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Apoptotic Caspases in Promoting Cancer: Implications from Their Roles in Development and Tissue Homeostasis

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Abstract

Apoptosis, a major form of programmed cell death, is an important mechanism to remove extra or unwanted cells during development. In tissue homeostasis apoptosis also acts as a monitoring machinery to eliminate damaged cells in response to environmental stresses. During these processes, caspases, a group of proteases, have been well defined as key drivers of cell death. However, a wealth of evidence is emerging which supports the existence of many other non-apoptotic functions of these caspases, which are essential not only in proper organism development but also in tissue homeostasis and post-injury recovery. In particular, apoptotic caspases in stress-induced dying cells can activate mitogenic signals leading to proliferation of neighboring cells, a phenomenon termed apoptosis-induced proliferation. Apparently, such non-apoptotic functions of caspases need to be controlled and restrained in a context-dependent manner during development to prevent their detrimental effects. Intriguingly, accumulating studies suggest that cancer cells are able to utilise these functions of caspases to their advantage to enable their survival, proliferation and metastasis in order to grow and progress. This book chapter will review non-apoptotic functions of the caspases in development and tissue homeostasis with focus on how these cellular processes can be hijacked by cancer cells and contribute to tumorigenesis.

Keywords

Apoptosis, Caspase, Non-apoptotic Function, Apoptosis-induced Proliferation, Development, Tissue Homeostasis, Cancer

1 Introduction

Apoptosis was first identified as a form of cell death by its distinct morphological characteristics including cellular shrinkage, chromosome condensation, nuclear fragmentation and formation of apoptotic bodies (Kerr et al., 1972, Jacobson et al., 1997). Studies in *C. elegans* then uncovered that apoptosis is genetically controlled and plays critical roles during development to remove unwanted or unnecessary cells (Ellis and Horvitz, 1986, Ellis et al., 1991). Such function of apoptosis further extends to maintenance of tissue homeostasis by eliminating damaged or unfit cells (Thompson, 1995, Miura, 2011). Apoptosis has therefore been viewed as a monitoring program to identify and kill potentially harmful cells that may develop into cancer. Consistent with this idea, evading apoptosis has been considered as a hallmark of cancer (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).

The key components of the apoptotic machinery are caspases, a family of cysteine proteases which cleave their substrates leading to cell death (Thornberry and Lazebnik, 1998, Hengartner, 2000). Recently however, in addition to their functions in apoptosis, caspases are becoming better understood in their multifunctional nature with an increasing number of non-apoptotic functions discovered. We acknowledge the abundance of high quality reviews which have described with clarity the non-apoptotic functions of caspases in the context of development and tissue regeneration (Kuranaga, 2011, Miura, 2012, Connolly et al., 2014, Shalini et al., 2015). This chapter therefore focuses on the roles of caspases in sustaining cancers and promoting their spread which seems to contradict what we know about their roles in apoptosis. There is now certainly a great wealth of evidence to show that the apoptotic

caspases actually have multiple functions other than executing cell death, and cancer cells can hijack these activities to directly promote their growth, metastasis and recurrence after therapy. Here we have synthesised the evidence present in the current literature supporting this claim, to highlight that the caspases do indeed have a role in progressing cancers. Issues that may exist in current cancer therapies for particular patient subsets are also discussed.

2 The apoptotic machinery: functions of the apoptotic caspases

Apoptosis is an evolutionarily conserved mechanism in multicellular organisms, allowing correct pattern formation during development and the removal of cells which are detrimental to the health and survival of the organism (Degterev and Yuan, 2008, Fuchs and Steller, 2011, Green et al., 2014, Yamaguchi and Miura, 2015). The pathways leading to apoptosis have been elucidated in many organisms, including *C. elegans*, *Drosophila* and mammals, which are summarised in figure 1. A noticeable family of key components in these apoptosis pathways are the caspases. By definition, caspases are cysteine-aspartic acid proteases. They cleave their substrates after the aspartic acid residue which features at the end of short tetrapeptide motifs (Earnshaw et al., 1999, Hengartner, 2000). In addition to their functions in apoptosis, caspases are also well known for their roles in inflammatory responses (Lamkanfi et al., 2007, Lamkanfi and Dixit, 2014, Vanaja et al., 2015). For examples, there are 18 known mammalian caspases among which caspase-2, -3, -6, -7, -8, -9 and -10 function in apoptosis and thus have been classified as apoptotic caspases (Eckhart et al., 2008, Shalini et al., 2015). This review focuses on these caspases especially the caspases-3, -7, -8 and -9 due to their multiple non-apoptotic functions reported.

Under normal cellular conditions all apoptotic caspases are present as inactive pro-caspases, called zymogens, which consist of a prodomain, a small subunit and a large subunit (Degterev et al., 2003, Earnshaw et al., 1999). They require cleavage in apoptotic cellular conditions to become activated. Based on the structure of N-terminal prodomains, apoptotic caspases can be subdivided into the initiator (or apical) and effector (or executioner) caspases. The initiator caspases have elongated prodomains which contain either the death effector domains (DED, e.g. for caspase 8) or the caspase-recruitment domains (CARD, e.g. for caspase 9). In contrast, the effector caspases have small prodomains. These caspases also have distinct functions and substrates during the process of apoptosis (Kumar, 2007, Chowdhury et al., 2008, Yan and Shi, 2005). The initiator caspases cleave inactive pro-effector caspases and activate them. They are therefore also called apical caspases. In contrast, effector caspases, once activated by the initiator caspases, further cleave their broad range of cellular proteins leading to execution of cell death. They therefore have another name as executioner caspases. For simplicity, terms of initiator and effector caspases are used in this review.

2.1 Apoptosis in *C. elegans*

The caspases were first identified in *C. elegans* in which 131 cells undergo apoptosis during development by the action of a simple and linear pathway (Fig.1A) (Ellis and Horvitz, 1986, Ellis et al., 1991, Fuchs and Steller, 2011). Before an apoptotic stimulus is detected by a cell, CED-4, a homolog of the mammalian adaptor protein apoptosis activating factor 1 (Apaf-1), exists as a dimer, which is sequestered on the outer leaflet of the outer membrane of the mitochondria by contact with a Bcl-2 family member called CED-9 (Lettre and Hengartner, 2006). Upon apoptotic stimulus, Egl-1, a pro-apoptotic BH3-only protein (Bcl-2 homology 3), is expressed, binding CED-9, thus releasing CED-4. Ced-4 is then free to form a tetramer.

Once the CED-4 tetramer is assembled, it can cleave and activate the caspase CED-3, which in turn activates other downstream apoptotic effector proteins leading to cell death (Fig.1A) (Conradt and Xue, 2005, Lettre and Hengartner, 2006).

