

Cite this: *Integr. Biol.*, 2011, **3**, 279–296

www.rsc.org/ibiology

## REVIEW ARTICLE

## Apoptotic cell signaling in cancer progression and therapy†

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Received 23rd November 2010, Accepted 24th January 2011

DOI: 10.1039/c0ib00144a

Apoptosis is a tightly regulated cell suicide program that plays an essential role in the development and maintenance of tissue homeostasis by eliminating unnecessary or harmful cells. Impairment of this native defense mechanism promotes aberrant cellular proliferation and the accumulation of genetic defects, ultimately resulting in tumorigenesis, and frequently confers drug resistance to cancer cells. The regulation of apoptosis at several levels is essential to maintain the delicate balance between cellular survival and death signaling that is required to prevent disease. Complex networks of signaling pathways act to promote or inhibit apoptosis in response to various cues. Apoptosis can be triggered by signals from within the cell, such as genotoxic stress, or by extrinsic signals, such as the binding of ligands to cell surface death receptors. Various upstream signaling pathways can modulate apoptosis by converging on, and thereby altering the activity of, common central control points within the apoptotic signaling pathways, which involve the BCL-2 family proteins, inhibitor of apoptosis (IAP) proteins, and FLICE-inhibitory protein (c-FLIP). This review highlights the role of these fundamental regulators of apoptosis in the context of both normal apoptotic signaling mechanisms and dysregulated apoptotic pathways that can render cancer cells resistant to cell death. In addition, therapeutic strategies aimed at modulating the activity of BCL-2 family proteins, IAPs, and c-FLIP for the targeted induction of apoptosis are briefly discussed.

## Introduction

Apoptosis is a tightly regulated form of cell death that plays a critical role in normal development and tissue homeostasis by eliminating unnecessary and unwanted cells.<sup>1,2</sup> Proper apoptotic signaling is vitally important in maintaining a healthy balance between cell survival and cell death and in safeguarding the integrity of the genome, as highlighted by the establishment of the evasion of apoptosis as a prominent hallmark of cancer.<sup>3</sup> In response to severe DNA damage, such

as that found in precancerous lesions, induction of apoptosis *via* activation of the DNA damage checkpoint pathway can serve to remove potentially harmful DNA-damaged cells and thereby block cancer development.<sup>4,5</sup> In line with apoptosis acting as a barrier to tumorigenesis, cancer cells typically harbor alterations that result in impaired apoptotic signaling, which facilitates tumor development and metastasis.<sup>3,6,7</sup>

Importantly, dysregulation of the apoptotic pathways can not only promote tumorigenesis, but can also render cancer cells resistant to conventional anti-cancer agents, since chemotherapy- and radiotherapy-induced killing of cancer cells is mainly mediated through activation of apoptosis.<sup>8–10</sup> Furthermore, TRAIL, a candidate for targeted therapy that has gained much attention for its ability to preferentially kill cancer cells, is frequently hampered by various mechanisms of resistance of cancer cells to apoptosis.<sup>11,12</sup> TRAIL is a cytokine that induces apoptosis by binding one of its cognate death

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† Published as part of an Integrative Biology themed issue in honour of Mina J. Bissell: Guest Editor Mary Helen Barcellos-Hoff.

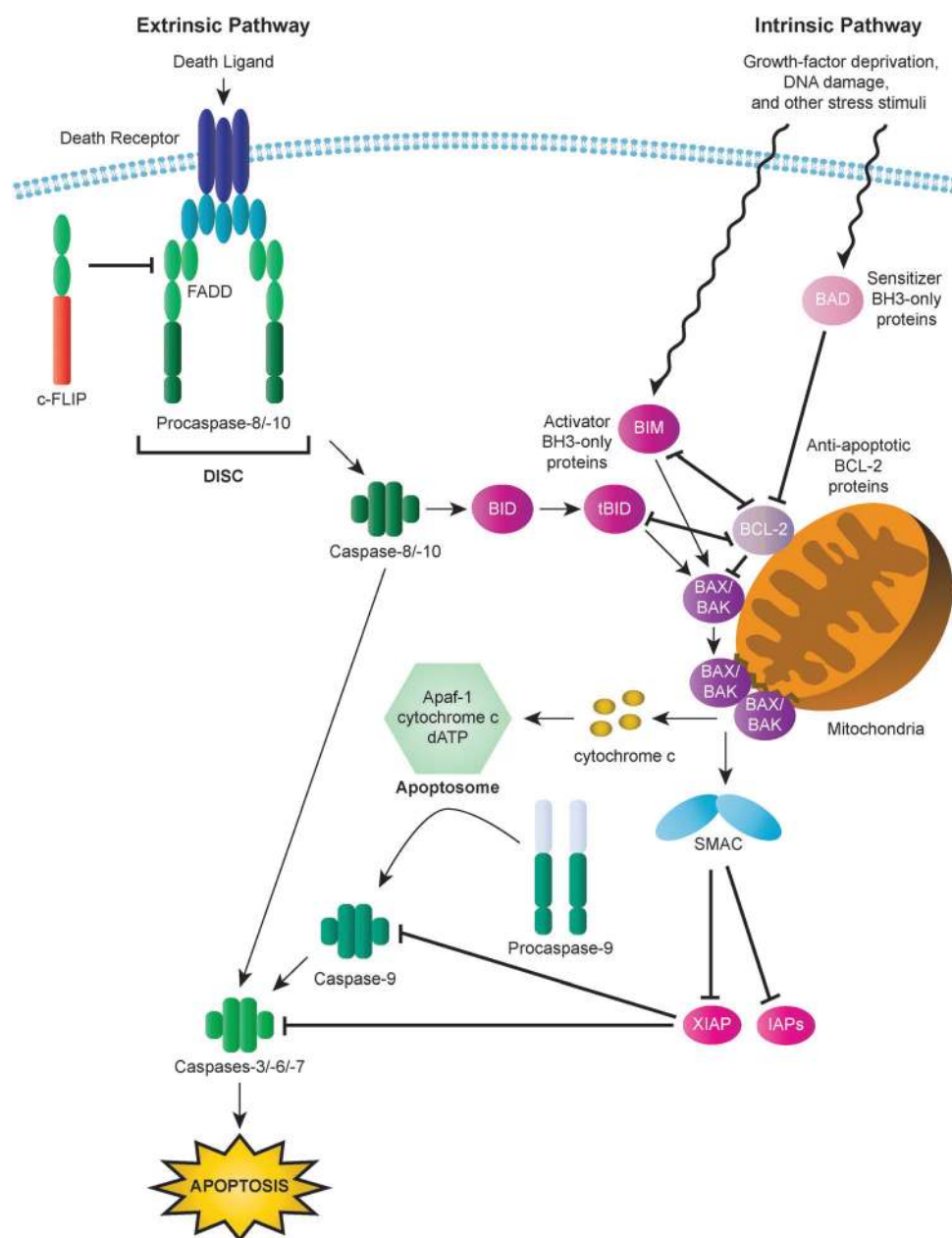
## Insight, innovation, integration

Apoptosis plays an essential role in the development and maintenance of tissue homeostasis and its deregulation results in a variety of diseases including tumorigenesis. Moreover, evasion of apoptosis is one of the hallmarks of cancer and also confers drug resistance to cancer cells. Here,

we will highlight the role and therapeutic values of various regulators of apoptosis. We will also briefly discuss the importance of preclinical models utilizing three dimensional systems and genetically engineered mouse models in validation of novel apoptosis-based cancer therapeutics.

receptors and is known to play an important role in immune surveillance against tumors.<sup>11–13</sup> Nevertheless, in many types of cancer cells, effective anti-tumor activity of TRAIL requires the suppression of aberrantly expressed negative regulators of

apoptosis.<sup>11,12</sup> To enhance cancer cell sensitivity to apoptosis, and thus overcome treatment failure, therapeutic strategies targeting molecules implicated in apoptosis resistance have been developed.<sup>8–10</sup>



**Fig. 1** Intrinsic and extrinsic apoptotic pathways. In the intrinsic (mitochondrial) pathway, mitochondrial outer membrane permeabilization (MOMP) results in the release of cytochrome *c* and other apoptogenic factors from mitochondria into the cytosol and the ensuing formation of the apoptosome, which triggers activation of the apoptosis-inducing caspase cascade *via* activation of caspase-9. Interactions among BCL-2 family proteins play a critical role in the mediating MOMP induction and consequent apoptosis. BH3-only proteins (activator BH3-only proteins are represented here by BIM and tBID and sensitizer BH3-only proteins are represented by BAD) can relay apoptotic signals to the mitochondria through activation of BAX or BAK, the principal effectors of the intrinsic apoptotic pathway. In contrast, anti-apoptotic BCL-2 proteins (represented by BCL-2) serve to inhibit apoptosis by blocking BAX/BAK activation. In the extrinsic (death receptor) pathway, binding of death receptors such as FAS or TRAIL receptors (TRAILR1, TRAILR2) by their cognate ligands triggers the recruitment of death domain (DD)-containing adaptor proteins (represented by FADD) and procaspases with a death effector domain (DED), specifically procaspase-8 and procaspase-10. The resulting complex is known as the death inducing signaling complex (DISC). High levels of active caspase-8 generated by large amounts of procaspase-8 processing at the DISC lead to the activation of executioner caspases, including caspase-3, and the induction of apoptosis. Activation of caspase-8 can also result in the cleavage of the BH3-only protein BID to generate the activated BID fragment tBID, which serves to transmit the death signal from the extrinsic to the intrinsic signaling pathway.

As regulation of apoptosis relies on multiple cell signaling mechanisms, cancer cells can employ a number of different strategies to suppress a protective apoptotic response.<sup>3,6,7</sup> Numerous signaling pathways, including survival signaling pathways<sup>14,15</sup> and stress-induced signaling pathways,<sup>16,17</sup> are linked to the apoptotic machinery, directly modulating components of the apoptotic machinery itself or key regulatory molecules within the core apoptotic signaling pathways. Notably, various upstream signaling pathways often impinge on the same few central apoptosis control points, which involve the critical apoptosis regulators in the BCL-2 family of proteins; inhibitor of apoptosis (IAP) proteins; and FLICE-inhibitory protein (c-FLIP).<sup>6,7,10,18,19</sup> In this review, we provide an overview of mechanisms by which these regulatory molecules govern apoptosis in normal cells and describe modes of apoptotic dysregulation based on alterations in their function that facilitate the evasion of apoptosis in cancer cells. We will also briefly discuss the development of some promising cancer treatment strategies aimed at restoring the apoptotic response.

