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Attenuating Apoptosis in Chinese Hamster Ovary Cells for Improved Biopharmaceutical Production

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Abstract

Chinese Hamster Ovary (CHO) cells are the predominant host cell line for the production of biopharmaceuticals, a growing industry currently worth more than $188 billion USD in global sales. CHO cells undergo programmed cell death (apoptosis) following different stresses encountered in cell culture, such as substrate limitation, accumulation of toxic by-products, and mechanical shear; hindering production. Genetic engineering strategies to reduce apoptosis in CHO cells have been investigated with mixed results. In this review, a contemporary understanding of the real complexity of apoptotic mechanisms and signalling pathways is described; followed by an overview of anti-apoptotic cell line engineering strategies tested so far in CHO cells.

Keywords: CHO Cell Line Engineering, Apoptosis, Biopharmaceutical Production, Programmed Cell Death

Introduction

The global biotherapeutics market was valuated at $188 billion USD in 2017 (Walsh, 2018) and continues to grow. Chinese hamster ovary (CHO) cells are the most widely used mammalian production cell line in the biopharmaceutical industry due to their robustness, scalability, ability to generate human-like post-translational modifications, and proven regulatory track record (Jayapal, Wlaschin, Hu, & Yap, 2007; Matasci, Hacker, Baldi, & Wurm, 2009; Walsh, 2010; Wurm, 2004). However, cell death

arising from stresses encountered during the cell culture process remains an issue for industrial biopharmaceuticals production.

Cell death reduces product yield in cell culture (Arden & Betenbaugh, 2004), and can negatively affect product quality (Dorai & Ganguly, 2014). Cell death results in increased release of proteases, glycosylases and sialidases, which degrade proteins and alter protein glycans structure, especially at the later stages of batch culture (Arden & Betenbaugh, 2004; Birch & Racher, 2006; Curling et al., 1990; Gramer & Goochee, 1993; S. Weikert et al., 1999; Shuler & Kargi, 2002; Wong, Wong, Nissom, Heng, & Yap, 2006). Moreover, debris from cell death is known to complicate downstream processing (Aboulaich et al., 2014; Gagnon et al., 2014; Singh et al., 2016). Ultimately, these issues can lead to the early harvest of the culture, and reduce final product titres by as much as 30%, as observed for monoclonal antibodies (mAb) (Shuler & Kargi, 2002).

Several bioprocessing strategies have been used to extend cell life and increase yields in culture, such as medium modifications, substrate feeding, and bioreactor design optimisation (Arden & Betenbaugh, 2004; Becerra, Berrios, Osses, & Altamirano, 2012; Ferrer & Valero, 2016; Kelly, Clarke, Clynes, & Barron, 2014; Vives, Juanola, Cairó, & Gòdia, 2003). However, an intrinsically death-resistant cell line is desirable for reliable product quality and yield in the biopharmaceutical industry.

The two main methods by which CHO cell death may occur are apoptosis and necrosis. Apoptosis has been shown to be the primary cell death mechanism in CHO cell culture and hence forms the subject of this review (Moore et al., 1995). Apoptosis is defined as programmed cell death in response to extra and intracellular signals (Goswami et al., 1999). Apoptosis-inducing signals include, but are not limited to,

nutrient limitation, hypoxia, hypertonicity, pH, and increased protein synthesis load via the unfolded protein response (UPR) (Al-Rubeai & Sing, 1999). Apoptosis is physiologically characterized by the orderly loss of cellular volume, membrane blebbing, nucleus condensation, and DNA fragmentation (Al-Rubeai & Sing, 1999; Elmore, 2007; Krampe & Al-Rubeai, 2010; Taylor, Cullen, & Martin, 2008). Multicellular organisms, including Chinese hamsters, benefit from such a programmed death response as it allows for the destruction of cancerous or infected cells. However, in the context of cell culture for biopharmaceutical production, these cellular mechanisms are undesirable events that conflict with the ultimate bioprocess objective of producing high levels of recombinant proteins with desired post-translational modifications.

Apoptosis signalling pathways

Apoptosis is initiated and controlled by numerous signalling pathways which can be broadly classified as either extrinsic: responding to extracellular stresses, or intrinsic: responding to intracellular stresses (Figure 1).

Classically, all apoptosis pathways converge on the activation of caspases, a group of proteases otherwise referred to as the ‘central executioners’ of apoptosis (Hail, Carter, Konopleva, & Andreeff, 2006). During apoptosis, initiator caspases (caspases-8,9,10) (M. Chen & Wang, 2002) cleave and activate the less pathway-specific executioner caspases (caspases-3,6,7) (Elmore, 2007). The action of executioner caspases lead to the systematic deconstruction of cellular organelles and ultimately its own destruction. The ubiquity of caspases in apoptotic pathways and their highly efficacious method of action, have led to this ‘caspase-centric’ view of apoptosis. However, numerous caspase-independent apoptotic mechanisms have been observed

(Lartigue et al., 2009; Momeni, 2011; Tait & Green, 2008; Takano et al., 2005). It should thus be noted that apoptosis is a complex, multi-nodal process that is yet to be comprehensively understood.

The extrinsic apoptosis pathway

The extrinsic, or death receptor, pathway (Figure 2) is triggered through the binding of a death ligand, of the tumour necrosis factor (TNF) family, to an associated receptor (TNF receptor, or TNFR) on the cell surface. The ligand-receptor complex on the intracellular membrane side forms the Death-Inducing Signalling Complexes (DISCs). Two well-characterized pairings of TNFs and TNFRs are the TNF-related apoptosis-inducing ligand (TRAIL, also known as APO2L) and receptors (TRAIL-R1 and TRAIL-R2), and the first apoptosis signal ligand (FASL) and receptor (FAS also known as APO-1 or CD95) (Ashkenazi, 2002; Curtin & Cotter, 2003). Binding of the receptors to their respective ligand activates the protein FAS-Associated Death Domain (FADD), which subsequently converts pro-caspase-8 to caspase-8. Once activated, caspase 8 triggers caspase-3 activation promoting apoptosis (Ashkenazi, 2002). Caspase-8 is also able to initiate BID activation, leading to BAX and BAK translocation to the mitochondria and initiation of the mitochondria mediated apoptotic pathway (Ashkenazi, 2002) (see the Mitochondria-mediated apoptosis section below). FAS is also able to activate a caspase-independent apoptotic pathway through the JNK cascade (Figure 2) (Ashkenazi, 2002; Charette, Lavoie, Lambert, & Landry, 2000; L. Y. Chen & Chen, 2003; Curtin & Cotter, 2003).

