A fate worse than death: apoptosis as an oncogenic process

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Preface

Apoptotic cell death is widely considered a positive process that serves to both prevent and treat cancer. While undoubtedly playing a beneficial role, paradoxically, apoptosis can also cause unwanted effects that may even promote cancer. In this opinion article we highlight some of the ways through which apoptosis can exert oncogenic function. We argue that fully understanding this dark-side will be required in order to optimally engage apoptosis, thereby maximising tumour cell kill while minimizing unwanted effects.

Introduction

Apoptosis is a major type of regulated cell death in our bodies. This evolutionary conserved process plays crucial roles that range from tissue sculpting during embryonic development through to execution of immune effector functions [1]. Moreover, too much or too little apoptosis has been implicated in diverse diseases including neuro-degeneration and auto-immunity [2, 3]. Abundant evidence supports a role for inhibition of apoptosis in the promotion of cancer and blunting of therapeutic responses [4-6]. Accordingly, significant excitement surrounds engaging apoptosis in a tumour-specific manner in order to treat cancer; to this end, highly promising apoptosis-inducing therapies called BH3-mimetics are in advanced clinical trials for the treatment of chronic lymphocytic leukemia (CLL) [7]. Therefore, apoptosis is widely considered a positive process that serves to both inhibit and treat cancer. In this opinion article we present a more nuanced view of apoptosis, namely that it can also have cancer-promoting functions. We begin our discussion by providing a general overview of apoptotic signaling before briefly reviewing its role in restraining cancer. From this basis, we highlight the varied means by which apoptotic signaling can exert oncogenic effects; these include extrinsic effects of the dying cell, intrinsic effects due to surviving apoptotic stress or due to non-apoptotic functions of proteins typically considered apoptotic. We propose that improved understanding of these effects and their importance in cancer should optimise our ability to fully exploit apoptosis as a therapeutic mechanism.

Apoptotic signaling pathways

Caspase protease activity is essential for the morphological and biochemical hallmarks of apoptosis [8]. In broad terms, caspases can be activated through one of two pathways - the extrinsic (also called death-receptor) pathway and the intrinsic (also called mitochondrial) pathway (Figure 1). As the name implies, the extrinsic pathway requires external stimulation, this occurs via a death receptor-family member, such as TRAIL-R1 (DR4), TRAIL-R2 (DR5), CD95 (FAS) or TNF-R1, located at the plasma

membrane. Following ligand binding, death receptors activate caspases leading to widespread substrate protein cleavage and rapid cell death [8].

The intrinsic apoptotic pathway is engaged by a wide array of stimuli that are sensed intra-cellularly including cytokine deprivation, DNA damage and endoplasmic reticulum (ER) stress (Figure 1). As we will discuss, the intrinsic apoptotic pathway is often deregulated in cancer. These diverse apoptotic stresses converge to trigger one critical event - mitochondrial outer membrane permeabilisation or MOMP [9]. The extrinsic and intrinsic pathways also crosstalk through caspase-8 cleavage of the BH3-only protein BID - this generates the active, truncated form of BID (tBID) that triggers MOMP [10, 11]. Following mitochondrial permeabilisation cytochrome *c* is released from the mitochondrial intermembrane space and induces caspase activation via a cytoplasmic complex termed the apoptosome [9]. Importantly, because MOMP often kills irrespective of caspase activity, it is considered a cellular death sentence [12]. Nevertheless, there are notable exceptions; for example some types of neurons can circumvent the lethal effect of MOMP by inhibiting caspase activity through various means including low expression of APAF-1 or degradation of cytochrome *c* [13, 14]. Moreover, proliferating cells under caspase-inhibited conditions can also survive MOMP dependent upon glycolytic metabolism and autophagy [15].

Because of this pivotal role in dictating life and death, MOMP is highly regulated through interactions between Bcl-2 family members [16]. There are three subfamilies of Bcl-2 proteins: anti-apoptotic Bcl-2 proteins (such as BCL-2 or BCL-xL), pro-apoptotic BH3-only proteins (including PUMA, BID and BIM) and pro-apoptotic effector proteins (BAX, BAK and BOK). BH3-only proteins relay diverse apoptotic signals to activate BAX and BAK at the mitochondrial outer membrane whereupon BAX and BAK trigger MOMP [16]. Anti-apoptotic Bcl-2 proteins inhibit MOMP and cell death either by directly binding BH3-only proteins or by binding activated BAX and BAK [17]. This is therapeutically important since competitive disruption of these interactions - thereby sensitizing to apoptosis- forms the mechanistic basis of BH3-mimetic action [18].

Apoptosis in the prevention and treatment of cancer

Apoptosis has long-been considered a process that must be evaded in order to allow cancer to develop [4]. This view is supported by a wealth of data, including: 1) demonstration that inhibition of cell death, in combination with mitogenic oncogenes, can promote cancer in experimental models [19-21] 2) discovery that many oncogenic pathways inhibit apoptosis, whereas tumour suppressors, such as p53, can engage apoptosis [22] 3) positive correlation between tumour cell apoptotic sensitivity and therapeutic efficacy in patient derived cancer cells [23, 24] 4) frequently observed up-regulation of anti-apoptotic proteins in cancer [25]. Nevertheless, while inhibition of apoptosis may promote cancer, cancer cells are often not inherently apoptosis-resistant. Indeed, as we will highlight, some human tumour types are actually more sensitive to apoptosis than normal tissue [26, 27]. Counter-intuitively, higher levels of apoptosis in cancer patients have also been shown to correlate with poorer prognosis in some cancer types [28-33]. Finally, and again paradoxically, high-levels of anti-apoptotic proteins correlate with better prognosis in certain cancers. For example, as shown in Table 1, a high level of anti-apoptotic Bcl-2 protein expression associates with favourable outcome in some human cancers. Notably, increased anti-apoptotic Bcl-2 protein expression does not necessarily translate to decreased apoptotic sensitivity, indeed the opposite can hold true. For example, some cancers, such as CLL, express high levels of anti-apoptotic Bcl-2 proteins to buffer intrinsic pro-apoptotic Bcl-2 activity [34]. Cancer cells in this state are referred to as “primed for death” and are highly sensitive to apoptosis-inducing therapies [26]. Whereas increased-expression of anti-apoptotic BCL-2 proteins can associate with good prognosis, high-expression of pro-apoptotic BAX can correlate with a poor outcome (Table 1). As we now discuss, these findings and others challenge the view that apoptotic signaling serves solely to inhibit cancer, arguing instead that apoptosis also has a dark-side that can actually promote cancer.

Calling from beyond the grave: cell extrinsic effects of tumour cell apoptosis

While apoptotic cell death is a cell-autonomous event, its effects are not; dying cells impact on their surrounding environment in various, yet not fully understood, ways. These can include stimulating the proliferation of neighbouring cells, impacting on intra-tumoural cell competition as well as exerting paracrine effects on the tumour microenvironment. By serving to promote cancer progression, they also represent potential nodes for therapeutic intervention.

