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Non-lethal roles of the initiator caspase Dronc in *Drosophila*

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The role of caspases, or cysteine-aspartic proteases, in apoptosis has been well-studied across multiple organisms. These apoptotic caspases can be divided into initiator and effector caspases, with the former cleaving and activating the latter to trigger cell death. However, emerging evidence is supporting non-lethal roles of caspases in development, tissue homeostasis and disease. In comparison to effector caspases, less is known about the non-apoptotic functions of initiator caspases because of their more restricted activities and fewer known substrates. This review focuses on some recent findings in *Drosophila* on non-lethal roles of the initiator caspase Dronc. We discuss their biological importance, underlying regulatory mechanisms, and implications for our understanding of their mammalian counterparts. Deciphering the non-apoptotic functions of Dronc will provide valuable insights into the multifaceted functions of caspases during development and in diseases including cancer.

KEYWORDS

apoptosis, initiator caspase, Dronc, non-lethal role, non-apoptotic function, *Drosophila*

Introduction

The caspase family is comprised of cysteine proteases which are found as inactive zymogens, but once activated, cleave their substrates mainly at aspartate residues (McIlwain et al., 2013; Julien and Wells, 2017). Roles of caspases have been intensively studied in apoptosis, a major form of programmed cell death (Vandenabeele et al., 2010). In addition to apoptosis, caspases also play critical roles in immune responses by cleaving and activating inflammatory cytokines and stalling bacterial replication (Jiménez Fernández and Lamkanfi, 2015; Ross et al., 2022). Therefore, caspases are categorized as apoptotic and inflammatory caspases. Apoptotic caspases are further divided into initiator or effector caspases depending on their structures and functions in apoptosis (Riedl and Shi, 2004). Initiator caspases have long N-terminal domains, including the death effector domain (DED) or the caspase activation and recruitment domain (CARD). In contrast, effector caspases have short or no N-terminal prodomains. Once activated, initiator caspases cleave and activate downstream effector caspases, which in turn cleave a substantial number of substrates to execute apoptosis. Intriguingly, caspases also possess a broad spectrum of non-lethal functions (Arama et al., 2021). Advanced genetic tools developed in the model organism *Drosophila* have revealed widespread and sublethal activation of caspases, particularly effector caspases, during development (Tang et al., 2015; Ding et al., 2016). This supports widely observed non-lethal caspase functions. Compared to effector caspases, initiator caspases have more restricted activities and fewer known substrates. However, their non-lethal roles are also emerging. This review will discuss evidence for this by focusing on several recent studies in *Drosophila*.

