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The initiator caspase Dronc is subject of enhanced autophagy upon proteasome impairment in *Drosophila*

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A major function of ubiquitylation is to deliver target proteins to the proteasome for degradation. In the apoptotic pathway in *Drosophila*, the inhibitor of apoptosis protein 1 (Diap1) regulates the activity of the initiator caspase Dronc (death regulator Nedd2-like caspase; caspase-9 ortholog) by ubiquitylation, supposedly targeting Dronc for degradation by the proteasome. Using a genetic approach, we show that Dronc protein fails to accumulate in epithelial cells with impaired proteasome function suggesting that it is not degraded by the proteasome, contrary to the expectation. Similarly, decreased autophagy, an alternative catabolic pathway, does not result in increased Dronc protein levels. However, combined impairment of the proteasome and autophagy triggers accumulation of Dronc protein levels suggesting that autophagy compensates for the loss of the proteasome with respect to Dronc turnover. Consistently, we show that loss of the proteasome enhances endogenous autophagy in epithelial cells. We propose that enhanced autophagy degrades Dronc if proteasome function is impaired.
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There are two major catabolic pathways in eukaryotic cells that degrade the bulk of cellular proteins, the ubiquitin–proteasome system (UPS) and macro-autophagy, hereafter referred to as autophagy.[1–4] In the UPS, poly-ubiquitylated proteins are delivered to the 26S proteasome for degradation. The 26S proteasome consists of a 20S catalytic core, flanked by two 19S regulatory complexes.[5,6] The 20S catalytic core is composed of a total of 28 α- and β-type subunits, which are organized in a barrel with four stacked rings. The outer two rings are formed by seven α-type subunits each, the two inner rings by seven β-type subunits each.[5] All α- and β-type subunits are needed for structural integrity of the proteasome.[7] Three β-type subunits, β1, β2 and β5, have proteolytic activity.[6] The 19S regulatory particle is composed of at least 19 subunits involved in recognition of ubiquitin-conjugated substrates, ATP hydrolysis, de-ubiquitination, protein unfolding and feeding of the substrates into the 20S catalytic core for degradation.[5] Genetic studies in yeast and *Drosophila* have revealed that mutations in many subunits of the 20S core and the 19S regulatory domains impair proteasome function.[8,9] Genetic analysis of proteasome function is also of clinical importance as proteasome inhibition may be used as potential antitumor strategy, especially for treatment of multiple myeloma.[10–13]

Autophagy is characterized by the formation of double-membrane vesicles termed autophagosomes.[14,15] During autophagosome maturation, cytosolic proteins and entire organelles are trapped and delivered to the lysosome for degradation. Two ubiquitin-like conjugation pathways (autophagy-related-8 (Atg8)/light chain 3 (LC3) and Atg12) are active during maturation of autophagosomes.[14,15] Atg7 is an enzyme 1 (E1)-activating enzyme involved in both conjugation pathways[14] and essential for autophagy.[16] The incorporation of Atg8 fusion proteins (for example, with green fluorescent protein (GFP) and/or mCherry) into autophagosomes is often used as a marker for autophagosomes[17] and autophagic flux.[18]

Although it was initially assumed that the UPS and autophagy are independent of each other, recent evidence has suggested that there is crosstalk and feedback between the two[1,19–27] (reviewed by Park and Cuervo,[3] Wojcik[28] and Lamark and Johansen[29]). This is mostly due to the observation that autophagy can also degrade ubiquitylated proteins.[30–34] Mechanistically, adaptor proteins with ubiquitin-binding domains and LC3-interacting regions (LIRs) link poly-ubiquitylated proteins to LC3/Atg8 at the autophagosome.[30,35] Loss of autophagy can lead to the formation of protein aggregates composed of poly-ubiquitylated proteins and ubiquitin-binding proteins. These protein aggregates are frequently associated with neurodegenerative diseases in humans.[36–38]

Apoptosis is the major form of cell death and evolutionarily conserved from flies to humans.[39,40] Caspases are highly

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Abbreviations: Atg-7,-8a, autophagy-related-7, -8a; β2, beta 2; cCsp-3, cleaved caspase-3; da-Gal4, daughterless-Gal4; Diap1, *Drosophila* inhibitor of apoptosis protein; DrICE, death-related ICE-like protease; Dronc, death regulator Nedd2-like caspase; DTS7, dominant temperature sensitive 7; E1, enzyme 1; ey, eyeless; Flp, Flippase; FRT, Flp recombination target; GFP, green fluorescent protein; IAP, inhibitor of apoptosis protein; IκBα, inhibitor kappa B alpha; L3, 3rd larval stage; LC3, light chain 3; LIR, LC3-interacting region; MARCM, mosaic analysis with a repressible cell marker; NcF, Nedd2-like caspase forward primer; NcR, Nedd2-like caspase reverse primer; Prosβ2, proteasome subunit beta 2; qPCR, quantitative polymerase chain reaction; RING, really interesting new gene; RNAi, ribonucleic acid (RNA) interference; Ubi-GFP, ubiquitous GFP; UPS, ubiquitin–proteasome system; XIAP, X-linked IAP

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specific Cys-proteases and are the main effectors of apoptosis. They are produced as inactive zymogens that are activated either through incorporation into large protein complexes, such as the apoptosome (initiator caspases), or by proteolytic processing (effector caspases).[41,42] After activation in the apoptosome, initiator caspases such as caspase-9 and its *Drosophila* ortholog Dronc (death regulator Nedd2-like caspase), cleave and activate effector caspases, such as caspase-3 and its *Drosophila* ortholog DrICE (death-related ICE-like protease).[40,43]

The activity of caspases is controlled at multiple levels. In addition to zymogen production, apoptosome-mediated activation of caspase-9/Dronc and proteolytic processing of caspase-3/DrICE, caspases are also controlled by ubiquitylation, mediated by inhibitor of apoptosis proteins (IAPs), most notably X-linked IAP (XIAP) in mammals and *Drosophila* IAP1 (Diap1).[43] IAPs carry a RING (really interesting new gene) domain, which has E3 ubiquitin ligase activity.[44] In *Drosophila*, because the RING domain of Diap1 ubiquitylates Dronc,[45–48] it is commonly assumed that this ubiquitylation targets the caspase for proteasome-mediated degradation.[45,46] However, *in vivo* this has not been observed. On the contrary, we have shown that loss or gain of Diap1 activity does not affect the protein levels of Dronc in surviving cells.[49] A similar observation has been reported for DrICE.[48] Furthermore, a mouse mutant deleting the RING domain of XIAP does not significantly affect caspase protein levels.[50] Therefore, it is currently unclear how the protein levels of Dronc are controlled in living cells to avoid deleterious accumulation and autoprocessing of these potentially dangerous proteins.

