The International Journal of Biochemistry & Cell Biology 44 (2012) 1622–1631

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Small heat shock proteins HSP27 (HspB1), αB-crystallin (HspB5) and HSP22 (HspB8) as regulators of cell death☆,☆☆

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ARTICLE INFO

Article history:
Received 13 January 2012
Received in revised form 2 April 2012
Accepted 4 April 2012
Available online 13 April 2012

Keywords:
Heat shock proteins
Cell death
Apoptosis
Chaperone
Cancer

ABSTRACT

Hsp27, αB-crystallin and HSP22 are ubiquitous small heat shock proteins (sHsp) whose expression is induced in response to a wide variety of unfavorable physiological and environmental conditions. These sHsp protect cells from otherwise lethal conditions mainly by their involvement in cell death pathways such as necrosis, apoptosis or autophagy. At a molecular level, the mechanisms accounting for sHsp functions in cell death are (1) prevention of denatured proteins aggregation, (2) regulation of caspase activity, (3) regulation of the intracellular redox state, (4) function in actin polymerization and cytoskeleton integrity and (5) proteasome-mediated degradation of selected proteins. In cancer cells, these sHsp are often overexpressed and associated with increased tumorigenicity, cancer cells metastatic potential and resistance to chemotherapy. Altogether, these properties suggest that Hsp27, αB-crystallin and Hsp22 are appropriate targets for modulating cell death pathways. In the present, we briefly review recent reports showing molecular evidence of cell death regulation by these sHsp and co-chaperones.

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1. Introduction

Small heat shock proteins (sHsp) comprise the most widespread but also the most poorly conserved family of molecular chaperones. sHsp are found in most organisms where they are induced upon stress and are involved in protecting cells from various unfavorable conditions (Van Montfort et al., 2001; Haslbeck et al., 2005). The human genome encodes ten members of sHsp family (HspB1–HspB10) diverse in size, in sequence and in the N- and C-terminal regions but sharing the following structural and functional characteristics (Fig. 1): (1) a low molecular mass of 17.0–28.4 kDa, (2) a conserved α-crystallin domain of ~90 residues, (3) the capacity to form large oligomers, (4) a dynamic quaternary structure and (5) induction by stress conditions. Numerous studies have shown that sHsp display different chaperone activities in suppressing protein aggregation (Fontaine et al., 2003; Kappe et al., 2003; Basha et al., 2010; Van Montfort et al., 2001). For instance, HspB8 (Hsp22) stimulates autophagy-mediated degradation of aggregates in an eIF2 alpha-dependent manner (Carra et al., 2008a,b, 2009). Whereas, HspB1 (Hsp27) has been shown to suppress huntingtin-induced toxicity without having a major effect on its aggregation, rather the formation of reactive oxygen species (Wyttenbach et al., 2002; Rogalla et al., 1999). Finally, under stress, HspB5 (αB-crystallin) acts as a molecular chaperone by suppressing desmin and myosin abnormal aggregation by maintaining protein folding (Melkani et al., 2006; Wang et al., 2003; Nicholl and Quinlan, 1994).

Unlike other heat shock proteins, sHsp are ATP-independent chaperones and that are able to form oligomers than can reach 800 kDa. Oligomeric complexes, reaching up to 800 kDa, result from interaction of the α-crystallin domains of two units forming a dimer, the molecular base of the oligomeric complex (Lentze and Narberhaus, 2004). The oligomerization potential, and thus biological function of sHsp, is regulated by phosphorylation (Kato et al., 2002; Koteiche and McHaourab, 2003; Martin et al., 1999; Webster, 2003; Garrido, 2002). sHsp oligomerization is thought to be required for substrate binding and chaperone function, although oligomer roles vary from one sHsp to another (Sun and MacRae, 2005). Precisely, previous studies have shown that individual

☆ This article is part of a Directed Issue entitled: Small HSPs in physiology and pathology.
☆☆ P. Rocchi's team is supported from grants by the French Cancer Institute (InCa, PAIR prostate program), l'Institut National de la Santé et de la Recherche Médicale (Inserm), l'Association pour la Recherche sur le Cancer (ARC), l'Association pour la Recherche sur les Tumeurs de la Prostate (ARTP), the French Research Ministry (FRM), the National Research Agency (ANR), the Mediterranean University and the competitivity pole Eurobiomed.
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http://dx.doi.org/10.1016/j.biocel.2012.04.002

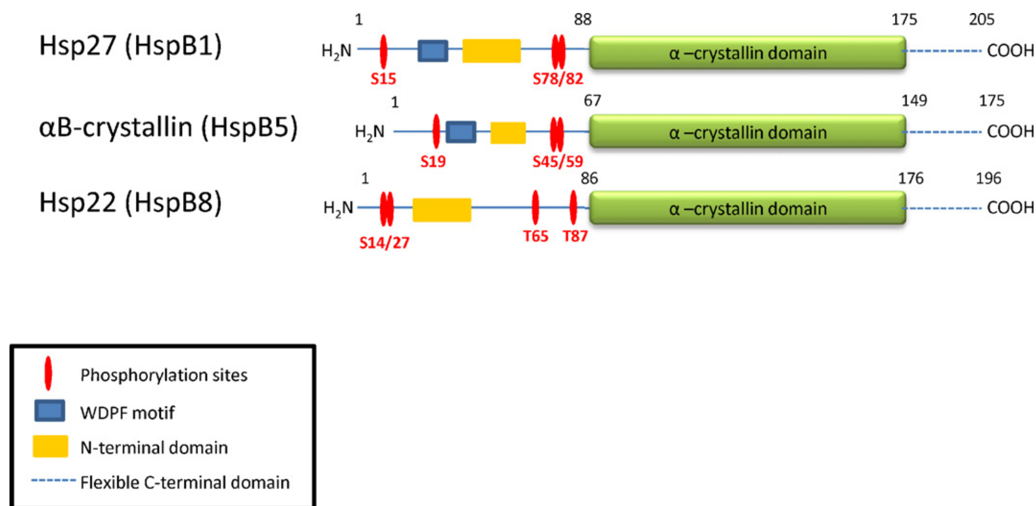

Fig. 1. Structural domains of HSP27, HSP22 and αB-crystallin human proteins. Schematic representation of Hsp27, αB-crystallin and Hsp22 structures indicating the N-terminal domain, flexible C-terminal domain, WDPF domain and the α-crystallin domain as well as the serine (S) and threonine (T) phosphorylation sites.

αB-crystallin subunits fail to interact with unfolded proteins (Avilov et al., 2004) and that phosphorylated Hsp27 exhibits significantly decreased oligomeric size and chaperone activity (Hayes et al., 2009; Rogalla et al., 1999). Conversely, Hsp22 and Hsp20 apparently never form homo-oligomers, yet they have shown to exhibit chaperone activity *in vitro* (Chowdary et al., 2004; Kim et al., 2004; Bukach et al., 2004). From a functional point of view, sHsp are involved in cell resistance to heat shock and other extracellular stress factors such as anticancer agents, therefore playing a fundamental role in cell survival (Haslbeck et al., 2005). At a molecular level, sHsp are involved in preventing aggregation of partially misfolded proteins (HspB1, HspB7) (Stromer et al., 2003; Vos et al., 2011). They have also been described to be involved in regulating redox state enzymes activity and activation of caspase-3 (HspB1) (Haslbeck et al., 2005; Stromer et al., 2003; Arrigo, 2001). Previously, sHsp have been shown to intervene in modulating cell death pathways by interacting with various components of the cell death machinery upstream and downstream of the mitochondrial apoptotic events and can prevent apoptosis in different lethal stress situations (HspB1, HspB5, HspB6) (Kanagasabai et al., 2010; Lanneau et al., 2008; Velotta et al., 2011; Zeng et al., 2010). Homeostatic and pathological cell death can occur through various different cell mechanisms including apoptosis (programmed cell death), autophagy and necrosis. The exact nature of the molecular switches that determine which pathway the cell follows remain elusive. Moreover, contributions of the different cell death machineries to avoid cancer or degenerative disease have recently started to be defined. In cancer, for example, cell death defects are prevalent and survival of cancer cells enables progression and treatment resistance. In such cases, restoration of cell death pathways is an important and challenging therapeutic aim. Moreover, sHsp are overexpressed in many types of malignant tumors where they are involved in tumor growth and treatment resistance (Modem et al., 2011; Rocchi et al., 2004; Zeng et al., 2010; Baylot et al., 2011). For instance, inhibition of Hsp27 expression in prostate cancer cells by OGX-427 has shown successful cancer cell growth arrest and induction of apoptosis (Rocchi et al., 2005, 2006). Interestingly, OGX-427 is now in clinical trial phase II to treat prostate and bladder cancer (Hotte et al., 2009; Hirte et al., 2010). Altogether, these data suggest that sHsp are promising targets for modulating cell death pathways. With the view to understand the wider roles of sHsp in cell death, in the present, we briefly review how the three major sHsp, Hsp22, Hsp27 and αB-crystallin participate in the cell's "life-or-death" decisions.

2. HspB8 (Hsp22)

2.1. Tissue distribution, structure and properties of HspB8 (Hsp22)

HspB8 is expressed in the majority of tissues but its mRNA was found to be highly expressed in the human skeletal and smooth muscle, the spinal cord, the motor and sensory neurons, heart, brain, prostate, lung and kidney (Benndorf et al., 2001; Taylor and Benjamin, 2005; Irobi et al., 2004). HspB8 was first identified in human melanoma cells, where it was described as a H11 protein kinase (Smith et al., 2000). In estrogen-responsive breast cancer cells, HspB8 was observed to be expressed in response to estrogen and was designated as a product of E2IG1 gene, coding a new member of the family of sHsp (Charpentier et al., 2000). HspB8, also called Hsp22 due to its molecular mass close to 22 kDa, contains the conserved α-crystallin domain, common among all sHsp and is closely related to other sHsp such as HspB1 (Hsp27) (Benndorf et al., 2001; Sun et al., 2004). Hsp22 is thought to belong to the group of "intrinsically disordered proteins" involved in recognition, regulation and cell signaling (Uversky et al., 2005) and possesses a flexible structure. Indeed, Hsp22 is enriched in β-strands and it lacks the β2 strand detected in other sHsp (Kasakov et al., 2007; Kazakov et al., 2009). The intracellular localization of Hsp22 is not well characterized. It interacts with the intracellular leaflet of the cell outer membranes (Chowdary et al., 2007; Smith et al., 2000) and tends to form low-molecular-mass oligomers in the cell cytoplasm and outside mitochondria where it has been shown to colocalize with various proteins, such as glycolytic enzymes and different protein kinases (Chowdary et al., 2007; Kim et al., 2004). Moreover, Hsp22 has been shown to form hetero-oligomeric complexes with HspB1 (Hsp27), HspB2 (MKBP), HspB3, HspB5 (αB-crystallin), HspB6 (Hsp20) and HspB7 (Fontaine et al., 2005; Sun et al., 2004) and these interactions seem to be dependent on the phosphorylation state of the partner sHsp (Sun et al., 2006). Recently, it has been described that phosphorylation of Hsp22 by cAMP-dependent and ERK1 protein kinases affects the structure and decreases chaperone-like activity of HSP22 *in vitro* (Shemetov et al., 2008a, 2011).

Very often, Hsp22 was found to colocalize with aggregates formed by partially denatured or improperly folded proteins which suggests a protective role of Hsp22 from accumulation of insoluble aggregates either by preventing aggregation or by promoting degradation of misfolded proteins (Carra et al., 2005; Simon et al.,

2007; Shemetov and Gusev, 2011). The above properties of Hsp22 account for its biological functions, namely the chaperone-like activity and intrinsic kinase activity, the regulated proteolysis of unfolded proteins (by the proteasome or by macroautophagy) (Shemetov and Gusev, 2011; Carra et al., 2008b, 2009; Hedhli et al., 2008) and its involvement in regulation of cell proliferation, cardiac hypertrophy, apoptosis, carcinogenesis and different neuromuscular diseases.

2.2. Chaperone-like activity, macroautophagy and cytoprotection

Hsp22 chaperone activity assuring the proteolytic degradation of unfolded proteins can be mediated either by proteasomes or by macroautophagy (Arndt et al., 2010). Hsp22 seems to be in the crossroad of both mechanisms, playing a dual role in cell survival (Shemetov et al., 2008b). The molecular mechanisms underlying the opposing role of Hsp22 in cell apoptosis remain enigmatic and complex and seem to be dependent on cell type and the extent of Hsp22 expression. Indeed, it is thought that Hsp22 expression regulation (for example by promoter methylation) helps achieve efficient regulation of cell proliferation, differentiation and apoptosis (Gober et al., 2003; Hase et al., 2005; Bany and Schultz, 2001; Charpentier et al., 2000). Moreover, previously mentioned data indicate that Hsp22 chaperone-like activity seems to be important for anti-apoptotic effects, whereas interaction with and regulation of certain protein kinases by Hsp22 could lead to pro-apoptotic effects (Gober et al., 2003, 2005; Shemetov et al., 2008b).

Toward a pro-survival goal, Hsp22 efficiently prevents or diminishes aggregation of partially unfolded or denaturated proteins in cells (Shemetov et al., 2008b). This chaperone-like activity was largely demonstrated *in vitro* (Chowdary et al., 2004). For example, Hsp22 has been demonstrated to prevent formation of aggregates formed by R120G mutant of αB-crystallin (Chavez Zobel et al., 2003). Moreover, overexpressing Hsp22 was accompanied by reduction in amyloid aggregates leading to improvement in cardiac function and survival (Sanbe et al., 2009). Two natural mutations of Hsp22, K141E and K141N have been shown *in vivo* and *in vitro*, leading to decrease its chaperone-like activity and accumulation of protein aggregates. Interestingly, expression of K141N or K141E mutants of HSP22 correlates with development of distal hereditary motor neuropathy and/or Charcot–Marie–Tooth disease (Carra et al., 2005; Kim et al., 2006; Irobi et al., 2004; Fontaine et al., 2006). The chaperone-like activity of Hsp22 is also mediated through regulation of proteasome stability and intracellular location leading to activation of proteolytic degradation of certain proteins (Hedhli et al., 2008). This hypothesis has been strongly suggested in the case of cardiac hypertrophy, induced by Hsp22 overexpression, during which the catalytic activity of 20S subunit of proteasome was significantly increased and proteasome subunits were redistributed in the cytoplasm (Hedhli et al., 2008). Studies in the human hibernating myocardium have shown that up-regulation of Hsp22 was found to be involved in cytoprotection and apoptosis inhibition (Depre et al., 2004).

As previously mentioned Hsp22 displays a dual and opposing role in cell survival depending on its levels of expression. In fact, an in-depth investigation of cardiac myocytes strikingly revealed that low expression levels of Hsp22 resulted in increased cell size, by direct binding and activation of phosphoglucomutase and inhibition of casein kinase 2, an anti-apoptotic protein, whereas high levels of Hsp22 induced apoptosis (Hase et al., 2005; Wang et al., 2004). Recent data have also indicated that Hsp22 interacts with Bag3 (Fig. 2), a stimulator of macroautophagy, forming a heteromultimeric complex formed by Hsp22, Bag3, Hsc70 and CHIP (chaperon-associated ubiquitin ligase) (Arndt et al., 2010; Carra et al., 2008b). Formation of this complex, promotes autophagic removal of misfolded proteins providing a cell clearing of aggregated proteins (Crippa et al., 2010; Carra et al., 2008a, 2009, 2010). In addition, by a mechanism still unknown, this complex also leads to phosphorylation of eIF2α, inhibition of protein synthesis and stimulation of macroautophagy (Carra, 2009; Carra et al., 2009). It has been well described that depending on specific cellular circumstances, autophagy can either protect cells against death or mediate cellular self-destruction (Galluzzi et al., 2008; Tsujimoto and Shimizu, 2005; Maiuri et al., 2007). As a pro-survival process, organelles and/or portions of the cytoplasm are engulfed within double-membraned autophagic vacuoles for degradation ensuring the physiological turnover of old/damaged organelles, providing cells with metabolic substrates to satisfy energy demands under stress (Maiuri et al., 2007). However, accumulation of these vacuoles and further activation of the autophagic pathway, represent an alternative pathway of autophagic cell death by cellular atrophy and function collapse (type II cell death). Autophagy can also engage apoptotic (type I) or necrotic (type III) cell death programs by activation of common regulators such as proteins of the Bcl-2 family (Galluzzi et al., 2008; Tsujimoto and Shimizu, 2005). It is thought that the pro-apoptotic effect of Hsp22 can be in part explained by its interaction with Bag3 (Rosati et al., 2011). Even though it has never been demonstrated, it is thought that when Hsp22 is overexpressed, it forms tight complexes with Bag3 and by this means releases Bcl2 from the complex with Bag3 which could therefore lead to apoptosis (Kroemer et al., 2010; Maiuri et al., 2007).

All the above described data, clearly demonstrate that the additional complexity characterizing Hsp22 functional role is associated with the nature and the quantity of the proteins that interact with overexpressed Hsp22, the effects are either pro- or anti-apoptotic.

2.3. Hsp22 in carcinogenesis and apoptosis regulation

In various types of cancer cells Hsp22 is overexpressed and possesses an anti-apoptotic activity (Gober et al., 2005). For instance, treatment of human breast cancer cell line MCF-7 with 17b-estradiol and cyclin D1 induced up-regulation of certain genes including Hsp22 through erB1/erB2 signaling (Yang et al., 2006; Charpentier et al., 2000). In agreement with these observations, numerous data have described Hsp22 overexpression association with tumor proliferation and protection from apoptosis of melanoma, glioblastoma, breast cancer cells by cell cycle regulation (Sui et al., 2009; Charpentier et al., 2000; Modem et al., 2011; Smith et al., 2000). Precisely, in melanocytes, Hsp22 has been shown to display transforming potential during the G1 cell cycle stage, which could involve activation of growth-associated transcription factors such as E2F and/or cyclin-dependent kinase such as cdk4 (Smith et al., 2000; Bartkova et al., 1996; Halaban et al., 1998). In glioblastoma cells, Hsp22 is inversely correlated to Sam68 (Src-associated protein in mitosis 68 kDa) regulating Sam68 expression thus cells proliferative potential mediated by increased S phase population and enhanced expression of cycle regulatory proteins such as cyclin E and A, ribonucleotide reductase (RNR), and proliferating cell nuclear antigen (PCNA), which are required for the transition of cells from G1 to S phase (Modem et al., 2011). In cardiac cells, Hsp22 induced cell growth and survival through activation of the PI3K/Akt pathway mediated by the bone morphogenetic protein signaling. Moreover, it was shown to be accompanied by anchorage-independent growth in HEK 293 cells stably transfected with Hsp22 (Smith et al., 2000; Yang et al., 2006). Nevertheless, in certain tumor tissues and cells lines (prostate, colon, sarcoma cell lines), the expression on Hsp22 was shown to be reduced as compared to normal cells (Gober et al., 2003). Moreover, treating these cell lines by DNA demethylating agents or transiently transfecting with Hsp22 induced apoptosis that was both caspase- and $p38^{MAPK}$-dependent (Gober et al., 2003). Thus, depending on the

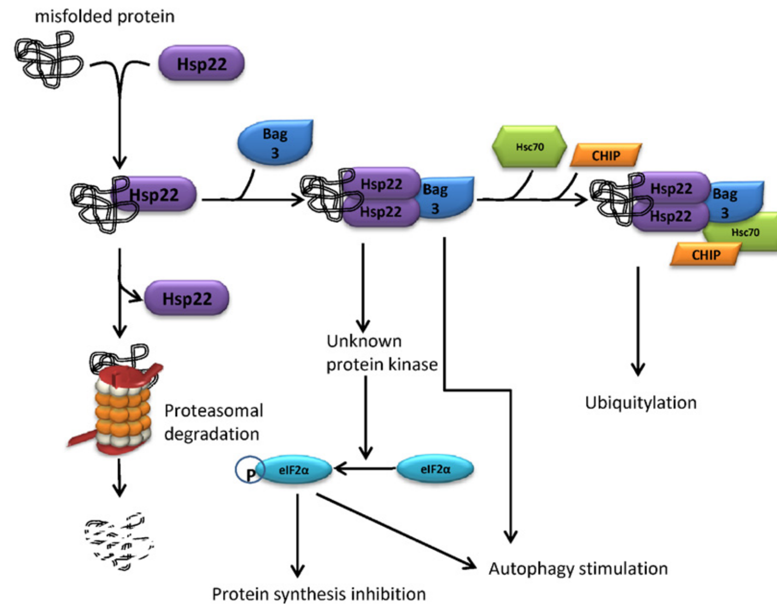

Fig. 2. Hsp22 involvement in regulated proteolysis of misfolded proteins. HSP22 is involved in the ubiquitin-proteasome degradation of misfolded proteins, protein synthesis inhibition and autophagy regulation. This Hsp22 chaperone activity participates in cell survival regulation.

cell type and the level of expression, Hsp22 is associated to either pro- or anti-apoptotic effects (Gober et al., 2003, 2005).

3. HspB1 (Hsp27)

3.1. Structure and expression of Hsp27

Human Hsp27 also called HSPB1, HSP25 or HSP28 was described in 1980 when HeLa cells, cultured at high temperature, were accompanied by the production of an unknown protein with a molecular weight of 27 kDa (Hickey and Weber, 1982). The primary structure of HSPB1 is highly homologous to other members of the sHsp family, containing the conserved α-crystallin domain and differing in the C- and N-terminal regions (Fig. 1). Hsp27 is expressed in all human tissues, including astrocytes and primary neuronal cells but mainly in skeletal, smooth and cardiac muscles (Sugiyama et al., 2000) and just like the majority of sHsp, Hsp27 possesses chaperone-like activity, preventing aggregation of improperly folder or partially denatured protein (Bryantsev et al., 2007; Jakob et al., 1993). Expression of Hsp27 is induced during ontogenesis (Lutsch et al., 1997), in various pathologies and in many types of tumors (Ciocca and Calderwood, 2005; Huang et al., 2010).

3.2. Cell death regulation by Hsp27

Among cell death pathways, Hsp27 has been shown to participate mainly in apoptosis regulation. In fact, Hsp27 can prevent apoptosis by protecting cells against heat shock, apoptosis effectors, oxidatives stress, and ischemia. Hsp27 can also inactivate Bax and block the release of Smac and cytochrome C (Arrigo, 2007; Garrido et al., 2006) (Fig. 3). Jacques Landry et al., were the first to demonstrate a relationship between Hsp27 and thermoresistance by the study of cell survival (Landry et al., 1989). Later, the cytoprotective effect of Hsp27 was demonstrated against various apoptotic effectors (Mehlen et al., 1996). It is important to note at this point that in cells treated by apoptotic effectors that act on different pathways, Hsp27 has diverse localizations, oligomeric sizes and phosphorylation states leading to negative regulation of apoptosis. More precisely, the two apoptotic effectors, ectoposide and Fas antibody, have the tendency to increase Hsp27 native sizes reflecting medium sized and large oligomers accumulation, while staurosporine and cytochalasin D induced Hsp27 in small oligomer (Paul et al., 2010). In fact, in murine L929 cells, Hsp27 expression prevents Fas/APO-1 receptor from inducing cell death. Moreover, Hsp27 expression blocks apoptosis normally induced by staurosporine (Mehlen et al., 1996).

It is noteworthy, that Hsp27 also displays neuroprotective effect. In fact, in astrocytes and in primary neuronal cells, it has been demonstrated that Hsp27 transfer protects cells from apoptosis induced by oxidative stress *in vitro* and *in vivo* (An et al., 2008). In addition, in a model of neurodegenerative disorder, Hsp27 transfer induced cell protection against pro-apoptotic activity of mutant SOD1 (a mutant of peroxide dismutase, SOD) (An et al., 2008). Moreover, in amyotrophic lateral sclerosis, mice resulting from cross between mice overexpressing Hsp27 and SOD1 mice, present an increase in spinal motor neurons (Schmitt et al., 2007). Interestingly, the function of Hsp27 is highly influenced by post-translational modifications. Recently, it has been shown that overexpression of phosphorylated Hsp27 induced neuroprotection against ischemic neuronal injury. In addition, this phosphorylation essential for the anti-apoptotic effect of Hsp27 is induced by protein kinase D (Stetler et al., 2012). In non-neuronal cells, Hsp27 also acts on cell survival by mechanisms that seem to be cell-type dependent. In fact, Hsp27 overexpression in liver decreases cell apoptosis induced by ischemia (An et al., 2008; Ye et al., 2011). Precisely, Hsp27 acts by regulating partner proteins involved in cell death pathways. Under stress condition, in renal epithelial cells, Hsp27 indirectly inactivates Bax and its translocation to mitochondria. This is due to an increase of PI3-kinase activity that activates Akt (a pro-survival kinase) and promotes interaction between Akt and Bax (Havasi et al., 2008). Moreover, in polymorphonuclear leukocytes, Hsp27 also regulates apoptosis by interacting with Akt and promotes Akt/MK2 (protein kinase 2) binding (Wu et al., 2007). In myeloma cell lines, it has been reported that Hsp27 activation blocks release of Smac (second mitochondria-derivated activator of caspase) from mitochondria (Arrigo et al., 2007; Chauhan et al., 2003). All these reports, and others, account for the role of Hsp27 in apoptotic cell death inhibition (Havasi et al., 2008;

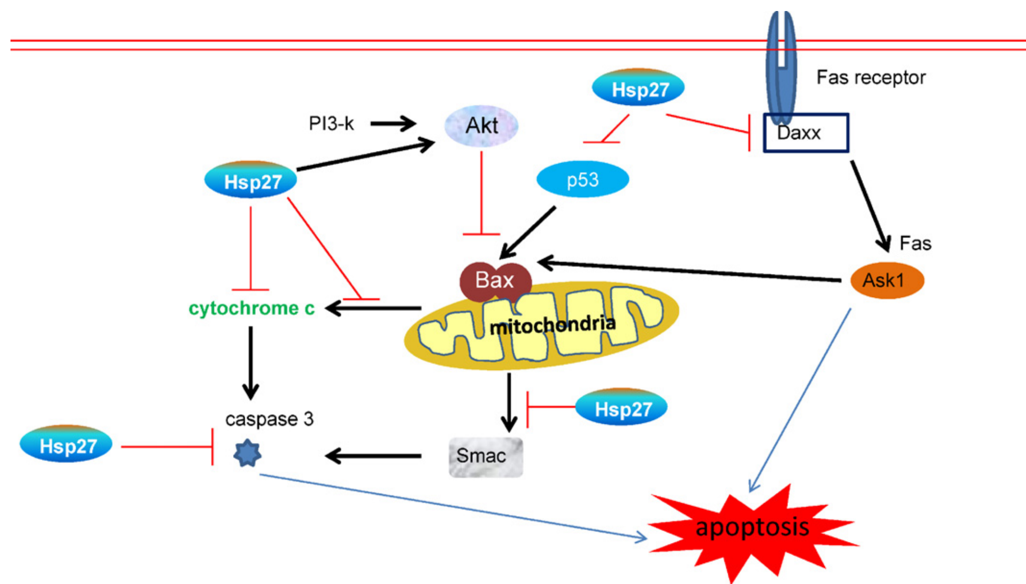

Fig. 3. Involvement of Hsp27 in apoptosis regulation. Hsp27 regulates apoptosis by blocking mitochondrial intrinsic pathway of apoptosis through interactions with cell death regulation factors. Inhibition: ⊣ and activation: →.

