

Executioner caspase is proximal to Fasciclin 3 which facilitates non-lethal activation in *Drosophila* olfactory receptor neurons

Masaya Muramoto, Nozomi Hanawa, Misako Okumura, Takahiro Chihara, Masayuki Miura ,
Natsuki Shinoda 

Department of Genetics, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan • Program of Biomedical Science, Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8526, Japan • Program of Basic Biology, Graduate School of Integrated Sciences for Life, Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8526, Japan

 https://en.wikipedia.org/wiki/Open_access

 Copyright information

Abstract

The nervous system undergoes functional modification independent on cell turn over. Caspase participates in reversible neuronal modulation via non-lethal activation. However, the mechanism that enables non-lethal activation remains unclear. Here, we analyzed proximal proteins of *Drosophila* executioner caspase in the adult brain using TurboID. We discovered that executioner caspase Drice is, as an inactive proform, proximal to cell membrane proteins, including a specific splicing isoform of cell adhesion molecule Fasciclin 3 (Fas3), Fas3G. To investigate whether sequestration of executioner caspase to plasma membrane of axons is the mechanism for non-lethal activation, we developed a Gal4-Manipulated Area-Specific CaspaseTracker/CasExpress system for sensitive monitoring of caspase activity near plasma membrane. We demonstrated that *Fas3G*-overexpression promotes caspase activation in olfactory receptor neurons without killing them, by inducing expression of initiator caspase Dronc, which also comes close to Fas3G. Physiologically, *Fas3G*-overexpression facilitated non-lethal activation suppresses innate olfactory attraction behavior. Our findings suggest that subcellularly-restricted caspase activation, defined by caspase proximal proteins, is the mechanism for non-lethal activation, opening the methodological development of reversible modification of neuronal function via regulating caspase proximal proteins.

eLife assessment

This **important** study combines multiple techniques to investigate how caspase activity regulates non-lethal caspase-dependent processes. Through a combination of various approaches, and the development of new techniques, the authors provide **compelling** evidence supporting the claim that Fas3G-overexpression promotes non-lethal caspase activation in olfactory receptor neurons.

<https://doi.org/10.7554/eLife.99650.1.sa2>

Introduction

During and even after development, the nervous system undergoes functional modification usually not depending on cell turn over. Caspase, best known as a cysteine aspartic acid protease involved in cell death, participates neuronal functional modulation through non-lethal activation (Mukherjee and Williams, 2017 [↗](#)). In zebrafish embryo, non-lethal caspase activity promotes axon arbor dynamics in retinal ganglion cells (Campbell and Okamoto, 2013 [↗](#)). In *Drosophila*, caspase activation promotes engulfment of pruned dendrites during metamorphosis (Williams et al., 2006 [↗](#)). Furthermore, non-lethal caspase activity promotes the maturation of olfactory sensory neurons during development in mice (Ohsawa et al., 2010 [↗](#)). In the mature nervous system, non-lethal caspase activity induces long-term depression by AKT cleavage in mice (Li et al., 2010 [↗](#)). These processes are termed caspase-dependent non-lethal cellular processes (CDPs) (Aram et al., 2017 [↗](#)). While a growing number of CDPs have been identified (Nakajima and Kuranaga, 2017 [↗](#)), relatively less is known about its regulatory mechanisms that enable specific non-lethal functions without causing cell death especially in nervous systems (Mukherjee and Williams, 2017 [↗](#)). One potential mechanism is the restriction of the extent and spread of activated caspases (Mukherjee and Williams, 2017 [↗](#)), yet experimental evidence is limited.

Proteins that interact with initiator caspases are among the key regulators of CDPs. Tango7, the *Drosophila* translation initiation factor eIF3 subunit m, acts as an adapter and regulates the localization of the initiator caspase Dronc. During sperm individualization, Tango7 directs Dronc to the individualization complex (D'Brot et al., 2013 [↗](#)). In salivary glands, Tango7 directs Dronc to the plasma membrane (Kang et al., 2017 [↗](#)). In both cases, caspase activity promotes cellular remodeling without inducing cell death (D'Brot et al., 2013 [↗](#); Kang et al., 2017 [↗](#)). CRINKLED, a *Drosophila* unconventional myosin, functions as an adaptor of Dronc and facilitates the cleavage of Shaggy46 (Orme et al., 2016 [↗](#)). Shaggy46, activated by caspase-mediated cleavage, suppresses the emergence of ectopic sensory organ precursors (Kanuka et al., 2005 [↗](#)). During apoptosis-induced proliferation (AiP), Myo1D, another *Drosophila* unconventional myosin, facilitates Dronc translocation to the basal side of the plasma membrane of imaginal discs to promote AiP (Amcheslavsky et al., 2018 [↗](#)). Thus, initiator caspase-interacting proteins regulate caspase activity in CDPs. However, given the importance of executioner caspases, which are activated by the initiator caspase to cleave a broad range of substrates and are essential for apoptosis (Julien and Wells, 2017 [↗](#); Kumar, 2007 [↗](#)), little is known about the subcellular compartmentalization and interacting proteins of those involved in CDPs. This lack of understanding is partly due to the absence of protein-protein interaction domains (e.g., DED and CARD) in the N-terminus of executioner caspases (Kumar et al., 2022 [↗](#)).

To understand the molecular mechanism regulating caspase activity involved in CDPs, we focused on the proximal proteins of executioner caspases using TurboID, a proximity labeling technique that analyzes protein proximity *in vivo* (Branon et al., 2018 [↗](#); Qin et al., 2021 [↗](#)). We previously

established C-terminal TurboID knocked-in caspase fly lines for Dronc (initiator), Drice (executioner), and Dcp-1 (executioner) (Shinoda et al., 2019). Importantly, we have demonstrated that one of the preferred substrates of Drice, BubR1, is proximal to Drice *in vivo* (Shinoda et al., 2023), suggesting that substrate preference is exploited by protein proximity. Therefore, the proximity of executioner caspases to proteins is emerging as a means of regulating specific non-lethal functions.

To investigate the regulatory mechanisms of non-lethal function of caspase in the nervous system, we conducted comprehensive analysis of proximal proteins of executioner caspase Drice. We discovered that Drice is, as an inactive proform, primarily proximal to cell membrane proteins, including cell adhesion molecule Fasciclin 3 (Fas3). Notably, Drice is proximal to the specific alternative splicing isoforms of Fas3, Fas3G. To ascertain whether Fas3G modulates caspase activity, we developed a Gal4-Manipulated Area Specific CaspaseTracker/CasExpress (MASCAT) system, which permits the monitoring of caspase activity near plasma membrane with high sensitivity and simultaneous genetic manipulation in the cells of interest. Using MASCAT, we demonstrated that *Fas3G* overexpression enhances caspase activation without killing olfactory receptor neurons through the induction of the expression of initiator caspase Dronc, which also comes proximal to Fas3G. Subsequently, we showed that *Fas3G* overexpression-facilitated non-lethal caspase activation in olfactory receptor neurons suppresses innate olfactory attraction behavior. Collectively, our findings suggest that caspase activation is subcellularly restricted, the platform of which is defined by caspase proximal proteins, for non-lethal functions. In contrast to lethal activation, suppressing neuronal activity with non-lethal caspase activation is reversible. By regulating caspase proximal proteins, reversible modification of neuronal functional by non-lethal caspase activation will be achieved.

Results

Drice is the major executioner caspase expressed in the adult brains

Apoptotic stimuli activate initiator caspases that trigger a proteolytic cascade, leading to the activation of executioner caspases and culminating in apoptosis (Green, 2019). In *Drosophila*, the caspase-mediated apoptotic signaling pathway is highly conserved (Figure 1A) (Nakajima and Kuranaga, 2017). The activity of executioner caspases, specifically Drice and Dcp-1, is regulated by apoptotic signaling pathways, including the initiator caspase Dronc (Figure 1A). To investigate the expression patterns of *Drosophila* caspases, we employed *Caspase::V5::TurboID* knocked-in fly lines (Shinoda et al., 2019). We found low levels of expression of the initiator caspase Dronc in adult heads (Figure 1B). Conversely, we observed high expression of the executioner caspase Drice, whereas Dcp-1 was expressed at lower levels in adult heads (Figure 1B). The proximal proteins of each caspase were labeled with TurboID in adult heads by administration of 100 μ M biotin (Figure 1B). Furthermore, we investigated the expression patterns of caspases using histochemical analysis by examining streptavidin (SA) signal. Consistent with the western blotting findings, Drice showed higher expression levels than Dronc and Dcp-1, which were expressed at lower levels (Figure 1C). We found that Drice was expressed in distinct regions, including the mushroom bodies (MBs), suboesophageal ganglions (SGs), optic lobes (OLs), and antennal lobes (ALs), which showed the highest levels of expression (Figure 1C). The *Drosophila* ALs comprise synaptic contacts between the axons of olfactory receptor neurons (ORNs), the dendrites of projection neurons (PNs), and local interneuron (LNs) processes. Neuron-type-specific Gal4s were used to knockdown *Drice* and the expression patterns were subsequently examined. We found that Drice was expressed predominantly in ORNs in the AL (Figure 1D). These results showcase that Drice is not expressed in the entire brain, but rather in a specific subset of neurons in the adult brains. Comparisons of the expression patterns of Drice were

consistent across both males and females (**Figure 1–figure supplement 1A**), indicating an absence of sex-based differences, which justifies the use of males for functional simplicity in this study.

Drice is proximal to cell membrane proteins rather than cytosolic proteins in the adult brains

To comprehensively identify proximal proteins of Drice, we employed a proximity labeling approach. Specifically, we used *Drice::V5::TurboID* knocked-in flies fed 100 μ M biotin. We confirmed biotinylation of Drice proximal proteins in the adult brains (**Figure 1E**). After purifying the biotinylated proteins using NeutrAvidin magnetic beads, we performed LC-MS/MS analysis of the samples (**Figure 1F**). We identified 643 proteins (**Figure 1G**, Table S1), of which 158 were highly specific to *Drice::V5::TurboID* flies compared to wild-type flies (*Drice::V5::TurboID/w*¹¹¹⁸ > 10; **Figure 1G**, Table S1). Gene ontology analysis revealed that Drice proximal proteins were enriched in the plasma membrane, synaptic vesicles, postsynaptic membranes, and adherens junctions (**Figure 1H**, Table S2). This finding suggests that Drice is localized primarily in cell membrane compartments rather than in the cytoplasm of the adult brain. Additionally, because the SA signal of *Drice::V5::TurboID* is mainly observed in the AL where the axons of ORNs project (**Figure 1C**), the enrichment of cell membrane proteins in the proximal proteins of Drice is reasonable.

A specific splicing isoform of Fasciclin 3 is in proximity to Drice

During our analysis of proteins proximal to Drice, we discovered that the expression patterns of Fasciclin 3 (Fas3) in the adult brain closely resembled those of Drice, especially within the OLs, SGs, and ALs (**Figure 2A**, B). Thus, we focused on Fas3 (Chiba et al., 1995), a transmembrane protein containing immunoglobulin-like domains that regulates synaptic target recognition and axon fasciculation (Kose et al., 1997). Fas3 is encoded by seven splicing isoforms, resulting in the production of five protein isoforms that share extracellular domains but differ in intracellular domains with low-complexity sequences (**Figure 2C**, D). Only one specific protein isoform, Fas3 isoform G (Fas3G), was identified in the LC-MS/MS analysis as a Drice-proximal protein in the adult brain (**Figure 1G**, Table S1). Using AlphaFold2-Multimer (Evans et al., 2021; Mirdita et al., 2022), Drice was predicted to interact with Fas3A (ipTM = 0.456) and Fas3G (ipTM = 0.372) rather than Fas3C (ipTM = 0.298), Fas3D/E (ipTM = 0.220) and Fas3B/F (ipTM = 0.207). Among Fas3 isoforms, only Fas3G is predicted to interact with Drice with 2 intracellular regions (**Figure 2–figure supplement 1A**). To validate this result, we generated an antibody specific to Fas3G (**Figure 2C**, E). We confirmed that only the Fas3G was labeled with *Drice::V5::TurboID* and purified using NeutrAvidin magnetic beads as detected using western blotting (**Figure 2F**). To further confirm the isoform specificity to Drice proximity, we overexpressed 3xFLAG-tagged Fas3 isoforms A, B/F, C, D/E, and G in olfactory receptor neurons using *Pebbled-Gal4* in a *Drice::V5::TurboID* knocked-in background. After proximity labeling with TurboID, followed by NeutrAvidin purification, we found that the Fas3G was highly enriched compared to the other isoforms (**Figure 2G**). These findings suggested that Drice is proximal to a specific isoform of Fas3 in olfactory receptor neurons, regardless of its protein expression level. We also investigated whether Drice and Fas3G statically interact by co-immunoprecipitation. Although Fas3G was labelled by *Drice::V5::TurboID*, indicating its proximity to Drice within the ORNs of adult brains, Drice did not co-immunoprecipitate with Fas3G (**Figure 2–figure supplement 2A**). This result suggests that while Drice and Fas3G are proximate, their interaction is not static, highlighting the utility of proximity labeling as a superior technique to conventional co-immunoprecipitation for identifying proteins that are spatially close.