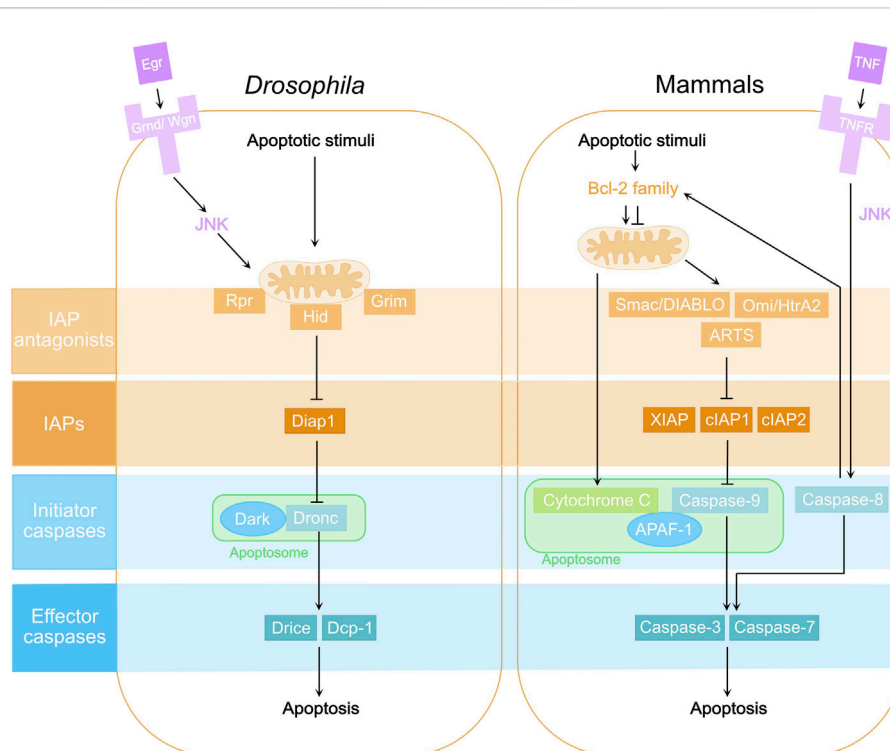


FIGURE 1

Core components of the apoptosis pathway in *Drosophila* and mammals. In *Drosophila*, apoptotic stimuli trigger the expression of IAP antagonists, mainly Rpr, Hid and Grim, which localize to mitochondria and inhibit the *Drosophila* inhibitor of apoptosis protein 1 (Diap1). Consequently, the initiator caspase Dronc, the *Drosophila* homolog of Caspase-2 and -9, is released from the inhibition of Diap1. Dronc then binds to *Drosophila* Ark (Dark), the Apaf-1 homolog, forming the apoptosome and becoming activated. Activated Dronc further cleaves and activates effector caspases DrICE and Dcp-1 leading to apoptosis. Eiger (Egr), the *Drosophila* Tumor Necrosis Factor (TNF), can trigger apoptosis via its receptors Grindelwald (Grnd) and Wengen (Wgn) and activation of c-Jun N-terminal Kinase (JNK) signaling. Their counterparts in the mammalian apoptosis pathway are highlighted in the same color.

Caspases in *Drosophila*

The core components, including caspases, of the apoptotic machinery are well conserved between mammals and *Drosophila* (Figure 1) (Fuchs and Steller, 2011; Hounsell and Fan, 2021). There are seven caspases in *Drosophila*: the initiator caspases Dronc, Dredd, and Strica (Chen et al., 1998; Dorstyn et al., 1999a; Meier et al., 2000; Doumanis et al., 2001), and the effector caspases DrICE, Dcp-1, Damm, and Decay (Fraser and Evan, 1997; Song et al., 1997; Dorstyn et al., 1999b; Harvey et al., 2001). The major apoptotic caspases in *Drosophila* somatic tissues are Dronc, DrICE and Dcp-1 (Chew et al., 2004; Xu et al., 2005; Xu et al., 2006). Dronc is the *Drosophila* ortholog of Caspase-2 and -9 in mammals and, like its counterparts, contains a CARD prodomain. In response to apoptotic stimuli such as heat, hypoxia and toxins, expression of pro-apoptotic proteins Hid, Rpr and Grim antagonizes the *Drosophila* inhibitor of apoptosis proteins (Diap1) (Fuchs and Steller, 2011; Hounsell and Fan, 2021). Like the mammalian IAPs, Diap1 suppresses caspases. Once released from Diap1 inhibition, Dronc associates with Dark, the *Drosophila* equivalent of the adaptor protein Apaf-1, to form the apoptosome. Dronc then auto-cleaves and becomes activated. Activated Dronc further cleaves and activates the effector caspases DrICE and Dcp-1, leading to the execution of apoptosis.

Similarly to mammals, apoptosis can also be triggered by Eiger (Egr), the *Drosophila* Tumor Necrosis Factor (TNF), through activation of c-Jun N-terminal kinase (JNK) signaling resulting in the expression of pro-apoptotic genes (Figure 1). In addition to apoptosis, Dronc also plays a role in other forms of cell death, such as in TNF-induced necrosis (Li et al., 2019).