Sanchez-Nino et al., 2011). Interestingly, numerous studies describe that the caspase cascade inactivation is a consequence of Hsp27 binding with caspase-3 and cytochrome-c released from mitochondria (Bruey et al., 2000; Garrido et al., 1999, 2006; Schmitt et al., 2007; Voss et al., 2007). These data were also confirmed in acute myeloid leukemia (AML) where Hsp27 prevents apoptosis and induces resistance to chemotherapy (VP-16) through phosphorylation of p38 (the mitogen-activated protein kinases), c-Jun, the release of cytochrome-c and so apoptosis (Schepers et al., 2005). In addition, Hsp27 overexpression in human mammary epithelium cells (MCF10A cells) has been shown to inhibit senescence (characterized by growth arrest) and apoptosis of cells treated by doxorubicin. This effect is due to the inhibitory effect of Hsp27 on p53, the regulator of the cyclin-dependent kinase inhibitor p21 (also called waf1) (O'Callaghan-Sunol et al., 2007). In addition to cell proliferation inhibition, p21 acts as an regulator of apoptosis in a number of systems by cell cycle arrest or by binding and inactivation of cyclin A/Cdk2 (Gartel and Tyner, 2002). Moreover, p21 functions depend of its localisation and the expression of Hsp27. In fact, Hsp27 expression increases p21 phosphorylation and its translocation to the cytoplasm leading to cell survival. Conversely, Hsp27 inactivation prevents p21 translocation and enhances cell death (Kanagasabai et al., 2010). Previous studies demonstrated that phosphorylation levels of Hsp27 increased in advanced tumors and are correlated to treatment resistance. In fact, in pancreatic and prostate cancer cells, cytoprotection induced by Hsp27 is due, at least in part, to its interaction with eIF4E (eukaryotic translational initiation factor 4E) that increased when Hsp27 is phosphorylated. Hsp27 interaction protects eIF4E from its ubiquitin-proteasome dependent degradation process, leading to apoptosis resistance induced by castration and chemotherapy (Baylot et al., 2011; Andrieu et al., 2010). A similar mechanism was previously described involving cooperative interactions between ligand-activated androgen receptor and Hsp27 phospho-activation that enhance androgen receptor stability, shuttling, and transcriptional activity, thereby increasing prostate cancer cell survival (Zoubeidi et al., 2007). Finally, a caspase-independent apoptosis effect of Hsp27 has been demonstrated. Indeed Hsp27 interacts with Daxx (death domain-associated protein 6), preventing its binding with Ask1 (apoptosis signal-regulated kinase 1) and Fas (a regulator of cell death) in the cytoplasm which leads to inhibition of the apoptotic effects of these proteins (Charette and Landry, 2000; Charette et al., 2000).

Very few data demonstrate the role of Hsp27 in cell death by necrosis or autophagy. Necrosis is a cell death pathway that occurs when cells are exposed to extreme variance of physiological conditions. Hsp27 was shown to be involved in necrosis stimulation of human alveolar epithelial cell line A549, as its inactivation reversed the switch from necrosis to apoptosis (Lim et al., 2010). In addition, Hsp27 is known to be involved in the cytoprotective effect of the unfolded protein response (UPR) (a cellular stress response) that contributes to the cisplatin-induce apoptosis (Garrido et al., 1997). Recently it was shown in hepatocellular carcinoma cells line, that Hsp27 protects cells from cisplatin-induced death through autophagy mechanism (Chen et al., 2011). The anti-apoptotic properties and the cytoprotective function of Hsp27 make this protein a potential target for anticancer therapy to induce tumor cell death. Inhibition strategies aiming at Hsp27 down-regulation could prevent its protective effect. In fact, recent studies on human tumors resistant to chemotherapeutic agents (SQ20B head-and-neck squamous carcinoma, PC-3 prostate cancer cell, U87 glioblastoma and human colorectal cancer cell lines) have shown that the decrease of Hsp27 expression induced apoptosis (Aloy et al., 2008; Choi et al., 2007). In addition, Hsp27 is upregulated under high glucose and angiotensine II that normally contributes to diabetic nephropathy, leading to apoptosis of podocytes cells (cells in the Bowman's capsule in the kidneys). Sanchez-Nino et al., demonstrated that Hsp27 down-regulation using small interference RNA (siRNA) increased the apoptosis rate of podocytes *in vitro* (Sanchez-Nino et al., 2011). This observation was also made in prostate cancer cells, where Hsp27 inhibition leads to caspase3/7 induction and decreases cell growth (Liu et al., 2009, 2012; Rocchi et al., 2006). Moreover, elevated levels of Hsp27 have been observed in lung cancer stem-like cells that could explain the resistance of these cells to apoptosis induced by traditional chemotherapy. *In vivo*, Hsp27 down-regulation using siRNA, antisens oligonucleotide (ASO) or small molecule inhibitors was shown to increase cell apoptosis and decrease tumor growth in mice (Hsu, 2010; Kamada, 2007; Xia, 2009; Xia, 2012; Baylot, 2011; Rocchi, 2005; Rocchi, 2004; Xia, 2012). More recently, Kaur et al. have developed a siRNA introduced into a lentiviral vector to improve the delivery and a permanent silencing of Hsp27 (Kaur et al., 2011). In fact, the use of this

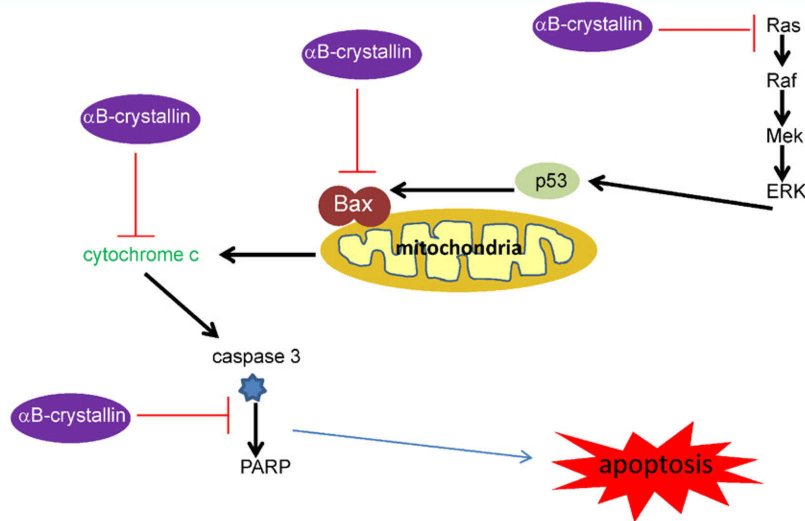

Fig. 4. Involvement of αB-crystallin in apoptosis regulation. Schematic representation of apoptosis regulation induced by αB-crystallin is mainly mediated by inhibition of caspase 3-dependant apoptosis. Inhibition: ┤and activation: →.

vector enhanced proteasome activity and induced the regression of established breast tumors (Nagaraja et al., 2012).

4. HspB5 (αB-crystallin)

4.1. Structure and expression of αB-crystallin

The alpha-basic-crystallin (αB-crystallin) protein also called HSPB5 is a chaperone discovered as a highly abundant protein in eye lens where it plays an essential role in maintaining the transparency of the ocular lens (Bloemendal, 1982). Later this protein was found to act as a molecular chaperone induced by a variety of stress stimuli, conferring cytoprotection by suppressing aggregation of denatured protein (Horwitz, 1992). Human αB-crystallin contains a N-terminal domain, the central α-crystallin domain and a C-terminal domain (Fig. 1) (Jehle et al., 2011) and is expressed in many tissue types, including muscle, brain, spleen, lung, kidney and skin. αB-crystallin has been shown to be involved in some diseases such cardiac myopathies and neurodegenerative diseases (Goldstein et al., 2003; Maddala and Rao, 2005; Vicart et al., 1998). In addition, αB-crystallin is highly expressed in various neurological diseases and diverse cancers like gliomas, breast, prostate and renal cell carcinomas (Pinder et al., 1994; Takashi et al., 1998). From a structural point of view, αB-crystallin can form homo- and hetero-oligomers depending on its phosphorylation state, with consequences on its chaperone-activity, as it will be described later (Ahmad et al., 2008).

4.2. Apoptosis regulation by αB-crystallin

Accumulating evidence suggests that αB-crystallin can provide anti-apoptotic protection. In fact, αB-crystallin protects cells against stress, TNF-α, apoptotic effectors, can inhibit caspase-3 and PARP and prevents the translocation of Bax and Bcl-2 from the cytosol (Fig. 4). In the nervous system, αB-crystallin is thought to play a neuroprotective role in neurodegenerative disease like in familial amyloidotic polyneuropathy where αB-crystallin is over-expressed (Magalhaes et al., 2010). In rat primary astrocytes, the protease-activated receptor-2 (PARP-2) increased phosphorylation of αB-crystallin at ser59. This is essential for the protective function of αB-crystallin that involves P38 and ERK activity (Li and Reiser, 2011). P38β-MAPK is induced in astrocytes after ischemia and was shown to protect cells from apoptosis and was seen to co-localize with αB-crystallin. This suggestion was based on the fact that inhibition of αB-crystallin suppressed this protective effect (Shin et al., 2011). In agreement with the previously mentioned data, in retinal ganglion cell degeneration, αB-crystallin transfer induces cell survival showing its neuroprotective effect (Munemasa et al., 2009). In addition, in human retinal pigment epithelial cells, αB-crystallin prevents apoptosis induced by staurosporine by interacting with Bax and Bcl-2 and inhibiting their translocation from the cytosol to the mitochondria (Mao et al., 2004). More recently, Parameswaran et al. have shown that αB-crystallin protects stressed retinal pigment epithelial cells from apoptosis by inhibiting caspase-3 and PARP (poly (ADP-ribose) polymerase) activation (Sreekumar et al., 2010). A previous study in agreement with this data has shown that αB-crystallin inhibits autocatalytic maturation of caspase-3 preventing apoptosis (Kamradt et al., 2001). In non-neuronal cells, αB-crystallin also acts on cell death regulation. Indeed, overexpression of α≡-crystallin in transgenic mice attenuates cardiomyocyte apoptosis and necrosis (Ray et al., 2001). It has been shown that in neonatal mouse cardiomyocytes, cardiac hypertrophy is the consequence of an active form of calcineurin A that protects against apoptosis through αB-crystallin activity (Bousette et al., 2010). More recently, in C2C12 (the mouse skeletal myoblast cell line), αB-crystallin was observed to prevent apoptosis induced by hydrogen peroxide treatment by interacting with p53 in the cytoplasm and preventing its translocation to mitochondria (Liu et al., 2007). In the same model, TNF-α pro-apoptotic treatment that normally induces apoptosis, also lead to αB-crystallin over-expression. This overexpression promoted NF-kB activation protecting cells against apoptosis. In this study, the anti-apoptotic effect of αB-crystallin has been associated with the elevated expression level of the anti-apoptotic protein Bcl-2 (Adhikari et al., 2011). αB-crystallin prevents apoptosis by acting on several effectors induced during apoptosis. In fact, in rabbit lens epithelial cells, the RAF/MEK/ERK pathway, activated by RAS, plays a key role in stress-activated apoptosis. In these cells, αB-crystallin overexpression decreases cell apoptosis by preventing RAS activation and inhibiting the RAF/MEK/ERK pathway (Cortese et al., 2005). Due to its anti-apoptotic effect, αB-crystallin is considered an oncoprotein in breast cancer because of its role in apoptosis resistance that could explain the aggressive character of cancer cells (Gruvberger-Saal and Parsons, 2006; Moyano et al., 2006). In fact, in breast epithelial carcinoma cells treated by anti-cancer agent (vinblastine), αB-crystallin is founded phosphorylated at

serine 59, and this status is positively correlated with apoptosis. More precisely, phosphorylated αB-crystallin interacts with the anti-apoptotic protein Bcl-2 and increase apoptosis of cells treated by anti-cancer agents. In contrast, overexpression of wild-type αB-crystallin induced resistance to vinblastine (Launay et al., 2010). In agreement with this study, it was shown, in cardiac myocytes after hypoxic stress, that phosphorylation state of αB-crystallin is necessary to protect cells from apoptosis (Morrison et al., 2003).

As Hsp27, αB-crystallin inhibition could represent a promising therapeutic strategy and further studies need to be done in that direction. The first model of αB-crystallin knockout in mice, has revealed abnormal morphology of lens fiber cells due to an increase of caspase activation and apoptosis (Morozov and Wawrousek, 2006). In addition, in human glioblastoma, αB-crystallin protein is highly expressed in invasive tumor cells resistant to apoptosis. In these tumors cells (U-373MG) inhibition of αB-crystallin sensitizes cells to apoptosis induced by actinomycin D (Goplen et al., 2010). Finally, another study in astrocyte and glioma cells expressing oncoprotein Bcl2L12 (Bcl2-Like12), has described that knock-down of αB-crystallin leads to a caspase-3 dependant apoptosis (Stegh et al., 2008). In addition, inhibition of αB-crystallin induces TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-associated apoptosis by caspase-3 activation (Kamradt et al., 2001, 2005). All the above data strengthen the anti-apoptotic effect of αB-crystallin.

5. Conclusion and future directions

The data we have presented in this review indicate that Hsp22, Hsp27 and αB-crystallin are involved in vital cell processes, finely regulating the balance between life and death of the cells by protecting cells under unfavorable conditions. Additional complexity in the investigation and understanding of the mechanisms underlying sHsp cell functions is due to the fine regulation of sHsp by posttranslational modifications, like phosphorylation and to their characteristic ability to form homo- and hetero-oligomeric complexes as well as interacting with a multitude of proteins. Such protein–protein interactions account for sHsp involvement in as diverse processes as cell protection from oxidative stress, apoptosis and proteolysis, proliferation, cell motility and muscle contraction. Although numerous reports unquestionably account for the role of sHsp in all the above processes, some of the aspects concerning the structure and molecular mechanisms underlying their functions need profound understanding. Future work should be directed toward analysis and definition of sHsp interactions with other chaperones and co-chaperones, protein kinases and elements of signaling pathways. Moreover, mutations of sHsp are often associated with human disorders and tissue-specific overexpression of sHsp has been closely linked to cardiovascular diseases and cancer. These proteins are increasingly considered as attractive therapeutic targets. Ongoing studies will indicate whether these proteins are useful targets for therapeutic manipulation of the apoptotic pathways, for example to induce cancer cells death or to sensitize them to current therapeutic approaches. Thus further efforts should be made for the development of drugs specifically targeting sHsp phosphorylation or oligomerization but also interaction with partner proteins.

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Recombinant protein folding and misfolding in *Escherichia coli*

François Baneyx & Mirna Mujacic

The past 20 years have seen enormous progress in the understanding of the mechanisms used by the enteric bacterium *Escherichia coli* to promote protein folding, support protein translocation and handle protein misfolding. Insights from these studies have been exploited to tackle the problems of inclusion body formation, proteolytic degradation and disulfide bond generation that have long impeded the production of complex heterologous proteins in a properly folded and biologically active form. The application of this information to industrial processes, together with emerging strategies for creating designer folding modulators and performing glycosylation all but guarantee that *E. coli* will remain an important host for the production of both commodity and high value added proteins.

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The enteric bacterium *Escherichia coli* is one of the most extensively used prokaryotic organisms for genetic manipulations and for the industrial production of proteins of therapeutic or commercial interest. Compared with other established and emerging expression systems[1], *E. coli* offers several advantages, including growth on inexpensive carbon sources, rapid biomass accumulation, amenability to high cell-density fermentations and simple process scale-up. Because of its long history as a model system, *E. coli* genetics are very well characterized and many tools have been developed for chromosome engineering and to facilitate gene cloning and expression. If heterologous proteins do not require complex post-translational modifications and are expressed in a soluble form, *E. coli* is usually first selected to obtain enough material for biochemical and/or structural studies and for the subsequent large-scale production of valuable gene products. It is, however, not uncommon that overexpressed recombinant proteins fail to reach a correct conformation and undergo proteolytic degradation or associate with each other to form insoluble aggregates of nonnative proteins known as inclusion bodies.

Over the past 20 years, there has been considerable progress in the fundamental understanding of the mechanisms used by *E. coli* to support *de novo* protein folding, manage stress-induced protein misfolding and decide whether misfolded polypeptides should be refolded or degraded. Here, we review this body of knowledge and how it has been exploited to promote the high-level production of heterologous proteins in a correct and bioactive conformation in the bacterial cytoplasm and periplasm.

Protein misfolding and inclusion body formation

In the crowded milieu of the *E. coli* cytoplasm where transcription and translation are tightly coupled and one protein chain is released from the ribosome every 35 seconds[2], an environment where macromolecule concentration can reach 300–400 mg/ml (ref. 3), protein folding is an extraordinary challenge. In general, small (<100 residues), single domain host proteins efficiently reach a native conformation owing to their fast folding kinetics, whereas large multidomain and overexpressed recombinant proteins often require the assistance of folding modulators. Folding helpers include molecular chaperones, which favor on-pathway folding by shielding interactive surfaces from each other and from the solvent, and folding catalysts that accelerate rate-limiting steps, such as the isomerization of peptidyl-prolyl bonds from an abnormal *cis* to a *trans* conformation and the formation and reshuffling of disulfide bonds.

For a heterologous protein, failure to rapidly reach a native conformation or to interact with folding modulators in a timely fashion has two possible consequences: partial or complete deposition into insoluble aggregates known as inclusion bodies or degradation. The likelihood of misfolding is increased by the routine use of strong promoters and high inducer concentrations that can lead to product yields exceeding 50% of the total cellular protein. Under such conditions, the rate of protein aggregation is often much greater than that of proper folding and folding modulators are rapidly titrated. A second factor contributing to inclusion body formation is the inability of bacteria to support all post-translational modifications that a protein requires to fold. For instance, the formation of intra- or intermolecular disulfides is not possible in the reducing cytoplasm of wild-type *E. coli*, which results in the aggregation of certain disulfide bond–rich proteins (e.g., Fab antibody fragments).

Inclusion bodies can accumulate in the cytoplasm or periplasm depending on whether or not a recombinant protein has been engineered for secretion. The target typically accounts for 80–95% of the inclusion body material and is contaminated by outer membrane proteins, ribosomal components and a small amount of phospholipids and nucleic acids that likely adsorb upon cell lysis[4]. Folding modulators (e.g., DnaK, GroEL and IbpA/B) are sometimes—but not always—associated with inclusion bodies[5,6]. Cytoplasmic inclusion

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Published online 4 November 2004; doi:10.1038/nbt1029

Table 1 Cytoplasmic chaperones

Family	Name	Cofactors	Function	Substrate specificity	ATP requirement
Hsp100 (AAA+)[a]	ClpB		Disaggregase	Segments enriched in aromatic and basic residues	+
Hsp90	HtpG		Possible folding/secretory chaperone	Unknown	+
Hsp70	DnaK	DnaJ, GrpE	Folding chaperone	Segments of four to five hydrophobic amino acids, enriched in leucine and flanked by basic residues	+
	HscA	HscB	Iron-sulfur cluster protein assembly	LPPVK motif in iron-sulfur cluster protein assembly IscU	+
	HscC	YbeV, YbeS	σ^{70} regulation	Unknown	+
Hsp60	GroEL	GroES	Folding chaperone	α/β folds enriched in hydrophobic and basic residues	+
Hsp33	Hsp33		Holding chaperone	Unknown	–
DJ-1 superfamily	Hsp31		Holding chaperone	Unknown	–[b]
Small Hsps	IbpA, IbpB		Holding chaperone	Unknown	–[c]
PPIase	TF		Holding chaperone, PPIase	Eight amino acid motif enriched in aromatic and basic residues	–
SecB	SecB		Secretory chaperone	Nine amino acid motif enriched in aromatic and basic residues	–

[a]AAA, ATPases associated with a variety of cellular activities. [b]ATP binding negatively regulates the chaperone activity of Hsp31 at high temperatures[23]. [c]ATP binding to certain small Hsps triggers conformational changes and substrate release[17].

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bodies are porous ovoids or cylinders with maximum characteristic length and volume of 1 m and 0.6 m^3, respectively[6–8]. However, hemispherical inclusion bodies of 0.5- m diameter have been observed in the periplasm[7]. In the cytoplasm, inclusion bodies grow from structured folding intermediates[9] at nearly constant rates and around nucleation cores that are mutually exclusive. Thus, multiple inclusions of different sizes may be present within a single cell[6]. Because inclusion bodies are resistant to proteolysis and contain large amounts of relatively pure material, their formation is often exploited for the production of proteins that are toxic, unstable or easy to refold. Finding optimal conditions for efficient refolding requires considerable optimization, but acceptable yields can usually be achieved using established strategies[10].

Cytoplasmic folding modulators

In *E. coli* and other systems, host protein misfolding is not uncommon. It may result from premature termination of translation, failure of a newly synthesized chain to reach a correct conformation or from loss of structure triggered by environmental stress. To cope with this situation, cells have evolved largely conserved mechanisms to favor proper *de novo* folding, refold partially folded proteins, dissolve aggregates and dispose of irretrievably damaged proteins.

Molecular chaperones, a ubiquitous class of folding modulators, play a central role in the conformational quality control of the proteome by interacting with, stabilizing and remodeling a wide range of nonnative polypeptides. Although constitutively expressed under balanced growth conditions, many chaperones are upregulated upon heat shock or other insults that increase cellular protein misfolding (including heterologous protein expression[11]) and are therefore classified as stress or heat shock proteins (Hsps). Mechanistically, molecular chaperones rely on the differential exposure of structured hydrophobic domains to the solvent to bind nonpolar segments that would normally be buried within the core of their substrates. Although there are subtle differences in the composition of client protein recognition sequences (and thus some degree of selectivity in substrate capture), the typical chaperone target is a short unstructured stretch of hydrophobic amino acids flanked by basic residues and lacking acidic residues (**Table 1**). The fact that such motifs are common explains why chaperones are so promiscuous.

Molecular chaperones can be divided into three functional subclasses based on their mechanism of action (**Fig. 1**). 'Folding' chaperones (e.g., DnaK and GroEL) rely on ATP-driven conformational changes to mediate the net refolding/unfolding of their substrates. 'Holding' chaperones (e.g., IbpB) maintain partially folded proteins on their surface to await availability of folding chaperones upon stress abatement. Finally, the 'disaggregating' chaperone ClpB promotes the solubilization of proteins that have become aggregated as a result of stress.

In the *E. coli* cytoplasm, *de novo* folding involves three chaperone systems: trigger factor (TF), DnaK-DnaJ-GrpE and GroEL-GroES (reviewed in refs. 12,13). TF, a three-domain protein that binds ribosomes with moderate affinity in the vicinity of the peptide exit site, is ideally positioned to interact with short nascent chains. Although the central domain of TF exhibits peptidyl-prolyl *cis/trans* isomerase (PPIase) activity, proline residues are not necessary for substrate capture[14] and native TF clients are primarily large (>60 kDa) multidomain proteins, which represent 10–20% of the *E. coli* proteome[15]. Longer nascent chains or newly synthesized proteins may alternatively be captured by DnaK, a chaperone whose substrate pool overlaps with that of TF[15]. DnaK is targeted to high-affinity sites by the cochaperone DnaJ, which activates tight substrate binding by triggering hydrolysis of DnaK-bound ATP. Substrate ejection is controlled by GrpE-catalyzed ADP/ATP exchange. Once released, a newly synthesized protein may reach a native conformation, undergo additional cycles of interactions with DnaK (and possibly TF) until it folds, or be transferred to the downstream GroEL-GroES system which handles about 10% of newly synthesized host proteins[16]. GroEL is an ≈800-kDa oligomer organized as two stacked homoheptameric rings, one of which is always bound by the cochaperone GroES[12]. GroEL substrates, which consist of structured but nonnative proteins up to 60-kDa in size, are bound by the free ring and allowed to fold at infinite dilution within the central chamber in a process controlled by reversible GroES capping and conformational changes orchestrated by ATP binding and hydrolysis[12].

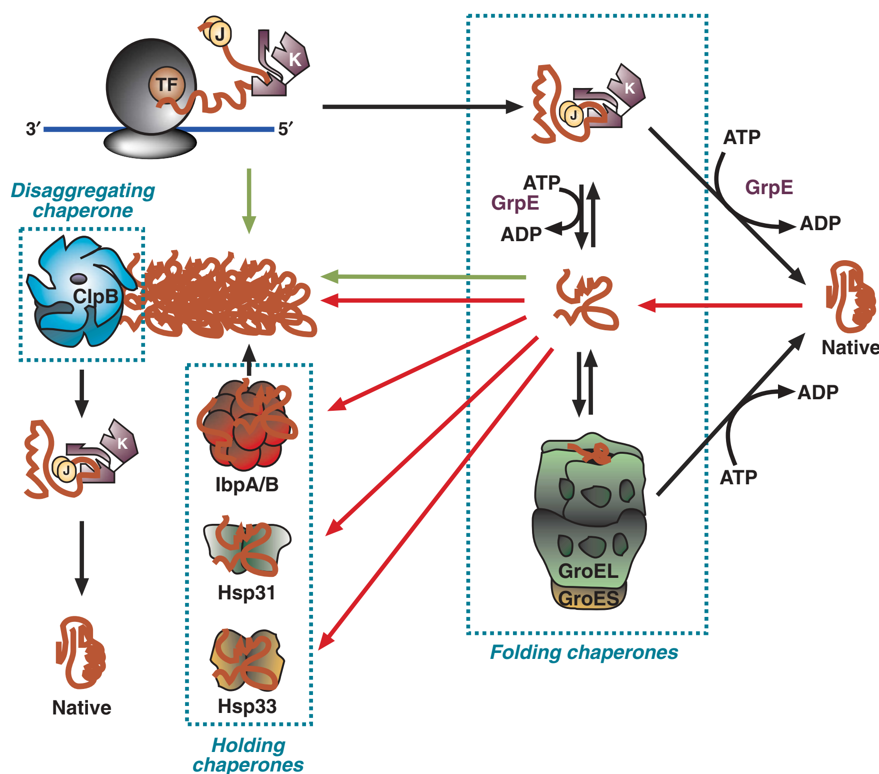

Figure 1 Chaperone-assisted protein folding in the cytoplasm of *E. coli*. Nascent polypeptides requiring the assistance of molecular chaperones first encounter TF or DnaK-DnaJ. Both chaperones engage solvent-exposed stretches of hydrophobic amino acids, shielding them from the solvent and each other. After undocking from TF- or GrpE-mediated release from DnaK, folding intermediate may reach a native conformation, cycle back to DnaK-DnaJ or be transferred to the central chamber of GroEL for folding at infinite dilution upon GroES capping. In times of stress (red arrows), thermolabile proteins unfold and aggregate. IbpB binds partially folded proteins on its surface to serve as a reservoir of unfolded intermediates until folding chaperones become available and intercalates within large aggregates. The holding chaperones Hsp33 and Hsp31 become important under oxidative and severe thermal stress, respectively. ClpB promotes the shearing and disaggregation of thermally unfolded host proteins and cooperates with DnaK-DnaJ-GrpE to reactivate them once stress has abated. Recombinant proteins that miss an early interaction with TF or DnaK/DnaJ, that undergo multiple cycles of abortive interactions with folding chaperones or titrate them out, accumulate in inclusion bodies (green arrows).

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In addition to mediating proper *de novo* folding, DnaK and GroEL refold host proteins that become unfolded when cells experience environmental stress. They are assisted in this task by holding chaperones (holdases) that stabilize partially folded proteins without actively promoting their remodeling (**Fig. 1** and **Table 1**). The most extensively characterized holdases belong to the small Hsp family[17]. The bacterial representatives, IbpA and IbpB, are two homologous 16-kDa proteins encoded on a single operon[5]. IbpB forms large oligomers and relies on temperature-driven exposure of structured hydrophobic domains to capture unfolded intermediates of denaturation-prone proteins on its surface[18]. Once stress abates, IbpB-bound species are engaged by DnaK, and if necessary transferred to GroEL, for refolding[19].

Hsp33, which was identified on the basis of its thermal induction, is also classified as a holdase[20]. The main function of this redox-regulated chaperone is to manage oxidative protein misfolding[20]. Under balanced conditions, Hsp33 is a reduced monomer that coordinates a zinc atom via four conserved cysteines. When cells are exposed to reactive oxygen species—a situation that often accompanies heat shock—the cytoplasm becomes more oxidizing, Hsp33 monomers form intramolecular disulfide bonds, which trigger zinc release, and the protein adopts a dimeric conformation exhibiting chaperone activity[20]. The thioredoxin and glutaredoxin systems (see below) rapidly reduce Hsp33 disulfides in a process that does not cause substrate release but primes the chaperone for fast inactivation. Upon return to nonstress conditions, DnaK-DnaJ engage the bound substrate and refold it alone or with the help of GroEL-GroES[21].

Hsp31, a recently characterized cytoplasmic folding modulator[22,23] also functions as a holdase that binds early unfolding intermediates in times of severe stress, thereby preventing overloading of the DnaK-DnaJ-GrpE system[24]. The interface of this homodimer of 31-kDa units contains a ≈20-Å hydrophobic bowl proximal to flexible linker-loop regions that shield large nonpolar patches on either side of the bowl[25,26]. Temperature-induced motion of the linker-loop domains allows efficient capture of unfolding intermediates by uncovering high-affinity binding sites adjacent to the bowl[27]. The linker-loop region may also play a role in substrate ejection by returning to its original position upon stress abatement. Although Hsp31 is not an ATPase, its chaperone activity is negatively regulated by ATP binding at high temperature[23], possibly to coordinate substrate capture with the needs of the chaperone network.