Here, we report that in epithelial cells of *Drosophila* eye imaginal discs, the protein levels of Dronc are unaffected by proteasome impairment. Similarly, loss of autophagy by itself does not affect Dronc protein levels. However, simultaneous impairment of the proteasome and autophagy causes accumulation of Dronc suggesting that autophagy can compensate for the loss of the proteasome with respect to Dronc turnover. Consistently, autophagy is enhanced in proteasome mutants in epithelial cells. In summary, these data identify Dronc as a common substrate for both the proteasome and autophagy.

Results

Accumulation of poly-ubiquitylated proteins is a convenient marker for proteasome dysfunction. Ubiquitylation of Dronc by Diap1 has previously been observed *in vitro*.[45,47] To directly test if ubiquitylated Dronc is degraded by the proteasome, we analyzed Dronc protein levels in two mutants affecting the proteasome. The first mutant affects the *prosβ2* gene, also known as *DTS7* in *Drosophila*,[51] which encodes the β2 subunit of the 20S catalytic core of the proteasome. The proteasome subunit beta 2 (Prosβ2) subunit provides both structural integrity to the proteasome and proteolytic activity.[7] The second mutant affects the *Mov34* gene (also known as p39B), which encodes a subunit in the 19S regulatory complex, corresponding to regulatory particle non-ATPase-8 (Rpn8) in yeast and S12 in the human regulatory complex.[52] Both mutants, $prosβ2^{EP3067}$ and $Mov34^{k08003}$, are caused by P element insertions in the first exon, which likely disrupt the transcripts. Both proteasome mutants behave identically in our assays (see below). As these proteasome mutants are homozygous lethal, we induced mutant clones of cells using the *ey-Flp*/*FRT* system.[53,54] Not unexpectedly, mutant clones affecting the proteasome are very small and are difficult to identify using negative selection with GFP (Figure 1a–c). However, we were able to positively mark and identify mutant clones using antibodies that recognize ubiquitin or ubiquitin-conjugated proteins. Antibodies raised against ubiquitin display increased immunoreactivity in *prosβ2* mutant clones (Figure 1a'). There is a perfect match in the areas lacking GFP, which mark the *prosβ2* mutant cells, and increased abundance of ubiquitin labeling (Figure 1a").

As poly-ubiquitin-conjugated proteins are often subject to proteasome-mediated degradation, we tested whether ubiquitin-conjugated proteins account for the accumulation of ubiquitin in *prosβ2* mutants. The FK1 and FK2 antibodies specifically recognize ubiquitin-conjugated proteins, but not unconjugated ubiquitin (FK1 labels poly-ubiquitylated conjugates, FK2 labels mono- and poly-ubiquitylated proteins).[55,56] As shown in Figures 1b' and c', the immunoreactivity of FK1 and FK2 antibodies increases in *prosβ2* mutant cells. We also find increased FK1 and FK2 labeling in *Mov34* mutant cells (Supplementary Figure S1). In wild-type (wt) control mosaics (wt clones in wt background), an accumulation of ubiquitin and conjugated ubiquitin (FK1 and FK2) is not observed (Supplementary Figure S2) suggesting that ubiquitin-conjugated proteins specifically accumulate in proteasome-deficient cells *in vivo*, consistent with the expectation.

Mutations in proteasome subunits result in elevated cleaved caspase-3. Proteasome inhibitors are used to induce cell death in cancer patients, including those with multiple myeloma.[11,13] Consistently, pharmacological inhibition of the proteasome by Bortezomib in whole flies resulted in DrICE cleavage after 4 days of treatment.[27] In contrast, tissue-specific inhibition of the proteasome in *Drosophila* fat body cells by ribonucleic acid (RNA) interference (RNAi) did not confirm such an apoptotic response.[57] As RNAi is known to cause partial loss-of-function phenotypes, we tested the strong *prosβ2* mutant allele in mosaic eye imaginal discs for apoptosis induction. We used FK2 labeling to positively mark and identify proteasome mutant cells, and examined the consequence of proteasome dysfunction for the survival of the affected cells. Indeed, we observed increased cleaved Caspase-3 (cCsp-3) antibody labeling in proteasome-deficient cells (Figure 2). However, not all mutant cells contain cCsp-3 staining suggesting that not all of them are apoptotic. Nevertheless, whether apoptotic or not, all proteasome-defective cell clones are very small indicating that the mutant cells do not grow very well.

Proteasome subunit mutant cells accumulate Diap1, but do not affect Dronc protein levels. As Diap1 can ubiquitylate Dronc *in vitro*,[45,47] it was predicted that Dronc is subject to proteasome-mediated degradation in surviving cells.[45,46] Therefore, it would be expected that the protein levels of

