 2 family, such as Bcl 2 itself, in mammals. Debcl is involved in the control of some developmental cell death processes as well as in the irradiation response (16, 19, 25).

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Most apoptotic processes in *Drosophila* involve the initiator caspase Dronc and its adaptor Dark (26). Dronc activation is tightly controlled by Diap1. Indeed, loss-of-function mutations in *diap1* are often sufficient to induce apoptosis (27-29). Diap1 has been widely studied and acts as an E3 ubiquitin ligase, targeting caspases, including Dronc, for proteasomal degradation. Diap1 is itself targeted for proteasomal degradation in response to pro-apoptotic RHG (Rpr, Hid, Grim) protein signaling. Therefore, the UPS plays a key role in regulating apoptosis in *Drosophila* by acting on caspases, IAPs or RHG proteins. However, no data are available concerning the degradation of *Drosophila* Bcl-2 family members by the proteasome and the mechanisms of action and regulation of Debcl and Buffy remain poorly documented as compared to our knowledge of their mammalian counterparts (30).

Therefore, we investigated the roles of Bcl-2 family members in *Drosophila*, using transgenic flies expressing the murine *bax* gene under control of the inducible UAS/Gal4 system (31). We have previously shown that mammalian Bax is functional in *Drosophila* where it interacts with the cell death machinery to induce an apoptosis involving mitochondrial events (32, 33). We have also shown that *bax*-induced apoptosis can be antagonized in *Drosophila* by overexpressing mammalian *bcl-2* (31, 32). Studies of *debcl* demonstrated that both *debcl*- and *bax*-induced cell death share common features. Indeed, the mitochondrion stands as a key component of *debcl*- and *bax*-induced apoptosis and both trigger two different cell death pathways. The first one is effector caspase-dependent while the second appears to resist p35 inhibition, suggesting the existence of an effector caspase-independent pathway. *Bax*- and *debcl*-induced apoptosis in the wing disc result in a wing phenotype and partially penetrant fly lethality. To identify suppressors of *bax*- and *debcl*-induced cell death, we screened a library of gain-of-function mutants (34) for modifiers of these phenotypes (Colin *et al.*, in preparation). One of the suppressors identified this way consists in a mutation of the gene encoding the unique ubiquitin activating enzyme, *Uba1*. Studies of this mutant provided insight into the proteasome-dependent regulation of Bcl-2 family members in *Drosophila*. We show here that Bax and its counterpart Debcl are degraded by the proteasome in *Drosophila*. We identified the E3 ubiquitin ligase Slimb (Slmb), the *Drosophila* homologue of β-TrCP, as the regulator of Debcl levels.

Materials and methods

Fly stocks

Flies were raised on standard medium at 25°C, except those used for phenotypic suppression tests involving *vg>bax* flies, which were performed at 18°C. *UAS-bax* flies were generated in our laboratory. The *yw^c*, *w^1118*, *vg-gal4*, *hs-gal4* and *da-gal4* strains are described in (31, 32). *ptc-gal4* flies were provided by L. Théodore. *UAS-debcl-HA* flies were generously provided by H. Richardson (35). The *slmb* mutants *slmb^8* and *slmb^41* were generously provided by B. Limbourg-Bouchon (36). The *UAS-slmb^DN-flag-myc* flies were generously provided by T. Murphy. In this strain, the UAS-slmb^DN transgene expresses dominant-negative Slmb protein containing five point mutations in the F-box domain that completely abolish interaction with SkpA, and includes a 3xFLAG-6xmyc N-terminal epitope made with the pTFMW vector (Drosophila Genomics Resource Center; T.D. Murphy, personal communication). *Uba1^UY3010* was generated by mobilizing *P[Mae-UAS.6.11]* (*P[UY]*). *Uba1^EP2375*, *morgue^EP1184* and *pros25^EP931* were obtained from Szeged Stock Center. *Uba1^s3484* and *skpA^EP1423* were obtained from Bloomington Stock Center. The *Δp54* mutant described by T. Szlanka (37) was generously provided by A. Udvardy. *UAS-PABP-flag* flies (#9420) were obtained from Boomington.

Wilcoxon test

vg>bax, *vg>rpr* and *ptc>(debcl)₂* virgins were crossed, in parallel, with the tested mutant and a control strain. We have previously verified that the presence of a *UAS-GFP* transgene does not affect *bax*- or *debcl*-induced phenotypes (data not shown). Therefore, there is no detectable GAL4 titration in our system. Thus, according to the genetic background of the tested mutant strains, control experiments were performed by crossing *vg>bax* or *ptc>Debcl* females with w^1118 males, except for the UY^3010 mutant strain for which ywc males were used. The progenies of each of these crosses were classified, according to the strength of the wing phenotype, into four phenotypic categories (from wild-type to strong phenotype) by the same blind observer to avoid changes in categorization. A Wilcoxon test was then used to compare the distributions of phenotypes between the two lineages (T: test *versus* C: control). Differences in distribution were considered significant if α < 10^{-3}. A positive Ws value indicates enhancement of the phenotype and a negative Ws value indicates suppression of the phenotype.

Identification of the *P*[UY] insertion in the *UY3010* mutant

For identification of the gene affected in the *UY3010* mutant, we used inverse PCR and sequencing of the DNA flanking the *P*[UY] element. Inverse-PCR was carried out as in the BDGP protocol (http://www.fruitfly.org/about/methods/inverse.pcr.html). We used genomic DNA from young UY3010 males and MspI for DNA digestions. The following primers were used: 5'-GCTTTCGCTTAGCGACGTGT-3', 5'-CAGATCGTAAGGGTTAATGTT-3', 5'-GAATTGAATTGTCGCTCCGT-3', and 5'-CTCTCAACAAGCAAACGTGC-3'.

Immunostaining

Wing discs were dissected from *vg>bax* or *vg>bax/Uba1*^{EP2375} third-instar larvae in PBS, pH 7.6 and fixed in 3.7% formaldehyde. Discs were labeled by incubation for 2 hours with anti-Bax Δ21 antibody (1/200) in 0.3% Triton X-100/10% NGS in PBS (PBT/NGS). After washing in 0.3% Triton X-100 in PBS, TRITC-conjugate was incubated for 1 hour in PBT/NGS. Images were captured with a conventional Leica DMRHC research microscope, using an N2.1 filter to detect TRITC fluorescence. The same exposure time was used for all images.

Immunoprecipitation

Two hundred embryos were collected, dechorionated in 100% sodium hypochlorite and rinsed in PBS. Embryos were then collected by centrifugation and washed once in PBS. Pellets were resuspended in 270 μl of ice-cold CHAPS buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 1% CHAPS, 1 mM DTT, Pefabloc) and homogenized on ice with a Wheaton glass homogenizer (until the suspension became milky). For control immunoprecipitation, 75 μl of lysate was incubated 4 hours at 4°C with 3 μg of IgG1 before the addition of 15 μl of protein G-agarose beads overnight at 4°C. For anti-Slmb immunoprecipitation, 75 μl of lysate was incubated overnight at 4°C with 5 μl of anti-FLAG M2 agarose beads (Sigma Aldrich). Immune complexes were then washed four times with ice-cold CHAPS buffer and resuspended in loading buffer. Samples were boiled for 5 min. and proteins were subjected to western blotting as described below.

Western blot

Western blotting was carried out with the INVITROGEN NuPAGE^{TM} system according to the manufacturer's instructions, with 4 to 12% Bis-Tris Gels and MES running buffer (50 mM MES, 50 mM Tris base, 0.1% SDS, 1 mM EDTA, pH 7.3). Proteins were electrotransferred onto PVDF membrane (Immobilon™-P, MILLIPORE) in transfer buffer (25 mM Bicine, 25 mM Bis-Tris , 1 mM EDTA, pH 7.2). Transfer efficiency was evaluated with Ponceau-S (0.3% Ponceau-S, 1% acetic acid). Membranes were saturated by incubation for one hour with skim milk before antibody staining.

Antibodies

Immunostaining was performed with anti-Bax Δ21 (Santa Cruz, 1/200) and TRITC-conjugate anti-rabbit (Jackson, 1/200) antibodies. Western blots were probed with anti-Bax 5B7 or N20 (Santa Cruz, 1/500 or 1/200 respectively), anti-HA (Babco, 1/1000), and anti-tubulin E7 (DSHB, 1/1000) antibodies. HRP-conjugate anti-mouse or anti-rabbit antibodies were obtained from Jackson Laboratories and used at a dilution of 1/10000. Immunodetection was performed by a chemoluminescence-based method (ECL, Amersham).

In Situ Proximity Ligation Assay (PLA)

Ptc>Debcl-HA,Slmb^{DN}*-flag-myc* and *Ptc>Debcl-HA,Pabp-flag* third instar larvae were dissected in PBS pH 7.6, fixed in PBS/formaldehyde 3.7%, washed three times for 5 min in PBT (PBS, 0.3% Triton). Wing imaginal discs were then dissected and incubated in PBT/FCS (PBS, 0,3% Triton, 10% FCS) at room temperature during 20 min. *In situ* PLA was performed using the Duolink® kit (Olink Bioscience) essentially according to manufacturer's instructions. Briefly, wing imaginal discs were stained with primary antibodies overnight at 4°C. The antibodies used for Duolink assays and their corresponding dilutions are: anti-HA antibody (ab9110, abcam®, 1/200) and anti-flag.M2 antibody (Agilent, 1/200). After washing, wing imaginal discs were incubated with the secondary oligonucleotide-linked antibodies (PLA probes: anti-Mouse PLUS and anti-Rabbit MINUS) provided in the kit. The oligonucleotides bound to the antibodies were hybridized, ligated, amplified, and detected using a fluorescent probe (Detection Kit 563). Discs were mounted in CitifluorTM (Biovalley) and observed with a Leica SP2 upright confocal microscope.