Because folding and holding chaperones fail to abrogate protein aggregation under severe or prolonged stress conditions, *E. coli* possesses a third line of defense to manage the deleterious effects associated with misfolding: active aggregate solubilization. Disaggregation is performed by ClpB, a member of the Hsp100 family of ring-forming ATPases that also include ClpA, ClpX and ClpY, three proteins whose primary function is in proteolysis[28] (see below). The structure of *Thermus thermophilus* ClpB suggests that solubilization may rely on both a 'crowbar' action involving a long surface exposed coiled-coil domain, as well as net unfolding of the substrate by threading through the ≈16-Å central pore of the chaperone[29,30]. ClpB-mediated disaggregation is facilitated by intercalation of small Hsps within the aggregates[31] but renaturation requires transfer of partially folded substrate from ClpB to DnaK[32,33]. Interestingly, DnaK-DnaJ can solubilize small aggregates *in vitro*[34] and their interaction with large aggregates may be necessary for the initial steps of ClpB-driven disaggregation[35].

More poorly characterized folding modulators include HtpG, which may play a role in *de novo* folding and secretion, the specialized DnaK paralogs HscA and HscC (**Table 1**), and SlpA and SlyD, two homologous PPIases of unclear function.

Protein export

Proteins synthesized in the cytoplasm may remain in this compartment, integrate within the inner membrane or translocate to the periplasm[36] (**Fig. 2**). In *E. coli*, the vast majority of proteins destined for export are secreted by the Sec-dependent pathway and are

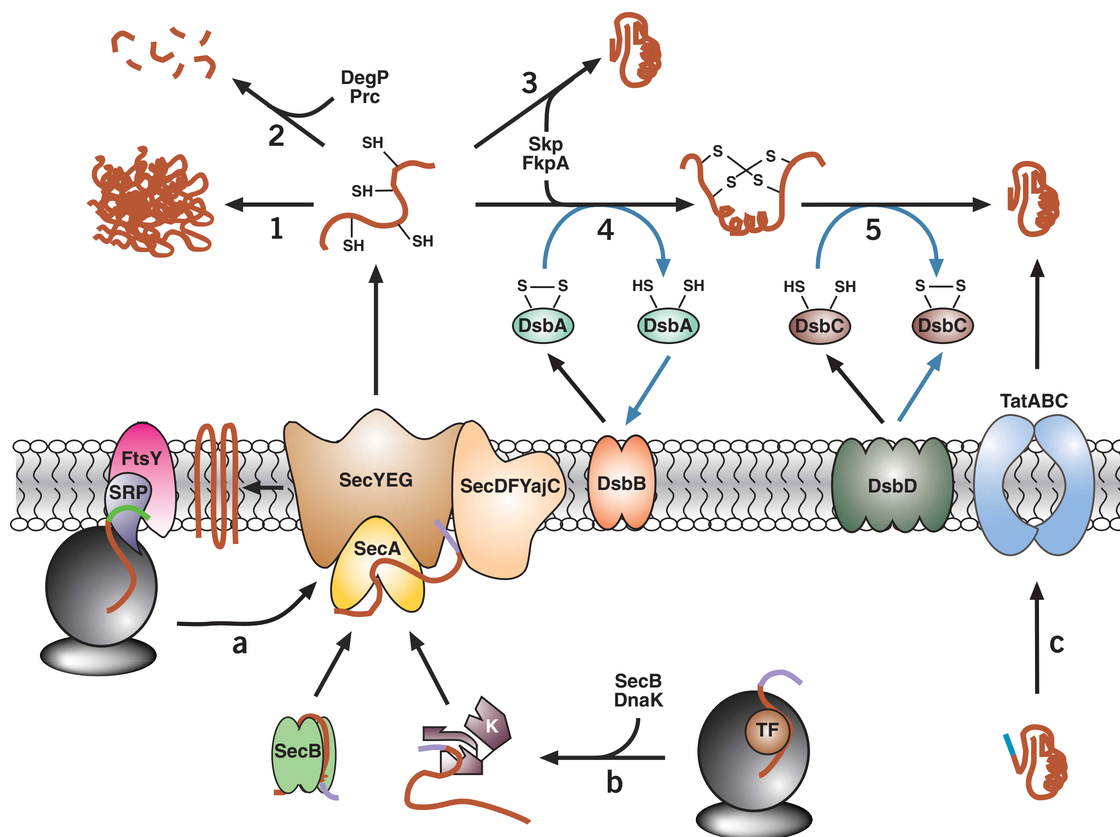

Figure 2 Export and periplasmic folding pathways. Proteins destined for export can be translocated across the inner membrane in three different fashions. (**a**) Preproteins with highly nonpolar signal sequences (green) or transmembrane segments of inner membrane proteins are recognized by SRP which, along with TF, scans nascent chains. SRP-dependent export involves delivery of the ribosome-nascent chain complex to FtsY and subsequent translocation through the SecYEG-SecDFYajC translocon. (**b**) The vast majority of preproteins have less hydrophobic signal sequence (lavender) and undergo Sec-dependent export. TF associates with the nascent polypeptide, halting cotranslational folding. As the chain grows, TF dissociates and the polypeptide is transferred to SecB or DnaK that maintain it in an extended conformation. Delivery to SecA and ATP-dependent translocation through SecYEG completes the process. (**c**) Preproteins with signal sequences containing the twin-arginine motif (cyan) are exported via the Tat-dependent pathway in a folded form. After cleavage of the signal sequence, partially folded periplasmic proteins may aggregate (1), undergo proteolysis (2) or reach a native conformation, possibly with the assistance of folding modulators (3). Cysteine pairs in proteins containing disulfide bonds are oxidized by DsbA (4) whereas incorrect disulfides are isomerized by DsbC (5). These oxidoreductases are reactivated by DsbB and DsbD, respectively. Black arrows show products obtained after each step, whereas blue arrows represent electron flow.

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synthesized with an amino-terminal signal sequence, 20–30 amino acids in length, that consists of a hydrophobic core followed by a proteolytic cleavage site. Efficient export of the resulting preproteins requires targeting to the membrane-associated translocation apparatus in an extended conformation. The homotetrameric secretory chaperone SecB maintains large (>200 residues) preproteins in an export-competent form by using two 70-Å-long hydrophobic channels running along its sides[37]. Although generic chaperone such as DnaK and GroEL can also perform this duty[38], SecB has the advantage of containing an acidic 'top' region that allows it to dock and transfer the protein cargo to the peripheral membrane protein SecA[37]. Through cycles of ATP-hydrolysis, SecA drives itself and the preprotein into the pore formed by the integral membrane proteins SecYEG. Translocation to the periplasm is dependent upon the proton motive force and facilitated by the SecDFYajC complex (**Fig. 2**, path a). In the process, the signal sequence is removed by the membrane-associated Lep or Lsp signal peptidases (the latter being specific for glyceride-modified prolipoproteins).

A subset of proteins is exported via the signal recognition particle (SRP)-dependent pathway[39] (**Fig. 2**, path b). Bacterial SRP, composed of a 48-kDa GTPase termed Ffh and the 114 nt-long 4.5S RNA, can bind either to the signal sequence of certain secretory proteins (provided that it is highly hydrophobic) or to transmembrane segments of inner membrane proteins as they emerge from the ribosome. The SRP-bound ribosome nascent chain complex (RNC) is then targeted to the membrane-bound receptor FtsY. Upon GTP hydrolysis by SRP and its receptor, the SRP-RNC-FtsY complex dissociates and the RNC is transferred to SecYEG for cotranslational translocation in a process that involves SecA[40].

Because of its ability to bind nascent proteins, TF also plays a role in both Sec- and SRP-dependent protein secretion. In the former pathway, TF seems to sequester nascent chains for relatively long periods of times (perhaps until half of a typical 30-kDa protein has been translated), thereby allowing larger, unstructured proteins to be efficiently engaged by SecB[41]. In the case of SRP-dependent export, TF and SRP, which share a common attachment site (the L23 ribosomal protein), both sample nascent chains exiting from the ribosome. The appearance of a highly hydrophobic signal sequence or transmembrane segment leads to high-affinity SRP binding, and subsequent interaction with FtsY results in TF ejection and initiation of cotranslational translocation[41].

Table 2 Periplasmic folding modulators

Classification	Protein	Substrates
Generic chaperones	Skp (OmpH)	Outer membrane proteins and misfolded periplasmic proteins
	FkpA	Broad substrate range
Specialized chaperones	SurA	Outer membrane proteins
	LolA	Outer membrane lipoproteins
	PapD (and its family)	Proteins involved in P pili biosynthesis
	FimC	Proteins involved in type 1 pili biosynthesis
PPIases	SurA	Outer membrane β-barrel proteins
	PpiD	Outer membrane β-barrel proteins
	FkpA	Broad substrate range
	PpiA (RotA)	Unknown
Proteins involved in disulfide bond formation	DsbA	Reduced cell-envelope proteins
	DsbB	Reduced DsbA
	DsbC	Proteins with nonnative disulfides
	DsbG	Proteins with nonnative disulfides
	DsbD	Oxidized DsbC, DsbG and CcmG
	DsbE (CcmG)	Cytochrome *c* biogenesis
	CcmH	Cytochrome *c* biogenesis

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Whereas both Sec- and SRP-dependent pathways handle preproteins that have not yet reached a native conformation, the twin-arginine (Tat)-dependent secretion pathway exclusively deals with folded or partially folded proteins. Proteins exported via the Tat pathway are produced with a signal sequence that contains a conserved—but not absolutely required[42]—twin arginine motif and most natural substrates are redox cofactor-binding proteins necessary for anaerobic respiration. Four integral membrane proteins, TatA, TatB, TatC and TatE, make up the Tat-export machinery (**Fig. 2**, path c). Although, the precise mechanism of Tat-dependent transport remains controversial[43], it has been postulated that the TatBC complex recognizes substrate proteins and delivers them to TatA, which forms a transport channel capable of accommodating substrates with diameters of up to 70 Å[44]. TatE seems to be interchangeable with TatA[45].

Periplasmic folding modulators

The periplasm contains a single bona fide chaperone termed Skp that captures unfolded proteins as they emerge from the Sec translocation apparatus (**Fig. 2**) and whose primary function is to assist the folding and membrane insertion of outer membrane proteins[46,47]. In the Skp homotrimer, α-helical tentacles extending from a β-barrel body define a central cavity that can accommodate nonnative substrates or folding modules up to ≈20-kDa in size[48]. Consistent with the absence of a periplasmic ATP pool, Skp chaperone activity is ATP independent[48] and this chaperone is likely a holdase.

Other periplasmic folding modulators include the PPIases SurA, FkpA, PpiA and PpiD (**Table 2**). Among these, FkpA has the most generic folding activity[49] and combines PPIase and chaperone functions[50]. FkpA is a V-shaped homodimer with N-terminal segments responsible for dimerization and chaperone activity and C-terminal PPIase domains[51]. FkpA is believed to cradle partially folded substrates within the hydrophobic cleft formed at the dimerization interface, allowing the flexible C-terminal domains easy access to prolyl bonds requiring isomerization.

SurA, which contains two parvulin-like PPIase domains, relies on its chaperone activity–rather than PPIase activity–to support the maturation of trimeric outer membrane proteins[52]. It resembles an asymmetric dumbbell and contains a deep cleft within its core module that may be responsible for substrate binding[53]. The fact that SurA preferentially recognizes an *Ar*-X-*Ar* motif (where *Ar* is an aromatic and X any residue) that is common in outer membrane proteins but infrequent in other polypeptides may explain its substrate specificity[54].

One of the features that distinguish the periplasm from the cytoplasm is its oxidizing environment. Indeed, in wild-type *E. coli*, stably disulfide-bonded proteins are only found in the cell envelope where disulfide formation and isomerization is catalyzed by a set of thiol-disulfide oxidoreductases known as the Dsb proteins[55,56]. DsbA, a soluble periplasmic protein containing a C-P-H-G active site embedded in a thioredoxin-like fold uses its highly reactive Cys30 to promote disulfide transfer to substrate proteins by the formation of mixed disulfide species (**Fig. 2**, path 4). It is kept in an active oxidized state by DsbB, an inner membrane protein exposing two loops to the periplasm, each containing two cysteines. DsbA recycling involves initial attack of the Cys104-Cys130 DsbB disulfide and coordinated involvement of all four DsbB cysteines before release of oxidized DsbA[56]. If incorrect disulfide bonds form in proteins containing more than two cysteines, the disulfide bond isomerase DsbC comes to the rescue (**Fig. 2**, path 5). This soluble V-shaped homodimer is structurally similar to Skp with N-terminal dimerization domains and C-terminal thioredoxin folds containing C-G-Y-C active sites[57]. DsbC is thought to capture folding intermediates within the uncharged cleft formed by its dimerization interface and to use its reduced Cys98 to attack disulfides in substrate proteins, thereby catalyzing isomerization in a process involving mixed disulfide intermediates. DsbC is maintained in a reduced state by the inner membrane protein DsbD at the expense of NADH oxidation in the cytoplasm[55,56]. DsbA, DsbC and DsbG all exhibit chaperone activity, presumably because a partially folded structure is needed to allow efficient disulfide formation and isomerization in substrate proteins.

Proteolysis

The degradation of misfolded proteins by host proteases guarantees that abnormal polypeptides do not accumulate within the cell and allows amino acid recycling. Targets for degradation include prematurely terminated polypeptides, proteolytically vulnerable folding intermediates that are kinetically trapped off-pathway, and partially folded proteins that have failed to reach a native conformation after multiple cycles of interactions with folding modulators.

In the cytoplasm, proteolytic degradation is initiated by five ATP-dependent heat shock proteases (Lon, ClpYQ/HslUV, ClpAP, ClpXP and FtsH) and completed by peptidases that hydrolyze sequences 2–5 residues in length. These proteases consist of a remodeling component or domain that binds substrate proteins and couples ATP hydrolysis to unfolding and transfer of the polypeptide to an associated protease domain or proteolytic component.

Lon is a tetrameric serine protease of 87-kDa subunits containing three functional domains. Its N-terminus is involved in substrate recognition and binding whereas its central and C-terminus domain

are responsible for ATPase and proteolytic activities, respectively. In addition to being responsible for bulk protein degradation[58,59], Lon also exerts a regulatory function by degrading a class of proteins that are designed to be unstable (e.g., SulA).

ClpA (84 kDa), ClpX (46 kDa) and ClpY (49 kDa) assemble into hexameric ATPase rings that recognize and actively unfold proteins destined for degradation or remodeling by threading them through a central channel in a process fueled by ATP-driven conformational changes[60]. Both ClpA and ClpX associate with the same serine proteolytic component ClpP (a protein organized as two stacked heptamers of 23-kDa subunits) via 'ClpP loops' containing a [L/I/V]-G-[F/L] tripeptide absent in ClpB[61]. Because ClpP presents two identical faces for ClpA/ClpX interactions, complexes consisting of one or two ClpA or ClpX rings bound to one ClpP double-ring as well as heteromeric ClpA:ClpP:ClpX complexes can all form *in vitro*[62]. It has been suggested that ClpA:ClpP:ClpA particles may be best suited to carry out the remodeling of a subset of substrate proteins that are released without degradation[62]. ClpY specifically binds to either or both ends of ClpQ, a proteolytic component organized as two stacked hexameric rings of 19-kDa subunits, to form a structure that resembles the eukaryotic 26S proteaseome[63].

Together with Lon, ClpYQ is thought to be primarily responsible for the degradation of abnormal proteins[58]. Although also involved in bulk proteolysis, ClpXP, and to a lesser extent ClpAP, specifically degrade prematurely terminated proteins that have been modified by attachment of an SsrA tag (AANDENYALAA) at their C terminus[64]. Shuttling of tagged substrates to ClpX requires binding of the adaptor protein SspB to the SsrA tag and its subsequent interaction with the N terminus of ClpX[65]. ClpAP does not appear to require such an usher protein. However, an adaptor protein termed ClpS that associate with the N terminus of ClpA redirects ClpAP protease activity from soluble proteins to aggregated species[66].

FtsH (HflB), the only ATP-dependent cytoplasmic protease associated with the inner membrane, is organized as an hexamer of 71-kDa subunits that associates with dimers or hexamers of the HflK-HflC inner membrane proteins to form 1-MDa complexes[67]. FtsH relies on its cytoplasmic metalloprotease active site to degrade both membrane-embedded and soluble cytosolic proteins including the heat shock sigma factor σ^{32} and SsrA-tagged proteins. The role of HflKC in FtsH function is poorly understood.

Accumulation of misfolded proteins also occurs in the cell envelope owing to temperature increase, oxidative stress or improper formation of disulfide bonds. The primary housekeeping periplasmic protease is DegP a hexamer formed by staggered association of trimeric rings[68]. The proteolytic sites of DegP are located within an inner cavity bounded by mobile side walls formed by PDZ domains. PDZ regions control access to the protease chamber and are likely involved in substrate binding[69]. At low temperatures, DegP switches function from protease to chaperone[70] but the physiological relevance of this activity remains unclear. A second generic periplasmic protease, Prc (Tsp), cleaves proteins with nonpolar C-termini, membrane proteins and unfolded polypeptides with broad primary sequence specificity[71]. Prc contains a single PDZ domain that has been implicated in substrate binding[72].

Additional cell envelope proteases include DegS, DegQ, Protease III and OmpT. DegS is an inner membrane homotrimer that senses protein misfolding by binding Y-X-F tripeptides (where X is any amino acid) exposed at the C-terminus of immature outer membrane porins. Motion of the PDZ domains results in the cleavage of RseA, a transmembrane protein that sequesters the extracytoplasmic factor σ^{24}, thereby allowing high-level production of proteins involved in combating protein misfolding in the cell envelope[73]. The more poorly characterized serine protease DegQ hydrolyzes denatured substrates at discrete V/I-X locations[74], whereas protease III (the *ptr* gene product) is involved in the degradation of both protein fragments and larger abnormal proteins[75]. OmpT is an outer membrane protein specific for paired basic residues, which is organized in a vase-like structure with a serine active site that faces the growth milieu[76]. Because OmpT readily adsorbs to inclusion bodies during cell lysis and remains active under highly denaturing conditions[77], *ompT* null strains should always be used if the refolding route is chosen.

Cytoplasmic folding pathways engineering

Many eukaryotic proteins of therapeutic or commercial interest possess complex tertiary and quaternary structures and often require the formation of multiple disulfide bonds and other post-translational modifications to reach a native, biologically active conformation. Producing these proteins in *E. coli* can be challenging because the cellular environment, folding machinery and conformational quality control checkpoints of prokaryotes are quite different from those of eukaryotes. Not surprisingly, inclusion body formation and proteolytic degradation are commonly observed upon heterologous protein overexpression in *E. coli*.

A traditional approach to alleviate these problems involves reducing the synthesis rate of the target gene product to promote proper folding. This can be achieved by using weaker promoters or by decreasing the concentration of gratuitous inducer. For promoters based on *lac*-derived control elements (e.g., the *tac* or *trc* promoters), isopropyl D-thiogalactopyranoside (IPTG) concentrations below 100 M are suitable for partial induction. However, because the P_{BAD} promoter operates in an 'all or none' fashion in wild-type cells, graded induction by subsaturating arabinose concentrations is only possible in strains that have been engineered to constitutively transport arabinose[78,79].

An alternative strategy is to decrease the temperature at which the recombinant protein accumulates. The use of low temperatures has the combined advantages of slowing down transcription and translation rates and of reducing the strength of hydrophobic interactions that contribute to protein misfolding. The drawback of this approach (as is the case with low inducer concentrations) is a reduction in productivity. Because traditional promoter systems exhibit reduced efficiency below 15°C, cold-inducible promoters, such as that of the major *E. coli* cold-shock gene *cspA*, are best suited for driving transcription at very low temperatures[80]. *cspA*-driven transcription is also useful for the expression of proteolytically sensitive and membrane-associated gene products[81] and companion strains that relieve promoter repression after prolonged incubation at low temperature are available[82]. Second generation *cspA*-based expression vectors have recently been described[83].

One of the most extensively used approaches to improve the yields of soluble proteins in the *E. coli* cytoplasm involves coexpression of molecular chaperones implicated in *de novo* protein folding. The beneficial effects of an increase in the intracellular concentration of TF, DnaK-DnaJ (with or without GrpE) and GroEL-GroES is well documented and a number of plasmids compatible with the routinely used ColE1-derived expression vectors are available[84]. DnaK-DnaJ or TF overexpression is suitable to increase the solubility of proteins requiring the assistance of chaperones in the early stages of their folding pathway[85]. For folding intermediates that rapidly transit through the TF/DnaK or require help at later folding stages, GroEL-GroES coexpression may be most beneficial. Technically, the GroEL-GroES encapsulation mechanism should limit the usefulness of this system to proteins smaller than ≈60 kDa. Nevertheless, larger proteins may also

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benefit from GroEL-GroES coexpression[86], presumably because GroES-independent stabilization of partially folded domains by GroEL facilitates correct folding of the remainder of the chain[87]. If aggregation-prone intermediates are formed at both early and late stages of the folding pathway, coordinated expression of DnaK-DnaJ (or TF) and GroEL-GroES may be required to maximize recovery of the target protein in a soluble form[85,88].

Nevertheless, there are many—and often unpublished—studies in which coexpression of folding modulators fails to improve recombinant protein solubility. The underlying mechanisms are unclear but may be related to the need for timely interactions with specific folding modulators or to the substrate folding pathway itself. Indeed, it was recently reported that binding of TF and DnaK to nascent firefly luciferase chains redirects the folding of this protein from an efficient eukaryotic cotranslational mode to a slower post-translational pathway that is accompanied by aggregation[89]. Consequently, certain multidomain eukaryotic proteins that have evolved to take advantage of cotranslational folding may not benefit from chaperone overproduction. It should finally be noted that chaperone overexpression may also reduce the overall yield of recombinant proteins[90] (defined as the sum of soluble and insoluble fractions), possibly by transiently stabilizing off-pathway intermediates that are subsequently degraded by host proteases. In such cases, an increase in the yields of soluble and bioactive product may be achieved in strains bearing mutations in chaperone systems[91].

Stable disulfide bonds do not form in the cytoplasm of *E. coli* owing to their rapid reduction by the combined action of thioredoxins and glutaredoxins[92]. Enzymes from both pathways share a thioredoxin fold and a C-X-X-C active site. In their reduced form, the thioredoxins TrxA and TrxC attack disulfides in substrate proteins and leave them reduced while becoming oxidized in the process. Thioredoxin reductase (TrxB) recycles oxidized TrxA/C by reducing active site disulfides in an NADPH-dependent manner. GrxA, GrxB and GrxC perform a similar disulfide bond–reductase function but belong to the glutaredoxin pathway. They are kept in a reduced state through the action of tripeptide glutathione (the product of the *gshA* and *gshB* genes), which is in turn reduced by glutathione reductase (Gor).

Identification of the members of thioredoxin and glutaredoxin pathways and subsequent elucidation of their roles in disulfide bond reduction has made it possible to manipulate the *E. coli* cytoplasm to rationally promote disulfide bond formation in heterologous proteins. Production of oxidized proteins in the cytoplasm was first demonstrated in *trxB* mutants[93] and later shown to be due to a reversal of function of TrxA and TrxC from reductases to oxidases, owing to their accumulation in a disulfide-bonded form in the absence of TrxB[94]. Later work showed that the cytoplasm could be made even more reducing by incubating *trxB* cells at low temperatures[90,95] or by combining *trxB* and *gshA* or *gor* null mutations[96]. Although aerobic growth of the double mutants was impeded[96], suppressor strains exhibiting good growth characteristics were isolated and shown to be suitable for enhancing disulfide bond formation in heterologous proteins[97,98]. The yields of properly disulfide-bonded proteins in *trxB gor* suppressor cells can be further increased by coexpressing folding modulators including TF, GroEL-GroES and variants of Skp and DsbC that remain cytoplasmic due to the removal of their signal sequences[97–99]. In the last case, the chaperone activity of DsbC, rather than its disulfide isomerase activity, is determining in enhancing folding[98].

Despite the usefulness of the strategies described in the above paragraphs, recombinant gene products are commonly sensitive to proteolysis. In such cases, strains lacking ATP-dependent cytoplasmic proteases may be useful expression hosts, particularly if a single protease is responsible for degradation and if production is carried out at the laboratory scale. However, this approach is not without drawbacks. For example, thermosensitive *ftsH* mutants exhibit poor growth characteristics and *E. coli* K-12 mutants lacking Lon are filamentous and unsuitable for high-density fermentations. The fact that the *E. coli* B strain BL21 retains good growth characteristics while lacking both *lon* and *ompT* explains the popularity of this host. It is, however, not known if the cells compensate for these deficiencies by upregulating the concentration of other proteases.

Export pathways engineering

Recombinant proteins can be targeted to the periplasmic space in a Sec-dependent fashion by fusing naturally occurring signal sequences (e.g., those of PelB, OmpA, MalE or PhoA) to their N terminus. Periplasmic expression has a number of advantages over cytoplasmic production. First, an authentic N terminus can be obtained after removal of the signal sequence by leader peptidases. Second, the periplasm is conducive to disulfide bond formation because of the presence of the Dsb machinery. Third, there are fewer proteases in the periplasm compared to the cytoplasm and many have specific substrates. Finally, because the periplasm contains fewer proteins and because its content can be selectively released by osmotic shock or other strategies[100,101] purification of the target protein is facilitated.

One of the difficulties associated with heterologous protein secretion is inefficient export, which manifests itself by the degradation or aggregation of preproteins in the cytoplasm, and, in the cases of highly hydrophobic or integral membrane proteins, by membrane jamming which is associated with toxicity and eventual cell death. The use of low temperatures[81] or the cooverexpression of chaperones involved in secretion (e.g., SecB, DnaK-DnaJ and GroEL-GroES) may alleviate these problems. However, the benefits of the latter approach are highly dependent on the signal-sequence/mature-protein combination[102].

E. coli mutants originally selected for their ability to support the export of proteins with defective signal sequences offer an alternative route to promote secretion. One such allele, *prlA4*, encodes a defective version of SecY that leads to enhanced translocation rates, increased affinity of SecA for the SecYEG translocon, reduced reliance of Sec transport on the proton motive force and export of preproteins with folded domains[103,104]. The observation that inactivation of TF (encoded by *tig*) accelerates protein export and reduces the dependency of preproteins on secretory factors such as SecB[105,106] suggests that Δ*tig* strains will also be useful to enhance heterologous protein secretion. Finally, an artificial increase in signal sequence hydrophobicity may redirect translocation from the Sec- to the SRP-dependent pathway and concomitantly eliminate toxicity effects associated with membrane jamming by tightly coupling secretion with translation[105].

Owing to its relatively recent characterization, the potential of the bacterial Tat-dependent export pathway for heterologous protein secretion has not yet been fully explored. However, successful translocation of several heterologous proteins (including single chain and Fab antibody fragments[107]) suggest that Tat-dependent secretion will be a valuable tool for the secretion of heterologous proteins that assume a folded or a partially folded form before reaching the Sec machinery. Already, it has been shown that coexpression of the phage shock protein PspA improves the secretory capacity of the Tat system[108].

Engineering of periplasmic folding pathways

As they emerge in an unfolded or partially folded form on the periplasmic side of the inner membrane, heterologous proteins also confront the task of reaching a native conformation. As previously mentioned, the two cell envelope chaperones exhibiting the widest

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substrate specificity are Skp and the PPIase FkpA (**Table 2**). Several studies have shown that coexpression of these folding helpers enhances heterologous protein folding and reduces degradation and periplasmic inclusion body formation[49,50,109–111]. Similarly, cooverproduction of DsbA or DsbC alone or in combination with DsbB and DsbD can facilitate the folding of proteins containing complex patterns of disulfide bonds[112–116]. It should finally be noted that, because the outer membrane is permeable to small solutes (<600 Da), an alternative way to reduce aggregation in the periplasm is to supplement the growth medium with small nonmetabolizable sugars, such as sucrose and rafinose[117,118]. By equilibrating within the periplasm, these sugars directly affect folding pathways, presumably by increasing protein chemical potentials via preferential exclusion effects[117].

The increased understanding of the specificity and mode of action of cell-envelope proteases suggests that a few simple precautions may go a long way in alleviating the problem of periplasmic degradation. First, because DegP is the major housekeeping protease and recognizes commonly occurring paired hydrophobic residues[69], *degP* null hosts should be routinely used. Second, proteins containing nonpolar C-terminal sequence should be expressed in *prc* (or perhaps *degS*) mutants, or as N-terminal fusions to carrier proteins. Finally, if proteolysis remains a problem, strains containing multiple mutations in the *degP*, *ompT*, *ptr* and *prc* genes may prove valuable. Nevertheless, the tradeoff associated with their reduced growth rates should be carefully considered[75,119].

