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Exploring caspase-dependent non-lethal cellular processes using *Drosophila*

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Caspases are cysteine aspartic acid proteases conserved in animals that not only execute apoptosis, but also regulate diverse cellular processes independent of apoptosis, which are termed caspase-dependent non-lethal cellular processes (CDPs). Owing to its strong genetics to detect and manipulate caspase activity in cells of interest *in vivo*, *Drosophila melanogaster* serves as an excellent model organism for analyzing CDPs. This is further supported by the fact that apoptotic signaling, as well as CDPs and their mechanisms, are, in part, conserved in other animals. Here, we present a review to guide researchers studying CDPs using *Drosophila*. In this review, we provide an overview of the current understanding of apoptotic signaling, which regulates caspase activation in *Drosophila* as well as available genetic tools and their characteristics for detecting and manipulating caspase activity so that researchers can choose appropriate tools for their own experimental settings. We also introduce the CDPs identified in *Drosophila*, including a brief description of their discovery and characterization as non-lethal processes. We further describe the underlying molecular mechanisms of several well-characterized CDPs, including the regulatory mechanisms that enable non-lethal caspase activation. Finally, we introduce the use of proximity labeling techniques, especially TurboID, for studying CDPs, which facilitates the analysis of underlying molecular mechanisms. Because caspases regulate various non-lethal cellular functions, their activation is no longer considered a point of no return in cell death. Understanding CDPs will advance our understanding of the states of living and dying cells, along with the intermediate states.

KEYWORDS

caspase, caspase-dependent non-lethal cellular processes, *Drosophila*, caspase activity probes, TurboID, mass spectrometry

1 Introduction

Caspases are cysteine aspartic acid proteases that are conserved in multicellular organisms, and primarily execute apoptosis by cleaving hundreds of substrates (Newton et al., 2024). For examples, caspases cleave inhibitor of caspase-activated DNase (ICAD) to activate caspase-activated DNase (CAD) for DNA fragmentation during apoptosis (Enari et al., 1998; Sakahira et al., 1998). Caspases also cleave phospholipid scramblase Xkr8 and phospholipid flippases ATP11A/C to externalize phosphatidylserine which acts as an "eat-me" signal of apoptotic cells (Suzuki et al., 2013; Segawa et al., 2014; Segawa et al., 2016). In addition, caspases also regulate a wide range of cellular processes, from cell differentiation to organismal aging, without inducing cell death during development or beyond. In 2017, these processes were termed caspase-dependent non-lethal cellular processes (CDPs)

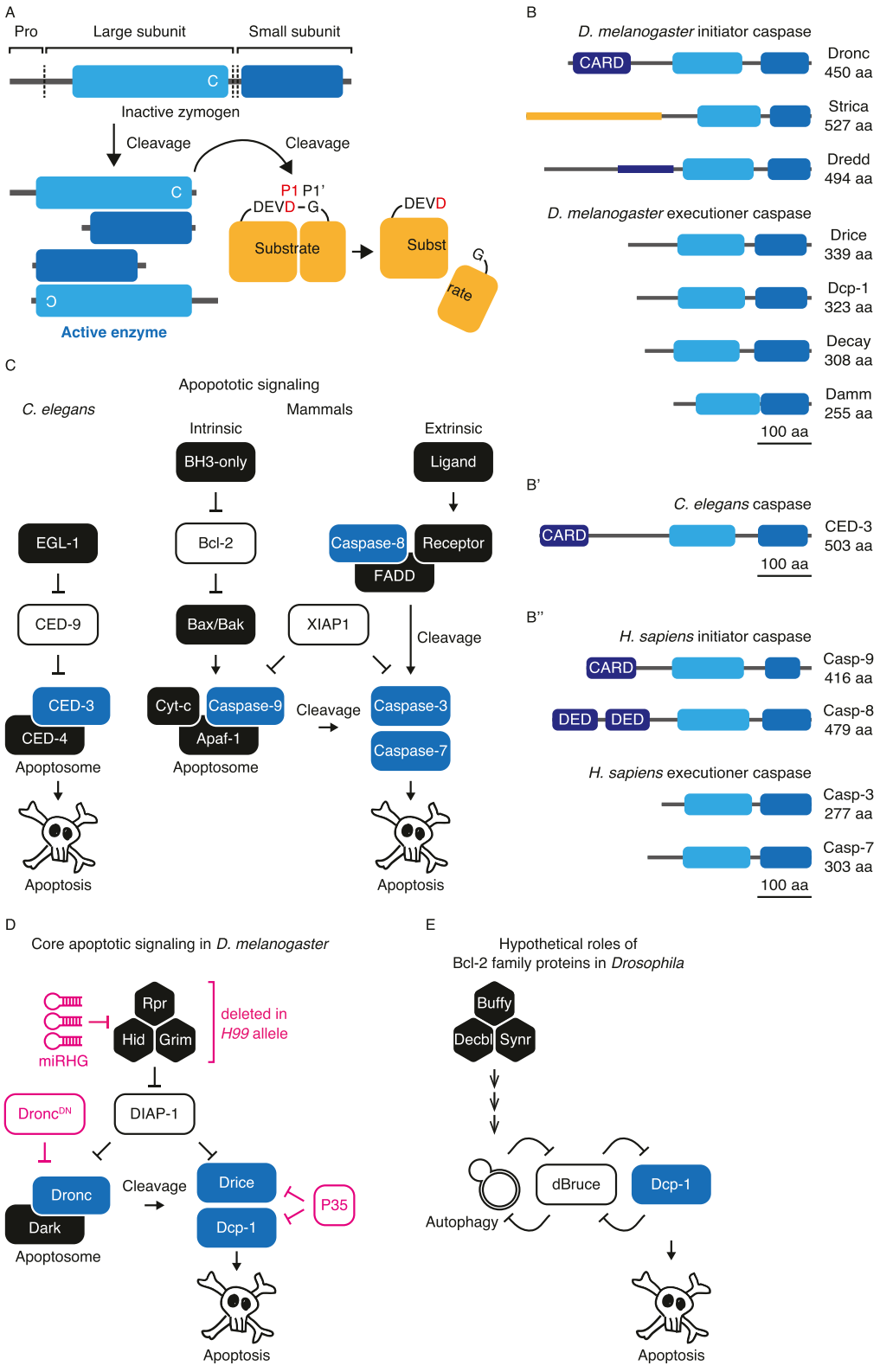

FIGURE 1
Caspases and apoptotic signaling in *Drosophila*. **(A)** Schematic structures and activation machinery of caspase. Inactive zymogen is activated by cleavages (dotted lines) followed by hetero-tetramerization which consists of two large subunits (light blue) and two small subunits (blue). Active enzyme typically cleaves C-terminus of aspartic acid at P1 position of substrates. **(B)** Schematic structures of seven *Drosophila* caspases **(B)**, *C. elegans* CED-3 **(B')**, and four representative mammalian caspases **(B")**. Three *Drosophila* caspases (Dronc, Strica, Dredd) which have long pro-domains are categorized as initiator caspases while four *Drosophila* caspases (Drice, Dcp-1, Decay, Damm) which have small pro-domains are categorized as executioner caspases. Purple boxes show CARD and DED domains. Light blue shows large subunits and blue shows small subunits. Domain information is from Pfam database [http://pfam.xfam.org/]. Although not in Pfam databse, Strica has serine-threonine rich region indicated by the orange line and Dredd has DID
(*Continued*)

FIGURE 1 (Continued)
domain indicated by the purple line at their N termini, respectively. **(C)** Apoptotic signaling in *C. elegans* and mammals. Caspases are shown in blue and their activators are shown in black while inhibiting components are shown in white. **(D)** Core apoptotic signaling in *Drosophila*. Caspases are shown in blue and their activators are shown in black while an inhibiting component is shown in white. Widely used genetic methods for manipulating the signaling are shown in magenta. **(E)** Hypothetical roles of Bcl-2 family proteins in *Drosophila*. Caspase is shown in blue and their activators are shown in black while an inhibiting component is shown in white.

(Aram et al., 2017). New CDPs are continually being discovered in diverse animals, although the underlying mechanisms that enable non-lethal caspase activation without causing cell death or downstream signaling of caspase activation are lagging.

Drosophila melanogaster is a widely used model organism for analyzing diverse biological processes, from development to aging. Caspases and their upstream apoptotic signaling in *Drosophila* are, in part, conserved among other animals (Kuranaga and Miura, 2007; Nakajima and Kuranaga, 2017). Thus, along with strong *Drosophila* genetics to detect and manipulate caspase activity in cells of interest *in vivo*, *Drosophila* serves as one of the best model organisms not only for describing CDPs but also for characterizing their underlying molecular mechanisms. A wide variety of CDPs have been discovered in *Drosophila* (Nakajima and Kuranaga, 2017). Notably, some CDPs and their mechanisms are conserved in other animals, further supporting the idea that *Drosophila* is an excellent model organism for studying CDPs.

In this review, we provide an overview of the current understanding of apoptotic signaling that regulates caspase activation in *Drosophila*. In the following section, we summarize the currently available genetic tools and their characteristics for detecting and manipulating caspase activity so that researchers can choose appropriate tools for their own experimental settings. We also introduce the currently identified CDPs in *Drosophila*, including a brief background on their discovery and characterization as non-lethal processes. We further describe the underlying molecular mechanisms of some intensively studied CDPs, including the regulatory mechanisms of non-lethal caspase activation. Finally, we introduce the use of proximity labeling techniques, especially TurboID, to study CDPs as a recently emerging method for analyzing caspase-proximal proteins, which facilitates the analysis of the molecular mechanisms of CDPs. We hope that researchers will find ways to study CDPs after reviewing this review.

2 Caspases and apoptotic signaling in *Drosophila*

2.1 Caspases and their classification in *Drosophila*

Caspases are cysteine aspartic acid proteases which execute apoptosis in multicellular organisms (Newton et al., 2024). Caspases primarily cleave their substrates at the C-terminal of aspartic acid at P1 position. Preference for P1' has been observed in smaller amino acids (glycine, serine, alanine) and amino acids with large aromatic rings (phenylalanine, tyrosine) (Timmer and Salvesen, 2007). Structurally, caspase, which is first synthesized as an inactive zymogen, consists of a pro-domain and large and small subunits. For full activation, caspases need to be cleaved by themselves or by other caspases, resulting in a heterotetramer composed of two large and two small subunits (Julien and Wells, 2017) (Figure 1A). In *Drosophila*, there are seven caspases which are divided into two groups (Kumar and Doumanis, 2000) (Figure 1B): initiator caspases (class I caspases) which have a large pro-domain, including Dronc (Dorstyn et al., 1999a), Strica (Doumanis et al., 2001), and Dredd (Chen et al., 1998), and executioner caspases (class II caspases) which have a short pro-domain, including Drice (Fraser and Evan, 1997), Dcp-1 (Song et al., 1997), Decay (Dorstyn et al., 1999b), and Damm (Harvey et al., 2001). Among the seven caspases, the initiator caspase Dronc (Chew et al., 2004; Daish et al., 2004; Xu et al., 2005) and the executioner caspases Drice and Dcp-1 (Xu et al., 2006; Kondo et al., 2006; Florentin and Arama, 2012) are well described in their requirements for executing apoptosis, where Drice is more effective in inducing apoptosis than Dcp-1 (Florentin and Arama, 2012).