2.2 The intrinsic apoptosis pathway in *Drosophila* and mammals

Unlike the linear pathway in *C. elegans*, apoptotic pathways of extrinsic and intrinsic origin have been identified in both *Drosophila* and mammals (Fig.1). The intrinsic pathway has been extensively studied in *Drosophila* (Fig.1B). Initially, apoptotic stimuli cause the expression of the pro-apoptotic genes of the RHG family: mainly *reaper*, *hid* (*head involution defective*) and *grim* (Kornbluth and White, 2005, Steller, 2008). These gene products act to relieve the repression exerted by the inhibitors of apoptosis (IAPs) (Wang et al., 1999, Lisi et al., 2000, Goyal et al., 2000), which, under normal cellular conditions, inhibits activities of the *Drosophila* initiator caspases such as Dronc (Meier et al., 2000, Wilson et al., 2002) and effector caspases such as DrICE and Dcp-1 (Hawkins et al., 1999, Yan et al., 2004). The major IAP in *Drosophila* is Diap1 whose function as an E3-ubiquitin ligase. Under no apoptotic stimuli, it binds to Dronc via its own BIR2 domain and causes ubiquitin to be tagged to Dronc (Wilson et al., 2002). Such ubiquitylation was believed to stimulate degradation of Dronc via the proteasome. However, a recent genetic analysis suggests that Diap1-mediated ubiquitylation blocks processing and activation of Dronc but does not lead to its protein degradation (Lee et al., 2011). When RHG proteins antagonise Diap1 by competitively binding to its BIR domains, Diap1 can no longer perform its function on inhibiting Dronc (Wilson et al., 2002, Ryoo et al., 2002, Yoo et al., 2002). From here released Dronc, although inactive, can induce formation of the apoptosome by the adapter protein Ark (Chai and Shi, 2014, Pang et al., 2015). Upon such interaction Dronc can autocleave and become activated. Activated Dronc further cleaves and activates its downstream effector

caspases, mainly DrICE and Dcp-1, leading to apoptosis (Fig.1B) (Hawkins et al., 2000, Dorstyn and Kumar, 2008, Snipas et al., 2008). Notably, pro-apoptotic proteins need to localize to the mitochondria and execute their apoptotic functions in *Drosophila* (Abdelwahid et al., 2007, Claveria et al., 1998, Claveria et al., 2002, Freel et al., 2008, Haining et al., 1999, Olson et al., 2003, Thomenius et al., 2011, Morishita et al., 2013). Two Bcl-2 family members, Debcl and Buffy, have been identified in *Drosophila* (Brachmann et al., 2000, Colussi et al., 2000, Igaki et al., 2000, Zhang et al., 2000, Quinn et al., 2003). Debcl is localized to the mitochondria and has pro-apoptotic functions, while Buffy may localize to endoplasmic reticula to carry out its own anti-apoptotic roles (Doumanis et al., 2007).

In the mammalian intrinsic pathway, the mitochondrion plays a central and more decidedly important role (Fig.1C). The Bcl-2 family proteins can be subdivided into three groups: the BH3-only proteins (such as Bid, Bad, Bik, Bim, Noxa and Puma), the pro-apoptotic Bax subfamily members (such as Bax, Bak and Bok) and the anti-apoptotic Bcl-2 family members (such as Bcl-2 and Bcl-XL) (Vander Heiden et al., 1997, Yang et al., 1997, Gabriel et al., 2003, Xiong et al., 2014). In response to apoptosis, BH3-only proteins either activate the Bax subfamily members or antagonize the anti-apoptotic Bcl-2 members to regulate mitochondrial outer membrane permeabilisation (MOMP) which then leads to release of cytochrome c (cyt c). Released cyt c binds to the adaptor protein Apaf-1, via the WD repeat domain at the carboxyterminus of Apaf-1, forming the apoptosome. Pro-caspase-9 can in turn interact with Apaf-1 in the apoptosome, via their mutual CARD domains (Srinivasula et al., 1998). Pro-caspase-9 then autocleaves and becomes active (Kumar and Colussi, 1999). The activated caspase-9 further cleaves its downstream effector caspases, caspase-3 and -7, to trigger apoptotic cell death (Xiong et al., 2014). In addition to cyt c, pro-apoptotic proteins such as Smac (or Diablo) and HtrA2 (or Omi) are also released from mitochondria during the process

of MOMP (Silke et al., 2000, Suzuki et al., 2001, Green and Kroemer, 2004). Similar to what happens in *Drosophila*, these pro-apoptotic proteins antagonize IAPs such as XIAP leading to activation of caspase-9, -3 and -7 and apoptosis. In addition to Smac and HtrA2, another mammalian IAP antagonist is ARTS which is not released from mitochondria (Larisch et al., 2000, Gottfried et al., 2004). Similar as the RHG proteins in *Drosophila*, it is localized to the mitochondrial outer membrane and inhibits XIAP (Edison et al., 2012).

2.3 The extrinsic apoptosis pathway in *Drosophila* and mammals

In contrast to the intrinsic pathway, the extrinsic pathway is initiated by the binding of a death ligand to a death receptor in the cell (Fig.1C). In mammals, examples of the death ligands are tumor necrosis factor (TNF) family members including Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (Ashkenazi and Dixit, 1998, Peter and Krammer, 1998, Lavrik et al., 2005). These ligands bind to their specific receptors Fas and DR4/5, forming complexes. Once such a ligand-receptor complex is formed, the adaptor protein Fas-associated Death Domain (FADD) can bind the cytosolic region of Fas and DR4/5. There, FADD acts as a platform on to which procaspase-8 can bind, by interaction of the death effector domain (DED) of FADD with the DED at the extended N-terminus of pro-caspase-8, forming the death-inducing signaling complex (DISC) (Medema et al., 1997, Juo et al., 1998, Varfolomeev et al., 1998). Due to receptor clustering in the plasma membrane, the pro-caspase-8 monomers are brought within close proximity of each other in DISC complexes, and once in this newly established close proximity they can autocleave and become activated (Chang et al., 2003, Guicciardi and Gores, 2009). Upon activation, caspase-8 can then cause the cleavage and activation of effector caspases caspase-3 and -7 leading to cell death (Hengartner, 2000, Chowdhury et al., 2008). Homologs of death ligands, receptors and their functions in apoptosis induction have also been found in *Drosophila* (Fig.1B).

There is only one TNF homolog, Eiger (Egr), identified in *Drosophila* so far (Moreno et al., 2002, Igaki et al., 2002). Two TNF receptors including Wengen (Wgn) and, more recently, Grindelwald (Grnd) have been reported (Kanda et al., 2002, Andersen et al., 2015). Activation of Egr triggers both apoptosis and a type of non-apoptotic cell death through the Jun N-terminal Kinase (JNK) pathway, a stress-response signaling pathway (Moreno et al., 2002, Igaki et al., 2002, Kanda et al., 2011, Ma et al., 2012). For the aspect of apoptosis, JNK induces expression of pro-apoptotic genes and activation of the apoptotic machinery (Moreno et al., 2002, Ma et al., 2012). Interestingly, in stress-induced apoptosis, the initiator caspase Dronc can activate not only effector caspases DrICE and Dcp-1 but also JNK which then feed back to the apoptosis pathway to further amplify it (Shlevkov and Morata, 2012). Notably, although different in their nature of inducing apoptosis, connections between the extrinsic and intrinsic pathways also exist in mammals. Caspase-8 can act on the pro-apoptotic BH3-only proteins such as BID leading to activation of the intrinsic pathway which further ensures a robust apoptotic response (Tang et al., 2000, Anto et al., 2002, Kantari and Walczak, 2011).