The road ahead

The growing understanding of the principles that govern protein folding and misfolding in *E. coli*, the availability of sophisticated tools for chromosome engineering and rapid progress in PCR and directed evolution methodologies, have opened many new avenues of research. Already, it is obvious that one is not limited to traditional systems because the chaperone activity either of periplasmic folding helpers (e.g., Skp and DsbC[98,99]) or of oxidoreductases (e.g., TrxA[120,121]) can be co-opted to improve the folding of cytoplasmic recombinant proteins. Progress in the study of the structure-function relationship of folding modulators will undoubtedly allow rational improvements and/or coordination of chaperone and catalytic activities to promote more efficient heterologous protein folding.

The 'irrational' route also holds enormous potential. In a landmark study, Weissman and coworkers[122] showed that successive rounds of *in vivo* screening and DNA shuffling can be used to evolve GroEL variants exhibiting greatly enhanced ability to fold green fluorescent protein. Similar strategies could be applied to other chaperone-substrate pairs to isolate designer folding modulators dedicated to the efficient folding of specific, high-value recombinant proteins. That ClpB possesses disaggregation activity raises the possibility that engineered or evolved variants could solubilize cytoplasmic inclusion bodies and release component proteins in a conformation committed to folding, thereby enhancing the overall yields of native species during fermentation.

Finally, it has recently become apparent that *E. coli* can be manipulated to achieve post-translational modifications that have long been considered to be beyond its reach. For instance, engineering of the *Campylobacter jejuni* glycosylation pathway into *E. coli* has allowed (non-eukaryotic) N-linked glycosylation of the model protein AcrA[123]. More recently, Schultz and coworkers[124] evolved an orthogonal synthetase-tRNA pair suitable for the insertion of an exogenously added glycosylated amino acid (N-acetylglucosamine-serine) in response to amber codons, and obtained good yields of recombinant myoglobin containing N-acetylglucosamine-serine. Because N-acetylglucosamine serves as a substrate for the synthesis of more complex carbohydrates, further strain engineering to coexpress glycosyltransferases may provide an economically viable route for the production of therapeutic glycoproteins. Clearly, progress on the above issues and unexpected new discoveries all but guarantee the future of *E. coli* as an expression host.

ACKNOWLEDGMENTS
This work was supported by National Science Fund award BES-0097430.

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

Published online at http://www.nature.com/naturebiotechnology/

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www.impactaging.com AGING, April 2016, Vol 8 No 4

Review

Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies

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Key words: *apoptosis, defective apoptotic pathways, cancer, small molecules, miRNAs, p53*
Received: *01/04/16; Accepted: 03/08/16; Published: 03/27/16*
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Abstract: **Apoptosis is a form of programmed cell death that results in the orderly and efficient removal of damaged cells, such as those resulting from DNA damage or during development. 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. Deregulation in apoptotic cell death machinery is an hallmark of cancer. Apoptosis alteration is responsible not only for tumor development and progression but also for tumor resistance to therapies. Most anticancer drugs currently used in clinical oncology exploit the intact apoptotic signaling pathways to trigger cancer cell death. Thus, defects in the death pathways may result in drug resistance so limiting the efficacy of therapies. Therefore, a better understanding of the apoptotic cell death signaling pathways may improve the efficacy of cancer therapy and bypass resistance. This review will highlight the role of the fundamental regulators of apoptosis and how their deregulation, including activation of anti-apoptotic factors (i.e., Bcl-2, Bcl-xL, etc) or inactivation of pro-apoptotic factors (i.e., p53 pathway) ends up in cancer cell resistance to therapies. In addition, therapeutic strategies aimed at modulating apoptotic activity are briefly discussed.**

INTRODUCTION

Apoptosis, the programmed cell death, is finely regulated at gene level resulting in the orderly and efficient removal of damaged cells such as those occurring following DNA damage or during development [1]. The machinery of apoptosis is complex and involves many signaling pathways. Apoptosis can be triggered in a cell through either the caspase-mediated extrinsic or intrinsic pathways. Both pathways converge to activate the effector apoptotic caspases resulting ultimately in morphological and biochemical cellular alterations, characteristics of apoptosis [2]. Usually, the balance between the pro-apoptotic and anti-apoptotic protein regulators is a critical key point to determine if a cell undergoes apoptosis. The induction of apoptosis as result of DNA damage in precancerous lesions can remove potentially harmful cells, thereby blocking tumor growth. Deregulation of this death process is associated with unchecked cell proliferation, development and progression of cancer and cancer resistance to drug therapies [3,4]. For that reason, deregulation of apoptosis is considered one of the hallmarks of cancer [5]. Therapeutic strategies targeting molecules involved in apoptotic resistance therefore represent a valid approach to be pursued in order to restore cancer cells sensitivity to apoptosis and overcome the ineffectiveness of the treatments [6,7]. This article focuses on the mechanisms of apoptosis, how defects along the

apoptotic pathway contribute to cancer development and drug resistance and, briefly, how apoptosis can be used as a vehicle of targeted treatment in cancer.

Morphological and biochemical changes in apoptosis

From the morphological point of view apoptotic cells show a characteristic cytoplasmic cell shrinkage, budding of plasma membrane, membrane exposure of phosphatidylserine (PS) on extracellular side, chromatin condensation and DNA fragmentation [8,9]. The plasma membrane is intact throughout the total process. The expression of PS in the outer layers of the cell membrane allows early recognition of dead cells by macrophages, resulting in phagocytosis without the release of proinflammatory cellular components [10]. At the later stage of apoptosis some of the morphological features include membrane blebbing, ultrastructural modification of cytoplasmic organelles and loss of membrane integrity [11]. Usually phagocytic cells engulf apoptotic cells before apoptotic bodies occur [12]. Apoptosis is primarily executed by a family of proteases known as the caspases (cysteinyl, aspartate-specific proteases) [13]. Caspases are central to the mechanism of apoptosis as they are both the initiators (caspase-2, -8, -9 and -10, primarily responsible for the beginning of the apoptotic pathway) and the executors (caspase-3, -6 and -7, responsible for the definite cleavage of cellular components) of cell death [14]. After being produced as inactive proteins (zymogens or pro-caspases), the initiator caspases auto-activate through auto-proteolysis, a process that is facilitated by their interaction with specific adapter molecules [15]. Once activated, the initiator caspases cleave off the executors caspases that perform critical cleavage of specific cellular substrates resulting in the final apoptotic cell death [16]. This caspases activity is responsible of the apoptotic hallmarks, such as chromatin condensation, plasma membrane asymmetry and cellular blebbing. The extensive and irreversible proteolytic activity mediated by executor caspases represents the ultimate outcome of both the extrinsic and the intrinsic apoptotic pathways (see below). Thus, both pathways converge on caspases-3, 6, or -7 that allow disruption of DNA and cellular components inducing the typical morphological changes in apoptosis [17]. Of note, caspases activity has been also extended to non-apoptotic functions such as cell differentiation/maturation suggesting that the caspase cascade may become activated independently of– or without inducing- an apoptotic cascade [18-20].

Extrinsic apoptotic pathway

The extrinsic apoptotic pathway (death receptor-dependent) is initiated by the interaction of cell surface exposed death receptors, belonging to the superfamily of tumor necrosis factor receptor (TNFR), with their respective protein TNF family ligands [21]. Death receptors are structurally defined by an intracellular protein-protein interaction domain, called the death domain (DD), which is critically involved in apoptosis-inducing signalling [22]. The more broadly characterized signaling systems of death receptor-ligands include TNFR1-TNFα, FAS (CD95, APO-1)-FasL, TRAILR1 (DR4)-TRAIL, TRAILR2 (DR5)-TRAIL. Upon death receptor stimulation by its corresponding ligand, the same receptor undergoes oligomerization and a conformational change to reveal its cytoplasmic DD to support homotypic interactions with other DD-containing proteins [21]. The role of adapter proteins (FADD/TRADD) is to sequester, at level of this protein complex, the initiator pro-caspase-8 and/or -10 resulting in the formation of the so-called death-inducing signaling complex (DISC), increasing the local concentration of pro-caspase and promoting the mutual auto-activation [23]. The activation of initiator caspases results in the processing of the downstream effector caspases-3, -6 and -7 whose activation leads to the cleavage of essential substrates for cell viability, inducing cell death (Figure 1) [17]. Some cells do not die in response to the extrinsic pathway alone and require an amplification step that is induced by caspase-8. In this situation, capase-8 targets the BH3-only protein Bid (BH3-interacting-domain death agonist) for cleavage and generate the activated fragment t-Bid; t-Bid then directly activates pro-apoptotic multi-domain proteins to induce mitochondrial outer membrane permeability (MOMP), so this co-engages the intrinsic pathway [3] (Figure 1) (see below).

Intrinsic apoptotic pathway

The intrinsic apoptotic pathway (mitochondria-dependent) is mediated by intracellular signals that converge at the mitochondrial level in response to different stress conditions (i..e, irradiation, treatment with chemotherapeutic agents, etc.) [24]. Internal stimuli such as irreparable genetic damage, hypoxia, extremely high concentrations of cytosolic Ca^{+} and severe oxidative stress are some triggers of the initiation of the intrinsic mitochondrial pathway [25]. Subsequent activation of pro-apoptotic BH3-only members of the Bcl-2 family (Bax, Bak) neutralizes the antiapoptotic proteins Bcl-2, Bcl-xL, and Mcl-1, leading to disruption of mitochondrial membrane outer membrane permeability (MOMP) so that proteins normally confined in the intermembrane space spread into the cytosol. These proteins include the so-called apopto-genic factors, such as cytochrome-*c*, which plays a

crucial role in activating the mitochondrial-dependent death in the cytosol [26]. Cytochrome-*c* binds to the cytosolic Apaf-1 (apoptosis protease activating factor-1) and triggers the formation of a complex named apoptosome, which recruits initiator pro-caspase-9 to its caspase recruitment domain (CARD), allowing auto-activation and then proteolysis. The process in turn activates downstream executor caspases-3, -6 and -7 for cleavage of cellular substrates leading to apoptotic cell death (Figure 1) [27,28].

The B-cell lymphoma 2 (Bcl-2) family proteins

The intrinsic pathway is closely regulated by the B-cell lymphoma 2 (Bcl-2) family of intracellular proteins. This proteins family regulates both pro-apoptotic and anti-apoptotic intrinsic pathways controlling the alteration of MOMP [29]. Therefore, by mediating permeabilization of the mitochondrial membrane, the Bcl-2 proteins serve as an "apoptotic switch" [30]. The Bcl-2 proteins are classified into three subgroups, one group with anti-apoptotic and two with pro-apoptotic function, depending on the composition of typical BH (Bcl-2 Homology) domains, listed from BH1 to BH4 [31,32] (Figure 2). Whereas the BH1 and BH2 domains of bcl-2 are required for dimerization with pro-apoptotic proteins, BH3 domain is crucially important to the interaction between pro-apoptotic and anti-apoptotic proteins and is contained by all family members. The amino-terminal BH4 domain is mainly found in the bcl-2 family members with death-repressing activity, but is also present in some pro-apoptotic molecules. The anti-apoptotic multi-domain group includes Bcl-2, Bcl-xL, Bcl-W, Mcl-1, A1, and Bcl-B, containing from three to four BH domains; the pro-apoptotic multi-domain group includes Bax, Bak and Bok proteins, containing three

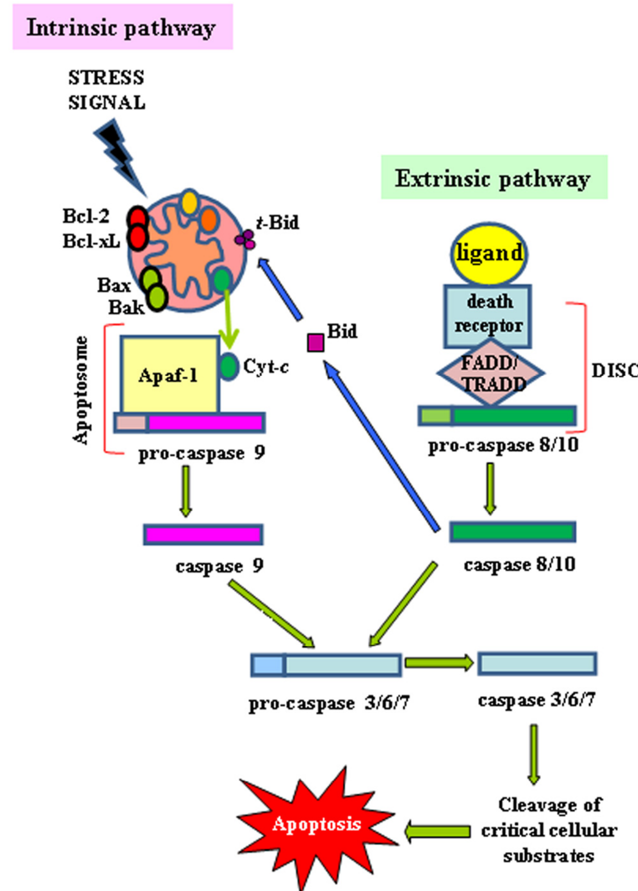

Figure 1. Intrinsic and extrinsic apoptotic pathways. The intrinsic (mitochondrial) and the extrinsic (ligands/death receptors) cell death pathways and their convergence through t-Bid are depicted (see text for details).

BH-domains (BH1, BH2 and BH3); and the pro-apoptotic BH3-only proteins group includes Bid (BH3 interacting-domain death agonist), Bim (Bcl-2-like protein 11), Bad (Bcl-2-associated death promoter), Puma (p53 upregulated modulator of apoptosis), Noxa, BMF, HRK and BIK (Figure 3) [33]. While the anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-*c*, the pro-apoptotic proteins act by promoting such release.

The balance and protein-protein interactions between Bcl-2 family members is required to determine whether a cell undergoes cell survival or apoptosis. The activation of Bax (cytosolic protein that translocates into mitochondria during induction of apoptosis), and Bak (integral membrane protein located in the mitochondria and endoplasmic reticulum) involves conformational changes that trigger the formation of homo-oligomeric protein complexes that end up altering

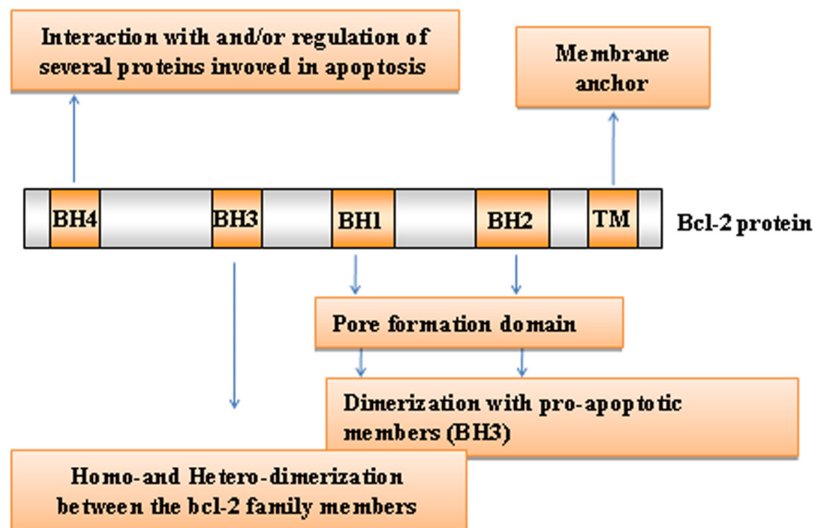

Figure 2. Bcl-2 family members domain composition and function. Typical BH (Bcl-2 Homology) domains, listed from BH1 to BH4, are shown. TM: transmembrane domain.

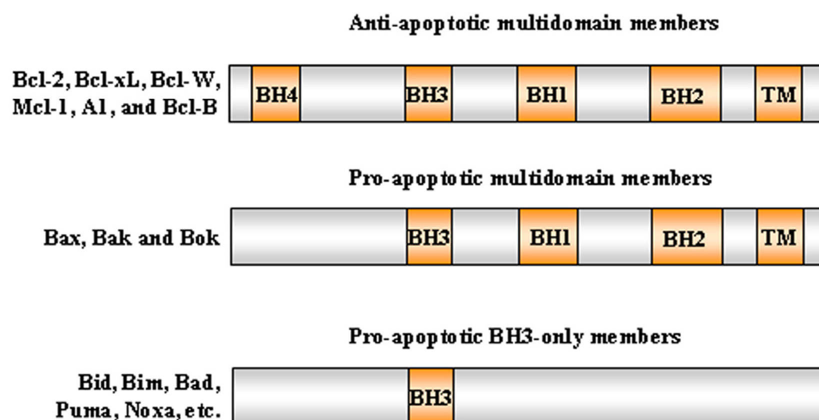

Figure 3. Bcl-2 protein subgroups. The Bcl-2 proteins are classified into three subgroups, one group with anti-apoptotic and two with pro-apoptotic function, depending on the composition of the typical BH domains, listed from BH1 to BH4. Representative members of each subfamily are shown.

the mitochondrial membrane permeability [34,35]. The pro-apoptotic BH3-only proteins act upstream of this event binding with high affinity to anti-apoptotic Bcl-2 family members thereby allowing Bax/Bak to elicit MOMP and activation of the caspase cascade [36,37]. Anti-apoptotic multidomain members of the Bcl-2 protein family not only counteract the pore-forming activity of Bax and Bak by engaging in direct inhibitory interactions, but also prevent the generation of pro-apoptotic cytosolic Ca^{2+} waves either by reducing capacity of endoplasmic reticulum (ER) Ca^{2+} storage, an effect that is antagonized by Bax and Bak or by interacting with inositol 1,4,5- trisphosphate (IP3) receptor [38,39]. Other apoptotic factors that are released from the mitochondrial intermembrane space into the cytoplasm include apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP Binding protein with Low pI (DIABLO) and Omi/high temperature requirement protein A (HtrA2) [40].

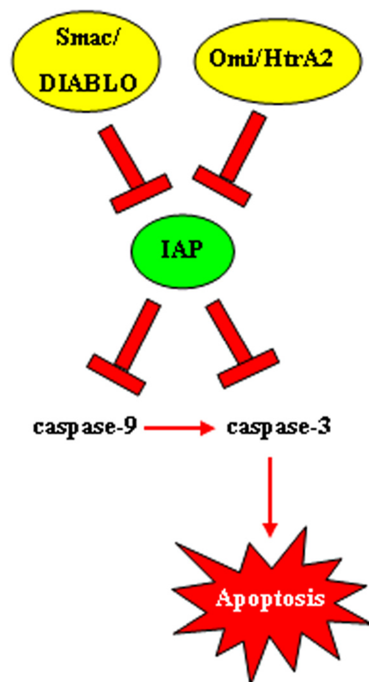

Figure 4. Function of inhibitors of apoptosis *proteins* (IAPs). IAPs are often overexpressed in cancer and they have the ability to bind and inactivate caspases 9 and 3. The activities of IAPs, on the other hand, may be suppressed by mitochondrial proteins, such as Omi/HtrA2 and Smac/DIABLO, released into the cytosol during apoptosis.

The inhibitors of apoptosis proteins (IAPs)

Considering that proteolysis is an irreversible process, strict control of caspases-mediated proteolytic cleavage is imperative to prevent inappropriate cell destruction [41]. Negative regulation of caspases function is achieved by IAP proteins family whose principal members in humans are NAIP (BIRC1), cIAP1 (BIRC2), cIAP2 (BIRC3), X-linked IAP (XIAP, BIRC4), Survivin (BIRC5), Apollon (BRUCE, BIRC6), Livin/ML-IAP (BIRC7), and IAP-like protein 2 (ILP2 – BIRC8) [42]. Their characteristic BIR (baculovirus IAP repeat) domain mediates the interaction with various proteins and gives them the ability to bind and inactivate caspases [43]. The activities of IAPs, however, may be suppressed by mitochondrial proteins, such as Omi/HtrA2 and Smac/DIABLO, released into the cytosol during apoptosis (Figure 4). These endogenous IAPs antagonists are able to bind to the BIR domain of IAPs reducing their ability to interact with caspase-3 or -9 thereby restoring their activity [44]. XIAP is the best characterized IAP so far and is generally recognized as the most potent endogenous caspase inhibitor. XIAP anti-apoptotic activity involves inhibition of active executor capsases as well as prevention of initiator caspase-9 activation [45].

Alterations of the apoptotic pathways

There are many ways through which both the extrinsic and the intrinsic apoptotic pathways may be altered, resulting in reduction of apoptosis or acquisition of apoptosis resistance. They include impaired death receptor signaling, disrupted balance between pro-apoptotic and anti-apoptotic proteins, reduced caspase function and impaired p53 function (Figure 5). Alteration of extrinsic apoptotic signaling has been associated with different types of human tumors, underscoring how the loss of activity of Fas-FasL system [46] or the aberrant expression of cytosolic components of this death receptor apoptotic pathway (i.e., FADD) [47] can contribute to the tumor transformation. Several genetic defects have been proven to contribute to the resistance of tumor cells to Fas-mediated apoptosis. Fas transcriptional silencing is a common oncogenic event in the epithelial transformation, while its mutation has been often associated with B-cell germinal center-derived lymphomas [48]. In acute myelogenous leukemia (AML) reduced or absent expression of FADD has been frequently observed, resulting in resistance to chemotherapy and poor patient prognosis [47,49]. Moreover, in several cancers including neuroblastoma, medulloblastoma, and small cell lung cancer (SCLC), absent or reduced expression of caspase-8 was reported

[50-52]. Another resistance mechanism reported in a variety of human tumors is the overexpression of anti-apoptotic protein c-Flip, recruited at the DISC level, that prevents the pro-caspase-8 auto-activation thereby rendering cell resistant to death receptor-mediated apoptosis [53-55].

As for the extrinsic pathway, alteration of some components of the intrinsic apoptotic pathway can play a fundamental role in the development of resistance to chemotherapy in different types of tumors. Disruption in the balance of anti-apoptotic and pro-apoptotic members of the Bcl-2 family results in deregulated apoptosis in the affected cells. This can be due to overexpression of one or more anti-apoptotic proteins or downregulation of one or more pro-apoptotic proteins or a combination of both. Anti-apoptotic Bcl-2 over-expression has been reported in several human cancers, including prostate cancer, diffuse large B-cell lymphoma (DLBCL), melanoma, etc. [56-58], resulting in protection of cancer cells from apoptosis or inhibition of TRAIL-induced apoptosis [59,60]. Overexpression of Bcl-xL has also been reported in colorectal cancer and Kaposi's sarcoma [61,62]. Such overexpression confers a multi-drug resistance phenotype in tumor cells and prevents them from undergoing apoptosis [63]. Thus, high expression levels of anti-apoptotic proteins Bcl-2 and Bcl-xL have been reported to correlate with cisplatin resistance and tumor recurrence in different cancers including non-small cell lung cancer (NSCLC), head and neck, ovarian, and breast [64-68]. On the other hand, mutations in the pro-apoptotic Bax gene have been reported in colorectal cancers and contribute to resistance to anticancer treatments [69]. Increased Bcl-2/Bax ratio has been reported in chronic lymphocytic leukaemia (CLL) patients. [70]. Other examples of alteration of the intrinsic pathway include reduced expression of the basic component of the apoptosome, Apaf-1, in melanomas [71,72], as result of promoter aberrant methylation. In addition, tumor cells resistance to apoptosis also occurs as a result of alteration of mediators that control the intrinsic apoptotic pathway downstream from the apoptosome formation, i.e. acting on caspase activity. In this regard, high level of IAPs expression has been found in different types of cancers, and this evidence is considered a marker of poor prognosis for patients [73,74].

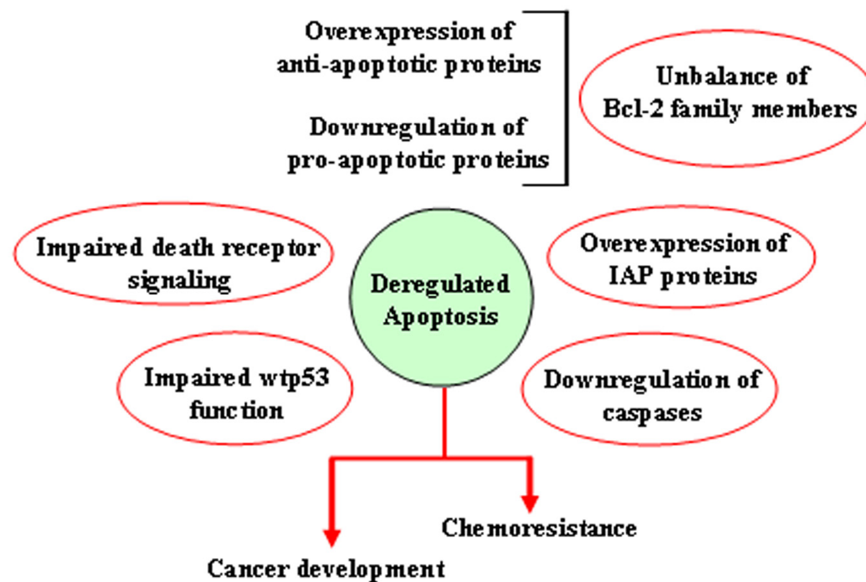

Figure 5. Mechanisms leading to deregulation of apoptosis. Schematic representation of the different ways through which both the extrinsic and the intrinsic apoptotic pathways may be altered, resulting in reduction of apoptosis or acquisition of apoptosis resistance.

Pharmacological targeting of the apoptotic pathways

Based on this evidence, restoration of apoptotic pathway by drugs targeting both apoptotic pathways constitutes a promising anticancer therapeutic approach. Regarding the extrinsic pathway, the down-regulation of c-Flip by metabolic inhibitors and the promotion of caspase-8 activation by interferon, are some examples of strategies aimed at making tumors responsive to death receptor-induced apoptosis, and more generally, to chemotherapy-induced apoptosis [55,75,76]. The therapeutic importance of inducing apoptosis through the extrinsic pathway also extends to cancer cells that do not show defects in components of that pathway. Indeed, inducing the apoptosis by stimulating the extrinsic pathway can overcome the resistance to therapeutic agents that act by causing DNA damage, as death receptor-dependent apoptosis may occur regardless of the stress response. An example of such therapeutic strategy is represented by the ligand TRAIL known to induce apoptosis in different tumor cell lines [77]. The preferential destructive effect against tumor cells and the apparent absence of systemic toxicity through TRAIL-induced apoptosis, led to the development of antibodies with agonistic activity against the TRAIL death receptors (DR4 and DR5) or soluble recombinant derivatives of TRAIL (sTRAIL) as promising chemotherapeutic agents [78]. An attractive strategy to sensitize resistant malignancies to TRAIL-induced cell death is the design of small molecules that target and promote caspase-8 activation. Through an *in silico* screening some authors successfully found a small molecule activator of caspase-8 [79]. Experimental validation performed in multiple cell lines, such as leukemic and prostate cells, revealed that CaspPro small molecule promotes caspase-8 activation, caspase-3 activation and PARP cleavage, in the presence of TRAIL, leading to cell death [79]. Owing to its different toxicity for transformed as opposed to normal cells, Apo2/TRAIL shows promise as potential cancer therapy agent [80,81].

As in the extrinsic pathway, mediators of the intrinsic pathway involved both in tumorigenesis and chemo-resistance, are targeted for therapeutic approaches. These anticancer strategies attempt to develop drug-designed inhibitors of anti-apoptotic proteins typically overexpressed in cancer cells, such as Bcl-2, Bcl-xL and IAPs [82]. Efforts to target Bcl-2 proteins involve the development of agents that disrupt Bcl-2 complexes. BH3 mimetics bind to the hydrophobic groove of antiapoptotic proteins mimicking the action of BH3-only proteins in binding to pro-survival proteins, leading to the release of BH3-only proteins from complexes and activation of BAX and BAK. So far, nearly a dozen BH3 mimetics are under investigation as Bcl-2 inhibitors in different phases of human clinical trials such as AT-101 (R-(-)-gossypol) [83,84], ABT-199 (venetoclax) [85], ABT-737 [86], ABT-263 (navitoclax, orally available derivative of ABT-737) [87,88], GX15-070 (obatoclax) [89,90] and TW37 [91]. The field of inhibitors of Bcl-2 family members is in continuous development [92,93], underscoring the importance of these molecules as potent anticancer agents. Moreover, targeting the specific BH4 domain of Bcl-2 is also emerging as a novel strategy for anticancer therapy [94]. Thus, Bcl-2, via its BH4 domain, cooperates with numerous proteins regulating different cellular pathways involved in tumor progression and chemoresistance such as hypoxia and angiogenesis [95-97]. Recently, a small molecule namely BDA-366 was discovered as a potent and effective BH4 domain antagonist, displaying remarkable anticancer activity in vitro and in vivo, thus providing the proof-of-concept of this approach [98]. Another innovative approach to inhibit Bcl-2 comes from the evidence that human bcl-2 gene contains a GC-rich sequence located in its promoter with the potential to form G-quadruplex structures [99] and functions as a transcriptional repressor element. Therefore, G-quadruplex-specific ligands can regulate the transcription of bcl-2 through stabilization of quadruplex structure [100,101].