Results

Isolation of the *UY3010* mutant

The expression of mammalian *bax* during development using the *vg-gal4* driver — which targets expression to the wing margin tissue — led to an apoptosis-induced variable notch phenotype in the adult wing (figure 1A-D and (31, 32)). Moreover, this excess of apoptosis led to an increase in fly lethality. We used these phenotypes to screen the mutants of the *P*[UY] collection (34), which present a random insertion of a UAS-containing *P*-element. Out of the 1475 tested mutants, only 17 modifier genes suppressing *bax*-induced apoptosis were identified, showing how stringent our screen was. One of the suppressors we identified was the *UY3010* mutation. This mutation attenuated both the wing phenotype and lethality due to *bax* expression and did not display any phenotype in absence of *bax* when driven by *vg-gal4* or *ptc-gal4* (data not shown). We carried out a phenotypic suppression test, validated by the statistical Wilcoxon test (see Materials & Methods) to confirm the suppressor effect of this mutation. The result of this phenotypic suppression test is presented figure 1E. In the control cross offspring (*Uba1^{UY3010}*C), expressing only *bax*, only 3.4% of the progeny displayed a weak phenotype. The percentage of weak phenotypes increased to 41% of the progeny in the test cross (*Uba1^{UY3010}*T), in which flies expressed *bax* in presence of the *UY3010* mutation. The Wilcoxon test gave a probability of 5×10^{-15} for this shift, which is highly significant ($\leq 10^{-3}$). We conclude that the *vg-gal4*-mediated induction of the *UY3010* mutation significantly suppresses the *bax*-induced phenotype.

Characterization of the *Uba1^{UY3010}* allele

We carried out inverse PCR on genomic DNA from *UY3010* flies to identify the genes flanking the *UY3010* insertion. We sequenced the PCR products and localized the *P*[UY] insertion to the 5'UTR region of the E1 ubiquitin activating enzyme gene, *Uba1*. The *P*[UY] was found to be inserted 258 bp upstream from the translation initiation codon and 554 bp downstream from the transcription start site in an orientation compatible with *Uba1* overexpression in the *UY3010* mutant in the presence of *gal4* (supplementary figure 1).

We checked whether *gal4* induction led to *Uba1* overexpression in the *UY3010* mutant, using the *hs-gal4* driver to trigger the *UAS*-dependent transcription of *Uba1* (supplementary figure 2). As expected, *Uba1* transcript levels present a 40 fold increase in heat shocked *hs>Uba1^{UY3010}* flies compared with *Uba1^{UY3010}* or wild type flies, showing that the *UY3010* mutation leads to UAS/Gal4-dependent *Uba1* overexpression.

We investigated whether this overexpression led to synthesis of a functional form of the Uba1 protein. Thus, we tested its ability to complement the null allele *Uba1^{s3484}*. The progeny of a genetic cross between flies carrying the *UY3010* mutation and *Uba1^{s3484}* flies, did not count any *Uba1^{s3484}/Uba1^{UY3010}* flies (table 1), showing that in the absence of Gal4 the UY3010 allele is a loss of function. In contrast, ubiquitous induction of *Uba1* using the *da-gal4* driver rescued heterozygous *Uba1^{s3484}/Uba1^{UY3010}* flies. Besides, we observed that decreasing Uba1 levels thanks to a background heterozygous for the *Uba1^{s3484}* null allele failed to modify *bax*-induced phenotypes (data not shown). Altogether, these data demonstrate that *UY3010*-mediated suppression of *bax*-induced phenotypes is due to overexpression of a functional form of Uba1.

Uba1 overexpression abolishes *bax*-induced apoptosis

The *UY3010* mutant was isolated based on its ability to suppress *bax*-induced phenotypes. We tried to reproduce this suppression by overexpressing *Uba1* with another allele reported to overexpress *Uba1* in a *gal4*-dependent manner, the *Uba1^{EP2375}* allele (38). This allele presents a *UAS*-containing *P*-element different from *P*[UY] but inserted in the same region. We verified that driving expression of *Uba1^{EP2375}* using *vg-gal4* or *ptc-gal4* did not lead to any wing phenotype. The *Uba1^{EP2375}* allele allowed a much stronger suppression of the *bax*-induced phenotype than *Uba1^{UY3010}*. The *Uba1^{UY3010}* allele gave weak phenotypes in about 40% of cases, but never gave rise to wild-type wings or wings with only a very small notch. By contrast, as shown in figure 2A, B and C, *Uba1^{EP2375}* gave mostly very weak phenotypes (wings with only a very small notch in the posterior region of the wing margin) and wild-type wings. Thus, *Uba1* overexpression from the *Uba1^{EP2375}* allele abolished the notched wing phenotype almost totally. This stronger suppression indicates that this allele may produce more Uba1 protein than *Uba1^{UY3010}*.

As previously described, *bax* expression induced apoptosis ==and an alteration of wing disc morphology== (figure 2D-E and (32)). To test whether Uba1 suppression of Bax-induced wing phenotypes is associated to a decrease of cell death, we stained apoptotic cells with acridine orange (AO) in third instar larvae imaginal discs. The number of apoptotic cells was strongly reduced when *Uba1* was co-expressed with *bax* (figure 2F-G).

We chose to express *bax* in the wing disc from the *vg-gal4* driver, because Bax is such a potent death inducer that most *gal4* drivers induce massive apoptosis leading to the embryonic death as observed with the ubiquitous *da-gal4* driver (31, 32). As *Uba1* overexpression efficiently suppresses *bax*-induced phenotypes, we tried to prevent *bax*-induced lethality by overexpressing the *Uba1^{EP2375}* allele with the *da-gal4* driver. This resulted in the survival of part of the offspring despite the ubiquitous overexpression of *bax* (data not shown).

Thus, *Uba1* overexpression inhibits the massive wave of apoptosis induced by *bax,* allowing embryos to develop to adulthood.

Uba1 overexpression suppresses *debcl*-induced cell death

Little is known about Debcl regulation in fruit flies and we decided to investigate whether *debcl*-induced cell death was also sensitive to *Uba1* overexpression. We carried out a phenotypic suppression test with *debcl*-expressing flies. Ectopic *debcl* expression in the wing disc with the *ptc-gal4* driver induced apoptosis along the antero-posterior frontier (supplementary figure 3). In adult wings, this apoptosis led to the L3 and L4 veins being brought closer together in the proximal region of the wing and the fusion of these veins at the anterior crossvein (figure 3B-B'). $Uba1^{EP2375}$ allele overexpression almost completely abolished this phenotype (figure 3C-C') and increased the percentage of wild-type phenotypes from 0 to 80% (figure 3H).

$Uba1^{UY3010}$ expression did not significantly suppress *debcl*-induced phenotypes (α=1.8x10^{-3}). However, $Uba1^{EP2375}$ gave efficient phenotypic suppression of *debcl*. The difference in the results obtained with these two alleles could lie in the Uba1 protein overproduction capacity of the two constructs. However, RT-qPCR did not reveal any significant difference between the *Uba1* mRNA levels observed in $Uba1^{UY3010}$ and $Uba1^{EP2375}$ strains (data not shown), possibly as the consequence of a limited number of cells expressing Gal4 in the imaginal disc when using a *ptc* driver. Nevertheless, these results show that *Uba1* overexpression inhibits the apoptotic processes induced both by Bax or Debcl.

Proteasome activity modulates *bax*- and *debcl*-induced apoptosis

We have shown that *Uba1* overexpression inhibits the apoptosis induced by *bax* or *debcl*. Therefore, we investigated the possible role of the proteasome in the regulation of *bax*- and *debcl*-induced cell death, by modifying proteasome activity and assessing the effects of these modifications on apoptotic processes. We carried out phenotypic suppression tests, using mutants with defective proteasomal subunits. We chose to use the $\Delta p54$ mutant, as this mutant has been well characterized (37) and displays no wing phenotype. It carries a deletion of the *p54/S5a/Rpn10* gene*,* which encodes a subunit of the 19S regulatory particle of the proteasome. This subunit links the base and lid subcomplexes of the 19S regulatory particle. It also acts as a "receptor" for the 26S proteasome by interacting with polyubiquitin chains. Szlanka *et al.* have shown that deletion of *p54* in *Drosophila* activates a regulatory feedback loop, leading to 26S proteasome overexpression (37). We used this mutant to increase proteasomal activity with this mutant. We also used the $pros25^{EP931}$ loss of function allele that carries a mutation affecting negatively the $\alpha 2$ subunit of the 20S core particle of the proteasome and does not lead by itself to any phenotype when driven by *vg-gal4* or *ptc-gal4*. We assessed the effects of these mutations on proteasome activity, by western blot analysis of the degradation of Armadillo, a known target of the proteasome (Supplementary figure 4). As expected, the $\Delta p54$ mutant stimulated the proteasomal activity whereas the $pros25^{EP931}$ allele inhibited proteasomal activity (39).

The $\Delta p54$ mutation shifted the distribution of *bax*-induced phenotypes toward weaker phenotypes (figure 4, compare $\Delta p54$C and $\Delta p54$T). Indeed, the progeny of the control cross included 49.5% strong phenotypes and only 0.5% weak phenotypes, whereas crosses with the $\Delta p54$ mutant gave 4.9% strong phenotypes and 45.7% weak phenotypes. By contrast, $pros25^{EP931}$ shifts the phenotypic distribution toward stronger phenotypes, doubling the percentage of strong phenotypes with respect to the control cross (figure 4 compare $pros25^{EP931}$ C and T). We can therefore conclude that enhancing the proteasomal activity suppresses *bax*-induced apoptosis, whereas turning it down increases the rate of *bax*-induced apoptosis.