3 Apoptosis, development and non-apoptotic functions of caspases

Apoptosis and development are interconnected. On the one hand, apoptotic caspases were originally identified as key players in the developmental program (Ellis and Horvitz, 1986). Their apoptotic functions are critical for removal of extra cells produced at the early stage of development and elimination of unwanted cells in tissue patterning and morphogenesis (Fuchs and Steller, 2011). A recent study on Apaf-1 knock-out mice suggests that apoptosis is required to remove Fgf8 morphogen-producing cells and terminate Fgf8 production at the correct developmental time, thus ensuring proper development of the forebrain (Nonomura et

al., 2013). Apoptotic cells can even actively drive epithelial folding during morphogenesis (Monier et al., 2015) and cell extrusion during tissue repair (Kuipers et al., 2014). These examples have certainly underlined the developmental role of apoptosis. On the other hand, it is also becoming clear that the developmental program can modulate cellular apoptotic responses. Many key components in the apoptosis pathway can be targeted by the developmental program to define distinct cellular susceptibilities to apoptosis. For example, in *Drosophila* 3rd instar larvae, a pulse of hormone ecdysone increases the whole organismal sensitivity to apoptosis by upregulating the basal level of Ark, Dronc and DrICE (Kang and Bashirullah, 2014). Furthermore, in the developing *Drosophila* eye tissue, multiple mechanisms were employed to control cellular levels of IAPs as well as pro-apoptotic proteins (Fan and Bergmann, 2014, Hilgers et al., 2010). Similarly, in mouse embryos, primed stem cells are very sensitive to apoptosis due to their low levels of BIM regulated by microRNAs (Pernaute et al., 2014). Therefore, cellular apoptosis susceptibility can be modulated by developmental programs. However, the links between apoptotic caspases and development are far beyond death. Increasing evidence is now demonstrating the actual, true multifunctional nature of the caspases with somewhat surprising and fascinating roles in diverse cellular processes. These functions include regulating immune responses, promoting cell proliferation, and regulating cell differentiation and fate specification which have been extensively reviewed elsewhere (Jacobson et al., 1997, Kuranaga and Miura, 2007, Lamkanfi et al., 2007, Chowdhury et al., 2008, Kuranaga, 2012, Miura, 2012, Connolly et al., 2014, Shalini et al., 2015). Here, we highlight some of these non-apoptotic functions, in particular roles of caspases in tissue homeostasis, in the context of cancer development.

4 Caspases in tissue homeostasis: apoptosis-induced proliferation (AiP)

Organisms are constantly exposed to environmental stresses. Damaged cells are frequently removed by apoptosis. Meanwhile, new cells are generated by proliferation to compensate for the cell loss thus to maintain tissue homeostasis. For example, up to 60% of cells in the developing *Drosophila* wing epithelial tissue can be lost in response to radiation without affecting final adult wing size and morphology (Haynie and Bryant, 1977). A similar phenomenon has also been found in the processes of wound healing and liver regeneration in mammals (Li et al., 2010c). Apparently, tissue homeostasis is important for tissue function to remain optimal and critical to organism survival. Evidence in multiple organisms including *Hydra* (Chera et al., 2009), *Drosophila* (Huh et al., 2004, Kondo et al., 2006, Fan and Bergmann, 2008b) and mouse (Li et al., 2010c) is now demonstrating that apoptotic caspases have non-apoptotic functions to trigger compensatory proliferation, a process therefore termed apoptosis-induced proliferation (AiP) or apoptosis-induced compensatory cell proliferation (Fan and Bergmann, 2008a, Bergmann and Steller, 2010, Ryoo and Bergmann, 2012, Mollereau et al., 2013). For simplicity we use the term apoptosis-induced proliferation (AiP) in this review. Recent studies in *Drosophila* provided mechanistic insights on how AiP occurs (Fig.2A,B) (Ryoo et al., 2004, Perez-Garijo et al., 2004, Huh et al., 2004, Wells et al., 2006, Kondo et al., 2006, Fan and Bergmann, 2008b, Fan et al., 2014). Intriguingly, depending on the developmental state of the affected tissue, i.e. proliferating versus differentiating tissues, either initiator or effector caspases drive distinct mechanisms of AiP in *Drosophila* (Fan and Bergmann, 2008b).

4.1 The initiator caspase-driven AiP in *Drosophila*

The molecular mechanism of AiP was first addressed in *Drosophila* by taking advantage of caspase inhibitors (Ryoo et al., 2004, Perez-Garijo et al., 2004, Huh et al., 2004). P35, a baculovirus inhibitor of apoptosis, acts as a peudosubstrate of *Drosophila* effector caspases

e.g. DrICE and Dcp-1 (Hay et al., 1994). Expression of P35 thus blocks activity of DrICE and Dcp-1 and execution of cell death. To determine how stress-induced apoptotic cells may contribute to compensatory proliferation, such cells were kept "undead" by P35 (i.e. the apoptotic machinery is activated but execution of cell death is blocked). Surprisingly, "undead" cells stimulate overgrowth of surrounding tissues despite the presence of P35 (Ryoo et al., 2004, Perez-Garijo et al., 2004, Huh et al., 2004). This suggests that dying cells release mitogenic signals to induce AiP independent of effector caspases. Further loss-of-function analyses revealed that the initiator caspase Dronc, which is not inhibited by P35, actually coordinates apoptosis and AiP (Fig.2A). It appears that, at least in the "undead" model of AiP, Dronc activates JNK in dying cells leading to activation of several mitogenic signaling pathways including the Wingless (Wg, a homolog of the mammalian Wnt) and Decapentaplegic (Dpp, a TGF-β-like homolog of the mammalian BMP) signaling pathways which are required for AiP (Ryoo et al., 2004, Perez-Garijo et al., 2009). *Drosophila* homolog of p53 is also required for AiP, probably through its role in a feedback regulatory loop including JNK, p53 and pro-apoptotic genes (Shlevkov and Morata, 2012, Wells et al., 2006). However, one concern of the "undead" model of AiP is that it may not represent what happens in the physiological process of AiP (Perez-Garijo et al., 2009, Martin et al., 2009, Bergantinos et al., 2010). For example, it has been suggested that Wg and Dpp are not required for AiP when there are no "undead" cells (Perez-Garijo et al., 2009). Nevertheless, a *Drosophila* model of regenerative growth without using P35 has identified Wg as an important factor which is induced in response to tissue damage and is required for tissue regeneration (Smith-Bolton et al., 2009). In addition to these, a recent genetic screen using both an "undead" model and a P35-independent regenerative model has discovered a role of EGFR signaling in AiP and tissue regenerative growth (Fan et al., 2014). In this process, JNK transcriptionally induces Spi, one of EGF ligands in *Drosophila*, in dying cells which then

activates proliferation of neighboring cells via EGFR signaling. JNK can also activate the transcription factor Yorkie (Yki) in the Hippo signaling pathway to regulate AiP in developing Drosophila wing tissues (Sun and Irvine, 2011, Sun and Irvine, 2013). Interestingly, such a role of Yki in AiP seems to be tissue-specific as it is not required for AiP in proliferating eye tissues (Fan et al., 2014).