Interestingly, it has been shown that the tumor suppressor p53, at least in part by transcription independent mechanisms, contributes to cell death induction by BH3 mimetic inhibitors of BCL-xL. In addition to mildly facilitating the ability of compounds to derepress BAX from BCL-xL, p53 also provides a death signal downstream of anti-apoptotic proteins inhibition that is independent from PUMA, as enhanced p53 can substitute for PUMA to promote BAX activation in response to BH3 mimetics [102]. It is thus of particular relevance that p53, even when expressed constitutively under conditions where it does not influence the expression of its pro-apoptotic transcription targets, enhances cell death induced by BCL-xL inhibition [102]. Such results suggest on one hand that BH3 mimetics may not totally substitute for the lack of an active p53 tumor suppressor in cancer cells; on the other hand, they imply that healthy tissues may be more harmed than anticipated when BCL-xL inhibitors are combined with chemotherapeutic agents that even mildly affect p53.

Among the therapeutic strategies targeting IAPs two approaches have being developed, that is the use of antisense oligonucleotides and of small-molecule inhibitors. The XIAP down-regulation through administration of antisense agents carried by an

adenoviral vector has been proven effective in inducing apoptosis in chemoresistant ovarian cancer cells [103] and sensitizing lung cancer cells to the radiation treatment [104]. Similarly, the inhibition of XIAP expression with specific oligomers has been shown to induce caspase-3 activity, boosting the apoptotic effect of cisplatin and TRAIL in human prostate androgen-insensitive cancer cells [105]. Moreover, preclinical studies have shown that Smac mimetics can directly trigger cancer cell death or sensitize tumor cells to various cytotoxic therapies, including conventional chemotherapy, radiotherapy, or novel agents. They promote activation of caspases by neutralizing XIAP-mediated caspase inhibition [106]. Therefore, the success of each therapeutic strategy depends mainly on the ability of the therapeutic tool to induce apoptosis either by targeting the overexpressed anti-apoptotic proteins or by stimulating the expression of the pro-apoptotic molecules.

However, it is worth to mention that the cancer genetic background may induce failure of apoptosis by drugs. In this regard, KRAS and the PI3K/AKT/mTOR pathway are frequently dysregulated in cancer and, for such reason, are the most critical targets in clinical oncology. Thus, direct targeting of KRAS has not been successful so far and, similarly, inhibition of the PI3K/AKT/mTOR pathway often results in apoptosis resistance. Using a panel of 20 human KRAS-mutant NSCLC (non-small cell lung cancer) cell lines Hata and collaborators show that most human KRAS-mutant cell lines fail to undergo marked apoptosis in response to AZD6244 (Selumetinib, a potent, selective, and ATP-uncompetitive inhibitor of MEK1/2 kinases) [107] in combination with GDC-0941 (an orally bioavailable inhibitor of class I PI3K) [108], thus suggesting that failure to induce apoptosis may limit the efficacy of combined MEK and PI3K inhibition for KRAS-mutant NSCLCs. This differential apoptotic response induced by MEKi/PI3Ki is not simply explained by variable inhibition of RAS effector pathways but results from differential ability of the MEK and PI3K pathways to modulate the BCL-2 family of apoptotic regulatory proteins [109]. Another recent study reveals that Bcl-xL upregulation is an important mechanism of apoptosis resistance in mutant KRAS cells. Concurrent induction of pro-apoptotic Noxa/Bik and antagonism of Bcl-xL have been shown to synergistically interact to overcome KRAS-mediated apoptosis resistance [110]. These findings highlight a promising therapeutic strategy to overcome apoptosis resistance in KRAS-mutant colorectal cancer cells. Moreover, Corcoran and collaborators identified, by a pooled shRNA-drug screen, a synthetic, lethal interaction of combined Bcl-xL and MEK inhibition to promote tumor regressions in KRAS mutant cancer models [111]. Therefore, a dual-targeted or multitargeted strategy may be more efficient to overcome the resistance due to cancer genetic background.

Oncosuppressor p53 and apoptosis

The tumor suppressor p53 is a transcription factor that, upon DNA damage, is activated to induce sequence-specific target genes involved in either cancer cell growth arrest or apoptosis [112]. Activation of wild-type (wt) p53 occurs in response to genotoxic stress essentially through posttranslational modifications, such as acetylation and phosphorylation, resulting in protein stabilization (by escape from proteasome-mediated degradation) and nuclear localization leading to binding to sequence-specific promoters of target genes as final outcome of its function as transcription factor [113]. The induction of apoptosis by p53 in response to cellular stress is its most conserved function and is crucial for p53 tumor suppression [114]. The apoptotic activation of p53 is central not only for preventing tumor transformation but also for efficient response to therapies aiming at tumor eradication. In response to cellular stress p53 regulates molecules involved in both the death receptor (extrinsic) and mitochondria-dependent (intrinsic) apoptotic pathways [115]. In response to multiple chemotherapeutic drugs two pro-apoptotic members of the TNFR superfamily, Fas/Apo1 and Killer/DR5, are regulated in a p53-dependent manner [116,117]. In addition to stimulating Fas transcription, activated p53 may enhance levels of Fas at the cell surface promoting trafficking of the Fas receptor from the Golgi [118]. Another membrane-bound protein that was identified as a p53 target gene is p53 apoptosis effector related PMP-22 (PERP), although the precise mechanism by which its induction occurs has not being fully elucidated [119] (Figure 6). Regarding the apoptotic function of the intrinsic pathway, p53 seems to play a pivotal role because it modulates both pro-survival and pro-apoptotic Bcl-2 family members. Indeed, a key subset of the Bcl-2 family genes are p53 targets, including *Bax*, *Noxa*, *PUMA* and *Bid* [120-122] (Figure 6). *PUMA* gene is extremely effective in inducing apoptotic cell death within few hours and, more importantly, knockout experiments in human colorectal cancer cells showed that *PUMA* is required for p53-induced apoptosis [123]. Moreover, p53 appears to promote the convergence of the intrinsic and extrinsic pathways through *Bid* regulation. Indeed, *Bid* gene has been found to be transcriptionally induced by p53 in response to γ-irradiation [124]. Interestingly, cellular chemo-sensitivity to the DNA-damaging agents doxorubicin and 5-fluorouracil appears to be critically dependent on

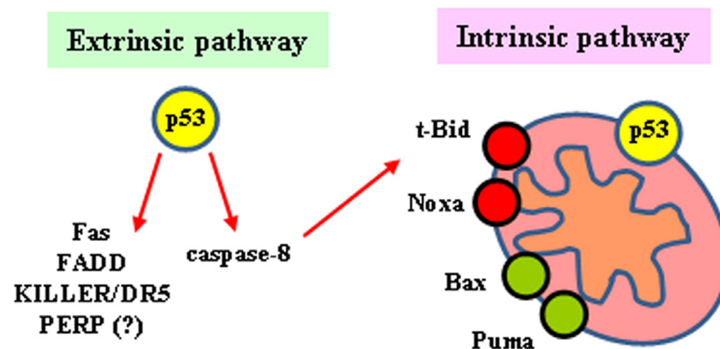

Figure 6. p53-mediated apoptosis. Role of p53 in both the extrinsic and the intrinsic pathway and their convergence through t-Bid.

the presence of wtp53 and Bid. Therefore, the induction of Bid by p53 helps to sensitize the cells to the toxic effects of chemotherapeutic drugs [124]. While the induction of some p53 target genes appears to be sufficient to initiate apoptosis, another class of p53 target genes (i.e., Apaf-1, caspase-6, and Bid) does not efficiently induce apoptosis per se but rather sensitizes cancer cells to the effects of chemotherapeutic agents, improving the apoptotic outcome [124-127]. Moreover, p53 also participates in apoptosis induction in a transcription-independent way by acting directly at mitochondria [128]; mechanistically, p53 interacts with anti-apoptotic Bcl-xL as well as pro-apoptotic Bcl-2 family proteins resulting in releasing of the pro-apoptotic effectors Bax/Bak that elicit cytochrome-*c* release and procaspase-3 activation [129].

Waking up the guardian: p53 as a druggable target

Because of its critical antitumor function, p53 is frequently targeted for inactivation and suffers disabling mutations or deletions in about 50% of all malignant tumors. The other half of human cancers express wild-type p53 protein that, however, can be inactivated by deregulation of regulatory proteins [130]. Stimulation of disabled p53 pathways has been suggested as a valuable anticancer strategy and, interestingly, activated wtp53 may target cancer cells though sparing the normal ones [131] which is an important concern in clinical studies. The p53 oncosuppressor protein is normally kept at low level because subject to negative regulation by MDM2-dependent proteasome degradation [132]; in response to genotoxic stress, however, p53 undergoes post-translational modifications that allow the protein to escape MDM2 control, accumulate, and become active [133]. The *mdm2* gene is amplified in a significant proportion of human tumor types, thereby contributing to tumor development by efficiently reducing the availability of a functional p53 protein [134]. The MDM2-negative regulation of p53 protein can be neutralized by specific protein modifications such as serine 46 (Ser46) phosphorylation [135], a key determinant in shifting the p53 pro-apoptotic transcription in response, for instance, to UV irradiation and chemotherapy [136,137]. In particular, p53-Ser46 phosphorylation by kinase HIPK2 is able to neutralize MDM2-mediated p53 inhibition rescuing p53 transcriptional activity of pro-apoptotic factors such as *p53AIP1*, *PIG3*, *Bax*, *Noxa*, *Puma* and *Killer/DR5* [138-142]. The interaction between p53 and MDM2 is a promising target in anticancer therapy [143]. To this aim, various peptidomimetic small molecules have been developed as protein-protein interaction blockers [144]. Among these is Nutlin-3, an imidazoline-based MDM2 antagonist that potentially inhibits the p53/MDM2 interaction though maintaining MDM2 E3 ligase activity [145]. The pharmacological action of Nutlin-3 is through both the transcription-dependent and -independent p53 apoptotic pathways [128,146,147]. MDM2 can also trigger, in response to low genotoxic damage, the downregulation of p53 apoptotic activator HIPK2 [148]. In agreement, the use of Nutlin-3 has been shown to mainly induce mitotic arrest rather than apoptosis [149]. Interestingly, co-treatment of cancer cells with zinc ion in the presence of Nutlin-3 can interfere with the interplay between HIPK2, p53 and MDM2 favoring HIPK2 stabilization and induction of p53 apoptotic activity through inhibition of MDM2 ligase activity [150]. In addition, p53 apoptotic activation can be achieved by zinc combination with

low-dose doxorubicin (ADR) that used alone does not achieve such effect; mechanistically, zinc supplementation reduces the p53 binding to MDM2, improving the low-dose drug-induced cytotoxic effect and cancer cell apoptosis [151]. Additionally, zinc ion restores the HIPK2/p53 apoptotic pathway that is inhibited by hypoxia [152]. Co-treatment with Nutlin-3 and Bcl-2 inhibitor ABT-737 has been shown to greatly enhance the sensitivity to apoptosis of cancer cells with high MDM2 levels [153], suggesting that the combined inhibition of MDM2 and Bcl-2 could be a multi-target-based anticancer strategy to trigger tumor death [154]. Some p53 activators as small-molecules MDM2 antagonist are in clinical trials [155] (https://clinicaltrials.gov). In contrast with the majority of the approaches that target the interaction between p53 and MDM2, a new method has been developed aimed at inhibiting the activity of the MDM2/MDM4 complexes by interfering with their heterodimerization [156]. The binding of the peptide mimicking the MDM4 C-terminus tail to MDM2 impairs MDM2-mediated p53 ubiquitination and activates p53-dependent transcription and oncosuppressive activities [156]. MDM4 (also known as HDM4, MDMX or HDMX) is a cytoplasmic protein with p53-activating function under DNA damage conditions. Particularly, MDM4 promotes mitochondrial localization of p53 phosphorylated at Ser46 through MDM4/HIPK2/p53 cytoplasmic assembly, uncovering coordinated repression of molecules with anti-apoptotic activity such as Bcl-2, release of cytochrome-*c* and apoptosis [157,158]. The existence of nuclear and cytoplasmic complexes able to stimulate the same p53 modification, that is $Ser46^{P}$, may indicate the presence of overlapping pathways to ensure the proper realization of a crucial process as the apoptosis. These findings highlight the potential therapeutic value of targeting the MDM2/MDM4 heterodimers for p53 apoptotic function.

Pharmacological reactivation of mutant (mut) p53 is an interesting field of research under continuous development aimed at designing new drugs. Some of them exploit the intrinsically unstable nature of mutp53 and therefore the possibility to stabilize the wild-type conformation thus restoring wild-type function and tumor response to therapies. Numerous findings about this subject have been shown and summarized in different reviews [159-161].

MicroRNA and apoptosis

MicroRNAs (miRNAs) are highly conserved, small noncoding RNA molecules, which post-transcriptionally regulate gene expression via inhibition of mRNA translation or inducing degradation of target mRNAs [162]. They are key regulators of many cell processes often deregulated in cancer, including apoptosis. Indeed, it is becoming clear that miRNAs might act as both anti-apoptotic and pro-apoptotic by directly targeting, respectively, pro- or anti-apoptotic mRNAs or their positive regulators [163]. The currently known apoptosis-regulating miRNAs list is expected to expand quickly and hopefully also their therapeutic use; therefore, we just highlight here some miRNAs involved in apoptosis regulation. Among the microRNAs involved in regulating the extrinsic apoptotic pathway, miR-20a, miR-21, miR-196b and miR-590 were reported as potential modulator of Fas/FasL system in different cancer types [164-167], while MiR-34a, miR-181c and miR-187 were shown to directly target TNF-α mRNA [168-170]. Among the microRNAs involved in regulating the intrinsic pathway there are the let-7 family, miR-15a, miR-16-1, miR-204, and miR-608, just to mention a few. The Let-7 family is highly conserved in sequence across animal species and is one of the first identified miRNA families. Let-7 miRNAs have been shown to negatively regulate Bcl-xL expression in human hepatocellular carcinomas and induce apoptosis in cooperation with anti-cancer drug targeting Mcl-1 [171]. Bcl-2 mRNA may be targeted by miR-204 with consequent increase in cells responsiveness to both 5-fluorouracil and oxaliplatin treatments and therefore to apoptotic cell death [172]. MiR-608 has been reported to target Bcl-xL in chordoma malignancy and lung cancer [173]. Notably, numerous miRNAs are also transcriptionally modulated by wtp53 [174] and among them is miR-34a [175,176], a member of the MiR-34 family implicated in cell death/survival signaling. MiR-34a is associated with G1 cell cycle arrest, senescence and apoptosis, thereby possessing a tumor suppressor activity. Deregulation of MiR-34a has been reported in several types of cancers [175,176]. Mutant (mut) p53 was also found to play a role in the regulation of miRNA processing. Garibaldi and collaborators demonstrate that mutp53 proteins modulate the biogenesis of several miRNAs in cancer cells directly interfering with Drosha-p72 association and promoting cell survival and cell migration [177]. They demonstrate a global impact of mutp53 on miRNA biogenesis and suggest that miRNAs are downregulated by mutp53 in order to inactivate tumor suppressive pathways. Of note they found that the endogenous wtp53 has an opposite effect on the expression of mutp53 repressed miRNAs on colon cancer cell lines confirming the contribution of mutp53 gain of oncogenic function (GOF) on miRNA repression [177]. Additional studies on a large scale would help in identifying the entire repertoire of miRNAs negatively downregulated by different mutp53 in different tumor models. According to the authors, the

characterization of the entire gene-regulatory networks governed by mutp53-miRNA cross-talks will offer a molecular basis for diagnostic and therapeutic strategies based on miRNA biology. In the meanwhile, developing strategies to block mutp53 GOF may have clinical impact in cancer treatment.

Delivery of miRNAs as synthetic miRNA mimics or antagomirs has emerged as a promising approach to treat cancer. Although different miRNAs are currently in the preclinical stage and ready to enter Phase 1 clinical trials, to date, only two miRNA therapeutics are registered for the treatment of cancers [https://clinicaltrials.gov]. The first therapeutic trial began in 2013 and is a Phase I, open-label, multicenter, dose-escalation study to investigate the safety, pharmacokinetics and pharmacodynamics of MRX34 in patients with unresectable primary liver cancer or advanced/metastatic cancer with or without liver involvement or in patients with hematologic malignancies (Mirna Therapeutics). MRX34 is based on the formulation of miR-34 mimic and the liposomal delivery technology SMARTICLES (Marina Biotech). The second one, began in early 2015, and is an early stage clinical trial of a new therapeutical approach for selected patients with malignant pleural mesothelioma or non-small cell lung cancer. The trial aims to test optimal dose of TargomiRs, an experimental medication consisting of three components, that is, miR-16-based microRNA mimic, a nanoparticle drug delivery system using nonliving bacterial minicells, and an anti-epidermal growth factor receptor antibody as a targeting moiety. The trial is being carried out in three different hospitals in Australia and the study is expected to be completed in mid 2016.

Concluding remarks

Intensive investigation in the last decades on the molecular mechanisms of apoptosis in cancer cells has led to the identification of the several molecules involved in both the intrinsic and the extrinsic apoptotic pathways. This is underscored by the extensive literature that those studies have produced in the last years. Those findings also reported how the many different alterations of key players of the apoptotic mechanisms are responsible of evasion from apoptosis and therefore of tumor development and resistance to therapies. For that reason, evasion from apoptosis is an hallmark of cancer and apoptotic proteins are interesting therapeutic targets. Therefore, this insight into the deregulation of apoptosis has focused the research attention towards the development of apoptosis-reactivating strategies, to be used in the treatment of various types of cancer, that hold great promise for the benefit of patients, although the mechanisms defining their mode of action still need to be unveiled, as recently highlighted [178]. Some molecules or therapeutic strategies are in preclinical trial, others are already in clinical trials, though underscoring the usefulness of such discoveries.

However, the study of apoptosis still presents challenges that should be addressed in future studies. They include, for instance, the study of 3-D cellular models, since most of the findings have been so far produced in 2-D cell culture systems. Knowing that the tumor is a three-dimensional entity and that the environment plays a big role in the cross-talk with cancer cells, it is likely that more physiological studying approach for the manipulation of the apoptotic machinery might give us novel insight into the mechanisms of tumor development and response to therapies. Moreover, additional studies on the development of drugs aiming at targeting, for instance, IAP proteins or mutp53 should take in consideration also the in vivo toxicity and the fact that they should selectively induce apoptosis in malignant and not in normal cells. In conclusion, there is little doubt that drugs that target the deregulated apoptotic pathways could have wide application in the treatment of cancer. The intense effort devoted lately to target the apoptotic pathway is encouraging and supportive for the development of new approaches to drug discovery and therapy.

Funding

This work was supported by AIRC Grant (IG 2015 Id.16742) to GDO.

Conflict of interest statement

The authors declare no conflict of interest.

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Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins

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Abstract | The loss of vital cells within healthy tissues contributes to the development, progression and treatment outcomes of many human disorders, including neurological and infectious diseases as well as environmental and medical toxicities. Conversely, the abnormal survival and accumulation of damaged or superfluous cells drive prominent human pathologies such as cancers and autoimmune diseases. Apoptosis is an evolutionarily conserved cell death pathway that is responsible for the programmed culling of cells during normal eukaryotic development and maintenance of organismal homeostasis. This pathway is controlled by the BCL-2 family of proteins, which contains both pro-apoptotic and pro-survival members that balance the decision between cellular life and death. Recent insights into the dynamic interactions between BCL-2 family proteins and how they control apoptotic cell death in healthy and diseased cells have uncovered novel opportunities for therapeutic intervention. Importantly, the development of both positive and negative small-molecule modulators of apoptosis is now enabling researchers to translate the discoveries that have been made in the laboratory into clinical practice to positively impact human health.

Death receptor
A subgroup of the tumour necrosis factor receptor (TNFR) superfamily that can activate the extrinsic apoptosis pathway via a conserved cytoplasmic signalling platform called the death domain. Prominent members of this family include FAS (also known as Apo1 and CD95), TNFR1 and TRAIL.

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https://doi.org/10.1038/s41580-018-0089-8

In order for us to remain alive, certain cells within our bodies must die. To maintain normal physiology and tissue function, cells that are damaged, dysfunctional or no longer necessary are constantly cleared via regulated cell death and ideally replaced by new, healthy cells[1–3]. When these normal processes of cell death go awry, the consequences can be disastrous. Many of the diseases that constitute the leading causes of death and disability worldwide, including neurodegenerative, cardiovascular, autoimmune and infectious diseases, involve either excessive or insufficient cell removal[4,5]. Furthermore, cytotoxic chemotherapies and ionizing radiation can induce cell death in healthy tissues, limiting the use of these potentially curative cancer therapies, especially in paediatric patients[6–8]. Despite the undeniable importance of maintaining the survival of healthy cells or eliminating those that are damaged or potentially dangerous, our understanding of cell death processes and their regulation is still nascent, especially in light of recent findings demonstrating the dynamic nature of cell death regulation during development, ageing and disease.

The apoptosis pathway is evolutionarily conserved across metazoans. In vertebrates, apoptosis is important for proper development[9,10], maintenance of tissue homeostasis[11,12] and cancer prevention[13]. Apoptotic cell death is associated with several conserved features (see Supplementary Box 1) and culminates in the activation of cysteine–aspartic proteases (caspases), which degrade cellular components to prepare dying cells for clearance by phagocytes with minimal stress to surrounding cells and tissues[14,15,16]. Importantly, in contrast to necrosis (an unregulated form of cell death frequently resulting from acute cell trauma[17]), apoptosis requires energy input and thus is an active process. In more detail, apoptosis is initiated by either internal or external stimuli and mediated via two distinct pathways: the intrinsic pathway (mitochondria mediated, a focus of this Review) and the extrinsic pathway (death receptor-mediated; see Supplementary Box 2).

The key to the regulation and execution of intrinsic apoptosis is the BCL-2 family of proteins, which includes both pro-apoptotic and pro-survival (anti-apoptotic) members (FIG. 1). The careful modulation of the balance between these two groups of BCL-2 proteins can largely determine cell fate decisions between life and death.

The state of apoptosis research today is particularly exciting given the recent development of BH3 mimetics — small molecules that mimic the activity of selected pro-apoptotic proteins and thus can sensitize cells to mitochondrial apoptosis (FIG. 1). Several different BH3 mimetics, targeting various BCL-2 proteins, have been developed and are being explored

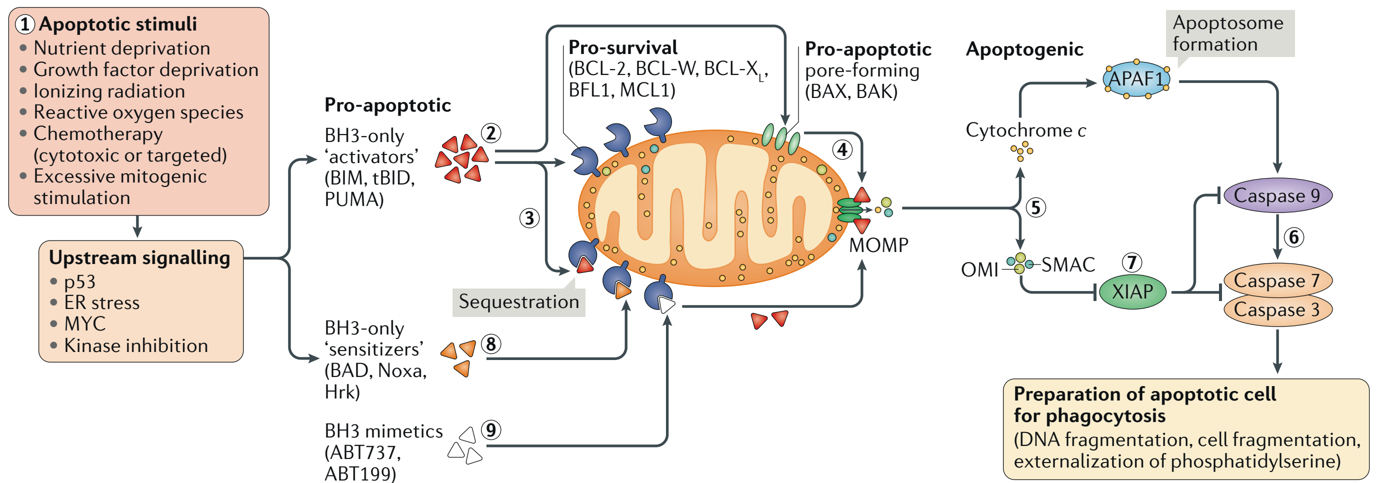


Fig. 1 | **The mitochondrial apoptosis pathway.** To initiate apoptosis, cellular stress or damage signals (step 1) typically unleash pro-apoptotic proteins (BH3-only 'activators' of apoptosis) via their upregulation (such as BCL-2-interacting mediator of cell death (BIM) or p53-upregulated modulator of apoptosis (PUMA)) or cleavage (BH3-interacting domain death agonist (BID) to form the active, truncated form (tBID)) (step 2). These pro-apoptotic activator proteins either can be bound and sequestered by pro-survival proteins such as BCL-2, B cell lymphoma extra large (BCL-X$_L$) or myeloid cell leukaemia 1 (MCL1) (step 3) or, when these pro-survival proteins are saturated or absent, can activate BCL-2-associated X protein (BAX) and/or BCL-2 antagonist/killer (BAK) (step 4). Activated BAX or BAK oligomerizes and forms pores to cause mitochondrial outer membrane permeabilization (MOMP), resulting in the release of apoptogenic molecules including second mitochondria-derived activator of caspases (SMAC), serine protease OMI and cytochrome *c* from the mitochondrial intermembrane space. Cytochrome *c* binds apoptotic protease-activating factor 1 (APAF1) in the cytosol to form the apoptosome (step 5), which serves as a platform for the activation of caspase 9, which then activates the effector caspases 3 and 7 (step 6) to dismantle the cell and prepare it for phagocytosis. Caspase activation can be blocked by the X-linked inhibitor of apoptosis protein (XIAP) (step 7), which in turn is inhibited by the released SMAC and OMI proteins from the mitochondria (step 5). Upstream damage or stress signalling can also activate BH3-only 'sensitizer' proteins that do not efficiently activate BAX and BAK but inhibit the activity of pro-survival BCL-2 family proteins to release any sequestered BH3-only activators, which trigger MOMP (step 8). BH3 mimetics are a novel class of agents that are able to sensitize cells to apoptosis by blocking the activity of pro-survival BCL-2 family proteins (step 9). BAD, BCL-2-associated agonist of cell death; BCL-W, B cell lymphoma W; BFL1, BCL-2-related isolated from fetal liver 1; Hrk, activator of apoptosis harakiri.

as potential therapeutics in pathological conditions caused by insufficient or excessive apoptosis (BOX 1). These agents have already demonstrated potent clinical utility for the treatment of blood cancers, including chronic lymphocytic leukaemia and acute myelogenous leukaemia, but their potential uses in other diseases, as discussed in this Review, are less established but nonetheless promising.

In this Review, we discuss the regulation of apoptosis by members of the BCL-2 family of proteins, including the modulation of BCL-2 proteins themselves in mammalian physiology as well as deregulation of BCL-2 protein balance in various pathologies. Although many other forms of cell death have been discovered and described, the intrinsic apoptosis pathway is physiologically dominant, leading to the demise of more than 60 billion of our cells each day[18], and it is the major focus of this Review.

Intrinsic pathway of apoptosis

Apoptosis is the most studied form of cell death and the primary mode of cell death involved in development and homeostasis[17,19]. Here, we describe the execution of apoptotic cell death via the intrinsic pathway, focusing on the dynamic regulation of the process by BCL-2 proteins.

BCL-2 proteins as regulators of intrinsic apoptosis. BCL-2 proteins are the key regulators of the intrinsic apoptosis pathway. Each member of this family contains one or more BCL-2 homology (BH) domains, BH1–BH4. Apoptosis is triggered by pro-apoptotic, BH3-only proteins, the name of which stems from the fact that these proteins contain only a single BH domain, in this case, BH3. The key effectors of apoptosis commitment are BH3-only 'activator' proteins[20]: BCL-2-interacting mediator of cell death (BIM), encoded by *BCL2L11*; BH3-interacting domain death agonist (BID), encoded by *BID*; p53-upregulated modulator of apoptosis (PUMA), encoded by *BBC3*; and potentially others (see below) that bind and activate either or both of the pro-apoptotic pore-forming proteins BCL-2-associated X protein (BAX), encoded by *BAX*, or BCL-2 antagonist/killer (BAK), encoded by *BAK1*. The activation of BAX or BAK at the mitochondrial surface results in an allosteric change in these proteins, enabling them to oligomerize and form macropores in this membrane, causing mitochondrial outer membrane permeabilization (MOMP).

