Dronc-independent basal executioner caspase activity sustains *Drosophila* imaginal tissue growth

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Caspase is best known as an enzyme involved in programmed cell death, which is conserved among multicellular organisms. In addition to its role in cell death, caspase is emerging as an indispensable enzyme in a wide range of cellular functions, which have recently been termed caspase-dependent nonlethal cellular processes (CDPs). In this study, we examined the involvement of cell death signaling in tissue-size determination using *Drosophila* wing as a model. We found that the *Drosophila* executioner caspases Dcp-1 and Decay, but not Drice, promoted wing growth independently of apoptosis. Most of the reports on CDPs argue the importance of the spatiotemporal regulation of the initiator caspase, Dronc; however, this sublethal caspase function was independent of Dronc, suggesting a more diverse array of CDP regulatory mechanisms. Tagging of TurboID, an improved promiscuous biotin ligase that biotinylates neighboring proteins, to the C terminus of caspases revealed the differences among the neighbors of executioner caspases. Furthermore, we found that the cleavage of Acinus, a substrate of the executioner caspase, was important in promoting wing growth. These results demonstrate the importance of executioner caspase-mediated basal proteolytic cleavage of substrates in sustaining tissue growth. Given the existence of caspase-like DEVDase activity in a unicellular alga, our results likely highlight the original function of caspase—not cell death, but basal proteolytic cleavages for cell vigor.

caspase-dependent nonlethal cellular processes | executioner caspase | tissue-size regulation | fluctuating asymmetry | TurboID

Caspase is best known as an enzyme involved in programmed cell death, which is conserved among multicellular organisms (1). The conserved cell death platform in multicellular organisms is an Apaf-1–Caspase-9 complex called apoptosome. A rise in the local concentration of procaspase-9 leads to its autoactivation and subsequent activation of downstream executioner caspases (2). In *Drosophila melanogaster*, the genetic architecture of apoptosis and its regulatory mechanisms are well characterized. Various apoptotic stimuli are transduced for the transcriptional up-regulation of proapoptotic genes *rpr*, *hid*, and *grim* (*RHG*), which antagonize DIAP-1 (mammalian inhibitors of apoptosis protein). This leads to formation of the apoptosome by complexation of Dronc (mammalian Caspase-9) with Dark (mammalian Apaf-1), thereby initiating a proteolytic cascade for activation of executioner caspases, Drice and Dcp-1 (mammalian Caspase-3/6/7), and finally leading to apoptosis (3) (Fig. 1*A*). Although both Drice and Dcp-1 preferentially target primary amino acid sequences of DEVD, Drice is more effective in inducing apoptosis (4). In addition to its role in cell death, caspase is emerging as an indispensable enzyme for a wide range of cellular functions, including partial cell destruction, cell fate determination, and cell migration (3, 5), which have recently been termed caspase-dependent nonlethal cellular processes (CDPs) (6). To date, studies in *Drosophila* caspase have demonstrated the importance of the spatiotemporal regulation of Dronc in various processes, including dendrite pruning (7), cell size expansion (8), apoptosis-induced proliferation (9), and sperm individualization (10, 11). However, events downstream of caspase activation and the mechanisms through which cells avoid death remain largely unexplored.

Tissue-size regulation is one of the most fundamental questions in the field of developmental biology. The size of a tissue is the integrated outcome of cell growth, division, and death. *Drosophila* wing imaginal disc (WD) is a well-established model system for studying tissue-size regulation (12). The WD emerges as a sac of 50 epithelial cells and grows to approximately 50,000 cells by the end of the third instar larval stage, accompanying cell death (13). Mutant flies lacking the irradiation-responsive enhancer region (IRER) of proapoptotic genes show increased wing size. Thus, developmental cell death, or at least *myc* overexpression-induced apoptosis, most likely negatively regulates tissue-size determination (14). However, there is an opposing observation that inhibition of caspase activity by overexpressing *p35* results in wing size reduction (15). Perturbation of cell death machinery has even led to a disturbance of the bilateral symmetry of wing blade sizes (16). Overall, it is likely that the cell death machinery plays a major role in tissue-size tuning and homeostasis; however, the underlying mechanisms remain unknown.

In this study, we examined the involvement of various caspases in wing size determination. We found that the relatively

Significance

Caspase is the enzyme involved in cell death, and its activation via the apoptosome is thought to represent irreversible cellular destruction. Furthermore, accumulating evidence suggests increasingly diverse functions of caspase beyond apoptosis. Here, using *Drosophila* wing as a model, we reveal that the specific executioner caspases, Dcp-1 and Decay, promote, rather than suppress by inducing apoptosis, tissue growth. These executioner caspases act independently of initiator caspase Dronc and apoptosis. We further show that the caspase-mediated cleavage of Acinus is important for sustaining tissue growth. Our research highlights the importance of executioner caspase-mediated basal proteolytic cleavage of substrates during tissue growth, and the findings hint at the original function of caspase—not apoptosis, but basal proteolytic cleavages for cell vigor.

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The authors declare no competing interest.