4.2 The effector caspase-driven AiP in *Drosophila*

A second form of AiP was identified in the differentiating *Drosophila* eye tissue which is a monolayer epithelium with differentiated photoreceptor cells at the apical side and cell-cycle-exited but unspecified cells at the basal side (Fan and Bergmann, 2008b). At the late 3rd instar larval stage, both types of cells have relatively low susceptibility to apoptosis presumably due to their post-mitotic status and protection of survival signals such as high Diap1 and the EGFR signaling (Fan and Bergmann, 2014). Therefore under apoptotic stresses, e.g. expression of the pro-apoptotic gene *hid*, these cells do not die immediately. Instead, the stressed photoreceptor neurons release Hedgehog (Hh), another evolutionarily conserved growth signaling ligand, to trigger cell cycle re-entry of unspecified cells (Fig.2B). Such an AiP event can be blocked by P35 or double mutants of DrICE and Dcp-1 suggesting an effector caspase-driven form of AiP is employed in the differentiating eye tissue (Fan and Bergmann, 2008b). Interestingly, mechanisms of AiP seem to be operated in a context-dependent manner. This is best shown in the developing *Drosophila* eye tissue. The late 3rd larval eye tissue consists of an anterior proliferating portion where all cells are actively dividing and a posterior differentiating portion where most of the cells present have exited the cell cycle. The initiator caspase-driven AiP appears to be employed in proliferating tissues, while the effector caspase-driven AiP is employed in differentiating tissues (Fan and Bergmann, 2008b). However, what controls such distinction is not yet known.

4.3 AiP in other organisms including mammals

In addition to *Drosophila*, roles of AiP in regeneration have also been implicated in other multi-cellular organisms particularly in *Hydra*, *Xenopus* and mouse (Chera et al., 2009, Tseng et al., 2007, Li et al., 2010c, Bergmann and Steller, 2010). In the freshwater *Hydra*, both head and foot can regenerate completely after bisection at the midgastric area. Massive localized apoptosis was observed for the head regenerating tip, but not the foot regenerating counterpart, preceding increase of cell proliferation (Chera et al., 2009). Interestingly, ectopic activation of apoptosis at the foot regenerating tip resulted in regeneration of head instead of foot. In this process, caspases activate Wnt3, a homolog of *Drosophila* Wg, in dying cells leading to regenerative proliferation (Chera et al., 2009). This study suggests that apoptosis can direct certain regenerative programs. Similar requirements of caspases in regeneration were also reported for *Xenopus* tadpole tail regeneration which is abolished by inhibiting caspase-3 (Tseng et al., 2007). Notably, in other regeneration models such as planaria and newt, massive apoptosis at the amputation site has been observed (Pellettieri et al., 2010, Pellettieri and Sanchez Alvarado, 2007, Vlaskalin et al., 2004). However, it is not yet clear whether apoptotic caspases actually drive release of mitogenic signals such as Wnt, TGF-β and Hh in these processes. More recently, roles of AiP in mammals were reported in mouse models of wound healing and liver regeneration (Li et al., 2010c, Jung et al., 2010). The rate of skin wound healing and liver regrowth after partial hepatectomy were significantly reduced in caspase-3 or -7 deficient mice due to impaired post-injury cell proliferation. It was further revealed that activated caspase-3 and -7 cleave calcium-independent phospholipase A2 (iPLA2) to increase its catalytic ability and promote synthesis of prostaglandin E2 (PGE2). Release of PGE2 from the dying cell then induces compensatory proliferation (Fig.2C) (Li et al., 2010c). Although detailed mechanisms on how PGE2 triggers

compensatory proliferation are not yet revealed, the link between PGE2 and the Wnt signaling cascade have been established in both zebrafish and mice (Goessling et al., 2009). PGE2 binds to the EP2, a G-protein coupled receptor, leading to activation of β-catenin, a key intracellular transducer of Wnt signaling (Castellone et al., 2005, Moon et al., 2004, Jager and Fearnhead, 2012).

5 Caspases in cancer development: non-apoptotic functions

Current cancer therapies such as chemo- and radiotherapies frequently aim to activate apoptosis of cancer cells. Therefore activating apoptosis has long been viewed as an "anti-cancer" process. However, increasing evidence is now suggesting that apoptotic caspases can play oncogenic roles through their non-apoptotic functions (Fig.3). Intriguingly, such "pro-cancer" functions of caspases seem not to be an invention by cancer cells. As discussed above the roles of the apoptotic caspases are essential in proper organism development and tissue homeostasis. Apparently such multi-functional nature of caspases needs to be tightly controlled and restrained by cellular contexts in order to prevent their detrimental effects. In the context of cancer, these non-apoptotic functions of caspases can be hijacked to ensure survival of cancer cells and promote their spread. Thus, the multifunctional nature of the apoptotic caspases is becoming clinically important.

5.1 Caspases promote cell survival and cell proliferation

The crucial function of caspases in cell survival and proliferation has been reported during development. Targeted disruption of caspase-8 in mice causes embryonic lethality, a feature not shared by the other caspases (Varfolomeev et al., 1998, van Raam and Salvesen, 2012).

Caspase-$8^{-/-}$ mouse embryos exhibited abnormal phenotypes prior to death, namely hyperemia, with the number of hematopoietic precursors significantly reduced (Varfolomeev et al., 1998). This suggests that caspase-8 is required for either maintenance or proliferation of hematopoietic precursors. As further supports for this view, depletion of caspase-8 in lymphoid tissues inhibits antigen-induced T and B lymphocyte proliferation (Salmena et al., 2003, Beisner et al., 2005, Su et al., 2005). Although it was originally thought that caspase-8 regulates cell proliferation in these cases, it is more likely that caspase-8 has pro-survival functions due to its inhibitory role on necroptosis, another form of programmed cell death (Oberst and Green, 2011, van Raam and Salvesen, 2012). The key factors involved in such regulation are caspase-8, the long isoform of cellular FLICE-like inhibitory protein ($FLIP_L$), and two kinases, RIPK1 and RIPK3, which are required for activation of necroptosis. $FLIP_L$ is structurally similar to caspase-8 but without its catalytic activity (Zhou et al., 2004). It can bind to pro-caspase-8, forming a heterodimer which prevents caspase-8 from completing its apoptotic functions by occupying all binding sites of caspase-8 in the DISC. This consequently prevents caspase-8 homodimer formation. Therefore, when the $FLIP_L$ levels are low, homodimerization of pro-caspase-8 occurs which activates caspase-8 for its apoptotic function. In contrast when $FLIP_L$ levels are high, e.g. triggered by survival signals mediated by a transcription factor NFκB, formation of the pro-caspase-8-$FLIP_L$ heterodimer does not trigger apoptosis. Instead, it can bind to the RIPK1-containing complex to suppress its activation on RIPK3 and necrotic cell death, although the underlying mechanism remains unclear (Oberst and Green, 2011). Hence, the level of $FLIP_L$ is crucial for caspase-8-regulated cell survival. Interestingly, an increase in $FLIP_L$ expression has been detected in a variety of tumor types, including B-cell chronic lymphocytic leukaemia, pancreatic cancer and ovarian cancer, amongst many others (Ghavami et al., 2009, Ili et al., 2013). Down-regulating $FLIP_L$ levels in tumors sensitises the cells to apoptosis (Ghavami et al., 2009,