We then performed the same experiments with *debcl*-expressing flies. As for *bax*-induced cell death, the $\Delta p54$ mutation was found to suppress *debcl*-induced apoptosis (figure 5, $\Delta p54$C and T) whereas the $pros25^{EP931}$ mutation enhanced this process (figure 5, $pros25^{EP931}$C and T, shows a shift from 31.4% strong phenotypes to 82.7%). The suppression of the *debcl*-induced phenotype by $\Delta p54$ is shown in figure 3 D-D' and the enhancement of this phenotype by $pros25^{EP931}$ in figure 3 E-E'. Thus, the apoptotic processes induced by Bax and Debcl are regulated by proteasomal degradation. Moreover, as increasing the proteasomal activity suppresses the apoptosis induced by these two proteins whereas decreasing it enhances cell death, the regulator degraded in these processes is presumably a pro-apoptotic molecule.

The Bax and Debcl proteins are degraded by the proteasome

Some members of the Bcl-2 family in mammals are subject to proteasome-mediated regulation. We therefore hypothesized that Bax and Debcl proteins were the pro-apoptotic regulators degraded by the proteasome in our system. To test whether the modulation of Bax- and Debcl-induced cell death involves a direct effect on the level of these proteins, we performed immunostaining and western-blot experiments.

First, wing discs of *vg>bax* third-instar larvae were labeled with an anti-Bax antibody (figure 6A, *vg>bax*) revealing a strong staining in the region in which *vg* drove *gal4* expression (right part of figure 6A, *i.e.* *vg-gal4*-mediated expression of GFP). High-intensity foci correspond to cells overexpressing *bax*. The same

immunolabeling was performed on *vg>bax, Uba1^{EP2375}* wing discs, which coexpress *bax* and *Uba1*. Staining of Bax in *Uba1^{EP2375}*-expressing discs was weaker, with fewer foci of *bax* overexpressing cells (figure 6A, *vg>bax, Uba1^{EP2375}*). This suggests that Bax protein levels are lower in discs overexpressing *Uba1*. To confirm that the decrease in immunodetection of Bax, when *Uba1* is overexpressed, is due to a decrease in Bax levels and not a loss of epitope accessibility, we carried out a western blotting analysis.

To this purpose, we expressed *bax* or *debcl* ubiquitously in embryos, using the *da-gal4* driver with or without overexpressing *Uba1* from the *Uba1^{EP2375}* allele. We then carried out western blotting to determine the amounts of Bax or Debcl in embryo lysates. When *Uba1* was overexpressed (figure 6B, *da>bax, Uba1^{EP2375}*), Bax became undetectable, even after long exposure times, whereas this protein was readily detectable in the control extract (figure 6B, *da>bax*, control). Similarly, as shown figure 6C, *Uba1* overexpression resulted in a strong decrease in Debcl protein levels, as a longer exposure time was required to detect Debcl (data not shown). Moreover, although the amount of Debcl was decreased by *Uba1* overexpression, Uba1 had no effect on the relative distribution of Debcl between cytosol and mitochondria. Debcl remained mainly detected in the mitochondrial fraction in the presence of *Uba1* overexpression (data not shown). We can therefore conclude that the ubiquitin-mediated regulation of Bax- and Debcl-induced cell death depended on a decrease in the level of Bax and Debcl proteins themselves.

The apoptotic processes induced by Bax and Debcl are regulated by an SCF^{Slmb} complex

As E3 ubiquitin ligases confer its specificity to the ubiquitination process, we tried to identify the E3 responsible for the regulation of *bax*- and *debcl*-induced cell death. Many signalization pathways involve SCF complexes. Thus, we first affected a common component to all SCF complexes SkpA. Overexpression of *skpA* using the *skpA^{EP1423}* allele under control of *vg-gal4* did not affect wing phenotypes. We thus overexpressed this allele in a *vg>bax* background. The progeny overexpressing both *bax* and *skpA*, displayed suppression of the *bax*-induced phenotype. The *skpA^{EP1423}* allele shifted the phenotype distribution toward weaker phenotypes (figure 4 *skpA^{EP1423}* C and T).

slmb (*supernumerary limb*) encodes an F-box protein homologous to mammalian β-TrCP, which is involved in Mcl-1 recognition in mammals. Slmb contains two functional domains: the F-box domain in the N-terminal half of the protein allowing assembling into the SCF complex through interaction with SkpA, and a C-terminal domain containing WD40 repeats, allowing interaction with substrates. We used *slmb^8* -a null allele of *slmb* which is viable at 18°C (*i.e.* the temperature used for the *bax*-induced cell death phenotypic suppression tests)- and *slmb^{41}* -a hypomorphic allele- to decrease the amount of Slmb (36). The *slmb^8* loss-of-function allele strongly enhanced the *bax*-induced apoptosis-associated phenotype (figure 4 *slmb^8* C and T). Indeed, strong phenotypes were significantly more frequent among the *slmb^8* mutants than in the absence of this mutation. The *slmb^{41}* allele also enhanced the *bax*-induced phenotype, although to a lesser extent (figure 4 *slmb^{41}* C and T). Thus, Slmb most likely targets a pro-apoptotic regulator to proteasomal degradation in our system.

To investigate whether the same SCF^{Slmb} E3 ligase was involved in regulating *debcl*-induced cell death, we carried out the phenotypic test with *debcl*-expressing flies. We tested the effect of a loss-of-function of *slmb* on *debcl*-induced cell death as it is the specific component of the SCF complex. The *slmb^8* mutant is partially lethal at 25°C (*i.e.* the temperature used for the *debcl*-induced cell death phenotypic suppression tests). We thus used the *slmb^{41}* allele for these tests and observed higher levels of *debcl*-induced cell death. A shift in the phenotype distribution from 5% (*slmb^{41}* C) to 50% of strong phenotypes (*slmb^{41}* T) was observed (figure 5). This enhancement was characterized by an increase in size of the fusion of the L3 and L4 veins in the region of the anterior crossvein (figure 3 F-F'). Therefore the SCF^{Slmb} E3 ubiquitin ligase is a negative regulator of *bax*- and *debcl*-induced apoptosis.

Finally, as a control, we investigated whether *morgue* affected *bax*- and *debcl*-induced apoptosis. Like Slmb, Morgue is an F-box protein but it is involved in the regulation of apoptosis through the targeting of Diap1 for proteasomal degradation. The hypomorphic *morgue^{EP1184}* allele (40) had no effect on the phenotype induced by Bax or Debcl, as shown figure 3 G-G' for the *debcl*-induced crossvein phenotype. As indicated in figures 4 and 5, no significant change in phenotype distribution was observed in either case (α values of 7×10^{-3} for *bax* and 2.4×10^{-2} for *debcl*). We also verified that this allele did not affect fly phenotypes in presence of *vg-gal4* or *ptc-gal4* drivers alone. The lack of effect of *morgue* suggests that Morgue is not the F-box protein involved in our system. Hence, the effects observed with *slmb* alleles reflect specific Diap1-independent regulation.

Given that Debcl is sensitive to the proteasome activity and that a mutation of *slmb* enhances the *debcl*-induced cell death, we speculated that Debcl physically interacts with Slmb. We performed two types of experiments with the aim of characterizing this physical interaction. In agreement with genetic data, Debcl co-immunoprecipitates with Slmb (figure 7A). We assessed the occurrence of this physical interaction *in vivo* by using a proximity ligation *in situ* assay (figure 7B). Red dots are specifically observed in *Ptc>Debcl-HA,Slmb^{DN}-flag-myc* wing discs, indicating that Debcl and Slmb colocalize within 40 nm. This approach confirmed that the two proteins can interact with each other *in vivo*. Together with our genetic data, these results show that a SCF^{Slmb} E3 ubiquitin ligase is involved in Debcl ubiquitination and subsequent proteasomal degradation.

Discussion

In this paper, using *Drosophila* as a model for genetic studies, we report the regulation of *bax*- and *debcl*-induced apoptosis by the ubiquitin-proteasome pathway. The stimulation of this pathway by overexpressing *Uba1*, which encodes the ubiquitin activating enzyme, leads to an almost complete loss of *bax*-induced cell death. This regulation seems conserved through evolution, as *debcl*-induced apoptosis is also regulated in this way. However, since Bax seems to necessitate Debcl in order to kill *Drosophila* eye cells (25), one could wonder whether suppression of Bax-induced cell death depends on the direct effect of *Uba1* on Debcl. Nevertheless, since we have shown that both Bax and Debcl proteins are degraded when *Uba1* is overexpressed, this seems unlikely unless Debcl stabilizes Bax.

Since Buffy inhibits autophagy in response to starvation (41), we can hypothesize that Debcl could induce an autophagic cell death. We have monitored autophagy in our system by using a UAS-Atg8-GFP transgene (42) and found that actually no autophagy could be detected upon Debcl expression (data not shown). Uba1 has been shown in the literature to be required for autophagy and reduction of cell size in the intestine (43). We show here that Debcl-induced cell death in the wing disc is not only suppressed by Uba1 but also by proteasome mutants. These data suggest that the UPS pathway is the main proteolytic pathway involved in the suppression of Bax and Debcl-induced apoptosis by Uba1. However, given that *slmb* has been shown to regulate Wg and Dpp pathways (36, 44-46), we cannot exclude that these pathways are partially involved in phenotypic suppression.

Studies of the proteasome-dependent regulation of members of the Bcl-2 family in mammals have only rarely led to the identification of the specific E3 ligases. We identified an SCF^{Slmb} complex as the E3 ubiquitin ligase that regulates the Debcl pathway and may target it to proteasomal degradation. It would be interesting to determine whether the mammalian homologue of Slmb, β-TrCP, targets Bax to the proteasome in mammalian cells.