## Caspases: the central effectors of apoptosis

Apoptosis is primarily executed by a family of proteases known as the caspases (cysteinyll, aspartate-specific proteases).<sup>20</sup> As fundamental players in death-inducing signaling pathways, the regulation of caspase activation must be subject to various layers of control to ensure apoptotic cell death is triggered only under appropriate conditions. Accordingly, caspases are synthesized in the cell as inactive zymogens with an N-terminal prodomain in addition to a p20 large subunit and a p10 small subunit that make up the catalytic domain.<sup>20,21</sup> In response to an apoptosis-inducing signal, the initiator caspases are recruited to oligomeric complexes by activating adaptor proteins; the consequent induced proximity of two caspase monomers then promotes dimerization and subsequent activation.<sup>21,22</sup> Activated initiator caspases, in turn, activate a second class of caspases, the executioner or effector caspases with short prodomains that occur as dimers, by proteolytic cleavage of the linker separating the two subunits of the catalytic domain.<sup>21</sup>

While the identification of apoptotic cells cannot be based on caspase activation alone,<sup>23</sup> and several studies have shown that caspases can be involved in biological processes other than cell death, including cell proliferation and differentiation,<sup>20,23</sup> the importance of caspases in executing the classical apoptotic cell death programs has been firmly established.<sup>24</sup> Once activated, caspases can dismantle the cell by cleaving a number of key proteins. Indeed, caspase activity may be required to induce the biochemical and morphological changes specific to apoptotic cells.<sup>25</sup> These characteristic alterations include blebbing of the plasma membrane, exposure of phosphatidylserine at the external surface of the cell membrane, shrinkage of the cytoplasm, chromatin condensation, and DNA fragmentation.<sup>25,26</sup> The morphological transformation of the apoptotic cell concludes with the formation of apoptotic bodies that are recognized and eliminated by specialized phagocytes and neighboring cells *via* phagocytosis.<sup>26,27</sup>

Significant advances toward the identification of the full set of caspase substrates, driven by the creation of a compiled list of reported caspase substrates (accessible *via* the CASBAH online database <http://bioinf.gen.tcd.ie/casbah/>)<sup>25</sup> as well as large-scale proteomic studies of proteolytic events following the induction of apoptosis,<sup>28</sup> have revealed that hundreds of proteins can be subject to caspase-mediated cleavage. Given the multitude of identified caspase substrates, the true challenge has become the ability to distinguish those cleavage events that are important for the apoptosis process and the accompanying biochemical and morphological changes from the cleavage of “innocent bystander” caspase substrates.<sup>25</sup> Evidence is lacking as to the functional importance of most of the hundreds of identified caspase substrates, with the cleavage of only a small number of these caspase substrates linked to the signature alterations of apoptotic cells.<sup>25</sup>

The irreversible and extensive proteolysis resulting from the activation of the executioner caspases, and the consequent induction of cell death, can be triggered by death receptor- or mitochondria-dependent apoptotic pathways, known as the intrinsic and extrinsic apoptotic pathways, respectively (Fig. 1).<sup>29</sup>

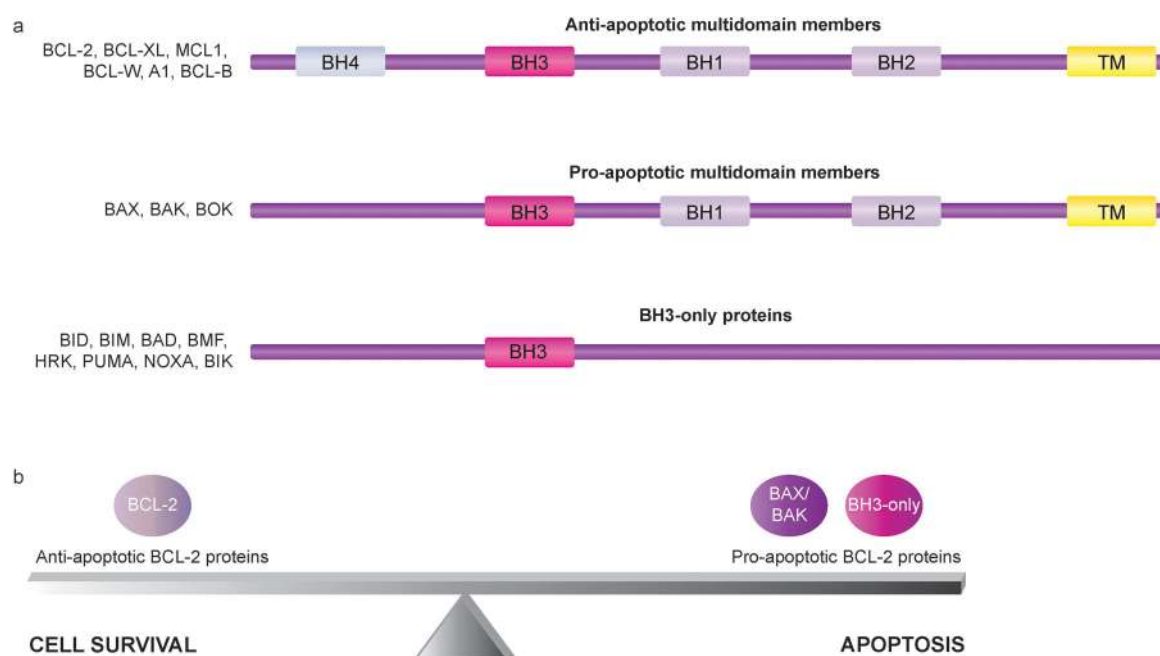
## The intrinsic apoptotic pathway

### Overview of the intrinsic apoptotic pathway

The intrinsic apoptotic pathway can be activated by a range of stress stimuli, including UV radiation, gamma irradiation, heat, viral virulence factors, growth-factor deprivation, most DNA-damaging agents, and the activation of some oncogenic factors (Fig. 1). These diverse stressors are recognized by multiple intracellular components that relay the signal to the mitochondria, resulting in mitochondrial outer membrane permeabilization (MOMP).<sup>26,30–32</sup> MOMP prompts various proteins, which are normally confined to mitochondrial intermembrane space (IMS), to diffuse into the cytosol. Among the proteins that escape from the IMS, a set of proteins, known as apoptogenic factors, play a vital role in the mitochondria-dependent initiation of the death-inducing caspase cascade.<sup>33</sup> In the cytosol, the apoptogenic factor cytochrome c binds to apoptosis protease activating factor-1 (Apaf-1) in a dATP-dependent manner to drive the formation of a complex known as the apoptosome that recruits procaspase-9, thus mediating the oligomerization and activation of caspase-9.<sup>34,35</sup> Activation of caspase-9 leads to the processing and activation of executioner caspases-3, -6 and -7 which bring about the execution of the cell.<sup>36</sup>

### The BCL-2 protein family

Molecules responsible for control of MOMP induction are central regulators of apoptosis, as MOMP is regarded as the critical event in the mitochondria-mediated apoptotic pathway that commits the cell to apoptosis.<sup>30</sup> Accordingly, the BCL-2 family of proteins serves as an “apoptotic switch” by mediating permeabilization of the mitochondrial membrane.<sup>37,38</sup> The BCL-2 family includes both pro- and anti-apoptotic regulators of the intrinsic apoptotic pathway. Each BCL-2 family member contains at least one of four conserved domains,



**Fig. 2** Subgroups of BCL-2 family members with representative members of each subfamily. (a) BCL-2 family members can be classified into three subgroups according to function and BH domain composition. All BCL-2 family members possess at least one of four BCL-2 homology (BH) domains, termed BH1, BH2, BH3, and BH4, and many also include a transmembrane (TM) domain. The anti-apoptotic multidomain members have three to four BH domains, with some members lacking a BH4 domain. Similar to the anti-apoptotic multidomain members, the pro-apoptotic multidomain members contain BH1, BH2, and BH3 domains. The BH3-only proteins are a subset of pro-apoptotic proteins that only bear a single BH motif, the BH3 domain. Some BH3-only proteins also include a TM domain. (b) BCL-2 proteins play a key role in mediating the delicate balance between cell survival and cell death. Disruption of this balance by cellular alterations that increase the functional activity of anti-apoptotic BCL-2 proteins relative to pro-apoptotic BCL-2 proteins can enable the evasion of apoptosis, which tips the balance to favor cell survival and thus promotes the development and progression of cancer.

known as BCL-2 homology (BH) domains (BH1–4). BCL-2 family members are broadly classified into three subgroups, one anti-apoptotic and two pro-apoptotic, based on function and BH domain composition (Fig. 2a).<sup>37,39,40</sup> Anti-apoptotic multidomain members, including BCL-2, BCL-XL, MCL1, BCL-W, A1, and BCL-B, contain three to four BH domains. Pro-apoptotic multidomain members, represented by BAX, BAK, and BOK, share substantial sequence similarities with anti-apoptotic BCL-2 proteins, as family members in both multidomain subgroups contain BH1, BH2, and BH3 domains.<sup>39,41</sup> BH3-only proteins, the third class of BCL-2 family members, are a subset of pro-apoptotic proteins that, as indicated by their name, only share strong sequence similarity with one another and multidomain BCL-2 proteins in a single region, the BH3 domain.<sup>37–39</sup> BH3-only family members include BID, BIM, BAD, BMF, HRK, PUMA, NOXA, and BIK.<sup>37,39</sup>