Sharp et al., 2005). This is most likely due to decreased ability for caspase-8-$FLIP_L$ heterodimers to form and increased ability of caspase-8 homodimerization, which can then activate caspase-3 and apoptosis. In a study of cervical cancers, high grade tumors were found to have higher expression of $FLIP_L$ (Ili et al., 2013). Moreover, increasing grade of lesions was directly associated to increased c-FLIP expression, where 12.5% of normal cervical epithelia stained positive for relevant expression of $FLIP_L$ compared to 82.1% of squamous cervical carcinomas stained positive for $FLIP_L$ (Ili et al., 2013). This shows the significance in correlation of uncontrolled caspase-8-$FLIP_L$ dimer formation and cancer progression. Interestingly, infection by high-risk human papillomavirus (HPV), particularly HPV-16, was highly significantly correlated with high expression of $FLIP_L$ (Wang et al., 2007). Although the viral infection does not explain the cause of high $FLIP_L$ expression in other cancer types, high expression of $FLIP_L$ was determined to be a marker of early cervical carcinogenesis and therefore has the potential to be utilised for early diagnosis (Ili et al., 2013, Wang et al., 2007). This evidence highlights that the caspase-8-$FLIP_L$ heterodimer can be hijacked by cancer cells to promote tumor survival, by avoiding the apoptotic functions of caspase-8.

In addition to the initiator caspases, the effector caspases have also been implicated in promoting cell survival and cell cycle progression. In cultured cancer cell lines with the origin of leukemia or hepatocellular or cervical carcinoma, caspase-3 and -7 are found to be required in cell cycle progression through the G1 and G2/M checkpoints (Hashimoto et al., 2011, Hashimoto et al., 2008). Overexpression of the BIR2 domain of XIAP inhibits caspase-3 and -7, and when added to cells also induced cell cycle arrest. In contrast, inhibition of caspase-9 by expression of the BIR3 domain of XIAP did not cause the same effect, which indicates that caspase-3 and -7 have functions independent of caspase-9 activity. Although it

is not yet clear how caspase-3 and -7 may promote cell cycle without being cleaved by caspase-9, the anaphase-promoting complex/cyclosome (APC/C), which regulates degradation of various cell cycle regulators through ubiquitylation, failed to form when caspase-3 and -7 were inhibited (Hashimoto et al., 2011). This suggests that pro-caspase-3 and -7 may contribute to cell proliferation. Interestingly, direct substrates of caspases including cell cycle regulators can also promote cell survival or cell cycle progression at least in some circumstances. For example, the cyclin-dependent kinase inhibitor $P27^{Kip1}$ can be cleaved by caspase-3 which then becomes activated and anti-apoptotic to protect human leukemic cells from death (Eymin et al., 1999). In addition to this, a more recent study suggested that caspase-3 can act as a sensor to extracellular stresses, therefore determining whether the cells live or not (Khalil et al., 2012). In this study, caspase-3-knockout mice become more sensitive to UV radiation with increased number of cells undergoing necrosis compared to the control animals. In response to doxorubicin, an anticancer drug inducing apoptosis of cardiomyocytes, the caspase-3 deficient mice also show significantly increased number of apoptotic cardiomyocytes which die through the caspase-7 instead (Khalil et al., 2012). Caspase-3, but not caspase-6 and -7, cleaves the p120 RasGAP protein in vitro to activate a kinase Akt leading to survival functions of the PI3K signaling (Yang and Widmann, 2002, Yang et al., 2004). Consistently, Akt activity, indicated by the level of phosphorylated Akt, increases in response to stresses such as UV radiation and doxorubicin injection. But such increase is strongly reduced in caspase-3-knockout mice. Knockin mice with a RasGAP mutant resistant to caspase-3 cleavage can restore their apoptotic sensitivity (Khalil et al., 2012). Given these findings of caspases in cell survival and proliferation, could they contribute to tumorigenesis? As discussed below (see Sect. 5.3), the answer becomes clear by discovering roles of AiP in tumor reoccurrence following cytotoxic cancer therapies.

5.2 Caspases and metastasis

Metastasis is a crucial process during cancer development as it is the cause of approximately 90% of cancer related deaths (Hanahan and Weinberg, 2011). Yet it is an incredibly complex process consisting of multiple key steps for a cancer cell, or a group of cancer cells, to progress through (Geiger and Peeper, 2009). These steps include breaking away from a bulk tumor, disseminating in the blood or lymph, exiting the circulation, then establishing and repopulating at a new site, where a secondary tumor forms. Interestingly, caspases have been implicated in aiding some of these steps through their non-apoptotic functions in cell migration, angiogenesis, and possibly cell dedifferentiation.

Caspases have been reported for their functions in controlling cell motility during development. In *Drosophila*, Dronc, the caspase-9 homolog, is required for migration of border cells in the ovary (Geisbrecht and Montell, 2004), a process critical for oocyte development. In mammals, caspase-$8^{-/-}$ mouse embryos die with a circulatory failure suggesting roles of caspase-8 in migration of endothelial cells (Varfolomeev et al., 1998, Kang et al., 2004). Similarly, in cancer specific studies, caspase-3 and its downstream targets have been implicated in causing tumor cell migration, thus contributing to achieving metastasis. Caspase-3 has been shown in ovarian cancer cells to be involved in the process of initiating cell migration, via activation of arachidonic acid, the precursor of PGE2 similarly as in the context of AiP described above (Fig. 2C) (Zhao et al., 2006, Jager and Fearnhead, 2012, Portela and Richardson, 2013). Ovarian cancer cells have strong migratory responses towards laminin-10/11, a protein component of the extracellular matrix. This is probably due to the high levels of β1 integrin in ovarian cancer cells because binding of laminin-10/11 to β1 integrin leads to a moderate increase of caspase-3 activity (Zhao et al., 2006). Although the intermediate molecules determining caspase-3 activation from integrin-laminin binding

are unknown, Zhao et al. (2006) determined the moderate increase of caspase-3 activity does not lead to apoptosis, instead, it cleaves iPLA2 and activate its enzymatic activity to produce arachidonic acid and then PGE2. Consistently, pan caspase inhibitors, caspase-3-specific inhibitors or knockdown of iPLA2 inhibits migration of these cells. Interestingly, cleaved iPLA2 also activates the Akt survival signaling to protect these cells from apoptosis (Zhao et al., 2006). This further enhances cancer cell migration. Not surprisingly the arachidonic acid has also been implicated to be the driving factor of cell migration in other cancers including prostate cancers (Brown et al., 2014). Further support to roles of caspase-3 in cell migration comes from a study on lung cancer metastasis (Cheng et al., 2008). In this study, however, a protease-independent function of caspase-3 was suggested to promote metastasis. The authors used A549 cells, derived from high malignancy lung adenocarcinoma cells with high levels of caspase-3, for their study. Knockdown of caspase-3 in A549 cells diminishes their metastatic activities in the lungs when these cells were injected into the nude mice via tail vein suggesting roles of caspase-3 in promoting metastasis. Consistently, ectopic expression of caspase-3 in MCF-7 cells, derived from caspase-3-deficient and low malignancy breast cancer cells, enhances metastatic ability of these cells. Following these, the authors found that high levels of caspase-3 actually lead to high activity of the extracellular signal-regulated kinases (ERK) which are required for the observed lung metastasis. But intriguingly, such increased ERK activity and cell migration are not affected by the caspase inhibitor Z-DEVD-FMK. Furthermore, expression of protease-dead mutants of caspase-3 in MCF-7 cells still enhances their migration through increased ERK activities. Although it is not yet clear, the acid sphingomyelinase and its downstream signal molecule ceramide were suggested to be the molecules linking caspase-3 and ERK (Cheng et al., 2008). Interestingly, another mechanism of caspase-3-dependent cell migration has been reported for the “undead” cells in *Drosophila* models (Rudrapatna et al., 2013). In this case, DrICE, a caspase-3 homolog in

Drosophila, activates JNK leading to cell migration and tissue invasion. Therefore, cellular contexts may determine how caspase-3 promotes cell motility.