Here, we proved Debcl to be a target of the UPS, finding a new regulation of apoptosis that differs from the control of Dronc, Drice and RHG protein levels by Diap1. Indeed, Diap1 is a key enzyme that decides of cell fate by degrading either pro-apoptotic regulators or itself, leading to either cell survival or apoptosis. Since Diap1 levels are downregulated by the F-box protein Morgue in presence of Rpr or Grim, we could hypothesize that the Morgue/Diap1 pathway is involved in *bax*- and *debcl*-induced apoptosis regulation. This does not seem to be the case because a hypomorphic allele of *morgue* did not increase *bax*- and *debcl*-induced apoptosis (data not shown). Furthermore, overexpression of *diap1* does not inhibit *bax*- and *debcl*-induced apoptosis (data not shown). Thus, the proteasome-dependent regulation we identified is independent of Diap1 and differs from the Morgue/Diap1 regulation of cell death. The existence of different UPS-modulated cell death pathways is also supported by the reported absence of genetic interaction between RHG pathway components and Debcl (25).

Our results indicate an anti-apoptotic role of *Uba1* in the wing tissue. In contrast, two studies revealed a pro-apoptotic role of *Uba1* in the eye; strong *Uba1* loss-of-function alleles lead to apoptosis and compensatory proliferation in the developing eye (47, 48). As previously shown in other systems, these processes seem to involve the RHG/Diap1/Dronc pathway (49, 50). Hypomorphic alleles of *Uba1* have shown opposite effects as they suppressed *hid*- or *grim*-induced apoptosis in the eye. These results are consistent with previous data indicating that *Uba1* overexpression, using the *Uba1^{EP2375}* allele, increases RHG-induced apoptosis. In principle, this apparent contradiction may result from either the cell death signal or the studied tissue. The use of a GMR-Gal4 driver shows that *Uba1* overexpression also inhibits *debcl*-induced apoptosis in the eye tissue (data not shown), which suggests that the Uba1 effect is specific of *debcl*-induced apoptosis. In contrast, *rpr*-induced cell death is enhanced by *Uba1* overexpression in the eye, whereas it is suppressed by Uba1 in the wing (data not shown). These results suggest that Debcl could be involved in *rpr*-induced cell death in the wing but not in the eye. RHG proteins are known to mediate their pro-apoptotic function by stimulating Diap1 degradation by the UPS while Debcl is a direct target of ubiquitination. Therefore, RHG-induced degradation of Diap1 through its ubiquitination by Morgue could explain the pro-apoptotic role of Uba1 in the eye whereas the anti-apoptotic Uba1 function mediated by Slmb in Debcl-induced cell death would rely on Debcl degradation. By showing that different pro-apoptotic pathways are regulated by the UPS in *Drosophila*, our work suggests that the tissue-dependent effect of the pleiotropic enzyme Uba1 must result from a change in the balance between UPS pro- and anti-apoptotic effects. We propose that this change relies on the availability in E3 enzymes, the incoming signals and relative amounts of pro- and anti-apoptotic regulators of cell fate.

8

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Figure legends

Figure 1. *vg-gal4*-mediated induction of the *UY3010* mutation significantly suppresses the *bax*-induced phenotype

(A) w^{1118} wild-type adult wing. (B-D) wings of *vg>bax* flies. *bax* expression leads to phenotypes that fall into three categories: weak (B), intermediate (C) and strong (D). Most specimens show strong or intermediate phenotypes (adapted from (32)). (E) Distribution of the offspring between the strong, intermediate and weak phenotype categories in *vg>bax* and *vg>bax/Uba1^{UY3010}* flies.

Figure 2. *Uba1^{EP2375}* overexpression efficiently suppresses *bax*-induced apoptosis
(A, B) *vg>bax/Uba1^{EP2375}* flies. *Uba1* overexpression leads to the appearance of wild-type (A) or very weak phenotypes, with only a small notch (B). (C) Distribution of the offspring between the strong, intermediate and weak phenotype categories in *vg>bax* and *vg>bax/Uba1^{EP2375}* flies.
(D, F) Acridine orange stainings on third-instar larval leg (asterisks) and wing (arrowheads) discs expressing *bax* in imaginal wing discs under control of the *vg-gal4* driver (*vg>bax*) or coexpressing *bax* and *Uba1* (*vg>bax + Uba1^{EP2375}*). (E, G) Nomarsky views of the upper panels discs.

Figure 3. Wings from *debcl*-expressing flies
(A-G) Adult wings and (A'-G') magnification of (A-G). (A, A') wild-type fly. (B, B') control fly of *ptc-gal4,UAS-debcl / +;UAS-debcl / +* genotype. (C, C') *ptc-gal4, UAS-debcl / Uba1^{EP2375};UAS-debcl / +.* (D, D') *ptc-gal4,UAS-debcl / +;UAS-debcl / Δp54.* (E, E') *ptc-gal4,UAS-debcl / +;UAS-debcl / pros25^{EP931}.* (F, F') *ptc-gal4,UAS-debcl / +;UAS-debcl / slmb^{41}.* (G, G') *ptc-gal4, UAS-debcl / morgue^{EP1184};UAS-debcl / +.* (H) Distribution of the offspring between the strong, intermediate weak and wild type phenotype categories in *ptc>debcl* and *ptc>debcl/Uba1^{EP2375}* flies.

Figure 4. Modulation of *bax*-induced ==wing== phenotype
(A) Distribution of the offspring between the strong, intermediate and weak phenotype categories. Each bar represents the progeny of a cross between *vg>bax* flies and a control (C) or a mutant (T) strain. Each cross (T) is presented with its control (C) that takes into account the specific genetic background of the tested strain. The percentage of phenotypes in each category is shown on the y-axis. (B) Every phenotypic test was validated by the statistical Wilcoxon test. The phenotype distribution of each test cross was compared with that of the corresponding control. Differences in distribution with a probability $\alpha < 10^{-3}$ were considered significant; Ws<0 indicates phenotype suppression and Ws>0 indicates phenotype enhancement; n is the number of flies counted.

Figure 5. Modulation of *debcl*-induced ==wing== phenotype
(A) Distribution of offspring between the strong, intermediate and weak phenotype categories. Each bar represents the progeny of a cross between *ptc>(debcl)$_2$* flies and a control strain (C) or a mutant strain (T). Each cross (T) is presented with its control (C). The percentage of phenotypes in each category is shown on the y-axis. (B). Every phenotypic test was validated by carrying out a Wilcoxon test as in figure 4.

**Figure 6.** Uba1 controls the level of Bax and Debcl proteins
(A) Bax immunostaining on third-instar larval wing discs expressing *bax* under control of the *vg-gal4* driver (*vg>bax*) or coexpressing *bax* and *Uba1* (*vg>bax + Uba1^{EP2375}*). The panel on the right shows GFP expression driven by *vg-gal4* in the wing pouch, along the dorso-ventral frontier. (B) Immunodetection of Bax in a *Uba1* overexpression background. Uba1 decreases Bax protein quantities down to an undetectable level. (C) Immunostaining of Debcl in a *Uba1* overexpression background. Uba1 reduces the amount of Debcl protein. (B, C) Immunodetection of tubulin was used as control.

Figure 7. Debcl and Slmb physically interact *in vivo*
(A) Embryos ubiquitously co-expressing Slmb^{DN}-flag-myc and Debcl-HA (*UAS-debcl-HA/+; da>slmb^{DN}-flag-myc/UAS-debcl-HA*) were prepared as described in "Materials & Methods" and subjected to immunoprecipitation (IP) with mouse anti-flag antibodies (Slmb) or IgG1 control (Irr). Western-blot analysis was then performed with an anti-myc mAb (Slmb) or anti-HA mAb (Debcl). The input lane contains 6% of the lysate while immunoprecipitates were obtained from 28% of the lysate. Debcl co-immunoprecipitates with Slmb. (B) Wing discs of *Ptc>Debcl-HA,Slmb^{DN}-flag-myc* and *Ptc>Debcl-HA,Pabp-flag* third instar larvae were

analysed by use of the proximity ligation *in situ* assay to monitor the Debcl/Slmb interaction. As expected, no signal was observed in *Ptc>Debcl-HA,Pabp-flag* discs whereas the red dots observed in *Ptc>Debcl-HA,Slmb^{DN}-flag-myc* indicate Slmb/Debcl colocalization.