The anti- and pro-apoptotic multidomain BCL-2 proteins, considered to be the core BCL-2 family members, have predicted secondary structures or experimentally determined 3D structures resembling one another, whereas BH3-only proteins, with the exception of BID, appear to lack structural similarities to the core BCL-2 family members.<sup>41</sup> Interestingly, structural analyses of seven core BCL-2 proteins, namely BCL-XL, BCL-2, BCL-W, MCL1, BAX, BAK, as well as the BH3-only protein BID, have revealed striking similar helical bundle structures, with no discernible difference

between anti- and pro-apoptotic BCL-2 family members.<sup>41</sup> The BH1, BH2, and BH3 domains of core multidomain BCL-2 proteins form a hydrophobic groove at the surface of the protein that has been shown to bind peptides corresponding to the alpha-helical BH3 domains of BH3-only proteins and the pro-apoptotic multidomain BCL-2 protein BAK.<sup>41,42</sup> Previous studies have firmly established that the heterodimerization of anti-apoptotic multidomain BCL-2 proteins and BH3-only proteins occurs *via* BH3-groove interactions,<sup>42,43</sup> and more recent studies have indicated that the formation of homodimers of BAX or BAK also involves BH3-groove interactions.<sup>43,44</sup> Importantly, protein–protein interactions among BCL-2 family members act to either prevent or promote apoptosis, and thus the BH3-groove mode of interaction plays a key role in the mechanisms by which BCL-2 proteins regulate apoptosis.<sup>43</sup>

While a predicted C-terminal transmembrane (TM) domain is common to most multidomain BCL-2 proteins, the subcellular localization of these proteins varies in healthy cells. For example, with regard to the well-studied pro-apoptotic multidomain BCL-2 proteins BAX and BAK, BAX is predominantly cytosolic and translocates to the mitochondria during apoptosis induction, whereas BAK is an integral membrane protein localized to the mitochondria and endoplasmic reticulum (ER).<sup>41</sup> Differences in the subcellular localization of anti-apoptotic multidomain BCL-2 proteins also exist, as constitutively membrane-bound BCL-2, with



the C-terminal tail anchored in the membrane and the remaining residues residing in the cytosol, is located at the mitochondria and ER, yet BCL-XL, BCL-W, and MCL1 are not tightly associated with membranes and thus can be located, at least in part, in the cytosol in healthy cells and translocate to the mitochondria during apoptosis.<sup>41</sup> In the cytosolic forms of BAX, BCL-XL, BCL-W, and MCL1, the characteristic hydrophobic pocket of multidomain BCL-2 proteins that can also bind BH3 domains is occupied by the C-terminal membrane-anchoring tail.<sup>42,45</sup> Mitochondrial translocation of these multidomain BCL-2 proteins involves disengagement of the C-terminal TM domain from the BH3 binding pocket and its insertion into the mitochondrial membrane.<sup>41,44,46</sup>

### BAX and BAK activation

In response to diverse cellular stressors, BH3-only proteins fulfil their role as the most apical regulators of the intrinsic apoptotic pathway by triggering the activation of BAX-like proteins.<sup>37–39</sup> The essential role of BAX-like proteins positioned downstream of BH3-only proteins in mitochondria-mediated apoptotic signaling has been established by studies that have shown that BH3-only proteins cannot induce apoptosis in cells deficient in both BAX and BAK.<sup>37–39</sup> The activation of BAX and BAK involves conformational changes that enable self-association, likely through formation of homodimers followed by generation of higher order oligomers, thereby leading to homo-oligomerization and induction of MOMP.<sup>44,47,48</sup> Despite the undisputed importance of BAX and BAK function in MOMP induction and consequent release of apoptogenic factors, the mechanism by which BAX and BAK are activated has been a matter of contention over the past several years. Previous studies have resulted in different conclusions as to whether a subset of BH3-only proteins can trigger apoptosis by directly binding to BAX/BAK and thus have led to the proposal of two different models of BAX/BAK activation, the indirect activation model and the direct activation model.<sup>37,39</sup>

The indirect activation model, also referred to as the displacement model, maintains that anti-apoptotic BCL-2 family members act primarily to bind and restrain BAX/BAK in inactive heterooligomeric complexes, while BH3-only proteins serve to neutralize anti-apoptotic BCL-2 proteins and thus can initiate apoptosis without directly interacting with BAX or BAK. In this scenario, BH3-only proteins bind to anti-apoptotic BCL-2 family members to allow the release of BAX/BAK, resulting in BAX/BAK homo-oligomerization and the consequent induction of MOMP.<sup>37,39,49</sup> According to the indirect model, this displacement mechanism is the principal means by which BH3-only proteins stimulate apoptosis, and the robust pro-apoptotic activity of BIM and BID can be attributed to their ability to strongly bind all anti-apoptotic BCL-2 proteins, whereas the other BH3-only proteins only engage subsets.<sup>39,49</sup>

The direct activation model contends that certain BH3-only proteins, termed activators, can directly bind and activate BAX/BAK. Activator BH3-only proteins (activator BH3s) include BIM, tBID (the activated, truncated form of BID), and perhaps others such as PUMA<sup>50–54</sup> (Fig. 1). In this model, promotion of cell survival by anti-apoptotic BCL-2 proteins

involves sequestering activator BH3s in addition to binding any activated BAX/BAK protein monomers that may exist.<sup>37,54</sup> BH3-only proteins that are not activators are categorized as sensitizers. The sensitizer BH3-only proteins are unable to directly interact with BAX/BAK, but instead bind to anti-apoptotic BCL-2 proteins (Fig. 1), freeing activator BH3s for binding to and activation of BAX/BAK.<sup>37,54</sup>

In recent years, compelling experimental evidence of BH3-only proteins directly interacting with BAX and consequent induction of BAX activation has been published.<sup>44,50,51</sup> For example, the NMR structure of a stabilized  $\alpha$ -helix of the BCL-2 domain (SAHB) peptide of the BH3 domain of BIM in complex with full-length BAX has been reported and binding of the BH3 ligand BIM SAHB was shown to trigger BAX activation.<sup>50</sup> Interestingly, the BIM SAHB-BAX interaction site defined by NMR analysis involves the two helices  $\alpha$ 1 and  $\alpha$ 6 of BAX and is located on the completely opposite face of the protein from the canonical BH3-groove interaction site characteristic of anti-apoptotic BCL-2 proteins.<sup>50</sup> Studies using Fluorescence (or Förster) Resonance Energy Transfer (FRET) analyses and liposomes to demonstrate the ordered series of events leading to tBid-induced oligomerization of BAX and subsequent membrane permeabilization have added further support for the direct activation of BAX by activator BH3s.<sup>51</sup> These studies also attest to the importance of the lipid membrane in BAX activation; the reported sequential steps for tBid-induced BAX-mediated MOMP entail protein–membrane interactions as well as protein–protein interactions, including the direct interaction between tBid and BAX, which occur primarily on or in the lipid membrane.<sup>51</sup> Previous evidence of the lipid membrane as a critical player in the regulation of membrane permeabilization by BCL-2 proteins formed the basis of the “embedded together” model.<sup>55</sup> While aspects of both the indirect and direct activation models are incorporated in the “embedded together” model, the focus of the model is on the importance of the membrane in governing conformational changes of BCL-2 proteins and interactions between them that mediate MOMP.<sup>55</sup>

New insights into the molecular mechanism of BAX/BAK activation revealed by recent studies have provided the basis for a proposed model of stepwise activation of BAX and BAK by activator BH3s.<sup>44</sup> According to this model, BAX activation is initiated by transient binding of an activator BH3 to the  $\alpha$ 1 helix of BAX, which has previously been determined by NMR analysis to be a component of a noncanonical BH3 binding site on BAX.<sup>50</sup> In the inactive cytosolic form of BAX, the  $\alpha$ 1 helix maintains the engagement of the C-terminal  $\alpha$ 9 helix in the hydrophobic pocket, thereby preventing mitochondrial translocation of BAX. Interaction between an activator BH3 and the  $\alpha$ 1 helix of BAX results in conformational changes that involve exposure of the N-terminal  $\alpha$ 1 helix of BAX with consequent release of the C-terminal TM domain from the hydrophobic pocket and thus promotion of mitochondrial targeting of BAX.<sup>44</sup> Subsequent to mitochondrial translocation, the activator BH3 remains associated with BAX, though rearrangement of the interaction occurs and results in the association of the activator BH3 with the BH1 domain of BAX, which drives homo-oligomerization of BAX.<sup>44</sup> Given that BAK is an integral membrane protein with its N-terminal

$\alpha$ 1 helix constitutively exposed, activation of BAK bypasses the first activation step of mitochondrial targeting promoted by activator BH3-induced conformational changes. Nevertheless, akin to activation of mitochondrially translocated BAX, activator BH3s are critical for homo-oligomerization of BAK activation, and both BAX and BAK require BH1 and BH3 domains for homo-oligomerization.<sup>44</sup> Previously, a major role for activator BH3s in driving the activation of BAX/BAK *via* direct interactions has been challenged, with arguments against direct activation including the failure to detect stable interactions between BH3-only proteins and soluble BAX in its inactive monomeric form.<sup>49</sup> However, recent studies demonstrating the transient and dynamic nature of interactions between activator BH3s and BAX offer an explanation for the difficulty encountered in earlier studies in observing these interactions, and provide clear evidence validating direct activation of BAX/BAK by activator BH3s.<sup>44,50</sup>

## The extrinsic apoptotic pathway

### Overview of the extrinsic apoptotic pathway

Stimulation of the extrinsic apoptotic pathway is initiated by the association of cell surface death receptors, a subset of the tumor necrosis factor (TNF) receptor superfamily, with their respective activating cytokine ligands (also known as death ligands), which are members of the TNF family of proteins.<sup>13,26,56</sup> TNF family members are mostly synthesized as type-II transmembrane proteins that assemble into biologically active homotrimers.<sup>13,29</sup> Widely studied death receptor-ligand signaling systems include TNF receptor 1 (TNFR1)-TNF (also known as TNF- $\alpha$ ), FAS (APO-1, CD95)-FASL, TNF-related apoptosis inducing ligand (TRAIL) receptor 1 (TRAILR1, DR4)-TRAIL, and TRAIL receptor 2 (TRAIL-R2, DR5)-TRAIL.<sup>13,29</sup>