Although caspases are best known at executing cell death, they also have non-lethal functions. Effector caspases, in particular, have been reported to have various roles in processes such as cell migration, growth control, neurite pruning, and tissue remodeling (Williams et al., 2006; Gorelick-Ashkenazi et al., 2018; Shinoda et al., 2019; Villars et al., 2022). In contrast, non-lethal roles of initiator caspases are less understood. Here, we will discuss the reported non-lethal roles of Dronc during *Drosophila* development and tissue homeostasis, as well as their implications for tumorigenesis.

Roles of Dronc during development and morphogenesis

During development of the central nervous system (CNS) in *Drosophila*, neuroblasts undergo asymmetric cell division to self-

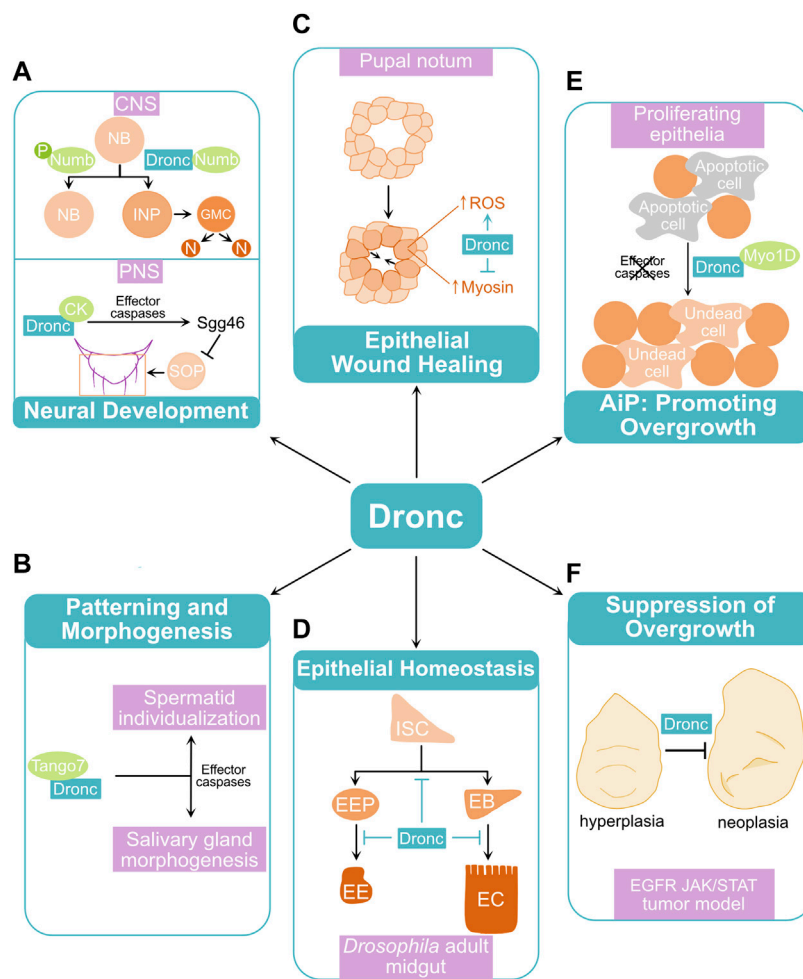


FIGURE 2

Examples of non-lethal roles of Dronc in *Drosophila*. **(A)** Roles of Dronc in neural development. In the larval central nervous system (CNS), Dronc interacts with Numb to promote cell differentiation from neuroblasts (NB) to intermediate neural precursors (INP), ganglion mother cells (GMC), and differentiated cells such as neurons (N). Phosphorylation (P) of Numb abolishes the Numb–Dronc interaction and promotes excess neuroblast proliferation. In the peripheral nervous system (PNS), Dronc promotes cleavage of Shaggy kinase 46 (Sgg46) via its interaction with Crinkled (CK) and effector caspases. This cleavage activates Sgg leading to division of sensory organ precursors (SOP) and formation of sensory organs such as macrochaetae. **(B)** Tango7 binds to Dronc and controls its function during spermatid individualization and salivary gland morphogenesis, via catalytic activities of effector caspases. **(C)** In the pupal notum, Dronc counteracts wound closure by promoting production of reactive oxygen species (ROS) and inhibiting myosin accumulation at the wound sites. **(D)** During gut epithelial homeostasis, Dronc contributes to maintenance of quiescent intestinal stem cells (ISC) and inhibits differentiation of enteroblasts (EB) into enterocytes (EC) and enteroendocrine progenitor cells (EEP) into enteroendocrine cells (EE). **(E)** In larval proliferating epithelia, Dronc drives tissue overgrowth via apoptosis-induced proliferation (AiP) when effector caspases are deficient or inhibited. **(F)** Dronc suppresses tumor progression in a *Drosophila* tumor model in which the EGFR and JAK/STAT signaling pathways are simultaneously activated. See the main text for detailed mechanisms regulating non-lethal roles of Dronc in these processes.