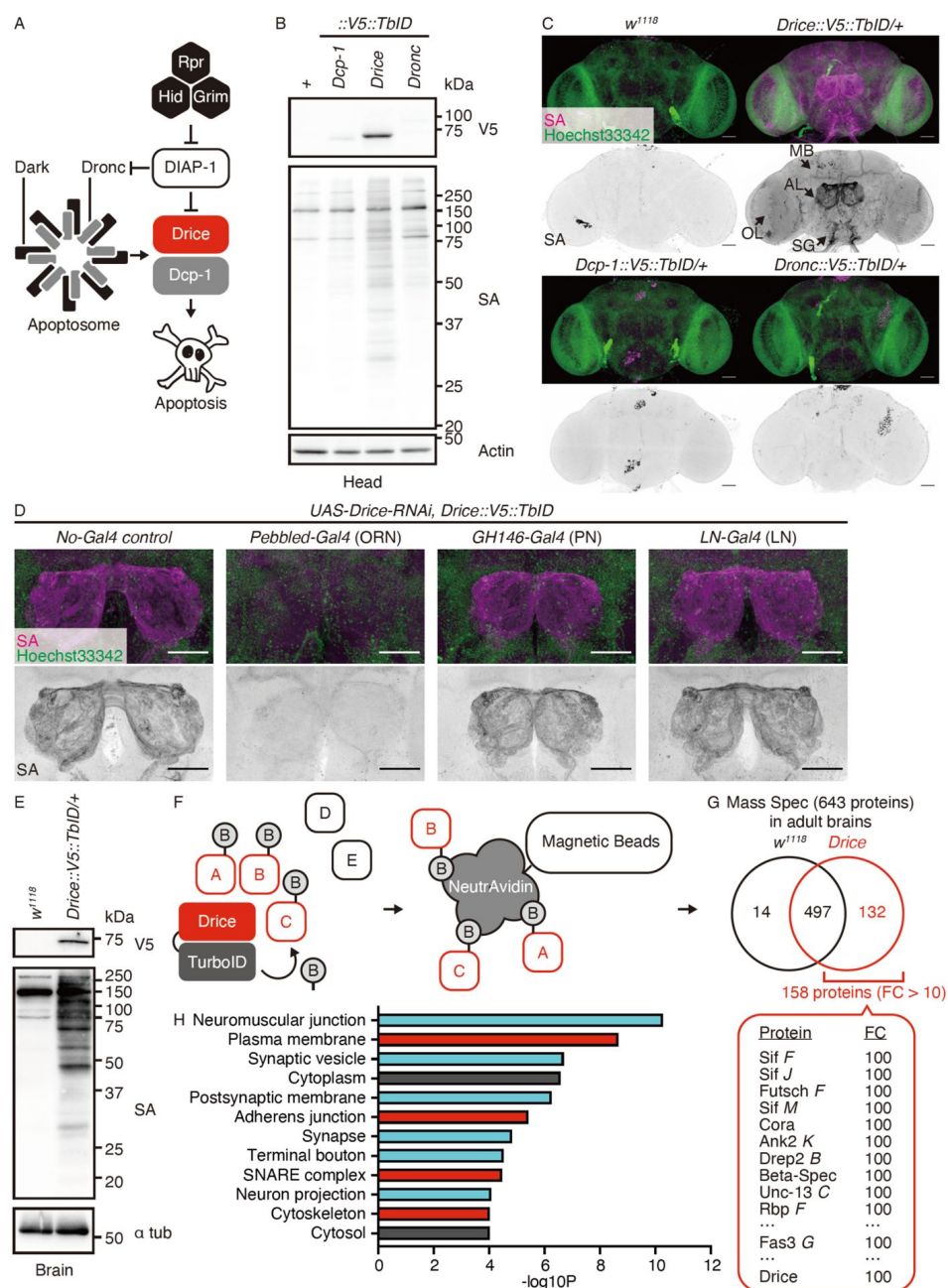


Figure 1.

Expression patterns and proximal proteins of Drice in the adult brain.

- (A) Schematic diagram of *Drosophila* apoptosis signaling.
- (B) Western blot of expression of caspases in adult male heads.
- (C) Representative images of adult male brains. Expression patterns of each caspase are visualized using Streptavidin (SA, magenta). Nuclei are visualized by Hoechst33342 (Green). Scale bar: 50 μ m.
- (D) Representative images of antennal lobes (ALs) in the adult female brains. Expression patterns of Drice are visualized using SA (magenta). Nuclei are visualized by Hoechst33342 (Green). Scale bar: 50 μ m.
- (E) Western blot of proteins extracted from adult male brains.
- (F) Schematic diagram of the TurboID-mediated identification of proximal proteins.
- (G) A summary of mass spectrometry analysis of proteins of the adult male brains. Partial list of Drice proximal proteins and their Fold Change (FC) are shown.
- (H) Gene Ontology analysis (Cellular Compartment) of 158 Drice proximal proteins (*Drice::V5::TurboID*/*w¹¹¹⁸* > 10). Cyan: neuronal-related fraction, Red: membrane fraction.

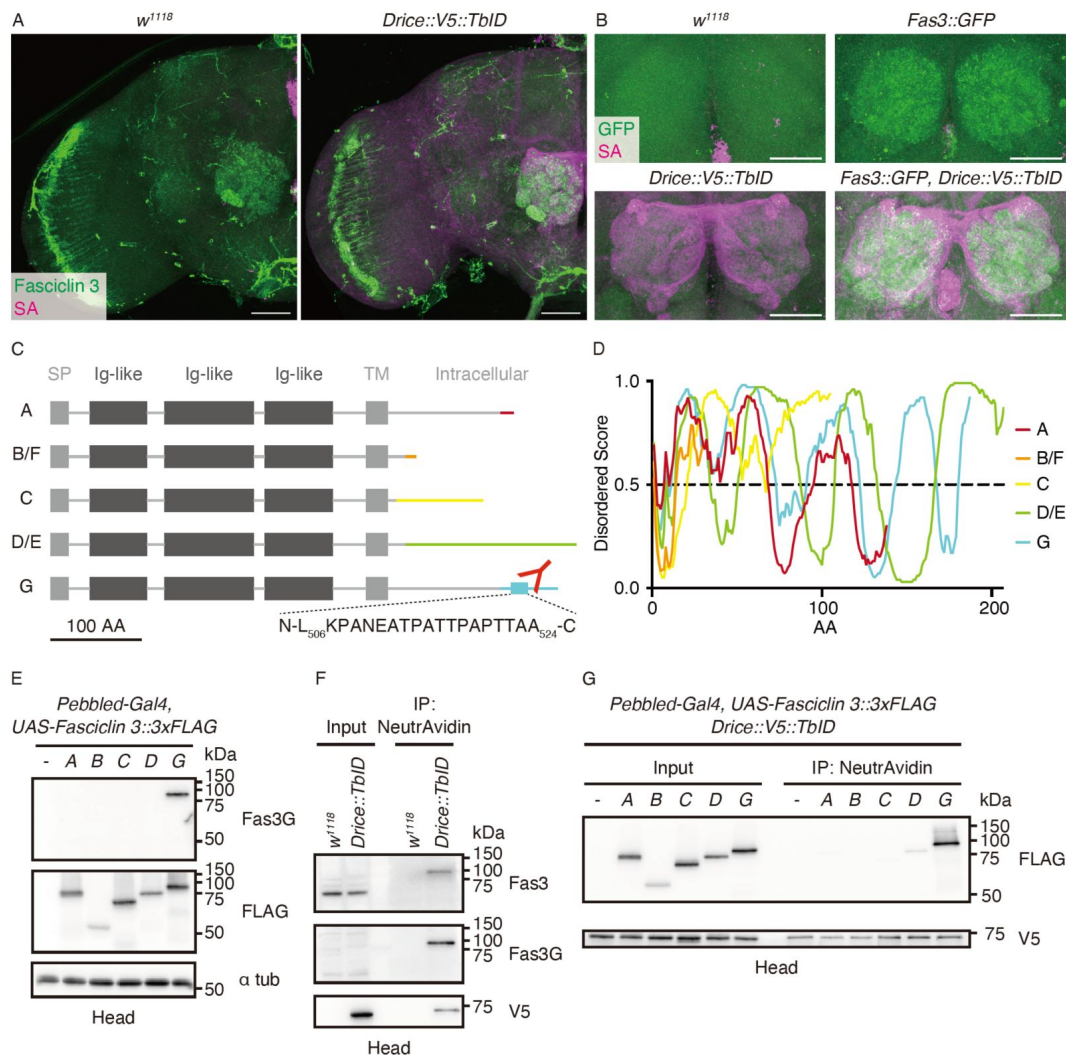


Figure 2.

A specific isoform of Fasciclin 3 is in proximity to Drice.

- (A) Representative expression patterns of Drice (Streptavidin (SA); magenta) and Fasciclin 3 (Fas3, anti-Fasciclin 3 antibody staining; green) in the adult male brains. Scale bar: 50 μ m.
- (B) Representative expression patterns of Drice (SA; magenta) and Fas3 (anti-GFP antibody staining; green) in the antennal lobes (ALs) of the adult male brains. Scale bar: 50 μ m.
- (C) Schematic protein structures of Fas3 isoforms. Intracellular regions differ from each other. A peptide region used to raise anti-Fas3G antibody is shown.
- (D) IDR predictions of intracellular regions of each protein isoform.
- (E) Western blot of proteins extracted from adult heads. Anti-Fas3G antibody specifically detects Fas3 isoform G.
- (F) Western blot of adult male heads. Drice proximal proteins were purified using NeutrAvidin.
- (G) Western blot of adult head proteins. Drice proximal proteins were purified using NeutrAvidin.

Fasciclin 3s are not substrates of caspase

It is often observed that proteins in close proximity to caspases are their preferred substrates (Shinoda et al., 2023 [DOI](#)). To investigate this, we examined whether any of the Fas3 isoforms are substrates for caspase by expressing all isoforms in *Drosophila* S2 cells. However, we found that none of the Fas3 isoforms were cleaved by caspase following the induction of apoptosis (**Figure 2-figure supplement 2B** [DOI](#)). Therefore, Fas3s are not substrates of caspase.

MASCaT: a cell type-specific highly sensitive non-lethal caspase activity reporter

We investigated whether Fas3G regulates caspase activity in lethal or non-lethal processes. CaspaseTracker/CasExpress is a highly sensitive reporter of non-lethal caspase activity that uses caspase-activatable Gal4 tethered to the membrane by mCD8 (Ding et al., 2016 [DOI](#); Tang et al., 2015 [DOI](#)). However, reliance on Gal4 to report their activity presents a challenge in reconciling Gal4/UAS-dependent gene manipulations. To overcome this limitation, we created a new reporter to capture non-lethal caspase activation independent of caspase-activatable Gal4. We replaced the caspase-activatable Gal4 with the caspase-activatable QF2 (*mCD8::DQVD::QF2*), where DQVD is a caspase cleavage sequence, and first expressed directly downstream of UAS sequence (**Figure 3-figure supplement 1A** [DOI](#)). However, the caspase-activatable QF2 induced mNeonGreen expression under *QUAS* sequence even either with a pan-caspase inhibitor, zVAD-fmk, or an uncleavable DQVA mutant probe (*mCD8::DQVA::QF2*, insensitive to caspase-mediated cleavage which serves as a negative control) (**Figure 3-figure supplement 1B** [DOI](#)), potentially because the Gal4/UAS binary system expressed excessive *mCD8::DQVD/A::QF2* probes that results in caspase activation-independent induction of QF2/*QUAS* system. Thus, to reduce the amount of probes expression, we generated *Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2*, simultaneously expressing *Flippase (FLP)* under *UAS* to restrict reporter expression to the cells of interest (**Figure 3A** [DOI](#)). *Drosophila* S2 cells undergo weak caspase activation only by transfection (Shinoda et al., 2023 [DOI](#)). Taking advantage of that, this system reports caspase activity through the caspase-activatable QF2/*QUAS* system, expressing mNeonGreen, which is not detected by caspase-non-activatable QF2 (**Figure 3B** [DOI](#)). We named this novel reporter a Gal4-Manipulated Area Specific CaspaseTracker/CasExpress (MASCaT; **Figure 3A** [DOI](#)). Using MASCaT, we detected non-lethal caspase activity, specifically in the neurons of adult brains, using *ELAV-Gal4* (**Figure 3C** [DOI](#)). Caspase activity was prominent in OLs (**Figure 3C** [DOI](#)), which is consistent with the previously reported activation pattern of caspase detected by CaspaseTracker/CasExpress (Ding et al., 2016 [DOI](#); Tang et al., 2015 [DOI](#)). Previously, we reported that a specific subset of ORNs, including Or42b, undergoes age-associated caspase activation (Chihara et al., 2014 [DOI](#)). Consistent with this finding, we detected age-dependent caspase activation in the AL (**Figure 3C** [DOI](#)). Therefore, our newly established reporter, MASCaT, enables the highly sensitive detection of caspase activity near the plasma membrane in cells of interest reconciling with Gal4/UAS-dependent gene manipulations.