While we primarily reviewed the role of non-lethal caspase functions of apoptotic components, it should be mentioned that few caspases are not involved in apoptosis. Dredd (Chen et al., 1998) is not involved in apoptosis but rather regulates innate immune pathways in *Drosophila*. The Toll (Valanne et al., 2011) and immune deficiency (IMD) pathways (Myllymäki et al., 2014) are the two major innate immune pathways in *Drosophila*. IMD pathway is activated by infection with gram-negative bacteria and eventually activate Relish, an ortholog of mammalian NF-κB, to induce antimicrobial peptide (AMP) expression. IMD protein binds to the bacterial peptidoglycan (PGN) receptor PGRP-LC, and the adaptor proteins FADD and Dredd. FADD-Dredd interaction is mediated through death-inducing domain (DID) (Hu and Yang, 2000). DIAP2 binds to Dredd and promotes K63 ubiquitination and activation. Activated Dredd cleaves IMD and Relish, and activates the IMD pathway (Meinander et al., 2012; Kleino and Silverman, 2014). C-terminal ankyrin repeat region of Relish is cleaved by Dredd and N-terminal NF-κB portion translocates to the nucleus to induce AMP expression. Dredd is activated by PGN in S2 cells. Dredd is not cleaved for activation and its activity cannot be inhibited by a baculovirus-derived protein, P35 (Kim et al., 2014).

2.2 Core apoptotic signaling in *Drosophila*

Apoptotic signaling for caspase activation in *Drosophila* is partially conserved among multicellular organisms (Kuranaga and Miura, 2007; Nakajima and Kuranaga, 2017) (Figures 1C, D). In *Caenorhabditis elegans*, caspase, CED-3 (Figure 1B'), is activated through the formation of apoptosome complex with CED-4. The apoptosome complex formation is inhibited by CED-9, which is further antagonized by EGL-1 upon apoptotic stimuli (Horowitz and Shaham, 2024) (Figure 1C). In contrast,

mammals have two apoptotic signaling pathways called intrinsic and extrinsic apoptotic signaling pathways. Similar to *Drosophila* caspases, mammalian caspases are also categorized into initiator caspases (Caspase-8 and Caspase-9) and executioner caspases (Caspase-3 and Caspase-7) (Figure 1B"). In both pathways, executioner caspases are activated by the initiator caspase. In intrinsic apoptotic signaling pathway, Caspase-9 is activated through the formation of apoptosome complex with apoptotic peptidase activating factor 1 (Apaf-1), an ortholog of CED-4, and mitochondria-derived cytochrome c, the release of which is mediated by mitochondrial outer membrane permeabilization (MOMP) by Bax and Bak. Bax and Bak are negatively regulated by B-cell lymphoma-2 (Bcl-2), an ortholog of CED-9, which is further antagonized by BH3-only proteins, orthologs of EGL-1, upon apoptotic stimuli. In extrinsic apoptotic signaling pathway, Caspase-8 is activated by extracellular death ligands-stimulated death receptors and its adaptor, FADD. In mammals, caspases are also negatively regulated by inhibitor of apoptosis proteins (IAPs), E3 ubiquitin ligases, including X-linked inhibitor of apoptosis protein (XIAP) (Newton et al., 2024) (Figure 1C).

Core apoptotic signaling in *Drosophila* is most similar to mammalian intrinsic apoptotic signaling pathway. Upon apoptotic stimuli, *Drosophila* Apaf-1-related killer (Dark)/homolog of Apaf-1 (Hac-1)/Dapaf-1 (Kanuka et al., 1999; Rodriguez et al., 1999; Zhou et al., 1999) forms apoptosome complex with the initiator caspase, Dronc, for activation via autocatalytic cleavage (Tian et al., 2024). Unlike mammals, apoptosome formation does not require mitochondria-derived cytochrome c (Dorstyn et al., 2002; Dorstyn et al., 2004; Yu et al., 2006). Activated Dronc cleaves the downstream executioner caspases Drice (Hawkins et al., 2000) and Dcp-1 to induce apoptosis. In living cells, caspase activation is negatively regulated by the *Drosophila* inhibitor of apoptosis protein 1 (DIAP1), an E3 ubiquitin ligase (Hay et al., 1995). DIAP1 ubiquitinates Dronc (Wilson et al., 2002), Drice, and Dcp-1 to inactivate them in a nondegradative (independent of proteasomal degradation) manner (Ditzel et al., 2008). Upon apoptotic stimuli, the pro-apoptotic IAP antagonists reaper (Rpr), head involution defective (Hid), and Grim, together called RHG proteins, the genes of which are deleted in *H99* allele (White et al., 1994), are synthesized. RHG proteins bind to DIAP1 to facilitate DIAP1 degradation through auto-ubiquitination (Ryoo et al., 2002). Developmental and stress-induced cell death is mostly suppressed in mutants of *RHG* (White et al., 1994), *Dronc* (Chew et al., 2004; Daish et al., 2004; Xu et al., 2005) and double mutants of *Drice* and *Dcp-1* (Kondo et al., 2006; Xu et al., 2006; Florentin and Arama, 2012), which are regarded as components of the core apoptotic signaling pathway in *Drosophila* (Figure 1D).

2.3 Bcl-2 family proteins in *Drosophila*

In addition to the well-characterized core apoptotic signaling pathway, *Drosophila* has two Bcl-2-like proteins, Death executioner Bcl-2 (Debcl)/Drob-1 (Colussi et al., 2000; Igaki et al., 2000) and Buffy (Quinn et al., 2003). In mammals, the Bcl-2 family proteins regulate apoptosis through the regulation of MOMP mediated by the pore-forming proteins, Bax and Bak (Figure 1C) (Singh et al., 2019). Since apoptosome formation in *Drosophila* does not require mitochondria-derived cytochrome c, the actions of Debcl/Drob-1 and Buffy seem to differ from those in mammals (Igaki and Miura, 2004). Recently, a BH3-only-like protein, Sayonara (Synr), whose overexpression induces caspase activation through autophagy, was described (Ikegawa et al., 2023). Synr-induced caspase activation is antagonized by *Debcl/Drob-1*, *Buffy* or *Dcp-1* knockdown. In addition, Debcl/Drob-1, Buffy, and Dcp-1 are all required for starvation-induced autophagy in *Drosophila* cell lines (Hou et al., 2008). Thus, it seems likely that the Bcl-2 family proteins in *Drosophila* regulate apoptosis through autophagy and the executioner caspase Dcp-1. Since autophagy degrades another giant IAP, dBruce, for caspase activation in nurse cells during oogenesis (Nezis et al., 2010), it is possible that autophagy activates caspase via dBruce degradation (Figure 1E). Importantly, both *Debcl/Drob-1*, *Buffy* double mutant (Sevrioukov et al., 2007) and *Synr* mutant (Ikegawa et al., 2023) are developmentally viable, suggesting that Bcl-2 family proteins in *Drosophila* act not as core apoptotic machinery, but rather only in a specific biological context such as starvation.

3 Detecting and manipulating caspase activation in *Drosophila*

3.1 Genetic manipulations of core apoptotic signaling in *Drosophila*

Using *Drosophila* strong genetics, we can detect and manipulate caspase activation in cells of interest to analyze functions and mechanisms of caspase activation *in vivo*. In addition to the mutant alleles and the Gal4/UAS system (Brand and Perrimon, 1993) -mediated knockdown of each apoptotic component, several other manipulations have been well established (Figure 1D). One such manipulation is the expression of the baculovirus-derived protein P35 which inhibits whole executioner caspase activation (Hay et al., 1994), however not initiator caspase activation (Meier et al., 2000). *P35* expression suppresses both Drice and Dcp-1, thereby avoiding the redundancy of each caspase to execute apoptosis. To inhibit all executioner caspase activities, combined RNAi against *Drice*, *Dcp-1*, *Decay*, and *Damm* is sometimes used to completely deplete their activity (Arthurton et al., 2020). The expression of *Dronc dominant-negative* ($Dronc^{DN}$), which harbors a mutation in the catalytic cysteine, is frequently used to suppress apoptosis (Meier et al., 2000). Furthermore, inhibition of upstream *RHG* genes by simultaneous knockdown using microRNAs (miRHG) strongly suppresses caspase activation (Siegrist et al., 2010). Genetic manipulations of caspase activity *in vivo* not only identify the role of apoptotic cells during development and homeostasis but also lead to the identification of numerous non-lethal caspase functions (see Section 4).

3.2 Antibody staining for detecting cleaved (activated) executioner caspases

One of the strong advantages of *Drosophila* in studying caspase function is the variety of biochemical and genetic tools for detecting

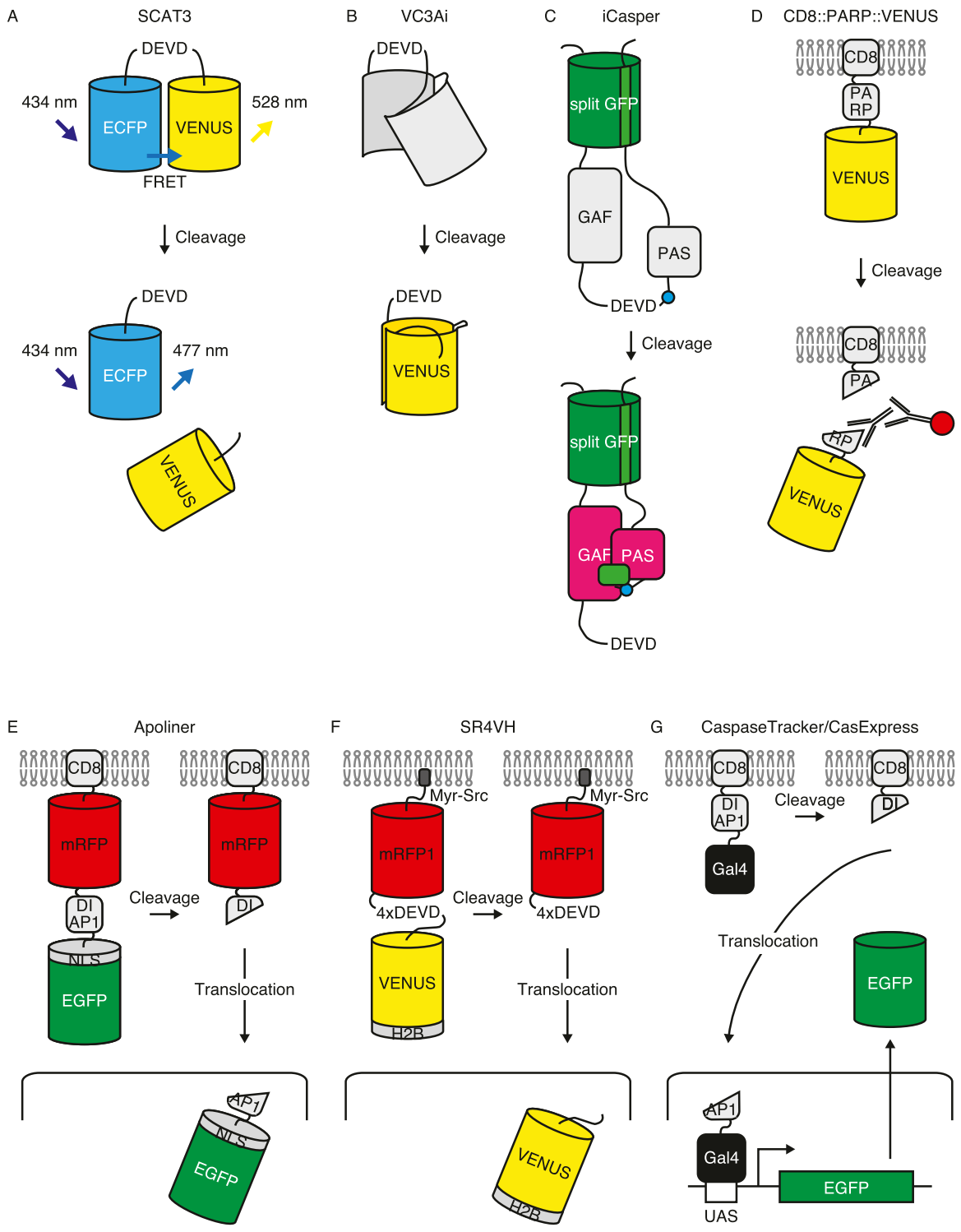

FIGURE 2
Genetically-encoded probes for detecting caspase activity in *Drosophila*. **(A)** Schematic structures of FRET-based probe, SCAT3. Caspase-mediated cleavage diminishes FRET between ECFP and VENUS. Caspase activity is measured by the reduction in VENUS/ECFP FRET ratio. The peak wavelengths for excitation/emission of fluorescent proteins are shown. **(B)** Schematic structures of fluorescent switch-on probe, VC3Ai. Caspase-mediated cleavage makes the probe fluorescent. Caspase activity is measured by the intensity of the probe. **(C)** Schematic structures of fluorescent switch-on probe, iCasper, which is tagged with a split GFP at new N′- and C′-terminal. A blue circle shows catalytic cysteine, while a green box shows biliverdin. Caspase-mediated cleavage makes the probe fluorescent. Caspase activity is measured by the intensity of the probe. Cells expressing iCasper are detected by split-GFP fluorescence. **(D)** Schematic structures of CD8::PARP::VENUS. Caspase-mediated cleavage generates neo-epitope of anti-cleaved PARP antibody. Caspase activity is measured by the intensity of the immunohistochemistry. **(E)** Schematic structures of fluorescent translocation probe, Apoliner. Caspase-mediated cleavage releases NLS::EGFP translocating to the nucleus. Caspase activity is measured by the intensity of NLS::EGFP in the nucleus.
(*Continued*)