(Unwanted) Life after death - apoptosis induced proliferation

Apoptotic cells can actively promote the proliferation of surrounding cells. As a physiological event, this may allow apoptotic cells within a tissue to signal their replacement either during normal turnover or serve as a healing response following extensive tissue damage. This process has been best studied in the fruit-fly *Drosophila melanogaster* where cells undergoing apoptosis in the imaginal disk were shown to activate mitogen-signaling, promoting the proliferation of neighboring cells [35-37].

Directly relevant to cancer, apoptosis-induced proliferation also occurs in mammalian settings. Apoptotic cells can stimulate the proliferation of stem cells in a caspase-dependent manner [38]. Moreover, genetic deletion of caspases-3 and -7 inhibits regeneration in two model systems - skin wound healing and liver regeneration following partial hepatectomy [38]. How can apoptotic cells stimulation proliferation? Various studies have implicated prostaglandin E_2 (PGE_2) as a key mediator of apoptosis-induced proliferation in mammalian systems. During apoptosis, caspases cleave and activate $iPLA_2$ (calcium-independent phospholipase A_2) increasing the production of arachidonic acid (AA), which is converted via cyclooxygenases 1 and 2 (COX1 and 2) to PGE_2 [39]. This may provide a mechanistic explanation to intriguing observations made in the 1950's that mixing of viable tumour cells together with cells destined to die actually accelerated tumour growth, a finding subsequently observed by others [40, 41] (Figure 2). Indeed recent data argue that signalling by apoptotic tumour cells, plays an important role in tumour re-growth following radiotherapy [42]. Tumour repopulation in a mouse xenograft model of breast cancer is promoted in a caspase-3 and $iPLA_2$

dependent manner, likely through the production of PGE_2 [42]. In bladder cancer, production of PGE_2 by apoptotic tumour cells promotes chemoresistance by stimulating cancer stem cell proliferation [43]. The therapeutic relevance of targeting apoptosis-induced proliferation is supported by the finding that neutralisation of PGE_2 signalling reduced the emergence of chemoresistance in a model of human bladder cancer [43]. Importantly, PGE_2 has pleiotropic functions, for example, not only does it promote proliferation, but it can also serve to skew immune responses towards a tumour-promoting, anti-inflammatory phenotype [44]. In doing so, generation of PGE_2 by apoptotic tumour cells could have dual tumour promoting functions - simultaneously driving proliferation and inhibiting anti-tumour immunity.

Apoptosis, cell competition and cancer

Many influences, including the availability of nutrients and growth factors, all combine to impact on a cell's ability to grow. Proliferating cells constantly compete with one another; "winner" cells outcompete "loser" cells either by, for example, taking-up nutrients more effectively - or through elimination of the competition. Apoptosis can contribute to the latter effect, generating a vacant niche into which cancerous cells can expand. The key tumour suppressor protein, p53 prevents cancer through multiple mechanisms [45]. In a transplantable mouse model of lymphoma, deletion of p53 from hematopoietic progenitors *per se* does not confer a competitive advantage over wild-type cells unless the system is stressed (in this case by irradiation) [46, 47]. p53-deficient cells survive irradiation whereas wild-type cells are eliminated by p53 activity (either through apoptosis, cell cycle arrest or senescence). This provides a vacant niche into which p53-deficient can proliferate, facilitating the accumulation of genetic lesions that lead to cancer.

Similarly, a direct role for apoptosis in promoting cancer by killing off the healthy competition has been shown in PUMA-deficient mice. The BH3-only protein PUMA is essential for p53-mediated apoptosis in thymocytes and its loss enhances *myc*-induced lymphomagenesis [48-51]. Counter-intuitively, loss of PUMA prevents rather than promotes thymic lymphoma following irradiation [52, 53]. Similarly, in a carcinogen-induced liver cancer model, deletion

of PUMA or overexpression of anti-apoptotic BCL-2 protein also delays tumour development [54, 55]. Finally, deletion of another pro-apoptotic BH3-only protein - BID - inhibits rather than promotes hepatocarcinogenesis following different chronic liver injury insults [56, 57]. Why would inhibition of apoptosis prevent rather than promote cancer? The answer likely relates to our earlier discussion of p53 and cell competition. As the authors propose, PUMA mediated apoptosis of healthy cells could remove the competitive pressure that normally acts to restrain proliferation of aberrant cells, in doing so this leads to cancer. In support, glucocorticoid treatment eliminates cells in a PUMA-independent manner and leads to thymic lymphoma in PUMA-deficient mice [52]. Relating to our earlier discussion, particularly in liver cancer, apoptosis may also promote cancer by actively engaging compensatory proliferation of neighbouring cells [54].

At least in mice, these studies strongly support a cancer-promoting role for apoptosis through an ability to generate a vacant niche. However, to what extent might the role of apoptosis in competition affect human malignancies? One possibility is in the development of myelodysplatic syndrome (MDS) that can progress to MDS related acute myeloid leukaemia (MDR-AML) [58]. MDS is a blood disease that leads to sudden blood cell cytopenia. In many cases its etiology is unknown but its increasing incidence with age strongly suggests that cumulative damage plays a major role. MDS is also a relatively common, secondary consequence of DNA-damaging cancer therapy. MDS leading to MDR-AML are malignancies that arise as a clonal disease of hematopoietic stem cells (HSCs). Through successive rounds of DNA-damage, proliferation and apoptosis, clones are selected that ultimately give rise to AML [58]. The role of apoptosis in this disease may be multi-faceted, but one possibility is that, similar to the PUMA models described above, a proportion of highly apoptosis-sensitive HSCs die leaving a vacant niche that mutated HSCs fill. In a ramped-up process of Darwinian selection, rounds of these events, may lead to the development of aggressive and apoptosis resistant AML. Indeed, supporting this idea, inhibiting apoptosis, either through ectopic BCL-2 expression or loss of PUMA expression delays leukemia progression in mouse models of MDS [59, 60]. As the authors suggest, if these findings hold

true in humans, inhibition of apoptosis may actually delay MDS progression. Through similar means, engagement of apoptosis by cancer therapies may also inadvertently promote more aggressive disease. In this scenario, killing of sensitive cancer cells (losers) may remove competitive pressure allowing aggressive clones to dominate and win (Figure 2).

Pro-oncogenic effects of apoptotic cells upon the tumour microenvironment

An estimated one million cells in our bodies undergo apoptosis every second [1]. Nevertheless, analysis at any given timepoint fails to reveal this wholesale carnage, largely because apoptotic cells are efficiently engulfed and destroyed by phagocytic cells. Caspase-dependent events actively orchestrate recruitment of phagocytes towards the apoptotic cell, by releasing "find-me" signals and promote the engulfment of the dying cell by exposing "eat-me" signals [61]. Several "find-me" signals released by apoptotic cells have been identified including the lipid lysophosphatidylcholine (LPC), nucleotides such as ATP, the proteins fractalkine (also called CX_3CL1 or FKN) and lactoferrin (LTF) (Figure 2). These signals may exert oncogenic function through pleiotropic effects that include but are not limited, to their role as "find-me" signals. For example, fractalkine can stimulate angiogenesis *in vitro*, hypoxia-induced proliferation of prostate cancer cells and enhance oncogenic ERBB2 receptor signaling [62,63]. Besides being a powerful chemokine released by apoptotic cells [64], ATP can also have a major influence on cancer. While numerous studies support an anti-tumorigenic effect of ATP, there is increasing realisation that adenosine (a degradation product of extracellular ATP), can be oncogenic, supporting tumour growth, angiogenesis and immune-escape [65]. Various data also support the idea apoptosis can promote tumourigenesis through the recruitment and activation of phagocytic macrophages at the tumour site. For example, different studies have found high levels of apoptosis or macrophage infiltration associate with poor prognosis for cancer patients [28-31, 66].