Fas3G overexpression activates caspase in a non-lethal manner

To investigate the regulatory role of Fas3 in caspase activity *in vivo*, we used Or42b neurons as a model, which have previously been shown to undergo age-dependent caspase activation (Chihara et al., 2014 [DOI](#)). Initially, we evaluated the effects of *Dronc* overexpression on apoptosis by quantifying cell body numbers in the antenna in young flies. *Dronc* induced cell loss while catalytically inactive *Dronc* (*Dronc^{CG}*) did not, suggesting that *Dronc* induce apoptosis in a catalytic-dependent manner (**Figure 4A** [DOI](#), B). We then evaluated the effects of the overexpression of the executioner caspases *Dcp-1* and *Drice*. Interestingly, although *Dcp-1* induced complete cell loss, *Drice* did not (**Figure 4A** [DOI](#), B), suggesting that *Drice* requires an initiator caspase for activation, whereas *Dcp-1* does not. These results indicate that Or42b neurons are competent in

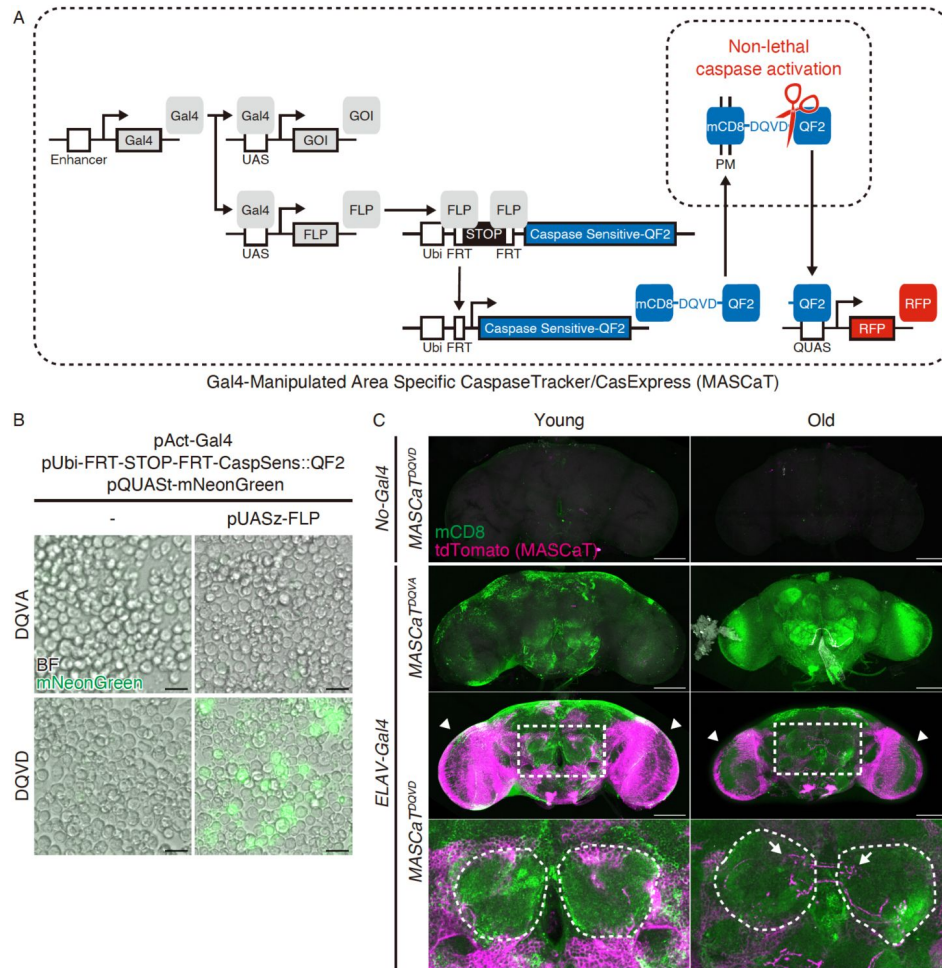


Figure 3.

A Gal4-Manipulated Area Specific CaspaseTracker/CasExpress (MASCaT).

(A) A schematic diagram of MASCaT expressed using the Gal4/UAS system with FLP-mediated recombination. Caspase sensitive-QF2 is activated by caspase-mediated cleavage at the plasma membrane.

(B) Representative images of S2 cells expressing caspase sensitive-QF2 probes by the Gal4/UAS system with FLP-mediated recombination. Scale bar: 20 μ m.

(C) Representative images of adult male brains of young (1 week old) and old (3 weeks old) flies raised at 29°C. Arrowheads indicate optic lobes. Magnified images (white rectangle) show antennal lobes (ALs; white circles). Arrows indicate age-dependent caspase activation at the ALs. Scale bar: 100 μ m.

caspase-mediated cell loss and thus serve as a model for evaluating apoptosis. However, the expression of *Fas3G*, even in combination with *Drice*, did not reduce the number of cells, implying that *Fas3G* overexpression did not induce apoptosis (**Figure 4C**, D).

Subsequently, we assessed the regulation of non-lethal caspase activity by *Fas3G* *in vivo*. We first confirmed that MASCaT probes were distributed in the axons of ORNs (**Figure 4E**). Using MASCaT, we observed weak non-lethal caspase activation even in the control group at a young age (**Figure 4F**, G). The expression of *Fas3G* significantly promoted caspase activation (**Figure 4F**, G), indicating that *Fas3G* regulates non-lethal caspase activation. Moreover, the simultaneous expression of *Drice* and *Fas3G* significantly enhanced caspase activation (**Figure 4F**, G), signifying the promoting effect of their proximity. Moreover, the concurrent expression of Diap-1, an E3 ligase known to inhibit caspase activation (Ditzel et al., 2008; Hay et al., 1995; Lee et al., 2011), mitigated the caspase activation induced by *Fas3G* overexpression (**Figure 4H**, I). This indicates that *Fas3G* overexpression-facilitated non-lethal caspase activation is dependent on the core apoptotic machinery. To test whether endogenous *Fas3G* regulates caspase activation, we generated *Fas3G*-specific *shRNA* expressing strain (**Figure 4-figure supplement 1A, B**). Knockdown of *Fas3* or *Fas3G* in Or42b neurons didn't suppress the inherent, weak caspase activation (**Figure 4-figure supplement 1C, D**). In agreement with this, *Drice*'s presence in the axon of ORNs with *Fas3G* was observed, yet its presence was not strictly contingent on *Fas3* or *Fas3G* (**Figure 4-figure supplement 1E**). Given the numerous other membrane proteins in proximity to *Drice* (**Figure 1G**, H and Table S1), it is probable that additional proteins may be responsible for recruiting *Drice* to the axon. These findings collectively imply that while *Fas3G* is not essential for mild endogenous caspase activation, overexpression of *Fas3G* does enhance non-lethal caspase activation in ORNs, thus providing a valuable model for exploring the molecular processes that facilitate caspase activation without leading to cell death.

Fas3G is proximal to Dronc which is required for non-lethal caspase activation

Next, we investigated the underlying mechanism that enhances non-lethal activation facilitated by *Fas3G* overexpression. Executioner caspases, *Drice* and *Dcp-1*, are activated by initiator caspases, *Dronc* (**Figure 1A**). We precisely analyzed expression pattern of *Dronc* in adult brains and found that while its expression was low in control, overexpression of *Fas3G* in ORNs markedly increased *Dronc* levels in those neurons (**Figure 5A**). Moreover, akin to *Drice*, *Dronc* was also found in close proximity to the overexpressed *Fas3G*, but not to the overexpressed *Fas3C* in ORNs (**Figure 5B**). Consequently, we examined whether *Dronc* is required for the non-lethal caspase activation enhanced by *Fas3G* overexpression. Employing MASCaT, we discovered that both knockdown of *Dronc* and the expression of the *dominant-negative form of Dronc* hindered the non-lethal caspase activation facilitated by *Fas3G* overexpression (**Figure 5C**, D), indicating a dependence on initiator caspase activity. Importantly, the genetic suppression of *Dronc* activity did not alter the proximity between *Drice* and *Fas3G* (**Figure 5E**), suggesting that the inactive *Drice* proform is primarily proximal to *Fas3G*. Solo overexpression of *Dronc* led to cell death in Or42b neurons (**Figure 4A**, B), whereas *Fas3G* overexpression elevated *Dronc* expression without causing cell death (**Figure 4C**, D, **Figure 5A**). Collectively, these findings imply that the non-lethal caspase activation driven by *Fas3G* overexpression is orchestrated by an increase in *Dronc* induced by *Fas3G*, bringing *Dronc* in close vicinity to *Fas3G* and thus enabling caspase activation near the plasma membrane (**Figure 5F**).

Non-lethal caspase activation regulates innate attraction behavior

Finally, we examined the function of *Fas3G* overexpression-facilitated non-lethal caspase activation. Apple cider vinegar (ACV) excites six glomeruli in the AL, including DM1, which is innervated by the axons of Or42b neurons (Sammelhack and Wang, 2009). We have previously reported that Or42b neurons undergo aging-dependent caspase activation and cell death, which

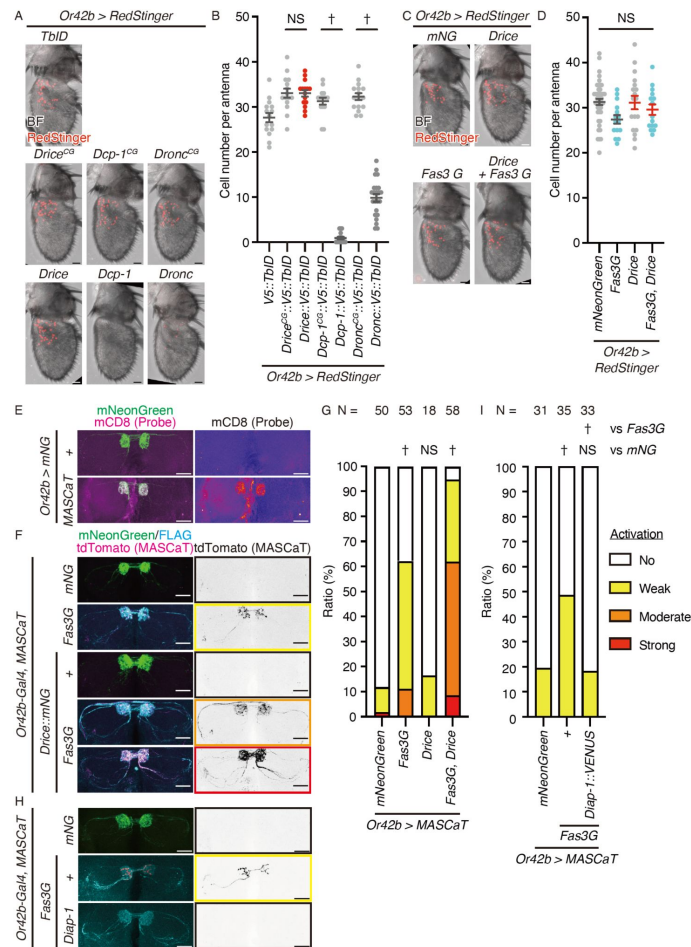


Figure 4.

***Fasciclin 3 isoform G* overexpression enhances non-lethal caspase activation.**

(A) Representative images of the adult male antennae of 1 weeks old flies raised at 29°C. Cell bodies are visualized using RedStinger (red). Scale bar: 20 μ m.

(B) Quantification of cell number of (A). Data are presented as mean $\pm$ SEM. *P*-values were calculated using one-way analysis of variance (ANOVA) with Bonferroni's correction for selected pairs. NS: *P* > 0.05, †: *P* < 0.05. Sample sizes: V5::TbID (N = 15), Drice^{CG}::V5::TbID (N = 15), Drice::V5::TbID (N = 15), Dcp-1^{CG}::V5::TbID (N = 15), Dcp-1::V5::TbID (N = 16), Dronc^{CG}::V5::TbID (N = 16), Dronc::V5::TbID (N = 23).

(C) Representative images of the adult male antennae of 1 week old flies raised at 29°C. Cell bodies are visualized using RedStinger (red). Scale bar: 20 μ m.

(D) Quantification of cell number of (C). Data are presented as mean $\pm$ SEM. *P*-values were calculated using one-way ANOVA with Bonferroni's correction for every pair. NS: *P* > 0.05, †: *P* < 0.05. Sample sizes: mNeonGreen (N = 44), Fas3G (N = 15), Drice (N = 18), Fas3G + Drice (N = 15).

(E) Representative images of the antennal lobes (ALs) of male brains of 1 week old flies raised at 29°C expressing MASCaT probe (mCD8, magenta). Scale bar: 30 μ m.

(F) Representative images of the ALs of brains of 1 week old flies raised at 29°C with caspase activation visualized by MASCaT. Scale bar: 30 μ m.

(G) Quantifications of caspase activity detected by MASCaT of (F). *P*-values were calculated using chi-square test with Bonferroni's correction using mNeonGreen as control. NS: *P* > 0.05, †: *P* < 0.05. Sample sizes are shown in the graph.

(H) Representative images of the ALs of brains of 1 week old flies raised at 29°C with caspase activation visualized by MASCaT. Scale bar: 30 μ m.

(I) Quantifications of caspase activity detected by MASCaT of (H). *P*-values were calculated using chi-square test with Bonferroni's correction using mNeonGreen and Fas3G as controls, respectively. NS: *P* > 0.05, †: *P* < 0.05. Sample sizes are shown in the graph.



reduces neuronal activity and attraction behavior in response to ACV (Chihara et al., 2014 [↗](#)). Inhibition of caspase activity in aged flies restores attraction behavior but does not affect that of young flies (Chihara et al., 2014 [↗](#)). Using a two choice preference assay with ACV (**Figure 6A** [↗](#)), we found that the overexpression of *Fas3G*, which activates caspases, did not impair attraction behavior in young flies (**Figure 6B** [↗](#), C). In contrast, simultaneous inhibition of *Fas3G* overexpression-facilitated non-lethal caspase activation promoted attraction to ACV (**Figure 6B** [↗](#), C). Overall, these results suggest that *Fas3G* overexpression-facilitated non-lethal caspase activation suppresses innate attractive behavior even without killing the cells.