Figure 1
Click here to download high resolution image
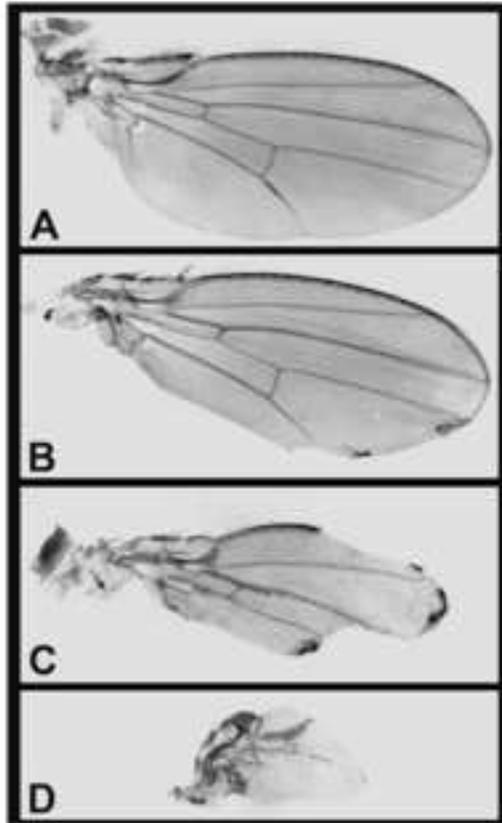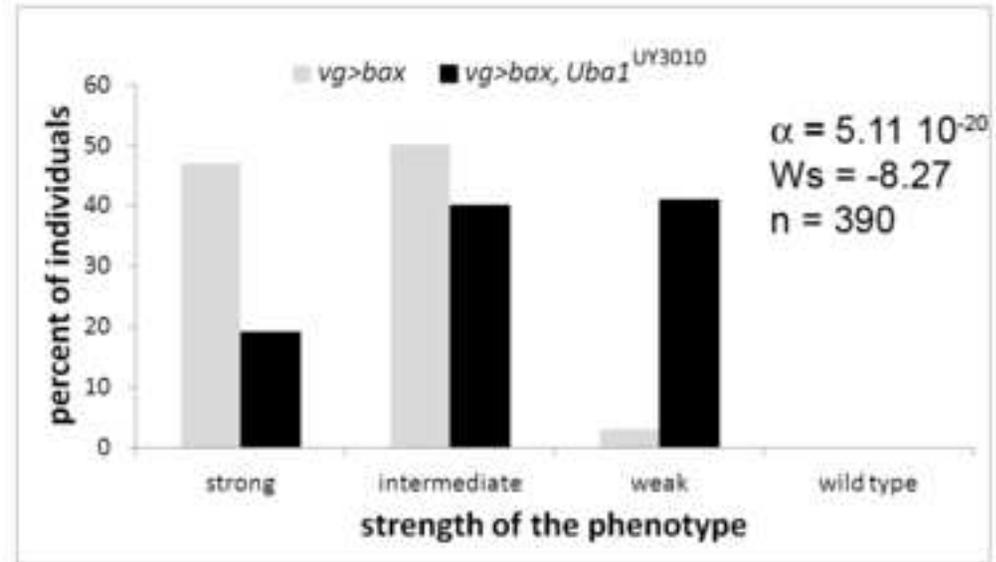
Figure 1
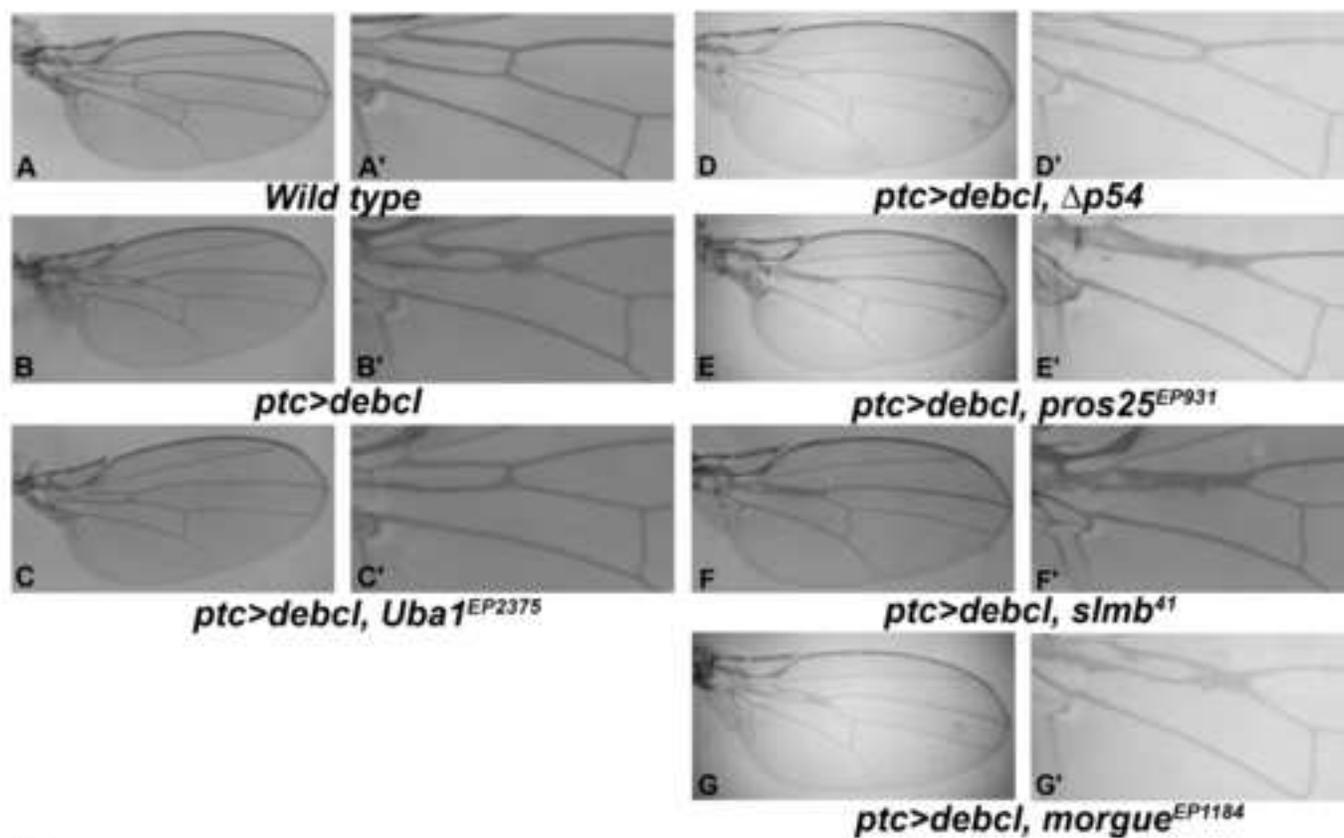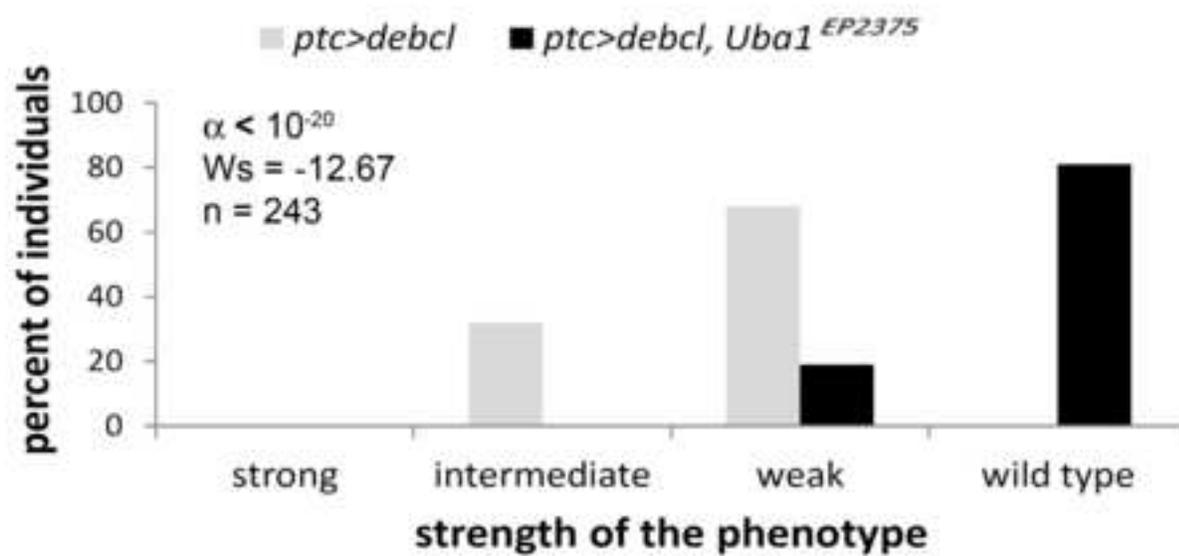
Figure 2
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A A' Wild type
B B' ptc>debcl
C C' ptc>debcl, Uba1^{EP2375}
D D' ptc>debcl, Δp54
E E' ptc>debcl, pros25^{EP931}
F F' ptc>debcl, slmb^{41}
G G' ptc>debcl, morgue^{EP1184}

H

$\alpha < 10^{-20}$
Ws = -12.67
n = 243

percent of individuals

strength of the phenotype

strong   intermediate   weak   wild type

ptc>debcl   ptc>debcl, Uba1^{EP2375}

A

Figure 4 panel A: bar chart showing % of phenotypes for proteasome (Δp54, pros25^EP931) and E3 (skpA^EP1423, slmb^8, slmb^41, morgue^EP1184) groups, with C and T conditions, categorized as strong, intermediate, weak, wild type.