Is there a relationship between tumour-associated macrophages (TAM), apoptosis and cancer progression? Similar to wound-healing

macrophages, TAM promote tissue-remodeling and angiogenesis while exerting anti-inflammatory effects. In doing so, TAM promote cancer progression [67]. Because apoptotic cells are in close proximity with TAM in the tumoural milieu, a possible functional interconnection is possible. Direct support for a causal effect has come from recent research demonstrating that inhibition of mitochondrial apoptosis in a mouse model of B-cell lymphoma impairs angiogenesis and tumour growth [68]. This implicates non-autonomous effects of apoptosis in the promotion of tumour cell growth (Figure 2). The pro-oncogenic effect of apoptotic cells also extended to different other cancer types since altering apoptotic tumour cell level in transplantable mouse models of λ-MYC B lymphoma and melanoma also affected tumourigenesis; removal of apoptotic cells from the transplant inoculum delayed tumourigenesis whereas addition of apoptotic cells had the opposite effect, enhancing tumourigenesis. Importantly, TAM infiltration within tumours directly correlated with levels of tumour cell apoptosis - in apoptosis proficient tumours TAM infiltration was much higher than in apoptosis deficient settings. These results, together with transcriptomic and *in vitro* co-culture experiments led the authors to propose a model whereby tumour cell apoptosis reprogram TAM towards a wound-healing type response that fuels tumourigenesis [68]. This study provides strong experimental evidence that apoptotic cells can promote tumourigenesis. Clearly many questions remain, not least in understanding how apoptotic tumour cells promote TAM activation.

Besides promoting tumour growth, apoptotic cells may also facilitate metastatic tumour progression [69]. It is well known that pregnancy reduces the overall risk of developing breast cancer; however when breast cancer is diagnosed in postpartum women it is often more aggressive and has a poorer prognosis compared to disease diagnosed in nulliparous women [70]. Potentially contributing to this, postpartum involution is characterised by massive cell death leading to extensive efferocytosis (clearance of dead cells). Implicating the clearance of apoptotic cells in cancer progression, a recent study in an MMTV-PyMT mouse model of breast cancer found that a high level of efferocytosis in parous animals promoted TAM infiltration,

stimulation of a wound-healing cytokine response and increased metastasis [69].

The enemy within: oncogenic effects of apoptosis signaling at the cell-intrinsic level

Here we address oncogenic effects of engaging apoptosis at the single cell level. The only way in which apoptosis can exert oncogenic potential in a cell intrinsic manner is if the cell in question can survive apoptosis signalling. Following our discussion of this, we present an overview of emerging evidence, which argues that proteins, generally considered apoptotic, can exert oncogenic functions through non-apoptotic mechanisms.

Failed apoptosis and cancer

Recent data show that cells which initiate apoptosis but do not die –we term this failed-apoptosis– sustain potentially oncogenic damage such as genomic instability and gene amplification (Figure 3) [71-73]. The failure to commit suicide is puzzling given what we know about apoptosis. First, activation of caspases can unleash a variety of feed-forward mechanisms to fully engage apoptosis [10, 11]. Secondly, live-cell imaging studies have shown that during apoptosis, MOMP itself is an explosive event that occurs in all mitochondria over a ten-minute window [74, 75]. Finally, the level of caspase activity that is required to kill a cell does not appear to be very high [76]. These points notwithstanding, various studies have found that cells can engage caspase activity and survive, sustaining damage in the process [71-73, 70]

Intense interest has surrounded the potential of TRAIL ligands as anti-cancer therapies because many tumour-types are selectively sensitive to TRAIL-mediated apoptosis [77]. One of the first studies that demonstrated engagement of caspases activity in the absence of cell death focused on TRAIL-induced apoptosis [78]. The authors found that under conditions where MOMP was inhibited and XIAP function was compromised (thereby facilitating executioner caspase activity) TRAIL treated cells could engage limited executioner caspase activation yet survive – an example of failed-apoptosis.

Supporting an oncogenic role for failed apoptosis following TRAIL-treatment, another study found that sub-lethal doses of TRAIL (and FAS ligand) led to caspase-dependent mutations and genomic instability in surviving cells [72]. Why might limited caspase activity prove oncogenic? The answer relates to specific caspase substrates; one caspase-dependent hallmark of apoptosis is extensive DNA fragmentation that is mediated by a protein called caspase-activated DNAse or CAD [79]. Under conditions of failed apoptosis, low-levels of caspases activity might trigger limited CAD activity leading to DNA damage. Indeed, glioma cells and mouse fibroblasts surviving TRAIL treatment engage DNA damage in a caspase and CAD-dependent manner [72] (Figure 3).

Most stimuli, including established anti-cancer therapies such as etoposide, paclitaxel or more recently developed BH3 mimetics, initiate apoptosis through the intrinsic pathway by engaging or sensitising to MOMP [80]. Based on live-cell imaging studies, the binary all-or-nothing nature of MOMP makes it difficult to envision how intrinsic stimuli could act in any other way but to kill a cell. However, several years ago we demonstrated that some mitochondria could resist MOMP, due to higher anti-apoptotic Bcl-2 protein expression, a situation we termed incomplete MOMP or iMOMP [81]. These findings revealed intracellular heterogeneity in the sensitivity of mitochondria to MOMP. Recently, we have found that different stresses applied at sub-lethal doses can engage MOMP in limited numbers mitochondria without killing the cell - we call this minority MOMP [71]. Importantly, minority MOMP triggers sub-lethal caspase activity (similar to TRAIL), causing DNA-damage and genome instability in a CAD-dependent manner (Figure 3). In such a manner, repeated engagement of sub-lethal stress was shown to promote transformation and tumourigenesis in a MOMP and caspase-dependent manner [71].

Other studies support oncogenic effects of failed apoptosis. For instance, mitotic arrest has been shown to trigger DNA damage in a MOMP and caspase-dependent manner [82, 83]. Moreover, transgenic expression of pro-apoptotic BAX has been found to promote lymphomagenesis - characterised by genomic instability- in a manner that is suppressed by co-

expressing anti-apoptotic BCL-2 [84]. Besides CAD, the mitochondrial nuclease Endo G has also been found to promote radiation-induced DNA damage and transformation [73]. Implicating a direct role for caspase-3, deletion of caspase-3 inhibited transformation following DNA-damage and reduced tumourigenesis in a chemically induced model of skin-cancer [73].