Signal transduction through death receptors entails receptor oligomerization involving aggregation of an intracellular motif common to the death receptor family members, known as the death domain (DD).<sup>13,57</sup> Trimerization of death receptors was initially thought to be prompted by ligand binding, but later studies have provided evidence in favor of the formation of preassembled receptor oligomers in a ligand-independent manner.<sup>13</sup> Ligand binding leads to the recruitment of DD-containing adaptor proteins, such as FAS-associated death domain protein (FADD) and TNFR1-associated death domain protein, to the aggregated receptor domains *via* DD–DD interactions.<sup>13,29</sup> These adaptor proteins include an additional homotypic protein interaction module, the death effector domain (DED), that sequesters the inactive zymogen of initiator caspases with a DED in their prodomain, namely caspases-8 and -10, to form the Death Inducing Signaling Complex (DISC) (Fig. 1).<sup>29</sup> DISC formation mediates the oligomerization and consequent activation of caspase-8.<sup>35</sup> Activation of the executioner caspases, including caspase-3, caspase-6, and caspase-7, by the activated upstream initiator caspases results in the cleavage of numerous caspase substrates and the subsequent demise of the cell.<sup>56,58,59</sup>

### Integration of the extrinsic and intrinsic apoptotic signaling pathways

In response to death receptor stimulation, the extent of caspase-8 activation at the DISC can be insufficient to elicit apoptosis and thus effective apoptotic signaling requires engagement of the mitochondria-mediated intrinsic pathway for full activation of executioner caspases, such as caspase-3, and consequent induction of apoptosis downstream of MOMP.<sup>29</sup> The cleavage and consequent activation of BID by caspase-8 to generate the activated BID fragment (tBID) serves as the conduit through which the apoptotic signal is relayed from the extrinsic to the intrinsic signaling pathway.<sup>60,61</sup> In this scenario, caspase-8 activity induced by death receptor-mediated signaling is adequate to cleave cytosolic, full-length BID to result in the production of tBID and its translocation to the mitochondria, which leads to the release of the apoptogenic factors (Fig. 1).<sup>26,29,60,61</sup> These pro-apoptotic molecules ultimately promote activation of executioner caspase-3 and subsequent activation of caspase-8, thereby generating a positive feedback loop to amplify the apoptotic response.<sup>29,62</sup>

The requirement of the intrinsic pathway to commit cells to death in response to FAS activation defines two types of cells, type I and type II. In type I cells, active caspase-8, generated by extensive processing of procaspase-8 at the DISC, directly activates the executioner caspases, including caspase-3, to carry out FAS-initiated apoptosis, whereas in type II cells a low concentration of active caspase-8 is generated at the DISC. Accordingly, although FAS-mediated death signaling triggers activation of the mitochondrial apoptotic pathway in both cell types, only type II cells depend on mitochondria to complete the apoptotic response induced by FAS activation.<sup>13,63</sup> This fundamental difference between cell types is evidenced by reports demonstrating that overexpression of BCL-2 or BCL-XL, anti-apoptotic BCL-2 family members which inhibit MOMP and cytochrome c release, blocks activation of caspase-8 and caspase-3 as well as FAS-mediated apoptosis in type II cells, but not type I cells.<sup>13,63</sup> Classification of cells based on the requirement of mitochondria for induction of apoptosis in response to death receptor signaling has been extended beyond FAS to other death receptors, including TRAIL death receptors.<sup>13,64</sup> Highlighting the important roles that BCL-2 proteins can play in mediating apoptosis, the rate of BID cleavage has been implicated as the determining factor in defining TRAIL-sensitive cells as mitochondria-dependent or independent.<sup>64</sup> Various other BCL-2 family members, both pro-apoptotic and anti-apoptotic, have been shown to promote or inhibit TRAIL-induced apoptosis, respectively, in a cell type-specific fashion.<sup>65</sup>

### Control points in the apoptotic pathways

#### Control point 1: apoptosis control *via* regulation of MOMP by BCL-2 family proteins

BH3-only proteins serve as sensitive detectors of apoptotic stimuli that transmit death signals to downstream BAX-like proteins, the principal effectors of the intrinsic apoptotic pathway, thereby promoting apoptosis.<sup>37–39</sup> The activation

of certain BH3-only family members can entail distinct transcriptional or post-translational modifications under specific conditions.<sup>39,41,54,66</sup> For example, the pro-apoptotic activity of BAD and BIM is controlled by phosphorylation, and as mentioned above, the full activity of BID is unleashed through proteolytic cleavage.<sup>67,68</sup> The relatively large number of BH3-only proteins, together with the unique activation mechanisms of particular BH3-only proteins, have been proposed to contribute to the ability of cells to decipher the signals from a wide range of apoptotic stimuli by enabling a specialized response to different death signals.<sup>39,66</sup> In addition, the concept that BH3-only proteins can act in a tissue- and/or signal-specific manner has been supported by genetic deletion studies.<sup>68</sup>

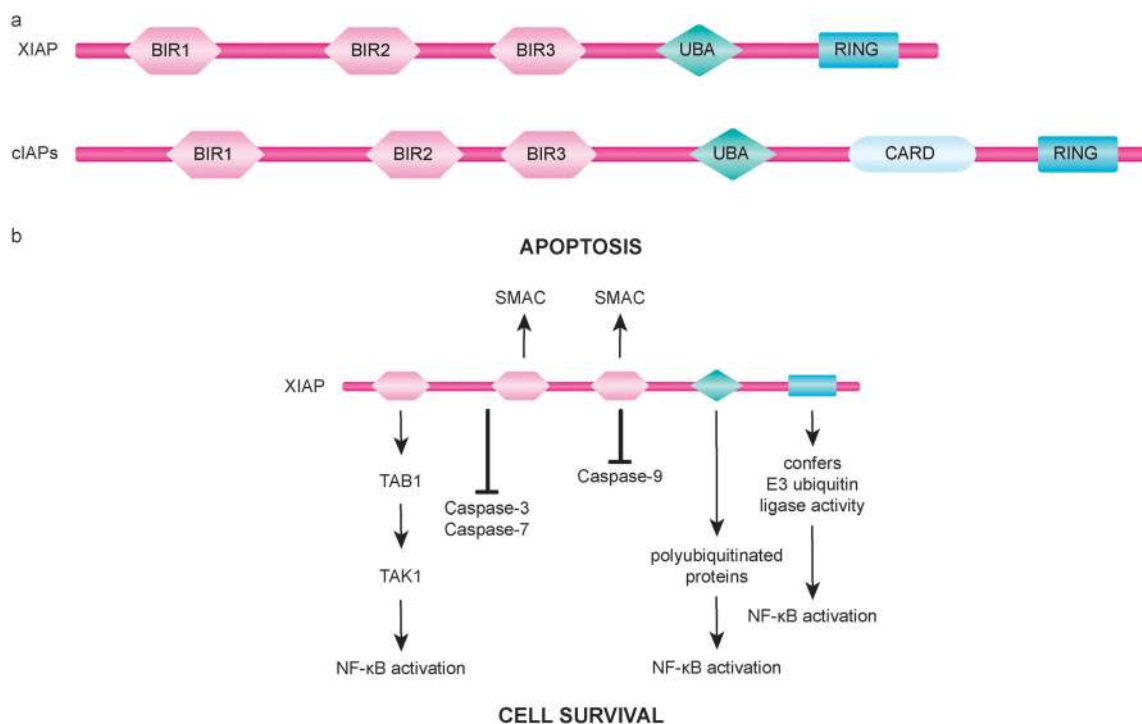
As with the BH3-only proteins, apoptotic signals can regulate the functional activity of multidomain BCL-2 proteins.<sup>68</sup> The regulatory mechanisms of BCL-2 family members can serve to alter their stability or their ability to bind other family members.<sup>68</sup> Given that interactions between BCL-2 family members govern BAX and BAK activation and consequent induction of MOMP, shifts in the binding activities of pro- and anti-apoptotic BCL-2 family members in response to many diverse stimuli play a fundamental role in determining whether a cell will live or die.<sup>39,54</sup> The specific contribution of an individual BCL-2 family member to promote or inhibit apoptosis is mediated by its binding profile. Anti-apoptotic proteins inhibit apoptosis by sequestering pro-apoptotic BCL-2 proteins, including BAX and BAK, as well as activator BH3s. For example, an investigation of the inhibition of tBID-mediated BAX activation by BCL-XL has provided evidence that multiple mechanisms, involving both BCL-XL-tBID and BCL-XL-BAX interactions, play a part in the anti-apoptotic function of BCL-XL.<sup>46</sup> BH3-only proteins promote apoptosis by neutralizing anti-apoptotic BCL-2 proteins and/or by binding to and thereby directly activating BAX and BAK. Characterization of the binding affinities and specificity of the interactions between BH3-only proteins and multidomain BCL-2 family members has revealed that BID, BIM, and PUMA can bind to all anti-apoptotic multidomain BCL-2 family members while other BH3-only proteins, such as NOXA and BAD, are only able to engage a certain subset of the anti-apoptotic BCL-2 proteins.<sup>66,68</sup> The differences in the observed potency of the BH3-only proteins have been attributed to the wide range of differences in their binding affinities.<sup>69–71</sup> Using a genomic approach to examine the pro-apoptotic function of BIM *in vivo*, the maximal pro-apoptotic potential of BIM, found to be significantly enhanced relative to other BH3-only proteins, seems to require its ability to bind all anti-apoptotic BCL-2 family members as well as directly interacting with BAX.<sup>71</sup>