FIGURE 2 (Continued)
(F) Schematic structures of fluorescent translocation probe, SR4VH. Caspase-mediated cleavage releases VENUS::H2B translocating to the nucleus. Caspase activity is measured by the intensity of VENUS::H2B in the nucleus. **(G)** Schematic structures of transcription factors activating probes, CaspaseTracker/CasExpress. Caspase-mediated cleavage releases Gal4 translocating to the nucleus to drive fluorescent protein expressions (e.g., EGFP) downstream of the *UAS* sequence. Caspase activity is measured by the intensity of the synthesized EGFP or other fluorescent proteins.

caspase activation. Because caspase activation relies on its own cleavage, anti-cleaved caspase antibodies, including anti-cleaved (activated) Caspase-3 (Asp175) antibody (#9661, Cell Signaling Technology), anti-cleaved (activated) Dcp-1 (Asp215) antibody (#9578, Cell Signaling Technology) and anti-active Drice antibody (Dorstyn et al., 2002), are frequently used to detect activated caspases and are thus sometimes used as markers for apoptosis. However, when using these antibodies, researchers must be careful while interpreting of the results. Because anti-cleaved Caspase-3 (Asp175) antibody immunoreactivity persists in *Dcp-1* and *Drice* (*Drosophila* orthologs of *Caspase-3*) double mutants and immunoreactivity is lost in both *Dronc* and *Dark* (*Drosophila* orthologs of *Caspase-9* and *Apaf-1*, respectively) mutants, this antibody is considered a marker of Dronc activity in *Drosophila* (Fan and Bergmann, 2010). Anti-cleaved Dcp-1 (Asp215) antibody immunoreactivity is lost in *Drice* and *Dcp-1* double mutants, but not in *Dcp-1* mutant, and thus serves as a specific marker for activated executioner caspases of Drice and Dcp-1 however is not specific to activated Dcp-1 in *Drosophila* (Li et al., 2019). Because immunohistochemistry using antibodies preserves the spatial information of caspase activation, sometimes even inside the cell, it is useful not only for detecting apoptotic cells within tissues, but also for examining the spatial patterns of caspase activation in non-lethal scenarios (Kang et al., 2017; Amcheslavsky et al., 2018; McSharry and Beitel, 2019). However, since antibody staining only captures a snapshot of caspase activation at a single point in time, it is unclear whether cells ultimately die. Thus, live cell monitoring of caspase activity using genetically-encoded caspase activity probes and reporters helps trace the fate of cells to characterize the processes as CDPs.

3.3 Genetically-encoded caspase activity probes and reporters

A more direct method for measuring caspase activity is the use of caspase activity probes and reporters. Since caspases recognize specific amino acid sequences with a high preference for aspartic acid at the P1 position (canonically, DEVD for executioner caspases) (Talanian et al., 1997), probes and reporters utilizing these properties are often developed in *Drosophila*. These include sensor for activated caspase-3 based on FRET (SCAT3) (Takemoto et al., 2003; Takemoto et al., 2007), VENUS-based variant of caspase-3-like proteases activity indicator (VC3Ai) (Zhang et al., 2013; Schott et al., 2017), infrared fluorescent executioner-caspase reporter (iCasper) (To et al., 2015), CD8::PARP::VENUS (Williams et al., 2006), Apoliner (Bardet et al., 2008), SR4VH (Pop et al., 2020), CaspaseTracker/CasExpress (Tang et al., 2015; Ding et al., 2016) and their variants (Baena-Lopez et al., 2018; Sun et al., 2020; Muramoto et al., 2024). As these probes are based on different rationales and have distinct characteristics, each probe has its own advantages and disadvantages in detecting caspase activity.

3.3.1 A FRET-based probe for detecting caspase activity

SCAT3 is a FRET-based caspase activity probe that detects caspase activation with high temporal resolution during live cell imaging (Figure 2A). SCAT3 is composed of ECFP and VENUS linked by 18-amino acids including the caspase recognition sequence DEVD. Caspase activity was detected by a reduction in the VENUS/ECFP FRET ratio due to the cleavage-mediated release of VENUS (Takemoto et al., 2003). SCAT3 is useful for detecting caspase activity in live cell/tissue imaging, such as in *Drosophila* pupal salivary gland, abdomen, male genitalia, and notum, with high temporal resolution (Takemoto et al., 2007; Koto et al., 2011; Kuranaga et al., 2011; Nakajima et al., 2011; Levayer et al., 2016; Fujisawa et al., 2019; Fujisawa et al., 2020). A nuclear-localized signal-tagged variant of SCAT3 (NLS::SCAT3) is also available (Takemoto et al., 2003; Koto et al., 2009), and is useful for the identification of individual cells in complex tissues. The drawback of SCAT3 is that FRET imaging requires an ECFP-exciting laser such as 448 nm diode laser, and post-acquisition image processing to calculate the FRET ratio, which needs to be prepared before experiments by researchers.

3.3.2 Fluorescent switch-on probes for detecting caspase activity

VC3Ai is a circularly permuted VENUS (cpVENUS) caspase activity probe that detects caspase activation by switching on VENUS fluorescence (Figure 2B). VC3Ai is composed of cpVENUS with its N- and C-terminal fused with caspase recognition sequence, DEVD, with a split *Npu* DnaE intein at new N′- and C′-terminal to enhance the efficiency of complementation upon cleavage. VC3Ai was originally synthesized as a non-fluorescent indicator that becomes fluorescent upon cleavage. In addition to the VENUS fluorescent protein, multi-fluorescent color variants of CC3Ai based on Cerulean, GC3Ai based on superfolder GFP, and RC3Ai based on mCherry are available (Zhang et al., 2013). VC3Ai is useful in detecting caspase activity with a standard fluorescent microscope system in live and fixed cell/tissue such as *Drosophila* imaginal discs (Schott et al., 2017; Matamoro-Vidal et al., 2024) and pupal notum (Fujisawa et al., 2019; Fujisawa et al., 2020; Villars et al., 2022). VC3Ai is good for detecting apoptosis mediated by strong caspase activation. However, it may lack the sensitivity to detect non-lethal caspase activation (Schott et al., 2017; Fujisawa et al., 2019).

iCasper is a cpIFP-based caspase activity probe that detects caspase activation using switched-on IFP fluorescence (Figure 2C). iCasper is composed of cpIFP with its N- and C-terminal fused with the caspase recognition sequence, DEVD, with a split GFP at the new N′- and C′-terminal to ensure that following caspase-mediated

cleavage, the cleaved probes remain intact. iCasper was originally synthesized as a non-IFP fluorescent indicator that becomes fluorescent upon cleavage, with split GFP fluorescence. Since IFP requires biliverdin at its chromophore, iCasper also requires it to be fluorescent (To et al., 2015). iCasper is useful for detecting caspase activity, at least in apoptotic cells in live and fixed cells/tissues, such as embryos, larval brains (To et al., 2015), and pupal nota (Canales Coutiño et al., 2020). Because the number of reports using iCasper is still limited, its characteristics, including sensitivity compared to other probes as well as the potential of this probe to be applicable for analyzing non-lethal caspase activation, should be described in the future.

3.3.3 Antibody that recognizes cleaved neoepitope of the probe for detecting caspase activity

CD8::PARP::VENUS is a probe that was developed to detect localized caspase activity in neurons. CD8::PARP::VENUS is composed of the extracellular and transmembrane domains of mouse CD8 fused to 40 amino acids from human PARP containing a caspase cleavage site and VENUS. Caspase activity was detected by immunohistochemistry using an anti-cleaved PARP antibody that recognizes cleaved fragments of the probe (Williams et al., 2006) (Figure 2D). Probe overexpression enhances the sensitivity of the immunoreaction to antibodies in the respective cells. CD8::PARP::VENUS is useful not only for detecting caspase activation in neurons (Williams et al., 2006; Chihara et al., 2014), but also in other tissues, including wing imaginal discs (Florentin and Arama, 2012; Shinoda et al., 2016; Shinoda et al., 2019; Gorelick-Ashkenazi et al., 2018). It is also used to detect local caspase activation in the pruned dendrites of neurons (Williams et al., 2006). However, because CD8::PARP::VENUS requires immunohistochemistry, it is not applicable to live cell/tissue imaging.

3.3.4 Fluorescent protein translocation probes for detecting caspase activity

Apoliner is an mRFP- and EGFP-based probe that detects caspase activation via translocation of EGFP into the nucleus (Figure 2E). Apoliner is composed of the transmembrane domain of mouse CD8 fused with mRFP, a caspase cleavage site from DIAP1, and EGFP with a nuclear localization signal of the large T antigen (NLS::EGFP). Apoliner resides on the membrane. Following caspase activation, NLS::EGFP is released via caspase-mediated cleavage and accumulates in the nucleus (Bardet et al., 2008). Apoliner is useful for detecting caspase activity in live and fixed cells of embryo (Bardet et al., 2008; Kolahgar et al., 2011).

SR4VH is an RFP- and VENUS-based probe that detects caspase activation with the translocation of VENUS into the nucleus, the rationale for which is similar to that of Apoliner. SR4VH is composed of a membrane-bound RFP (Src::RFP) fused with four tandem repeats of the amino acid sequences DEVD and VENUS tagged with Histone H2B (VENUS::H2B) (Figure 2F). Following caspase activation, VENUS::H2B is released by caspase-mediated cleavage, translocated, and accumulated in the nucleus. While the design was similar to that of Apoliner, the authors discussed that the myristoylation signal from Src differentiates its subcellular localization from that of Apoliner. They also reported that the nuclear localization signal from H2B allow for efficient sequestration, even in the late stages of apoptosis (Pop et al., 2020). SR4VH is useful for detecting caspase activity in live and fixed cells/tissues, such as larval brains (Pop et al., 2020), wing imaginal discs, and dendritic arborization neurons (Mukherjee et al., 2021). Both Apoliner and SR4VH have sufficient sensitivity to detect apoptosis. However, whether these probes can be used to analyze non-lethal caspase activation needs to be further tested.