The intrinsic apoptosis pathway

The intrinsic apoptosis pathway is a term which broadly applies to intracellular regulatory mechanisms which contribute to or signal the initiation of apoptosis. The

intrinsic apoptosis pathway ultimately triggers apoptosis through up to three interconnected signalling systems; the BCL-2 family proteins, calcium ions (Ca^{2+}), and reactive oxygen species (ROS). These systems are closely tied to the mitochondria and the endoplasmic reticulum (ER), and facilitate strong interactions between these organelles and the wider cell. These signals and organelles determine a pro-survival versus pro-apoptotic balance, which are categorised below as two responses: mitochondria-mediated apoptosis (MMA) and ER stress mediated apoptosis (Figures 3 and 5, respectively).

Mitochondria-mediated apoptosis

The mitochondria play a central role in apoptosis as effector of both the intrinsic and extrinsic pathways. MMA is achieved through the permeabilization of the mitochondrial outer membrane (MOMP), or by the permeabilization of both the inner (MIM) and outer membrane simultaneously by the mitochondria permeability transition (MPT).MOMP causes the release of apoptotic factors (Cyt-c, SMAC/DIABLO, OMI/HTRA2, AIF, and Endonuclease G (EndoG)) from the mitochondrial inter-membrane space into the cytoplasm (Korsmeyer et al., 2000; Luna-Vargas & Chipuk, 2016; Munoz-Pinedo et al., 2006; Rehm, Düßgmann, & Prehn, 2003; Scorrano & Korsmeyer, 2003; Wei et al., 2001; Wolter et al., 1997; Zong et al., 2003). Cyt-c directly activates apoptotic pathways in the cytosol through oligomerization with APAF-1 and caspase-9 to form the apoptosome (Korsmeyer et al., 2000; Pan, O'Rourke, & Dixit, 1998), in turn activating caspase-8 and subsequently executioner caspase-3 (Cai, Yang, & Jones, 1998; Liu, Kim, Yang, Jemmerson, & Wang, 1996). SMAC and OMI act indirectly by inhibiting 'inhibitor of apoptosis proteins' (IAPs), which would otherwise inhibit apoptosis (Flanagan et al., 2010). AIF and EndoG enter the nucleus causing DNA fragmentation and chromatin

condensation as direct parts of the coordinated cell death (Arnoult et al., 2003; Li, Luo, & Wang, 2001; Susin et al., 1996; Vařecha, Potěšilová, Matula, & Kozubek, 2012; Zamzami et al., 1996). (Figure 3).

MPT is concomitant with MOMP, but also releases concentrated Ca^{2+} and ROS from the MIM, subsequently causing oxidative damage in the cell (Briston et al., 2017; Crompton, 1999). Moreover, MOMP may occur independently of MPT through the oligomerization of BAK, BAX, and/or BOK (see BCL-2 family proteins). Both MOMP and MPTlead to cell death (Jourdain & Martinou, 2009; Landes & Martinou, 2011; Lartigue et al., 2009; Shoshan-Barmatz, Keinan, Abu-Hamad, Tyomkin, & Aram, 2010).

BCL-2 family proteins

BCL-2 family proteins (BFPs) possess up to four homologous regions; BH1, BH2, BH3 and BH4 (Oltvai, Milliman, & Korsmeyer, 1993). These BH regions are capable of interacting with one another, facilitating the activation or inhibition of the other family members (Reed, Zha, Aime-Sempe, Takayama, & Wang, 1996; Sato et al., 1994; Sedlak et al., 1995; Yin, Oltvai, & Korsmeyer, 1994). The function of all BFPs are related to apoptotic signalling, and categorised further as follows: effectors, activators, inhibitors, and sensitizers.

Effector BFPs are primarily BAK and BAX. These proteins may auto- or hetero-oligomerize with one another to form pore structures on the ER or mitochondrial outer membrane allowing the release of Ca2+ or pro-apoptotic signalling molecules respectively (Ghribi, DeWitt, Forbes, Herman, & Savory, 2001; Korsmeyer et al., 2000; N. S. Wang, Unkila, Reineks, & Distelhorst, 2001; Zha et al., 1996). A possible third effector BFP protein, BOK, has also been identified owing to its highly

homologous structure (Brem & Letai, 2016; Carpio et al., 2015; Chipuk & Luna-Vargas, 2016; Echeverry et al., 2013; Ke et al., 2013). Activator BFPs (Bid, BIM and PUMA) facilitate the localisation, oligomerization, or otherwise activation of the effector proteins. Inhibitor BFPs (BCL-2, BCL-w, BCL2A1, BCL-xL, and MCL-1) inhibit or disallow the oligomerization of effector BFPs. Sensitizer BFPs (BMF, BIK, HRK and NOXA) are capable of blocking the inhibitor class proteins, but not of activating effector proteins, and thus 'sensitize' the effectors to activator proteins (Kale, Osterlund, & Andrews, 2018; Shamas A, Kale J, Leber B, 2013) (Figure 4).