B
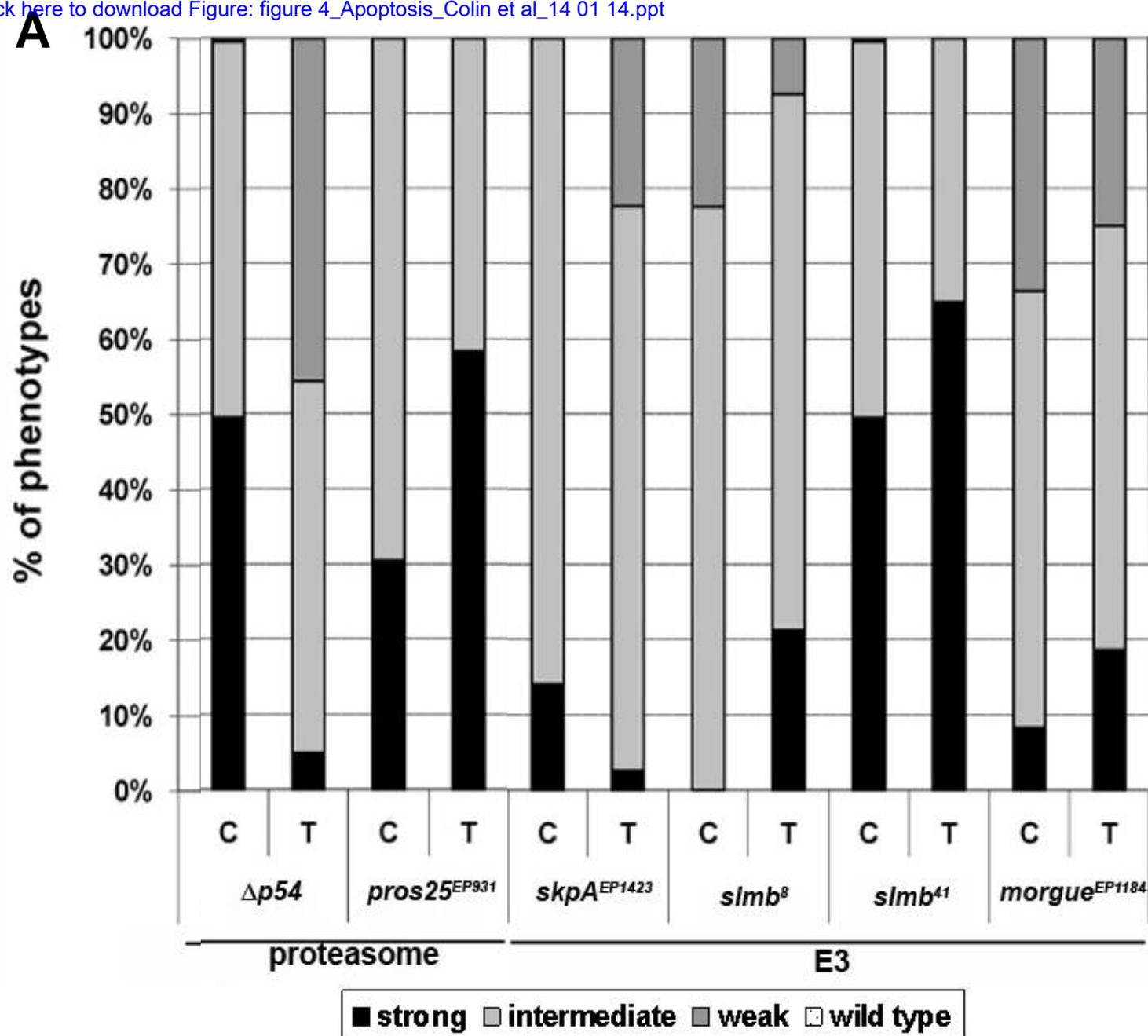
	$\Delta p54$	$pros25^{EP931}$	$skpA^{EP1423}$	$slmb^8$	$slmb^{41}$	$morgue^{EP1184}$
α	0	3.21×10^{-5}	1.68×10^{-7}	2.08×10^{-7}	1.24×10^{-3}	7.22×10^{-3}
Ws	-14.30	4.17	-5.30	5.25	3.23	2.69
n	578	272	190	236	542	349

A

B
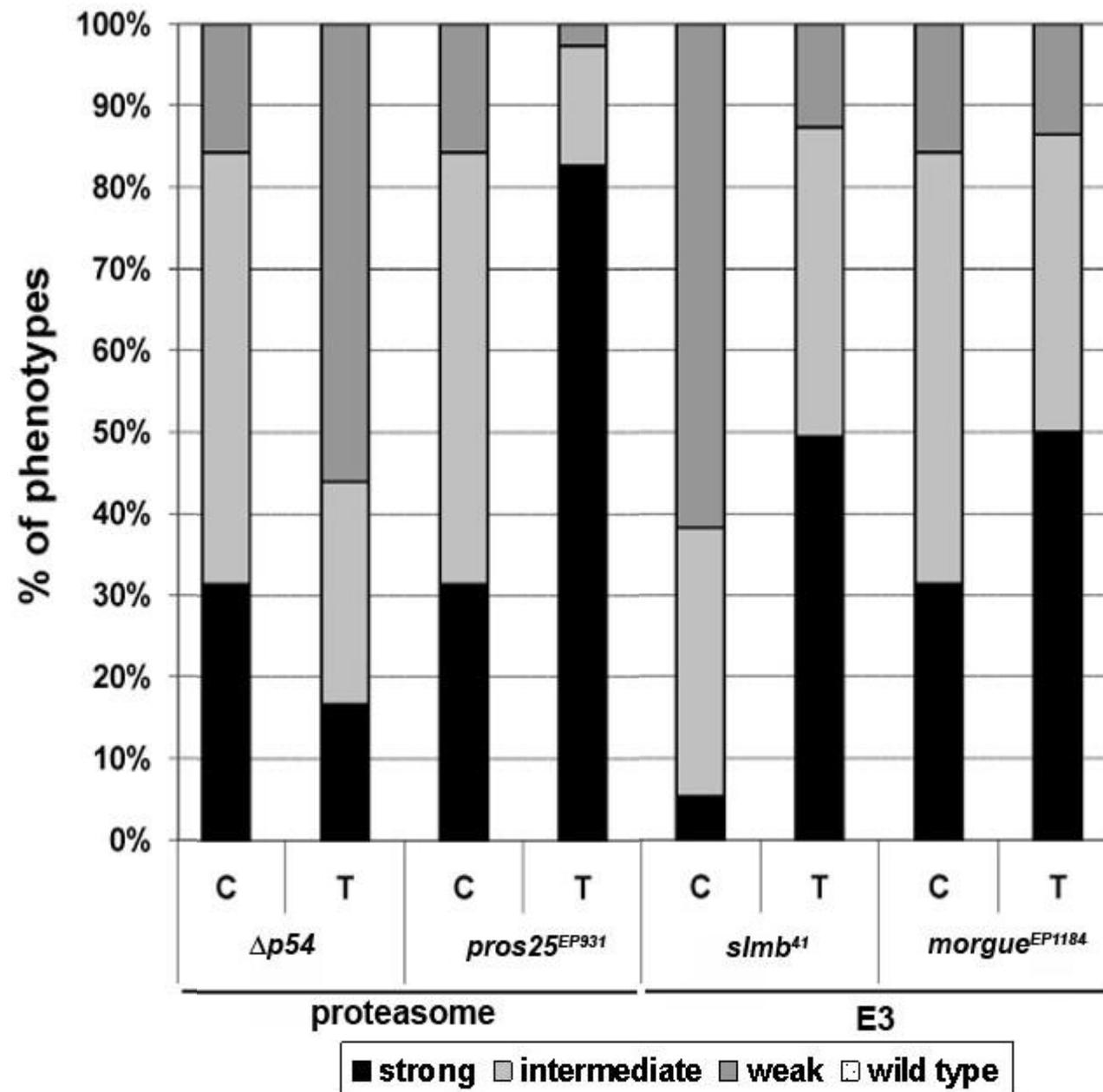
	$\Delta p54$	$pros25^{EP931}$	$slmb^{41}$	$morgue^{EP1184}$
α	2.29×10^{-9}	1.69×10^{-11}	8.50×10^{-14}	0.024
Ws	-6.11	6.965	7.83	2.25
n	272	215	181	214
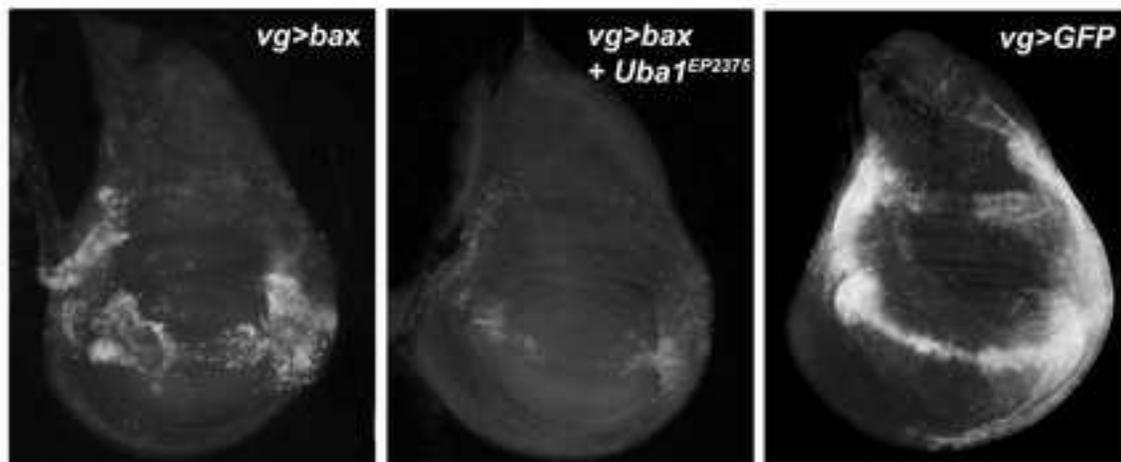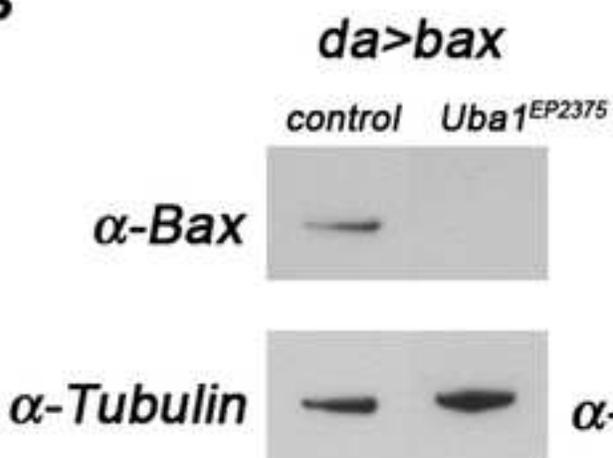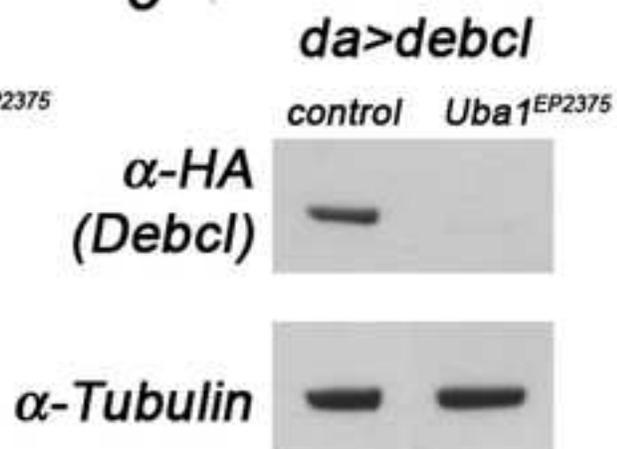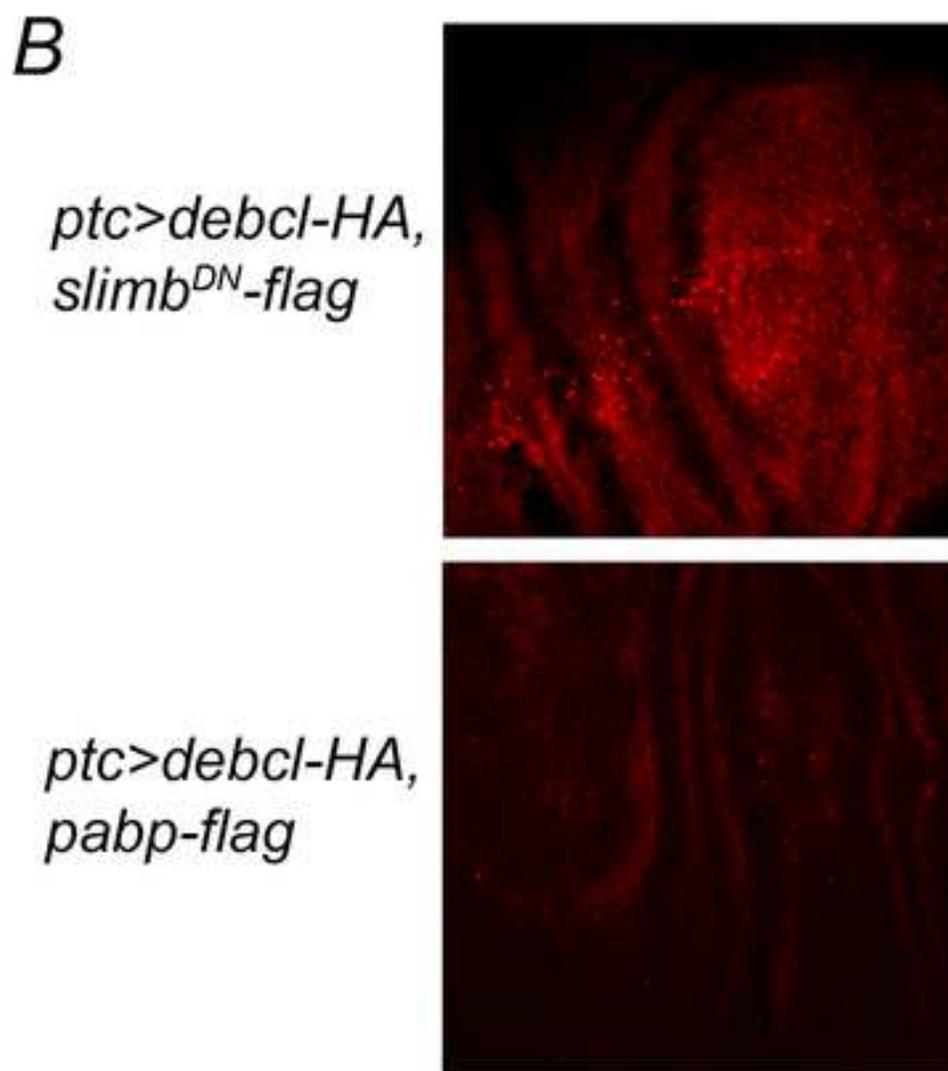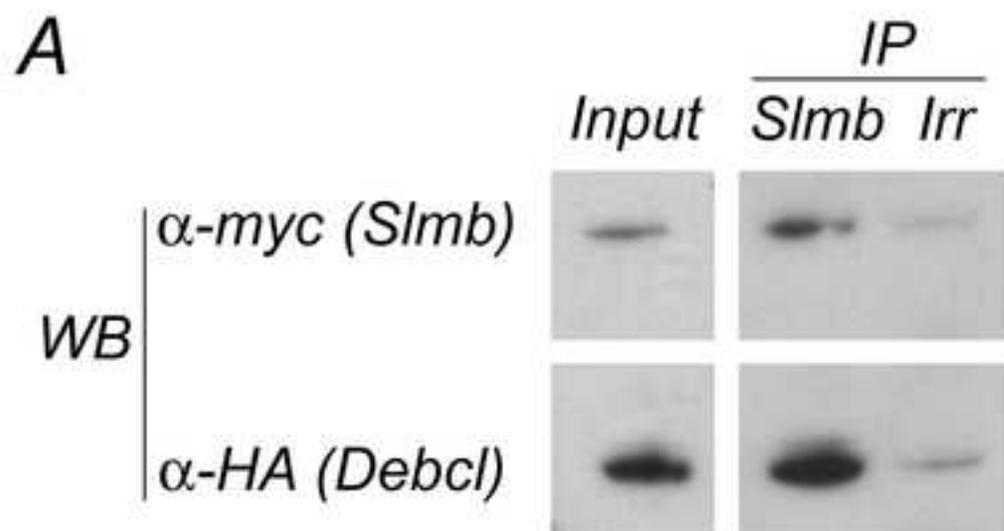
Figure 6
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A