3.3.5 Transcription factors activating probes for detecting caspase activity

CaspaseTracker/CasExpress is a caspase-activated transcription factor-based reporter that drives the expression of downstream fluorescent proteins for visualization. The caspase-activatable transcription factor is composed of a transmembrane protein of mouse CD8 fused with a caspase-binding and cleavage domain from DIAP1 and the transcription factor Gal4. In the systems, caspase activatable-transcription factor is ubiquitously expressed using ubiquitin promoter. Following caspase activation, the cell membrane-sequestered Gal4 is released by caspase-mediated cleavage and Gal4 accumulates in the nucleus to drive gene expression downstream of *UAS* sequence (Tang et al., 2015; Ding et al., 2016) (Figure 2G). Because of its Gal4/UAS-mediated binary gene expression system, CaspaseTracker/CasExpress is highly useful for detecting both lethal and non-lethal caspase activation throughout the body with high sensitivity. Moreover, CaspaseTracker/CasExpress is more sensitive than VC3Ai and SCAT3 (Schott et al., 2017; Fujisawa et al., 2019). It may also be more sensitive than all other probes. By combining the probe with the lineage-tracing system G-TRACE, the authors identified a wide range of developmentally caspase-activated but surviving cells at the adult stage, suggesting diverse non-lethal caspase functions in *Drosophila* (Tang et al., 2015; Ding et al., 2016). However, because CaspaseTracker/CasExpress relies on the Gal4/UAS-mediated binary gene expression system, the temporal resolution should not be as high as that of SCAT3 and other probes. In addition, the reliance on Gal4 to report caspase activation in CaspaseTracker/CasExpress presents a challenge in reconciling Gal4/UAS-dependent gene manipulations, such as RNAi or overexpression. Thus, L-CasExpress, in which Gal4 is replaced with LexA, has been developed (Sun et al., 2020). Furthermore, to restrict the expression of the probe within the cells of interest, a Gal4-Manipulated Area Specific CaspaseTracker/CasExpress (MASCaT) has been developed, where Gal4 was replaced by QF2 and probe expression was regulated by FLP/FRT-mediated excision of the STOP cassette downstream of Gal4/UAS expression system (Muramoto et al., 2024). Both systems further improve the usability of CaspaseTracker/CasExpress. Similar to CaspaseTracker/CasExpress, which detects executioner caspase activity, a Drice-based sensor (DBS), specifically CD8::Drice^{C211A}-short (DBS-S), was developed to detect the activity of initiator caspase, Dronc. DBS-S is composed of a transmembrane domain of CD8 fused with an enzymatically inactive Drice, Drice^{C211A}, which retains only 16 amino acids downstream of the Dronc cleavage site and Histone2Av::GFP or transcription factor QF. Following caspase activation, cell membrane sequestered Histone2Av::GFP or QF is released by caspase-mediated cleavage and accumulates in the nucleus (Baena-Lopez et al., 2018). DBS-S::Histone2Av::GFP is

useful for detecting Dronc activity in live and fixed cells or tissues, such as wing imaginal discs (Baena-Lopez et al., 2018). In combination with the QF/QUAS-mediated binary gene expression system, DBS-S::QF is useful for detecting caspase activation throughout the body with high sensitivity. DBS-S::QF is useful for a variety of tissues, including imaginal discs (Baena-Lopez et al., 2018; Xu et al., 2022), midguts (Baena-Lopez et al., 2018; Arthurton et al., 2020), and ovaries (Galasso et al., 2023). CaspaseTracker/CasExpress and their variants have the best sensitivity for detecting caspase activation. Thus, they should be listed as the first-choice reporters for analyzing CDPs.

3.4 Monitoring DIAP1 protein status

In addition to directly detecting caspase activity, there are several ways to monitor the upstream components of apoptotic signaling. Increased or decreased DIAP1 expression is crucial for caspase activation and function. DIAP1 is an E3 ubiquitin ligase that is ubiquitinated and degraded by the proteasome when RHG expression is induced by apoptotic stimulation. A pre-apoptosis signal-detecting probe based on diap1 degradation (PRAP) was developed to monitor DIAP1 status *in vivo* (Koto et al., 2009). In this probe, VENUS is linked to mutant forms of DIAP1 that are unable to inhibit caspases because of mutations in the caspase-binding sites of the baculovirus IAP repeat (BIR) domains. VENUS fluorescence intensity monitors DIAP1 status. In larval epidermal cells, during metamorphosis, VENUS expression is rapidly reduced before nuclear condensation during apoptosis (Koto et al., 2009). PRAP is also useful for detecting DIAP1 status in live cell/tissue imaging, such as *Drosophila* pupal notum (Koto et al., 2009). Although direct examination of caspase activity is necessary for subsequent analyses because DIAP1 protein status does not always correlate with caspase activity, it is informative for analyzing the upstream signaling that regulates caspase activation.

3.5 Monitoring RHG genes status

DIAP1 protein levels are regulated by RHG proteins whose expression is transcriptionally induced by apoptotic stimulation. Thus, monitoring *RHG* genes expression using enhancer trap lines is useful for indirect detection of caspase activation patterns. Frequently used reporters include *Rpr-11-LacZ* (Nordstrom et al., 1996), hid^{05014}*-LacZ* (Grether et al., 1995), and $hid^{20\text{-}10}$*-LacZ* (Fan et al., 2010). In addition to each single *RHG* gene reporter, the irradiation-responsive enhancer region (IRER) mediates the expression of surrounding pro-apoptotic genes, including *Rpr*. The epigenetic status of the IRER correlates pro-apoptotic gene expression propensity (Zhang et al., 2008). Thus, IRER {ubi-DsRed} reporter, where DsRed expression is driven by the 2.2 kb upstream regulatory sequence of the *ubiquitin-63E* gene, is inserted into IRER, and can be used for monitoring pro-apoptotic genes expression propensity (Zhang et al., 2015). Similar to DIAP1, although a direct examination of caspase activity is necessary for subsequent analysis, it is informative for analyzing the upstream signaling that regulates caspase activation.

3.6 Summary

A wide variety of methods for detecting caspase activation and its upstream status *in vivo* have not only uncovered when and where cells undergo apoptosis within tissues, but also identified cells undergoing caspase activation without cell death. This led to the identification of diverse non-lethal caspase functions in *Drosophila* (see Section 4).

4 Caspase-dependent non-lethal cellular processes in *Drosophila*

In addition to their roles in apoptosis, caspases exhibit diverse non-lethal cellular functions in many organisms (Kuranaga and Miura, 2007; Nakajima and Kuranaga, 2017). CDPs identified in *Drosophila* include cell remodeling and shaping (Arama et al., 2003; Huh et al., 2004b; Oshima et al., 2006; Williams et al., 2006; Koto et al., 2009), tissue morphogenesis (Kang et al., 2017; McSharry and Beitel, 2019; Ojha and Tapadia, 2022), cell migration (Geisbrecht and Montell, 2004; Gorelick-Ashkenazi et al., 2018; Fujisawa et al., 2019), cell differentiation (Kanuka et al., 2005; Verghese and Su, 2018; Arthurton et al., 2020; Galasso et al., 2023; Maurya et al., 2024), cell proliferation (Huh et al., 2004a; Pérez-Garijo et al., 2004; Ryoo et al., 2004; Kondo et al., 2006; Shinoda et al., 2019; Xu et al., 2022) (Table 1). A single caspase family regulates various cellular processes. The regulatory mechanisms of CDPs are not unique, but rather diversified for different outcomes. In this section, we introduce the CDPs identified in *Drosophila* with a background of their discovery and characterization as non-lethal processes. We also introduce the functions and mechanisms of the CDPs to understand the diversity of non-lethal caspase functions. As observed with respect to several CDPs, we introduce similar examples observed in animals apart from *Drosophila*, to understand that CDPs are not limited to *Drosophila*.

4.1 Cell remodeling and shaping

4.1.1 Sperm individualization

One representative CDP in *Drosophila* is sperm individualization (Arama et al., 2003; Huh et al., 2004b). Spermatogenesis occurs within individual units known as cysts, which contain 64 spermatids that remain connected via cytoplasmic bridges after meiosis. During terminal differentiation, round spermatids transform into thin spermatozoa. In the final stage of spermatogenesis, termed individualization, the cytoplasmic bridges are disconnected, and most of the cytoplasm is expelled, leading to individual sperms, events that are reminiscent of apoptosis. Using anti-active mouse caspase-3 antibody (also known as CM1 (IDUN Pharmaceuticals) which is no longer available (Fan and Bergmann, 2010)) and anti-active Drice antibody (Dorstyn et al., 2002), the executioner caspase was found to be activated at the onset of individualization. In addition, they showed that the inhibition of caspase activity by zVAD-fmk, a broad-spectrum caspase inhibitor, or *P35* expression results in defects in individualization, suggesting that caspase activity

TABLE 1 List of CDPs in *Drosophila*.

CDP type	CDP	Cell/Tissue	Method	Caspase	Regulator	Substrate	Reference
Cell Remodeling and Shaping	Sperm Individualization	Spermatid	Antibody	Dronc, Dredd, Exe. Caspase	Cyt-c-d, Dark, Tango7, [Not CRINKLED], dBruce, Cullin-3, Klhl10, Roc1b, Scotti, A-Sβ		Arama et al. (2003), Arama et al. (2007); Huh et al. (2004b), Kaplan et al. (2010), D'Brot et al. (2013), Aram et al. (2016), Orme et al. (2016)
	Phagocytic Removal of Pruned Dendrites	ddaC Neuron	CPV	Dronc, Exe. Caspase, [Not Drice]	Dark, [Not Dark], Tango7, DIAP1		Williams et al. (2006), Kang et al. (2017)
	Bristle Elongation	Shaft Cell		Dronc, [Not Exe. Caspase]	DIAP1, IKKε		Koto et al. (2009)
	Arista Branching	Arista		Dronc, [Not Exe. Caspase]	Hid, Dark, CRINKLED, DIAP1, IKKε		Cullen and McCall (2004), Oshima et al. (2006), Orme et al. (2016)
Tissue Morphogenesis	Salivary Gland Elasticity	Salivary Gland	Antibody	Dronc, Dcp-1, [Not Drice]	Reaper, [Not Dark], Tango7, [Not Myo1D], DIAP1		Kang et al. (2017), Amcheslavsky et al. (2018)
	Tracheal Dorsal Trunk Elongation	Tracheal Dorsal Trunk	Antibody	[Not Dronc], Drice	DIAP1		Robbins et al. (2014), McSharry and Beitel (2019)
	Malpighian Tubule Morphogenesis	Malpighian Tubule		Drice			Ojha and Tapadia (2022)
Cell Migration	Border Cell Migration	Border Cell		Dronc, [Not Exe. Caspase]	Dark, CRINKLED, DIAP1, IKKε	Shaggy46	Geisbrecht and Montell (2004), Oshima et al. (2006), Orme et al. (2016)
	Epithelial Thorax Closure	Pupal Notum	CasT/CasExs	Dronc, Exe. Caspase			Fujisawa et al. (2019)
	Epithelial Wound Healing	Pupal Notum	CasT/CasExs	Dronc, [Not Exe. Caspase]	DIAP1		Fujisawa et al. (2019)
	Irradiation-Induced Cell Migration	Imaginal Disc	CPV	Exe. Caspase, Dcp-1			Gorelick-Ashkenazi et al. (2018)
Cell Differentiation	SOP Cell Specification	Wing Imaginal Disc	SCAT3 (Imaging)	Dronc, Exe. Caspase	Dark, CRINKLED, DIAP1 IKKε	Shaggy46	Kanuka et al. (2005), Kuranaga et al. (2006), Orme et al. (2016)
	Intestinal Progenitor Cell Quiescence	ISC/EB in Midgut	DBS-S::QF	Dronc, [Not Exe. Caspase]	[Not RHG, Dark]		Arthurton et al. (2020)
	Ovarian Somatic Cell Differentiation	Ovarian Somatic Cell	DBS-S::QF	Dronc, Exe. Caspase	RHG, Dark		Galasso et al. (2023)
	Plasmatocyte Differentiation	Intermediate Progenitor Cell in Lymph Gland	Antibody, GC3Ai, Apoliner, CasT/CasExs, L-CasExs, DBS-S::H2Av::GFP	Dronc, Drice, Dcp-1	RHG, Dark	Drep1	Maurya et al. (2024)
	Cell Fate Changes in Tissue Regeneration	Hinge Cell in Wing Imaginal Disc		Dronc, Exe. Caspase	RHG		Verghese and Su (2018)
Cell Proliferation	Imaginal Tissue Growth	Imaginal Disc	SCAT3 (WB)	[Not Dronc, Drice], Dcp-1, Decay	RHG, [Not Dark], DIAP1	Acinus	Shinoda et al. (2019)
	Wound-like Tumor Growth	Wing Imaginal Disc	DBS-S::QF	Dronc, [Not Exe. Caspase]	[Not RHG], Dark, [Not Tango7, Myo1D], DIAP1		Xu et al. (2022)

(Continued on following page)

TABLE 1 (*Continued*) List of CDPs in *Drosophila*.