Endoplasmic reticulum-mediated apoptosis

Endoplasmic reticulum-mediated apoptosis is triggered by sustained ER stress through the Unfolded Protein Response (UPR) (Tabas & Ron, 2011). The UPR is an adaptive feedback system that monitors and manages ER redox, protein load, and folding homeostasis (Cao & Kaufman, 2012; Diehl, Fuchs, & Koumenis, 2011; Hampton, 2000; Hetz, 2012; Kaufman, 2002; Ron & Walter, 2007; Schröder & Kaufman, 2005; Walter & Ron, 2011). Unusually high levels of polypeptide folding events contribute to ER-mediated apoptosis through the accumulation of unfolded proteins, changes in the ER oxidation state, or the generation of ROS (Malhotra & Kaufman, 2007; Malhotra et al., 2008; Puthalakath et al., 2007; Rutkowski & Hegde, 2010). Although the precise sensing mechanisms remain unclear, it is understood that ER-stress is monitored by three UPR sensors: ATF6 (Ye et al., 2000), PERK (Harding, Zhang, Bertolotti, Zeng, & Ron, 2000) and IRE1 (Hetz et al., 2006; Ron & Walter, 2007; Rutkowski & Hegde, 2010; Walter & Ron, 2011), which in turn activate the transcription factors ATF6, ATF4, and XBP1. These transcription factors increase ER protein folding capacity (ATF6, XBP1), or decrease global translation and hence the protein-folding load on the ER (PERK), (Walter & Ron, 2011) in an

attempt to respond to abnormal protein-folding loads. However, failing a timely return to homeostasis, these factors ultimately trigger the apoptotic cascades by contributing to a feed-forward amplification of redox 'death' signals (Ron & Walter, 2007; Rutkowski et al., 2006; Rutkowski & Hegde, 2010; Tabas & Ron, 2011; Urra, Dufey, Lisbona, Rojas-Rivera, & Hetz, 2013) (Figure 5).

The ability of the UPR to act as a 'death' timer if ER stress remains unresolved is facilitated by CHOP ('control loop' 1 of the UPR, Figure 5). Once activated, CHOP restores global translation by upregulating GADD34 and ERO1 (Ron & Habener, 1992; Urra et al., 2013). If ER stress remains unresolved, restoring global translation ultimately contributes to increased ROS production and depletion of glutathione levels (Marciniak et al., 2004; Urra et al., 2013; Zinszner et al., 1998). CHOP has also been shown to downregulate BCL-2 (an anti-apoptotic factor) (Mccullough et al., 2001), further enhancing its dual-role.

IRE1 ('control loop' 2 of the UPR, Figure 5) binds with TRAF2 and ASK1, triggering JNK phosphorylation and caspase-12 activation ('control loop' 3 of the UPR, Figure 5) (Nishitoh et al., 2002; Yoneda et al., 2001), subsequently promoting BAK and BAX facilitated pore-formation through BCL-2 inhibition (Scorrano et al., 2003).

In addition to MOMP, BAK and BAX form pores on the ER, deregulating calcium homeostasis. The ER is the most important store of intracellular Ca^{2+}, with levels around 1,000 times higher than in the cytoplasm (Gorlach, Klappa, & Kietzmann, 2006). Ca^{2+} is a messenger involved in intra- and extracellular signalling cascades and plays an essential role in deciding cell life or death through its ability to regulate ion gradients (Giorgi, De Stefani, Bononi, Rizzuto, & Pinton, 2009; Görlach, Bertram, Hudecova, & Krizanova, 2015). Ca^{2+} release from the ER ensues if the UPR fails to

resolve stress, stimulating the opening of the MPTP causing an influx of Ca^{2+} ions into the mitochondria (Jacobson & Duchen, 2002). This stimulates ATP production, and at higher levels, pro-apoptotic factors which leads to apoptosis. The increased Ca^{2+} content in the mitochondria also leads to increased mitochondrial ATP and ROS production (Dykens, 1994). An ER Ca^{2+} importer, known as the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) is upregulated to restore Ca^{2+} homeostasis in the ER – but this importer requires ATP to function, which may further increase load on the mitochondria (Berridge, Bootman, & Roderick, 2003; Chemaly, Troncone, & Lebeche, 2018). Thus, the interplay between H_2O_2 and Ca^{2+} levels eventually leads to a breakdown of the mitochondrial membrane and cell death ('control loop' 3, Figure 5). (Malhotra & Kaufman, 2007).

Apoptosis inhibitors

A number of factors in the cell promote survival by inhibiting key steps in the pathways of apoptosis. Some of these inhibitors of apoptosis can function to inhibit multiple pathways simultaneously. Two families of such inhibitors, studied previously in CHO cells, are the heat shock proteins (HSPs) and the aptly-named 'inhibitor of apoptosis proteins' (IAPs).

Heat shock proteins

The HSPs are induced in response to low levels of stress in what is known as the 'stress response' (Concannon, Orrenius, & Samali, 2001). Many members of the family play a role as chaperones for unfolded proteins (Concannon et al., 2001) and act to stabilise folded proteins in general. Heat Shock Protein-27 (HSP27), a 27 kDa chaperone protein, plays an important role in modulating cytoskeleton stability, protein synthesis, redox potential, and inhibiting apoptosis (Concannon, Gorman, &

Samali, 2003; Cuesta, Laroia, & Schneider, 2000). HSP27 inhibits the caspase independent arm of the FAS-activated extrinsic pathway (Charette et al., 2000). HSP27 is also able to inhibit caspase-3 activation (Concannon et al., 2001). Another member of the heat shock protein family, HSP70, inhibits mitochondria associated apoptosis by inhibiting BAX translocation from the cytosol to the mitochondria (Stankiewicz, Lachapelle, Foo, Radicioni, & Mosser, 2005). HSP70 has also been shown to inhibit JNK activation (Gabai et al., 1997) on overexpression, which has implications for both the extrinsic and intrinsic pathways. HSP27 and HSP70 are shown in Figures 2, 3 and 4 beside the apoptotic factors they inhibit.

Inhibitor of apoptosis proteins

Members of the IAP family are defined by N-terminal baculovirus IAP repeat (BIR) sequences, and are also referred to as BIR-containing (BIRC) proteins. This family of proteins are able to inhibit caspase activity, and therefore greatly hinder caspase-dependent apoptotic pathways. The most well studied of these, XIAP, is known to bind and inhibit executioner caspases 3 and 7, and caspase 9 (Altieri, 2010; Scott et al., 2005; Shiozaki et al., 2003). Members of the IAP family are also involved in MMA (Figure 3).