vg>bax

vg>bax
+ Uba1^EP2375

vg>GFP

B

da>bax

control   Uba1^EP2375

α-Bax

α-Tubulin

C

da>debcl

control   Uba1^EP2375

α-HA
(Debcl)

α-Tubulin

Figure 7
Click here to download high resolution image

A

IP

Input   Slmb   Irr

WB

α-myc (Slmb)

α-HA (Debcl)

B

ptc>debcl-HA,
slimb^DN-flag

ptc>debcl-HA,
pabp-flag

The Drosophila Bcl-2 family protein Debcl is targeted to the proteasome by the β-TrCP homologue Slimb

Jessie Colin, Julie Garibal, Amandine Clavier, Aurore Rincheval-Arnold, Sébastien Gaumer, Bernard Mignotte and Isabelle Guénal
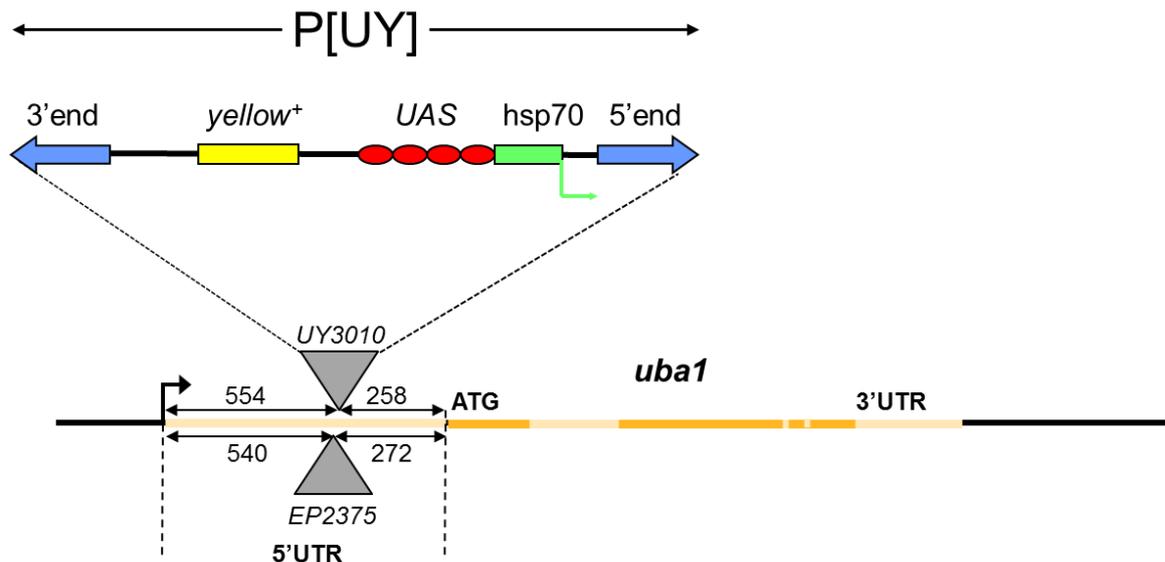

Supplementary figure 1

In the *UY3010* line, P[*UY*] is inserted in the 5'UTR region of *Uba1*.
Schematic diagram of the P[*UY*] insertion in the *UY3010* mutant. P[*UY*] is inserted in the 5'UTR region, 258 bp upstream from the start codon and 554 bp downstream from the transcription start site of the *Uba1* gene. The orientation of P[*UY*] is compatible with *UAS*-mediated *Uba1* overexpression in the presence of Gal4. The insertion of the P[*EP*] in the Uba1[EP2375] line is 272 bp upstream from the start codon and also compatible with *UAS*-mediated *Uba1* overexpression.