Disruption of the intricate balance between pro- and anti-apoptotic BCL-2 proteins can render cells resistant to apoptotic stimuli and thereby promote cancer cell survival (Fig. 2b).<sup>9</sup> As oncogenic signaling is linked to apoptotic signaling pathways through mechanisms involving critical apoptosis regulators such as BCL-2 family members,<sup>72</sup> alterations that increase the functional activity of anti-apoptotic BCL-2 proteins relative to pro-apoptotic BCL-2 proteins can significantly contribute to cancer development

and progression by enabling cells to escape from apoptosis. Both suppressed activation of pro-apoptotic members<sup>73–76</sup> and overexpression of anti-apoptotic members<sup>9,77</sup> have been reported in various cancers. Genetic and epigenetic alterations targeting genes encoding pro-apoptotic BCL-2 proteins, including BAX as well as several BH3-only proteins, have been identified in several types of cancer cells. Frameshift mutations along with functional inactivation of BAX have been found in colorectal cancers with microsatellite instability,<sup>78,79</sup> and these mutations have been suggested to be associated with disease progression.<sup>80</sup> DNA methylation has been reported to downregulate the expression of BAD, BAK, BIK, and BAX in a multiple myeloma cell line.<sup>74</sup> Silencing of BIM by homozygous deletions and promoter hypermethylation has been observed in mantle cell lymphoma (MCL)<sup>73,81</sup> and Burkitt lymphoma (BL),<sup>73</sup> respectively. The significance of frequent BIM inactivation in BL has been demonstrated by the reversal of BL chemoresistance upon restoration of BIM function.<sup>82</sup> Similarly, the critical role of a specific BH3-only protein in governing a death response has been shown in pancreatic ductal adenocarcinoma (PDAC) cells, as reversal of epigenetically-mediated NOXA silencing sensitizes PDAC cells to apoptosis by induced by etoposide.<sup>83</sup>

Besides genetic and epigenetic changes, another mechanism of gene silencing involves microRNAs (miRNAs), small non-coding RNA molecules (20–24 nucleotides long) that base-pair to target mRNAs and generally repress protein synthesis.<sup>84</sup> miRNAs known to be aberrantly expressed in a variety of cancers have been linked to multiple oncogenic processes, including inhibition of apoptosis,<sup>85,86</sup> and have been found to regulate the expression of many key factors involved in the apoptotic pathways, including BCL-2 family proteins.<sup>87,88</sup> For example, several miRNAs have been found to mediate the suppression of BIM expression in a number of cancers;<sup>87</sup> BIM has been reported to be targeted by miRNAs encoded by the *miR-17-92* cluster in neuroblastoma<sup>89</sup> and lymphomas<sup>90,91</sup> and the *miR-106b-25* cluster, specifically miR-25, in hepatocellular carcinoma (HCC)<sup>92</sup> and esophageal adenocarcinoma.<sup>93</sup> Moreover, as with other mechanisms of BIM silencing, treatment with agents that upregulate BIM expression, such as inhibitors of BIM-targeting miRNA, can induce apoptosis in therapy-resistant cancer cells.<sup>89</sup>

In addition to inactivation of pro-apoptotic BCL-2 family proteins, overexpression of anti-apoptotic family members is a prominent mechanism of apoptosis dysregulation in cancer.<sup>7,94</sup> Increased expression of BCL-2 has been observed in many human malignancies, including acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), diffuse large B-cell lymphoma (DLBCL), glioblastoma, melanoma, and cancers of the prostate and lung.<sup>95–102</sup> BCL-XL overexpression has also been reported in a number of cancers, including multiple myeloma, Kaposi's sarcoma, HCC, colorectal adenocarcinoma, and pancreatic cancer.<sup>103–108</sup> Elevated levels of BCL-2 and BCL-XL have frequently been associated with aggressive disease and/or chemoresistance.<sup>101,106,109–114</sup> Furthermore, given that some cell types, namely type II cells, require activation of the mitochondria-mediated intrinsic pathway for commitment to apoptosis in response to TRAIL, the anti-apoptotic effect of overexpression of anti-apoptotic



**Fig. 3** Domain organization and function of inhibitors of apoptosis (IAP) proteins. (a) XIAP, a well studied human IAP family member, and the structurally similar family members cIAP1 and cIAP2 (cIAPs) each have three tandem BIR domains followed by an ubiquitin-associated (UBA) domain and a C-terminal RING finger domain. cIAPs also possess a caspase recruitment domain (CARD) of unknown function located between the UBA and the RING domains. (b) The BIR2 domain of XIAP, along with residues in its N-terminal flanking linker region, mediates the binding and inhibition of caspase-3 and caspase-7. Inactivation of caspase-9 by XIAP involves the BIR3 domain of XIAP binding to caspase-9. In addition to blocking caspase activity, XIAP can also promote cell survival through regulation of important cellular signaling pathways, including signaling mechanisms of NF- $\kappa$ B activation. IAP-binding motif (IBM)-containing proteins, such as SMAC, interact with the BIR2 and BIR3 domains of XIAP to neutralize its anti-apoptotic activity.

BCL-2 proteins can extend to the death-receptor mediated pathway and render these cells resistant to TRAIL-induced apoptosis.<sup>12,65,115</sup>

The widespread overexpression of BCL-2 in human cancer arises from several different types of cellular defects.<sup>116</sup> The involvement of BCL-2 in the t(14;18) chromosomal translocation, which frequently occurs in follicular lymphomas, led to its initial identification<sup>117</sup> and is now known to drive elevated BCL-2 expression.<sup>101</sup> Gene amplification has also been implicated as an important mechanism for BCL-2 overexpression in DLBCL<sup>118</sup> and small cell lung cancer (SCLC).<sup>119</sup> Furthermore, loss or reduced expression of the tumor suppressor miRNAs miR-15a and miR-16-1 through the deletion or down-regulation of their encoding gene cluster, the *miR-15a-miR-16-1* cluster, can serve as yet another mechanism promoting elevated levels of BCL-2 expression in cancer cells.<sup>120</sup> Markedly reduced levels of miR-15a and miR-16-1 have been observed in approximately 70% of chronic lymphocytic leukemia (CLL) patient samples, as compared with their normal counterparts,<sup>121</sup> along with inversely correlated levels of BCL-2 expression.<sup>122</sup> Indeed, dysregulated posttranscriptional repression of BCL2 by miR-15a and miR-16-1 is currently regarded as one of the main causes of BCL-2 overexpression in CLL.<sup>120</sup>

The known tumor suppressor function of miR-15a and miR-16-1 in CLL is supported by their ability to target multiple oncogenes, including multiple negative regulators of

apoptosis, as indicated by the report that these miRNAs downregulate the expression of another anti-apoptotic BCL-2 family member, MCL-1.<sup>123</sup> Compelling evidence indicates that aberrantly low levels of miR-15a and miR-16-1 may significantly contribute to the pathogenesis of various other cancers as well.<sup>120</sup> Moreover, within the last few years, several studies have implicated the dysregulated expression of other miRNAs that have also been suggested to play a tumor suppressor role as an important contributing factor to the frequent aberrant expression of BCL-2 and/or MCL-1 expression in cancer cells.<sup>86</sup> For example, inactivation of miR-34 family members (miR-34a, miR-34b, and miR-34c) has been found to occur in many cancers;<sup>124</sup> miR-34 family members have been shown to target BCL-2 in numerous types of cancer cells, including neuroblastoma,<sup>125</sup> non-small cell lung cancer (NSCLC),<sup>126</sup> gastric cancer,<sup>127</sup> and pancreatic cancer cells;<sup>128</sup> and ectopic expression of miR-34a can induce apoptosis.<sup>124</sup> In addition, the miR-29 family members (miR-29a, miR-29b, and miR-29c), particularly miR-29b, have been proposed to serve as tumor suppressors in various cancers, as restoration of miR-29b in cancer cells exhibiting downregulation of this miRNA has been shown to induce a pro-apoptotic effect that is associated with its ability to target, and thereby repress the expression of, MCL-1 in cholangiocarcinoma<sup>129</sup> and AML<sup>130</sup> and both BCL-2 and MCL-1 in HCC.<sup>131</sup>



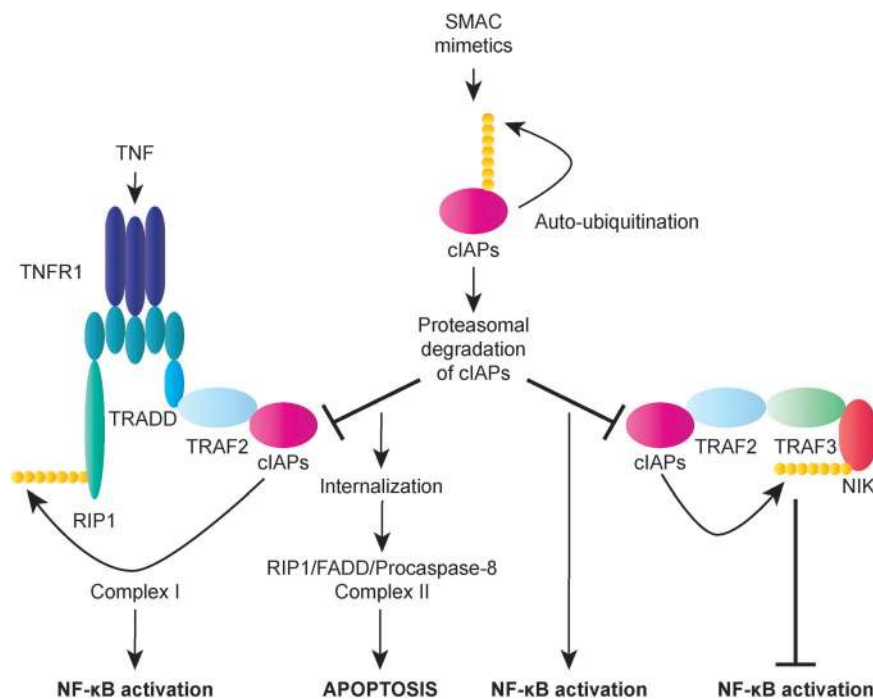
## Control point 2: IAP-mediated inhibition of apoptosis