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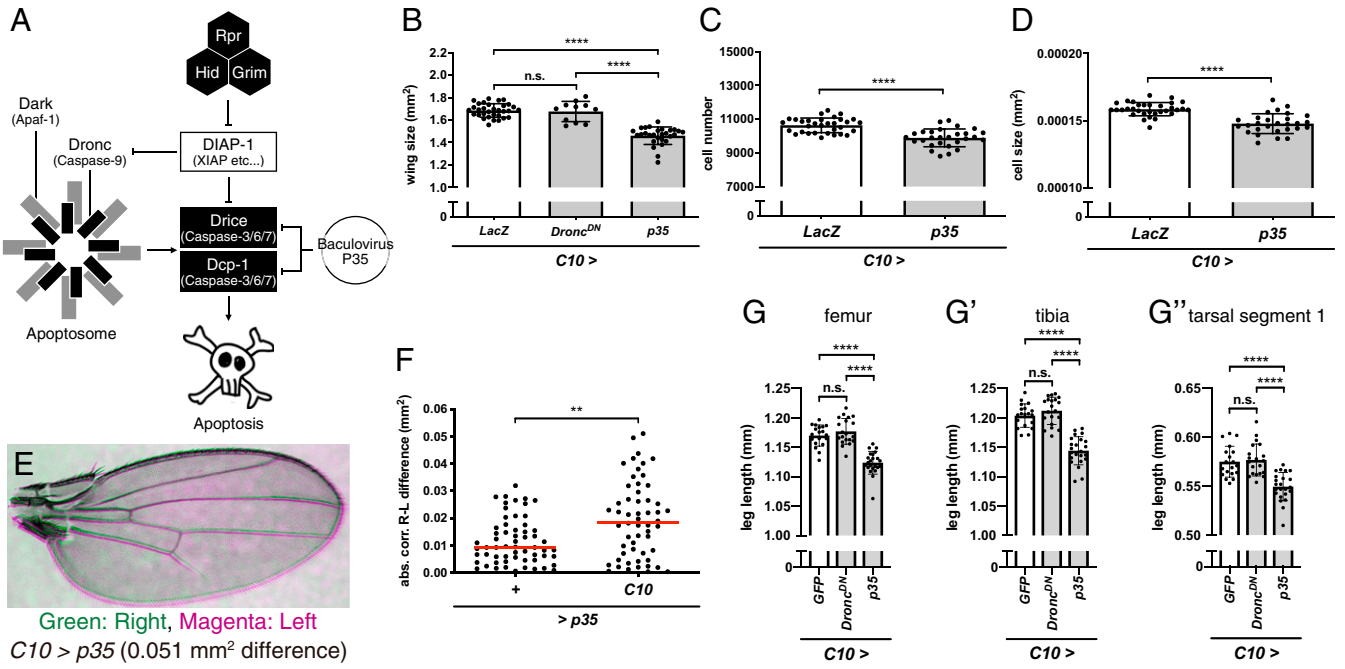

Fig. 1. Cell death signaling inhibition reduces *Drosophila* imaginal tissue size. (*A*) Schematic diagram of *Drosophila* apoptosis signaling. (*B*) Wing sizes under control (*C10 > LacZ*; $n = 32$) and caspase-inhibited conditions (*C10 > Dronc*^{DN}, $n = 11$; *C10 > p35*, $n = 28$). (*C* and *D*) Cell sizes (*C*) and cell numbers (*D*) of *B*. (*E*) Representative image of a bilaterally asymmetric *Drosophila* wing pair of *C10 > p35*. Green, right wing; magenta, left wing. (*F*) Wing size differences between right and left wings within the same flies in control (+ > *p35*, $n = 60$) and caspase-inhibited conditions (*C10 > p35*, $n = 56$). Red bars indicate the median of each group. (*G*) Femur, (*G′*) tibia, and (*G″*) tarsal segment 1 lengths in the control (*C10 > GFP*, $n = 19$) and caspase-inhibited conditions (*C10 > Dronc*^{DN}, $n = 20$; *C10 > p35*, $n = 23$). For all graphs except *F*, data are mean ± SD. Statistical analyses were performed with Tukey's multiple comparison test after 1-way ANOVA (*B*, *G*, *G′*, and *G″*), unpaired Student's *t* test (*C* and *D*), or the Mann–Whitney *U* test (*F*). n.s., $P > 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$. n.s., not significant.

minor *Drosophila* executioner caspases Dcp-1 and Decay, but not Drice, promoted wing growth independently of apoptosis. In addition, we found that the basal cleavage of one of the executioner caspase substrates, Acinus (Acn), was important in promoting wing growth. Collectively, our findings highlight the importance of executioner caspase-mediated basal proteolytic cleavage of substrates in sustaining tissue growth.