Other apoptosis inhibitors studied in CHO cells are: Fas apoptotic inhibitory molecules (FAIMs), the cytokine response modifier A (CRMA), AVEN, and E1B-19K. FAIMs inhibit the activation of FAS-mediated apoptosis by upregulating the expression of c-FLIP (Huo, Xu, Guo, Zeng, & Lam, 2009; Schneider, Fischer, Donohoe, Colarusso, & Rothstein, 1999), which restricts the activation of caspase 8 (Scaffidi, Schmitz, Krammer, & Peter, 1999) (Figure 2). CRMA from cowpox virus (Ray et al., 1992) binds and inhibits caspases 1 and 8 (Komiyama et al., 1994;

Sauerwald, Oyler, & Betenbaugh, 2003; Takahashi et al., 1997; Zhou et al., 1997). AVEN is an inhibitor of APAF-1 which is necessary to form the apoptosome and activate caspase-9 downstream of MOMP (Chau, Cheng, Kerr, & Hardwick, 2000) (Figure 3). E1B-19K is an adenoviral Bcl-2 homologue protein which inhibits BAK and is also implicated in counteraction of the cellular p53 response (Cuconati, Mukherjee, Perez, & White, 2003) (Figure 4).

Genetic engineering strategies for apoptosis reduction

Many of the proteins previously discussed have been explored as engineering targets to attenuate apoptosis in CHO cells (Figure 6). The overall trends are discussed below. Results are generally consistent across different experimental conditions; however variables such as cell line, culture method, and product influence the results in some cases. For a comprehensive summary of all relevant studies please refer to Supplementary Tables S1-S7.

In order to be able to compare the multiple studies, we have used quantitative metrics: viability, viable cell density (VCD) and productivity. We further defined the terms: culture duration, and drop onset and drop rate for viability and VCD. Culture duration refers to the total culture time with more than 50% viability. Drop onset, the delay in viability or VCD drop of a treatment group over a control and drop rate to how quickly a culture dies. For a more precise explanation of novel terms, see the Supplementary Method S1. The parameters defined here could be used as a framework for comparing future experiments.

BCL-2 family proteins

To our knowledge, the extent of the literature on CHO cell line engineering of the BCL-2 family has been limited to either the overexpression of BCL-2, MCL-1, or

BCL-xL (all inhibitors of BAK/BAX) or the simultaneous knock-out of BAK and BAX (Figure 6). Therefore all efforts in this area are broadly categorised as limiting or preventing MOMP and subsequent MMA.

Both the overexpression of the negative regulators of apoptosis (BCL-2, BCL-xL, MCL-1) and the knock-out of the positive regulators of apoptosis (BAK and BAX) generally decreased the rates of drop off in VCD and viability, and sometimes improved maximum VCD, thereby extending cell culture duration (Table S1 and Figure 6). In many of these studies also caspase-3 activity was reduced upon treatment, agreeing with the current model of the BCL-2 family and MOMP (Tables S1 and S2).

Though not directly assessed, it can be inferred that the integral of VCD (IVCD) of cultures in which either the negative regulator (BCL-2, BCL-xL, MCL-1) was overexpressed or the positive regulator (BAK and BAX) knocked-out or knocked-down was consistently positively affected. Most studies showed increased titres, ranging from 35% (Lim et al., 2006) to 500% (Cost et al., 2010), with only one exception (Meents, Enenkel, Eppenberger, Werner, & Fussenegger, 2002). Specific productivities, were reported in two instances; a large positive increase of 100% (Grav et al., 2015), and a decrease of 35% (Misaghi, Qu, Snowden, Chang, & Snedecor, 2013). In the few studies where product quality was indicated, quality was shown to be preserved (Kim, Kim, Mohan, & Lee, 2009; Kim et al., 2011; Majors, Betenbaugh, Pederson, & Chiang, 2008).

The BAK/BAX knock-out studies (Table S2), are an example of the relationship between productivity and improved culture performance (Cost et al., 2010; Grav et al., 2015; Misaghi et al., 2013). A 3-fold increase in maximum VCD and titre was

achieved in one study (Cost et al., 2010). In another study, multiple clones were assessed for productivity, culture performance, and titre. IVCDs were found to have increased over the controls (with no increase in specific productivity (q_p)) (Misaghi et al., 2013). Nonetheless, in one case the maximum VCD of the knock-out decreased by 35%, but with an associated doubling of q_p (Grav et al., 2015). In this study, two other clones were selected which were triple knocked-out for BAX, BAK and FUT8. Out of those clones, one conserved the q_p of the parental cell line and the other one had a doubling in q_p. Therefore, the differences in q_p and VCD might be attributed to genomic differences between clones. The same group later addressed this issue with the establishment of an isogenic cell line (Grav et al., 2018).

In a recent study these results were further confirmed by knocking-down either BAK,or BAX through a Crispr interference technique, resulting in up to 1.5 fold improvement in VCD for each individual gene (Xiong et al., 2019).

Taken together, these studies indicate that modifications of the BCL-2 pathway tends to have positive impacts on culture performance rather than directly influencing productivity.

Caspases

Caspases are a logical target for anti-apoptosis engineering due to their role in executing caspase-dependent apoptosis (Figure 1 and Table S3). Caspases 3, 7, 8 and 9 have been targeted in CHO cells by knock-down (Kim & Lee, 2002; Sung, Hwang, & Lee, 2005; Sung, Lee, Park, Koo, & Lee, 2007; Wong et al., 2006; Xiong et al., 2019; Yun et al., 2007), or through the overexpression of the caspase-inhibitors XIAPs (Kim, Kim, & Lee, 2009; Sauerwald, Betenbaugh, & Oyler, 2002; Sauerwald et al., 2003) or CRMA (Sauerwald et al., 2003).