Discussion

Proximal proteins of executioner caspases regulate non-lethal caspase activity

It is important to identify the proximal proteins of executioner caspases, which cleave a wide range of substrates and are essential for apoptosis (Julien and Wells, 2017 [↗](#); Kumar, 2007 [↗](#)), as proximity often dictates substrate specificity (Shinoda et al., 2023 [↗](#)). In humans, caspase-3 and caspase-7 have been shown to share but also have discrete substrate preferences (Walsh et al., 2008 [↗](#)). Caspase-7 leverages an exosite to facilitate interaction with RNA and enhance the proteolysis of RNA-binding proteins (Boucher et al., 2012 [↗](#); Desroches and Denault, 2019 [↗](#)). Thus, protein proximity can be used to precisely regulate specific cellular functions by selectively cleaving a limited pool of substrates in non-lethal scenarios compared with lethal cleavages where all the substrates are potentially cleaved during apoptosis. We showed that executioner caspases, before activation as proforms, are exclusively sequestered in specific subcellular domains, including the plasma membrane. We found that *Fas3G* overexpression facilitates non-lethal activation, underscoring the importance of protein proximity not only in substrate cleavage, but also in localizing non-lethal activation to the vicinity of plasma membrane (**Figure 5F** [↗](#)). Our results suggest that subcellularly restricted caspase activation mediated by caspase proximal proteins is one of the potential mechanisms regulating caspase activation without inducing cell death.

MASCaT as a highly sensitive reporter for identifying non-lethal caspase regulators

Various caspase activity reporters have been developed, including SCAT3, a FRET-based reporter with superior temporal resolution (Takemoto et al., 2003 [↗](#)), CaspaseTracker/CasExpress, Caspase-activatable Gal4-based reporters with superior sensitivity (Ding et al., 2016 [↗](#); Tang et al., 2015 [↗](#)). However, to date, no reporter has been developed that is sensitive enough to identify non-lethal regulators of caspases in cells of interest. In this study, we developed a novel caspase reporter, MASCaT, that can detect non-lethal caspase activation with high sensitivity in a cell type-specific manner following gene manipulation. Using MASCaT, we identified previously overlooked regulators of non-lethal activation of caspases in a cell type-specific manner. Proximal proteins of executioner caspases are potential non-lethal regulators, and combining TurboID-mediated identification of these proteins will pave the way for identifying non-lethal components and broaden our understanding of the molecular mechanism of non-lethal caspase activation.

Non-lethal caspase activation regulates neuronal activity and behaviors

We previously reported that age-dependent caspase activation reduces neuronal activity and innate attraction behavior (Chihara et al., 2014 [↗](#)). Here, we found that, while *Fas3G* overexpression did not impair attraction behavior by itself, caspase activation upon *Fas3G* overexpression suppressed attraction behavior toward ACV (**Figure 6A** [↗](#), B). In mice, L1CAM, an

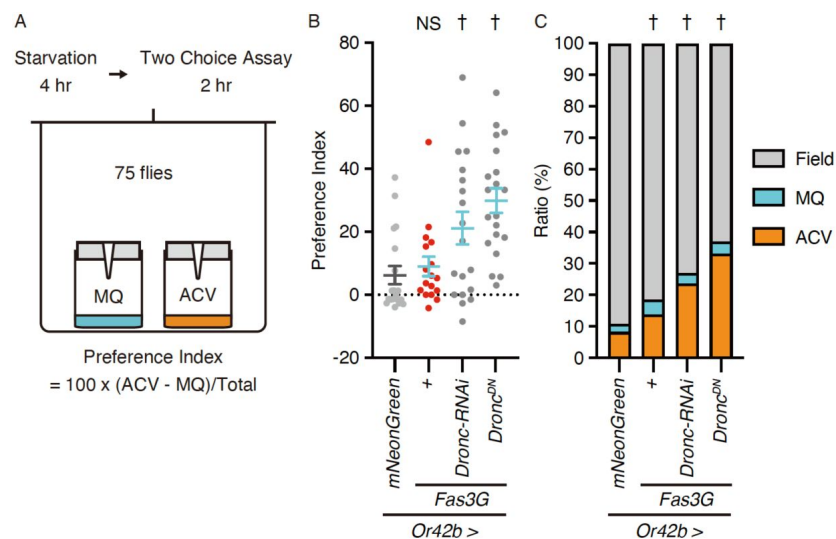


Figure 6.

***Fasciclin 3 isoform G* overexpression-facilitated caspase activation regulates attraction behavior.**

(A) Schematic diagram of two choice preference assay. Flies are starved for 4 hours before the assay. Flies are left for 2 hours in the bottle. Preference index is calculated by counting the number of flies left in MQ, ACV and other (field) compartments.

(B) Preference index of 1 week old male flies raised at 29°C in response to 1% apple cider vinegar in two choice preference assay. Data are presented as mean $\pm$ SEM. *P*-values were calculated using one-way ANOVA followed by Dunnett's multiple comparison test using mNeonGreen as a control. NS: *P* > 0.05, †: *P* < 0.05. Sample sizes: mNG (N = 19 (1327 flies)), Fas3G (N = 17 (1191 flies)), Fas3G + Dronc-RNAi (N = 19 (1295 flies)), Fas3G + Dronc^{DN} (N = 20 (1320 flies)).

(C) The ratio of flies in the given conditions in the two choice preference assay of (B). *P*-values were calculated using chi-square test with Bonferroni's correction using mNeonGreen as control. †: *P* < 0.05. Sample sizes: mNG (N = 1327 flies), Fas3G (N = 1191 flies), Fas3G + Dronc-RNAi (N = 1295 flies), Fas3G + Dronc^{DN} (N = 1320 flies).

immunoglobulin-like cell adhesion molecule, facilitates neuronal excitability by regulating the voltage-gated Na⁺ channels (Valente et al., 2016). Thus, we hypothesize that non-lethal caspase activation antagonizes the neuronal excitability promoted by Fas3G. In neurons, caspases have diverse non-lethal functions, including axon degeneration and macro- and micro-pruning, to regulate synaptic plasticity (Dehkordi et al., 2022; Mukherjee and Williams, 2017). In presynaptic terminals, it has been recently reported that Caspase-3 modulates synaptic vesicle pools via autophagy (Gu et al., 2021). Thus, it is possible that non-lethal activation regulates synaptic pools and neuronal activity in a reversible manner without killing the cells. Analyzing the functions mediated by Drice and Fas3G proximity, including the identification of specific substrates, will pave the way for identifying diverse CDPs and understanding their molecular mechanisms.

While suppressing innate attraction behavior does not seem beneficial by itself, it should be a great advantage in decision-making. To survive, flies must constantly make appropriate behavioral decisions (e.g., feeding versus mating) depending on their physiological conditions. In males, a subset of neurons regulated by tyramine controls feeding versus mating behaviors (Cheriyamkunnel et al., 2021). In regard of that, it is important to adequately promote or suppress neuronal activity, which controls behaviors of the organisms, by precisely controlling caspase activation in a subcellularly restricted manner in neurons. Compared with lethal activation which irreversibly removed the neurons of interest, suppressing neuronal activity with non-lethal reversible caspase activation is beneficial for decision makings depending on their varying physiological conditions. As regulating caspase proximal proteins enables non-lethal activation which is not achieved by directly manipulating caspase expression, our results open the possibility of the methodological development for reversible manipulation of neuronal activity via non-lethal caspase activity.

Proximal proteins of executioner caspases define the site of caspase activation “hotspot”

Although the core apoptotic machinery has been well characterized during the last 30 years (Nakajima and Kuranaga, 2017), the precise regulation of CDPs, remains incompletely elucidated. In this study, we identified *Fas3G* overexpression activates Drice. Mechanistically, we found that *Fas3G* overexpression induced the expression of initiator caspase Dronc, which also comes close to Fas3G, regulating non-lethal caspase activation. Direct overexpression of Dronc alone results in cell death within olfactory receptor neurons (Figure 4A, B). However, when Dronc expression is upregulated due to *Fas3G* overexpression, it does not lead to cell death in ORNs (Figure 4C, D). The exact process by which Dronc expression is increased in response to *Fas3G* overexpression remains to be elucidated. It is known that Dronc can be upregulated via the ecdysone signaling pathway (Dorstyn et al., 1999) and the Hippo signaling pathway (Verghese et al., 2012). Therefore, the overexpression of *Fas3G* might potentially activate Dronc expression through one or both of these signaling pathways. Fas3 is an immunoglobulin-like domain-containing transmembrane protein that regulates axonal fasciculation and neuronal projections (Chiba et al., 1995). NCAM, a mammalian homologue of Fascicilin 2, is an immunoglobulin-like domain-containing cell adhesion molecule. NCAM can physically bind to caspase-8, and depending on NCAM clustering, caspase-8 and -3 are activated and contribute to neurite outgrowth by cleaving its substrate spectrin (Westphal et al., 2010). Thus, it seems likely that cell adhesion molecule-mediated subcellularly restricted caspase activation is conserved among species. Indeed, caspase-9/Apaf-1-dependent caspase-3 activation, without resulting in cell death, has also been observed in the axon of olfactory sensory neurons during mouse development (Ohsawa et al., 2010).

While the restriction of the extent and spread of activated caspases is proposed as one of the potential molecular mechanisms for non-lethal activation (Mukherjee and Williams, 2017), experimental evidences are limited. A critical insight from our research is that the executioner

caspase is pre-compartmentalized in proximity to a specific set of proteins prior to activation, poised for the initiator caspase to approach. Consequently, the “hotspot” for non-lethal caspase activity appears to be determined by these neighboring proteins of executioner caspases. While the “hotspot” located in the axon of the ORN may exert an inhibitory effect on neuronal activity, it could also play a role in avoiding cell death by localizing caspase activity, especially during development.

Recently, a protein interactome analysis in humans showed that tissue-preferentially expressed proteins that interact with known apoptosis regulators participate in apoptosis sensitization (Luck et al., 2020 [DOI](#)). Given that CDPs are often cell- and tissue type-specific processes, the identification of modulators of context-dependent apoptotic pathways will aid in understanding the precise molecular regulation of CDPs. Importantly, alternative splicing can diversify protein-protein interaction networks. A recent report showed that minor spliceosomes regulate alternative splicing, including Fas3. A specific isoform of Fas3 has distinct functions and cannot be substituted by other isoforms during neuronal development (Li et al., 2020 [DOI](#)). Our results support the idea that alternative splicing may not only diversify the protein itself, but also its interaction networks to regulate module-dependent activation. Further interactome analysis using TurboID will reveal the regulatory mechanisms of the non-lethal activation of caspases.

Methods

Fly strains and rearing conditions

We raised the flies in standard *Drosophila* medium at 25°C. For in vivo biotin labeling experiments, flies were raised in standard *Drosophila* medium supplemented with 100 µM (+)-biotin (#132-02635, WAKO). The following fly strains were used in this study: *w¹¹¹⁸*, *Drice::V5::TurboID* (Shinoda et al., 2019 [DOI](#)), *Dcp-1::V5::TurboID* (Shinoda et al., 2019 [DOI](#)), *Dronc::V5::TurboID* (Shinoda et al., 2019 [DOI](#)), *Pebbled-Gal4* (BL80570), *LN-Gal4* (BL63325), *GH146-Gal4* (BL30026), *UAS-Drice-RNAi* (V28065), *Fas3::EGFP::FLAsH::StrepII::TEV::3xFLAG* (BL59809), *UASz-Fas3A::3xFLAG* (this study), *UASz-Fas3B::3xFLAG* (this study), *UASz-Fas3C::3xFLAG* (this study), *UASz-Fas3D::3xFLAG* (this study), *UASz-Fas3G::3xFLAG* (this study), *Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2* (this study), *Ubi-FRT-STOP-FRT-mCD8::DQVA::QF2* (this study), *UAS-FLP* (K107788), *QUAS-tdTomato::3xHA* (BL30005), *ELAV-Gal4* (BL8765), *Or42b-Gal4* (BL9971), *UAS-RedStinger* (BL8547), *UASz-myc::mNeonGreen* (this study), *UASz-Drice::myc::mNeonGreen* (this study), *UASz-V5::TurboID* (this study), *UASz-Drice::V5::TurboID* (this study), *UASz-Drice^{CG}::V5::TurboID* (this study), *UASz-Dcp-1::V5::TurboID* (this study), *UASz-Dcp-1^{CG}::V5::TurboID* (this study), *UASz-Dronc::V5::TurboID* (this study), *UASz-Dronc^{CG}::V5::TurboID* (this study), *UAS-Diap-1::VENUS* (Koto et al., 2009 [DOI](#)), *UAS-Dronc-RNAi* (Kanuka et al., 2005 [DOI](#)), *UAS-Dronc^{DN}::EGFP* (Igaki et al., 2002 [DOI](#)), *UAS-LacZ-RNAi*, *UAS-Fas3-RNAi#1* (V940), *UAS-Fas3-RNAi#2* (V3091),

UAS-Fas3G-RNAi (this study). BL, Bloomington *Drosophila* Resource Center; K, Kyoto Stock Center; V, Vienna *Drosophila* Resource Center.