Results

Executioner Caspase Activity Inhibition Leads to Reduction in Wing and Leg Size. The WD experiences caspase activation, which induces both apoptosis and CDPs, during development (13, 17). To examine the contribution of caspase activation to wing growth, we first inhibited caspase activity by overexpressing either *p35*, a baculovirus-derived caspase inhibitor, or *Dronc Dominant Negative form* (*Dronc*^{DN}) (*Drosophila* apoptosis signaling in Fig. 1*A*) in the entire WD using a *C10-Gal4* driver (*SI Appendix*, Fig. S1) and examined their effects on wing size (*SI Appendix*, Fig. S2*A*). We found that wing size decreased significantly under *p35* overexpression but did not change under *Dronc*^{DN} overexpression (Fig. 1*B*). We also checked the cell number and cell size (*SI Appendix*, Fig. S2*B*) and found that both the cell size and number decreased under *p35* overexpression (Fig. 1 *C* and *D*). Interestingly, comparison of the bilateral symmetry of wing size in *C10 > p35* caspase-inhibited flies revealed increased bilateral asymmetry (Fig. 1 *E* and *F*). On the other hand, we found no reduction in overall body size under *p35* overexpression (*SI Appendix*, Fig. S3 *A* and *B*). In addition, there was no difference in the developmental duration of larval stage in

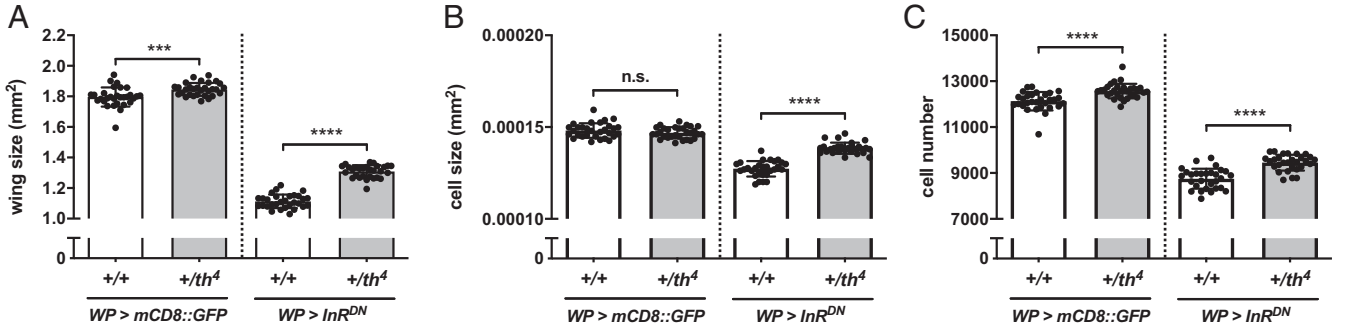

Fig. 2. Enhanced cell death signaling increases *Drosophila* wing size. (*A*) Wing size in control (*WP > mCD8::GFP*, $n = 30$; *th*^{4}, *WP > mCD8::GFP*, $n = 30$) and wing size-reduced conditions (*WP > InR*^{DN}, $n = 30$; *th*^{4}, *WP > InR*^{DN}, $n = 29$). (*B* and *C*) Cell sizes (*B*) and cell numbers (*C*) of *A*. For all graphs, data are mean ± SD. Statistical analyses were performed with unpaired Student's *t* test. n.s., $P > 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$. n.s., not significant.

p35-overexpressing flies (*SI Appendix*, Fig. S3*C*). Taken together, these results suggest that caspase mainly regulates the growth rate of the WD in a tissue-autonomous manner, without affecting the duration of larval development or entire body size.

We also inhibited caspase activity by knocking down the *RHG* genes (Fig. 1*A*), using *UAS-RHG microRNA* (*UAS-miRHG*). Wing size decreased on knockdown of *RHG* genes (*SI Appendix*, Fig. S4*A*). In this condition, cell numbers decreased, whereas cell size increased slightly (*SI Appendix*, Fig. S4 *B* and *C*). Thus, these results, together with those of *p35* overexpression, suggest that caspase promotes wing growth mainly by regulating cell number. We further tested the effect of caspase inhibition on the length of hind legs (*SI Appendix*, Fig. S2*C*). We found that leg length decreased under *p35* overexpression but not under $Dronc^{DN}$ overexpression (Fig. 1 *G*–*G*″). We also found that leg length decreased on knockdown of *RHG* genes (*SI Appendix*, Fig. S4 *D*–*D*″). These results support the notion that the growth-promoting effect of caspase is rather general, at least in imaginal tissues. Furthermore, we examined the effect of caspase inhibition on WD size. Using *apterous-Gal4* (*ap-Gal4*), which is expressed in the dorsal part of wing pouch, we found that *p35* overexpression significantly reduced the volume of the dorsal part of the WD (*SI Appendix*, Fig. S5 *A*–*C*). Thus, we concluded that caspase activity promotes WD growth.

Increased Caspase Activity in *diap1* Heterozygous Mutant Results in Increased Wing Size. To further validate that caspase activity promotes wing growth, we attempted to increase caspase activity without inducing massive apoptosis. We used a *diap1* heterozygous mutant of the th^4 allele, which is known to exhibit increased caspase activity for both apoptosis and CDPs in WD (18). As we expected, the *diap1* mutant showed increased wing size (Fig. 2*A*). Using *WP-Gal4*, which is expressed in the wing pouch of the WD (19), we overexpressed *Insulin Receptor Dominant Negative form* (InR^{DN}), which leads to a significant reduction in wing size, to sensitize the effect of caspase on tissue growth (Fig. 2*A*). The growth-promoting effect of caspase was more evident in the sensitized condition (Fig. 2*A*). Cell size increased only in the sensitized condition (Fig. 2*B*), while cell number increased in the *diap1* heterozygous mutant in both normal and sensitized conditions (Fig. 2*C*). This result further supports the idea that caspase activity promotes the growth of imaginal tissue by increasing cell number and, in part, cell size.