The caspase knock-down studies in CHO cells generally led to small improvements in VCD or viability of up to 40% (Yun et al., 2007), with one outlier of 360% (Sung et al., 2005). Large increases in titre and q_p (Sung et al., 2005) of up to 3-fold and 1.5-fold respectively were also observed with similar studies finding complimentary trends (Kim & Lee, 2002a; Sung et al., 2005, 2007; Wong et al., 2006); however, specific productivity was only directly assessed in one study (Sung et al., 2007).

The overexpression of caspase inhibitors XIAP and CRMA in cultures exposed to the apoptosis inducer Sindbis virus showed similar culture dynamics to controls but with a consistently lower drop rate in viability (Sauerwald et al., 2002, 2003). Another group found no substantial differences in culture or titre when sodium butyrate (NaBu) was used to stimulate apoptosis (Kim, Kim, & Lee, 2009). These conflicting results confound the assessment of this strategy (Figure 1 and Table S4).

Overall, it appears that targeting caspases, or their inhibitors, is poorer than would be expected at attenuating apoptosis from a 'caspase-centric' view (Figure 6 and Table S4). This is perhaps in part due to the downstream role of caspases, meaning that cells may have already lost functionality (e.g. through MOMP) prior to the activation of caspases. Furthermore, caspases are not required for caspase-independent apoptosis.

Heat shock proteins

HSP27 was overexpressed in IgG producer CHO cells (Lee et al., 2009; Tan et al., 2015) (Figure 1 and Table S5). Culture duration was extended by 2 to 4 days in both batch and fed-batch culture by this strategy (Lee et al., 2009; Tan et al., 2015). Furthermore, a 2-fold increase in maximum VCD, with an associated improvement in titre of 3-fold was also observed in fed-batch culture (Tan et al., 2015).

Similar trends were observed with the overexpression of HSP70 (Lee et al., 2009). Co-overexpression of both HSP27 and HSP70 also showed improved culture performance, albeit with a smaller improvement in maximum VCD (of around 2-fold), but still with a near 2-fold increase in titre. These improved culture performances were, in all cases, supplemented with evidence of reduced caspase-2, 3, 8 and 9 activities in order to demonstrate apoptosis attenuation.

Cell cycle and AKT signalling pathway factors

Regulation of cell growth is a strategy to reduce apoptosis and cellular stress, and a number of factors have been tested through overexpression strategies (Figure 1 and Table S6). These factors often affect a variety of cell functions. Protein kinase B (AKT) regulates multiple cellular mechanisms including protein synthesis, cell proliferation and cell survival (Brazil & Hemmings, 2001). One study tested the overexpression of human CA-AKT (a constitutively active AKT) in batch culture (Hwang & Lee, 2009). The overexpression resulted in a delay of the onset of apoptosis and autophagy and an increase in mAb titre by 30%.

The cMYC transcription factor regulates multiple cellular processes including cell cycle progression, proliferation, cell growth, apoptosis, and macromolecular synthesis (Dang, 1999). The overexpression of human cMYC in CHO cells consistently increased maximum VCD, often 2-fold or higher (Ifandi & Al-Rubeai, 2003, 2005), and extended culture duration in some cases (Crea, Sarti, Falciani, & Al-Rubeai, 2006; Ifandi & Al-Rubeai, 2003, 2005; Kuystermans & Al-Rubeai, 2009). The rates of VCD and viability drop increased frequently in these cultures, possibly due to nutrient depletion or waste product build-up resulting from the substantial increases in IVCD. Specific productivity was assessed by only one study (Kuystermans & Al-

Rubeai, 2009), and showed a slight reduction of less than 15%, but the improvement in max VCD of around 72% would mean titre should improve overall (although this was not reported). cMYC overexpression tends to increase apoptotic fractions when assayed; likely due to its role in upregulating various apoptotic activators, including, but not limited to, p53 (Prendergast, 1999; Reisman, Elkind, Roy, & Beamon, 1993). This effect is mitigated somewhat by co-overexpression with BCL-2 (Ifandi & Al-Rubeai, 2005).

Overexpression of MDM2, E3 ubiquitin-protein ligase which degrades p53, increased maximum VCD by up to 2-fold whilst delaying the onset of both viability and VCD drop by around 1 day. Interestingly, the rate of VCD and viability drops were either unchanged (batch) or increased (batch using spent media), similar to the trends with cMYC overexpression. Effects on productivity were not assessed but assays indicated reduced apoptotic cell fractions (Arden, Majors, Ahn, Oyler, & Betenbaugh, 2006).

The E2F-1 cell cycle transcription factor stimulates the transition between G1 and S phases (Johnson, Schwarz, Cress, & Nevins, 1993). As well as stimulating proliferation, dysregulation of E2F-1 has also been shown to promote apoptosis, likely though the p53 pathway (Qin, Livingston, Kaelin, & Adams, 2006). E2F-1 overexpression (Majors, Arden, et al., 2008) showed a slight increase in maximum VCD of 20% and reduction in VCD drop rate, but viability and productivity were not assessed.

mTOR, a kinase with many roles including regulation of cell cycle and proliferation (Fingar et al., 2004), has been overexpressed and shown to extend culture duration for up to 4 days through reduction of the drop rate of VCD, leading to considerable increases in IgG titre of 2- to 3-fold (Dreesen & Fussenegger, 2011).