Detailed genotypes

Figure 1B, C [DOI](#) (Left to Right, Top to Bottom)

w¹¹¹⁸/Y

w/Y; Dcp-1::V5::TurboID/+

w/Y;; Drice::V5::TurboID/+

w/Y;; Dronc::V5::TurboID/+

Figure 1D [↗](#) (Left to Right)

w; UAS-Drice-RNAi/+; Drice::V5::TurboID/+

w, Pebbled-Gal4/w; UAS-Drice-RNAi/+; Drice::V5::TurboID/+

w; UAS-Drice-RNAi/GH146-Gal4; Drice::V5::TurboID/+

w; UAS-Drice-RNAi/+; Drice::V5::TurboID/LN-Gal4

Figure 1E [↗](#) (Left to Right)

w¹¹¹⁸/Y

w/Y;; Drice::V5::TurboID/+

Figure 1G [↗](#) (Left to Right)

w¹¹¹⁸/Y

w/Y;; Drice::V5::TurboID/+

Figure 1–figure supplement 1A (Left to Right, Top to Bottom)

w¹¹¹⁸/Y

w¹¹¹⁸

w/Y;; Drice::V5::TurboID/+

w/w¹¹¹⁸;; Drice::V5::TurboID/+

Figure 2A [↗](#) (Left to Right)

w¹¹¹⁸/Y

w/Y;; Drice::V5::TurboID/+

Figure 2B [↗](#) (Top to Bottom, Left to Right)

w¹¹¹⁸/Y

w/Y;; Drice::V5::TurboID/+

w/Y; Fas3::EGFP::FLAsH::StrepII::TEV::3xFLAG/+

w/Y; Fas3::EGFP::FLAsH::StrepII::TEV::3xFLAG/+; Drice::V5::TurboID/+

Figure 2E [↗](#) (Left to Right)

w, Pebbled-Gal4/w or Y

w, Pebbled-Gal4/w or Y;; UASz-Fas3A::3xFLAG/+

w, *Pebbled-Gal4/w* or *Y*; *UASz-Fas3B::3xFLAG/+*

w, *Pebbled-Gal4/w* or *Y*; *UASz-Fas3C::3xFLAG/+*

w, *Pebbled-Gal4/w* or *Y*; *UASz-Fas3D::3xFLAG/+*

w, *Pebbled-Gal4/w* or *Y*; *UASz-Fas3G::3xFLAG/+*

Figure 2F [↗](#) (Left to Right)

*w*¹¹¹⁸/*Y*

w/Y; *Drice::V5::TurboID/+*

Figure 2G [↗](#) (Left to Right)

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/+*

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/UASz-Fas3A::3xFLAG*

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/UASz-Fas3B::3xFLAG*

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/UASz-Fas3C::3xFLAG*

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/UASz-Fas3D::3xFLAG*

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/UASz-Fas3G::3xFLAG*

Figure 2–figure supplement 2A (Left to Right)

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/+*

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/UASz-Fas3C::3xFLAG*

w, *Pebbled-Gal4/w* or *Y*; *Drice::V5::TurboID/UASz-Fas3G::3xFLAG*

Figure 3C [↗](#) (Top to Bottom)

w/Y; *Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/+*; *UAS-FLP*, *QUAS-tdTomato::3xHA/+*

w/Y; *Ubi-FRT-STOP-FRT-mCD8::DQVA::QF2/ELAV-Gal4*; *UAS-FLP*, *QUAS-tdTomato::3xHA/+*

w/Y; *Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/ELAV-Gal4*; *UAS-FLP*, *QUAS-tdTomato::3xHA/+*

Figure 4A [↗](#), B (Top to Bottom, Left to Right)

w/Y; *Or42b-Gal4/+*; *UAS-RedStinger/UASz-V5::TurboID*

w/Y; *Or42b-Gal4/+*; *UAS-RedStinger/UASz-Drice^{CG}::V5::TurboID*

w/Y; Or42b-Gal4/+; UAS-RedStinger/UASz-Drice::V5::TurboID

w/Y; Or42b-Gal4/+; UAS-RedStinger/UASz-Dcp-1^{CG}::V5::TurboID

w/Y; Or42b-Gal4/+; UAS-RedStinger/UASz-Dcp-1::V5::TurboID

w/Y; Or42b-Gal4/+; UAS-RedStinger/UASz-Dronc^{CG}::V5::TurboID

w/Y; Or42b-Gal4/+; UAS-RedStinger/UASz-Dronc::V5::TurboID

Figure 4C, D [↗](#) (Top to Bottom, Left to Right)

w/Y; Or42b-Gal4/UASz-3xFLAG::mNeonGreen; UAS-RedStinger/+

w/Y; Or42b-Gal4/+; UAS-RedStinger/UASz-Fas3G::3xFLAG

w/Y; Or42b-Gal4/UASz-Drice::myc::mNeonGreen; UAS-RedStinger/+

w/Y; Or42b-Gal4/UASz-Drice::myc::mNeonGreen; UAS-RedStinger/UASz-Fas3G::3xFLAG

Figure 4E [↗](#) (Top to Bottom)

w/Y; Or42b-Gal4/UASz-3xFLAG::mNeonGreen

w/Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UASz-3xFLAG::mNeonGreen;

UAS-FLP, QUAS-tdTomato::3xHA/+

Figure 4F, G [↗](#) (Top to Bottom, Left to Right)

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UASz-3xFLAG::mNeonGreen; UAS-FLP,

QUAS-tdTomato::3xHA/+

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/+; UAS-FLP, QUAS-

tdTomato::3xHA/UASz-Fas3G::3xFLAG

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UASz-Drice::myc::mNeonGreen; UAS-

FLP, QUAS-tdTomato::3xHA/+

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UASz-Drice::myc::mNeonGreen; UAS-

FLP, QUAS-tdTomato::3xHA/UASz-Fas3G::3xFLAG

Figure 4H [↗](#), I (Top to Bottom, Left to Right)

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UASz-3xFLAG::mNeonGreen; UAS-FLP,

QUAS-tdTomato::3xHA/+

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/+; UAS-FLP, QUAS-

tdTomato::3xHA/UASz-Fas3G::3xFLAG

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UAS-Diap-1::VENUS; UAS-FLP, QUAS-

tdTomato::3xHA/UASz-Fas3G::3xFLAG

Figure 4—figure supplement 1C, D (Top to Bottom, Left to Right)

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UAS-LacZ-RNAi; UAS-FLP, QUAS-tdTomato::3xHA/+

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/+; UAS-FLP, QUAS-tdTomato::3xHA/UAS-Fas3-RNAi #1

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/+; UAS-FLP, QUAS-tdTomato::3xHA/UAS-Fas3-RNAi #2

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UAS-Fas3G-RNAi; UAS-FLP, QUAS-tdTomato::3xHA/+

w, Pebbled-Gal4/Y; UASz-Drice::myc::mNeonGreen/+

w, Pebbled-Gal4/Y; UASz-Drice::myc::mNeonGreen/+; UASz-Fas3G::3xFLAG/+

w, Pebbled-Gal4/Y; UASz-Drice::myc::mNeonGreen/+; UAS-Fas3-RNAi #1/+

w, Pebbled-Gal4/Y; UASz-Drice::myc::mNeonGreen/+; UAS-Fas3-RNAi #2/+

w, Pebbled-Gal4/Y; UASz-Drice::myc::mNeonGreen/UAS-Fas3G-RNAi

Figure 5A [↗](#) (Left to Right)

w, Pebbled-Gal4/Y

w, Pebbled-Gal4/Y;; Dronc::V5::TurboID/+

w, Pebbled-Gal4/Y;; Dronc::V5::TurboID/UASz-Fas3G::3xFLAG

Figure 5B [↗](#) (Left to Right)

w, Pebbled-Gal4/Y;; Dronc::V5::TurboID/+

w, Pebbled-Gal4/Y;; Dronc::V5::TurboID/UASz-Fas3G::3xFLAG

w, Pebbled-Gal4/Y;; Dronc::V5::TurboID/UASz-Fas3C::3xFLAG

Figure 5C [↗](#), D (Top to Bottom, Left to Right)

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UASz-3xFLAG::mNeonGreen; UAS-FLP, QUAS-tdTomato::3xHA/+

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/+; UAS-FLP, QUAS-tdTomato::3xHA/UASz-Fas3G::3xFLAG

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UAS-Dronc-RNAi; UAS-FLP, QUAS-tdTomato::3xHA/UASz-Fas3G::3xFLAG

w/w or Y; Or42b-Gal4, Ubi-FRT-STOP-FRT-mCD8::DQVD::QF2/UAS-Dronc^{DN}; UAS-FLP, QUAS-tdTomato::3xHA/UASz-Fas3G::3xFLAG

Figure 5E [↗](#) (Left to Right)

w, Pebbled-Gal4/Y;; Drice::V5::TurboID/+

w, Pebbled-Gal4/Y;; Drice::V5::TurboID/UASz-Fas3G::3xFLAG

w, Pebbled-Gal4/Y; +/UAS-Dronc-RNAi; Drice::V5::TurboID/UASz-Fas3G::3xFLAG

w, Pebbled-Gal4/Y; +/UAS-Dronc^{DN}; Drice::V5::TurboID/UASz-Fas3G::3xFLAG

Figure 6B [↗](#), C (Left to Right)

w/Y; Or42b-Gal4/UASz-3xFLAG::mNeonGreen

w/Y; Or42b-Gal4/+; +/UASz-Fas3G::3xFLAG

w/Y; Or42b-Gal4/UAS-Dronc-RNAi; +/UASz-Fas3G::3xFLAG

w/Y; Or42b-Gal4/UAS-Dronc^{DN}; +/UASz-Fas3G::3xFLAG

Protein preparation from Drosophila tissues

Adult heads or brains of the given genotype were dissected in phosphate-buffered saline (PBS) and lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM Sodium Chloride, 0.5 w/v% Sodium Deoxycholate, 0.1% w/v Sodium Dodecyl Sulfate, and 1.0% w/v NP-40) supplemented with cOmplete ULTRA EDTA-free protease inhibitor cocktail (#05892953001, Roche). Samples were homogenized and centrifuged at 20,000 ×g, 4°C for 10 min. The supernatants were collected and snap-frozen in liquid nitrogen. Protein concentrations were determined using the BCA assay (#297-73101, WAKO) following the manufacturer's protocol. The samples were mixed with 6x Laemmli buffer, boiled at 95°C for 5 min, and then subjected to purification of biotinylated proteins and western blot analysis.

Western blotting

Each sample was separated using SDS-PAGE. The proteins were then transferred onto Immobilon-P PVDF membranes (#IPVH00010; Millipore) for immunoblotting. Membranes were blocked with 4% skim milk diluted in 1× TBST. Immunoblotting was performed using the antibodies mentioned below diluted in 4% skim milk diluted in 1× TBST. Signals were visualized using Immobilon Western Chemiluminescent HRP Substrate (#WBKLS0500; Millipore) and FUSION SOLO. 7S. EDGE imaging station (Vilber-Lourmat). Contrast and brightness adjustments were applied equally using Fiji (ImageJ) software (NIH Image).

The primary antibodies used included mouse anti-V5 monoclonal antibody (1:5,000, #46-0705, Invitrogen), mouse anti-FLAG M2 monoclonal antibody (1:5,000, Sigma), mouse anti-alpha tubulin (DM1A) monoclonal antibody (1:10,000, #T9026, Sigma), mouse anti-Actin monoclonal antibody (1:5,000; #A4700, Sigma), mouse anti-Fasciclin 3 (7G10) antibody (1:50, #AB_528238, DSHB), rabbit anti-Fasciclin3 isoform G antibody (1:50, this study), mouse anti-Drosophila Lamin B monoclonal antibody (1:1000, #ADL67.10, DSHB). The secondary antibodies used included goat anti-rabbit IgG HRP-conjugated antibody (1:5,000, #7074S, CST) and goat/rabbit/donkey anti-mouse IgG HRP Conjugate (1:5,000, #W402B, Promega). The membranes for streptavidin blotting were blocked with 3% BSA diluted in 1× TBST. Streptavidin blotting was performed using streptavidin-horseradish peroxidase (1:10,000, #SA10001; Invitrogen) diluted in 3% BSA diluted in 1× TBST.

Co-immunoprecipitation

Adult heads were lysed on ice using DDM IP lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% N-Dodecyl- β -D-maltoside (DDM) (#341-06161, WAKO)) containing cOmplete ULTRA EDTA-free protease inhibitor cocktail. The samples were adjusted to 150 μ g protein/150 μ L DDM IP lysis buffer. The samples were incubated overnight at 4 °C with 10 μ L anti-FLAG M2 Magnetic Beads (#M8823-1ML, Millipore) equilibrated with the DDM IP lysis buffer. The beads were washed three times in wash buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% DDM) and boiled for 5 min at 95 °C with 50 μ L 1 $\times$ Laemmli buffer. The samples were magnetically separated, and the supernatants were subjected to SDS-PAGE.

Immunohistochemistry

Adult brains were dissected in PBS and fixed in 4% paraformaldehyde (PFA) at room temperature for 30 min. Adult brains were washed three times with 0.3% PBST (Triton-X100) for 10 min. After blocking with 5.0% normal donkey serum (#S30, Millipore)/PBST (PBSTn) for 30 minutes at room temperature, tissues were incubated with primary antibody/PBSTn overnight at 4°C. The samples were then washed with PBST three times for 10 min each and incubated with secondary antibody/PBSTn for 2 h at room temperature. After incubation, the tissues were washed with PBST three times for 10 min. Streptavidin and Hoechst 33342 were added during secondary antibody incubation. The samples were mounted on glass slides with SlowFade Gold antifade reagent (#S36939, Invitrogen). Images were captured using a Leica TCS SP8 microscope (Leica Microsystems). Images were analyzed and edited using the Fiji (ImageJ) software. For MASCaT analysis, newly eclosed adults were raised at 29°C for 1 week. Classification of the MASCaT signal was performed by optically assessing the tdTomato intensity. For cell number analysis, newly eclosed adults were raised at 29°C for 1 week. The antennae were dissected as previously described (Karim et al., 2014 [DOI](#)), fixed with 4% PFA at room temperature for 30 min, and washed with 0.4% PBST (Triton-X100) three times for 10 min. Quantification of cell numbers in antenna was performed using Fiji (ImageJ) software using “3D object counter” for RedStinger positive cells.