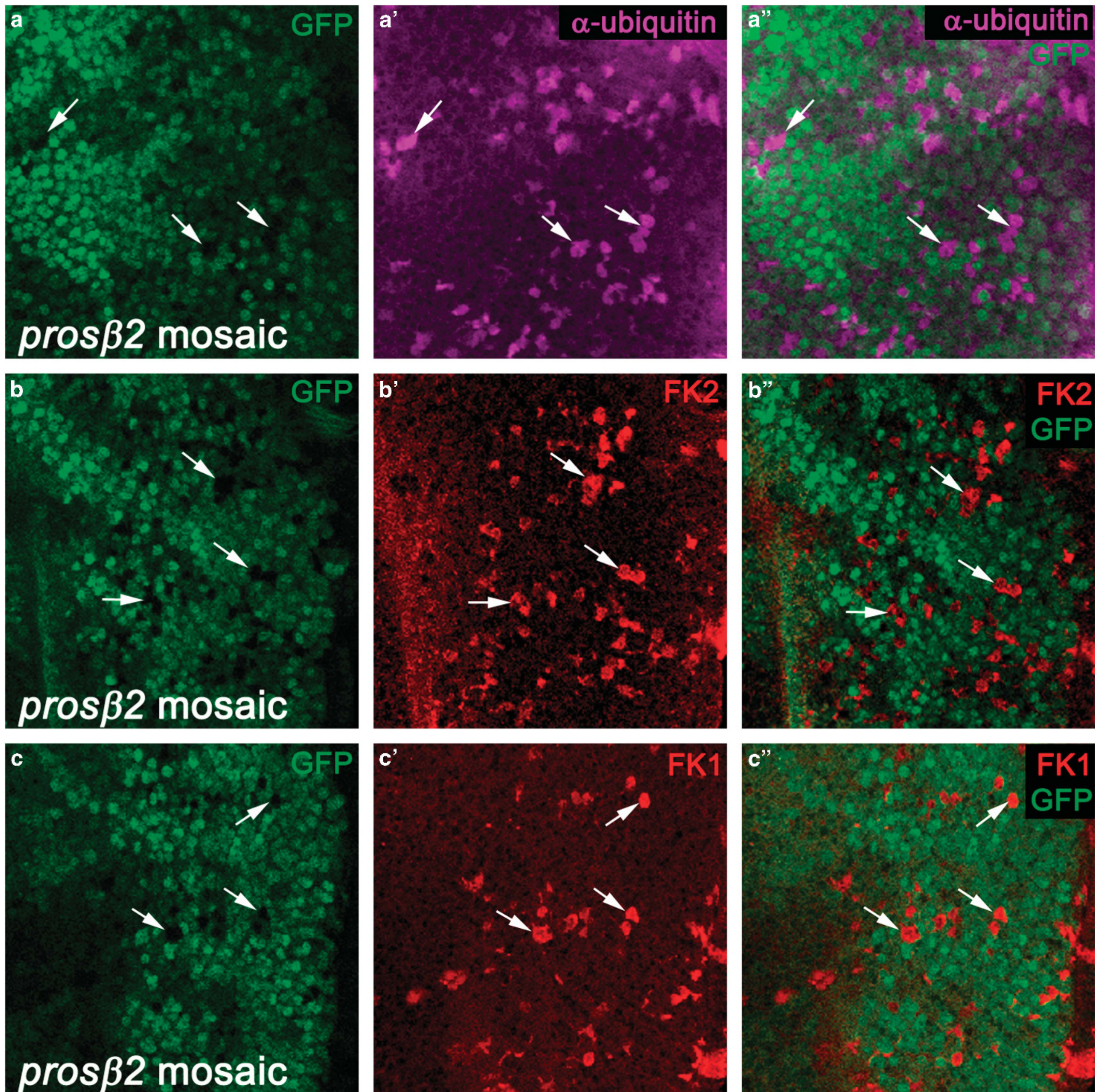

Figure 1 *prosβ2* mutant cells accumulate ubiquitin-conjugated proteins. Shown are high magnification images (x100) of the posterior compartment of *prosβ2* mosaic eye imaginal discs labeled for ubiquitin (**a**), FK2 (**b**) and FK1 (**c**). In this and all other figures, posterior is to the right. The FK2 and FK1 antibodies detect ubiquitin-conjugated proteins, but not free unconjugated ubiquitin. The left panels indicate the positions of the proteasome mutant cell clones by absence of GFP, the middle panels show the experiment (in magenta), and the right panels are the merged images of left and middle panels. White arrows mark a few cell clones as examples. Similar data were obtained for *Mov34* mosaic discs (Supplementary Figure S1). Wild-type mosaic control discs do not show accumulation of ubiquitin (Supplementary Figure S2). Genotype: *ey-FLP*; *prosβ2*^{EP3067} *FRT80/ubi-GFP FRT80*

Dronc would accumulate in proteasome mutants. We tested this expectation by analyzing Dronc protein levels in *prosβ2* and *Mov34* mutant cells located in the developing posterior eye imaginal disc of third instar larvae. We chose the larval posterior eye imaginal disc in these analyses because under normal conditions, there is no developmental apoptosis in this tissue, thus avoiding complications with physiological apoptosis. Surprisingly, Dronc protein levels are not detectably altered in *prosβ2* and *Mov34* mutant cells (Figures 3a–b"). We also obtained the same results using a different *prosβ2* mutant allele, the commonly used *DTS7* allele, which we used in genetic mosaics (Supplementary Figure S3). However, under these conditions, the *DTS7* allele appears to be weaker than the allele used in Figure 3a, because it did not cause accumulation of poly-ubiquitylated proteins (FK1 antibody labeling) and the mutant clones grow much larger