renew while also giving rise to more fate-specified cells such as the intermediate neural precursors (INP) and ganglion mother cells (GMC), depending on the context (Homem and Knoblich, 2012; Harding and White, 2018). GMC can further divide and produce two differentiated cells such as neurons and glial cells. Numb is a membrane-associated protein during asymmetric cell division (Knoblich et al., 1997). It segregates into the more differentiated daughter cell and inhibits Notch signaling, contributing to divergent cell fates. Interestingly, Dronc was revealed as a binding partner of Numb, and this interaction is abolished by Numb phosphorylation (Ouyang et al., 2011). Expression of a phospho-mimetic form of Numb in the larval brain produces supernumerary neuroblasts but

fewer differentiated neurons. Moreover, expression of a catalytically inactive form of Dronc (Dronc^{C>A}) (Meier et al., 2000), but not the effector caspase inhibitor P35 (Hay et al., 1994), attenuates this phospho-Numb-induced neuroblast production (Ouyang et al., 2011). Therefore, Dronc has a non-apoptotic role in the maintenance of neuroblast homeostasis via its interaction with Numb (Figure 2A). The catalytic activity of Dronc, but not effector caspases, is required in this process.

In the *Drosophila* peripheral nervous system (PNS), typical sensory organs on the adult thorax include large (macrochaete) and small (microchaete) bristles (Simpson and Marcellini, 2006). Each consists of four cells, including a socket cell, a shaft (hair) cell, a

sheath cell, and a neuron, which are derived from asymmetric divisions of a sensory organ precursor (SOP). Wild-type flies have two pairs of macrochaetes on the scutellum of the notum (Figure 2A) (Kanuka et al., 2005; Koto et al., 2009). Interestingly, *dark* mutants, expression of a dominant negative mutant form of Dronc (Dronc^{DN}), or RNAi knockdown of Dronc in the scutellum frequently gave rise to flies with extra macrochaetes (Kanuka et al., 1999; Rodriguez et al., 1999; Kanuka et al., 2005). These suggest a role for caspases in bristle pattern formation. An isoform of Shaggy kinase Sgg46, an antagonist of Wg/Wnt signaling during macrochaete formation, was identified as a substrate of effector caspases (Kanuka et al., 2005). Dronc regulates this process through its interaction with Crinkled (CK), a non-conventional myosin acting as an adaptor of Dronc to facilitate the cleavage of Sgg46 (Figure 2A) (Orme et al., 2016). This regulatory role of CK on Dronc has also been implicated in antenna arista morphogenesis and border cell migration during oogenesis (Geisbrecht and Montell, 2004; Kiehart et al., 2004; Orme et al., 2016).