Executioner Caspases Dcp-1 and Decay Are Responsible for Wing Growth. To gain mechanical insights into the observed phenomena, we screened the responsible caspase(s), as *p35* overexpression and *RHG* knockdown simultaneously inhibit the activation of multiple caspases (20) (Fig. 1*A*). *D. melanogaster* is known to have 7 caspases (1). We used the curly-up wing phenotype and the opaque wing phenotype as indicators of growth inhibition and apoptosis inhibition, respectively (more details in *SI Appendix*, Fig. S6 *A*–*F*). From the RNA interference (RNAi) screening, we identified Dcp-1 and Decay, but not Dronc or Drice, as the most prominent candidates of growth-promoting caspases (more details in *SI Appendix*, Figs. S6 *G*–*L* and S7). Of note, while *dark*, *dronc*, and *drice* RNAi showed an opaque wing phenotype without the curly wing phenotype, *dcp-1* and *decay* RNAi showed the curly wing phenotype without the opaque wing phenotype, suggesting the Dronc- and apoptosis-independent nature of the wing growth-promoting effect. To evaluate the screening results, we directly examined the growth-promoting effects of Dark, Dronc, Drice, Dcp-1, and Decay on wing sizes using *C10-Gal4*. Introduction of *C10-Gal4* resulted in 2.48% (against w^{1118}) and 2.52% (against *LacZ* RNAi) reductions in wing size compared with *No-Gal4* control (Fig. 3 *A* and *B*). Similar extents of reduction were observed with *dark*, *dronc*, and *drice* RNAi (2.28%, 1.98%, and 3.31% respectively; Fig. 3 *A* and *B*). In contrast, *dcp-1* and *decay* RNAi led to large reductions in wing size (7.07%, 5.64%, and 4.92%, respectively; Fig. 3 *A* and *B*). Importantly, Dcp-1 is relatively minor caspase for apoptosis compared with Drice (4). In addition, Decay is not required for apoptosis in WD (21). Overall, our screening results support the idea that the non-apoptotic caspase activity of *Drosophila* cell death signaling, especially Dcp-1 and Decay, but not Drice or Dronc, is required for promoting wing growth.

Growth-Promoting Effect of Executioner Caspase Activity Is Independent of Apoptosis. Next, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to examine the involvement of apoptosis for promoting wing growth. Compared with *LacZ* RNAi (*SI Appendix*, Fig. S8 *A* and *G*), *p35* overexpression and *RHG* genes RNAi showed reduced TUNEL signals (*SI Appendix*, Fig. S8 *B*, *C*, and *G*), while *dcp-1* RNAi and *decay* RNAi showed no significant reduction (*SI Appendix*, Fig. S8 *D*–*G*). We also used the genetically encoded mCD8::PARP::VENUS probe (7) to detect strong DEVDase activity in the WD (4, 18). Expression of the probe in the entire WD using *C10-Gal4* yielded similar results to