The primary antibodies used were mouse anti-Fasciclin 3 (7G10) antibody (1:20, #AB_528238, DSHB), rat anti-GFP (GF090R) monoclonal antibody (1:100, #04404-26, ncalai Tesque), mouse anti-FLAG M2 monoclonal antibody (1:500, F1804, Sigma), and rat anti-mCD8 monoclonal antibody (1:50, #MCD0800, Invitrogen). The secondary antibodies used included Alexa Fluor 488-conjugated donkey anti-mouse IgG antibody (1:500, #A-21202, ThermoFisher Scientific), Alexa Fluor 488-conjugated donkey anti-rat IgG antibody (1:500, #A-21208, ThermoFisher Scientific), Alexa Fluor 647-conjugated donkey anti-mouse IgG antibody (1:500, #A-31571, ThermoFisher Scientific), and Alexa Fluor 633-conjugated goat anti-rat IgG antibody (1:500, #A-21094, ThermoFisher Scientific). The nuclei were stained with Hoechst 33342 (8 μ M, #H3570, Invitrogen). Streptavidin-Cy2 (1:500, #016-220-084, Jackson ImmunoResearch) and Streptavidin-Cy5 (1:500, #016-170-084, Jackson ImmunoResearch) were used to stain the biotinylated proteins.

Experimental design for LC-MS/MS analyses

Two biological replicates were analyzed for each experimental condition to determine the relative abundance of *Drice::V5::TurboID/w¹¹¹⁸*.

Purification of biotinylated proteins

Samples were prepared as previously described (Shinoda et al., 2023 [DOI](#)). Briefly, 100 μ g of biotinylated protein-containing lysate (from 100 brains or 20 heads) was subjected to FG-NeutrAvidin bead (#TAS8848 N1171, Tamagawa Seiki) purification. The FG-NeutrAvidin beads (25 μ L, approximately 500 μ g) were washed three times with RIPA buffer. Benzoylase-treated biotinylated protein samples suspended in 1 mL RIPA buffer were incubated overnight at 4°C. Conjugated beads were magnetically isolated and washed with 500 μ L of an ice-cold RIPA buffer

solution, 1 M KCl solution, 0.1 M Na₂CO₃ solution and 4 M urea solution. For western blot analyses, the purified samples were mixed with Laemmli buffer, boiled at 95°C for 5 min, and then subjected to western blot analysis. For LC-MS/MS analysis, the purified samples were washed with 500 µL ultrapure water (#214-01301, WAKO) and 500 µL of 50 mM ammonium bicarbonate (AMBC). The samples were then mixed with 50 µL of 0.1% Rapigest diluted in 50 mM AMBC, and 5 µL of 50 mM TCEP was subsequently added. Samples were incubated at 60°C for 5 min, and then 2.5 µL 200 mM MMTS was added. One microgram sequence-grade modified trypsin (#V5111, Promega) was then added for on-bead trypsin digestion at 37°C for 16 h. The beads were magnetically isolated and 60 µL of the supernatant was collected. Then, 3 µL 10% TFA was added to the supernatants, and the samples were incubated at 37°C for 60 min with gentle agitation. The samples were then centrifuged at 20,000 ×g, 4°C for 10 min. The peptides were desalinated and purified using a GL-tip SDB (#7820-11200, GL Sciences) following the manufacturer's protocol. The samples were speed-backed at 45°C for 30 min and dissolved in 25 µL of 0.1% formic acid. The samples were then centrifuged at 20,000 ×g, 4°C for 10 min, and the supernatants were collected. The peptide concentrations were determined using the BCA assay. Finally, 500 ng of the purified protein was subjected to LC-MS/MS analysis.

LC-MS/MS analyses

LC-MS/MS analyses were performed as previously described (Shinoda et al., 2023 [\[link\]](#)). Briefly, samples were loaded onto Acclaim PepMap 100 C18 column (75 µm x 2 cm, 3 µm particle size and 100 Å pore size; #164946, ThermoFisher Scientific) and separated on nano-capillary C18 column, (75 µm x 12.5 cm, 3 µm particle size, #NTCC-360/75-3-125, Nikkyo Technos) using EASY-nLC 1200 system (ThermoFisher Scientific). The elution conditions are listed in Table S3. The separated peptides were analyzed using QExactive (ThermoFisher Scientific) in data-dependent MS/MS mode. The parameters for the MS/MS analysis are listed in Table S4. The collected data were analyzed using Proteome Discoverer (PD) 2.2 software with the Sequest HT search engine. The parameters for the PD 2.2 analysis are described in Table S5. Peptides were filtered at a false discovery rate of 0.01 using the Percolator node. Label-free quantification was performed based on the intensities of the precursor ions using a precursor-ion quantifier node. Normalization was performed using the total amount of peptides in all average scaling modes. Proteins with 10 folds or higher abundance relative to the control (w^{1118}) were considered for further analysis. MS proteomic data were deposited in the ProteomeXchange Consortium via the jPOST partner repository with the dataset identifier PXD042922 (Okuda et al., 2017 [\[link\]](#)).

Gene Ontology analysis

Gene Ontology (GO) analysis of cellular components was performed using DAVID (Huang et al., 2009 [\[link\]](#)).

Generation of rabbit polyclonal anti-Fasciclin 3 isoform G antibody

The peptide corresponding to the 506–524 AA (N-LKPANEATPATTAPTAA-C) of Fas3G was used by Eurofins Inc. to immunize rabbits to raise a polyclonal antibody. The antibody was affinity purified using the same peptide provided by Eurofins, Inc.

IDR prediction

Amino acid sequences were obtained from the FlyBase database [<https://flybase.org/> [\[link\]](#)]. Disordered intracellular regions of Fas3 isoforms were predicted using the PSIPRED protein sequence analysis workbench in DISOPRED3 [<http://bioinf.cs.ucl.ac.uk/psipred/> [\[link\]](#)].

Protein complexes prediction with AlphaFold2-Multimer

Amino acid sequences were obtained from the FlyBase database [<https://flybase.org/>]. Protein complexes were predicted using ColabFold v1.5.5: AlphaFold2 using MMseqs2 (Mirdita et al., 2022) with the following settings: template_mode:none, msa_mode: mmseqs2_uniref_env, pair_mode: unpaired_paired, model_type: alphafold2_multimer_v3, num_recycles: 3. PAE plots of the structural models ranked first among five models were selected as representatives.

Molecular cloning

For all isoforms (A, B/F, C, D/E, G) of *pUASz-Fasciclin 3::3xFLAG*, the coding sequences of Fasciclin 3s were PCR-amplified using 3xFLAG-tag harboring primers from *Drosophila* cDNA and were ligated into the *Bam*HI/*Kpn*I-digested *pUASz1.0* vector (#1431, *Drosophila* Genomics Resource Center (DGRC)) (DeLuca and Spradling, 2018) using In-Fusion (#Z9648N, TaKaRa). For all isoforms of *pAc5-Fasciclin 3::3xFLAG*, the coding sequence of *Fasciclin 3s::3xFLAG* were PCR-amplified from corresponding *pUASz-Fasciclin 3::3xFLAG* vectors and were ligated into the *Eco*RI/*Xho*I-digested *pAc5-STABLE2-neo* vector (#32426, Addgene) (González et al., 2011) using NEBuilder HiFi DNA Assembly (#E2621L, NEB). For *pUASz-3xFLAG::mNeonGreen*, the coding sequence of mNeonGreen was PCR-amplified using 3xFLAG-tag harboring primers from *pAc5-V5::mNeonGreen* (Shinoda et al., 2023) and ligated into the *Xho*I-digested *pUASz1.0* vector using In-Fusion. For *pUASz-Drice::myc::mNeonGreen*, the coding sequence of mNeonGreen was PCR-amplified from *pAc5-V5::mNeonGreen* using myc-tag-harboring primers. The coding sequence of Drice was PCR-amplified from *Drosophila* cDNA. The two fragments were then ligated into the *Xho*I-digested *pUASz1.0* vector using In-Fusion. For *pUASz-V5::TurboID* and *pUASz-Caspase::V5::TurboID*, the coding sequences of *Dcp-1*, *Drice* and *Dronc* were PCR amplified from *Drosophila* cDNA. The coding sequence of V5::TurboID was PCR-amplified from *V5-TurboID-NES_pCDNA3* (#107169; Addgene). The two fragments were then ligated into the *Xho*I-digested *pUASz1.0* vector using In-Fusion. Catalytic dead mutations (*Dcp-1*: C196G (TGC to GGC), *Drice*: C211G (TGC to GGC), and *Dronc*: C318G (TGC to GGC)) were introduced by PCR-based site-directed mutagenesis, and the corresponding fragments were ligated into the *Xho*I-digested *pUASz1.0* vector using In-Fusion. For *pJFRC-mCD8::DQVD/A::QF2*, the coding sequence of *mCD8::DQVD/A* were PCR-amplified from genomic DNA of *Caspase-Sensitive/Insensitive-Gal4* (Tang et al., 2015). The coding sequence of *QF2* was PCR-amplified from *pBPQU* vector (Kashio et al., 2016). These fragments were ligated into the *Xho*I/*Xba*I-digested *pJFRC-28K* vector (a gift from H. Kazama) using In-Fusion. For *pUbi-FRT-STOP-FRT-mCD8::DQVD/A::QF2*, the FRT-STOP-FRT cassette sequence was PCR amplified from *pJFRC201-10xUAS-FRT-STOP-FRT-myr::smGFP-HA* (#63166; Addgene). The coding sequence of *mCD8::DQVD/A* was PCR-amplified from the genomic DNA of *Caspase-Sensitive/Insensitive-Gal4* (Tang et al., 2015). The coding sequence of *QF2* was PCR amplified from the *pBPQUw* plasmid (Kashio et al., 2016). The three fragments were ligated into *Eco*RI-digested *pUbi83* vector (a gift from D. Umetsu) using In-Fusion. For *pQUAST-mNeonGreen*, the coding sequence of mNeonGreen was PCR-amplified from *pAc5-V5::mNeonGreen* and ligated into the *Eco*RI-digested *pQUAST* (#24349, Addgene) vector using In-Fusion. For *pUASz-FLPo*, the coding sequences of *FLPo* were PCR-amplified from *pQUAST-FLPo* (#24357, Addgene) and ligated into the *Xho*I-digested *pUASz1.0* vector using In-Fusion. For *pWALIU20-Fas3G-shRNA*, the top and bottom oligonucleotides including mRNA sequence specific to isoform G (5'-AATGAACCAAAGCAAGACAAA-3') were annealed in annealing buffer (10 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA). The annealed fragment was ligated into *Nhe*I/*Eco*RI-digested *pWALIU20* (#1472, DGRC) vector using DNA Ligation Kit <MIGHTY Mix> (#6023, TaKaRa).

All established plasmids were sequenced by Eurofins, Inc. The detailed plasmid DNA sequences are available upon request. The established plasmids were injected into $y^1, w^{67c23}; P[CaryP]attP40$ (*pUASz-3xFLAG::mNeonGreen*, *pUASz-Drice::myc::mNeonGreen*, *pUbi-FRT-STOP-FRT-mCD8::DQVD/A::QF2* and *pWALIU20-Fas3G-shRNA*) or $y^1, w^{67c23}; P[CaryP]attP2$ (*pUASz-Fasciclin*

3s::3xFLAG, *pUASz-V5::TurboID* and *pUASz-Caspase::V5::TurboID*) for PhiC31-mediated transgenesis (BestGene Inc.). Each red-eye-positive transformant was isogenized. Integration of the attP landing site was confirmed using genomic PCR.

Cell culture

Drosophila S2 cells were grown at 25°C in Schneider's *Drosophila* medium (#21720001, GIBCO) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (#168-23191, WAKO). Cells were seeded in 24-well plates and transfected with the desired plasmids using Effectene Transfection Reagent (#301427, QIAGEN) following the manufacturer's protocol. Sixteen hours after transfection, the cells were reseeded into CELL CULTURE DISH, PS, 35/10 MM (#627870, Grainer BIO-ONE) and incubated for 24 h. Images were obtained using a Leica AF6000 DMI6000 B microscope (Leica Microsystems). Images were analyzed and edited using Fiji software (NIH Image).

For the substrate cleavage assay, cells were seeded in 12-well plates and were transfected with desired *pAc5-Fas3::3xFLAG* plasmids using Effectene Transfection Reagent following the manufacturer's protocol. After 40 h of incubation, the cells were treated with 10 µg/mL cycloheximide (CHX, #C7698, Sigma) for apoptosis induction. After 6 h of incubation, cells were collected using RIPA buffer supplemented with cOmplete ULTRA EDTA-free protease inhibitor cocktail depending on the cell status. For apoptotic cell lysate collection, cells were resuspended by pipetting and were collected into a 1.5 mL tube. The cells were then centrifuged at 800 ×g, 4 °C for 5 min, resuspended in PBS, and centrifuged again using the same parameters. The cell pellets were then lysed with 100 µL RIPA buffer. For the other experimental conditions, the cells were washed thrice with PBS and directly lysed on the plate with 100 µL RIPA buffer. The supernatants were collected and snap-frozen in liquid nitrogen. Protein concentrations were determined using the BCA assay following the manufacturer's protocol. The samples were mixed with 6x Laemmli buffer, boiled at 95°C for 5 min, and then subjected to western blot analysis.

For the validation of Fas3G-shRNA, cells were seeded in 12-well plates and were co-transfected with *pActin-Gal4*, desired *pUASz-Fas3::3xFLAG* and *pWALIU20-empty* or *pWALIU20-Fas3G-shRNA* plasmids using Effectene Transfection Reagent following the manufacturer's protocol. After 40 h of incubation, the cells were washed thrice with PBS and directly lysed on the plate with 100 µL RIPA buffer. The supernatants were collected and snap-frozen in liquid nitrogen. Protein concentrations were determined using the BCA assay following the manufacturer's protocol. The samples were mixed with 6x Laemmli buffer, boiled at 95°C for 5 min, and then subjected to western blot analysis.