Intriguingly, another adaptor protein, Tango7, was found to mediate non-lethal roles of Dronc in regulating patterning and morphogenesis, specifically during spermatogenesis and development of the salivary gland (Figure 2B) (D'Brot et al., 2013; Kang et al., 2017). During spermatid individualization, a large proportion of cytoplasm is removed and cytoplasmic bridges between spermatids are broken, resulting in the formation of individual sperms (Arama et al., 2003; Huh et al., 2003). This non-apoptotic process requires localized activation of the apoptosome and effector caspases which depends on a physical binding of Tango7 to both Dronc and Dark (D'Brot et al., 2013). A similar role of Tango7 was also observed during development of the salivary gland, which becomes morphologically stretched due to an enlarged lumen at late larval stage (Kang et al., 2017). During this morphogenetic process, Dronc localizes to and dismantles the cortical actin meshwork leading to its redistribution to the cytoplasm. Tango7, but not Dark, is required for the localization and proteolytic function of Dronc in this process. In contrast, the cytoplasmic localization and activation of Dronc depends on Dark and is critical for cell death that occurs in salivary glands starting at early pupal stage. These suggest that Dark and Tango7 determine the subcellular localization of Dronc and its lethal versus non-lethal functions.

The identification of Numb, CK and Tango7 as binding partners and mediators of Dronc function during development and morphogenesis is intriguing. Notably, MYO7A, the mammalian ortholog of CK, can selectively bind to Caspase-8 and regulate its non-apoptotic cleavage activity (Orme et al., 2016). Therefore, adaptor proteins modulating caspase specificity appears to be an evolutionarily conserved phenomenon. However, the mechanisms controlling the context-specific interactions between caspases and their unique adaptor proteins remain elusive.

Roles of Dronc in epithelial wound healing and homeostasis

Tissue regeneration and repair frequently rely on wound healing and stem cell renewal. During *Drosophila* pupal development, two wing imaginal discs, the epithelial primordia of adult notum and wings, fuse anteriorly to form an intact thorax (Zeitlinger and

Bohmann, 1999). This process, termed thorax closure, has been used as a model of epithelial wound closure. During this process, non-apoptotic caspase activity was detected at the leading edge cells (Fujisawa et al., 2019). Similarly, a sublethal caspase activation was observed close to the wound sites following laser ablation of pupa notum. Interestingly, expression of Diap1, *dronc*^{RNAi}, or Dronc^{DN}, but not P35, promoted wound healing. In agreement with this finding, co-expression of Rpr and P35, hence activating Dronc but inhibiting effector caspases, resulted in slower wound closure and flies with abnormal thoraxes. Therefore, Dronc, but not effector caspases, negatively regulates epithelial wound closure. Notably, active JNK signaling is required for wound healing. However, Dronc does not regulate JNK in this process. In contrast, production of reactive oxygen species (ROS) at wound sites, which regulates wound closure, depends on Dronc. Moreover, the level of myosin, a motor protein critical for actin cytoskeleton remodeling and wound closure, is significantly increased in response to loss of *dronc*. These support an inhibitory role of Dronc in wound healing by promoting ROS production and inhibiting myosin accumulation (Figure 2C).

The adult *Drosophila* midgut, a functional equivalent of the mammalian small intestine, is an established model for the study of stem cell biology and epithelial tissue homeostasis (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Jiang and Edgar, 2011). In the midgut, multipotent intestinal stem cells (ISC) can self-renew and give rise to enteroblasts (EB) or enteroendocrine progenitor cells (EPP) (Hung et al., 2021). EBs can further differentiate into absorptive enterocytes (EC), while EEPs can develop into hormone-secreting enteroendocrine cells (EE) (Figure 2D). Dronc is critical for gut epithelial tissue homeostasis as a defective intestinal barrier function was observed in survived *dronc* homozygous mutants, which are strong semi-lethal (Lindblad et al., 2021). In support of this, expression of Dronc was detected in progenitor cells including EBs (Arthurson et al., 2020; Lindblad et al., 2021). A conditional knockout of Dronc in both ISCs and EBs or RNAi knockdown of Dronc specifically in EBs resulted in a hyperplastic phenotype characterized by an increased number of both progenitor cells such as EBs and differentiated cells including ECs and EEs. Therefore, Dronc contributes to the maintenance of quiescent progenitor cells as well as inhibition of cell differentiation (Figure 2D). Moreover, this hyperplastic phenotype was rescued by expressing wild-type Dronc, but not Dronc^{C>A}, suggesting that Dronc catalytic activity is required in this process. However, blocking apoptosis by downregulating key apoptosis pathway components including Dark, DrICE and Dcp-1 or expression of P35 in EBs did not replicate the phenotype observed with loss of *dronc*. Hence, Dronc appears to have apoptosis cascade-independent roles in regulating gut epithelial tissue homeostasis.