Considering that proteolysis is an irreversible process, strict control of caspase-mediated proteolytic cleavage is imperative to prevent inappropriate cell destruction.<sup>21,24</sup> Accordingly, cells use multiple layers of regulation to modulate caspase activation.<sup>132</sup> One such layer of caspase regulation involves inhibition of caspase activity by IAP proteins.<sup>132</sup> All members of the IAP family of proteins (with the human IAP family including; NAIP, XIAP, cIAP1, cIAP2, ILP2, livin, survivin, and BRUCE) contain at least one baculovirus IAP repeat (BIR) domain and many have multiple copies of this defining motif of IAPs.<sup>132,133</sup> Three tandem BIR domains (BIR1-BIR3) are present in the N-terminal region of the mammalian IAPs XIAP, cIAP1, and cIAP2.<sup>133</sup> In addition to BIR domains, these IAPs also contain a C-terminal RING finger domain that confers E3 ubiquitin ligase activity and an ubiquitin-associated (UBA) domain for binding ubiquitinated proteins (Fig. 3a).<sup>134</sup> Furthermore, cIAP1 and cIAP2 include a caspase recruitment domain (CARD) domain of unknown function. BIR domains of IAPs can mediate interactions with various proteins and their ability to directly bind to caspases can serve as a mechanism for IAP-mediated caspase inhibition.<sup>133</sup>

XIAP is recognized as the most potent IAP family member with respect to its anti-apoptotic activity, which involves inhibition of active executioner caspases as well as prevention of initiator caspase-9 activation (Fig. 3b).<sup>94,133</sup> In XIAP, the linker region between BIR1 and BIR2, along with BIR2,

contributes to the direct binding and inhibition of caspase-3. As with caspase-3, the same linker region of XIAP also binds to and thus obstructs the substrate-binding groove of caspase-7.<sup>133</sup> Although structural studies have found some contacts between the BIR2 domain of XIAP and caspase-3, but not caspase-7, the inability of the linker region alone to effectively inhibit caspases suggests that BIR2 does play a role in binding.<sup>133</sup> A distinct mode of binding occurs between XIAP and caspase-9; the BIR3 domain of XIAP mediates interactions with caspase-9 to keep it in an inactive monomeric state.<sup>133</sup>

Notably, cIAP1 and cIAP2 are able to bind caspases but lack critical elements required for direct caspase inhibition.<sup>135,136</sup> In fact, although direct inhibition of caspase activity was previously thought to be a significant function of several IAP proteins, later studies suggest that XIAP is the only IAP family member that serves as a direct caspase inhibitor.<sup>136</sup> Nevertheless, IAP family members have been shown to be key regulators of signaling pathways important in determining whether cells live or die.<sup>132,133</sup> In particular, several IAPs have been found to play a complex role in modulating the signaling pathways that activate NF- $\kappa$ B transcription factors.<sup>132,134,137</sup> The upstream signaling mechanisms leading to their activation can generally be classified as classical (also known as canonical) or alternative (also known as non-canonical) NF- $\kappa$ B signaling pathways.<sup>137,138</sup> Members of the NF- $\kappa$ B family of transcription factors are key regulators of a range of physiological processes, including innate and adaptive immune responses, inflammation,



**Fig. 4** Regulation of NF- $\kappa$ B signaling by cIAP1 and cIAP2 (cIAPs). cIAPs play a key role in TNF-induced NF- $\kappa$ B activation by functioning as E3 ligases that ubiquitinate RIP1, leading to the stabilization of complex I and activation of NF- $\kappa$ B. In the presence of SMAC mimetics, cIAPs undergo auto-ubiquitination and proteasomal degradation. The loss of cIAPs sensitizes cells to apoptosis in response to TNF by allowing the formation of complex II, which requires deubiquitination of RIP1. Complex II contains RIP1, FADD, and procaspase-8 and serves to trigger apoptosis by promoting activation of caspase-8. Interestingly, cIAPs can also act as negative regulators of NF- $\kappa$ B activation by targeting NF- $\kappa$ B-inducing kinase (NIK) for proteasomal degradation. In this context, the loss of cIAPs induced by SMAC mimetics promotes NIK stabilization and consequent NF- $\kappa$ B activation.

and cell survival, and their established ability to induce the expression of genes encoding anti-apoptotic proteins has been recognized as an important means of suppressing apoptosis and promoting cell survival.<sup>137</sup> Interestingly, while XIAP, cIAP1, and cIAP2 have been reported to mediate NF- $\kappa$ B activation, these IAPs are also among the recognized anti-apoptotic target genes of NF- $\kappa$ B, thus indicating a positive feedback loop for activation of NF- $\kappa$ B.<sup>134,137</sup>

The ability of XIAP overexpression to induce NF- $\kappa$ B activation has been commonly observed in previous studies, yet the mechanistic details involved have not been fully elucidated and the physiological role of XIAP in regulating NF- $\kappa$ B activity is not yet well understood.<sup>134,139</sup> Nevertheless, reports using ectopic expression of XIAP provide evidence that XIAP can induce NF- $\kappa$ B activation through the activation of transforming growth factor- $\beta$  (TGF- $\beta$ )-activated kinase (TAK1),<sup>134,139</sup> a major player in classical NF- $\kappa$ B signaling.<sup>138,140</sup> Direct interaction of the BIR1 domain of XIAP with the adaptor protein TAK1-binding protein (TAB1) has been reported to play a central role in XIAP-mediated TAK1 and NF- $\kappa$ B activation (Fig. 3b).<sup>134,139</sup> In at least some contexts, efficient activation of NF- $\kappa$ B by XIAP has been shown to require functionally intact UBA and RING finger domains.<sup>134,139</sup> In addition to TAK1, the ubiquitin ligase activity of XIAP has also been implicated as an important regulator of other components involved in signaling mechanisms of NF- $\kappa$ B activation.<sup>139,141</sup>

While the role of XIAP in cellular signaling pathways is less well-defined,<sup>139</sup> a number of studies within the last few years have confirmed the physiological involvement of cIAP1 and cIAP2 in receptor-initiated signaling mechanisms.<sup>132,134</sup> These studies have established cIAP1 and cIAP2 as critical regulators of both classical and alternative NF- $\kappa$ B signaling pathways that act in a functionally redundant manner.<sup>132,134</sup> Interestingly, cIAPs are essential for efficient activation of classical NF- $\kappa$ B signaling in response to stimulation of the death receptor TNFR1, yet they also function as vital participants in the suppression of alternative NF- $\kappa$ B signaling in resting cells (Fig. 4).<sup>132,134</sup> The ubiquitin ligase activity of cIAP1 and cIAP2, along with their BIR1-mediated binding to the adaptor protein TNF receptor-associated factor 2 (TRAF2), plays a major role in their function as both positive regulators of TNF-mediated NF- $\kappa$ B activation and negative regulators of alternative NF- $\kappa$ B signaling pathways.<sup>132–134</sup>

Based on a number of recent findings and reassessment of previous data, a newly revised model of NF- $\kappa$ B activation by TNF involves the recruitment of the protein kinase receptor-interacting protein 1 (RIP1) as well as TRADD and TRAF2, which in turn recruit cIAP1, to the activated TNFR1 receptor to form the membrane-bound complex I (Fig. 4).<sup>142</sup> Subsequently, cIAP ubiquitinates RIP1 as well as other components of complex I, which triggers the recruitment of additional components to complex I, thus stabilizing it and activating kinases that lead to NF- $\kappa$ B activation.<sup>142</sup> While TNFR1 signaling typically serves to stimulate inflammation and promote cell survival, under certain circumstances, TNF can elicit a death response.<sup>13</sup> Importantly, cIAP-mediated K63-linked polyubiquitination of RIP plays a central role in protecting cells from TNF-induced apoptosis.<sup>132,134</sup> The

pro-apoptotic potential of TNF can be switched on through the formation of complex II, a cytoplasmic complex that is derived from the internalization of complex I in a temporally distinct step of TNFR1 signaling; with the loss of both cIAP1 and cIAP2, complex II can recruit FADD and procaspase-8 to induce apoptosis in a manner that requires deubiquitination of RIP1 (Fig. 4).<sup>13,134,143</sup> Accordingly, cIAPs can significantly contribute to cell survival by promoting NF- $\kappa$ B activation as well as preventing caspase-8-mediated apoptosis in response to TNF. Nevertheless, previous studies have also elucidated an important role for cIAPs in maintaining low levels of NF- $\kappa$ B-inducing kinase (NIK), a central mediator of alternative NF- $\kappa$ B signaling, thereby restraining NF- $\kappa$ B activation in resting cells (Fig. 4).<sup>134,137</sup> Constitutive degradation of NIK occurs through its recruitment to the adaptor protein TNF receptor-associated factor 2 (TRAF3), which also binds to the complex TRAF2-cIAP1 and/or TRAF2-cIAP2, resulting in the targeting of NIK for proteasomal degradation by cIAP-mediated K48-linked polyubiquitination.<sup>134,137</sup> Given their multifaceted function in regulating signal transduction pathways, IAPs may play a role in both promoting and antagonizing cell survival, depending on the cellular context.<sup>132</sup>

In response to apoptotic stimuli that trigger MOMP, endogenous inhibitors of IAPs, including SMAC (also known as DIABLO) and OMI (also known as HTR2A), are released from the mitochondria to counter the activity of IAPs and thereby promote apoptosis.<sup>26,29</sup> In their mature form, these proteins contain a conserved N-terminal IAP-binding motif (IBM) that interacts with a conserved groove found in the BIR2 and BIR3 domains of IAPs (Fig. 3b).<sup>133,136</sup> SMAC has been found to function as a homodimer, with the four residues (AVPI) that constitute the IBM of each SMAC molecule acting in a bivalent manner so that one IBM binds to BIR2 and the other to BIR3 of a single XIAP molecule to block caspase inhibition.<sup>132,144</sup> In addition to XIAP, SMAC has been shown to bind most IAPs,<sup>132</sup> and IAP antagonists that mimic the IBM of SMAC (SMAC mimetics) have been found to effectively kill cancer cells through a mechanisms involving auto-ubiquitination and proteasomal degradation of cIAP1 and cIAP2 (Fig. 4).<sup>133,134</sup>