Following invasion and migration of cancer cells, angiogenesis is essential to further cancer progression, enabling tumor growth above a diameter of 1mm and metastasis (Geiger and Peeper, 2009). Knockdown of caspase-8 suppressed the vascular endothelial growth factor (VEGF)-mediated angiogenic signaling (Na et al., 2014). Interestingly, such requirement of caspase-8 in promoting angiogenesis is not affected by Ac-IETD-cho, a caspase-8 inhibitor that maintains high levels of pro-caspase-8. In contrast, the same study also showed that caspase-8 is required in TRAIL signaling to antagonize angiogenesis which can be inhibited by Ac-IETD-cho (Na et al., 2014). Therefore, pro-caspase-8 and caspase-8 appear to have distinct functions during angiogenesis mediated by VEGF.

Another cellular process that can potentially impact on cancer metastasis is cell dedifferentiation. Although it is still a subject of debate, existence of "cancer stem cells", a small fraction of stem cell-like cancer progenitor cells, may facilitate or even establish the metastatic colonies for cancer progression (Geiger and Peeper, 2009). If this is true, maintenance and reprograming, thus dedifferentiation, of cancer cells may be crucial in the process of metastasis which, again, may involve caspases. Notably, both caspase-8 and caspase-3 are required for the dedifferentiation of murine fibroblasts to form induced pluripotent stem cells (iPSCs) in vitro (Li et al., 2010a). Activation of caspase-8 and -3 are induced by expression of Oct-4, one of the four transcription factors used to program iPSCs. By inhibiting caspase-8 the cells were completely unable to develop into iPSCs, whereas some could if only caspase-3 was inhibited suggesting potential roles of other effector

caspases such as caspase-7 in induction of iPSCs. The authors further showed that the caspases act upon retinoblastoma susceptibility protein (Rb), but how from here the phenotype of a pluripotent stem cell is produced is unknown although p53 and its downstream cell cycle regulator p21 have been implicated in the process (Li et al., 2010a). Interestingly, studies of human tumors in relation to their Oct-4 expression showed that tumors expressing high levels of Oct-4 resulted in increased metastases, shorter survival and furthered disease progression in comparison to tumors low in Oct-4 expression (Chang et al., 2002). A recent study further sorted murine breast cancer 4T1 cells with either high or low Oct-4 expression and tested their tumorigenic potential in vivo by injecting sorted cells into the mouse mammary glands (Kim and Nam, 2011). The results support that Oct-4 can enhance cancer stem cell properties. This fits in vitro data and hypotheses theorising on the capacity of cancer stem cells in disease progression, though of course more studies are required in a greater range of tumor types.

5.3 Caspases in tumor repopulation following cytotoxic cancer therapies

Cytotoxic therapies exert their anti-tumor properties by inducing apoptosis as a result of DNA damage (Fulda and Debatin, 2006). As discussed above, AiP is a process utilised in non-cancerous tissue in order to maintain tissue homeostasis that allows tissue regeneration and recovery from damage. Consequently, this means that cytotoxic therapies can potentially induce not only cell death but also the AiP pathway which may in fact counteract cancer treatment. Tumors, to some extent, are comparable to standard developmental tissues (Dick, 2008), and, conceivably, when damaged they can respond in the same way to regenerate and to compensate for the inflicted damage, thus to repopulate and reoccur. Recent studies on AiP in cancer models suggested this is the case. In one study, experiments were conducted to find out how caspase-3 is responsible for promoting accelerated tumor repopulation following

cytotoxic therapy in 4T1 murine breast cancer cells (Huang et al., 2011). It was found that the AiP pathway, activated in either cancer cells or stromal cells, could become hijacked by cancer cells following radiotherapy, causing accelerated tumor repopulation *in vitro* and *in vivo*, in nude mice. These were also confirmed with human breast cancer cell lines in the nude mice (Huang et al., 2011). The results gained in this study were further developed upon in studies on metastatic melanoma, showing that chemotherapy too can result in AiP and tumor repopulation (Donato et al., 2014). As in the tissue regeneration mouse model, it is PGE_2 which is secreted from apoptotic cells and stimulates recipient living cells to proliferate in the tumor repopulation model (Fig.2C) (Donato et al., 2014, Huang et al., 2011). The authors also found that tumors with elevated caspase-3 were more resistant to radiotherapy than those with reduced caspase-3 (Huang et al., 2011). This at first seems paradoxical however with regards to the AiP model this observation seems to be logical. Higher caspase-3 expression allows for greater production of PGE_2, which in turn stimulates the increased growth rate of surviving cells, thus ensuring the maintenance of a larger tumor mass. Huang et al. found that the therapy sensitive cells were induced to undergo apoptosis, and the release of prostaglandins from the therapy sensitive cells caused the therapy resistant cells to proliferate at an accelerated rate and repopulate the treated tumor (Huang et al., 2011).

Statistical studies have been conducted to give a measure of how higher expression of particular caspases in tumors can affect outcome and survival likelihood. In a study of breast cancer, 103 out of 137 tumors were deemed to have high levels of caspase-3, although some activity was noticed in all of the tumors (Nakopoulou et al., 2001). Increased caspase-3 level significantly correlated with worsened survival of the patients sampled and, in the tumors sampled, caspase-3 was only found in the cytoplasm, not the nucleus where the apoptotic target of caspase-3 resides. This suggests a possible mechanistic block preventing the effector

caspase-3 from reaching its target molecule, the inhibitor of caspase-activated DNase (iCAD), to free caspase-activated DNase (CAD) which can cause DNA fragmentation and subsequent cell death (Nakopoulou et al., 2001). Another study assessing implications of caspase-3 in gastric, ovarian, cervical and colorectal cancers concluded that patients possessing tumors which expressed higher caspase-3 had shortened survival time and also found that caspase-3 expression was significantly associated with tumor stage (Hu et al., 2014). Both studies concluded that higher caspase-3 expression resulted in worsened prognosis. Notably, participants of these studies had not undergone any form of therapy. However, these findings of statistical significance were further confirmed by Huang et al. (2011), on patients who had undergone radiotherapy or chemotherapy.