Notably, similar roles of initiator caspases in wound healing and intestinal tissue homeostasis have been reported in mammals. For example, Caspase-8 deficiency in mouse skin resulted in elevated wound closure due to hyperproliferation of epidermal cells and enhanced local immune responses (Lee et al., 2009). Downregulation of Caspase-9 was associated with less differentiated, or higher grade, colon cancer (Xu et al., 2013). However, it is not yet known how caspase expression is locally

regulated and what caspase substrates mediate these processes. Answers to these questions will provide novel insight into tissue-specific functions of initiator caspases.

Roles of Dronc in apoptosis-induced proliferation and tumorigenesis

Apoptosis-induced proliferation (AiP) is a mechanism of compensatory cell proliferation in response to apoptotic stress (Fan and Bergmann, 2008a; Mollereau et al., 2013). Caspases play critical roles in the activation of AiP in multiple organisms including hydra, *Drosophila*, and mouse (Ryoo and Bergmann, 2012). AiP was first reported in the *Drosophila* larval wing imaginal disc, a proliferating tissue in which most cells are dividing (Huh et al., 2004; Pérez-Garijo et al., 2004; Ryoo et al., 2004). It was later discovered that initiator and effector caspases can trigger different mechanisms of AiP in a context-dependent manner (Fan and Bergmann, 2008b). In response to apoptotic stress, DrICE and Dcp-1 drive AiP *via* activation of Hedgehog signaling in differentiating tissues, in which most cells have exited the cell cycle. In contrast, Dronc activates mitogenic signals, such as Wg/Wnt, Dpp/TGF- β , and Spi/EGF signaling, leading to AiP in proliferating tissues. Myo1D, an unconventional myosin, physically interacts with and relocates cytoplasmic Dronc to the plasma membrane, allowing Dronc to activate Duox, a NADPH-oxidase, leading to production of extracellular ROS (Fogarty et al., 2016; Amcheslavsky et al., 2018). These ROS attract hemocytes, the *Drosophila* macrophages, which secrete Egr to activate JNK signaling non-cell autonomously at the apoptotic or damage sites. Interestingly, a similar role for Myo1D-Dronc interaction was observed in old ECs contributing to midgut tissue homeostasis (Amcheslavsky et al., 2020). Furthermore, Myo1D mediates Dronc-dependent regulation of F-actin dynamics which is critical for AiP, through controlling ROS production and JNK activation in apoptotic cells (Farrell et al., 2022). Therefore, Myo1D is a key adaptor which mediates distinct roles of Dronc in AiP (Figure 2E). Moreover, expression of *dark*^{RNAi} or Dronc^{C>A} suppresses AiP, suggesting that apoptosome formation and Dronc catalytic activity are required (Huh et al., 2004; Fan et al., 2014). Notably, expression of P35 or knockdown of both DrICE and Dcp-1 suppresses apoptosis, but not Dronc-induced AiP, in the proliferating eye and wing tissues, leading to tissue overgrowth (Kondo et al., 2006; Fan et al., 2014). Hence, the role of Dronc in AiP can be tumorigenic. Indeed, AiP has been implicated in promoting tumor growth and cancer recurrence in mammals (Huang et al., 2011; Ford et al., 2015). However, compared to effector caspases, roles of initiator caspases in these processes are less known.