Aberrant expression or activity of IAPs has been reported in numerous types of human cancer.<sup>132,144,145</sup> Elevated IAP expression levels have frequently been observed in a variety of hematological malignancies as well as solid tumors and have been associated with advanced disease and poor prognosis.<sup>132,144,145</sup> An examination of the NCI 60 cancer cell line panel has revealed high mRNA levels of XIAP in nearly all of the cell lines.<sup>146</sup> Increased expression of XIAP has also been reported in several different types of tumor samples and has been linked to chemoresistance, tumor progression, and/or shorter survival in some cancers, including HCC, renal cell carcinoma, colorectal, ovarian and prostate cancer.<sup>132,145,147–149</sup> Various mechanisms can play a role in the dysregulation of XIAP in cancer cells.<sup>145</sup> One proposed mechanism of enhanced XIAP activity involves co-expression of survivin, another IAP family member, which can bind to SMAC to block SMAC-mediated inhibition of XIAP.<sup>145,150</sup> Furthermore, survivin can interact with XIAP to form a complex that increases XIAP stability against ubiquitin-mediated proteasomal

degradation and enhances caspase inhibition in a synergistic manner.<sup>150</sup> Besides its function as a potent inhibitor of apoptosis, which has been associated with accelerating tumor growth,<sup>151</sup> this IAP–IAP complex has also been reported to promote the metastasis of tumor cells *via* activation of NF- $\kappa$ B signaling.<sup>152</sup> Notably, survivin, unlike other IAPs, is overexpressed in nearly all human cancers but is generally undetectable in most adult tissues.<sup>132,150</sup> While the prognostic value of XIAP has been found to vary with tumor type,<sup>132,147</sup> overexpression of survivin has consistently been implicated as a negative prognostic factor.<sup>132,145,150</sup> As with XIAP, multiple mechanisms have been described by which survivin expression can be upregulated in cancer.<sup>150</sup> The prominent overexpression of XIAP and survivin, along with many studies demonstrating that inhibition of these IAPs can promote apoptosis and sensitize cancer cells to various therapies, strongly suggests that their anti-apoptotic activity can significantly contribute to cancer pathogenesis.<sup>132,145</sup>

cIAP1 and cIAP2 expression is also commonly dysregulated in cancer. A chromosomal region containing the genes encoding cIAP1 (*BIRC2*) and cIAP2 (*BIRC3*), 11q22, has been found to be amplified in several cancers,<sup>134</sup> with overexpression of cIAP1 and/or cIAP2.<sup>153–155</sup> Additional genetic evidence in support of an oncogenic role for cIAPs includes the involvement of *BIRC3* in the t(11q21:18q21) translocation, the most common translocation event in mucosa-associated lymphoid tissue (MALT) lymphoma.<sup>156</sup> The resulting cIAP2–MALT1 fusion protein triggers constitutive activation of classical NF- $\kappa$ B signaling, leading to B cell transformation and lymphoma progression.<sup>134</sup> Although the aberrant expression of IAPs that has been associated with tumor-promoting process typically involves their upregulation, inactivating genetic mutations of cIAPs have also been linked to oncogenesis.<sup>134,145</sup> Specifically, biallelic deletions and correlated reduced expression levels of cIAP1 and cIAP2, resulting in NIK stabilization and activation of alternative NF- $\kappa$ B signaling, has been identified in multiple myeloma.<sup>137,157</sup>

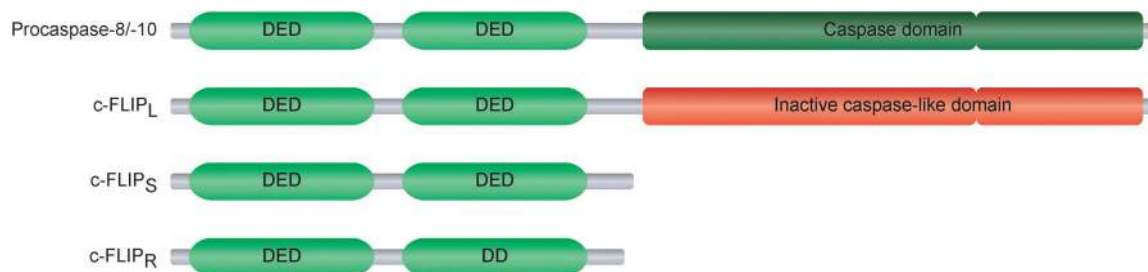
### Control point 3: regulation of apoptosis by c-FLIP

c-FLIP is a DED-containing protein that is recruited to the DISC and regulates the activation of caspase-8 in death receptor signaling pathways (Fig. 1).<sup>18</sup> Three c-FLIP protein isoforms derived from distinct mRNA splice variants have been identified, namely c-FLIP<sub>L</sub>, c-FLIP<sub>S</sub>, and c-FLIP<sub>R</sub>.<sup>18,158</sup>

c-FLIP<sub>L</sub>, the long c-FLIP isoform, is structurally similar to procaspase-8, with two tandem DEDs at its N-terminus and a catalytically inactive caspase-like domain at its C-terminus.<sup>18,158</sup> The short c-FLIP isoforms c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> also possess two tandem DEDs at their N-termini, but differ structurally from procaspase-8 at their C-termini, which are defined only by a short stretch of amino acids (Fig. 5).<sup>18,158</sup>

c-FLIP was initially characterized as a potent inhibitor of death receptor-mediated apoptosis.<sup>159</sup> At high expression levels, all three isoforms are recruited to the DISC *via* DED interactions and subsequently impair death receptor-induced apoptotic signaling by blocking the activation of caspase-8.<sup>18,158,159</sup> Nevertheless, some early studies reported conflicting evidence regarding the function of c-FLIP<sub>L</sub> in mediating apoptosis.<sup>160</sup> Later studies have clarified the previous, seemingly contradictory, findings by clearly revealing a dual functional role for c-FLIP<sub>L</sub> as an inhibitor or activator of caspase-8 activation, depending on various factors, including its expression level relative to that of caspase-8.<sup>18,160,161</sup> In contrast to its anti-apoptotic role at high levels of expression, c-FLIP<sub>L</sub> has been shown to act at the DISC to enhance caspase-8 activation and promote apoptosis at low and more physiologically relevant expression levels.<sup>160,161</sup> Modulation of caspase-8 activity by c-FLIP<sub>L</sub> is further complicated by evidence that in certain contexts c-FLIP<sub>L</sub> may induce only moderate procaspase-8 activation, which results in a level of caspase-8 activity that is too low to trigger apoptosis but is sufficient for cleavage of c-FLIP<sub>L</sub>, ultimately leading to the activation of NF- $\kappa$ B survival signaling and other non-apoptotic signaling pathways.<sup>161,162</sup> Still, the function of c-FLIP in blocking caspase-8 activation at the level of the DISC is recognized as an important check on caspase activity to protect against unwanted cellular destruction.<sup>21,24</sup>

Despite the identified dual functionality of c-FLIP<sub>L</sub> as a pro- or anti-apoptotic factor in normal tissues, c-FLIP<sub>L</sub> has generally been shown to act as a key negative regulator of apoptosis in human cancer cells.<sup>18,163</sup> Increased expression of c-FLIP has been commonly found in a number of human malignancies, including melanoma, hepatocellular carcinoma,<sup>164</sup> non-small cell lung carcinoma,<sup>165</sup> endometrial,<sup>166</sup> colon,<sup>167</sup> and prostate cancer.<sup>168–170</sup> Previous studies have clearly demonstrated that in various types of cancer cells, elevated c-FLIP expression levels serve to block caspase-8 activation at the DISC, thereby rendering cells resistant to death



**Fig. 5** Structural features of the FLICE-inhibitory protein (c-FLIP) isoforms. Each of the three c-FLIP isoforms, c-FLIP<sub>L</sub>, c-FLIP<sub>S</sub>, and c-FLIP<sub>R</sub>, contain two tandem death effector domains (DEDs) at its N-terminus. c-FLIP<sub>L</sub>, the long c-FLIP isoform, is similar in overall structure to procaspase-8, but its caspase-like domain is functionally inactive. Other than the two DEDs, the short c-FLIP isoforms c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> only include a short C-terminus.

receptor-mediated apoptosis.<sup>18</sup> Notably, c-FLIP overexpression has been associated with disease progression and/or poor prognosis in BL, HCC, ovarian, endometrial, colon, and prostate cancer.<sup>18,163,164,171</sup>

Besides blocking apoptotic signaling, c-FLIP has been implicated in contributing to tumor-promoting processes through its influence on key cellular survival signaling mechanisms.<sup>163</sup> In a number of diverse cellular contexts, death receptor stimulation triggers nonapoptotic signaling cascades with important roles in cell proliferation, migration, and survival.<sup>13</sup> Overexpression of c-FLIP<sub>L</sub> has been reported to bind proteins involved in these signaling pathways, including TRAF1, TRAF2, RIP, and RAF1, and thereby promote the activation of the downstream signaling molecules NF- $\kappa$ B and ERK.<sup>163</sup> In TRAIL-resistant NSCLC cells, c-FLIP and RIP have been shown to be essential for TRAIL-induced formation of the DISC in non-raft domains of the plasma membrane and consequent activation of NF- $\kappa$ B and ERK cell survival signals. In contrast, knockdown of c-FLIP has been found to redistribute the DISC to lipid rafts and switch DISC signaling to TRAIL-induced apoptosis.<sup>172</sup> Furthermore, c-FLIP<sub>L</sub>-mediated ERK activation has been shown to be responsible for the capacity of TRAIL to induce proliferation of TRAIL-resistant glioma cells.<sup>173</sup> Recently, c-FLIP<sub>L</sub> has also been reported to modulate signaling downstream of the pro-survival kinase AKT by means of direct interaction with AKT.<sup>174</sup> A novel mechanism of c-FLIP<sub>L</sub>-mediated TRAIL resistance in cancer cells has been proposed based on evidence indicating that c-FLIP<sub>L</sub> overexpression markedly reduces phosphorylation of a major AKT substrate, GSK3 $\beta$ , resulting in reduced levels of p27 and caspase-3 expression along with decreased sensitivity to TRAIL-induced apoptosis.<sup>174</sup>

Given that c-FLIP expression in cancer cells can be regulated at the transcriptional level by a variety of transcription factors, as well as at the translational and post-translational levels,<sup>18,163</sup> numerous opportunities exist for dysregulation of this key apoptosis regulator. One noteworthy regulatory mechanism by which c-FLIP expression can be upregulated involves activation of NF- $\kappa$ B transcriptional activity.<sup>175,176</sup> As NF- $\kappa$ B constitutive activation has been linked to the pathogenesis of many human cancers<sup>137</sup> and inhibition of NF- $\kappa$ B-induced transcription of c-FLIP has been shown to sensitize cells to death receptor-mediated apoptosis,<sup>13,177</sup> NF- $\kappa$ B-mediated upregulation of c-FLIP is implicated as an important factor in the evasion of cell death by cancer cells.