Factors p21 and p27, also involved in cell cycle regulation, have been individually overexpressed (Astley, Naciri, Racher, & Al-Rubeai, 2007; Mazur, Fussenegger, Renner, & Bailey, 1998) and co-overexpressed with anti-apoptotic proteins, such as BCL-xL and C/EBP-alpha (Fussenegger, Schlatter, Datwyler, Mazur, & Bailey, 1998; Mazur et al., 1998). These strategies were shown to consistently improve q_p by up to an order of magnitude, generally by arresting cell growth.

miRNA mediated knock-down strategies

MicroRNAs (miRNA) are small non-coding RNA molecules involved in the regulation of several biological processes including cell development, differentiation, proliferation and apoptosis (Bartel, 2004; He & Hannon, 2004; Krol, Loedige, & Filipowicz, 2010). Some miRNAs have been shown to function as oncogenes, whereas others function as tumour suppressors (Calin & Croce, 2006). Downregulation or overexpression of certain miRNAs have been tested in CHO cells to delay apoptosis (Kelly, Gallagher, Clynes, & Barron, 2015; Strotbek et al., 2013). Knock-down of mmu-miR-466h in batch culture limited apoptosis by reducing caspase-3 and caspase-7 activity and by up-regulating the anti-apoptotic genes BCL2L2, DAD1, BIRC6, STAT5A and SMO (Druz et al., 2011). Downregulation of miR-34a/b/c decreased maximum cell density by half and increased SEAP titre by 2-fold (Kelly et al., 2015). On the other hand, overexpression of miR-557 and miR-1287 increased q_p and mAb titre by 1.3-fold (Strotbek et al., 2013) (Figure 6 and Table S7).

Other targets

Other targets have been investigated for their ability to reduce apoptosis and improve productivity. Three groups of targets are discussed briefly here owing to the number of publications or systematic nature of the approach used. Other miscellaneous targets

(ATG69A (J. S. Lee & Lee, 2012); COX15 (Jaluria, Betenbaugh, Konstantopoulos, & Shiloach, 2007); NKIAMRE (Jaluria et al., 2007); ALG-2, FAIM, FADD, and Requiem (Wong et al., 2006); GADD153 (Mohan, Sathyamurthy, & Lee, 2012), VCP (Doolan et al., 2010) are only presented in Table S7.

The 30K proteins from *Bombyx mori* silkworm hemolymph are a group of plasma proteins, called 'storage proteins' with a molecular weight of approximately 30,000 Da. Expression of 30Kc6 in CHO-K1 was shown to reduce caspase 3 activity (Kim, Rhee, & Park, 2004). Viable cell density and culture duration were shown to increase across all studies (Choi, Rhee, Kim, & Park, 2006; Kim et al., 2004; Koo, Park, Park, & Park, 2009; Z. Wang, Ma, Fan, et al., 2012; Z. Wang, Ma, Zhao, Fan, & Tan, 2012). Rates of viability and VCD drop were mixed. Interestingly, product titres were shown to improve greatly, almost always more than 2-fold, and coupled with increases in q_p of around 2-fold. Wang et al. found a reduction in BAX translocation to mitochondria, resulting in the reduced release of Cyt-c from the mitochondria, and increases in ATP production and mTOR expression (Z. Wang, Ma, Zhao, et al., 2012). Under hyperosmotic pressure (410 mOsm/kg), the same cells had an increased q_p of around 2-fold compared to the control. The reduction in apoptosis was thus attributed to an increase in mitochondrial membrane potential (Z. Wang, Ma, Zhao, et al., 2012). This approach has shown very promising results, but may have niche applications due to its poorly-defined composition and naturally-derived origin.

Several studies have expressed AVEN and E1B-19K individually, together, and/or with other anti-apoptotic factors such as XIAP and BCL-xL (Dorai et al., 2010, 2009; Figueroa et al., 2007; Figueroa, Chen, Oyler, Hardwick, & Betenbaugh, 2004). Expressed individually, AVEN extended culture duration by one day and reduced

drop rate in viability (Figueroa et al., 2007). When exposed to a variety of apoptotic insults, AVEN-expressing cultures generally had slower reductions in viability, except in a batch culture with spent medium in one study (Figueroa et al., 2004). Cell culture performance was further improved by co-overexpression of AVEN and BCL-xL (Figueroa et al., 2004).

E1B-19K overexpression extended culture duration by 2 days (Figueroa et al., 2007) and improved VCD by nearly 2-fold when co-expressed with AVEN (Dorai et al., 2009). It was also later shown to reduce caspase-3 and 7 activity (Dorai et al., 2010). Co-overexpression of AVEN and E1B-19K presented conflicting results. Substantial delays in the onset of cell death and decreases in rates of viability and VCD drop have been observed (Figueroa et al., 2007), leading to a four day extension of cell culture, with no improvement in titre or q_p. The same strategy by a different group was found to nearly double maximum VCD in batch culture, but VCD began to decrease one day earlier and at an accelerated rate, with no negative effects on the viability of the culture (Dorai et al., 2009). The differing results may be attributed to different glucose supplementation concentrations between the studies.

Overexpression of AVEN, E1B-19K and EAX197 together were found to double maximum VCD in batch culture, but also increased VCD drop rate and failed to extend the life of the culture (Dorai et al., 2009). In the fed-batch cultures, culture duration was extended by five days, lower viability and VCD drop rates were observed (Dorai et al., 2009). Finally overexpression of AVEN, E1B-19K and XIAP increased maximum VCD and delayed onset and drop rate of VCD, improving titre approximately 5-fold and showing reduced caspase activity (Dorai et al., 2010). Ultimately the results of overexpressing these factors have given generally positive

but mixed results, possibly due to other cell culture parameters and clone selection bias.

Future directions

There has been much work done over the last 30 years to understand how apoptosis affects bio-therapeutics production in CHO cells. The nuances of CHO cell biology, and the lack of a universal framework for comparison obscures our understanding in some cases. It is recommended that future scientists adopt a uniform metric for assessing cell performance, such as those suggested here, to facilitate the direct comparison of different genetic targets. The direct comparison of cells at later phases of growth is particularly troublesome, and it is therefore recommended that attempts be made to normalise for nutrient levels and cell density. Nevertheless, the outcome of genetic experiments typically do converge across studies, which supports these strategies for rationally improving the phenotype of CHO cells. In summary, the largest improvements in cell culture performance have been the knock-out studies of BAK and BAX, with more modest results generated by overexpressing their inhibitors, namely, Bcl-2 and Bcl-xL. Cell cycle arrest factors have been shown to greatly improve q_p at the expense of a reduced growth rate.