MOMP results in the release of apoptogenic proteins from the intermembrane space (FIG. 1). In the cytoplasm, released mitochondrial proteins are involved in caspase activation either directly (as is the case

Cytochrome *c*
An essential component of the electron transport chain within mitochondria, where it carries electrons. When released from mitochondria as a result of BCL-2-associated X protein (BAX) and/or BCL 2 antagonist/killer (BAK) activation, cytochrome *c* has a prominent role in controlling the commitment to apoptosis — it binds to apoptotic protease-activating factor 1 (APAF1) to form an apoptosome, which activates caspases.

for cytochrome *c*, which binds to the scaffold protein apoptotic protease-activating factor 1 (APAF1) to form the apoptosome) or indirectly (as exemplified by second mitochondria-derived activator of caspases (SMAC)[21] and serine protease OMI (also known as HTR2A), which neutralize caspase-inhibitory proteins such as X-linked inhibitor of apoptosis protein (XIAP)). These events promote the activation of the initiator caspase (caspase 9) and executioner caspases (caspases 3, 6 and 7) for dismantling of the cell. Note that although caspase activation is a largely ubiquitous downstream event in apoptotic cell death, it is not the main commitment point for apoptosis as the prevention of caspase activation does not, under typical physiological circumstances, rescue dying cells after MOMP[22,23]. This finding may explain why previous efforts to use caspase inhibitors in cells undergoing apoptosis to maintain tissue function were largely unsuccessful — cells with permeabilized mitochondria cannot continue to function metabolically; thus, caspase inhibition only delays some of the morphological and biochemical changes that are observed after MOMP in dying cells (see Supplementary Box 1). Overall, MOMP is the critical step at which a cell irreversibly commits to undergoing apoptotic cell death[24] and represents a cellular point of no return. If, however, the commitment to MOMP is incomplete (only a minor subset of mitochondria undergo MOMP)[25,26], cells may prevent more widespread activation of BAX or BAK and retain their clonogenic potential. Interestingly, cells with so-called minority MOMP can be potentially tumorigenic owing to the activity of post-MOMP-activated proteins, such as DNases that can mutate DNA to facilitate neoplastic transformation[26,27].

A new detail in this pathway has recently emerged: apart from causing the release of mitochondrial proteins, BAX and/or BAK macropores in the mitochondrial membrane also facilitate the release of mitochondrial DNA into the cytoplasm where, in the absence of caspases[28], it can activate pro-inflammatory signalling, likely via the activation of cGAS–STING (cyclic GMP–AMP synthase–stimulator of interferon genes)

Box 1 | BH3 mimetics under development

As outlined in this Review, modulating cell death can have a potentially profound impact on the treatment of many human diseases, especially cancers. The recent development of novel small-molecule inhibitors of pro-survival proteins from the BCL-2 family, called BH3 mimetics, enables the direct and selective activation of mitochondrial apoptosis in cells that are highly dependent on one or more pro-survival proteins (because of inherent or stress-induced signalling). These agents work by inhibiting the activity of pro-survival proteins (see the figure) and, in the process, freeing any pro-apoptotic protein that is actively being sequestered. The pro-apoptotic protein is then able to carry out its primary function in activating apoptosis[73] (see also FIG. 4).

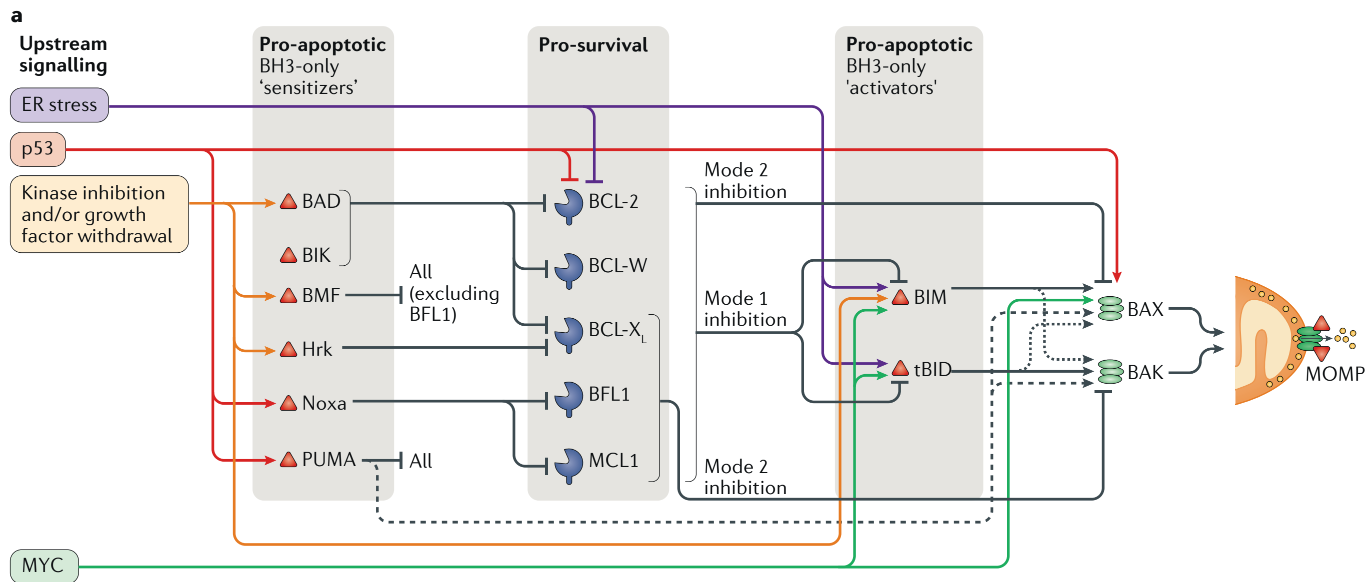
The US Food and Drug Administration recently approved the BCL-2 inhibitor venetoclax (ABT-199) for use in chronic lymphocytic leukaemia on the basis of its excellent clinical activity, including in patients who have relapsed after multiple rounds of therapy and those with mutated p53 (REF.[198]). Agents targeting other major pro-survival proteins (B cell lymphoma extra large ($BCL\text{-}X_L$) and myeloid cell leukaemia 1 (MCL1)) are also undergoing clinical evaluation[73,199,200]. Although these agents have already shown great potential in haematological cancers, their deployment in solid cancers and non-malignant diseases has been challenging owing to an insufficient understanding of apoptotic dependencies and how to identify and exploit them safely and effectively in the clinic.

Although these agents have on-target toxicities that can be managed clinically (such as thrombocytopenia due to platelet apoptosis in response to $BCL\text{-}X_L$ inhibition[201]), they would ideally be administered only to patients with cancers that are dependent on the specific pro-survival protein being inhibited to avoid unnecessary toxicity and maximize activity. As one might expect, the expression level of a pro-survival protein within cells is not itself a strong predictor of whether apoptosis will occur upon its inhibition[202] because the active sequestration of pro-apoptotic molecules ultimately determines the response, which is more difficult to ascertain. This is particularly true of clinical biopsy specimens, which are most commonly formalin fixed and paraffin embedded, making most biochemical and functional assays impossible. However, the clinical development of these agents is being complemented by intense research and development of the biomarkers that may guide their use.

As highlighted throughout this Review, there are exciting opportunities to block apoptosis for potential therapeutic benefit. The recent development of small-molecule inhibitors of BCL-2-associated X protein (BAX) and BCL-2 antagonist/killer (BAK) (see the figure) may finally enable clinical evaluation of such approaches. There are concerns that blocking apoptosis could facilitate tumorigenesis, because it would inhibit death of preneoplastic cells in response to accumulating DNA damage. However, we envision that inhibitors of BAX and BAK would not be used chronically, thereby limiting this risk. In addition, it is possible that even transient inhibition of apoptosis may provide cells with a prolonged opportunity to repair DNA damage or re-establish homeostasis and return to an optimal, healthy state; however, this needs to be formally tested clinically or in animal models.

Overall, as the understanding of BCL-2 family regulation advances beyond their role in cancer, agents such as those shown in the figure, both positive and negative regulators of apoptosis, may be extended for therapeutic use in non-malignant diseases, as proposed in this Review.

	ABT-199	ABT-263	ABT-737	AZD4320	BM-1197	S44563	S55746	BCL2-32	A-1155463	A-1331852	WEHI-539	A-1210477	AMG176	Compound 9	Compound 34	S63845	UMI-77	BTSA1	MSN-125
Promoters of apoptosis																			
Pro-survival BCL-2 family protein inhibitors (e.g. BAD mimetics)																			
BCL-2	✓	✓	✓	✓	✓	✓	✓	✓											
BCL-W		✓	✓																
$BCL\text{-}X_L$		✓	✓	✓	✓	✓		✓	✓	✓	✓								
BFL1																			
MCL1												✓	✓	✓	✓	✓	✓		
Direct activators of BAX or BAK (e.g. BIM mimetics)																			
BAX																		✓	
BAK																			
Inhibitors of apoptosis																			
Direct inhibitors of BAX or BAK (e.g. $BCL\text{-}X_L$ mimetics)																			
BAX																			✓
BAK																			✓



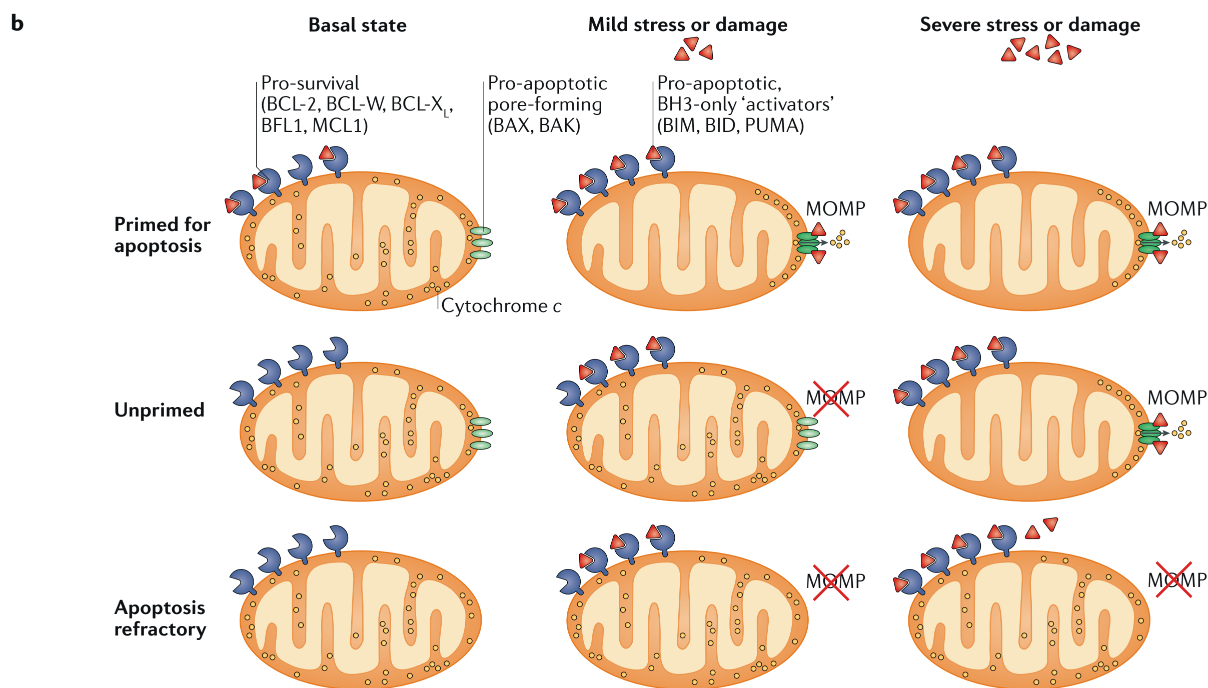
Interactions between BCL-2 family proteins

		Pro-apoptotic, BH3-only 'activators'		Pro-apoptotic, BH3-only 'sensitizers'						
Protein or BH3 peptide		BID (tBID)	BIM	BAD	BIK	None	BMF	Hrk	Noxa	PUMA
Drug (BH3 mimetic)		None	None	ABT-263	ABT-263	ABT-199	None	WEHI-539	S63845	None
Pro-apoptotic, pore-forming	BAK									
	BAX									
Pro-survival	BCL-2									
	BCL-W									
	BCL-XL									
	BFL1									
	MCL1									

Pro-apoptotic, pore-forming	
BAK	BAX

Weak or no direct activation — Strong activation

Weak or no direct inhibition — Strong inhibition



◀ Fig. 2 | **Interactions among BCL-2 family proteins and apoptotic priming.** **a** | BCL-2 family proteins interact in several key ways. BH3-only 'activator' proteins (BCL-2-interacting mediator of cell death (BIM), truncated and active form of the BH3-interacting domain death agonist (tBID) and, to a lesser extent, p53-upregulated modulator of apoptosis (PUMA) (dashed lines)) can activate BCL-2-associated X protein (BAX) and/or BCL-2 antagonist/killer (BAK) to result in mitochondrial permeabilization (tBID most efficiently activates BAK, whereas BIM shows stronger affinity for BAX). However, pro-survival proteins can also bind and sequester the activator BH3-only proteins via Mode 1 inhibition. BH3-only 'sensitizer' proteins can bind and inactivate specific pro-survival proteins, which would result in the release of any BH3-only activators that are actively being sequestered. Finally, pro-survival proteins can also bind and sequester BAX or BAK directly via Mode 2 inhibition, preventing their oligomerization. Damage and stress-induced signalling pathways trigger apoptosis via distinct mechanisms involving modulation of the expression levels or activity of BCL-2 family proteins. The table below is a graphic depiction of the different interactions and their affinities established between BCL-2 family proteins. These different interactions result in differential regulation of apoptosis, depending on expression levels of the different components. In addition, BH3 mimetics of sensitizer proteins can be used to further modify the BCL-2 protein interactome. **b** | Cells can exhibit widely differing susceptibility to apoptosis. Cells primed for apoptosis express high levels of pro-apoptotic activator proteins at a basal state, and even mild stress that causes a modest increase in the expression of pro-apoptotic factors will trigger mitochondrial outer membrane permeabilization (MOMP). Unprimed cells can activate apoptosis but require more damage or stress to overwhelm pro-survival proteins that are not actively sequestering pro-apoptotic molecules. Finally, cells that are apoptosis refractory do not express sufficient levels of the pro-apoptotic pore-forming protein BAX or BAK and are unable to trigger MOMP even under severe stress. BAD, BCL-2-associated agonist of cell death; BCL-W, B cell lymphoma W; BCL-X_L, B cell lymphoma extra large; BFL1, BCL-2-related isolated from fetal liver 1; BIK, BCL-2-interacting killer; BMF, BCL-2-modifying factor; Hrk, activator of apoptosis harakiri; MCL1, myeloid cell leukaemia 1.

Apoptosome
A quaternary protein complex composed of cytoplasmic cytochrome *c*, apoptotic protease-activating factor 1 (APAF1) and dATP that recruits and activates the normally inactive pro-caspase 9, which then activates effector caspases to prepare the cell for phagocytosis.

Second mitochondria-derived activator of caspases
(SMAC). Also known as DIABLO. A protein that is released from mitochondria during mitochondrial outer membrane permeabilization to bind and inactivate X-linked inhibitor of apoptosis protein (XIAP) to promote caspase activation.

X-linked inhibitor of apoptosis protein
(XIAP). A member of the inhibitor of apoptosis family of proteins that can prevent caspase activation. XIAP binds and inactivates caspases 3, 7 and 9 via its baculovirus IAP repeat BIR2 and BIR3 domains.

signalling[29,30]. This finding has possible implications for cancer immunotherapy — the activation of cGAS–STING signalling can activate an anticancer immune response[31]. Presumably, blocking downstream caspase activity in cancers that are undergoing mitochondrial apoptosis in response to therapy may greatly facilitate the activation of cGAS–STING signalling and the production and release of type-I interferons, which serve as damage-associated molecular pattern (DAMP) molecules[32] to encourage an anticancer immune response.

Countering this pro-apoptotic chain of events are the pro-survival (anti-apoptotic) BCL-2 family proteins: BCL-2, encoded by *BCL2*; B cell lymphoma extra large (BCL-X_L), encoded by *BCL2L1*; B cell lymphoma W (BCL-W), encoded by *BCL2L2*; BCL-2-related isolated from fetal liver 1 (BFL1), also known as BCL-2A1, encoded by *BCL2A1*; and myeloid cell leukaemia 1 (MCL1), encoded by *MCL1*. These proteins contain all BH domains (BH1–BH4) and can block apoptosis by binding and sequestering monomeric-activated BAX or BAK or BH3-only activator or sensitizer proteins (see below)[33] (FIGS 1,2). These specific interactions occur via the binding of the hydrophobic face of the BH3 domain on the pro-apoptotic protein into a hydrophobic groove formed by the BH1–BH3 domains on the anti-apoptotic protein. For apoptosis to occur, pro-survival proteins within the cell must be overwhelmed and BAX and/or BAK activated; this is where the balance between pro-survival and pro-apoptotic BH3-only proteins comes into play.

To date, at least eight different BH3-only proteins have been discovered and validated to have canonical pro-apoptotic activity (binding and modulating the activity of pro-survival BCL-2 family proteins, BAX and/or BAK) in mammalian cells. These proteins include BIM; BID; BCL-2-associated agonist of cell death (BAD), encoded by *BAD*; BCL-2-interacting killer (BIK), encoded by *BIK*; BCL-2-modifying factor (BMF), encoded by *BMF*; activator of apoptosis harakiri (Hrk), encoded by *HRK*; Noxa (Latin for 'damage'), also known as PMA-induced protein 1, encoded by *PMAIP1*; and PUMA. Of these proteins, BIM, tBID (truncated and active form of BID, generated by caspase-mediated cleavage) and PUMA are the most potent activators of BAX and BAK[20,34–37]. Because they are less efficient at activating BAX or BAK, the remaining BH3-only proteins are referred to as 'sensitizers', and their main function is considered to be in the inhibition of pro-survival proteins[20,38]. Each of these eight proteins contains a single 9–13 amino acid BH3 domain, which is required for the interactions with pro-survival BCL-2 proteins and/or with BAX or BAK[40]. This binding is highly selective, and BH3-only proteins have varying binding affinities for different pro-survival and pro-apoptotic effector proteins[33,40–45] (FIG. 2). Although BAX and BAK activation resulting from direct, detectable[35,47] interactions with activator BH3-only proteins is a widely accepted view of apoptosis induction, recent work demonstrating BAX and BAK activation even in the absence of all known BH3-only proteins[48] suggests that other direct activators may exist (including the mitochondrial outer membrane itself) when all pro-survival proteins are inactivated.

Apoptosis initiators. Intrinsic apoptosis can be triggered by diverse stimuli, including genotoxic damage (that is, ionizing radiation[45,48] and DNA-targeting cytotoxic chemotherapies[49]), damage to cellular organelles or signalling platforms (endoplasmic reticulum stress (ER stress)[50,51], mitochondrial damage[52] and inhibition of intracellular signalling pathways[53]), excessive mitogenic stimulation (activation-induced cell death[54,55]) or oncogene-induced cell death[56] (of note, this type of cell death has been thoroughly established only for the *Myc* oncogene, and the activation of other mitogenic oncogenes may increase apoptotic sensitivity of cells but potentially only through the requisite activation of MYC for cell growth and proliferation[45,56–60]). Cells may be driven into apoptosis even by the lack of proper stimulus (that is, growth factor withdrawal[61] or nutrient deprivation[62]) (FIG. 1). The diversity in apoptosis initiators is mirrored by the diversity in the levels and types of pro-apoptotic effector unleashed, as highlighted below. For example, DNA damage from ionizing radiation or genotoxic chemotherapeutics induces apoptosis by activating p53 to drive transcription of its target genes including *BAX*, *PUMA* and *Noxa*[63,64] and repression of BCL-2 (FIG. 2a). This contrasts with apoptosis induced by kinase inhibition or growth factor withdrawal, which involves activation of BIM[65,66], BAD (via dephosphorylation[67]), BMF[68] and Hrk[69]. The specificity of these responses may potentially be leveraged to control cell fate decisions in cells that are damaged or stressed.

Box 2 | **Regulation of BCL-2 proteins**

Although the final decision of whether a cell should undergo apoptosis is binary and switch-like, the regulation of BCL-2 family proteins is graded and highly complex. Multiple positive feedback loops ensure that when the balance of BCL-2 family proteins shifts towards apoptosis, it cannot be reversed. For example, activated caspases cleave and activate BH3-interacting domain death agonist (BID)[203] to facilitate further BCL-2-associated X protein (BAX) and/or BCL-2 antagonist/killer (BAK) activation to ensure that mitochondrial outer membrane permeabilization (MOMP) is complete and irreversible. In turn, the inhibition of the X-linked inhibitor of apoptosis protein (XIAP) by second mitochondria-derived activator of caspases (SMAC) and serine protease OMI following MOMP fuels rapid and potent caspase activation[204,205]. Ultimately, cellular susceptibility to apoptosis is determined by the abundance, stability, activity and localization of BCL-2 family proteins. Levels of BCL-2 family proteins are, in large part, determined by their transcriptional regulation. For example, transcription of pro-apoptotic *BAX* mRNA is regulated by p53 (REF.[206]) and MYC[45,92] (see also FIG. 2a) to drive apoptotic competence in damaged or highly proliferative cells, respectively. Similarly, transcription factors typically associated with immune cell activation such as NF-κB and STAT3 can upregulate BCL-2-related isolated from fetal liver 1 (BFL1) (REF.[207]) and BCL-2 (REF.[208]), respectively, to help promote cell survival, which likely supports immune system homeostasis and facilitates immune responses. This transcriptional regulation is balanced by protein degradation via the ubiquitin–proteasome system[209]. In addition, BCL-2 protein activity can be affected by interactions with other proteins — including interaction between BCL-2 family members (FIG. 2a) and interactions between various non-BCL-2 family proteins — and by various post-translational modifications (reviewed elsewhere[33,209–211]). Moreover, because the loss of mitochondrial outer membrane integrity is the key cellular event committing the cell to apoptosis, the regulation of pro-apoptotic protein localization to this membrane needs to be tightly regulated[212]. The regulation of BCL-2 family proteins at the mitochondrial surface has been comprehensively reviewed elsewhere[120,209]. A prominent example is the regulation of BAX and B cell lymphoma extra large (BCL-X_L), which are continuously shuttled between the mitochondrial membrane and the cytosol. Thus, BAX seemingly requires a two-step activation system: the first step induces a change in BAX conformation by the binding of a pro-apoptotic BH3 domain to a non-canonical binding site and recruits it to the mitochondrial surface[213,214]; the second step enables BAX to oligomerize and form pores in the mitochondrial outer membrane[34]. The redundant levels of regulation help to ensure that apoptosis occurs only when intended, helping to maintain organismal homeostasis. Importantly, the ways in which each BCL-2 family protein is regulated can vary in different physiological contexts and in disease, as described in this Review.

cGAS–STING
Cyclic GMP–AMP synthase (cGAS) is a DNA-sensing molecule that activates innate immune responses through production of the second messenger, cyclic GMP–AMP (cGAMP), which then binds to and activates the adaptor protein stimulator of interferon genes (STING). Importantly, cGAS is activated by double-stranded DNA that can be foreign or self.

Damage-associated molecular pattern
(DAMP). A signal that initiates and perpetuates immune activation in response to tissue damage, trauma or ischaemia regardless of whether pathogens are present at the site of injury.

BCL-2 protein levels and apoptotic priming. Proteins of the BCL-2 family are regulated by multiple mechanisms (BOX 2), and their expression levels differ widely depending on tissue type, cell lineage, developmental stage, organismal age and any stress or damage that is experienced[45,70] (FIG. 3). The balance between pro-apoptotic and pro-survival proteins determines whether a cell will live or die[65,71–73]. To illustrate, a cell that maintains its survival by expressing only enough pro-survival proteins to barely buffer existing pro-apoptotic proteins is considered primed for apoptosis (FIG. 2b). A primed cell readily undergoes MOMP in response to even a weak pro-apoptotic signal, which can be elicited by cellular stress or damage. A cell that expresses a surplus of pro-survival proteins that can buffer against existing and potentially additional pro-death molecules is less primed, or unprimed. Finally, a cell that does not express sufficient levels of the key pore-forming proteins BAX and BAK is unable to undergo MOMP (and therefore unable to die via the mitochondrial apoptosis pathway but may still be able to die via the extrinsic apoptosis pathway; see Supplementary Box 2) and is designated as apoptosis refractory. Although expressed here categorically for illustration, priming exists on a continuum, and cells can exist at any point along this scale. Recently, a functional assay called BH3 profiling was developed (BOX 3), which is used to measure apoptotic priming[71,74].

Apoptosis pathway in physiology

An important mechanism that ensures organismal homeostasis is the removal of damaged or superfluous cells via apoptosis, and in the former case, their replacement from stem-cell-derived progeny. Although not absolutely essential for animal development or survival, apoptotic competence ensures proper tissue development and homeostasis (FIG. 3).

Heightened apoptotic sensitivity as a hallmark of developing tissues. Development is associated with high cell proliferation. Apoptosis has long been considered essential for normal development[75,76] because it enables the removal of superfluous or damaged cells generated as a result of this high proliferation as well as the shaping of tissues by removing unwanted cells. However, mounting evidence supports the view that although apoptotic cell death has a major role in proper organ and limb formation and in cell specialization and differentiation, it is not absolutely essential for mammalian development. Although mice lacking both BAX and BAK have profound resistance to apoptosis and exhibit phenotypes indicative of loss of developmental apoptosis, such as interdigital webbing and imperforate vagina (see also below), they are able to survive into adulthood if they successfully pass the crucial perinatal period at which the vast majority of double-knockout mice die, reportedly owing to difficulties with proper feeding[10,77]. It is possible that the extrinsic apoptosis pathway or another pro-apoptotic, pore-forming protein, such as the non-canonical BCL-2 family member BCL-2-related ovarian killer (BOK, encoded by *BOK*), replaces the need for BAX and BAK during development. Indeed, the loss of BOK has been recently shown to exacerbate the phenotypes associated with loss of BAX and BAK[10] and to phenocopy many of the profound abnormalities of *Apaf1*-knockout mice, which are considered completely null for intrinsic apoptosis because of the inability to form functional apoptosomes[78]. However, a small subset of triple-knockout mice (*Bax*, *Bak1* and *Bok*) are able to survive to adulthood (similarly to *Apaf1*-knockout mice), highlighting that although intrinsic apoptosis is important for proper development, it is not absolutely essential for mammalian life. In addition, a recent report showed that concomitant loss of ATG5 made the phenotypes associated with *Bax* and *Bak1* double knockout even more pronounced, implying that autophagic cell death may compensate for the loss of competence for intrinsic apoptosis[79].

Despite these findings, apoptosis is important for the optimal formation and maturation of tissues and organs. For instance, perinatal brain development is associated with massive proliferation of neural progenitors[80], followed by a wave of apoptosis that eliminates approximately half of cells that are either excessive or have not established appropriate synaptic connectivity[81]. The death of neuronal cells during this period is

almost entirely BAX-dependent, as demonstrated by the accumulation of excess neurons in *Bax*-knockout (but not *Bak1*-knockout) animals[77,82] as well as in BCL-2-overexpressing animals[61] (FIG. 3). Apart from BAX, other pro-apoptotic proteins, including BIM and BID, are also highly expressed in the developing brain. Importantly, the high expression of pro-apoptotic proteins that facilitates neuronal pruning also renders the developing brain highly sensitive to pro-apoptotic signalling (FIG. 3), and therefore this tissue is classified as 'primed for apoptosis' by BH3 profiling[45] (BOX 3). The extremely high apoptotic sensitivity of the developing brain is likely responsible for the hypersensitivity of this tissue to sources of damage or stress, including chemotherapy or ionizing radiation, at this stage of life[7,45,83,84]. Importantly, pro-apoptotic proteins are strongly downregulated after the early postnatal period and thus protect terminally differentiated and largely irreplaceable neurons from unwanted apoptosis later in life (FIG. 3). In fact, we propose that the increased sensitivity of the developing brain to a great variety of environmental toxins (such as lead[85] or arsenic[86]) or medically necessary agents (such as anaesthetics[87] or glucocorticoids[88]) and perinatal injuries (such as ischaemia–reperfusion injury[89] or traumatic brain injuries[90]) may be due to the increased propensity of developing neural cells to undergo apoptosis compared with the mature neural cells of adults.