### Therapeutic strategies modulating the induction of apoptosis

Over the past decade, significant advances have been made in discovery and validation of several novel cancer therapeutics. However, inevitably resistance to therapy leads to treatment failures. As highlighted in the preceding section, regulators of apoptosis are major factors contributing to resistance to chemotherapy. Considering the established ability of cellular alterations that lead to the evasion of apoptosis to contribute to the development and progression of cancer as well as resistance to various anticancer therapies,<sup>6,7,94</sup> these advances have provided new drug targets for promising apoptosis-inducing therapeutic

strategies that can be used most likely in combination with other apoptosis-inducing therapeutic agents. Several review articles have called attention to the great potential of apoptosis-targeted therapies,<sup>8,10,178</sup> some of which focus on BCL-2 family proteins,<sup>9,179,180</sup> IAPs,<sup>144,181,182</sup> or c-FLIP<sup>18,183</sup> and the approaches that have been used to modify their activity to reactivate apoptosis and thus eradicate cancer cells. We refer readers to these reviews for specific details regarding the development of novel therapeutic agents directed against these targets, which have been shown to demonstrate enhanced apoptotic killing and sensitize resistant cancer cells to antineoplastic agents. Nevertheless, we outline below a few of the most promising therapeutic strategies for the targeted induction of apoptosis.

The recognition of the association between overexpression of BCL-2 and BCL-XL and aggressive disease and/or chemoresistance has marked these proteins as attractive therapeutic targets and spurred efforts to develop pharmacological interventions aimed at downregulating anti-apoptotic BCL-2 family members.<sup>9,179</sup> Strategies to inhibit anti-apoptotic BCL-2 proteins include reducing protein expression by targeting the corresponding mRNA with an antisense oligonucleotide compound as well as blocking anti-apoptotic activity by targeting at the protein level.<sup>8,9,179</sup> Small-molecule inhibitors that mimic the BH3 domain of BH3-only proteins and thus act to directly bind and neutralize anti-apoptotic BCL-2 proteins can inhibit multiple BCL-2 family members and seem to be especially promising anti-cancer therapeutics, both alone and in combination with other therapies, based on preclinical and early clinical study results.<sup>179</sup>

As with anti-apoptotic BCL-2 proteins, IAPs have also been strongly pursued as therapeutic targets for apoptosis-inducing anti-cancer strategies<sup>184</sup> due to evidence of their overexpression in many cancers and their association with poor prognosis and chemoresistance in some of these cancers.<sup>132,144,145</sup> Various IAP-targeted therapies have been developed, including antisense oligonucleotides against XIAP and survivin<sup>8,144</sup> and small-molecule inhibitors that bind to the BIR2 or BIR3 domain of XIAP, cIAP1, and cIAP2.<sup>184</sup> Most small-molecule IAP antagonists are SMAC mimetics, several of which are in advanced preclinical or early clinical development.<sup>132,184</sup> Bivalent SMAC mimetics, which bind to both the BIR2 and BIR3 domains within a single molecule of XIAP as well as the BIR3 domains of two cIAP molecules to induce their dimerization and subsequently trigger their proteasome-mediated degradation, have been shown to be significantly more potent than their monovalent counterparts.<sup>132,184</sup> Despite evidence indicating that SMAC mimetics show great promise for cancer therapy,<sup>132,184</sup> their potential value as anti-cancer therapeutics has been called into question as a result of their established ability to induce loss of cIAP1 and cIAP2, resulting in NIK stabilization and consequent NF- $\kappa$ B activation (Fig. 4).<sup>132,137</sup>

Downregulation of c-FLIP appears to be a highly promising therapeutic strategy to lower the apoptotic threshold of cancer cells, as evidenced by several reports demonstrating that c-FLIP<sub>L</sub> overexpression can protect cancer cells from both TRAIL- and chemotherapy-induced apoptosis, while small interfering RNA (siRNA)-mediated downregulation of



c-FLIP is able to sensitize cancer cells to FASL, TRAIL, and chemotherapeutic agents.<sup>18,163,183</sup> FLIP-targeted siRNAs have also been shown to induce spontaneous apoptosis in a variety of cancer cell types, underscoring their potential as therapeutic agents.<sup>183</sup> Nevertheless, obstacles against the effective and safe systemic delivery of siRNA-based therapies pose a significant challenge to their widespread use in clinical settings.<sup>185</sup> Despite this, intense research efforts have been focused on the development of RNA interference (RNAi) therapeutics, leading to important advances in the field.<sup>186,187</sup> In May 2008, the first Phase I clinical trial for the treatment of solid tumors involving systemic delivery of siRNA *via* targeted nanoparticles was initiated (ClinicalTrials.gov identifier: NCT00689065).<sup>187</sup> Recently, data from this trial demonstrated that systemically delivered siRNA can reduce mRNA and protein levels of a specific gene through an RNAi mechanism in humans.<sup>188</sup> The exciting breakthrough offers great hope for the future application of siRNA as a gene-specific therapeutic for cancer treatment. Given that therapies aimed at inhibiting the expression of anti-apoptotic proteins are expected to improve the effectiveness of cancer treatment, in addition to c-FLIP, other anti-apoptotic proteins are promising targets for the development of RNAi therapeutics.<sup>183</sup> Indeed, as with c-FLIP, RNAi-mediated downregulation of BCL-2, BCL-XL, XIAP, and survivin has also been shown to sensitize cancer cells to a range of cancer therapies, including chemotherapeutic drugs and TRAIL.<sup>8,183,189</sup>

### Challenges with validating the therapeutic strategies that modulate apoptosis

The low rate of FDA approval for cancer therapeutics in the past several years necessitates more clinically relevant models for preclinical studies for validation and development of novel targeted therapeutics, including Bcl-2, IAP and FLIP inhibitors. The major concerns with the current preclinical studies are that the cell-based assays and animal models that are being used do not closely resemble the human disease.<sup>190–192</sup> The majority of cell-based studies used for analysis of apoptotic pathways and their modulators are carried out in two-dimensional models, which differ significantly from tumors *in vivo*. It is now well established that tumors behave like organs, consisting of tumor, stroma, inflammatory and endothelial cells with complex interactions.<sup>193</sup> Moreover, it is now apparent that 3D models developed for a variety of cancers, including breast and melanoma, more closely resembles the *in vivo* setting.<sup>194,195</sup> Indeed, studies using 3D models (*i.e.*, breast cancer) indicate that additional levels of regulation of the apoptotic machinery exist.<sup>196–200</sup> For instance, various components of the extracellular matrix, including integrins, also modulate the response of cells to apoptotic stimuli.<sup>192,200,201</sup> Therefore, in therapeutic consideration of TRAIL, TRAIL-receptor agonist antibodies, modulators of the Bcl-2 family of proteins, inhibitors or IAPs and FLIPs, we should bear in mind the complexity of tumor microenvironment and investigate the validity and efficacy of these therapeutics in 3D models that more closely resemble the physiological conditions.<sup>192,196</sup>

Another concern in the validation of apoptosis-inducing agents as therapeutics is the use of *in vivo* animal models.

Currently, preclinical *in vivo* studies for efficacy and mechanism of action of therapeutic targets are carried out mostly in xenograft and sometimes in orthotopic mouse tumor models. However, these models do not adequately represent human cancer, as the interaction between tumor and its microenvironment and the multi-step tumor progression is not recapitulated.<sup>191</sup> Recent evaluation of genetically engineered mouse models (GEMMS) as platforms for testing cancer therapeutics has provided solid evidence to the value of these models in preclinical studies.<sup>190</sup> It is now apparent that the GEMMS can more accurately predict the efficacy and mechanism of action of anti-cancer therapeutics. Despite the high upfront cost of GEMMS for preclinical validation, these models can potentially significantly reduce the enormous cost of unsuccessful clinical trials and will allow selection of therapeutics that will have greater potential of success in the clinic. Therefore, the utility of these models needs to be considered in evaluation of anti-cancer agents that modulate apoptosis.

### Conclusion

Evasion of apoptosis has been established as a hallmark of cancer and the impaired apoptotic signaling characteristic of cancer cells is frequently linked to tumor development and progression as well as resistance to treatment. Intense research efforts to elucidate the mechanisms by which cancer cells are protected from apoptosis have led to the identification of several alterations that result in the aberrant activity of key apoptosis regulators, including BCL-2 family proteins, IAPs, and c-FLIP. These insights into the dysregulation of apoptosis in cancer have fuelled the pursuit of various targeted therapeutic strategies for reactivating apoptosis, which hold great promise for translation into anti-cancer therapeutics that will significantly benefit cancer patients. Nevertheless, the heterogeneity among tumors, even of the same type, necessitates a continued effort to further investigate mechanisms of apoptosis dysregulation in distinct cancer cell types. We are confident that future advances in our understanding of the underlying cellular defects that are responsible for cancer cell resistance to apoptosis and use of more stringent preclinical models will lead to more effective cancer treatments.

### Acknowledgements

We thank Jane Hayward, media specialist, Beth Israel Deaconess Medical Center, for assistance with figure preparation and Susan Glueck for editing this manuscript. J.P. is supported by a T32, HL007893. O.B. received a fellowship from the Lady TATA Memorial Trust, London, U.K. This work was also supported by the National Institutes of Health grants CA105306, CA131664, HL080192 awarded to R.K. R.K. is the recipient of a Melanoma Research Alliance Award and Harry Lloyd Trust Award.

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