Is there any evidence that DNA damage induced by failed apoptosis actually promotes cancer? One possibility is the following. The mixed lineage leukaemia (*MLL*) gene encodes a histone methylating enzyme (KMT2A) that functions as an epigenetic regulator. The *MLL* locus is highly susceptible to breakage and rearrangement, this can generate oncogenic MLL fusion proteins which lack methyltransferase activity [85]. Rearrangements in *MLL* are recurrent oncogenic drivers in a variety of leukaemias including acute myeloid leukaemia (AML) and acute lymphoid leukaemia (ALL) [86]. Importantly from the perspective of this discussion, *MLL* is the most commonly re-arranged gene in therapy-related neoplasms including AML and MDS [86]. While *MLL* rearrangements are associated with topoisomerase II inhibitor treatment, several lines of evidence argue that this is not solely through inhibition of topoisomerase II function; in these settings apoptosis may play a role in generating MLL rearrangements. Indeed, a variety of studies have shown that inhibition of caspase or CAD function reduces the incidence of *MLL* rearrangements following topoisomerase II inhibitor treatment [87-89]. This suggests a model whereby failed apoptosis, by causing caspase and CAD-dependent breakpoints in the *MLL* gene, promotes oncogenic re-arrangements in surviving cells.

How else might failed apoptosis-induced DNA damage/genome-instability impact on cancer? In inflammation-associated cancers, while DNA-damage is required for tumourigenesis, often the cause of DNA-damage remains unclear [90]. Potentially, engagement of failed apoptosis could serve to induce DNA-damage under inflammatory conditions. Another possibility may be by promoting acquired resistance to apoptosis-inducing anti-cancer therapies. Supporting this idea, repeated culturing of cells in the BCL-2 targeting BH3 mimetic ABT-199, led to the development of acquired

resistance [91]. Surprisingly, resistance was found to be due to a mutation in BCL-2 that allowed it to maintain anti-apoptotic function but prevented its inhibition by ABT-199 [91]. DNA damage engaged by failed apoptosis may potentially have given rise to the acquisition of resistance in this case, although it also remains formally possible that a pre-existing clone harboring these BCL-2 mutations may have been selected for by drug treatment. Finally, it is also important to note that DNA damage engaged during failed apoptosis may also have protective, anti-cancer functions. For example, BH3-mimetic treatment can engage senescence - where cells irreversibly enter cell cycle arrest - in a caspase-dependent manner [92]. This and other studies, support a role for DNA damage induced by failed apoptosis in feeding forward to engage p53 transcriptional responses [82, 83, 92]. By doing-so, p53 can exert tumour suppressor function. Importantly, non-lethal functions for caspases have also been described in various physiological processes including differentiation, memory and neurite pruning [93]. However it is unknown whether caspase activity also engages DNA-damage during these processes and, if not, how cells would suppress these effects.

Non-canonical oncogenic roles for "apoptotic" proteins

Besides their role in engaging apoptosis, a variety of non-apoptotic roles have been ascribed to almost all proteins classically viewed as apoptotic [94-96]. Some of these non-apoptotic functions may be oncogenic. For brevity, we have restricted our discussion of non-apoptotic protein functions and oncogenesis to death-receptor signalling. Bcl-2 family proteins have been implicated in a variety of non-apoptotic functions that may also impinge on tumourigenesis. These include regulation of calcium homeostasis, metastasis and autophagy - although how they regulate the latter remains controversial [97-100].

Focusing upon the death-receptors TRAIL and FAS, our discussion highlights that any consideration of pro-oncogenic effects of apoptosis signaling must also take into account these non-canonical functions (Figure 3). As discussed, many tumour types are selectively sensitive to TRAIL-induced apoptosis due to selective expression (relative to many normal

tissues) of TRAIL-receptor [101]. While provides therapeutic opportunities, it suggests that cancer cells must gain some advantage from expressing TRAIL receptor. Addressing this, a recent study has shown that TRAIL receptor (TRAIL-R) signaling can promote cancer independent of its role in canonical apoptosis signaling [102]. Here the authors found *in vivo* that TRAIL-R signaling could promote a variety of effects including invasion, proliferation and migration independently of its apoptotic function but dependent upon PI3K signaling. In line with functional importance, prevention of TRAIL-R signaling (through deletion of TRAIL-R) reduces metastasis while increasing survival in a KRAS-driven mouse model of pancreatic adenocarcinoma [102]. In clinical support, high TRAIL receptor 2 expression correlates with reduced metastasis-free survival in human KRAS-driven cancers [102]. In other *in vitro* settings, TRAIL-R signaling has also been found to stimulate cell migration in a caspase-dependent manner [103]. In a process dependent on failed apoptosis, TRAIL-R signaling can lead to caspase-dependent cleavage of ROCK1 kinase, causing activation of Rho GTPase, membrane blebbing and cell migration [103, 104]. In sum, these findings suggest that inhibition of TRAIL-R signaling may provide therapeutic benefit in some settings whereas TRAIL-R agonists could engage unwanted side effects in specific tumour types.

Engagement of FAS receptor (also called CD95 or APO-1) triggers extrinsic apoptosis in a manner similar to TRAIL. Like TRAIL, a variety of non-apoptotic, pro-oncogenic functions have also been described for FAS signaling, including stimulation of proliferation and migration [105]. Increased levels of FAS ligand are observed in a variety of cancers, and inhibition of FAS signalling (via deletion of FAS) inhibits tumourigenesis in different mouse tumour models [106]. Besides having pro-proliferative effects, FAS signaling can also exert pro-survival functions. This paradoxical role has been revealed in various cell types, including cancer stem cells, where elimination of FAS signalling actually causes cell death [107, 108]. The mechanism(s) underlying this type of cell death, termed *d*eath *i*nduced by *C*D95R/L *e*limination (DICE), is unclear but does not appear to involve any known regulated pathway of cell death [108].

Modeling oncogenic effects of apoptosis

Mouse models have proven invaluable in helping define the role of apoptosis in tumour suppression, anti-cancer therapy and as a *bone fide* therapeutic target [19, 20, 109-111]. Investigating the pro-oncogenic potential of apoptosis, we propose that mouse models should be extended to address the following interrelated questions: 1) can apoptosis exert a pro-oncogenic function during tumourigenesis ? 2) do we observe correlations between sub-lethal caspase activation, tumourigenesis and treatment responses?

I order to ask if apoptosis can exert tumour promoting functions, given that apoptosis has clear tumour suppressor functions, a key challenge will be to develop models that permit temporal inhibition or induction of apoptosis during cancer progression. Nevertheless, inducible systems based around tamoxifen/ER type control methods or degron-based approaches should facilitate this approach [112, 113]. In a complementary approach, experimentally engaging different levels of tumour cell apoptosis *in vivo* would directly allow one to test the effects of tumour cell apoptosis on tumour progression. To this end, we have developed a method called mito-priming, which should prove useful [114].