Owing to the high expression of pro-apoptotic proteins in the developing brain[45,76], this tissue is dependent on the expression of the pro-survival protein BCL-X_L to keep these death-inducing proteins in balance. In fact, knockout of the pro-survival gene *Bcl2l1* (encoding BCL-X_L), results in embryonic lethality due to massive cell loss within the developing central nervous system (and in the haematopoietic system)[91], thus highlighting the tight regulation of apoptosis during neurogenesis and development.

The heightened apoptotic sensitivity of tissues during development is not limited to the brain. The heart, kidneys and liver are also more sensitive to pro-apoptotic stimuli at the perinatal and early postnatal stages than at the adult stage[45,76]. Importantly, this heightened apoptotic sensitivity is frequently associated with active proliferation of tissues and expression of the growth- and division-supporting transcription factor MYC, suggesting that active proliferation is tightly coupled to expression of the machinery necessary for apoptosis induction. MYC actively drives the transcription of prominent pro-apoptotic genes including BAX, BID and BIM in proliferating tissues, promoting a state of high susceptibility to apoptosis[45,92]. Note that this process is also active in cancers, as discussed below. A major exception to this rule linking high proliferation with high apoptotic sensitivity is peripheral resting B and T lymphocytes, which are not actively cycling but are extremely primed for apoptosis and consequently are highly sensitive to many types of cytotoxic chemotherapy and ionizing radiation[93] (see also next subsection).

The normal development of germ cells and reproductive tissues is also highly dependent on balanced regulation of mitochondrial apoptosis by BCL-2 family proteins, potentially serving to eliminate male germ cells that carry genetic dysfunctions in apoptosis. For instance, *Bax* knockout[11] (as well as the knockout of multiple other pro-apoptotic genes, such as *Apaf1* (REF.[78]), or overexpression of pro-survival MCL1 (REF.[94]), BCL-2 or BCL-X_L (REF.[95])) impairs germ cell differentiation, resulting in male sterility. Although this process is incompletely understood, blocking the naturally occurring apoptosis that regulates germ cell number seems to lead to an imbalance of germ cells with supporting Sertoli cells, resulting in a massive loss of germ cells via non-apoptotic cell death (FIG. 3). In female mice, blocking apoptosis via deletion of BAX and BAK[77] or overexpressing BCL-2 (REF.[96]) prevents the normal hormonally driven opening of the vaginal cavity to the skin at 5 weeks of age, impinging on their reproductive capacity.

The role of MCL1 in mammalian development is less clear than that of BCL-2 or BCL-X_L because of the very early (at embryonic day 3.5) lethality of this knockout, which is associated with a trophectoderm defect that impairs implantation of the embryo[97]. Although this early lethality may be due to a strong requirement for MCL1 in counteracting apoptosis, it may alternatively be due to potential physiological roles of MCL1 beyond regulating apoptosis: it has been shown that an amino-terminally truncated isoform of MCL1 is imported into the mitochondrial matrix where it facilitates respiration and ATP production[98,99]. However, in cancer cells, the loss of MCL1 has no major impact on cell growth beyond regulating apoptosis[47]. It is still entirely possible that any non-apoptotic functions of MCL1 may be detected only in certain contexts, especially in non-transformed cells that are less proficient at adapting to changing growth conditions.

As many tissues mature and reach a largely post-mitotic state, most apoptosis-regulating proteins (both those that are pro-survival and those that are pro-apoptotic) are strongly downregulated to foster apoptosis resistance[7,45,76]. Although the aetiology of this downregulation is unclear, it ostensibly prepares non-replaceable cells for lifelong survival by removing the proteins that can potentially cause their demise. It is possible that the apoptosis pathway becomes reactivated in certain physiological or pathological circumstances, including those mentioned below in disease-specific settings, such as in neurodegenerative disorders.

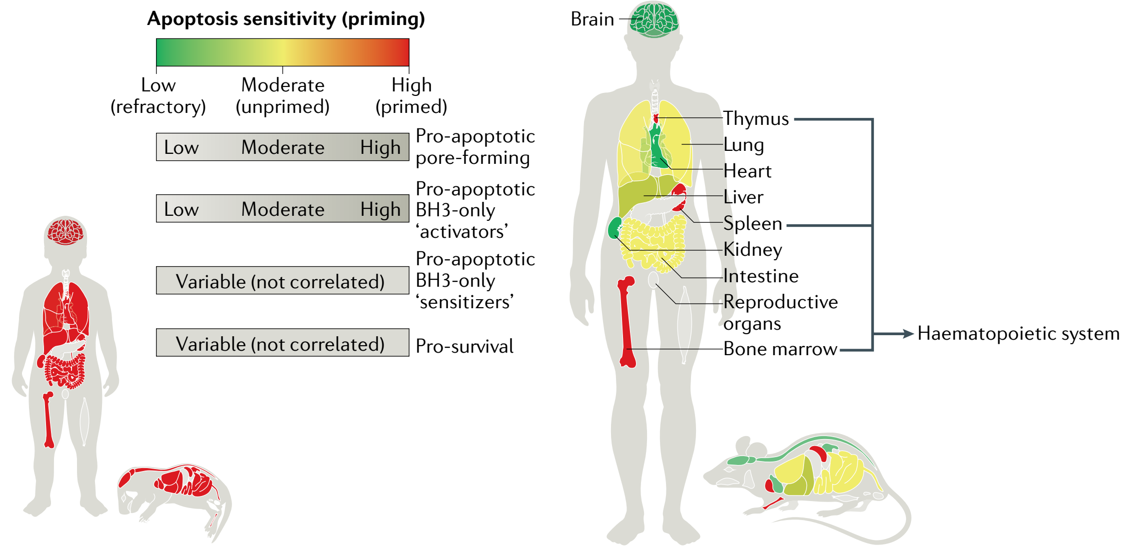
Importance of apoptosis for tissue homeostasis. In mammals, the blood system contains multiple types of differentiated cells with highly specialized functions. Many types of mature blood cell are extremely short-lived and are replaced at an estimated rate of more than 60 million cells per minute in an adult human. For example, neutrophils — the most common nucleated cell type in peripheral blood — have a lifespan of only 5.4 days[100], meaning that roughly 20% of neutrophils die via apoptosis each day in an adult human. The short half-life of these cells is likely due to their prominent role as phagocytes for invading pathogens; mammals have presumably evolved to allow these cells (together with their microbial load) to self-destruct via apoptosis and be replaced instead of using a detoxification strategy. Interestingly, a similar mechanism of self-destruction

Endoplasmic reticulum stress
(ER stress). A response of the ER to aberrations of protein folding (and other stresses), which is aimed at clearing unfolded proteins and restoring ER homeostasis. In cases when this cannot be accomplished, cellular functions degenerate and often result in apoptosis.

Activation-induced cell death
A programmed cell death process initiated in immune cells (especially T cells) by repeated stimulation of their T cell receptors that helps to maintain peripheral immune tolerance.

Ischaemia–reperfusion injury
A type of tissue damage resulting from initial ischaemia or hypoxia, followed by re-oxygenation, as seen in myocardial infarction, ischaemic stroke and other traumas.

Sertoli cells
Elongated cells of the seminiferous tubules within the testis to which spermatids attach during spermatogenesis for support and nourishment.



Apoptosis in homeostasis	Deregulation of apoptosis	Apoptosis in disease
Developing nervous system • High BAX, BIM, BID • BAX-dependent apoptosis to cull superfluous neurons or progenitors (Apoptotically primed neuron or progenitor; IR; Apoptotic neuron)	• ↑ Apoptosis: embryonically lethal; extensive cell death in the central nervous system and massive neuronal loss • ↓ Apoptosis: excess neurons; potential cognitive and behavioural dysfunction	High priming causes cells to die via apoptosis in response to damage or stress such as IR
Adult nervous system • Low BAX, BAK, etc. • Apoptosis is suppressed to maintain survival of post-mitotic neurons (Apoptotically refractory neuron; Disease-induced stress; Lifelong survival; Additional disease-induced or secondary stress)	• ↑ Apoptosis: loss of irreplaceable neurons causing neurodegeneration	Increased apoptosis of adult neurons is associated with neurodegenerative disorders
Male reproductive organs • High BAX, BAD • Germ cell apoptosis ensures optimal Sertoli-to-germ-cell ratio (Sertoli cell; Primordial male germ cell; IR)	• ↑ Apoptosis: germ cell loss leading to sterility • ↓ Apoptosis: germ cells overwhelm Sertoli cells to cause sterility	Chemotherapy and radiation therapy induce increased germ cell apoptosis, causing sterility
Bone marrow • High BAX, BAK, BIM, BID • Apoptosis deletes autoreactive B and T cells (HSC (high MCL1 to suppress apoptosis); Stress (e.g. inflammation), ageing; Loss of HSCs; Multiple progenitors; Autoreactive T cell (high BIM); Failure to undergo apoptosis (upregulation of BCL-2 or BCL-X_L); Self cell; Elimination; Autoimmunity)	• ↑ Apoptosis: bone marrow failure, thrombocytopenia • ↓ Apoptosis: accumulation of self-reactive lymphoid and myeloid cells to cause autoimmunity	• Excessive apoptosis causes immunodeficiency (e.g. in ageing or caused by HIV infection) • Defective apoptosis can drive autoimmune diseases including SLE
Adult liver • Low BAK • Apoptosis is suppressed in quiescent hepatocytes (Hepatocyte (non-cycling, low BAK); Liver injury; Dying hepatocyte; Liver regeneration; Proliferating hepatocyte (expression of MYC and its pro-apoptotic targets))	• ↑ Apoptosis: liver failure • ↓ Apoptosis: hepatomegaly	• Excessive hepatocyte apoptosis can be caused by alcohol, viral infection and excessive fatty acids and leads to liver disease • Decreased apoptosis may be involved in development of hepatomegaly

◀ Fig. 3 | **Apoptosis and apoptotic priming in physiology.** Apoptosis is differently and dynamically regulated across the mammalian lifespan. Tissues that are highly proliferative or have the potential to become highly proliferative (developing tissues, adult haematopoietic system) are typically primed for apoptosis (red). High apoptotic priming in these tissues makes them highly sensitive to various insults. Tissues that are largely postmitotic are apoptosis refractory (green), whereas tissues that are characterized as unprimed (such as those of the gastrointestinal system and lungs of adults) (yellow) typically contain highly heterogeneous cell types that may differ in apoptosis sensitivity at a cell-by-cell level. The level of priming within cells or tissues is dependent on the expression of BCL-2 family proteins, with the strongest determinants being expression of the pro-apoptotic, pore-forming molecules BCL-2-associated X protein (BAX) and/or BCL-2 antagonist/killer (BAK). Pro-survival and sensitizer BH3-only proteins can be expressed at variable levels and are not as clearly associated with a specific level of priming. Changes in the levels of BCL-2 proteins will, inevitably, impose changes on apoptotic susceptibility, leading to increased or insufficient apoptosis, which can result in pathology. BAD, BCL-2-associated agonist of cell death; BCL-X_L, B cell lymphoma extra large; BID, BH3-interacting domain death agonist; BIM, BCL-2-interacting mediator of cell death; HSC, haematopoietic stem cell; IR, ionizing radiation; MCL1, myeloid cell leukaemia 1; MOMP, mitochondrial outer membrane permeabilization; PUMA, p53-upregulated modulator of apoptosis; SLE, systemic lupus erythematosus.

exists in hepatocytes within the liver, which accumulate toxins and undergo apoptosis. Apoptotic hepatocytes release cytokines, which stimulate liver regeneration and hepatocyte proliferation, allowing the tissue to recover[101].

The continuous death of neutrophils requires the constant proliferation of haematopoietic progenitor cells and, upstream, haematopoietic stem cells (HSCs). The continuous yet variable proliferation of these cells is correlated with their levels of apoptotic priming: higher rates of proliferation in more differentiated progenitors[102] are associated with higher levels of priming[103]. Similarly, HSCs in young animals are more primed for apoptosis than those in aged individuals, which corresponds to the higher proliferative potential of young HSCs[103]. Strong evidence for the role of BCL-2 family proteins in maintaining homeostasis of the haematopoietic system can be observed in mouse models of apoptotic dysfunction. For example, *Bax*-knockout mice, and especially *Bax* and *Bak1* double-knockout[77] mice, accumulate mature T and B lymphocytes[11], which indicates that these cells, similarly to neutrophils, are normally cleared via apoptosis at the end of their useful lifespans, upon clearance of active infection and during the maturation process in the thymus when auto-reactive lymphocytes are removed (FIG. 3). In addition, genetic ablation or pharmacological inhibition of BCL-X_L causes accelerated apoptosis in platelets, which rely on BCL-X_L to counteract their high apoptotic susceptibility[104], causing thrombocytopenia and complicating the clinical translation of BH3 mimetics targeting this pro-survival protein for cancer treatment[105] (BOX 1). These regulatory mechanisms have recently been reviewed elsewhere[106,107].

Although some adult somatic cells within other tissues can die via apoptosis in response to genotoxic damage or other stress (for example, cells within the intestinal crypts[108]), the role of apoptosis in maintaining major organ systems, other than the haematopoietic system at steady state, is less clearly defined[109]. For example, the knockout of *Bax*, *Bak1* or *Bok* (or even all three[10]) genes has limited impact on the homeostasis of the gastrointestinal system, suggesting that apoptotic cell death is not a highly prominent feature of that tissue. However, the importance of apoptotic susceptibility may become apparent only when the tissue is damaged and requires repair or large-scale physiological remodelling, as is the case for mammary gland involution following lactation[110]. Indeed, cellular sensitivity to apoptosis changes during repair and regeneration[45,111], highlighting the link between tissue proliferation and apoptotic competence. For example, in the liver, hepatocyte loss due to alcohol toxicity, viral infection or excessive fatty acids[112] can stimulate surviving hepatocytes to proliferate and regenerate lost tissue, which is associated with the upregulation of MYC and its pro-apoptotic targets in dividing cells (FIG. 3). In this proliferative state, hepatocytes are transiently sensitized to apoptosis[45] and are potentially more vulnerable to cell death in the event of a subsequent insult (FIG. 2b).

Suppression of apoptosis in disease

As with many other biological, physical and chemical systems within complex organisms, apoptotic cell death is kept in a crucial balance to maintain equilibrium. When cells that are intended to die by apoptosis fail to do so, they can accumulate and cause detriment to the host via several distinct mechanisms, some of which are outlined below.

Cancer cell survival. There is no other set of diseases in which apoptosis has been better characterized than cancers. In fact, the namesake of this family, BCL-2, was discovered owing to its frequent translocation and overexpression in B cell lymphomas, where BCL-2 is frequently grossly overexpressed[113]. In addition, the ready access to clinical material and widespread use and utility of in vitro cancer cell lines have greatly accelerated studies of apoptosis regulation in cancer.

Because it is associated with accumulating DNA damage, aberrant growth signals and excessive pressure to support cell growth, the transformation of a cell to a malignant state is inherently stressful[114,115]. In addition, many of the genes necessary for active growth and proliferation are controlled by MYC, thus helping to explain why nearly all cancers deregulate this transcription factor[116]. This deregulation, however, comes at the cost of increased expression of pro-apoptotic genes, which are also controlled by MYC, resulting in heightened apoptotic priming and frequent apoptosis of premalignant cells (FIG. 4). The evasion of apoptosis at this stage of cellular transformation is vital for the continued proliferation of cancer cells and tumour formation, and the ability to suppress apoptosis is referred to as one of the hallmarks of cancer[117]. Indeed, blocking apoptosis has been shown across many model systems to facilitate malignant transformation and cancer development[118,119]. The means by which cells evade apoptosis vary greatly between cancer types and even within the same cancer type, resulting in heterogeneous expression of and dependence on pro-survival BCL-2 family proteins. This topic has recently been reviewed by several groups[73,120–122] and thus is addressed only briefly in this Review.

The need to actively suppress apoptotic cell death is particularly important for cancers that originate from

Double-negative T cells
(DNTCs). T cells expressing the T cell receptor but lacking CD4, CD8 or natural killer (NK) cell markers.

the haematopoietic system (which is particularly primed for apoptosis, as discussed above), and the expression of BCL-2 family members is frequently altered in these cancers. The mechanisms most typically employed include upregulation of pro-survival proteins including BCL-2, MCL1, BCL-X_L, BFL1 and BCL-W; downregulation or inactivation of pro-apoptotic proteins including BIM, BID, BAX, Noxa, PUMA and Hrk via mutation (observed only rarely[123], potentially owing to the large protein–protein interaction surfaces found in BCL-2 family proteins), phosphorylation[68,124] or other post-translational[125] or post-transcriptional[97] mechanisms; or downregulation or inactivation of the pore-forming proteins BAX and BAK (FIG. 4). Interestingly, aged HSCs in mammals are less adept at repairing DNA damage, and, as discussed above, they are also less primed for apoptosis than their younger counterparts[103]. These mechanisms may contribute to the outgrowth and survival of malignant clones in the haematopoietic system and may provide a mechanistic basis for the large increases in the prevalence of most lymphomas and leukaemias as we age[126].

Cancer cells must only suppress enough pro-apoptotic signalling via the above mechanisms to maintain survival during daily fluctuations in nutrient and environmental stressors, and so generally, they remain highly primed for apoptosis when unchallenged by exogenous perturbations. Thus, the evasion of apoptosis during carcinogenesis does not necessarily yield an apoptosis-resistant cell, and for this reason, cancer cells, particularly those of haematopoietic origin, show high sensitivity to cytotoxic chemotherapy and ionizing radiation[71,72,115,127,128] (FIG. 4). However, cancer cells can develop further resistance to these treatments (including via one of the aforementioned mechanisms), and for too many patients, the inability to eradicate 100% of malignant cells results in the selection and expansion of such resistant cells and thus drives therapy resistance.

Although solid tumours frequently exhibit changes in BCL-2 family proteins similar to those listed above for haematological malignancies, they typically employ fewer and less dramatic alterations to stave off apoptosis. This conservative approach may be due to the inherently lower levels of apoptotic sensitivity evident in cells of non-haematological lineages. For example, mature cells from the adult brain and kidneys are profoundly resistant to apoptosis[45], and thus the malignant cells that emerge from those tissues (which likely originate from stem cells within the tissue but ostensibly share many lineage-associated characteristics such as apoptosis regulation) are likely to be more resistant to apoptosis as well. Furthermore, the genes that can drive proliferation in cancers are also tissue-type specific[129], which may help explain why solid tumours are, in general, less sensitive to chemotherapy and radiation therapy than haematopoietic cancers.

Development of autoimmune diseases. Beyond cancer, the most compelling evidence of insufficient apoptosis contributing to disease is seen in autoimmune disorders. Although these defects may involve perturbations to the extrinsic pathway[130], the intrinsic pathway is also implicated. For instance, mice lacking BIM[131] or overexpressing BCL-2 (REF. 132) spontaneously produce autoantibodies to nuclear antigens and develop a systemic autoimmune disease. In humans, abnormally high expression of BCL-2 in peripheral blood B and T lymphocytes has been observed in patients diagnosed with systemic lupus erythematosus (SLE) — a prototypical autoimmune disease characterized by inflammation and organ damage in association with the expression of autoantibodies against double-stranded DNA. Such high BCL-2 levels may be responsible for the abnormal survival of self-reactive lymphocytes driving SLE.

Autoimmune lymphoproliferative syndrome (ALPS) is a recently characterized disorder caused predominantly by inherited mutations in the gene that encodes tumour necrosis factor receptor superfamily member 6 (FAS), which is implicated in extrinsic apoptosis, leading to reduction in apoptosis potential and expansion of double-negative T cells (DNTCs) — a hallmark of ALPS.

Box 3 | **BH3 profiling and its applications**

Owing to the large number of BCL-2 family proteins, their complex interactions and post-translational modifications, it is difficult to measure the apoptosis sensitivity of cells via biochemical approaches. The BH3 profiling assay overcomes this difficulty by functionally tracking cellular responses to pro-apoptotic signals delivered directly to the BCL-2 family proteins at the mitochondria. This integrated measure avoids the need for quantification of individual BCL-2 family proteins and can be used as a platform to test several important parameters.

Measuring the level of apoptotic priming within healthy or diseased cells and tissues
Cells differ in their sensitivity to apoptosis, ranging along a continuum that is defined from being primed for apoptosis (highly sensitive) to being apoptosis refractory (unable to undergo apoptotic cell death)[43,45,74,215,216] (see also FIG. 2b). Apoptotic priming defines how close cells are to the threshold of apoptosis. Owing to the complexity in regulation of BCL-2 family proteins and the varied upstream signalling pathways activated by stress or damage, delivering a dose of pro-apoptotic signals directly to mitochondria is the only manner in which measurement of apoptotic priming can be made accurately. Such measurements have been used previously to identify apoptotic priming as a major determinant of cancer responses to chemotherapy in the clinic[215,217,218] and as healthy tissue responses to ionizing radiation and chemotherapy[45].

Measuring cellular dependence on specific pro-survival proteins
Certain BH3 peptides, which mimic the activity of sensitizer BH3-only proteins, have very specific interactions with pro-survival proteins (see also FIG. 2a). For example, the BCL-2-associated agonist of cell death (BAD) BH3 peptide inhibits BCL-2, B cell lymphoma extra large (BCL-X_L) and B cell lymphoma W (BCL-W), whereas the activator of apoptosis harakiri (Hrk) BH3 peptide inhibits only BCL-X_L. Using these peptides, the dependence of a given cell on any of the pro-survival proteins can be directly assessed by measuring the extent of cytochrome *c* loss. These measurements have been used to identify dependencies on pro-survival proteins in chronic lymphocytic leukaemias (BCL-2)[192,219], certain acute myeloid leukaemias (BCL-2), subtypes of healthy T cells and their transformed counterparts (BCL-2 or BCL-X_L (REF. 220)) and multiple myelomas (myeloid cell leukaemia 1 (MCL1) and BCL-2).

Uncovering novel interaction dynamics among apoptosis-regulating proteins
The direct application of BH3 domain peptides to monitored mitochondria allows for the careful dissection of interaction dynamics between BCL-2 family proteins. This application has previously been used to characterize the selectivity of BH3 domains and the proteins that bear them for their pro-survival interaction partners[20] or pro-apoptotic effectors[40].

Predicting how patients will respond to chemotherapy in the clinic
The level of apoptotic priming within a tumour cell before therapy can be a major determinant of therapy response[215]. BH3 profiling has been shown to have potential clinical utility to predict how patients respond to both cytotoxic chemotherapies[215,221,222] and personalized use of BH3 mimetics[192,223].

Although the accumulation of T cells due to dysfunctional FAS-mediated apoptosis does not directly involve BCL-2 family proteins, it has been reported that DNTCs frequently upregulate pro-apoptotic BIM, which is counteracted by the overexpression of at least one pro-survival BCL-2 family protein (BCL-2 or BCL-X_L). The important role of intrinsic apoptosis deregulation in ALPS is supported by the sensitivity of ALPS-associated DNTCs to BH3 mimetics[102].

Finally, gastrointestinal autoimmune diseases such as Crohn's disease and ulcerative colitis are believed to occur as a consequence of chronic inflammation and consequent death of intestinal epithelial cells. This inflammation is established by hyperproliferating T cells that normally would be eliminated by cell death[133]. However, in these conditions, an increase in pro-survival BCL-2 prevents T cell apoptosis.

Propagation of intracellular pathogens. Pathogenic microorganisms must evade detection or must prevent the self-destruction of the host's cells for a period long enough to replicate and spread. To accomplish this, certain types of infectious pathogen have evolved the ability to produce factors that suppress apoptosis. It is important to note that, here, we focus exclusively on BCL-2 family proteins; however, virus-mediated and bacteria-mediated effects on the extrinsic apoptosis pathway, which are widely involved in responses to infection, are also common (reviewed elsewhere[134,135]).

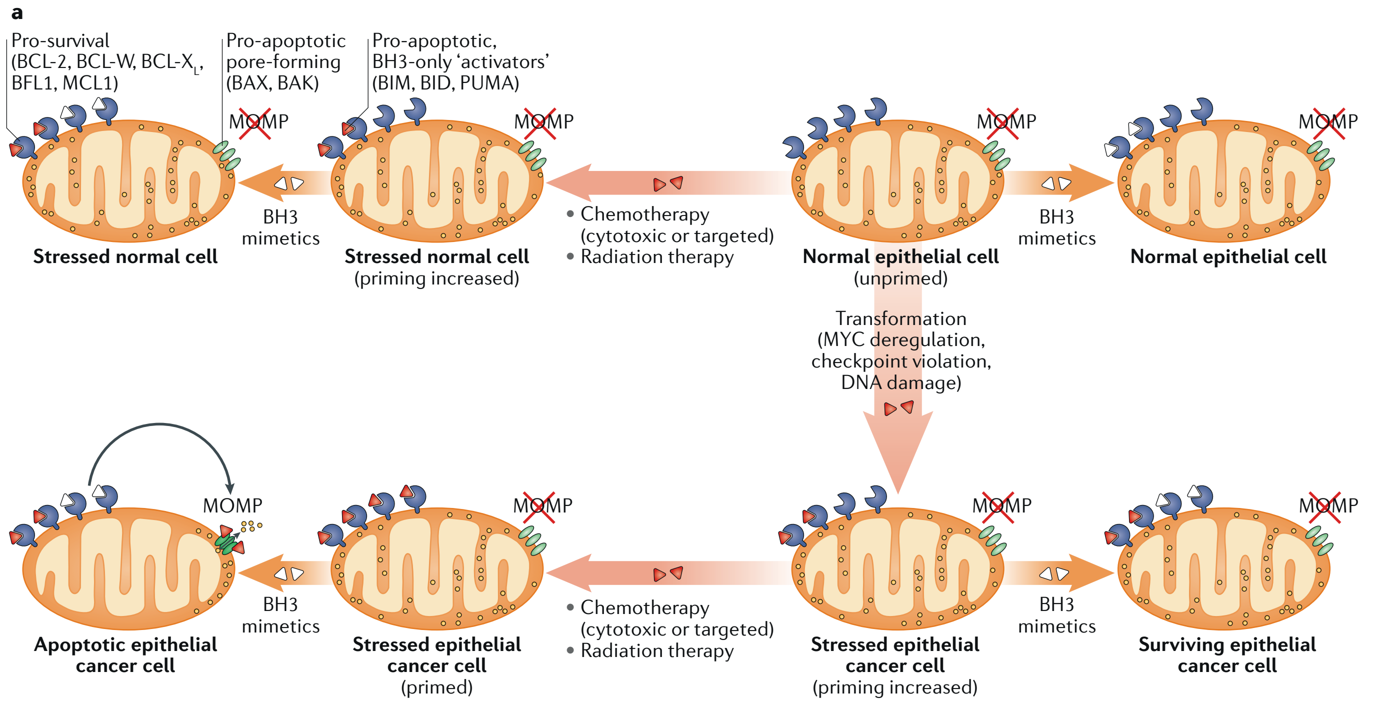
One-fourth of the world's population is infected with *Mycobacterium tuberculosis*, leading to more than 10 million symptomatic illnesses and more than 1.7 million deaths each year. Infection with *M. tuberculosis* initiates when lung macrophages phagocytize the bacteria in the alveolar spaces. Notably, the internalized bacteria are able to survive and replicate within these host cells. Once detected, the host attempts to use autocrine or paracrine signalling to trigger extrinsic apoptosis in infected macrophages. However, *M. tuberculosis* cell wall components enhance the activity of the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) to drive production of pro-survival protein MCL1, which inhibits both extrinsic and intrinsic apoptosis, thereby supporting further replication of the pathogen[136]. Finally, when the threshold of approximately 20 bacteria per cell is reached, the cell undergoes a form of cell death termed high-multiplicity of infection (MOI) apoptosis[136], which shares features of apoptosis and necrosis and may be the preferred mode of self-destruction from the perspective of pathogen spreading because of the non-immunogenic nature of apoptotic cell death. Once released from the dying cells, the bacteria are able to infect additional cells and continue the cycle or spread to other individuals via aerosolized transmission. A similar mechanism is employed by *Legionella pneumophila*, which secretes a protein (the phosphoinositide phosphatase, SidF) into infected host cells that blocks the activity of several pro-apoptotic proteins to prevent the mitochondrial apoptosis that typically serves to limit pathogen expansion[137].