Dronc also possesses a tumor suppressive role independent of apoptosis. This was observed recently in a *Drosophila* tumor model with synergistic activation of EGFR and JAK/STAT signaling (EJS) in wing imaginal discs (Xu et al., 2022). In EJS tumors, a high level of Dronc activity was observed. Inhibition of Dronc activity, RNAi knockdown, or a conditional knockout of Dronc resulted in an enhanced tumor overgrowth phenotype characterized by increased cell proliferation and cell sizes. In contrast, reduction of the pro-apoptotic proteins Rpr, Hid and Grim or expression of P35 suppressed apoptosis but did not cause elevated EJS tumor growth. Therefore, Dronc activity counteracts EJS tumor progression independent of apoptosis (Figure 2F). Loss of *dronc* further enhances JNK activity. This promotes tumor progression by

inducing the expression of Unpaired (Upd) ligands, which activate JAK/STAT signaling. Activation of JNK also leads to a higher number and proliferation of hemocytes, which attach to the EJS tumor cells and secrete Upd to further enhance tumor growth. Therefore, Dronc regulates both EJS tumor and its microenvironment by modulating JNK activity.

These examples in *Drosophila* suggest both pro- and anti-cancer activities of Dronc. In mammals, Caspase-8 promotes tumorigenesis in gliomas and is associated with poor patient prognosis (Fianco et al., 2017). In contrast, Caspase-2 deficiency in mouse models of lymphoma and lung cancer results in greater chemotherapeutic resistance, but also enhances tumor growth (Ho et al., 2009; Terry et al., 2015). Therefore, mammalian initiator caspases also have pro- or anti-oncogenic functions. However, how these initiator caspases are directed to execute their vital, but not lethal, roles in these processes remains elusive.

Discussion

The initiator caspase Dronc in *Drosophila*, like its counterparts Caspase-2, -8 and -9 in mammals, appears to exert its diverse functions in a context-dependent manner. These roles could be determined by the adaptor proteins with which Dronc interacts, the subcellular localization and activation of Dronc, or the Dronc-mediated cleavage of non-lethal substrates. Notably, these mechanisms are frequently interlinked and integrated. For example, the membrane-associated Numb interacts with Dronc to promote neuroblast differentiation *via* asymmetric localization and segregation of Numb (Figure 2A). Similarly, non-conventional myosins such as CK, Tango7 and Myo1D physically interact with Dronc and potentially regulate its subcellular localization for non-lethal functions (Figures 2A, B, E). However, it remains to be seen whether similar adaptors exist to mediate the functions of Dronc in wound healing, gut epithelial homeostasis, and tumor growth control (Figures 2C, D, F).

Another factor regulating the context-dependent activation of Dronc is its upstream inhibitor Diap1. For instance, Hippo signaling, a growth and tissue size control signal, monitors macrochaete formation by regulating Diap1 expression (Wang and Baker, 2019). Consistent with this finding, a dynamic expression of Diap1 was observed during sensory organ development, affecting bristle morphogenesis (Koto et al., 2009). Moreover, *diap1* expression and its protein stability are subject to the control of multiple cell type-specific mechanisms (Fan and Bergmann, 2014). Therefore, Diap1 can be a critical factor modulating sublethal Dronc activities. It is worth noting that anastasis, a phenomenon referring to cell recovery from activation of apoptotic caspases, can also contribute to caspase activation widely observed during development, regeneration, and tumorigenesis (Tang et al., 2015; Ding et al., 2016; Sun et al., 2020). Although physiological roles of anastasis remain to be clarified, it poses a challenge for future studies to distinguish it from the non-lethal roles of caspases.

Finally, the question of which substrates confer context-dependent functions of Dronc remains to be answered. Bioinformatics searches to identify Dronc substrates are not currently feasible as cleavage sites of Dronc are highly variable (Hawkins et al., 2000; Snipas et al., 2008). This, together with the spatial and temporal nature of Dronc activation, presents another challenge for future studies. However, recent technical advance on

developing sensitive caspase reporters and proximity labeling of Dronc *in vivo* has offered unique opportunities to address this challenge in *Drosophila* (Baena-Lopez et al., 2018; Shinoda et al., 2019). Therefore, studies using *Drosophila* models, which have uncovered the versatility of Dronc in processes underpinning tissue homeostasis and development, will continue to provide valuable insights into our understanding of caspase biology and its implication for human diseases.

Author contributions

DD and YF conducted literature research and wrote the manuscript.

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