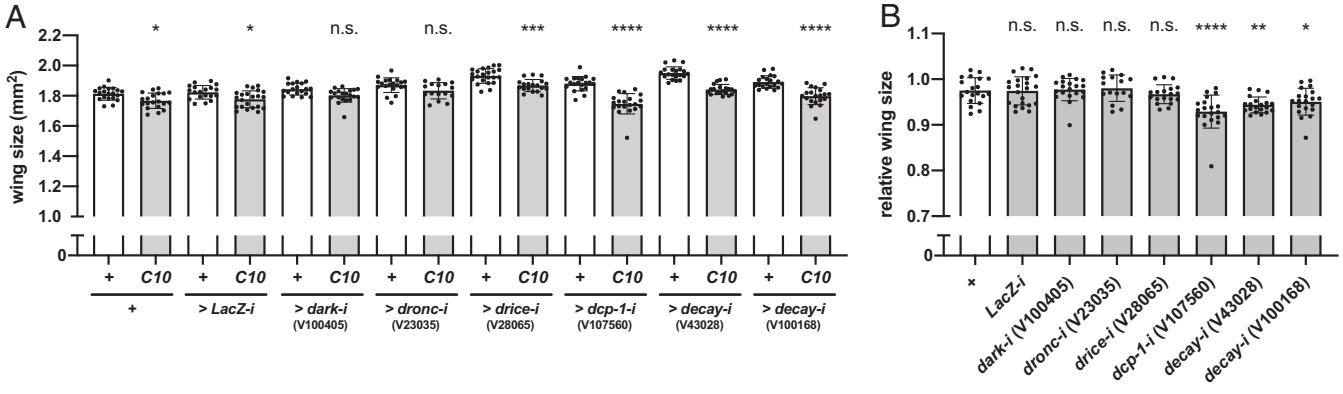

Fig. 3. Dcp-1 and Decay, but not Dronc or Drice, are required for promoting wing growth. (*A*) Wing size in *No-Gal4* controls (+ > +, $n = 20$; + > *LacZ-i*, $n = 19$; + > *dark-i*^{V100405}, $n = 19$; + > *dronc-i*^{V23035}, $n = 20$; + > *drice-i*^{V28065}, $n = 22$; + > *dcp-1-i*^{V107560}, $n = 20$; + > *decay-i*^{V43028}, $n = 20$; + > *decay-i*^{V100168}, $n = 20$) and RNAi groups (*C10* > +, $n = 20$; *C10* > *LacZ-i*, $n = 22$; *C10* > *dark-i*^{V100405}, $n = 20$; *C10* > *dronc-i*^{V23035}, $n = 16$; *C10* > *drice-i*^{V28065}, $n = 19$; *C10* > *dcp-1-i*^{V107560}, $n = 20$; *C10* > *decay-i*^{V43028}, $n = 20$; *C10* > *decay-i*^{V100168}, $n = 20$) for screening results validation. (*B*) Relative wing sizes of *A* normalized to the mean of corresponding *No-Gal4* control. For graphs *A* and *B*, data are mean ± SD. Statistical analyses were performed with unpaired Student's *t* test with Bonferroni correction for *A* and Dunnett's multiple comparison test against control (*C10* > +) after 1-way ANOVA for *B*. n.s., $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. n.s., not significant.

those of the TUNEL experiment; compared with *LacZ* RNAi (*SI Appendix*, Fig. S8 *H* and *N*), the signals were sharply reduced following *p35* overexpression and *RHG* RNAi (*SI Appendix*, Fig. S8 *I*, *J*, and *N*) and showed no reduction with *dcp-1* and *decay* RNAi (*SI Appendix*, Fig. S8 *K*–*N*). These results showed no correlation between the amount of apoptosis and wing size reduction, suggesting that the growth-promoting effect of executioner caspase activity is independent of its function in cell death.

Executioner Caspase Activity Exists Independently of Dronc during Larval Development. It is widely accepted that executioner caspase activation requires activation of the initiator caspase, Dronc. However, our results suggest that Dronc is not involved in the wing growth phenotype. This raises the possibility that the executioner caspases, Dcp-1 and Decay, might be activated independently of Dronc. To test this hypothesis, we took advantage of caspase activity probe SCAT3 (22) (Fig. 4*A*) and examined whether the cleaved SCAT3 bands were present in the $dronc^{\Delta A8}$ null mutant. We first confirmed that weak cleaved SCAT3 probe bands were detected on UV irradiation (*SI Appendix*, Fig. S9). Using a 10-fold higher concentration of the anti-myc antibody, we found faint cleaved SCAT3 bands even in the absence of Dronc (Fig. 4*B*). The detected bands got stronger on UV irradiation, were weaker (or even absent) by expressing *p35*, and were not detected in $SCAT3^{DEVG}$-negative control probe, all supporting the idea that the detected bands were produced by the proteolytic cleavage by DEVDases (Fig. 4*B*). These results show that executioner caspase can be basally activated in the absence of Dronc.

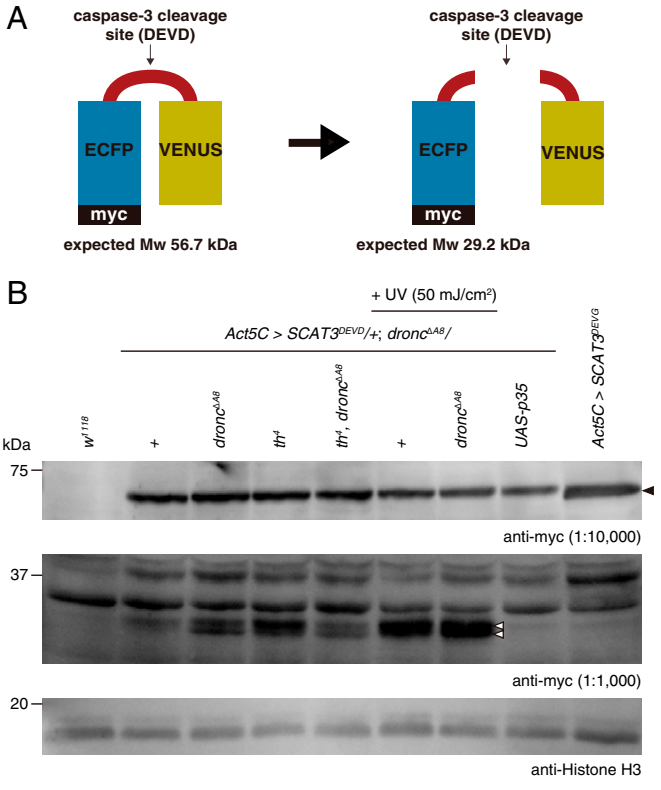

Fig. 4. Dronc-independent executioner caspase activity in *Drosophila*. (*A*) Schematic diagram of SCAT3 probe. (*B*) Western blotting against full-length SCAT3 probe (anti-myc antibody, dilution 1:10,000) and cleaved SCAT3 probe (anti-myc antibody, dilution 1:1,000) in the absence of Dronc. Genotypes are described in the figure. Black arrowhead indicates full-length SCAT3; white arrowheads, cleaved SCAT3.