The differences in outcomes across studies are partially explained by the variation in cell lines, products and culture conditions. Moreover clonal variation play a crucial role. The CHO genome is inherently unstable (Pilbrough, Munro, & Gray, 2009; Xu et al., 2011), and traditional random plasmid integration does not control the genomic location of insertion. CRISPR/Cas9 is able to generate site directed ‘knock-ins’ (Bachu, Bergareche, & Chasin, 2015; J. S. Lee, Grav, Pedersen, Lee, & Kildegaard, 2016). However, there is a chance of off-target effects and of aberrant recombination. The best approach moving forward would be to establish an isogenic cell line using

recombinase-mediated cassette exchange into a stable genomic location (Grav et al., 2018).

Gene overexpression strategies have historically been more common, owing to the robustness of plasmid vector systems for gene overexpression and the relative difficulty of engineering knock-out cell lines. However, with the advent of CRISPR/Cas9, it is expected that many more gene knock-out strategies will be tested in the near future. The genome editing tool CRISPR/Cas9 will accelerate the testing of knock-outs (Cong et al., 2013; Grav et al., 2015; Shin et al., 2015), even allowing the targeting of multiple genes simultaneously. The ability of this tool for multi-gene editing will allow for the investigation of synergistic effects, especially when more than one gene may be contributing to an anti-apoptotic phenotype. Moreover, recently new CRISPR tools for CHO cells have been developed to modulate transcription levels of endogenous genes (Karottki et al., 2019; Xiong et al., 2019). CRISPR/Cas9 will become a mainstream tool in the near future as workflows become more streamlined, and as precision continues to improve allowing faster validation of apoptotic strategies (Kleinstiver et al., 2016).

Apoptosis is a complex process, and targeting one or few key genes may not result in the desired attenuation, due to the presence of redundant pathways or other key mechanisms, some of which undoubtedly remain to be discovered. The unpredictable responses to pathway modifications could ultimately require several rounds of iterative cell line development and thorough characterization, which is usually impractical in an industrial setting due to the time and material costs involved. A multi-omics approach is a powerful method to interrogate complex cellular interactions, and aid in the identification of novel cell line engineering targets (Datta,

Linhardt, & Sharfstein, 2013; Korke et al., 2002; Lewis, Abu-Absi, Borys, & Li, 2016). Regulatory and metabolic information can be extracted from omics comparisons of different cell lines (with varying q_p) and culture conditions, offering a blueprint to reverse engineer desirable phenotypes. Many CHO omics studies have already been performed and can be of great value to find new gene targets to improve cells lines for biopharmaceutical production (Baik et al., 2006; Birzele et al., 2010; Carlage et al., 2009; Han et al., 2017; Joon, Gatti, Philp, Yap, & Hu, 2008; Lee, Kim, Kim, & Lee, 2003; Meleady et al., 2008; Nissom et al., 2006; Orellana, Marcellin, Munro, Gary, & Nielsen, 2015; Orellana et al., 2018; Shen et al., 2010; Yee, Gerdtzen, & Hu, 2009). Furthermore, the latest CHO genome scale reconstruction (Hefzi et al., 2016) is a valuable tool to perform *in silico* analysis on some of the discussed targets. The use of these tools will facilitate the implementation of standards for apoptosis attenuation strategies for industrial use.

Ultimately, the engineering of anti-apoptotic cell lines would have the strongest impact for high cell density, low growth processes, where cell death is highly detrimental to the production run, such as in perfusion culture.

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Abbreviations

AIF, apoptosis inducing factor; **AKT,** - also known as Protein kinase B (PKB); **ANXA-1,** annexin – 1; **APAF-1,** apoptotic protease – activating factor 1; **APO-1,**

apoptosis antigen 1; **ARF,** ADP ribosylation factor; **ASK1,** apoptosis signal-regulating kinase 1; **ATF4,** activating transcription factor 4; **ATF6,** activating transcription factor 6; **ATP,** adenosine triphosphate; **BAK,** BCL-2 homolous antagonist killer; **BAX,** Bcl-2 associated X protein; **BCL-2,** B cell lymphoma gene 2; **BCL2L2,** bcl-2 like protein 2; **BCL-xL,** B-cell lymphoma-extra large; **BFP,** Bcl-2 family proteins; **BH1,2,3,4,** bcl-2 homology domains 1,2,3,4; **BID,** BH3 interacting-domain death agonist; **BIK,** bcl-2 interacting killer; **BIM,** bcl-2 like protein 11; **BIR,** baculovirus IAP repeat; **BIRC,** N-terminal baculovirus IAP repeat containing; **BIRC6,** Baculovirul IAP repeat containging protein 6; **BOK,** Bcl-2 ovarian killer; **Ca^{2+},** calcium ion; **Caspase,** cysteine-aspartic proteases; **CD95,** cluster of differentiation 95; **CHO,** Chinese Hamster Ovary; **CHOP**, GADD153, CAAT/enhancer binding protein; **CIAP1,** cellular inhibitor of apoptosis protein -1; **CIAP2,** cellular inhibitor of apoptosis protein -2; **c-MYC,** myelocytematosis gene overexpressed in cancer; **CRISPR,** clustered interspaced short palindromic repeats; **CRMA,** cytokine response modifier A; **Cyt-c,** cytochrome – c; **DAD1,** defender against cell death – 1; **DAXX,** death domain associated protein - 6; **DIABLO / SMAC,** direct IAP binding protein with low pI – second mitochondria-derived activator of caspases; **DISCs,** death-inducing signalling complexes; **DNA,** deoxyribonucleic acid; **E1b-19K,** E1b 19-kilodalton protein; **E2F-1,** E2F transcription factor 1; **EIF2,** eukaryotic initiation factor 2; **EndoG,** endonuclease G; **EPO,** erythropoietin; **ER,** Endoplasmic Reticulum; **ERO1,** endoplasmic reticulum oxidoreductase -1; **FADD,** Fas-associated death domain; **FASL,** first apoptosis signal ligand; **FASR,** first apoptosis signal receptor; **GADD34,** DNA damage-inducible protein 34 (or Ppp1r15a); **GCLM,** glutamate cysteine ligase regulatory subunit; **H_2O_2,** hydrogen peroxide; **HEK293,** human embryonic kidney cell 293; **HILP2,** IAP-