Similarly to intracellular bacteria, viruses require the host cells to replicate and thus must prevent their self-destruction upon detection of infection. Cytomegalovirus (CMV), for example, produces multiple proteins that suppress apoptosis, including the viral mitochondria-localized inhibitor of apoptosis (vMIA, also known as pUL37x1), which is structurally similar to BCL-X_L despite considerable sequence differences[138]. Mitochondrial apoptosis can be blocked by vMIA via mechanisms reminiscent of BCL-X_L, including the binding and sequestration of BAX at the mitochondrial membrane to block MOMP. In addition, a product of the m41.1 open reading frame of CMV encodes a mitochondria-localized inhibitor of BAK oligomerization (v-IBO), which, in combination with vMIA, completely inhibits the intrinsic apoptosis pathway[138]. Interestingly, CMV also causes ER stress during active infection and actively produces the viral protein UL38 to block ER-stress-induced apoptosis. Similar mechanisms can be observed in cells infected with Epstein–Barr virus (EBV) and Kaposi sarcoma gamma-herpesviruses (KSHVs), which encode the pro-survival BCL-2 viral homologues BHRF1 and KSHV–BCL-2, respectively, which can bind and sequester BIM[139,140]. In addition, the expression of the EBV-encoded latent membrane protein 1 (LMP1) upregulates expression of several pro-survival proteins including BCL-2, MCL1 and BFL1 (REFS[141–143]) to suppress apoptosis during viral replication, making it a promising therapeutic target for the treatment of EBV infection. Note that expression of these virus-associated pro-survival proteins can suppress apoptosis during malignant transformation, facilitating the development of a wide range of cancer types[144].

Promoting apoptotic cell death for therapeutic benefit. On the basis of our understanding of apoptosis regulation in cancers, great effort across many academic and industry laboratories has been dedicated to the development of agents that can inhibit pro-survival protein function, with an aim of promoting apoptosis in cancer cells (BOX 1).

The involvement of BCL-2 family proteins in several types of autoimmune disorder suggests that inhibiting pro-survival protein function may reverse the abnormal accumulation of cells that would typically be destined for deletion. BH3 mimetics may, thus, have therapeutic efficacy in these diseases, and promising results have emerged from preclinical studies. For example, in the mouse model of SLE, treatment with the BCL-2 inhibitor venetoclax reduced the numbers of peripheral B cells and dampened phenotypes associated with the disease[145,146]. Based on the success in mouse studies, clinical trials are now underway to evaluate venetoclax in patients diagnosed with SLE. This treatment seems to show good tolerability[147], but therapeutic data are not yet available.

As highlighted above, infectious pathogens frequently suppress apoptosis in host cells as part of their infectious strategy. Theoretically, inhibiting the pro-survival signalling in these situations would enable apoptosis to proceed as intended and potentially could prevent expansion of infectious pathogens in the host. Thus far, BH3 mimetics have been shown to be effective during *L. pneumophila* infection. In this case, the bacterium blocks host cell protein synthesis, causing a



b

MOMP

Normal haematopoietic cell (highly primed)

BH3 mimetics

Apoptotic haematopoietic cell

1 Evasion of apoptosis via BIM mutation

Mutant BIM

Unable to bind

Unable to bind

Haematopoietic cancer cell (unprimed)

Haematopoietic cancer cell (unprimed)

2

3

Transformation (MYC deregulation, checkpoint violation, DNA damage)

Apoptotic haematopoietic cell

4 Evasion of apoptosis via BCL-2 or MCL1 upregulation

Haematopoietic cancer cell (highly primed)

Apoptotic haematopoietic cancer cell

Evasion of apoptosis via BAX downregulation

Haematopoietic cancer cell (apoptosis refractory)

Haematopoietic cancer cell (apoptosis refractory)

Evasion of apoptosis via BIM downregulation

Haematopoietic cancer cell (highly primed)

Apoptotic haematopoietic cancer cell

◀ Fig. 4 | **Apoptotic dependencies in cancer cells and their responsiveness to therapy.** **a** | During the process of neoplastic transformation, oncogene-driven abnormal growth signals and cell-cycle checkpoint violation lead to cellular stress and upregulation of pro-apoptotic proteins. This upregulation results in higher apoptotic priming of malignant cells at the basal state than normal cells. Because healthy adult tissues are mostly refractory to apoptosis or unprimed (see also FIG. 3), this increase in apoptotic priming of aberrant cells can be exploited therapeutically using BH3 mimetics, particularly when combined with standard anticancer therapies such as radiation or chemotherapy. **b** | Haematopoietic cells are naturally highly primed for apoptosis. Hence, oncogenic stress and the resulting upregulation of pro-apoptotic factors frequently result in the removal of pre-malignant cells derived from this lineage (middle arrow). Several mechanisms associated with BCL-2 protein deregulation, including BCL-2-interacting mediator of cell death (BIM) mutations (step 1), downregulation of BCL-2-associated X protein (BAX) (step 2) or BIM (step 3) and upregulation of BCL-2 or myeloid cell leukaemia 1 (MCL1) (step 4) support the emergence of haematopoietic cancers, which depend on these mechanisms for their survival. Notably, these mechanisms are mainly employed to keep malignant cells alive, and haematopoietic cancers typically remain primed for apoptosis and hence are susceptible to therapy and respond well to treatment with BH3 mimetics. However, certain mechanisms, including mutations of sensitizer proteins (step 1) and downregulation of mitochondrial outer membrane permeabilization (MOMP) pore-forming components (step 2) yield cells that are unprimed or even apoptosis refractory and hence resistant to therapy. Solid tumours can employ similar mechanisms to boost their survival, but these dependencies are much less pronounced than in haematopoietic cancers. Nevertheless, these mechanisms may underlie the development of resistance to treatment and disease relapse. BAK, BCL-2 antagonist/killer; BCL-W, B cell lymphoma W; BCL-X_L, B cell lymphoma extra large; BFL1, BCL-2-related isolated from fetal liver 1; BID, BH3-interacting domain death agonist; PUMA, p53-upregulated modulator of apoptosis.

Cell-cycle checkpoint violation
The failure of a cell to stop at specific checkpoints in the cell cycle when it would normally examine internal and external cues to determine whether to advance with cell division.

Amyloid-β
(Aβ). A peptide produced through the proteolytic cleavage of a transmembrane protein, amyloid precursor protein (APP), by β-secretases and γ-secretases. Accumulation of Aβ in the brain is thought to be an early event in the pathogenesis of Alzheimer disease.

Tau
The major microtubule-associated protein (MAP) of a normal postmitotic and mature neuron. Tau has six molecular isoforms that are generated by alternative splicing and is thought to promote the assembly of tubulin into microtubules. In Alzheimer disease and other tauopathies, tau is hyperphosphorylated and aggregates into neurofibrillary tangles to impair neuronal function.

Neurofibrillary tangles
(NFTs). Aggregates of hyper-phosphorylated tau proteins within neurons that cause dysfunction.

reduction in expression of high-turnover MCL1 and sensitizing cells to BCL-X_L inhibition[148]. Although using BH3 mimetics to target mammalian BCL-2 family proteins can be efficacious in certain contexts, developing agents that block the function of the pathogen-encoded counterparts would likely further widen the therapeutic index. A potential challenge here is the development of small molecules that inhibit the activity of pro-survival proteins expressed by the pathogen but not that of their mammalian counterparts, and we need to learn more about how to target these pathogenic proteins.

Excessive apoptosis in disease

Similarly to insufficient apoptosis, the loss of cells due to inappropriate or excessive apoptosis may be deleterious. The most notable example includes non-regenerative tissues, such as the brain, which are particularly susceptible to the loss of cells. Excessive apoptosis can also be caused by pathogens.

Neuronal cell death in neurodegenerative diseases. Prominent neurodegenerative diseases including Alzheimer disease, amyotrophic lateral sclerosis (ALS), Parkinson disease and Huntington disease are all characterized by the accumulation of misfolded proteins[149,150], which eventually disrupt the nervous system by aggregating and depositing within cells or the extracellular matrix, culminating in the dysfunction or loss of neurons[5]. Although apoptosis is important for perinatal brain development as discussed above, the vast majority of cells in the adult nervous system are postmitotic and profoundly resistant to apoptosis (apoptosis refractory) owing to insufficient expression of pre-MOMP and post-MOMP pro-apoptotic proteins[45,151–153] (FIGS 2,3). Despite this, apoptosis-associated proteins may be upregulated during the pathogenesis of these diseases and, as discussed below, may represent a potential novel therapeutic target for neurodegeneration.

In Alzheimer disease, the accumulation of amyloid-β (Aβ) peptides and tau-containing neurofibrillary tangles (NFTs) leads to the dysfunction and death of neurons, resulting in a progressive dementia that is eventually fatal[5]. Although the exact mechanisms by which neurons undergo cell death in response to Aβ peptides and tau-NFTs is unclear, it has been shown that synthetic Aβ peptides can activate caspase 3 and induce apoptosis in cultured neurons[154]. Interestingly, several groups have reported that caspases may also directly cleave tau and drive formation of NFTs[154], potentially linking Aβ to tau-NFT formation and to Alzheimer disease pathogenesis. Other studies have provided evidence that Aβ kills neurons by first inducing membrane-associated oxidative stress, resulting in lipid peroxidation and the production of 4-hydroxynonenal[155] and ceramide[156], which activate the intrinsic mitochondria-dependent apoptosis pathway. Most pertinent to this Review, Aβ has been reported to induce apoptosis by downregulating BCL-2 and upregulating BAX in addition to inducing oxidative stress and apoptotic cascades in synapses and dendrites[157]. However, it is important to note that in vitro cultures of primary neurons are typically derived from embryos or neonates, and these cells are more sensitive to apoptotic cell death than adult neurons[45] (see above), thus making generalization to human disease challenging.

Several mouse lines expressing human Aβ precursor (APP) show age-dependent accumulation of Aβ in the hippocampus and cerebral cortex, decreased neurogenesis and increased neurodegeneration along with markers of apoptosis[158]. In support of the contribution of intrinsic apoptosis to the pathology of Alzheimer disease, increased expression of pro-apoptotic BAX and BAK was shown in hippocampal neurons in the majority of brains obtained from individuals with Alzheimer disease, along with changes in other BCL-2 family proteins[4,159]. Furthermore, the potential benefit of inhibiting mitochondrial apoptosis has been directly demonstrated through the use of a triple-transgenic (overexpressing APPswe, tau-P301L and Presenilin-1 (PS1-M146V)) Alzheimer disease mouse model wherein overexpression of anti-apoptotic BCL-2 blocked activation of caspases 9 and 3; in these conditions, the degree of caspase cleavage of tau was limited, the formation of plaques and NFTs was inhibited and memory retention was improved[160].

The pathological hallmark of Parkinson disease is progressive and selective dopaminergic neuronal degeneration in the substantia nigra pars compacta (SNpc), the brain region that controls motor activity[161]. Although the mechanisms underlying the selective loss of nigrostriatal dopaminergic neurons in this disease have not yet been elucidated, it has been shown that Parkinson disease is associated with mitochondrial dysfunction, and several recent human post-mortem studies have suggested that dopaminergic neurons die by apoptosis[161]. To date, at least three genes have been found to be associated with Parkinson disease; these genes encode α-synuclein, parkin and PTEN-induced kinase 1 (PINK1)[161]. Deletion of PINK1 in human and mouse

neurons sensitizes them to apoptosis, as evidenced by decreased overall viability and increased caspase 3 activation in response to the classical apoptosis-inducing pan-kinase inhibitor staurosporine[162]. Parkin deficiency, which is a prominent cause of familial Parkinson disease, may contribute to heightened apoptotic sensitivity of cultured cells of neural lineage[163]. Adding further evidence to the causative role of apoptosis in Parkinson disease, experimental mouse models of the disease have shown that p53-mediated upregulation of BAX is a necessary step in SNpc dopaminergic neuron apoptosis caused by mitochondrial dysfunction (inhibition of respiratory complex I)[164].

ALS is a common motor neuron disease initiated by the loss of motor neurons in the brain and spinal cord[163], which leads to muscle weakness, paralysis and ultimately death due to respiratory failure. Several gene mutations have been identified that contribute to this disorder, with more than 20% of cases of familial ALS being linked to mutations in the gene encoding copper/zinc superoxide dismutase 1 (SOD1)[165]. Alterations in the expression of different BCL-2 family members have been described in the spinal cord of transgenic mice expressing mutant SOD1 and in humans with ALS[163]. Moreover, survival of SOD1-mutant mice is prolonged by the overexpression of BCL-2 (REF.[163]). Importantly, it has been shown that although BAX deletion only slightly increases the survival of SOD1-mutant mice, it strongly protects motor neurons against apoptosis and improves motor function by preventing neuronal loss and degeneration, slowing the onset of ALS symptoms, including paralysis[166].

Huntington disease is an inherited neurodegenerative disease characterized by chorea, personality changes, dementia and early death[167] that results from the selective death and dysfunction of specific neuronal subpopulations within the central nervous system. The gene responsible for these effects encodes huntingtin, and contains a polymorphic stretch of repeated CAG trinucleotides, encoding a polyglutamine tract. Huntingtin gene in healthy people possesses fewer than 20 repeats, and huntingtin with more than 35 repeats results in a higher probability of developing Huntington disease. In vitro models of Huntington disease show clear signs of apoptosis, as indicated by neurite loss or destruction, chromatin condensation and nuclear pyknosis and fragmentation[167]. In support of the role of apoptotic cell death in neurodegeneration in Huntington disease, brains of individuals expressing mutant huntingtin reproducibly stain for apoptotic markers (terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) positivity and activation of caspases 1, 3, 6 and 8), and these effects can be inhibited in mice by co-expression of BCL-2, BCL-X_L or caspase inhibitors[168].

In summary, although these different neurodegenerative diseases are driven by highly diverse mechanisms, they broadly involve the loss of neurons, often through apoptosis, which then results in disease-associated phenotypes. In each case, direct and potent inhibitors of BAX and BAK would potentially be effective at preventing the loss of neurons and delaying disease progression and mortality. However, the degree to which these strategies would be effective therapeutically depends on whether the host would be better served by keeping damaged or dysfunctional neural cells in place or perhaps allowing them to die via apoptosis. This question can be answered only by assessing functional outcomes including, for example, motor function, cognition and long-term survival upon apoptosis inhibition. Although some evidence, as outlined above, indicates that blocking mitochondrial apoptosis can have therapeutic benefit in neurodegeneration, other studies have demonstrated mixed results[169].

Acute brain injuries. Cerebral ischaemia has traditionally been considered to induce an exclusively necrotic cell death in affected neural cells[170]. However, more recent work has demonstrated that, although cells within the ischaemic core indeed die via necrosis, those within the penumbra may remain metabolically active and die via apoptosis at a later point, which exacerbates tissue loss and dysfunction[171]. Several reports connect BCL-2 family proteins directly to ischaemia–reperfusion injury in the brain, including evidence of BID cleavage (indicative of activation) following ischaemia in brain tissue and observations that loss of BID reduces the extent of infarcted brain tissue[172]. However, there are few data that show that inhibition of mitochondrial apoptosis may be effective at minimizing ischaemia–reperfusion-associated dysfunction in adult brain tissue[173]. Nevertheless, on the basis of the increased expression of apoptosis-related genes in the developing brain, we propose that mitochondrial apoptosis is most involved in ischaemia–reperfusion injury in young humans (especially during the perinatal period and infancy) and not in adults. As expected, BAX and downstream caspases are activated in the neonatal model of ischaemia–reperfusion injury[174].

Similar to cerebral ischaemia, there seems to be a greater role for mitochondrial apoptosis in neonatal traumatic brain injuries than in adult brain injuries[175,176]. This difference would suggest that cells primed for apoptosis within the brain respond to certain injuries with pro-apoptotic signalling that can effectively activate BAX. It is thus likely that brains of young mammals would be hypersensitive to any type of damage or stress that induces apoptotic signalling because of their heightened BAX expression and apoptotic priming (FIG. 3).

Cell death induced by pathogens. In contrast to the mechanisms inhibiting cell death in host cells upon infection mentioned above, which are only starting to emerge, the important role of apoptosis in the deleterious effects of infection in humans is well recognized. Here, we describe two notable examples.

In Nobel-prize-winning work, *Helicobacter pylori* was shown to be directly responsible for the development of gastritis and eventually peptic ulcers[136], along with gastric lymphomas and adenocarcinomas. *H. pylori* contains several virulence factors; among these, vacuolating cytotoxin (VacA) has been shown to directly induce mitochondrial apoptosis in gastric epithelial cells and is thought to play a crucial role in the development of ulcers in the duodenum[177]. Mechanistically, VacA can induce receptor-like protein tyrosine phosphatase-β (RPTPβ)

4-Hydroxynonenal
(4-HNE). A product of lipid peroxidation that can induce apoptosis.

Ceramide
A lipid that acts as a second messenger in activating apoptosis within the sphingomyelin pathway, which is initiated by the hydrolysis of the plasma membrane phospholipid sphingomyelin.

Presenilin-1
The catalytic subunit of γ-secretase, which is a protease that cleaves a variety of type 1 transmembrane proteins, most notably amyloid precursor protein. Mutations in the *PSEN1* gene encoding presenilin 1 are the most common cause of familial Alzheimer disease.

Chorea
A movement disorder that causes involuntary, unpredictable body movements.

TUNEL
(Terminal deoxynucleotidyl transferase dUTP nick end labelling). An assay that detects fragmented DNA, which is one of the hallmarks of apoptotic cell death.

Penumbra
In medicine, the area surrounding the focal point of an ischaemic event.

to cause activation of BAX and BAK[178]. In addition, this toxin inhibits JAK–STAT3 signalling, causing downregulation of pro-survival proteins BCL-2 and BCL-X_L, leading to apoptosis[179].

As another example, prolonged infection with HIV causes AIDS, owing to progressive loss of $CD4^+$ T cells, which involves BCL-2 family proteins at several levels. First, HIV triggers apoptosis in T cells upon infection by producing HIV proteases that specifically cleave and inactivate BCL-2 (REF.[180]), thereby shifting the balance of pro-survival and pro-apoptotic proteins towards apoptosis. Furthermore, infected cells also secrete viral proteins such as gp120, TAT and NEF into the extracellular environment. gp120 binds to various receptors on T cells, which activate the extrinsic and intrinsic apoptosis pathways in bystander (not necessarily infected) T cells[181]. The intrinsic apoptosis of bystander T cells is proposed to be driven by gp120-mediated upregulation of BAX[182] along with concurrent downregulation of BCL-2 (REF.[183]).

It is important to note that pathogen-induced death of infected cells may also occur via necrosis. This process is especially likely to occur at the lytic phase of infection, during which extensive bacterial or viral replication has been completed and when necrotic cell death facilitates the maximal spread of infectious material to additional host cells and, eventually, additional host organisms[104,184].

Inhibiting apoptotic cell death for therapeutic benefit. The strong evidence for apoptosis having a role in many diseases associated with abnormal cell loss has prompted efforts to drug this pathway for therapeutic benefit. However, the lack of potent and selective inhibitors of apoptosis, especially at the level of the BCL-2 family, has prevented more rigorous evaluation of the therapeutic potential in this space. Early studies tested the therapeutic efficacy of pan-caspase inhibitors such as Z–VAD–FMK that could block the key executioner caspases. Importantly, the use of these agents is expected to have limited clinical benefit for direct apoptosis inhibition because they work downstream of MOMP and cannot reverse the profound damage to mitochondrial function that is induced by BAX-mediated and BAK-mediated pore formation. Nevertheless, pharmacological inhibition of caspases is able to reduce caspase activation in response to ischaemia and traumatic injuries in adult brain tissue and can provide a therapeutic effect, in particular when combined with antagonists of the *N*-methyl-D-aspartate (NMDA) receptor, which reduce excitotoxicity[185]. It is possible that caspase inhibitors are acting via mechanisms outside of apoptosis prevention in these scenarios, such as the prevention of caspase-mediated cleavage of targets unrelated to apoptosis, as is the case for tau[154]. In addition, because of the involvement of caspases in key processes such as inflammation, these inhibitors may be blocking inflammatory pathways that have pathogenic roles such as neuroinflammation[186]. In neonates, several studies closely examined the use of caspase inhibitors for neuroprotection, reporting that they reduce caspase activation, TUNEL positivity and tissue damage[187–189]. Crucially, however, when functional outcomes were tested, the administration of these agents did not lead to improvement in post-injury motor or executive function[187], again suggesting that inhibiting caspases is unlikely to be sufficient to completely block injury-associated dysfunction if the primary mechanism is mitochondrial apoptosis.

Excitotoxicity
A process whereby cells in the nervous system are killed by excessive neurotransmitter stimulation.

More recently, agents that directly inhibit the activity of BAX have been reported based on strong in vitro evidence of binding and cell death inhibition[190] (BOX 1). Excitingly, these agents can protect neurons from excitotoxic damage in vitro[190] and from ischaemia injury in vivo[191] but likely require further optimization before clinical testing. Regardless, the development of these and additional agents is expected to provide new opportunities to reverse the progression of diseases that are driven by excessive apoptosis.

Conclusions and perspective

Accumulating evidence suggests that deregulated (increased or decreased) apoptosis is involved in the pathological depletion or accumulation of cells in many human disorders. However, there is an unclear understanding of the molecular mechanisms that drive deregulation of apoptosis during the onset of these diseases and how the apoptotic pathway may be modulated for clinical benefit. Continued research in this space should seek to define the cellular and molecular targets for controlling apoptosis — keeping it in check or enhancing, according to need — and to explore their potential for clinical translation. Of crucial importance in this space is the use of functional and physiological assessments of cellular survival as measurements of therapeutic efficacy of potential therapeutic agents instead of simply the preservation or elimination of cells. For example, a cardiomyocyte that does not activate caspases in response to ischaemia–reperfusion injury may appear to be alive by typical biochemical measurements but may be functionally inert. In fact, the presence of a functionally inert and damaged cell may be more deleterious to the host than a cell that has committed itself to apoptotic cell death and, as a result, has been cleared via non-immunogenic processes and perhaps even replaced. In addition, therapeutic efforts must consider our growing knowledge of the pivotal roles of apoptosis in maintaining organismal homeostasis and the dynamic nature of its regulation in different tissues at different ages; the modulation of apoptosis pharmacologically is expected to have drastically different effects in young individuals versus adults[45,84,152] and potentially in humans of advanced age (in which apoptotic potential might change, as exemplified by the changes in apoptotic susceptibility in the HSC niche and platelets)[103]. In addition, although the roles of BCL-2 family proteins in adult tissues have been studied and are thought to be established, the effects of cellular damage, stress or disease-associated factors are difficult to predict and have not been comprehensively characterized. Thus, it is likely that the function of some BCL-2 family proteins can become apparent only in specific physiological or pathological contexts. Furthermore, although our understanding of BCL-2 family regulation by cell-autonomous processes is steadily improving, how interactions between cells and their microenvironment (including immune cells, vasculature, growth factors and

nutrient availability) can affect apoptotic signalling is highly complex and may not be clear for quite some time.

Despite these challenges, research into how BCL-2 family proteins regulate and deregulate physiology is proceeding at a rapid pace, and additional opportunities for clinical translation are constantly being identified. In cancer, where the modulation of apoptosis has a clear and strong rationale, the biggest challenge is identifying which patients are most likely to benefit from treatment with BH3 mimetics and how to combine these mimetics with existing therapies to maximize efficacy and limit toxicity. As these agents have on-target toxicities (for example, thrombocytopenia due to BCL-X_L inhibition), the clinical development of biomarker assays that can measure cancer cell dependence on specific pro-survival BCL-2 family proteins is needed. A strong fundamental understanding of BCL-2 family protein function has been built, and methods for assigning therapies[192,193], monitoring responses[194] and predicting mechanisms of therapy resistance[195–197] are all now possible. Putting the efforts of so many into practice, the first-ever use of BH3 mimetics for cancer therapy has been approved[115], with many future successes in the modulation of BCL-2 family proteins for clinical benefit on the horizon (BOX 1).

Published online 17 January 2019

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Acknowledgements
The authors acknowledge the many researchers who contributed to their understanding of apoptosis and apologize that they could not cite all the relevant research because of space restrictions. The authors thank B. Croker, G. Joshi, A. Presser, J. Spetz, K. Webster and T. Gershon for critical feedback and helpful discussions. The authors gratefully acknowledge funding from the Alex's Lemonade Stand Foundation for Childhood Cancers Young Investigator Award (K.S.), Andrew McDonough B+ Foundation Childhood Cancer Research Grant (K.S.), Harvard T.H. Chan School of Public Health Dean's Fund for Scientific Advancement (K.S.), Making Headway Foundation St Baldrick's Research Grant (K.S.) and NIH/NCI grant R00CA188679 (K.S.).

Author contributions
The authors contributed equally to all aspects of the article.

Competing interests
The authors declare no competing interests.

Publisher's note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary information
Supplementary information is available for this paper at https://doi.org/10.1038/s41580-018-0089-8.

nature REVIEWS MOLECULAR CELL BIOLOGY

SUPPLEMENTARY INFORMATION
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Regulation of apoptosis in health and disease: the balancing act of BCL-2 family proteins

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https://doi.org/10.1038/s41580-018-0089-8

Supplementary Box 1: Detecting apoptotic cell death

As a cell dies via apoptosis, there are several morphological and molecular characteristics that can be detected and accurately measured, including:

Morphological changes:

1. Membrane blebbing (bulky protrusions from the plasma membrane): detected by microscopy, flow cytometry
2. Cytoplasm and cytoskeleton compaction (cell shrinkage): detected by microscopy, flow cytometry
3. Chromatin condensation (indicative of DNA degradation): detected by a) TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) staining and microscopy; b) DNA laddering assay
4. Formation of apoptotic bodies (membrane-bound vesicles containing portions of the cytoplasm): detected by microscopy.

Molecular markers:

1. Caspase 3/7 activation (serine proteases that degrade cellular contents and prepare cell for phagocytosis): detected by a) immunoblotting for cleaved caspases; b) caspase activity assays; c) immunohistochemistry or immunofluorescence for cleaved caspases
2. Mitochondrial outer membrane permeabilization (MOMP; pores in the outer membranes of mitochondria that are formed by activated BAX or BAK that mediate release of cytochrome c): detected by a) loss of mitochondrial potential; b) efflux of cytochrome c from mitochondria into the cytoplasm by microscopy, flow cytometry or cell fractionation followed by western blotting to compare levels in mitochondrial versus cytoplasmic fractions
3. Externalization of phosphatidylserine (“eat-me” signal for phagocytosis): detected by Annexin V staining and microscopy or flow cytometry
4. PARP-1 cleavage (one of several well-established substrates of caspases): detected by immunoblotting for cleaved PARP-1; b) immunohistochemistry or immunofluorescence for cleaved PARP-1
5. DNA degradation (DNA is cleaved into nucleosome-sized fragments by caspase-activated DNase): detected on agarose gels after ethidium bromide staining
6. BAX and BAK activation (BAX and BAK undergo conformational changes and oligomerize upon activation): detected by a) immunoblotting for activation-specific antibodies to BAX and BAK; b) immunoblotting for BAX and BAK oligomers after crosslinking.

Supplementary Box 2: Extrinsic apoptosis pathway.

Although the intrinsic pathway plays a pivotal role in controlling normal tissue homeostasis, the extrinsic pathway also contributes to tissue maintenance, especially within the haematopoietic system[1,2] and there is substantial cross-talk between the two pathways. Extrinsic apoptosis is activated by extracellular death ligands that bind and activate transmembrane death receptors (DRs), which are members of the tumour necrosis factor receptor superfamily. Each of these death receptors, which includes Fas/CD95, TNFR-1, DR3, DR4/TRAILR1 and DR5/TRAILR2, contain a cytoplasmic "death domain" that is responsible for transmitting the ligand signal to the apoptotic machinery[3,4]. Upon binding of their respective death ligands (such as FasL, TRAIL, and TNFα), the death receptors trimerize and recruit adapter proteins to the death domain, forming the death-inducing signalling complex (DISC)[5–7]. Initiator caspases (e.g. caspase 8 and caspase 10) are then recruited to the DISC via their death effector domains (DEDs) or caspase recruitment domains (CARDs), and are subsequently cleaved into their mature, active forms[1,5,8–12]. In cells that are designated as "type I", the activated initiator caspases are able to cleave and fully activate downstream effector caspases (i.e. caspase 3, caspase 6 and caspase 7) that prepare the cell for engulfment[10]. However, in "type II" cells, caspase 8 activation alone is insufficient to fully activate the effector caspases and requires a mitochondrial amplification loop[13]: active caspase 8 cleaves and activates BID (forming truncated tBID), which then activates BAX or BAK at the mitochondria to cause MOMP and activate caspase 9 and, subsequently, effector caspases via the intrinsic pathway[14,15]. These states are not completely permanent in a given cell type, as evidenced in T cells. For instance, just after activation but prior to proliferation, T cells have been shown to be type II cells yet long-term activated and proliferating T cells appear to be type I cells[16] due to higher expression of DISC components and highly efficient DISC formation upon stimulation with Fas[16,17].

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