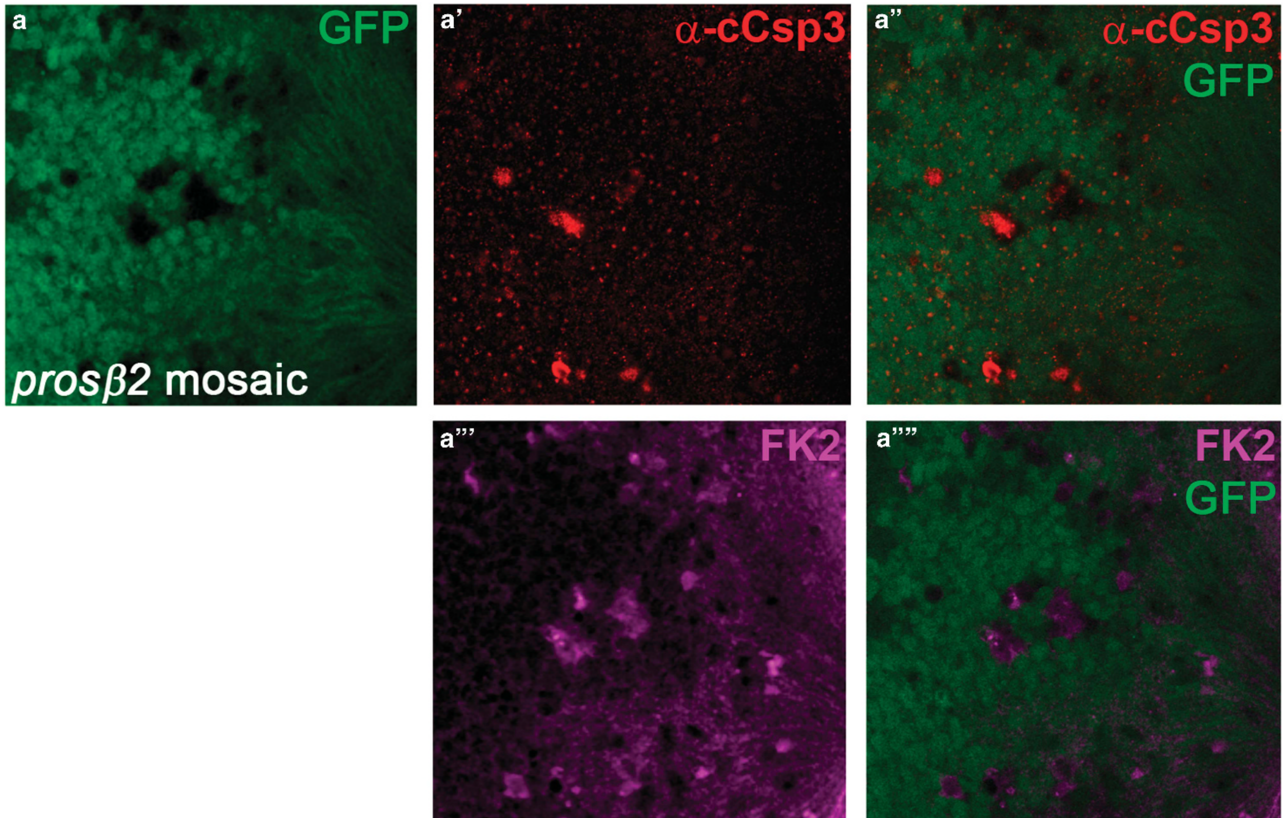

Figure 2 Mutations in proteasome subunits result in cell death. Shown are high magnification images (x100) of the posterior compartment of *prosβ2* mosaic eye imaginal discs labeled for cCsp-3 (**a'**, **a"**) and FK2 (**a'''**, **a''''**) to identify *prosβ2* mutant cell clones. cCsp-3 labeling is increased in some, but not all, *prosβ2* mutant cells (red in **a'** and **a"**). Genotype: *ey-FLP*; *prosβ2*^{EP3067} *FRT80*/*ubi-GFP FRT80*

(Supplementary Figure S3). The specificity of this Dronc antibody was previously established in *dronc* mosaic eye and wing imaginal discs, and this antibody can also detect accumulation of Dronc protein.[49]

To confirm the lack of Dronc accumulation in proteasome mutants, we analyzed the protein levels of Flag-tagged Dronc[58] expressed in proteasome mosaic eye imaginal discs. Using the Flag antibody as a tool to monitor Dronc levels, we also did not detect an accumulation of Flag-Dronc in *Mov34* mutant cells (Supplementary Figure S4).

As a positive control, we analyzed Diap1 protein levels in proteasome mutant cells as Diap1 degradation has been demonstrated *in vivo*.[59–62] Consistently, we observe accumulation of Diap1 in *prosβ2* and *Mov34* mutant cells (Figure 3c–d"). Thus, this assay can detect accumulating proteins *in vivo*.

Combined, these data indicate that protein levels of Dronc in living cells are either not regulated by the UPS, or there are compensatory mechanisms operating that turn over Dronc upon proteasome impairment.

Loss of the autophagy gene *Atg7* does not affect Dronc protein levels. In addition to the UPS, autophagy is a cellular catabolic process that is known to degrade proteins.[1,4,15] We considered the possibility that autophagy may regulate the protein levels of Dronc. The autophagy gene *Atg7* encodes the E1-activating enzyme for the two ubiquitin-like conjugation systems and is an important regulator for autophagy.[14,16] As proteasome impairment does not affect the protein levels of Dronc in epithelial disc cells, we tested the possibility that autophagy may control it. We downregulated *Atg7* function by RNAi or inactivated *Atg7* in mutant cell clones in eye imaginal discs. However, similar to proteasomal dysfunction, impaired *Atg7* function does not affect Dronc protein levels in mosaic eye imaginal discs (Figures 4a–b").

Simultaneous inactivation of both the proteasome and autophagy triggers accumulation of Dronc protein. We examined the possibility that the protein levels of Dronc are coordinately regulated by both the proteasome and autophagy. To address this question, we inactivated autophagy by *Atg7* RNAi in *prosβ2* mutant cell clones using the mosaic analysis with a repressible cell marker (MARCM) method.[63] Indeed, simultaneous impairment of both the proteasome and autophagy causes strong accumulation of Dronc protein in epithelial cells of eye imaginal discs (Figures 5a–a"). Similar observations were also made in wing imaginal discs, another epithelial tissue (Figures 5b–b"). The accumulation of Dronc in proteasome/autophagy double-deficient cells is not because of a transcriptional upregulation of *dronc* transcripts (Supplementary Figure S5). Together, these data suggest