Aging experiment

Adult male flies were raised in vials containing 15 fly/vial, and incubated at 29°C, 65% humidity with 12h/12h light/dark cycle. The flies were transferred every 2–4 days to vials containing fresh food.

Two choice preference assay

Newly eclosed adult male flies were raised in vials containing 25 fly/vial, and incubated at 29°C, 65% humidity with 12h/12h light/dark cycle. The flies were transferred every 2–4 days to vials containing fresh food for 1 week. The flies were starved in MilliQ (MQ) for 4 h prior to the assay. About 75 flies were put in 1000-mL glass beaker, which contained two standards bottles filled with 500 µl 1% ACV (Mizkan) and 500 µl MQ. The bottles were then covered with sponge plugs containing pipette tips. The pipette tip was cut to increase the size of the opening such that only one fly could climb into the bottle at a time. The glass beaker was covered with a mesh fabric. The beakers were set in an incubator at 29°C for 2 hr for the assay. The experiments were evaluated only if at least one of the tested flies entered either of 2 bottles. The preference index equaled the

number of flies in the ACV minus the number of flies in the MQ, divided by the number of total live flies in both inside and outside of the bottles, and multiplied by 100. Values greater than zero indicate a preference for 1% ACV and values less than zero indicate aversion. We repeated the same experiment with different sets of flies and the number of times is indicated by N.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 8 software (MDF). Data are shown as the means $\pm$ standard error of the mean. *P*-values were calculated using a one-way analysis of variance (ANOVA) with Bonferroni correction (**Figure 4B** [↗](#) for selected pairs, **Figure 4D** [↗](#) for every pair). *P*-values were calculated using the chi-square test with Bonferroni's correction, with mNeonGreen as a control (**Figure 4G** [↗](#)), LacZ-RNAi as a control (**Figure 4** [↗](#)– figure supplement 1D), mNeonGreen and Fas3G as controls (**Figure 4I** [↗](#), 5D), and mNeonGreen as a control (**Figure 6C** [↗](#)). *P*-values were calculated using one-way ANOVA with Dunnett's multiple comparison test (**Figure 6B** [↗](#), mNeonGreen as a control). NS: $P > 0.05$, †: < 0.05 .

Acknowledgements

We would like to thank Dr. D. Umetsu, Dr. H. Kazama, Addgene and *Drosophila* Genomics Resource Center for providing plasmids, Dr. Y. Nitta, Dr. A. Sugie, and Dr. T. Ichinose for helpful discussions, and the Bloomington *Drosophila* Stock Center, Vienna *Drosophila* Resource Center and Kyoto Stock Center for providing the fly strains. We thank Dr. S. Hirayama, Dr. S. Murata, and the one-stop sharing faculty center for future drug discovery at the Graduate School of Pharmaceutical Sciences, University of Tokyo, for the LC-MS/MS analysis. We thank Miura's lab members for their technical assistance and discussions; in particular, K. Takenaga for preparing the fly food, and R. Takamoto for experimental support. The MASCaT was established by MASaya Muramoto with critical advice from members of Shu MASuda at MASayuki Miura Laboratories. We would like to thank Editage (www.editage.com [↗](#)) for English language editing of this manuscript. This work was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (KAKENHI Grant Numbers 19K16137, 21K15080, 22H05586, and 23K05747 to N.S. and 21H04774, 21K19206 and 23H04766 to M.Mi.), the Japan Agency for Medical Research and Development (AMED; Grant number JP21gm5010001 to M.Mi.), and grants from the Takeda Science Foundation and Sumitomo Foundation to N.S.

Author Contributions

M.Mi. and N.S. designed the study; M.Mu., N.H. and N.S. conducted all the experiments and analyzed all the data; M.O. and T.C. supervised the two choice preference assay; M.Mu., N.H., M.Mi. and N.S. wrote the manuscript.

Conflict of Interests

The authors declare that they have no conflict of interest.

Figure 1–figure supplement 1.

Expression patterns of Drice in the adult brain in males and females.

(A) Representative images of adult male and female brains. Expression patterns of Drice are visualized using Streptavidin (SA, magenta). Nuclei are visualized by Hoechst33342 (Green). Scale bar: 50 μ m.

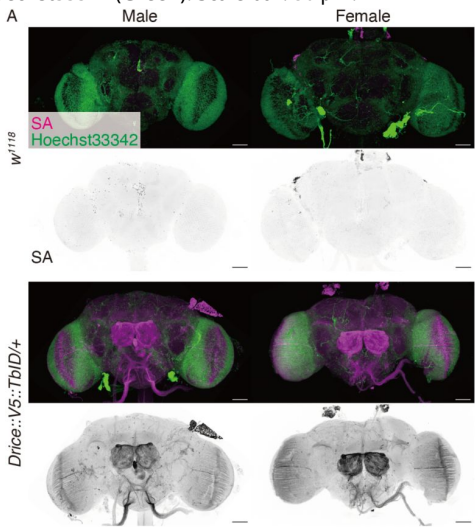


Figure 2–figure supplement 2.

Predicted protein complexes of Drice and each Fas3 isoform

(A) Predicted aligned error (PAE) plots of protein complex of Drice and each Fas3 isoform generated by AlphaFold2-Multimer. A represents Drice and B represents each Fas3 isoform. Numbers in X axis represent amino acid position. The intracellular region of each Fas3 isoform is represented by the orange bar. Potential interaction sites predicted by low PAE scores in Drice-Fas3A/G are highlighted by blue rectangles.

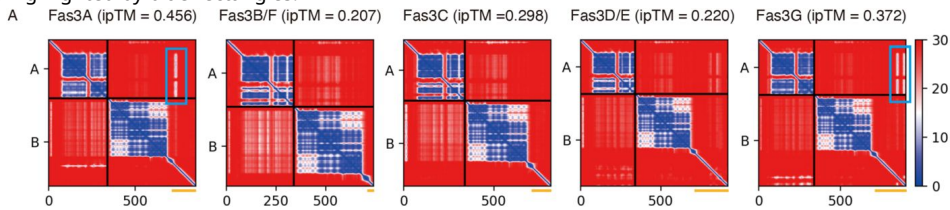


Figure 2-figure supplement 2.

Biochemical property of Fas3s with caspases.

(A) Co-immunoprecipitation of 3xFLAG-tagged Fas3s with V5-tagged Drice extracted from adult male heads.
(B) Western blot for 3xFLAG-tagged Fas3s expressed in *Drosophila* S2 cells. Apoptosis was induced by treating S2 cells with cycloheximide (CHX).

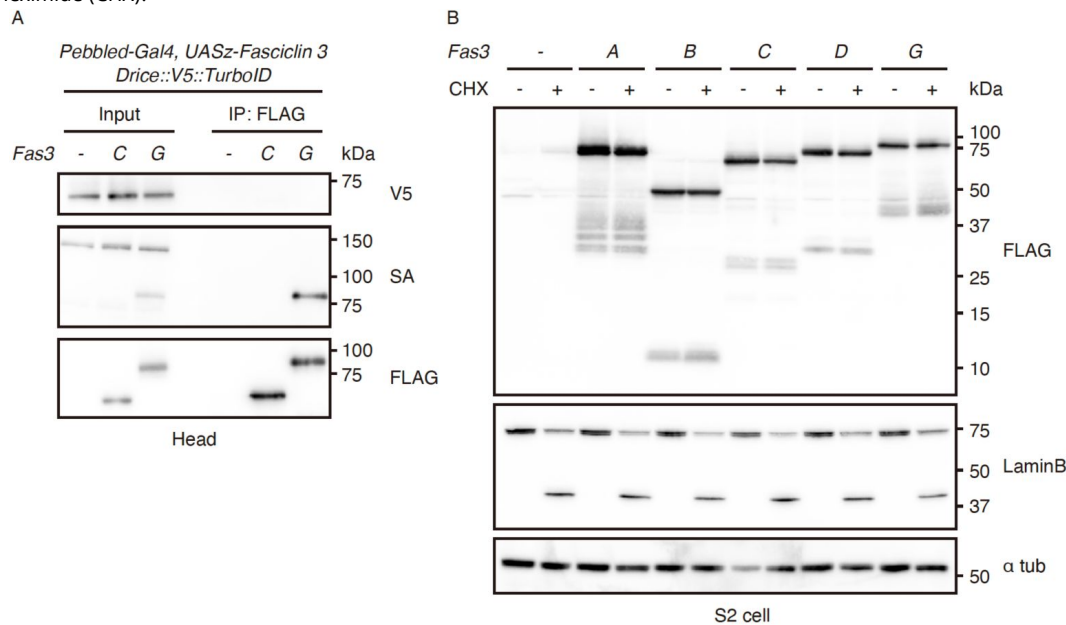
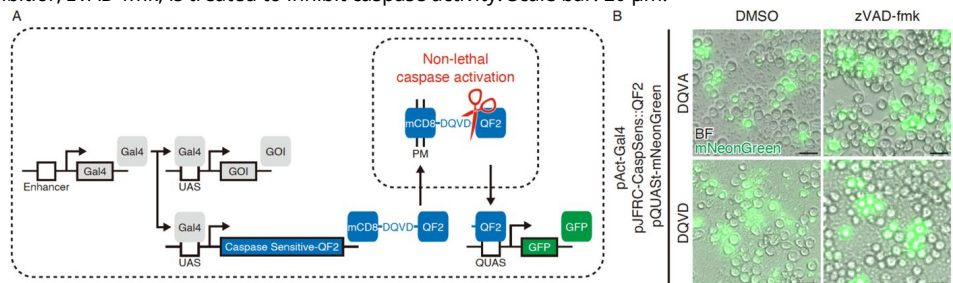


Figure 3-figure supplement 1.

Overexpression of *mCD8::DQVD::QF2* probes in *Drosophila* S2 cells.

(A) A schematic diagram of MASCaT expressed directly using the Gal4/UAS system. Caspase sensitive-QF2 is activated by caspase-mediated cleavage at the plasma membrane.
(B) Representative images of S2 cells expressing caspase sensitive-QF2 probes directly by the Gal4/UAS system. A pan-caspase inhibitor, zVAD-fmk, is treated to inhibit caspase activity. Scale bar: 20 μ m.



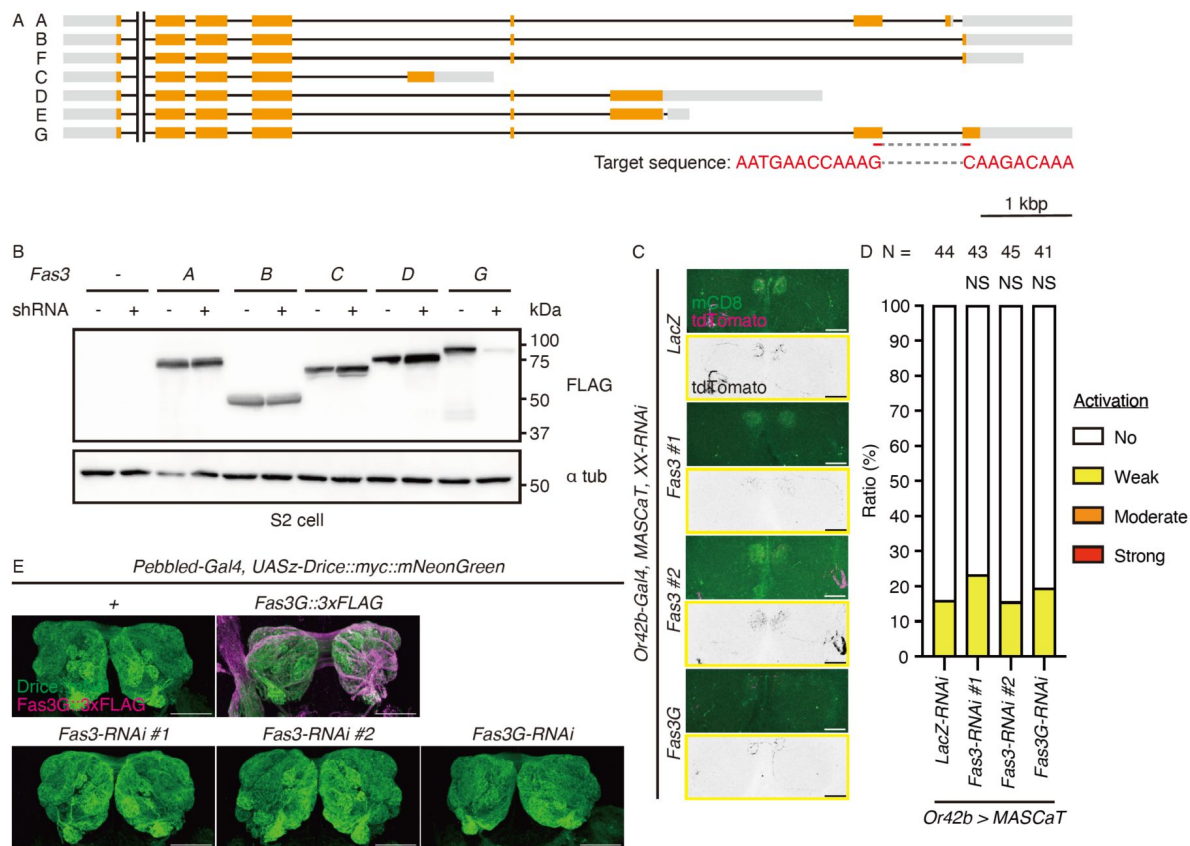


Figure 4-figure supplement 1

Loss of function analysis of Fas3G.