In parallel, it will be necessary to develop ways to report processes such as failed apoptosis or sub-lethal caspases activation *in vivo* to

ascertain whether these correlate with tumour progression. Along these lines, fluorescent reporters have recently been developed that accurately detect caspase activity even at sub lethal levels [71, 115]. While apoptosis has been extensively imaged *in vitro* very few studies have extended these analyses to an *in vivo* setting [116-118]. Particularly with respect to a possible role for apoptotic/immune cell interactions in promoting cancer, we speculate that real-time intravital imaging of tumour cell death may provide compelling new insights into this process.

Targeting apoptosis - pushing over the edge and pulling back from the brink

How can we better target apoptosis by improving tumour cell killing while preventing unwanted off-target effects? One possibility might be to induce intrinsic apoptosis while inhibiting caspase activity (Figure 4). Although this may seem counter-intuitive, as previously discussed, inhibition of caspase function does not ultimately protect against cell death following MOMP [12]. Triggering apoptosis in the presence of caspase inhibitors may prevent against detrimental caspase-dependent effects. Supporting this idea, some studies have shown a positive effect of caspase-inhibition in combination with chemo- or radiotherapy [119, 120]. These potentiating effects may be due to various mechasnisms including inhibition of tumour vascularization and enhanced anti-tumour immunity [119, 120]. Nevertheless, inhibition of caspase function may have untoward and potentially unwanted effects. For example, MOMP in the absence of caspase activity can engage interferon responses dependent upon activation of the STING pathway [121, 122]. The role of STING-dependent interferon signaling in cancer is complex such that it can exert both pro- and anti-tumourigenic effects [123, 124]. Moreover, caspase inhibitors can completely block extrinsic apoptosis and are thereby unsuitable when this is

expected to exert a therapeutic benefit. Another strategy might be to target specific, caspase-dependent effects, for example by blocking the activity of PGE_2 generated by apoptotic cells, using inhibitors such as celecoxib [43].

The overall balance between cell proliferation and death dictates whether a tumour grows or regresses. Besides tumour-intrinsic mechanisms (e.g. apoptotic sensitivity) affecting this, cell death triggered by anti-tumour immunity also plays an important role in regulating this balance [125]. It is now widely accepted that immune cells can be both anti- and pro-tumourigenic [125]. First, they can prevent cancer by eliminating pre-cancerous lesions (immune surveillance) and increased tumour infiltration by T and NK cells correlates with improved prognosis for a number of malignancies [126]. Secondly, the same immune response can also promote cancer growth [126]. Regarding the later effect, tumour cell death can activate macrophages towards a tumour promoting state. Understanding how this occurs should reveal possible ways to block it. Additional approaches might be combining apoptosis-inducing anti-cancer therapies with modulators of macrophage (TAM) signaling. For example, inhibition of CSF-1 signaling (the main trophic support factor for macrophages) has been shown to have multiple beneficial effects by either depleting TAM or switching them towards a more tumour suppressor mode [127, 128].

In cancer cells that have engaged caspase activity but survived, how do we push them over the edge and kill them? One avenue may be by antagonising endogenous caspase inhibitors such as XIAP using inhibitors called SMAC mimetics [129] (Figure 4). However, there are also various other XIAP-independent mechanisms whereby caspase activity can be restrained [9, 130]. Another possibility could be to enhance caspase cleavage of specific substrates, thereby triggering death. A prime candidate might be BID, of which minimal cleavage is required to kill a cell [131]. A potentially targetable possibility is using inhibitors for the kinase(s) that phosphorylate BID and inhibit its caspase-mediated activation [132, 133]. Finally, cancer cells undergoing sub-lethal caspase activity may be more sensitive than healthy tissue to

additional caspase activity providing a therapeutic window for direct small-molecule caspase activators [134].

Outlook

Clearly, apoptosis has abundant beneficial effects in restraining and treating cancer - the progression of BH3 mimetics to late-stage clinical trial attests to this. Nevertheless, as we have discussed, apoptosis is responsible for a variety of effects that may be tumour promoting. As such, tumour progression may be governed not only by the balance between proliferation and cell death, but also by the balance between tumour suppressor and oncogenic functions of apoptotic signalling. Going forward, the relative importance of these oncogenic versus tumour suppressor effects should be defined *in vivo*. The importance of oncogenic apoptotic effects may also be tumour-stage or tumour-type specific; in this respect, studies investigating apoptotic levels and/or BCL-2 protein expression with prognostic outcome (Table 1) should help guide investigation to specific tumour types. Finally, the end-goal is to investigate clinical relevance of these effects in primary patient samples. Beyond defining this dark side of apoptosis, some of these effects also offer promising potential to improve cancer cell killing; for example, understanding how cancer cells tolerate failed apoptosis and survive could provide new strategies to subvert this process in order to kill them.

Besides apoptosis, it is likely that other forms of cell death similarly impact on cancer in a multi-faceted manner. For example, inflammation associated with non-regulated, necrotic cell death can have both tumour promoting and inhibitory effects [135, 136]. Taking all these considerations into account, we envision that an improved understanding of the role of apoptosis in cancer will allow us to fully harness its potential as a therapeutic target.

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Figure Legends

Figure 1. Extrinsic and intrinsic apoptotic signalling pathways

In the extrinsic apoptotic pathway, upon binding their cognate ligand, death receptors such as TRAIL and FAS activate initiator caspases-8 and -10 through dimerisation mediated by adaptor proteins like FADD. Active caspase-8/-10 then cleave and activate the effector caspases 3 and 7, leading to apoptosis. The intrinsic (or mitochondrial) pathway of apoptosis requires mitochondrial outer-membrane permeabilisation (MOMP). Bcl-2 family proteins regulate mitochondrial permeabilisation. Cell stresses engage BH3-only protein activation leading to BAX and BAK activity that triggers MOMP. Anti-apoptotic BCL-2 proteins counteract this. Following MOMP, mitochondrial intermembrane space proteins such as SMAC and cytochrome *c* are released into the cytosol. Cytochrome *c* interacts with APAF-1 triggering apoptosome assembly that activates caspase-9. Active caspase-9, in turn, activates caspase-3 and -7 leading to apoptosis. Mitochondrial release of SMAC facilitates apoptosis by blocking the caspase inhibitor XIAP. Caspase-8 cleavage of BH3-only protein BID allows crosstalk between the extrinsic and intrinsic apoptotic pathways.

Figure 2. Cell extrinsic pro-oncogenic effects of apoptotic cell death

A. During apoptosis, caspases cleave and activate iPLA2 leading to the generation of PGE_2. PGE_2 can have pleiotropic effects on surrounding cells including pro-proliferative and immuno-silencing effects. **B**. Apoptosis of healthy cells can provide a vacant niche into which aberrant cells can proliferate ultimately leading to cancer. **C**. Apoptotic cells release a variety of “eat me” and “find me” molecules (FKN, ATP, LTF) to signal their removal by phagocytes. These signals can have a variety of pro-tumourigenic effects including turning tumour-associated macrophages (TAM) towards a pro-oncogenic state. More precisely, TAM can stimulate angiogenesis, tumour cell motility and dissemination, prepare the pre-metastatic niche and silence immune surveillance by preventing NK and T cells to attack cancer cells.