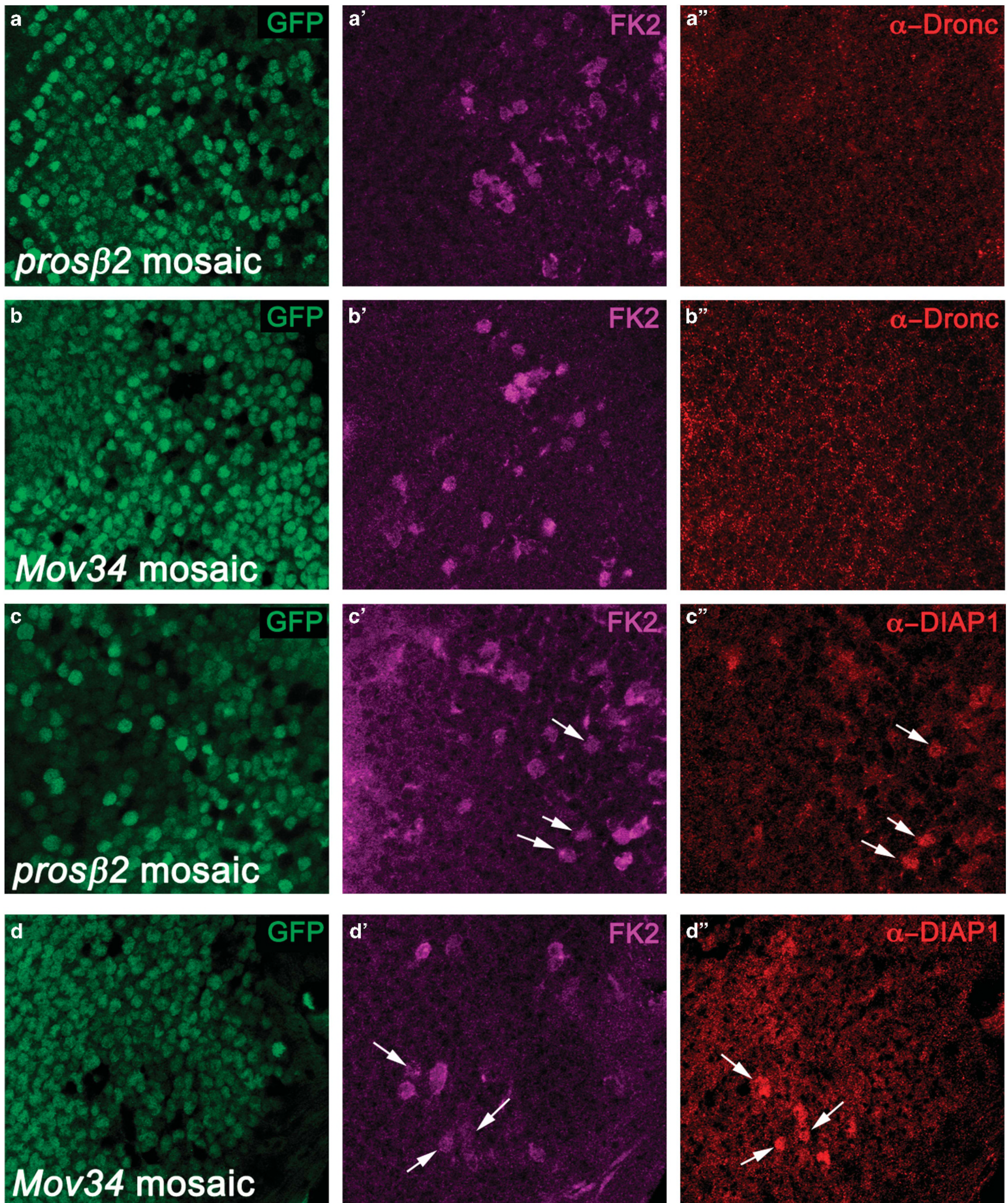

Figure 3 Diap1, but not Dronc, accumulate in proteasome mutant clones. Shown are high magnification images (x100) of the posterior compartment of *pros*β*2* (**a** and **c**) and *Mov34* (**b** and **d**) mosaic eye imaginal discs labeled for Dronc (**a** and **b**) and Diap1 (**c** and **d**). FK2 labeling was used to identify mutant clones. The left panels indicate the positions of the proteasome mutant cell clones by absence of GFP. In the middle panels, the proteasome mutant cell clones are positively marked by FK2 labeling (in magenta). The right panels show the Dronc (**a"** and **b"**) and Diap1 labelings (**c"** and **d"**) in red. White arrows mark a few cell clones as examples. See also related Supplementary Figures S3 and S4. Genotype in (**a** and **c**): *ey-FLP*; $pros\beta2^{EP3067}$ *FRT80*/P[*ubi-GFP*] *FRT80*. Genotype in (**b** and **d**): *ey-FLP*; *FRT42D* $Mov34^{k08003}$/*FRT42D* P[*ubi-GFP*]