CDP type	CDP	Cell/Tissue	Method	Caspase	Regulator	Substrate	Reference
	Apoptosis-induced Proliferation	Proliferating Cell in Imaginal Disc	Antibody	Dronc, [Not Exe. Caspase]	Dark, Myo1D		Huh et al. (2004a), Kondo et al. (2006), Fan and Bergmann (2008), Fan et al. (2014), Amcheslavsky et al. (2018)
		Differentiating Cell in Eye Imaginal Disc		Dronc, Drice, Dcp-1			Fan and Bergmann (2008)

Each CDP, was categorized as a CDP type based on its cellular function. The observed cells/tissues, methods for detecting non-lethal caspase activation, responsible and non-responsible caspases, regulators and non-responsible regulators, substrates, and references have been described for each CDP. The components that were not responsible are shown in []. CPV, represents CD8::PARP::VENUS. CasT/CasExs represents CaspaseTracker/CasExpress. Exe. caspase represents the total executioner caspase activity, the requirement of which is determined mostly by the expression of *P35*.

non-lethally regulates sperm individualization. Furthermore, the authors showed that a specific cytochrome c, cyt-c-d, is required for caspase activation, and that dBruce is required to protect spermatids during individualization (Arama et al., 2003). The molecular mechanisms that enable caspase activation without causing cell death are intensively studied by Eli Arama and others, which is described in the following section (see Section 5).

4.1.2 Phagocytic removal of pruned dendrites

Another CDP for cell remodeling and shaping is the phagocytic removal of pruned dendrites from neurons (Williams et al., 2006). During metamorphosis, the dendritic arborizing (da) sensory neuron ddaC undergoes dendrite pruning while the soma remains intact. The authors first showed that inhibition of the apoptotic machinery using $Dronc^{DN}$, *P35*, and *DIAP1* expression, as well as using *Dronc* and *Dark* mutant alleles, suppressed the removal of pruned dendrites however did not suppress dendrite severing by itself. Subsequently, using the CD8::PARP::VENUS probe, they showed that caspase activation was locally observed in severed branches, but not in branches attached to the soma (Williams et al., 2006). These results suggest that non-lethal local caspase activation at pruned dendrites facilitates ddaC neuronal remodeling through phagocytic removal of dendrites.

4.1.3 Bristle elongation

CDPs also participate in cellular shaping during bristle-length regulation (Koto et al., 2009). During *Drosophila* sensory organ development, the socket and shaft cells are generated by asymmetric division from the same precursor cell, pIIa. Live imaging analysis using PRAP, an indicator of DIAP1 status, revealed that DIAP1 levels decreased during asymmetric division and then increased in both socket and shaft cells. While high DIAP1 level is maintained in the socket cell, DIAP1 is degraded by IKKε, which promotes DIAP1 degradation through phosphorylation (Kuranaga et al., 2006) during elongation in the shaft cell. *IKKε* knockdown reduces the elongation, which is further suppressed by simultaneous expression of $Dronc^{DN}$, however not by *P35*, suggesting that non-lethal Dronc activation promotes shaft cell elongation under *IKKε* knockdown, thus regulating bristle length (Koto et al., 2009). These results suggest that the dynamics of DIAP1 protein status control the timing of non-apoptotic caspase activation and function.

4.1.4 Arista branching

DIAP1, IKKε, and Dronc also regulate cellular morphogenesis in branching of arista, a feather-like structure at the tip of the antenna (Cullen and McCall, 2004; Oshima et al., 2006). It was first reported that arista branching reduced in *DIAP1* mutant, but increased in *Hid* mutant (Cullen and McCall, 2004). The following study shows that IKKε negatively regulates F-actin assembly. Moreover, when IKKε is suppressed, arista showed increased lateral branching. Apoptotic component including Dronc, Dark, and DIAP1 genetically interact with the phenotype under the inhibition of IKKε activity. Specifically, reducing Dronc activity, but not inhibiting executioner caspase activity by *P35* overexpression, resulted in a further increase in lateral branching (Oshima et al., 2006). These results suggest that caspase, specifically Dronc, non-lethally suppressed ectopic branching of arista under *IKKε* knockdown.

4.1.5 CDPs for cell remodeling and shaping in other animals

Caspase also regulates cell remodeling and shaping in other animals. During rat lens fiber differentiation, the authors first found that differentiating lens fibers were TUNEL-positive and PARP was cleaved for denucleation, suggesting that caspase was activated. In addition, they found that zVAD-fmk, a broad-spectrum caspase inhibitor, blocked the denucleation process during lens fiber differentiation in cultured explants, suggesting that caspase non-lethally regulates the denucleation process in mammals (Ishizaki et al., 1998). During retinal ganglion cell axon pathfinding in zebrafish embryos, caspases are locally activated at the branch tip of dynamic arbors, which can be detected using SCAT3. Genetic inhibition of caspase using an antisense morpholino results in a reduction in axon arbor dynamics, suggesting that caspase non-lethally promote axon arbor dynamics during development (Campbell and Okamoto, 2013). As shown, CDPs for cell remodeling and shaping are not specific to *Drosophila* but are rather conserved among animals.

4.2 Tissue morphogenesis

4.2.1 Salivary gland elasticity

As caspases can shape cell morphology, it is not surprising that caspases can also regulates tissue morphogenesis. In the larval salivary glands, caspases regulate tissue elasticity (Kang et al.,

2017). Just before pupation, when no cell death occurs in the salivary gland, a weak pulse of ecdysone induces weak pro-apoptotic *Rpr* expression in the larval salivary gland. This leads to the activation of caspase, specifically in the cell cortex, which is detected by the anti-cleaved Dcp-1 antibody. Using *Dronc* and *Dcp-1* mutants, the authors showed that caspase activation in the cortex is mediated by Dronc and Dcp-1. Moreover, using *Dronc* and *Dcp-1* mutants, both caspases were required for the dismantling of cortical F-actin. However, Drice was not required for these processes. The dismantling of cortical F-actin was also suppressed by *Rpr* mutant and knockdown, *Dronc* knockdown, and *Diap1* and *P35* overexpression. Cortical caspase activation requires Tango7, which physically interacts with Dronc (D'Brot et al., 2013), but does not require Dark, suggesting that Tango7 regulates cortical caspase activation. Furthermore, using *Dcp-1* mutant, the authors showed that caspase activation is required for luminal expansion of the tissue, which allows the secretion of mucin-like glue protein into the lumen (Kang et al., 2017). These results suggest that caspase activity in the cortex regulates tissue morphogenesis by regulating tissue elasticity.

4.2.2 Tracheal dorsal trunk elongation

Caspases also regulate the tracheal dorsal trunk elongation (Robbins et al., 2014; McSharry and Beitel, 2019). The tracheal system delivers oxygen to the tissues through a network of epithelial tubes. The tracheal dorsal trunk tubes are elongated by changing the cell shape and junctions without changing the cell number during the late stages of embryonic development. The authors first showed that *Drice* mutant, as well as *P35* and *DIAP1* overexpression, however not *Dronc* mutant, reduced tracheal dorsal trunk length. The overall tracheal cell number was not influenced by these conditions, suggesting that caspases, especially Drice, non-lethally elongate the tracheal dorsal trunk, potentially downstream of the Hippo signaling pathway (Robbins et al., 2014). In a subsequent study, this was further supported by the fact that *Drice* overexpression in the tracheal system elongated the dorsal tank without inducing apoptosis, suggesting that Drice promotes elongation non-lethally. Using an anti-cleaved Drice antibody, they showed that numerous cleaved Drice puncta were enriched at the apical surface of the tracheal cells, which partially colocalize with Clathrin. Furthermore, the abundance and trafficking of several cargo proteins required for tracheal size control are altered in *Drice* mutant (McSharry and Beitel, 2019). These results suggest that caspases, particularly Drice, non-lethally regulate tracheal dorsal trunk elongation by regulating endocytic trafficking.

4.2.3 Malpighian tubule morphogenesis

In addition, Drice regulates tissue morphogenesis in malpighian tubules (Ojha and Tapadia, 2022). Malpighian tubule, a tissue responsible for excretion and osmoregulation, shows no apoptosis at the larval and pupal stages of development, although it does express apoptotic proteins (Shukla and Tapadia, 2011). The authors showed that *Drice* mutant exhibited deformed malpighian tubule morphology with reduced tubule length and a disorganized cytoskeleton in the third-instar larvae, suggesting that Drice non-lethally regulates malpighian tubule morphogenesis. This mutant also showed deposition of uric acid crystals suggesting the functional breakdown of malpighian tubule (Ojha and Tapadia, 2022). Since salivary glands, tracheal system, and malpighian tubules are all tubular tissues, caspases may regulate tubular tissue morphogenesis in other animals.

4.3 Cell migration

4.3.1 Border cell migration

As caspases regulate the actin cytoskeleton, caspases can also regulate cell migration in a non-lethal manner. During oogenesis in the *Drosophila* egg chamber which consists of 15 nurse cells and one oocyte, 6–10 cells emerge from the surrounding follicle cells to form border cell clusters. The cluster migrates through the center of the egg chamber of the nurse cells to the anterior border of the oocyte. The migration process, called border cell migration, starts during stage 9 and is completed by stage 10 (Montell, 2003). Migration is inhibited when *dominant-negative form of a small GTPase, Rac* (*RacN17*), is ectopically expressed in the border cells. Through genetic screening, the authors found that *DIAP1* overexpression rescued the migration defects caused by *RacN17* overexpression. Conversely, *DIAP1* hypomorphic mutant shows border cell migration defects without excess cell death. Since *IKKε* overexpression causes border cell migration defects, this also supports the involvement of DIAP1 for the regulation of border cell migration (Oshima et al., 2006). Furthermore, the authors showed that inhibiting Dronc activity, however not executioner caspase activity by *P35* overexpression, also rescued the migration defect mediated by *RacN17* overexpression. Thus, caspases, specifically Dronc, suppresses border cell migration (Geisbrecht and Montell, 2004).

4.3.2 Epithelial thorax closure and wound healing

Caspases also regulate cell migration during epithelial thorax closure and wound healing (Fujisawa et al., 2019). During thorax closure in the pupal stage, where a pair of wing imaginal disc joints form the adult thorax, caspase activation is specifically observed in the leading-edge cells using CaspaseTracker/CasExpress, mostly not undergoing delamination, as revealed by live imaging. Inhibition of Dronc or executioner caspase activity results in an increase in the speed of thorax closure, suggesting that caspases non-lethally attenuate thorax closing speeds. In addition, the authors found that numerous caspase-activating cells, mostly without delamination as revealed by live imaging, emerged around the wound site ablated by the laser at the developing pupal notum. Genetic inhibition of Dronc activity and *DIAP1* overexpression, but not *P35* overexpression, promotes wound closure, which is different from thorax closure in executioner caspase-dependency (Fujisawa et al., 2019). Therefore, the suppression of cell migration by caspases, specifically Dronc, seems rather general in *Drosophila*, whereas its dependency on executioner caspases differs depending on the situation.