- (A) Gene structure of Fas3. CDS (orange) and UTR (grey) are shown in boxes. Target sequence of Fas3G-shRNA is shown in red.
- (B) Western blot for 3xFLAG-tagged Fas3s expressed in S2 cells with Fas3G-shRNA.
- (C) Representative images of the ALs of brains of 1 week old flies raised at 29°C with caspase activation visualized by MASCaT. Scale bar: 30 μm.
- (D) Quantifications of caspase activity detected by MASCaT of (C). *P*-values were calculated using chi-square test with Bonferroni's correction using *LacZ-RNAi* as control. NS: *P* > 0.05. Sample sizes are shown in the graph.
- (E) Representative images of the ALs of brains of 1 week old flies expressing mNeonGreen-tagged Drice (green) and 3xFLAG-tagged Fas3G (magenta). Scale bar: 50 μm.

References

- Amcheslavsky A, Wang S, Fogarty CE, Lindblad JL, Fan Y, Bergmann A (2018) **Plasma membrane localization of apoptotic caspases for non-apoptotic functions** *Dev Cell* **45**:450–464
- Aram L, Yacobi-Sharon K, Arama E (2017) **CDPs: caspase-dependent non-lethal cellular processes** *Cell Death Differ* **24**:1307–1310
- Boucher D, Blais V, Denault J-B (2012) **Caspase-7 uses an exosite to promote poly(ADP-ribose) polymerase 1 proteolysis** *Proc Natl Acad Sci U S A* **109**:5669–5674
- Branon TC, Bosch JA, Sanchez AD, Udeshi ND, Svinkina T, Carr SA, Feldman JL, Perrimon N, Ting AY (2018) **Efficient proximity labeling in living cells and organisms with TurboID** *Nat Biotechnol* **36**:880–887
- Campbell DS, Okamoto H (2013) **Local caspase activation interacts with Slit-Robo signaling to restrict axonal arborization** *J Cell Biol* **203**:657–672
- Cheriyamkunnel SJ, Rose S, Jacob PF, Blackburn LA, Glasgow S, Moorse J, Winstanley M, Moynihan PJ, Waddell S, Rezaval C (2021) **A neuronal mechanism controlling the choice between feeding and sexual behaviors in Drosophila** *Curr Biol* **31**:4231–4245
- Chiba A, Snow P, Keshishian H, Hotta Y (1995) **Fasciclin III as a synaptic target recognition molecule in Drosophila** *Nature* **374**:166–168
- Chihara T, Kitabayashi A, Morimoto M, Takeuchi K-I, Masuyama K, Tonoki A, Davis RL, Wang JW, Miura M (2014) **Caspase inhibition in select olfactory neurons restores innate attraction behavior in aged Drosophila** *PLoS Genet* **10**
- D’Brot A, Chen P, Vaishnav M, Yuan S, Akey CW, Abrams JM (2013) **Tango7 directs cellular remodeling by the Drosophila apoptosome** *Genes Dev* **27**:1650–1655
- Dehkordi MH, Munn RGK, Fearnhead HO (2022) **Non-canonical roles of apoptotic caspases in the nervous system** *Front Cell Dev Biol* **10**
- DeLuca SZ, Spradling AC (2018) **Efficient expression of genes in the Drosophila germline using a UAS promoter free of interference by Hsp70 piRNAs** *Genetics* **209**:381–387
- Desroches A, Denault J-B (2019) **Caspase-7 uses RNA to enhance proteolysis of poly(ADP-ribose) polymerase 1 and other RNA-binding proteins** *Proc Natl Acad Sci U S A* **116**:21521–21528
- Ding AX, Sun G, Argaw YG, Wong JO, Easwaran S, Montell DJ (2016) **CasExpress reveals widespread and diverse patterns of cell survival of caspase-3 activation during development in vivo** *Elife* **5** <https://doi.org/10.7554/eLife.10936>
- Ditzel M *et al.* (2008) **Inactivation of effector caspases through nondegradative polyubiquitylation** *Mol Cell* **32**:540–553

- Dorstyn L, Colussi PA, Quinn LM, Richardson H, Kumar S (1999) **DRONC, an ecdysone-inducible Drosophila caspase** *Proc Natl Acad Sci U S A* **96**:4307–4312
- Evans R *et al.* (2021) **Protein complex prediction with AlphaFold-Multimer** *bioRxiv* <https://doi.org/10.1101/2021.10.04.463034>
- González M, Martín-Ruiz I, Jiménez S, Pirone L, Barrio R, Sutherland JD (2011) **Generation of stable Drosophila cell lines using multicistronic vectors** *Sci Rep* **1**
- Green DR (2019) **The coming decade of cell death research: Five riddles** *Cell* **177**:1094–1107
- Gu Q, Jiao S, Duan K, Wang Y-X, Petralia RS, Li Z (2021) **The BAD-BAX-caspase-3 cascade modulates synaptic vesicle pools via autophagy** *J Neurosci* **41**:1174–1190
- Hay BA, Wassarman DA, Rubin GM (1995) **Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death** *Cell* **83**:1253–1262
- Huang DW, Sherman BT, Lempicki RA (2009) **Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists** *Nucleic Acids Res* **37**:1–13
- Igaki T, Kanda H, Yamamoto-Goto Y, Kanuka H, Kuranaga E, Aigaki T, Miura M (2002) **Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway** *EMBO J* **21**:3009–3018
- Julien O, Wells JA (2017) **Caspases and their substrates** *Cell Death Differ* **24**:1380–1389
- Kang Y, Neuman SD, Bashirullah A (2017) **Tango7 regulates cortical activity of caspases during reaper-triggered changes in tissue elasticity** *Nat Commun* **8**
- Kanuka H, Kuranaga E, Takemoto K, Hiratou T, Okano H, Miura M (2005) **Drosophila caspase transduces Shaggy/GSK-3beta kinase activity in neural precursor development** *EMBO J* **24**:3793–3806
- Karim MR, Endo K, Moore AW, Taniguchi H (2014) **Whole mount immunolabeling of olfactory receptor neurons in the Drosophila antenna** *J Vis Exp* <https://doi.org/10.3791/51245-v>
- Kashio S, Obata F, Zhang L, Katsuyama T, Chihara T, Miura M (2016) **Tissue nonautonomous effects of fat body methionine metabolism on imaginal disc repair in Drosophila** *Proc Natl Acad Sci U S A* **113**:1835–1840
- Kose H, Rose D, Zhu X, Chiba A (1997) **Homophilic synaptic target recognition mediated by immunoglobulin-like cell adhesion molecule Fasciclin III** *Development* **124**:4143–4152
- Koto A, Kuranaga E, Miura M (2009) **Temporal regulation of Drosophila IAP1 determines caspase functions in sensory organ development** *J Cell Biol* **187**:219–231
- Kumar S (2007) **Caspase function in programmed cell death** *Cell Death Differ* **14**:32–43
- Kumar S, Dorstyn L, Lim Y (2022) **The role of caspases as executioners of apoptosis** *Biochem Soc Trans* **50**:33–45

- Lee TV, Fan Y, Wang S, Srivastava M, Broemer M, Meier P, Bergmann A (2011) **Drosophila IAP1-mediated ubiquitylation controls activation of the initiator caspase DRONC independent of protein degradation** *PLoS Genet* **7**
- Li L, Ding Z, Pang T-L, Zhang B, Li C-H, Liang A-M, Wang Y-R, Zhou Y, Fan Y-J, Xu Y-Z (2020) **Defective minor spliceosomes induce SMA-associated phenotypes through sensitive intron-containing neural genes in Drosophila** *Nat Commun* **11**
- Li Z, Jo J, Jia J-M, Lo S-C, Whitcomb DJ, Jiao S, Cho K, Sheng M (2010) **Caspase-3 activation via mitochondria is required for long-term depression and AMPA receptor internalization** *Cell* **141**:859–871
- Luck K *et al.* (2020) **A reference map of the human binary protein interactome** *Nature* **580**:402–408
- Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M (2022) **ColabFold: making protein folding accessible to all** *Nat Methods* **19**:679–682
- Mukherjee A, Williams DW (2017) **More alive than dead: non-apoptotic roles for caspases in neuronal development, plasticity and disease** *Cell Death Differ* **24**:1411–1421
- Nakajima Y-I, Kuranaga E (2017) **Caspase-dependent non-apoptotic processes in development** *Cell Death Differ* **24**:1422–1430
- Ohsawa S, Hamada S, Kuida K, Yoshida H, Igaki T, Miura M (2010) **Maturation of the olfactory sensory neurons by Apaf-1/caspase-9-mediated caspase activity** *Proc Natl Acad Sci U S A* **107**:13366–13371
- Okuda S *et al.* (2017) **jPOSTrepo: an international standard data repository for proteomes** *Nucleic Acids Res* **45**:D1107–D1111
- Orme MH *et al.* (2016) **The unconventional myosin CRINKLED and its mammalian orthologue MYO7A regulate caspases in their signalling roles** *Nat Commun* **7**
- Qin W, Cho KF, Cavanagh PE, Ting AY (2021) **Deciphering molecular interactions by proximity labeling** *Nat Methods* **18**:133–143
- Semmelhack JL, Wang JW (2009) **Select Drosophila glomeruli mediate innate olfactory attraction and aversion** *Nature* **459**:218–223
- Shinoda N, Hanawa N, Chihara T, Koto A, Miura M (2019) **Dronc-independent basal executioner caspase activity sustains Drosophila imaginal tissue growth** *Proc Natl Acad Sci U S A* **116**:20539–20544
- Shinoda N, Horikoshi M, Taira Y, Muramoto M, Hirayama S, Murata S, Miura M (2023) **Caspase cleaves Drosophila BubR1 to modulate spindle assembly checkpoint function and lifespan of the organism** *FEBS J* **290**:4200–4223
- Takemoto K, Nagai T, Miyawaki A, Miura M (2003) **Spatio-temporal activation of caspase revealed by indicator that is insensitive to environmental effects** *J Cell Biol* **160**:235–243

Tang HL, Tang HM, Fung MC, Hardwick JM (2015) **In vivo CaspaseTracker biosensor system for detecting anastasis and non-apoptotic caspase activity** *Sci Rep* **5**

Valente P *et al.* (2016) **Cell adhesion molecule L1 contributes to neuronal excitability regulating the function of voltage-gated Na⁺ channels** *J Cell Sci* **129**:1878–1891

Verghese S, Bedi S, Kango-Singh M (2012) **Hippo signalling controls Dronc activity to regulate organ size in Drosophila** *Cell Death Differ* **19**:1664–1676

Walsh JG, Cullen SP, Sheridan C, Lüthi AU, Gerner C, Martin SJ (2008) **Executioner caspase-3 and caspase-7 are functionally distinct proteases** *Proc Natl Acad Sci U S A* **105**:12815–12819

Westphal D, Sytnyk V, Schachner M, Leshchyns'ka I (2010) **Clustering of the neural cell adhesion molecule (NCAM) at the neuronal cell surface induces caspase-8- and -3-dependent changes of the spectrin meshwork required for NCAM-mediated neurite outgrowth** *J Biol Chem* **285**:42046–42057

Williams DW, Kondo S, Krzyzanowska A, Hiromi Y, Truman JW (2006) **Local caspase activity directs engulfment of dendrites during pruning** *Nat Neurosci* **9**:1234–1236

Editors

Reviewing Editor

Jimena Berni

University of Sussex, Brighton, United Kingdom

Senior Editor

Albert Cardona

University of Cambridge, Cambridge, United Kingdom

Reviewer #1 (Public review):

Summary:

In this manuscript, Muramoto and colleagues have examined a mechanism by which the executioner caspase Drice is activated in a non-lethal context in *Drosophila*. The authors have comprehensively examined this in the *Drosophila* olfactory receptor neurons using sophisticated techniques. In particular, they had to engineer a new reporter by which non-lethal caspase activation could be detected. The authors conducted a proximity labeling experiment and identified Fasciclin 3 as a key protein in this context. While the removal of Fasciclin 3 did not block non-lethal caspase activation (likely because of redundant mechanisms), its overexpression was sufficient to activate non-lethal caspase activation.

Strengths:

While non-lethal functions of caspases have been reported in several contexts, far less is known about the mechanisms by which caspases are activated in these non-lethal contexts. So, the topic is very timely. The overall detail of this work is impressive and the results for the most part are well-controlled and justified.

Weaknesses:

The behavioral results shown in Figure 6 need more explanation and clarification (more details below). As currently shown, the results of Figure 6 seem uninterpretable. Also, overall

presentation of the Figures and description in legends can be improved.

<https://doi.org/10.7554/eLife.99650.1.sa1>

Reviewer #2 (Public review):

In this study, the authors investigate the role of caspases in neuronal modulation through non-lethal activation. They analyze proximal proteins of executioner caspases using a variety of techniques, including TurboID and a newly developed monitoring system based on Gal4 manipulation, called MASCaT. They demonstrate that overexpression of Fas3G promotes the non-lethal activation of caspase Dronc in olfactory receptor neurons. In addition, they investigate the regulatory mechanisms of non-lethal function of caspase by performing a comprehensive analysis of proximal proteins of executioner caspase Drice. It is important to point out that the authors use an array of techniques from western blot to behavioral experiments and also that they generated several reagents, from fly lines to antibodies.

This is an interesting work that would appeal to readers of multiple disciplines. As a whole these findings suggest that overexpression of Fas3G enhances a non-lethal caspase activation in ORNs, providing a novel experimental model that will allow for exploration of molecular processes that facilitate caspase activation without leading to cell death.

<https://doi.org/10.7554/eLife.99650.1.sa0>