Given these new insights of mechanisms causing tumor repopulation following cytotoxic therapy, if repopulation is to be prohibited in tumors, then the AiP pathway needs to be blocked while still allowing caspase-3 to carry out its apoptotic functions. As described for AiP (Fig.2C), PGE_2 is synthesised from arachidonic acid by cyclooxygenases (COX). Thus, COX inhibitors in theory should prevent the AiP pathway from progressing. This has been shown in practice, where administering a COX inhibitor in conjunction with cytotoxic therapy significantly decreases rate of tumor repopulation (Huang et al., 2011, Donato et al., 2014). Therefore, use of a COX inhibitor in conjunction with the cytotoxic therapy may benefit patients possessing tumors with high levels of caspase-3. Notably, caspase-3 may not be the only components in the apoptosis pathway that can promote cancer tumorigenesis as suggested by studies on lymphoma (Michalak et al., 2010, Labi et al., 2010). Further mechanistic understanding of AiP in various cellular contexts will be the key to maximize its clinical benefits. Interestingly, in addition to AiP, engulfment of apoptotic cells by macrophages can create a tumor-promotive microenvironment by releasing signaling

molecules (Steidl et al., 2010, Qian et al., 2011, Willems et al., 2014) and regulating various aspects of tumor progression (Ford et al., 2015). Again, caspases play key roles here. Activation of iPLA2 by caspase-3 leads to production of lysophosphatidylcholine (LPC), as well as PGE2, from dying cells (Lauber et al., 2003). LPCs, together with several other molecules such as sphingosine-1-phosphates (S1Ps) and the nucleotides ATP and UTP, recruit macrophages to engulf apoptotic cells (Ravichandran, 2011). Therefore, apoptotic caspases can promote tumorigenesis directly through AiP or indirectly through recruiting macrophages (Jager and Fearnhead, 2012). This is further discussed in the Chapter 3.

6 Concluding remarks

For many years the apoptotic function of caspases has been considered, both in developmental settings and in a cancer setting, where activation of apoptotic proteins is considered to be essential in causing cell death and reducing tumor burden (Hanahan and Weinberg, 2011). While these considerations of caspase function remain valid, increasing evidence suggested that non-apoptotic functions of the apoptotic caspases exist in a context-dependent manner. These functions are crucial in development and tissue homeostasis, where caspases have been implicated in stem cell pool maintenance by enhancing survival pathways and in AiP for tissue recovery upon cell loss, in which we have learned a lot from *Drosophila* models. Intriguingly, a wide range of non-apoptotic functions of caspases have been implicated in promoting tumor growth, metastasis and recurrence post-cytotoxic therapy (Fig.3). It is therefore worthwhile to consider not only how to kill the tumor cells, but also how to prevent tumour spread and repopulation. Further understanding of molecular mechanisms and cellular contexts leading to various non-apoptotic functions of caspases would certainly be beneficial.

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Figure 1

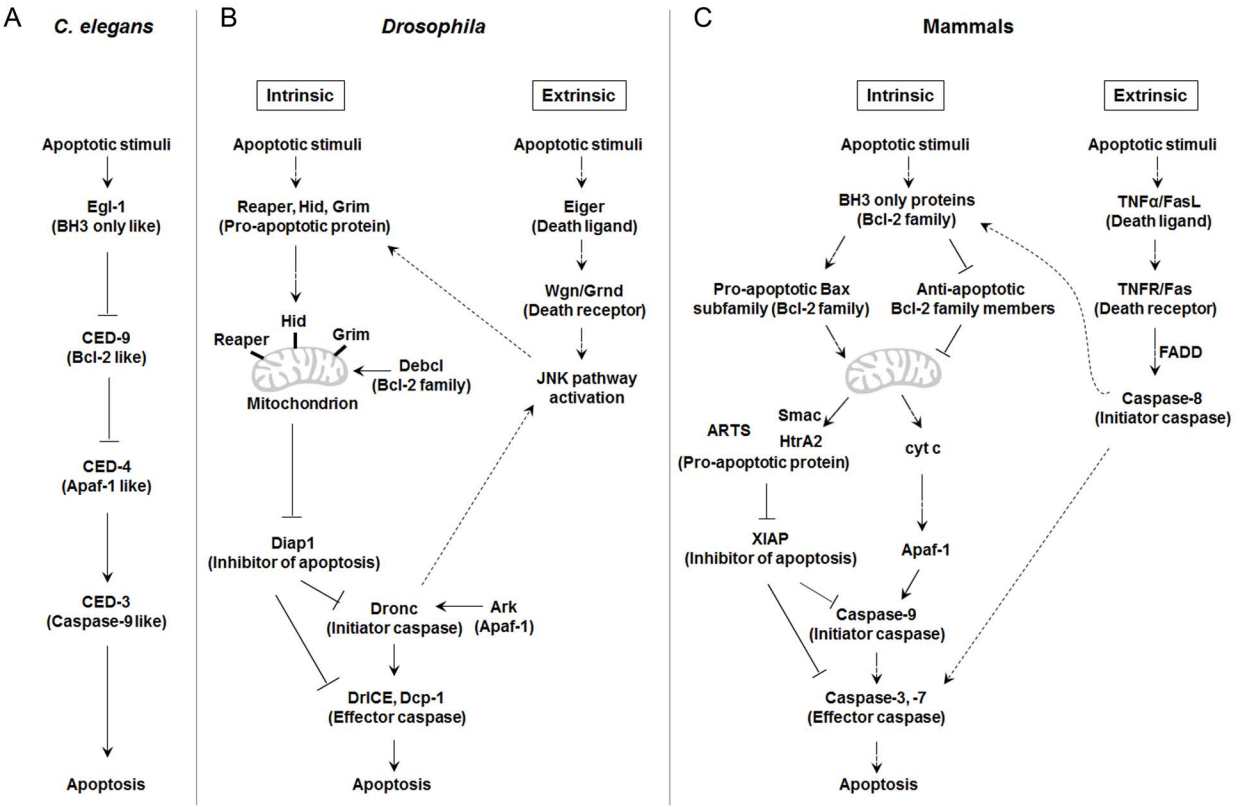

A
C. elegans
Apoptotic stimuli
Egl-1
(BH3 only like)
CED-9
(Bcl-2 like)
CED-4
(Apaf-1 like)
CED-3
(Caspase-9 like)
Apoptosis
B
Drosophila
Intrinsic
Extrinsic
Apoptotic stimuli
Reaper, Hid, Grim
(Pro-apoptotic protein)
Hid
Reaper
Grim
Debcl
(Bcl-2 family)
Mitochondrion
Diap1
(Inhibitor of apoptosis)
Dronc
(Initiator caspase)
Ark
(Apaf-1)
DrICE, Dcp-1
(Effector caspase)
Apoptosis
Apoptotic stimuli
Eiger
(Death ligand)
Wgn/Grnd
(Death receptor)
JNK pathway
activation
C
Mammals
Intrinsic
Extrinsic
Apoptotic stimuli
BH3 only proteins
(Bcl-2 family)
Pro-apoptotic Bax
subfamily (Bcl-2 family)
Anti-apoptotic
Bcl-2 family members
Smac
ARTS
HtrA2
(Pro-apoptotic protein)
cyt c
XIAP
(Inhibitor of apoptosis)
Apaf-1
Caspase-9
(Initiator caspase)
Caspase-3, -7
(Effector caspase)
Apoptosis
Apoptotic stimuli
TNFα/FasL
(Death ligand)
TNFR/Fas
(Death receptor)
FADD
Caspase-8
(Initiator caspase)