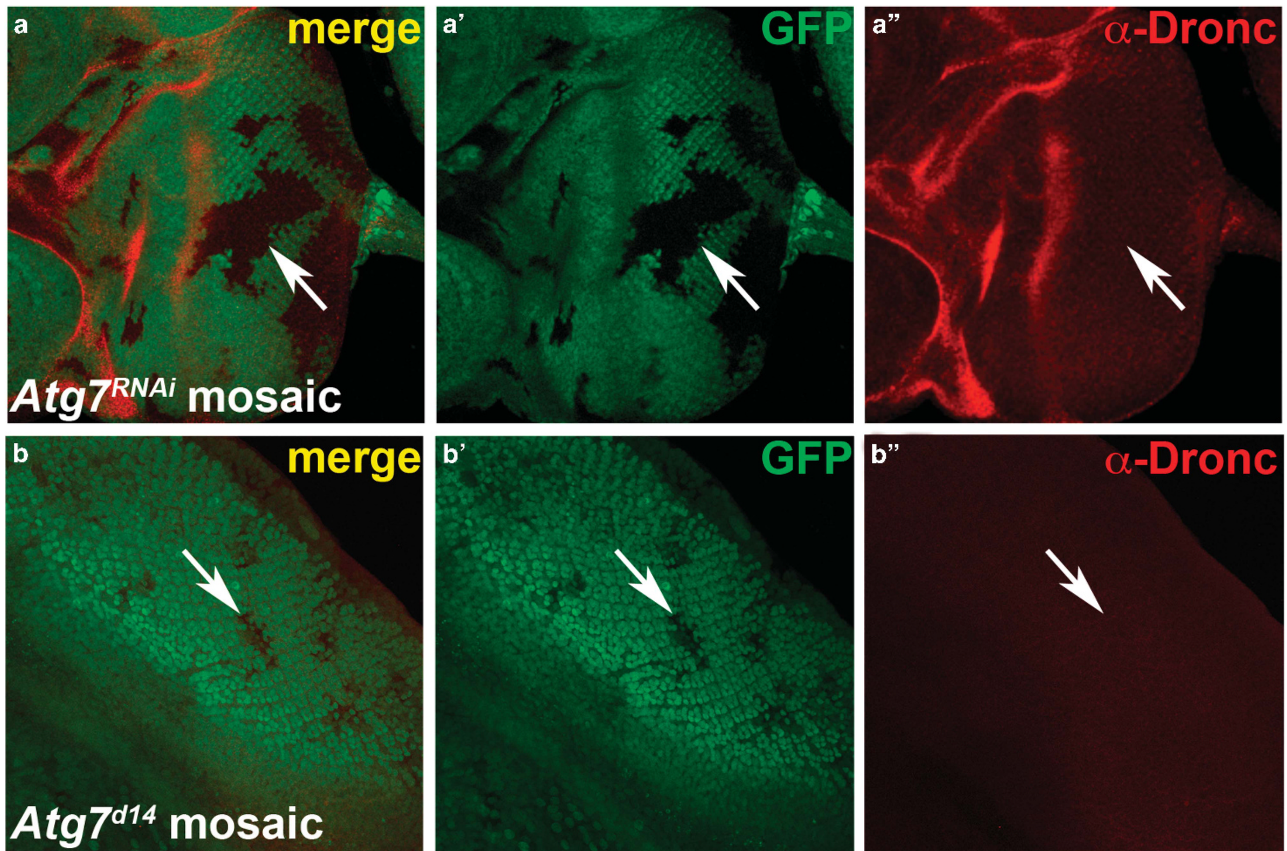

Figure 4 Loss of *Atg7* alone does not affect Dronc protein levels. Dronc labeling of *Atg7* mosaic eye imaginal discs. *Atg7* was either downregulated by RNAi (**a**) or genetically inactivated in mutant cell clones (**b**). *Atg7*-deficient clones are marked by absence of GFP (**a'**, **b'**). White arrows highlight one clone in each panel as example. Dronc protein levels are unaltered in *Atg7*-deficient clones (**a"**, **b"**). Genotypes: (**a**) *yw hsFLP*; *tub* > *GFP* > *Gal4*/*UAS-Atg7*^{RNAi} (> denotes FRT). (**b**) *yw eyFLP*; *FRT42D Atg7*^{d14}/*FRT42D* P[*ubi-GFP*]

that Dronc protein levels are coordinately regulated by both the UPS and autophagy.

Loss of proteasome function enhances autophagy in epithelial cells. To explain the synergistic control of Dronc protein levels by the UPS and autophagy, we considered that, because the UPS and autophagy are mechanistically linked, impairment of the UPS can enhance autophagy, which is often referred to as compensatory autophagy[1,19–27] (reviewed by Park and Cuervo,[3] Wojcik[28] and Lamark and Johansen[29]). For example, in *Drosophila*, compensatory autophagy after proteasome impairment has been reported in neurons, in fat body cells and in adult flies.[22,27,57] To examine this possibility in epithelial cells of eye imaginal discs, we monitored autophagy using a tandem fusion protein GFP-mCherry-Atg8a as reporter for autophagic flux.[18] This reporter is incorporated into autophagosomes, which mature into autolysosomes. In autolysosomes, fluorescence of the GFP moiety of the reporter is quenched, whereas mCherry signals persist. Therefore, this reporter is suited to monitor autophagic flux from autophagosomes into autolysosomes. Indeed, while weak GFP signals are present in a subset of *Mov34* mutant cell clones, there are also many clones where mCherry signals exist alone (Figures 6a" and a"') suggesting that at least in these clones autophagic flux from autophagosomes to autolysosomes is induced in response to proteasome impairment.

Discussion

This is the first report in which a clonal analysis of strong proteasome mutants was performed. Usually, dominant temperature sensitive (DTS) alleles of proteasome subunits (DTS5, DTS7, etc.), RNAi or pharmacological inhibition have been used to study proteasome function.[8,22,27,51,57,64] In other approaches, whole embryos mutant for proteasome subunits were characterized for defects in dendrite pruning in sensory neurons in *Drosophila*.[65–67] However, a specific analysis characterizing recessive alleles for defects in proteasome activity has not been reported. The reasons for this omission are obvious. Mutant animals are homozygous lethal and mutant clones in otherwise heterozygous animals are very small and difficult to identify. We found that cells mutant for proteasome function accumulate ubiquitin-conjugated proteins (Figure 1), consistent with the expectation. We used markers detecting ubiquitin-conjugated proteins to positively identify mutant clones. That enabled us to identify Diap1 as substrate of the proteasome, whereas control of Dronc protein levels appears to be independent of the proteasome (Figure 3). The proteasome alleles used in this study and our