4.3.3 Irradiation-induced cell migration

In addition to the initiator caspase, Dronc, executioner caspases also suppress cell migration under other conditions (Gorelick-Ashkenazi et al., 2018). In imaginal discs, which are composed of a monolayer of columnar epithelial cells, ionizing irradiation induces caspase activation, resulting in apoptosis (Florentin and

Arama, 2012). However, in *Drice* mutant, which only contains Dcp-1 executioner caspase, ionizing irradiation did not induce apoptosis, as detected by TUNEL staining, even though caspase activity was still detected using the CD8::PARP::VENUS probe. Under these conditions, irradiation-induced cell migration involving basal delamination was observed. Because cell migration is further enhanced by additional *Dcp-1* mutations, the authors suggested that non-lethal executioner caspase activity, especially that of Dcp-1, inhibits irradiation-induced cell migration (Gorelick-Ashkenazi et al., 2018). Since border cell migration and epithelial thorax wound healing are not dependent on executioner caspase activity, the underlying mechanisms seem different from those of irradiation-induced cell migration.

4.3.4 CDPs for cell migration in other animals

Caspases also regulate mammalian cell migration. For example, caspase-11-deficient leukocytes show defective migration, suggesting that caspase-11 promotes cell migration (Li et al., 2007). Caspase-8-deficient mouse embryonic fibroblasts exhibit reduced cell motility, suggesting that caspase-8 promotes cell motility (Helfer et al., 2006). Because caspases also negatively regulate cell migration in *Drosophila* as shown above (Geisbrecht and Montell, 2004; Gorelick-Ashkenazi et al., 2018; Fujisawa et al., 2019), caspases do not always regulate cell migration in a single direction, i.e., caspases can promote and suppress cell migration. Studying the downstream molecular mechanisms helps to understand their bidirectional ability to regulate cell motility, depending on the context and evolutionary conservation.

4.4 Cell differentiation

4.4.1 Sensory organ precursor cell specification

Caspases regulate cell differentiation in a non-lethal manner. *Drosophila* constantly produces 4 mechanosensory bristles called macrochaetae in their scutellum, which emerge from sensory organ precursor (SOP) cells selected within the proneural cluster of wing imaginal discs. Using *Dark* mutant as well as proneural cluster specific *P35* overexpression, the authors first noticed that ectopic bristles emerged in the adult scutellum. While no apoptosis was detected by TUNEL staining in the proneural cluster, caspase activity was detected using SCAT3 FRET imaging. Genetic screening revealed that Shaggy, especially the alternative isoform Shaggy46, which is a substrate of caspase whose cleavage activates Shaggy46 kinase, is responsible for the suppression of ectopic bristles. Thus, the authors concluded that caspase activity suppresses the emergence of ectopic bristles in the scutellum through non-lethal substrate cleavage of Shaggy46 for kinase activation (Kanuka et al., 2005). The ectopic bristle also emerges upon *DIAP1* overexpression (Kanuka et al., 2005) and *IKKε* knockdown (Kuranaga et al., 2006), suggesting that this phenomenon is regulated by IKKε-mediated DIAP1 degradation.

4.4.2 Intestinal progenitor cell quiescence

Caspases also preserve cells in quiescence (Arthurton et al., 2020). In intestinal progenitor cells, which consist of intestinal stem cells and intermediate progenitor cells termed enteroblasts, Dronc is non-lethally activated under normal conditions, as detected using DBS-S::QF. Using the conditional *Dronc* knockout allele, they found that Dronc activity is required to prevent an increase in the number of intestinal precursor cells as well as to prevent their unintended differentiation. This phenotype was not dependent on RHG, Dark, or executioner caspase activity, but was solely dependent on Dronc. Although the exact substrate has not been determined, Dronc activity limits Notch signaling to regulate cellular quiescence (Arthurton et al., 2020).

4.4.3 Ovarian somatic cell differentiation

Dronc also regulates ovarian somatic cell proliferation and differentiation (Galasso et al., 2023). In germarium, a tissue composed of germline cells and supporting somatic cells of *Drosophila* ovary, the authors first found Dronc activation in surviving somatic cells by using DBS-S::QF with lineage tracing. Using the conditional *Dronc* knockout allele, they found that Dronc was required for the proliferation and differentiation of ovarian somatic cells. In contrast to the role of Dronc in preserving intestinal progenitor cell quiescence (Arthurton et al., 2020), the entire apoptotic pathway, including RHG, DIAP1, Dark, and executioner caspase activity, is involved in these processes. Subsequently, Dronc deficiency reduced Hedgehog signaling through excess autophagy (Galasso et al., 2023), suggesting that caspases non-lethally prevent excess autophagy to sustain Hedgehog signaling for proliferation and differentiation.

4.4.4 Plasmatocyte differentiation

Caspases also participate in the differentiation of plasmatocyte, a macrophage-like cell in *Drosophila* (Maurya et al., 2024). During the larval stage, plasmatocytes differentiate from the lymph gland, the larval hematopoietic organ, which includes multipotent progenitors, intermediate progenitors, and differentiating cells. Using a wide variety of caspase activity probes, including GC3Ai, Apoliner, CaspaseTracker/CasExpress, L-CasExpress, DBS-S::Histone2Av::GFP as well as anti-cleaved Dcp-1 antibody staining, it was found that caspase is activated during differentiation, especially in intermediate progenitor cells. Moreover, using L-CasExpress combined with lineage tracing (L-Trace), the authors found that caspase-activated cells survived in the differentiation zone, suggesting that transiently activated caspases do not induce apoptosis. Subsequent analysis showed that caspase activation relies on the entire core apoptotic signaling pathway, including RHG, Dronc, Drice, and Dcp-1, and induces DNA damage through Drep4, a *Drosophila* ortholog of CAD, and Drep1, a *Drosophila* ortholog of ICAD, most likely through its cleavage. DNA damage is required for plasmatocyte differentiation, potentially by altering gene expression. Thus, developmentally programmed non-lethal caspase activation is required for plasmatocyte differentiation by inducing programmed DNA breaks (Maurya et al., 2024).

4.4.5 Cell fate changes in tissue regeneration

Caspases regulate cell fate changes during tissue regeneration (Verghese and Su, 2018). Imaginal discs are epithelial tissues capable of regeneration after damage (Hariharan and Serras, 2017). During the ionizing irradiation of wing imaginal discs, it was found that dorsal hinge cells were resistant to apoptosis (Verghese and Su,

2016). Moreover, hinge cells changed their fate and translocated to the wing pouch region during ionizing radiation-induced regeneration. This process was inhibited by the inhibition of caspase activity in the *H99* allele, in *P35* and $Dronc^{DN}$ overexpression (Verghese and Su, 2018), suggesting that caspase activation is non-lethally required for cell fate changes from hinge cells to wing pouch cells during regeneration.

4.4.6 CDPs for cell differentiation in other animals

Regulation of cell differentiation by non-lethal caspase activation is also common in other animals. Similar to plasmatocyte differentiation in *Drosophila*, caspase activation-mediated DNA damage is a conserved mechanism of cellular differentiation. These include skeletal muscle differentiation, which requires caspase activation and subsequent CAD activation (Fernando et al., 2002; Larsen et al., 2010) and Myc-induced oncogenic transformation of human mammary epithelial cells, which requires caspase activation and subsequent ENDOG-mediated DNA damage (Cartwright et al., 2017). In addition to cell differentiation, iPSC induction from human fibroblasts requires caspase activation, wherein retinoblastoma is cleaved for reprogramming (Li et al., 2010).

4.5 Cell proliferation

4.5.1 Imaginal tissue growth

While caspases can induce apoptosis to reduce the cell number in tissues, they increase the tissue size of growing epithelial tissues in a non-lethal manner (Shinoda et al., 2019). During imaginal disc growth, the inhibition of executioner caspase activity by overexpression of *P35*, but not by overexpression of $Dronc^{DN}$ or knockdown of *Dark*, reduces adult wing size. This effect is specific to the executioner caspases Dcp-1 and Decay, but not to Drice. Even in the absence of Dronc, basal caspase activity was detected by western blotting using a SCAT3 probe, suggesting that Dronc-independent basal executioner caspase activity exists. As *Dcp-1* or *Decay* knockdown did not result in any reduction in the apoptotic cell number detected by TUNEL staining, it was shown that basal executioner caspase activity, independent of Dronc activity, sustained imaginal tissue growth. Mechanistically, uncleavable Acinus, a Dcp-1 substrate (Nandi et al., 2014), showed reduced wing size (Shinoda et al., 2019). These results suggest that at least in part, Dcp-1-mediated basal Acinus cleavage sustains imaginal tissue growth.

4.5.2 Wound-like tumor growth

Caspases can limit tumor growth in a non-lethal manner, even in the same tissue (Xu et al., 2022). In wound-like tumors induced by simultaneous activation of the EGFR and JAK/STAT pathways in wing imaginal discs, most of the cells that survived underwent Dronc activation, which was detected using DBS-S::QF with lineage tracing. The authors showed that the tumor size increased upon genetic inhibition of Dronc activity, suggesting that Dronc limits tumor growth in a non-lethal manner. While this effect was observed with *Dark* knockdown and *DIAP1* overexpression, it was not observed with *RHG* knockdown or *P35* overexpression. Dronc activity limits JNK signaling, independent of Eiger and reactive oxygen species, which are required for tumor overgrowth (Xu et al., 2022).

4.5.3 Apoptosis-induced proliferation

Apoptosis-induced proliferation (AiP), which does not necessarily require apoptosis to induce nascent cell proliferation, is also categorized as a non-lethal process in which undead cells secrete mitogens into surrounding tissues, resulting in the over-proliferation of surrounding cells (Huh et al., 2004a; Pérez-Garijo et al., 2004; Ryoo et al., 2004; Kondo et al., 2006). Typically, AiP is induced by apoptosis induction, such as overexpression of pro-apoptotic genes, including *Rpr*, and simultaneous inhibition of apoptosis by expressing *P35* in wing imaginal discs. In proliferating cells, AiP requires Dronc, but not executioner caspase activity (Kondo et al., 2006). Using eye imaginal discs, a study showed that AiP in proliferating cells of eye imaginal discs is dependent on Dronc, but not on executioner caspase activity, while AiP in differentiating cells is dependent on executioner caspases Drice and Dcp-1, suggesting distinct mechanisms of AiP depending on their cell status (Fan and Bergmann, 2008). With respect to AiP in proliferating cells, Dark is required (Fan et al., 2014). However, the catalytic activity of Dronc may not be required because the substitution of the catalytic cysteine with alanine does not interfere with AiP if it simultaneously carries a substitution from lysine 78 to arginine, where inhibitory mono-ubiquitylation occurs (Kamber Kaya et al., 2017). With respect to Dronc-dependent AiP in proliferating cells, undead cells secrete mitogens, including Wingless and Decapentaplegic to stimulate tissue growth by inducing the proliferation of surrounding cells (Pérez-Garijo et al., 2009). The detailed molecular mechanism that enables the non-lethal function of caspase in AiP has long been pursued by Andreas Bergmann and Yun Fan, and is further described in the next section.

4.5.4 CDPs for tissue size regulation in other animals

Caspases non-lethally promote organ growth in the sebaceous glands of mice (Yosefzon et al., 2018). Caspase-3 cleaves α-Catenin which leads to the nuclear translocation of yes-associated protein (YAP), a vital regulator of organ size (Yosefzon et al., 2018). Conversely, examining the role of apoptosis in tissue size regulation by caspase inhibition should be considered, as caspase has non-lethal functions in regulating tissue size.