Figure 3. Oncogenic effects of engaging sub-lethal apoptotic signalling

Death receptors such as FAS and TRAIL can have non-apoptotic signalling functions that include promotion of growth, invasion and survival - all of which support cancer. Death receptors can also engage caspase dependent CAD activation leading to DNA-damage and mutagenesis. Intrinsic apoptotic stimuli can engage limited mitochondrial permeabilisation (minority MOMP) that activates effector caspases to sub-lethal levels. The endonucleases CAD (or Caspase-activated DNase) and EndoG (released following MOMP) cleave the dsDNA in the internucleosomal linker causing oncogenic mutations.

Figure 4. Enhancing apoptosis while minimising damage

Possible enhancers of apoptosis are in boxed in red, inhibitors of unwanted effects are boxed in green. Given they do not prevent death following widespread apoptotic MOMP, caspase inhibitors could prevent unwanted effects of caspase activity such as DNA-damage and PGE_2 production. Inhibitors of PGE_2 activity such as celecoxib could be applied in combination with apoptosis-inducing cancer therapies. Similarly, inhibiting CSF-1 signaling may either deplete TAM or switch them to an anti-tumourigenic phenotype. Following failed-apoptosis enhancement of caspase activity using SMAC-mimetics (to antagonise XIAP) or other means may promote apoptosis. Likewise, direct, caspase-activating molecules may preferentially promote death in tumour cells that have undergone failed apoptosis that already have a low-level of caspase activity. Examples of therapeutic agents and their clinical trail status (source: clinicaltrials.gov) are also depicted in the figure.

Table 1. Correlation of high anti-apoptotic BCL-2 levels with good prognosis and high pro-apoptotic BAX levels with bad prognosis in various human cancers

Correlation of increased anti-apoptotic BCL-2 expression and good cancer prognosis		
Cancer type	**Comments**	**Reference**
Breast cancer	Increased BCL-2 expression (IHC) is significantly associated with better DFS and OS	Berardo et al. 1998 [137]
	Following chemotherapy, higher BCL-2 expression (IHC) correlates with better survival rate	Vargas-Roig et al. 2008 [138]
	Increased BCL-2 expression (IHC) correlates with improved overall survival	Neri et al. 2006 [139]
	High BCL-2 (IHC) predicts better survival for early-stage cancers	Dawson et al. 2010 [140]
Colorectal cancer	BCL-2 expression (IHC) correlates with improved survival in Duke's B colon carcinoma	Meterissian et al. 2001 [141]
	Increased BCL-2 expression (IHC) in the context of p53-deficiency correlates with improved survival	Watson et al. 2005 [142]
NSCLC	High BCL-2 expression (IHC) correlates with better survival in lung cancer patients with lymph node infiltration	Tomita et al. 2003 [143]
	High BCL-2 (IHC) expressers have higher median survival	Anagnostou et al. 2010 [144]
	BCL-2 expression (IHC) is associated with increased survival	Renouf et al. 2009 [145]
Mesothelioma	Patients with high BCL-2 expression (IHC) confers a survival advantage	Pillai et al. 2013 [146]
Correlation of increased pro-apoptoic BAX expression and poor cancer prognosis		
Cancer type	**Comments**	**Reference**
AML	BAX and BAD high mRNA levels correlate with decreased survival	Köhler et al. 2002 [147]
ALL	Increased BAX/BCL2 ratio (mRNA) in patients with high-risk of relapse such as chromosomal abnormalities	Kaparou et al. 2013 [148]
	High BAX protein expression correlates with increased risk of relapse	Hogarth et al. 1999 [149]
Non-Hodgkin's lymphoma	BAX expression is associated with short survival	Bairey et al. 1999 [150]

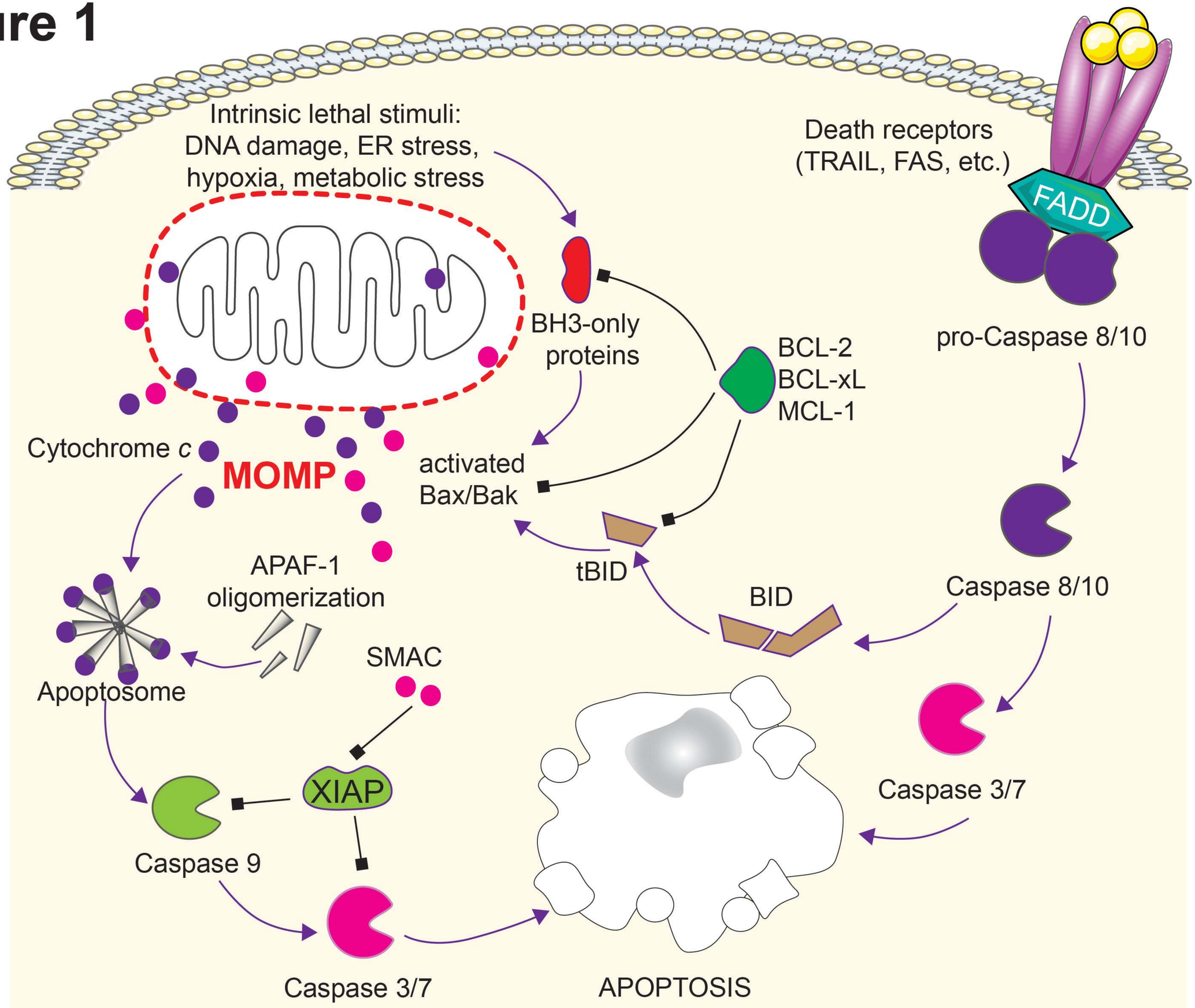
Author Biographies

Gabriel Ichim received his Ph.D. from Claude Bernard University in Lyon, France. From there, he continued his research as an EMBO Fellow in the lab

of Stephen Tait at the Beatson Institute where he characterized the oncogenic effects of failed apoptosis. He will establish his own group back in France in 2017.