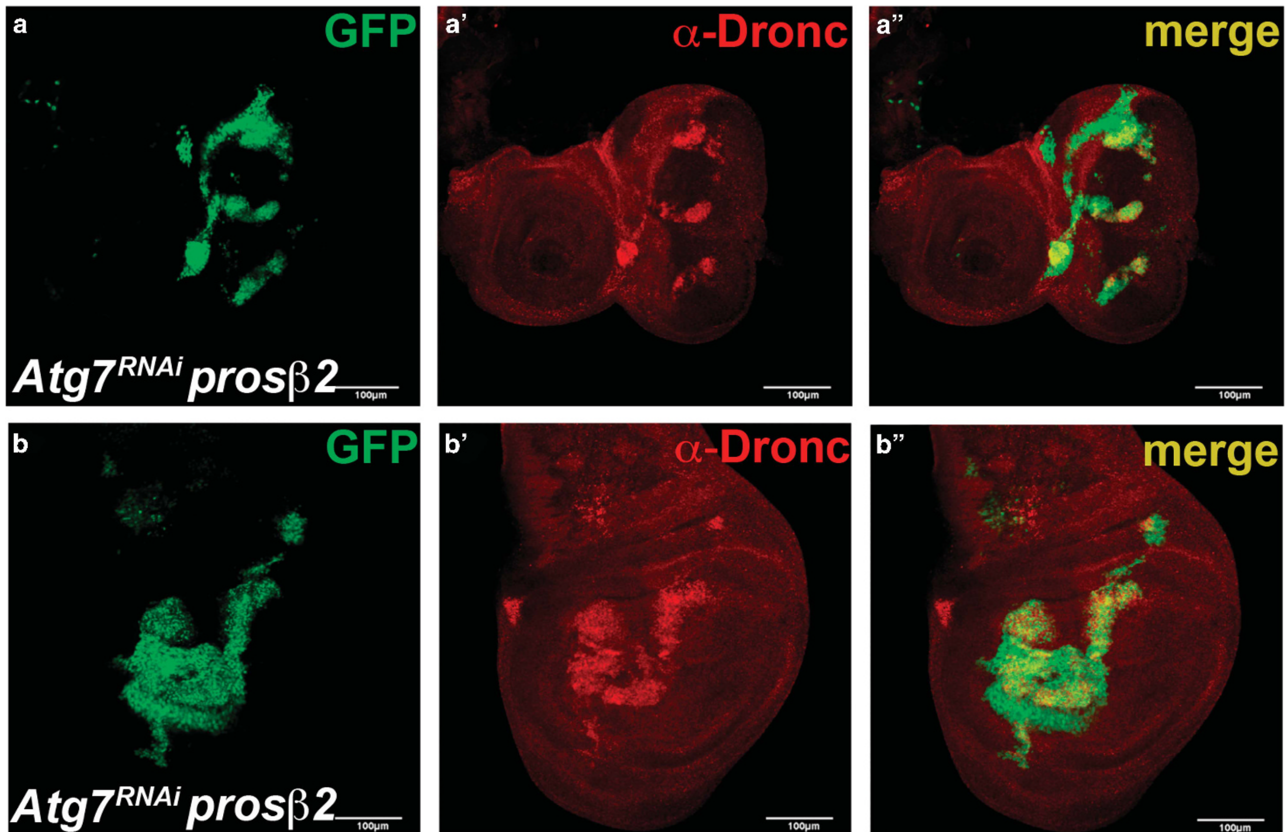

Figure 5 Dronc accumulates in clones simultaneously mutant for the proteasome and autophagy. Mosaic eye (**a**) and wing (**b**) imaginal discs doubly deficient for the proteasome and autophagy labeled for Dronc (red). *Atg7* knockdown was induced by RNAi in *prosβ2* mutant cell clones using the MARCM method. Mutant cell clones are positively labeled by GFP. Dronc accumulates in double-deficient cell clones. The cross was performed at 25 °C. Genotype: *hs-FLP UAS-GFP tub-Gal4; UAS-Atg7*^{RNAi}/+; *prosβ2*^{DTS7} *FRT80B/tub-Gal80 FRT80B*

approach to identify mutant clones will be of general use for future analysis of proteasome function.

Dronc also does not accumulate in autophagy-deficient cells in which the E1 encoding gene *Atg7* was mutant or downregulated by RNAi (Figure 4). However, simultaneous inactivation of the proteasome and *Atg7* resulted in strong accumulation of Dronc (Figure 5). In addition, autophagy is enhanced in proteasome mutants (Figure 6).

There are two possibilities to explain these results. First, both the UPS and autophagy degrade Dronc independently of each other and if one pathway is lost, the other one maintains Dronc at normal protein levels. Or second, under normal conditions, Dronc is degraded only by the UPS, but upon proteasome impairment, enhanced autophagy can compensate for this impairment and degrades Dronc instead. Although we cannot distinguish between these two possibilities, the lack of accumulation of Dronc protein in proteasome and autophagy mutants suggests a fail–safe mechanism that avoids accumulation of this potentially deleterious protein in cells.

Our data indicate that Dronc is a shared substrate for degradation by both the UPS and autophagy. There are not many substrates known which are common to both the UPS and autophagy. *α*-Synuclein is one substrate and mutant forms of this protein appear to poison both the UPS and autophagy causing Parkinson's disease.[68–70] Another shared substrate is inhibitor kappa B alpha (IκB*α*), the inhibitor of the transcription factor nuclear factor kappa B.[71] IκB*α* appears to be degraded by the UPS and autophagy in different cellular compartments with different rates.[71] In addition to these specific shared substrates, misfolded proteins are common substrates for both the UPS and autophagy.[29,72] Whether this relates to Dronc, is currently unknown.

It is unclear what distinguishes Dronc from Diap1 with respect to proteasome-mediated degradation and enhanced autophagy. The alternative question would be why Diap1 is not degraded by enhanced autophagy in proteasome-deficient cells. There may be specific ubiquitylation marks or other post-translational modifications that distinguish between these possibilities. Additional work is necessary to answer these questions in the future.

We observed that proteasome dysfunction triggers apoptosis in some, but not all, mutant cells (Figure 2). Given the accumulation of the anti-apoptotic protein Diap1 in proteasome-deficient cells, it is somewhat surprising that some of them undergo apoptosis. However, many other proteins likely also accumulate in proteasome-deficient cells, which combined will tilt the fate of the affected cells to either survival or death, depending on relative ratios. This consideration may explain why some cells are apoptotic and