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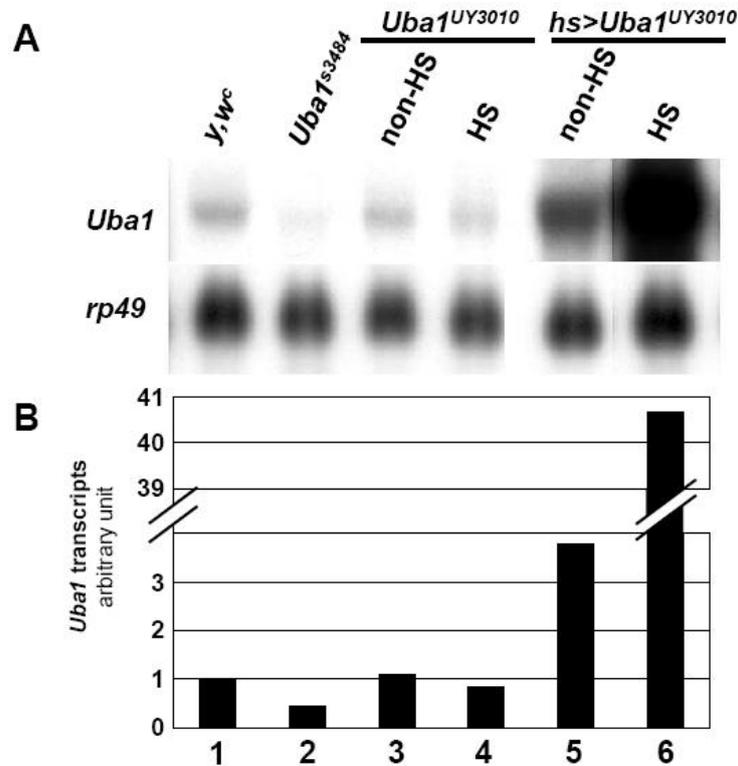
Supplementary figure 2

$Uba1^{UY3010}$ can be used to overexpress $Uba1$ under $gal4$ induction.
(A) Northern blot on total RNA from adult flies. Genotype of flies: y,w^c: wild-type; $Uba1^{s3484}$: $Uba1^{s3484}/CyO$ (loss-of-function allele of $Uba1$), $Uba1^{UY3010}$: $Uba1^{UY3010}/CyO$; $hs>Uba1^{UY3010}$: $hs\text{-}gal4,Uba1^{UY3010}/CyO$. Flies were subjected to heat shock treatment (HS) or not (non-HS). We used probes specific for $Uba1$ (top) or $rp49$ (bottom) as a control. (B) Quantification of $Uba1$ transcripts. $Uba1$ transcript levels were normalized with respect to $rp49$, with wild-type $Uba1$ transcript (y,w^c) levels set at 1. The loss-of-function allele of $Uba1$, $Uba1^{s3484}$, had half as many $Uba1$ transcripts as the wild-type. Use of the $hs\text{-}gal4$ driver and $Uba1^{UY3010}$ led to a 10 times increase in $Uba1$ transcript levels in response to heat shock treatment.

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*ptc>+*   *ptc>debcl*
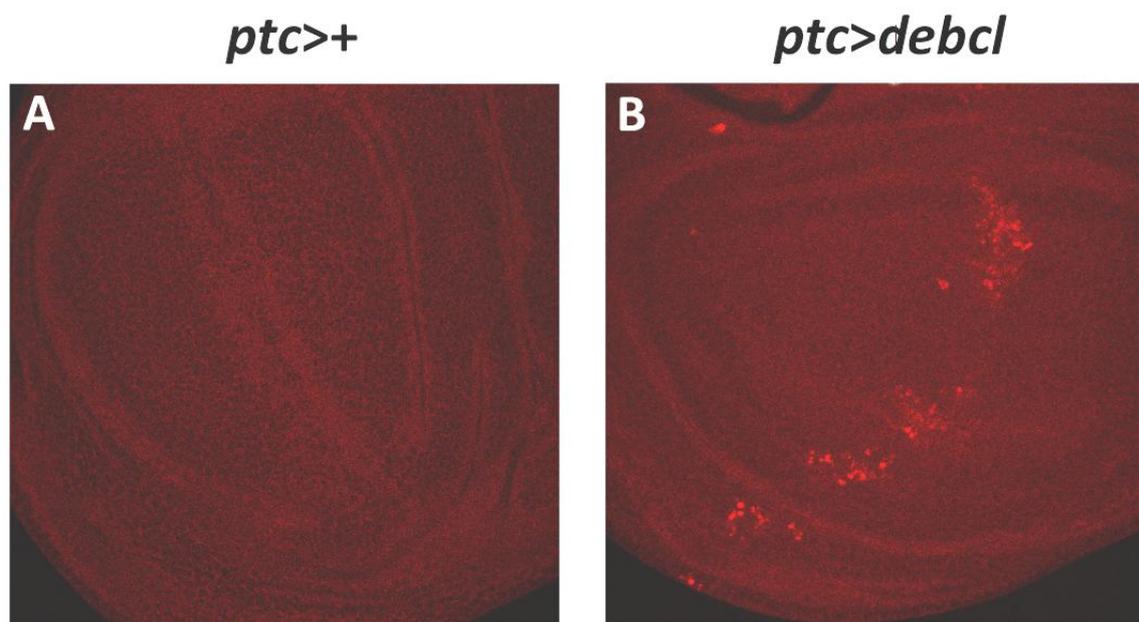
Supplementary figure 3

Expression of *UAS-debcl* in the wing imaginal disc thanks to the *ptc-gal4* driver induces apoptosis along the antero-posterior frontier
Apoptotic cells were visualized by TUNEL staining of wing imaginal discs of the genotype indicated at the top of the image.

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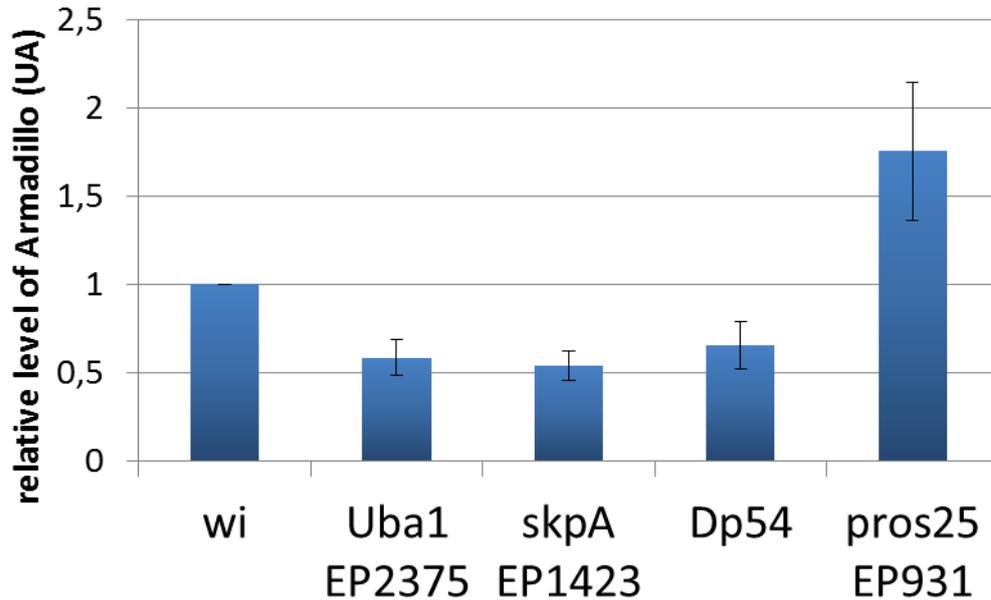
Supplementary figure 4

Effects of mutations on proteasome activity.
Cell lysates from Drosophila head of w[1118] (wi), GMR-Gal4>Uba1[EP2375], GMR-Gal4>skpA[EP1423], Dp54 and GMR-Gal4>pros25[EP931] flies were migrated on PAGE and probed with anti-armadillo (N2 7A1 from DSHB, 1/500) and anti-tubulin (E7 from DSHB, 1/1000) antibodies. Armadillo levels were normalized with tubulin levels and the ratio was set to 1 for wi flies. Results were from at least 3 independent experiments per genotype.

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Supplementary methods

Heat-shock treatment

Young males were placed in empty tubes at 36°C for 45 minutes. They were then transferred to a tube containing standard medium and incubated at 21°C for one day. This temperature shift was repeated 24 hours later. Flies were finally recovered five hours after heat shock and RNA was extracted.

Northern blot

Total RNA was extracted from 25 males by homogenization in Trizol (Life Technologies, phenol and guanidine isothiocyanate) and precipitated in isopropanol. The RNA was resuspended in water and its concentration evaluated by spectrophotometry (OD 260/280 nm). Northern blotting was then carried out with 30 µg of total RNA, using standard methods. The probes specific for *Uba1* or *rp49* (ribosomal protein, control for quantification), were generated by PCR, using the following primers:

5'-GTGTATTCCGACCAGGTTACA-3' (*rp49*, forward primer)
5'-ATACAGGCCCAAGATCGTGA-3' (*rp49*, reverse primer)
5'-TGTCGTCGCAATTCTATCTCAC-3' (*Uba1*, forward primer)
5'-TGGCAATCTGAGAATGATAGCC-3' (*Uba1*, reverse primer)

These probes were obtained by random priming and polymerization with the Klenow fragment of DNA polymerase I. The radioactive nucleotide used was $\alpha dCTP^{32}$. Quantification was achieved with a phosphorimager and normalized with respect to *rp49*.

TUNEL staining of wing imaginal discs

Discs were dissected and TUNEL staining was performed according to manufacturer's instructions (ApopTag® Red in situ apoptosis detection kit, Chemicon). Discs were mounted in CitifluorTM (Biovalley) and observed with a Leica SPE upright confocal microscope with an x63 objective.