4.6 Summary

As mentioned above, studies in *Drosophila* lead to the identification of wide variety of non-lethal caspase functions, mainly in developmental processes. Because several CDPs are also observed in other animals, and the mechanisms of which are conserved in some cases, *Drosophila* studies will serve as a guide for CDPs studies in other animals. From the pathological perspective, because CDPs include cell proliferation and cell migration, it is conceivable that caspases can non-lethally regulate cancer development. Indeed, it is reported that minority MOMP, where only a minority of mitochondria undergoes MOMP, induces sub-lethal caspase activation that causes DNA damage and genomic

instability, promoting transformation and tumorigenesis of primary mouse embryonic fibroblasts (Ichim et al., 2015). Thus, manipulation of caspase activity in both directions, suppressing the activity for reducing non-lethal effect and enhancing the activity for inducing cell death, can open therapeutic possibilities combined with anti-cancer regimes.

Important processes of CDPs studies include the detection of caspase activation without cell death (often analyzed by TUNEL staining), manipulation of the core apoptotic machinery at each level, and description of the cellular phenotype. Substrates are not often identified, which benefits the analysis of downstream events. Compared to the physiological functions of non-lethal caspase activation, the underlying mechanism that enables non-lethal caspase activation without cell death and regulates downstream events is rather lagging due to technical shortages in identifying substrates. As described above, the core apoptotic components, including caspases, are not always the same; some require both Dronc and executioner caspases, whereas others require only Dronc or executioner caspases. Thus, researchers must be aware that the underlying mechanisms differ depending on the analyzed CDPs. Yet, overlapping components are reproducibly used in some CDPs: IKKε-DIAP1-Dronc axis regulates wide range of phenotypes with respect to cytoskeleton dynamics. Thus, testing similarities will benefit the CDPs research. In the following section, we summarize several well-studied mechanisms that enable non-lethal caspase activation.

5 Mechanisms of controlling caspase activation without inducing cell death

The growing body of CDPs clearly show that their underlying molecular mechanisms are diverse. However, extensive analyses of several CDPs have revealed the underlying mechanisms and similarities between other CDPs. Specifically, evidence indicates the importance of restricting caspase activation to a specific compartment of cells shaped by caspase-interacting and proximal proteins. In this section, we introduce the underlying mechanisms that enable non-lethal caspase activation, specifically focusing on interacting proteins of caspase. In particular, analyses of sperm individualization, AiP, and SOP specification revealed subcellular restriction of caspase activation as a key regulator of non-lethal activation.

5.1 Restriction of caspase activation to the vicinity of mitochondrial outer membrane

Continuous analysis of non-lethal caspase functions in sperm individualization by Eli Arama and colleagues first identified the Cullin-3-dependent ubiquitin ligase complex from the genetic screening of a large collection of sterile male fly mutants. Mutations in the testis-specific isoform of Cullin-3, the small RING protein Roc1b, or a *Drosophila* ortholog of the mammalian BTB-Kelch protein, Klhl10, result in no caspase activation during sperm individualization (Arama et al., 2007). Subsequently, they conducted a yeast two-hybrid screen to identify the binding partners of Klhl10, an E3 substrate-recruitment subunit. They found that Scotti (Soti) is a binding partner of Klhl10. *Soti* mutant exhibited elevated caspase activation, suggesting that Soti antagonized this process. Moreover, caspase activation is not uniform in individualizing spermatids but is rather high in the head and low in the tail. Soti expression was inversely correlated with activation patterns. A giant IAP called dBruce, which competes with Soti, is a target of Cullin-3. This results in the degeneration of dBruce and graded activation patterns of caspase, showing that the distal part is protected from excessive caspase activation and cell death (Kaplan et al., 2010). Furthermore, using yeast two hybrid screening against Cullin-3, they found a Krebs cycle component, the ATP-specific form of the succinyl-CoA synthetase β subunit (A-Sβ) as an interacting partner. A-Sβ localizes at the mitochondrial outer membrane and restricts Cullin-3 to the proximity of the membrane, thereby limiting caspase activation to close proximity of the mitochondrial outer membrane (Aram et al., 2016). Thus, a series of studies by Eli Arama and colleagues provided a model for restricting caspase activation to a specific subcellular compartment; head-to-tail descending activation as well as proximal to the mitochondrial outer membrane activation are the keys to escape cell death caused by excessive caspase activation (Figure 3A, left).

5.2 Directing caspase activation at the individualization complex

Dronc-interacting protein Tango7, the *Drosophila* translation initiation factor eIF3 subunit m, is required for activation during sperm individualization. Tango7 physically interacts with Dronc and localizes to the individualization complex where caspase activity peaks (D'Brot et al., 2013). Therefore, by caspase-interacting proteins and not by IAP-mediated activity regulation, its direct localization to the site of interest is similarly important (Figure 3A, right). Tango7 is also required for regulating salivary gland cell elasticity, as well as phagocytic removal of pruned dendrites in ddaC neurons. Both phenotypes appear to be independent of Dark, suggesting that Dark is required for cytosolic Dronc activation, whereas Tango7 regulates cortical non-lethal Dronc activation (Kang et al., 2017).

5.3 Directing caspase activation at the basal side of the plasma membrane

As suggested, controlling the caspase activation site within cells appears to be the key to regulating non-lethal caspase activation. This has also been observed with respect to AiP. Andreas Bergmann and colleagues investigated the mechanism of AiP through genetic screening. A series of studies have identified an unconventional Myosin1D (Myo1D) that is required for AiP. Myo1D interacts physically with Dronc. They further found that endogenous Dronc proteins localized to the basal side of disc proper cells in undead conditions with Myo1D. Myo1D is required for the translocation of Dronc to the basal side of the plasma membrane in imaginal discs, where it activates Duox and induces AiP in surrounding cells. These results suggest that Myo1D localizes Dronc to the basal side of the plasma membrane for non-lethal activation (Amcheslavsky et al., 2018) (Figure 3B). Moreover,

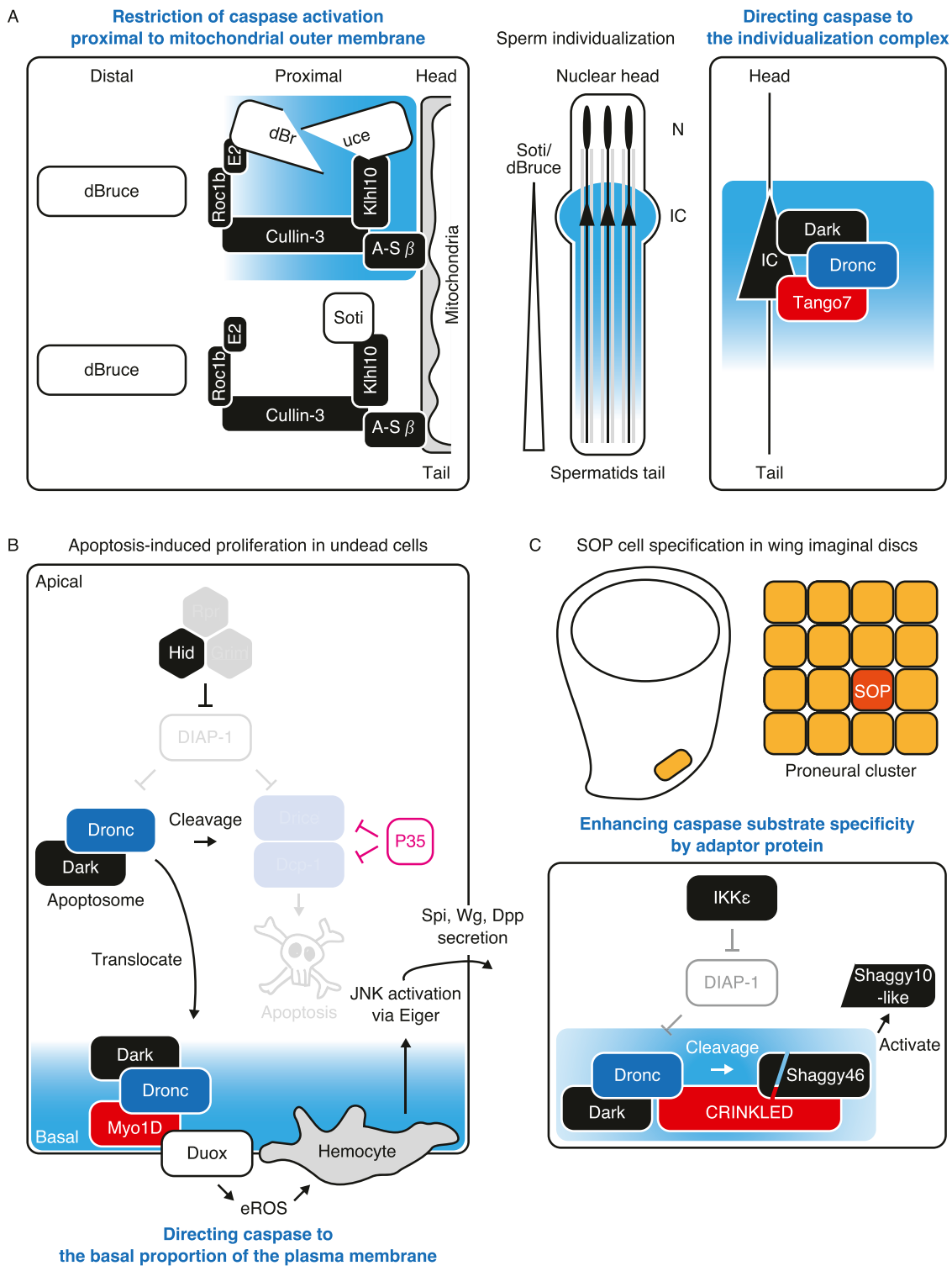

FIGURE 3
Mechanisms of controlling caspase activation without inducing cell death. **(A)** Regulation of non-lethal caspase activation during sperm individualization. During sperm individualization, caspase activation, which peaks in individualization complex (IC), forms descending gradient to the tail. This gradient activation is regulated by inverse gradient expression of Soti and dBruce which antagonize caspase activation. In addition, caspase activation is restricted to the outer membrane of mitochondria where Cullin-3 complex is tethered by A-Sβ (left). Alongside, Dronc is directed to the individualization complex by Tango7 (right). Caspase activating regions are shown in light blue. **(B)** Regulation of non-lethal caspase activation during apoptosis-induced proliferation in undead cells. Undead cells are induced by simultaneous activation of apoptotic signaling and inhibition of executioner caspase activity by *P35* expression. In undead cells, Dronc translocates to the basal side of the plasma membrane with help of Myo1D where activation is restricted. Dronc promotes activation of Duox to produce extracellular reactive oxygen species (eROS), which accumulates hemocyte for JNK activation
(*Continued*)

FIGURE 3 (Continued)
resulting in mitogen production in the undead cell for inducing nascent cell proliferation. Caspase activating region is shown in light blue. **(C)** Regulation of non-lethal caspase activation during sensory organ precursor (SOP) cell specification. During SOP cell (orange) specification from proneural cluster cells (yellow) (top), IKKε-mediated DIAP1 degradation induced caspase activation. CRINKLED, an adaptor protein of Dronc, proximates Dronc and Shaggy46, the cleavage of which by executioner caspases generates active Shaggy10-like kinase (bottom) and negatively regulates SOP cell specification, thus inhibiting the emergence of ectopic SOP cells. Caspase activating region is shown in light blue.