Figure 2

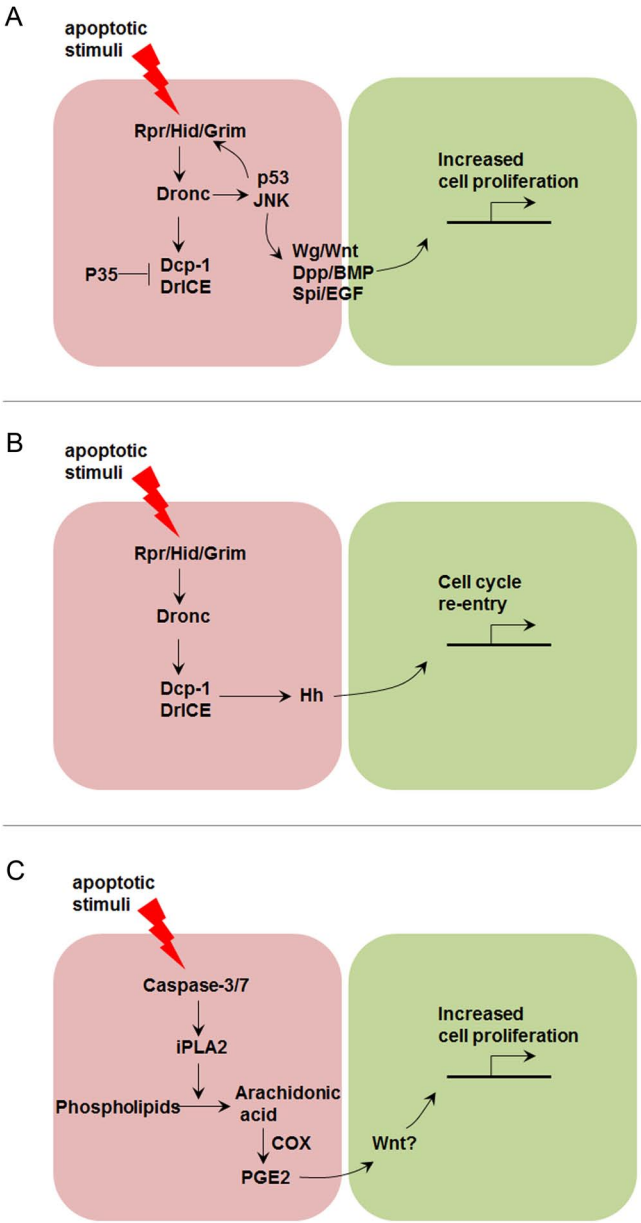
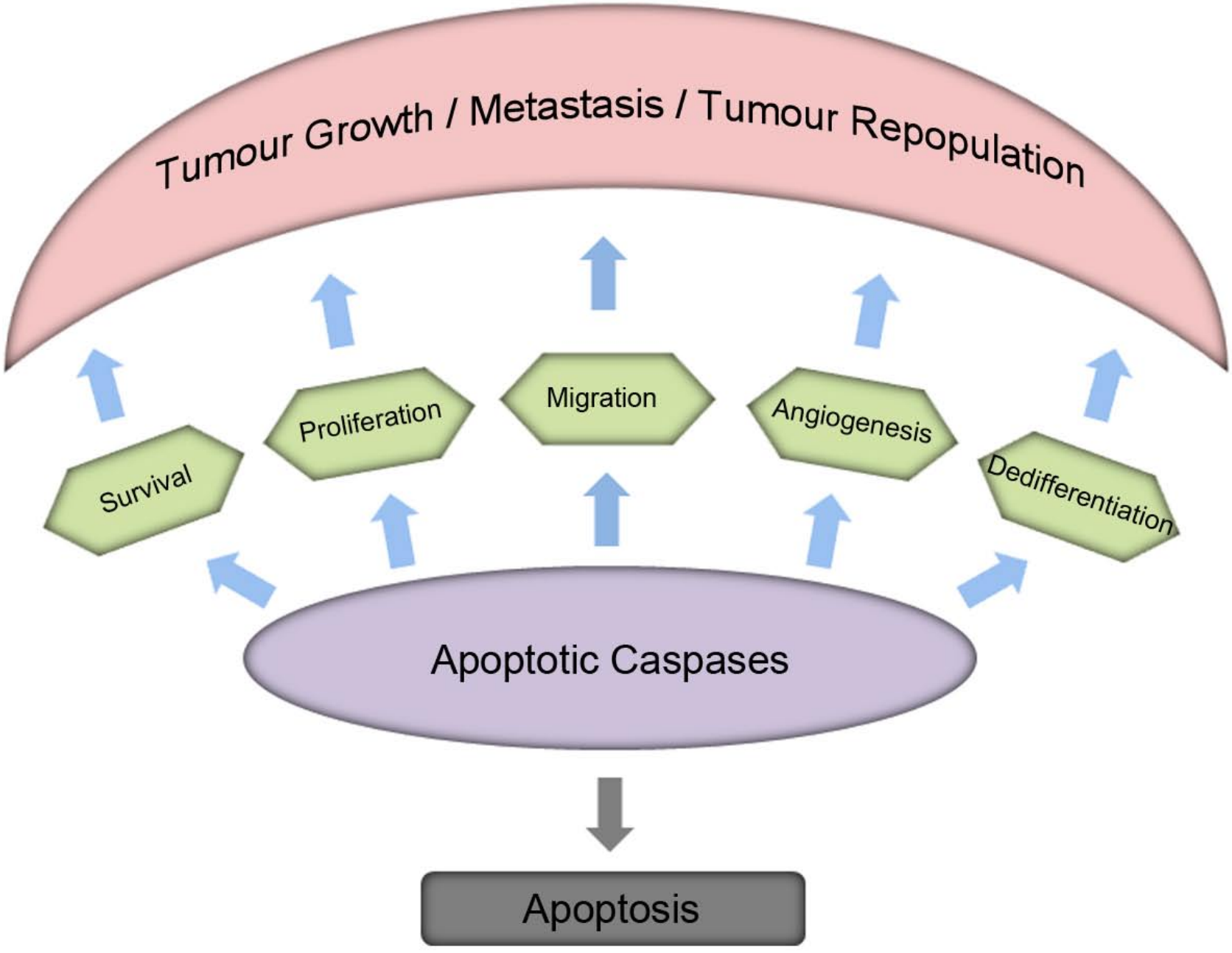

A
apoptotic stimuli
Rpr/Hid/Grim
Dronc
p53
JNK
P35
Dcp-1
DrICE
Wg/Wnt
Dpp/BMP
Spi/EGF
Increased cell proliferation
B
apoptotic stimuli
Rpr/Hid/Grim
Dronc
Dcp-1
DrICE
Hh
Cell cycle re-entry
C
apoptotic stimuli
Caspase-3/7
iPLA2
Phospholipids
Arachidonic acid
COX
PGE2
Wnt?
Increased cell proliferation

Figure 3


Tumour Growth / Metastasis / Tumour Repopulation
Survival
Proliferation
Migration
Angiogenesis
Dedifferentiation
Apoptotic Caspases
Apoptosis