Dcp-1, Drice, and Dronc Are Ubiquitously Expressed throughout the WD with Different Neighboring Proteins. Our results so far showed the potential differential activity of caspases on cellular phenomena. While Dronc and Drice efficiently induce apoptosis, Dcp-1 and Decay promote wing growth. To elucidate the cause of functional differences among caspases, we established V5::TurboID (23) knockin fly lines against Dcp-1, Drice, and Dronc at their C termini (Fig. 5 *A*–*C*). TurboID is an improved promiscuous biotin ligase applicable to *Drosophila* that biotinylates the proximal proteins (23). We first examined the spatial expression patterns of the 3 caspases during wing development. Staining with an anti-V5 antibody weakly detected the expression of Drice, Dcp-1, and Dronc in the WDs and pupal wings (Fig. 5 *D*–*I* and *SI Appendix*, Fig. S10 *A*–*H*). Importantly, staining with streptavidin produced highly improved signals for all 3 caspases, which were depleted on RNAi (Fig. 5 *D*–*I* and *SI Appendix*, Fig. S10 *A*–*H*). Western blot analysis confirmed high Drice and Dcp-1 expression and lower Dronc expression in the WDs (Fig. 5*J*). Importantly, streptavidin blotting to visualize the biotinylated proteins revealed the nonidentical patterns among the caspases (Fig. 5*J*). Of note, some bands were detected only in Dcp-1 but not in Drice and vice versa (Fig. 5*J*). These results suggest that functional differences among executioner caspases might originate from the differences in their neighboring proteins.

Caspase-Dependent Cleavage of Acn Promotes Wing Growth. To further characterize the molecular mechanisms downstream of caspase activation, we first determined the caspase inhibition-related changes in the overall transcriptome. However, we could not identify the change in the major signaling pathway for promoting tissue growth (more details in *SI Appendix*, Fig. S11). Thus, we looked at the caspase substrates. Acn, a nuclear protein that regulates alternative splicing (24) and basal autophagy (25), is reported to be a substrate for executioner caspase in *Drosophila* (26). A previous genetic analysis suggested that Acn is regulated mainly by Dcp-1, but not by Drice and Dronc (26). We examined the effect of Acn cleavage on wing size (Fig. 6*A*). Wing size measurements revealed a slight reduction in wing size in the caspase-cleavage resistant acn^{D527A}-carrying flies compared with control flies (Fig. 6*B*). We further tested the genetic interaction between *acn* and caspase activity. Introduction of *diap-1* heterozygous mutation significantly increased the difference in wing size between in acn^{WT} and acn^{D527A} flies (Fig. 6*B*). These results support the idea that the basal caspase activity of Dcp-1, and possibly that of Decay, restricts the basal proteolytic cleavage of Acn to sustain basal tissue growth.

As Acn is known to induce basal autophagy, we further tested whether the curly-up wing phenotype caused by *dcp-1* and *decay* RNAi can be rescued by the simultaneous knockdown of genes involved in autophagy. We performed RNAi of several autophagy-related genes, including *atg3*, *7*, *9*, *13*, and *18a*. We observed a partial rescue of the *dcp-1* and *decay* RNAi curly-up wing phenotype by some of the RNAi lines, including *atg18a* RNAi (*SI Appendix*, Fig. S12 *A*–*E*), suggesting the partial involvement of autophagy in Dcp-1- and Decay-mediated growth. Overall, our results suggest the importance of caspase-mediated basal proteolytic cleavage of their substrates, including Acn, to sustain basal tissue growth (*SI Appendix*, Fig. S13).

Discussion

The role of executioner caspases in promoting tissue growth is highly conserved among higher multicellular organisms. Mammalian studies showed that intrinsic cell death pathway-mediated caspase activation is essential for cardiomyocyte hypertrophy (27) and that the myocyte number is reduced in *caspase-3* and *caspase-7* double-knockout mice (28). Most recently, it has been shown in mouse sebocytes that caspase-3 mediates cell proliferation via the activation of YAP through the cleavage of α-catenin (29). Here we report an alternative mechanism in