Stephen Tait is a group leader at the Cancer Research UK Beatson Institute and Senior Lecturer, Institute of Cancer Sciences, University of Glasgow both in Glasgow, U.K. The Tait lab focuses on understanding mitochondrial regulation of cell death in the development and treatment of cancer.

Figure 1


Intrinsic lethal stimuli:
DNA damage, ER stress,
hypoxia, metabolic stress
Death receptors
(TRAIL, FAS, etc.)
FADD
pro-Caspase 8/10
BH3-only
proteins
BCL-2
BCL-xL
MCL-1
Cytochrome c
MOMP
activated
Bax/Bak
tBID
BID
Caspase 8/10
APAF-1
oligomerization
Apoptosome
SMAC
XIAP
Caspase 9
Caspase 3/7
Caspase 3/7
APOPTOSIS

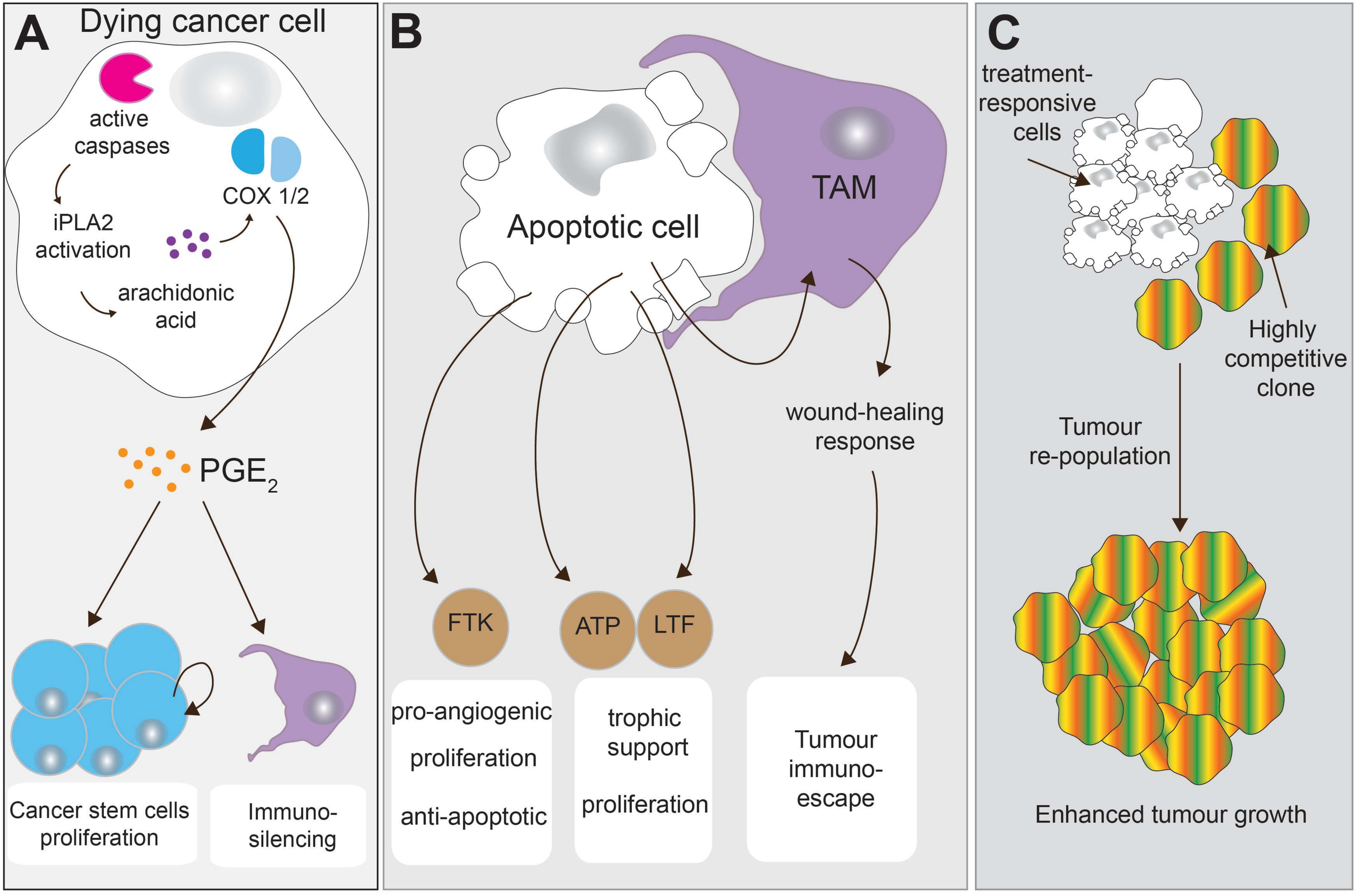

A
Dying cancer cell
active caspases
COX 1/2
iPLA2 activation
arachidonic acid
PGE2
Cancer stem cells proliferation
Immuno-silencing
B
Apoptotic cell
TAM
wound-healing response
FTK
ATP
LTF
pro-angiogenic
proliferation
anti-apoptotic
trophic support
proliferation
Tumour immuno-escape
C
treatment-responsive cells
Highly competitive clone
Tumour re-population
Enhanced tumour growth