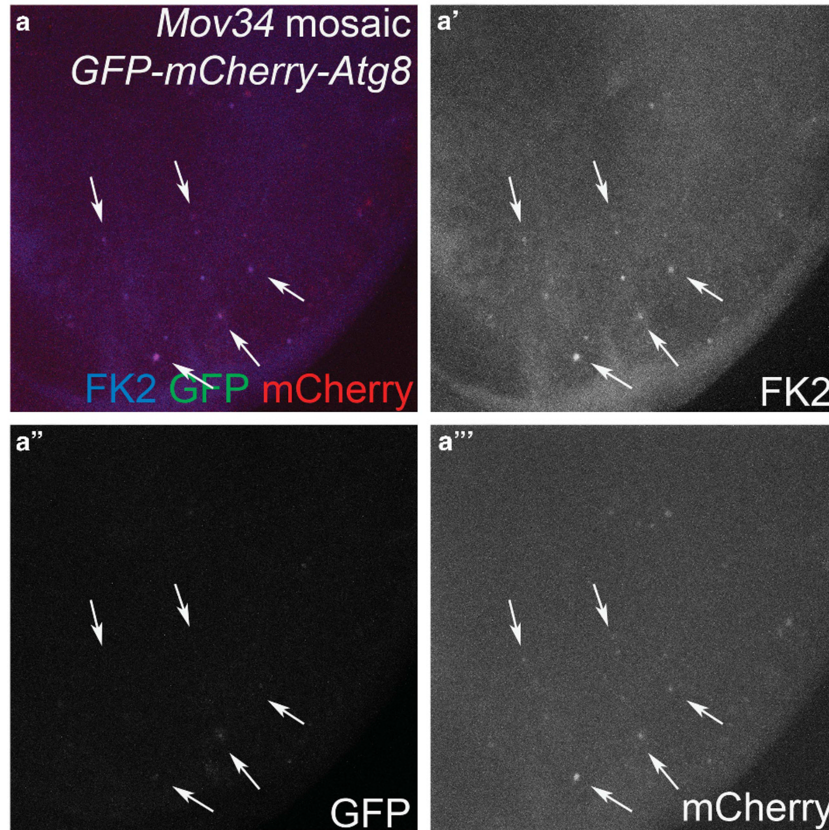

Figure 6 Impaired proteasome function induces autophagic flux. *Mov34* mosaic eye imaginal discs expressing *GFP-mCherry-Atg8a* as marker for autophagic flux. *Mov34* mutant cells were identified by FK2 labeling (blue in **a**; gray in **a'**). White arrows mark representative *Mov34* mutant cells as examples. Although there is little to no GFP labeling in *Mov34* mutant cell clones (**a''**), all clones contain increased mCherry labeling (**a'''**) suggesting that autophagic flux is enhanced in proteasome-deficient cells. Genotype: *ey-FLP*; *FRT42D Mov34^{k08003} /FRT42D ; patg8a> GFP-mCherry-Atg8a*

others are not. Another possibility is that the cCsp-3-negative cells in mutant cell clones will die later or are already dead. Consistent with the latter notion, we have previously shown that dead cells lack protein and DNA,[73] hence the cCsp-3 antibody would not label these cells. Importantly also, although not all proteasome-deficient cell clones may be apoptotic at the time of investigation, they do not grow very well and remain small. These considerations are important for the potential clinical use of proteasome inhibitors for treatment of cancer.

Materials and Methods

Fly stocks. *prosβ2^{EP3067}* is a P element transposon insertion in the first exon at base pair 63.[74] *Mov34^{k08003}* is a P element transposon insertion in the first exon at base pair 263.[65,67] Both insertions disrupt the transcripts. *prosβ2^{DTS7}* encodes a DTS allele of *prosβ2*; however, in this work, we used it in genetic mosaics using *FRT80B DTS7*, not applying a temperature-shift. *Atg7^{d14}* is a mutant allele as described.[16] *UAS-Atg7^{RNAi}* targets *Atg7* by RNAi.[17,75] *UAS-Flag-dronc* encodes a Dronc protein with an N-terminal Flag-tag.[58] It was expressed using *daughterless-Gal4* (*da-Gal4*) in *Mov34* mosaic background (Supplementary Figure S4).

Generation of mutant cell clones. Mutant clones of *prosβ2*, *Mov34* and *Atg7* were induced in eye imaginal discs using the *FLP/FRT*-induced mitotic recombination system using *ey-FLP*.[53,54] For this purpose, mutant alleles of *prosβ2* and *Mov34* were recombined on FRT80B and FRT42D bearing chromosomes, respectively. *FRT42D Atg7^{d14}* was used as described.[16] To generate wild-type control mosaics (Supplementary Figure S2), *ey-Flp*; *FRT42D* P[*ubi-GFP*] was used. Clones are marked by loss of GFP. To induce *Atg7* RNAi in *prosβ2* mutant clones, the MARCM method was used.[63] In this case, mutant cell clones are positively marked by GFP (Figure 5).

Immunohistochemistry. Eye imaginal discs from third instar larvae were dissected using standard protocols[76] and labeled with antibodies raised against the following antigens: ubiquitin (Sigma Aldrich, St. Louis, MO, USA); FK1 and FK2 (Biomol, Hamburg, Germany); cCsp-3 (Cell Signaling Technology, Danvers, MA, USA); Dronc (kind gift of Pascal Meier), Diap1 (a kind gift of Hermann Steller and Hyung Don Ryoo) and Flag (Sigma Aldrich). Cy3-conjugated and Cy-5 fluorescently conjugated secondary antibodies are obtained from Jackson ImmunoResearch (West Grove, PA, USA) and were used at dilutions of 1 : 400. In each experiment, multiple clones in 10–20 eye imaginal discs were analyzed, unless otherwise noted. Images were captured using Olympus Optical FV500 (Waltham, MA, USA) or Zeiss LSM700 confocal microscopes (Peabody, MA, USA).

Generation of the *GFP-mCherry-Atg8a* tandem reporter. A transgene encoding the tandem protein GFP-mCherry-Atg8a was generated and used to determine autophagic flux.[18] A region 2-kb upstream of *Atg8a (CG32672)* was inserted upstream of GFP-mCherry-Atg8a in the pCaSpeR4 *Drosophila* transformation vector, as was previously described for similar GFP-Atg8a and mCherry-Atg8a reporter lines.[77,78] The resulting plasmid pCaSpeR4-promoter-GFP-mCherry-Atg8a was used to generate transgenic *Drosophila* lines using standard procedures.

qPCR of *dronc* transcripts. Late 3rd larval stage (L3) larvae of control (*w^{1118}*) and experimental genotype (*w; UAS-Atg7^{RNAi}; da-Gal4/DTS7*) were shifted

to 29 °C until eclosion of adult flies. mRNA was extracted from 1 to 3 days old flies using RNAeasy (Qiagen, Hilden, Germany) and quantitative polymerase chain reaction (qPCR) was performed with the following *dronc*-specific primers: Nedd2-like caspase forward primer (NcF) 5'-CTCGCTAAACGAACGGAGAAC-3' and Nedd2-like caspase reverse primer (NcR) 5'-CAACGACACCCACATAAGGG-3', as described.[79] Tubulin was used for normalization.

Conflict of Interest

The authors declare no conflict of interest.

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