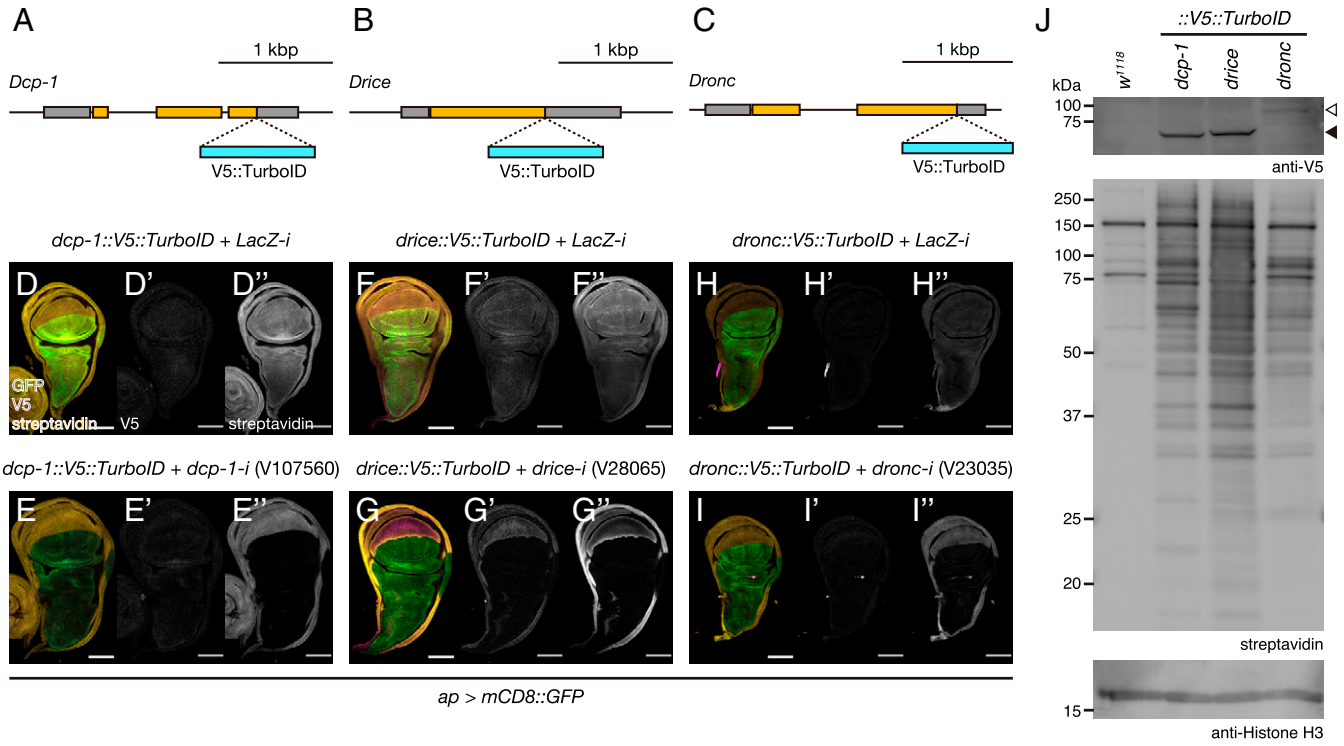

Fig. 5. The expression patterns of caspases during wing development. (*A*–*C*) Schematic diagrams of *dcp-1* (*A*), *drice* (*B*), and *dronc* (*C*) gene loci. TurboID with V5-tag was knocked in right before the stop codon. (*D*–*I*) Expression patterns of Dcp-1 (*D* and *E*), Drice (*F* and *G*), and Dronc (*H* and *I*) in WDs. (*Left*) Merged image of GFP (green), V5 (magenta), and streptavidin (yellow) staining. (*Middle*) V5 (gray). (*Right*) Streptavidin (gray). (Scale bar: 100 μm.) *dcp-1::V5::TurboID, ap > mCD8::GFP, LacZ-i* (*D*); *dcp-1::V5::TurboID, ap > mCD8::GFP, dcp-1-i^{V107560}* (*E*); *drice::V5::TurboID, ap > mCD8::GFP, LacZ-i* (*F*), *drice::V5::TurboID, ap > mCD8::GFP, drice-i^{V28065}* (*G*); *dronc::V5::TurboID, ap > mCD8::GFP, LacZ-i* (*H*); *dronc::V5::TurboID, ap > mCD8::GFP, dronc-i^{V23035}* (*I*). (*J*) Western blotting against V5-tagged caspases and their potential neighboring (biotinylated) proteins. The white arrowhead indicates Dronc::V5::TurboID; black arrowhead, Dcp-1::V5::TurboID and Drice::V5::TurboID.

which the Dronc-independent basal caspase activity promotes tissue growth, partly via the cleavage of Acn. We also confirmed that cell death signaling is related to wing bilateral asymmetry. Although cell death is currently thought to be important in adjusting bilateral asymmetry (16), our findings suggest the possibility that the growth-promoting effect of caspase might be important for achieving robust wing size. Our inference is supported by a report that dysregulation of growth regulating signals also resulted in bilaterally asymmetric body appendages (30).

The observation that executioner caspase could exert nondestructive proteolytic activity in the absence of the apoptosome component Dronc is noteworthy, as most of the observed caspase activation in both apoptosis and CDPs in *Drosophila* is acquired via Dronc. Given that the Dronc-independent basal caspase activity was not high enough to induce apoptosis, we believe that this is an alternative mechanism for cells to escape from cell death. Identifying how basal executioner caspase activity is regulated, especially from the perspective of posttranslational modification, is important for future research (*SI Appendix*, *Discussion*).