Figure 2

Figure 3

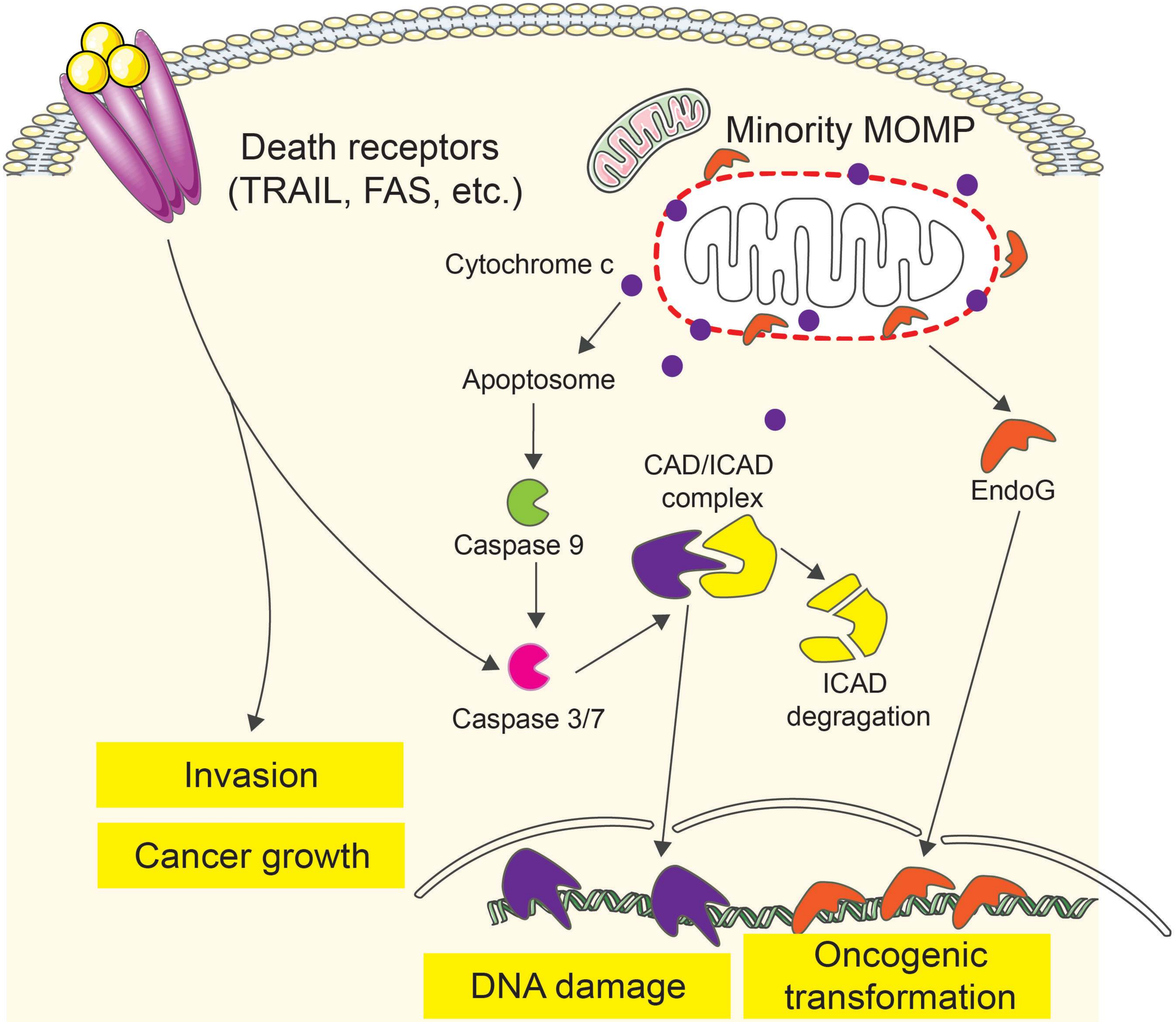
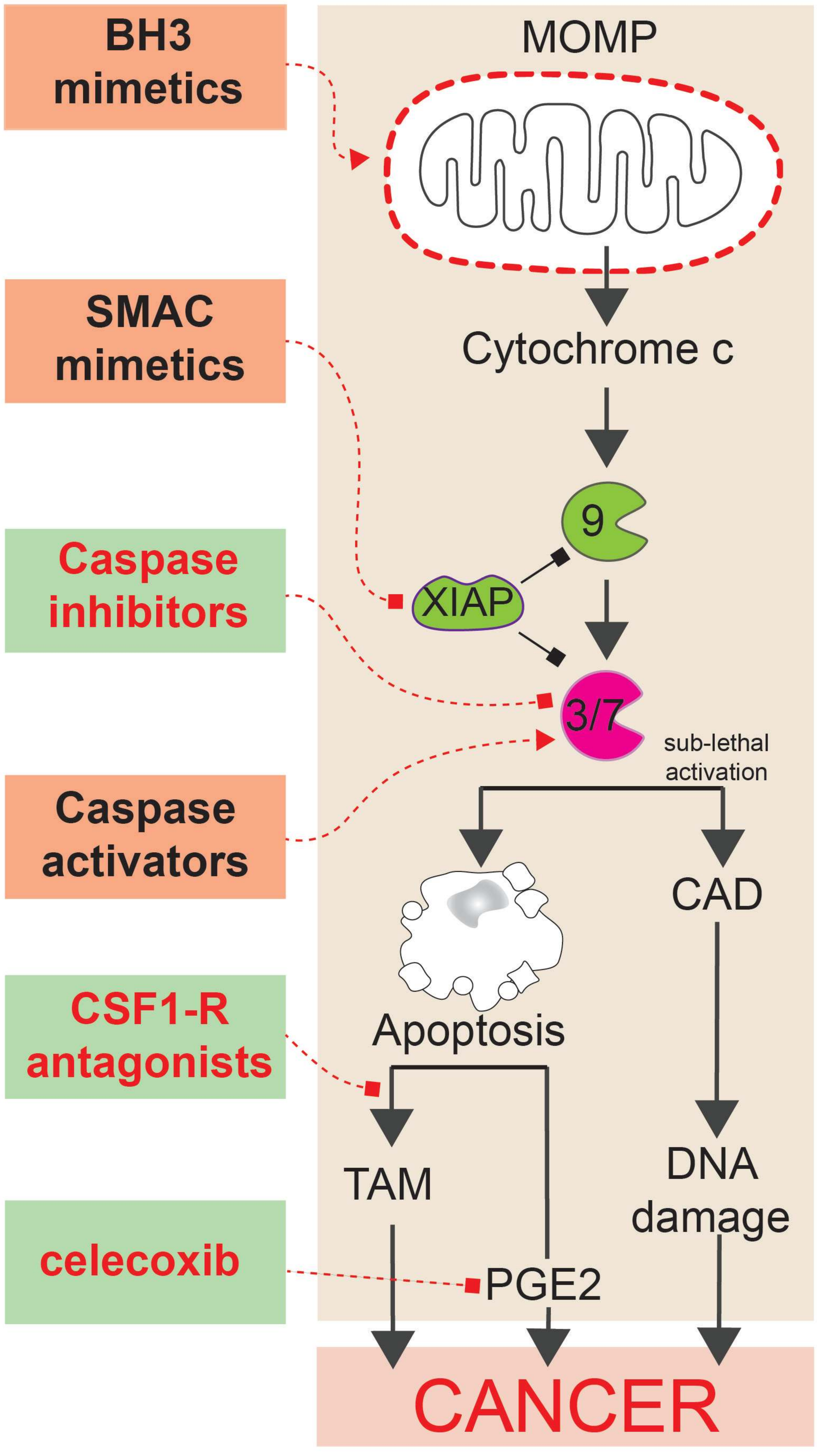

Death receptors
(TRAIL, FAS, etc.)
Minority MOMP
Cytochrome c
Apoptosome
Caspase 9
CAD/ICAD
complex
EndoG
ICAD
degragation
Caspase 3/7
Invasion
Cancer growth
DNA damage
Oncogenic
transformation

Figure 4


BH3 mimetics
SMAC mimetics
Caspase inhibitors
Caspase activators
CSF1-R antagonists
celecoxib
MOMP
Cytochrome c
9
XIAP
3/7
sub-lethal activation
CAD
Apoptosis
TAM
PGE2
DNA damage
CANCER