Myo1D works not only in imaginal discs of the AiP model, but also in midgut enterocytes, where they undergo transient non-lethal activation, suggesting that translocation of the caspase molecules to the restricted cellular compartment is key for non-lethal (transiently undead) activation (Amcheslavsky et al., 2020). Although Myo1D is expressed and localized on the basal side of the salivary gland and colocalizes with Dronc, it is not required for Dronc localization in the salivary gland, potentially because Dronc localization is regulated by Tango7 in this tissue under the normal condition. Alternatively, under undead condition in the salivary gland, the localization of Dronc to the basal side of the plasma membrane increases, which is dependent on Myo1D (Amcheslavsky et al., 2018). This suggests that Dronc mediates distinct non-lethal cellular processes by changing adaptor proteins to direct distinct subcellular compartments, depending on the conditions.

5.4 Enhancing substrate specificity by an adaptor protein

CRINKLED (CK), a *Drosophila* unconventional myosin, was identified in an IP-MS experiment using Dronc. Genetic analysis revealed that CK is required for efficient Dronc-dependent induction of apoptosis in response to high-level expression of IAP antagonists. In addition, further genetic analysis revealed that CK is involved in arista branching, SOP specification and border cell migration, a process which involves non-lethal caspase activation of Dronc, mediated by DIAP1 and IKKε (Geisbrecht and Montell, 2004; Kanuka et al., 2005; Kuranaga et al., 2006; Oshima et al., 2006). CK physically interacts with Shaggy46, a caspase substrate responsible for SOP cell specification (Kanuka et al., 2005). They showed that Shaggy46 is also involved in border cell migration. Thus, they proposed a model where CK interacts with both Dronc and Shaggy46 to induce specific substrate cleavage, providing that caspase-interacting proteins not only restrict their activity to a specific cellular compartment but also engage in substrate cleavage (Orme et al., 2016). IKKε also regulates SOP cell specification in a non-apoptotic scenario (Kuranaga et al., 2006). Thus, limiting activation level by IKKε-DIAP1 axis is necessary for regulating Dronc-CK-Shaggy46 mediated non-lethal functions (Figure 3C). CK is not involved in sperm individualization (Orme et al., 2016), further supporting the idea that Dronc mediates distinct non-lethal cellular processes by altering adaptor proteins.

5.5 Summary

Regulating caspase activity in restricted regions, as well as limiting the activation level or timing, is key to regulating non-lethal caspase activity. While their functions are different, caspases often exploit the same set of mechanisms for regulating caspase activation and function. Thus, it is possible that the molecular mechanisms of CDPs involve a pair of caspases and their adaptor proteins. It is noteworthy that although limiting tumor growth is mediated by Dronc, this CDP is not mediated by Tango7 or Myo1D (Xu et al., 2022), suggesting that there is another adaptor protein that mediates other types of CDPs. As in most cases, non-lethal caspase activity is regulated by its interacting and proximal proteins, identifying these proteins in cells of interest within living animals is critical. In the following section, we introduce a new approach to identify such components.

6 Analyzing caspase-proximal proteins for CDPs using TurboID

Proximity labeling techniques are emerging as means of detecting proximal proteins of interest inside cells or organisms (Qin et al., 2021). One such technique is APEX2, which is hydrogen peroxide-dependent proximity labeling with high resolution in a span of 1 min (Lam et al., 2015; Hung et al., 2016). However, the use of a toxic reagent, makes it difficult for its application in animals, including *Drosophila*. TurboID is another proximity labeling technique that uses ATP to convert biotin into the reactive intermediate biotin-AMP, which covalently labels the lysine residues of proximal proteins within 10 min. Because TurboID labeling is hydrogen peroxide-free, it also enables its application in *Drosophila* (Branon et al., 2018) (Figure 4A). In *Drosophila*, biotin is supplied through feeding. The labelled proteins were purified using streptavidin beads for further analysis, including mass spectrometry for proteomics.

Recently, we established C-terminal TurboID knocked-in strains in *Drosophila* with three major apoptotic caspases: Dronc, Drice, and Dcp-1 (Shinoda et al., 2019). As TurboID-tagged single caspase biotinylates numerous nearby proteins, we can examine the expression patterns of each caspase using streptavidin with high sensitivity (Shinoda et al., 2019; Arthurton et al., 2020; Muramoto et al., 2024). Executioner caspases are the "executioner" of cell death and other cellular processes as executioner caspases cleave wide ranges of substrate compared to initiator caspases. Because of the lack of protein-interacting motifs at their N-termini, the identification of their interacting proteins is rather limited. Moreover, caspase-proximal proteins are not identical between Drice and Dcp-1 in wing imaginal discs (Shinoda et al., 2019), suggesting that executioner caspases sometimes have distinct functions in CDPs even though they prefer similar primary sequences for cleavages. We showed that BubR1, one of the spindle assembly checkpoint components, is a substrate of caspase, the cleavage of which regulates organismal lifespan. Importantly, using the TurboID

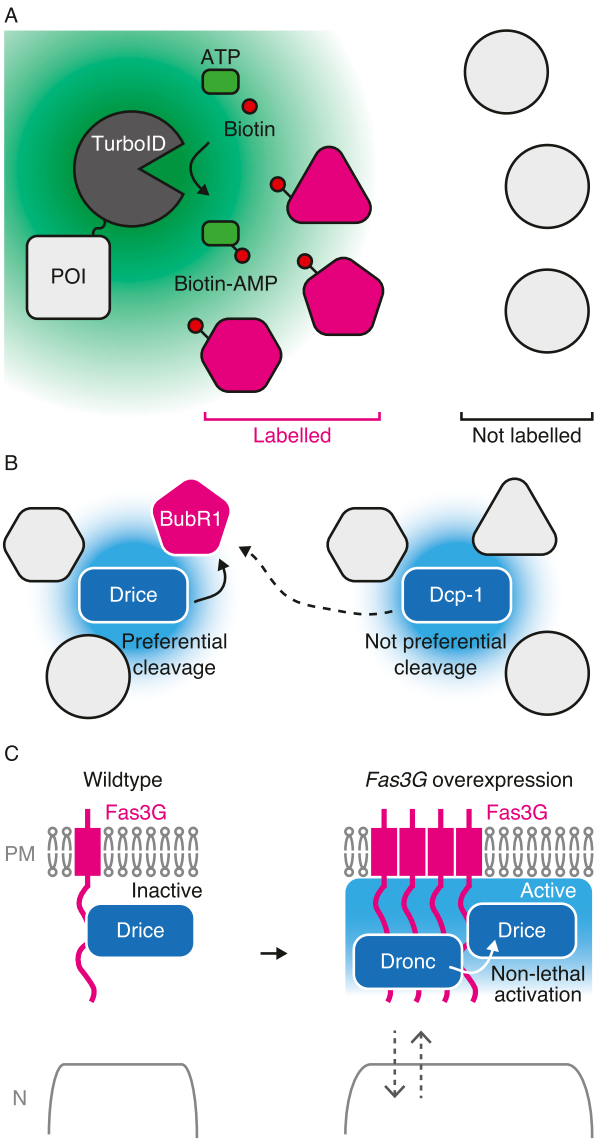

FIGURE 4
Analyzing caspase-proximal proteins in non-lethal scenario using TurboID. **(A)** Schematic diagram of TurboID-mediated biotinylation of proximal proteins. TurboID generates reactive biotin-AMP from ATP (green) and biotin (magenta), which conjugates to lysine residue of proximal proteins (magenta) within approximately 10 nm (green region) within 10 min. **(B)** Proximal proteins can be preferentially cleaved by proximal caspase. BubR1 (magenta), a substrate of caspase, is proximal to Drice rather than Dcp-1 which is preferentially cleaved by Drice rather than Dcp-1. Hypothetical caspase activating region of each caspase is shown in light blue. **(C)** Proximal proteins can regulate non-lethal caspase activation. Fasciclin 3 isoform G (*Fas3G*; magenta) is one of proximal proteins to Drice. Overexpression of *Fas3G* promotes Dronc expression which also comes close to *Fas3G* for facilitating non-lethal activation. Caspase activating region is shown in light blue.

technique, we showed that BubR1 is proximal to Drice but not to Dcp-1 in the ovaries, even though both are almost equally expressed. Accordingly, BubR1 is preferentially cleaved by Drice rather than Dcp-1 in S2 cells during non-lethal activation (Shinoda et al., 2023). These results indicate that caspase-proximal proteins include substrates of caspases that are preferentially cleaved by nearby proteins (Figure 4B). Because the cleavage of limited substrates is the key for non-lethal functions and as identifying responsible substrates is always challenging (Table 1), TurboID-mediated identification of CDP-responsible substrates helps in understanding the effectors of CDPs. In addition to CDPs, TurboID can also be applied for analyzing proteins related to cell death. Using BioID, a less efficient proximity labeling enzyme, to human Caspase-1, p62/sequestosome-1 is identified as a substrate which only interacts with Caspase-1 upon inflammasome activation (Jamilloux et al., 2018). Thus, TurboID is useful for identifying context-dependent substrates of caspases both in lethal and non-lethal scenarios.

We recently analyzed the proximal proteins of Drice in the adult brain. Although Drice is primarily a cytosolic protein, its proximity lies within the membrane fraction. Drice is only specifically proximal to the specific isoform of Fasciclin 3, isoform G, and not to other isoforms even though they are overexpressed. Thus, Drice might be compartmentalized to a specific microdomain of the membrane compartment. In addition, overexpression of one of the Drice proximal proteins, *Fasciclin 3 isoform G*, activates caspase in the olfactory receptor neurons without inducing cell death, suggesting that proximal proteins regulate non-lethal caspase activation. *Fasciclin 3 isoform G* overexpression induces Dronc expression, which also comes close to Fasciclin 3 isoform G. Dronc is required for caspase activation induced by *Fasciclin 3 isoform G* overexpression (Muramoto et al., 2024) (Figure 4C). In addition to the translocation of initiator caspase Dronc to the membrane for non-lethal activation, these results suggest that executioner caspase is pre-compartmentalized in proximity to a specific set of proteins prior to activation which defines the "hotspot" for non-lethal caspase activation. TurboID-mediated identification of proximal proteins, including substrates and regulators, in living animals will enhance our understanding of the regulation of caspase activation without inducing cell death in a wide range of CDPs in a variety of tissues.

7 Discussion

Diverse CDPs have been identified using advanced *Drosophila* genetic tools to detect and manipulate caspase activity in specific cells *in vivo*. Continuous analyses have revealed that non-lethal caspase activation can occur through limited activation in specific subcellular compartments, specifically mediated by caspase-interacting proteins. However, we still do not fully understand how the intensity and duration of caspase activation for non-lethal processes are regulated. To enhance our understanding, it is necessary to develop genetic tools capable of detecting and manipulating caspase activity with a high spatial resolution, potentially at the subcellular level, in a highly quantitative manner. Development of such tools will significantly advance our understanding of non-lethal functions mediated by caspase activity. Identifying critical substrates for non-lethal functions is equally important. TurboID will serve as a vital methodology for identifying critical substrates within cells of interest *in vivo*. In addition, it is critical to analyze the kinetics of substrates cleavage within

cells of interest *in vivo*. Given that enzymes with multiple substrates, such as caspase, are multifunctional, understanding the action of caspase will broaden our understanding of the actions of other multifunctional enzymes, both in cells and *in vivo*.

Because caspase regulates various non-lethal functions, its activation is no longer considered a point of no return in cell death. Understanding the non-lethal functions of caspases has also led to a deeper understanding of the molecular mechanisms of cell death, including the identification of critical substrates for the process of cell death. In the *Drosophila* pupal notum, caspase-mediated microtubule disassembly is the key rate-limiting step in cell extrusion, the process of expelling dying epithelial cells (Villars et al., 2022). Studying CDPs will advance our understanding of cell death. This will clarify the states of the living and dead cells, along with their intermediate states.

Author contributions

NS: Conceptualization, Funding acquisition, Writing–original draft, Writing–review and editing. MM: Conceptualization, Funding acquisition, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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