In this paper, we report the physiological function of Dronc-independent basal caspase activity in vivo using one of the caspase substrates, Acn, as an example. The mechanism through which stabilized Acn reduces wing size could not be determined in this study (*SI Appendix*, *Discussion*). Our findings also highlight the importance of precise analysis of caspase substrates in vivo. While more than 400 caspase proteolytic targets have been identified by degradomes in mammals (31, 32), there could still be many uncharacterized substrates with nonapoptotic caspase functions. In addition, we showed that executioner caspases have functional specificity for CDPs, possibly because of the difference in substrate specificity. A previous study has also shown that human executioner caspases differ in their substrate specificity (33). Such substrate specificity seems to be partly acquired via caspases' neighboring proteins; for example, CRINKLED contributes to the substrate specificity of Dronc (34). Furthermore, the neighboring proteins of caspases also regulate their

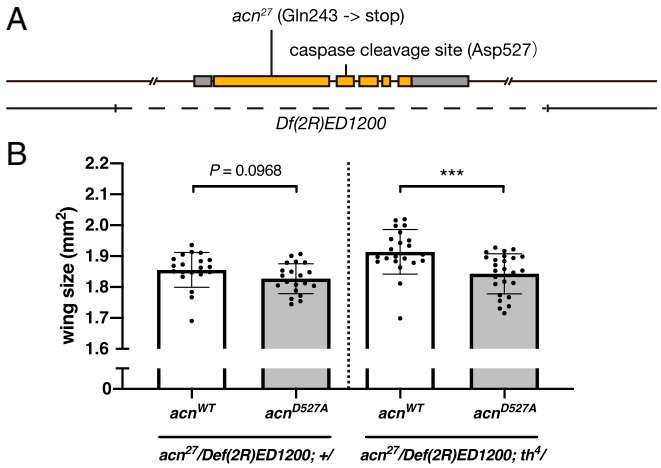

Fig. 6. Caspase-mediated cleavage of Acn increases wing size. (*A*) Schematic diagram of the *acn* gene locus. (*B*) Wing size in *acn^{WT}* (*acn^{27}/Df(2R)ED1200; acn^{WT}/+*, $n = 20$; *acn^{27}/Df(2R)ED1200; acn^{WT}/th^{4}*, $n = 22$), and *acn^{D527A}* (*acn^{27}/Df(2R)ED1200; acn^{D527A}/+*, $n = 20$; *acn^{27}/Df(2R)ED1200; acn^{D527A}/th^{4}*, $n = 25$) flies. Data are mean ± SD. Statistical analyses were performed using an unpaired Student's *t* test. ***$P < 0.001$.

localization; for example, Myo1D is needed for Dronc translocation to the plasma membrane (9). In this study, we generated TurboID knockin *Drosophila* lines for the major apoptotic caspases Dcp-1, Drice, and Dronc; the established fly lines facilitated examination of the molecular mechanisms of cell death and CDPs from the perspective of the innate neighboring proteins.

Our findings highlight some important aspects of caspase function from the standpoint of evolution and emergence of apoptosis. In metazoans, apoptosis requires a regulated, rapid, and strong activation of executioner caspase in the whole cytosolic fraction. In this case, the apoptosome—caspase activation and recruitment domain (CARD)-mediated Apaf-1/caspase-9 complex—plays a pivotal role in initiating the caspase-activating cascade. Nonmetazoans, including choanoflagellates, the closest living relatives of the metazoans, lack both the CARD domain and caspase (35); however, biochemical analysis shows the existence of caspase-like DEVDase activity in the unicellular alga *Dunaliella viridis* even in the absence of the CARD domain (36). These lines of evidence suggest that the original roles of caspase, or DEVDase, could be independent of the apoptosome and apoptosis. Thus, our present findings hint at the original function of executioner caspase in basal proteolytic cleavages for cell vigor, which in turn sheds light on the function of the apoptosome as an efficient cell death inducer acquired during the evolution of unicellular organisms into multicellular organisms.

In conclusion, we found that executioner caspase, originally identified as a cell death enzyme, promotes wing growth independently of cell death. Our data show that the basal caspase activity could be Dronc-independent; this result is in sharp contrast with the apoptotic function as well as with the non-apoptotic functions of caspase that have been revealed so far. Because the basal cleavage of Acn was in part responsible for promoting wing growth, our research highlights the importance of executioner caspase-mediated basal proteolytic cleavages of substrates in promoting tissue growth.

Materials and Methods

Detailed information on fly strains and rearing conditions (including detailed genotypes of the flies used in the figures); generation of *caspase::V5::TurboID* knockin alleles using CRISPR/Cas9 (including the sequence of pBac[3xP3-DsRed_polyA_Scarless_TK]); Gal4 expression check; wing size, cell size, cell number, and leg length measurements; pupal size measurement; fluctuating asymmetry measurement; pupation time measurement; curly up and opaque (cell remaining) fly wing scoring and screening; immunohistochemistry; quantification of wing imaginal disc volume; TUNEL assay; quantification of the TUNEL and cPARP ratio; Western blot analysis; microarray sample preparation and analysis; and statistical analysis are provided in *SI Appendix, Materials and Methods*.

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