like protein 2 (or Birc8 – Baculovirus containing IAP repeat containing 8, human); **HSP,** heat shock protein; **HSP27,** heat shock protein 27 kDa; **HSP70,** heat shock protein 70 kDA; **TERT,** telomerase reverse transcriptase; **IAPs,** inhibitor of apoptosis proteins; **IFN-γ,** interferon gamma; **IgG,** immunoglobulin G; **JNK,** c-Jun terminal kinases; **mAb,** monoclonal antibody; **MAPK,** mitogen-activated protein kinases; **MCL-1,** myeloid cell leukaemia cell differentiation protein – 1; **MDM2,** murine double minute 2 homologue; **MGI,** mouse genome informatics; **MiRNA,** micro RNA; **MIM**, mitochondrial inner membrane; **MMA,** mitochondria-mediated apoptosis; **MOMP,** mitochondrial outer membrane permeabilisation; **MPT**, mitochondrial permeability transition; **MPTP**, mitochondrial permeability transition pore; **NaBu,** sodium butyrate; **NF-κB,** nuclear factor kappa-light-chain enhancer of activated B cells; **NOD1,** nucleotide-binding oligomerization domain-containing protein – 1; **NOD2,** nucleotide-binding oligomerization domain-containing protein – 2; **NOXA,** Phorbol-12-myristate-13-acetate-induced protein 1; **PDI,** protein disulphide isomerase; **PERK,** protein kinase R (PKR) – like endoplasmic reticulum kinase; **PUMA,** p53 upregulated modulator of apoptosis; **q_P,** specific productivity; **REDOX,** reduction / oxidation; **RIP,** ribosome inactivating protein; **ROS,** reactive oxygen species; **SMAC,** second mitochondria – derived activator of caspases; **SMO,** gene which encodes the 'smoothened protein'; **STAT5A,** signal transducer sand activator of transcription 5 protein A; **TNF,** Tumour necrosis factor; **TNFR,** tumour necrosis factor receptor; **TRAIL,** TNF-related apoptosis inducing ligand; **TRAILR,** TNF-related apoptosis inducing ligand receptor; **UPR,** Unfolded Protein Response; **VCP,** vasolin containing protein; **XIAP,** X-linked IAP

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Figures

Figure 1: Apoptosis signalling pathways in CHO cells.

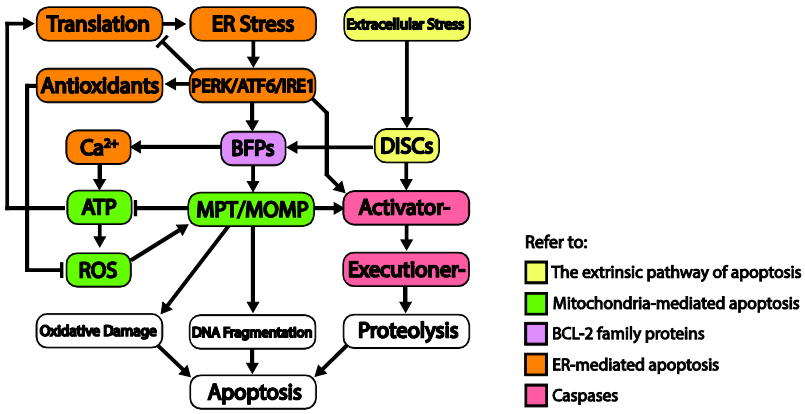

Figure 2: The Extrinsic Apoptosis Pathway and Pathway Inhibitors. Dashed lines and borders indicate simplified or partial interactions (e.g. between families); refer to corresponding sections for further detail.

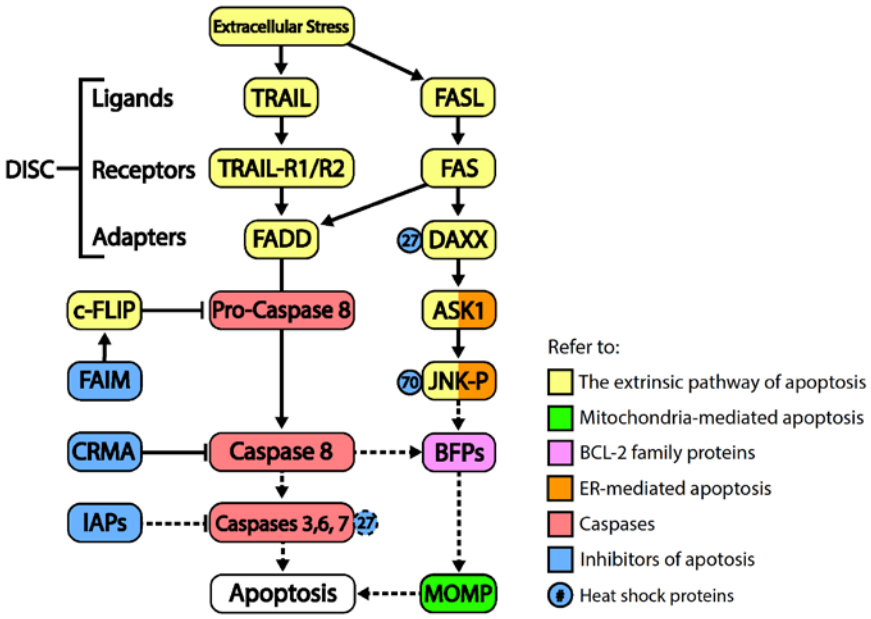

Figure 3: Mitochondria-Mediated Apoptosis. Dashed lines and borders indicate simplified or partial interactions (e.g. between families); refer to corresponding sections for further detail.

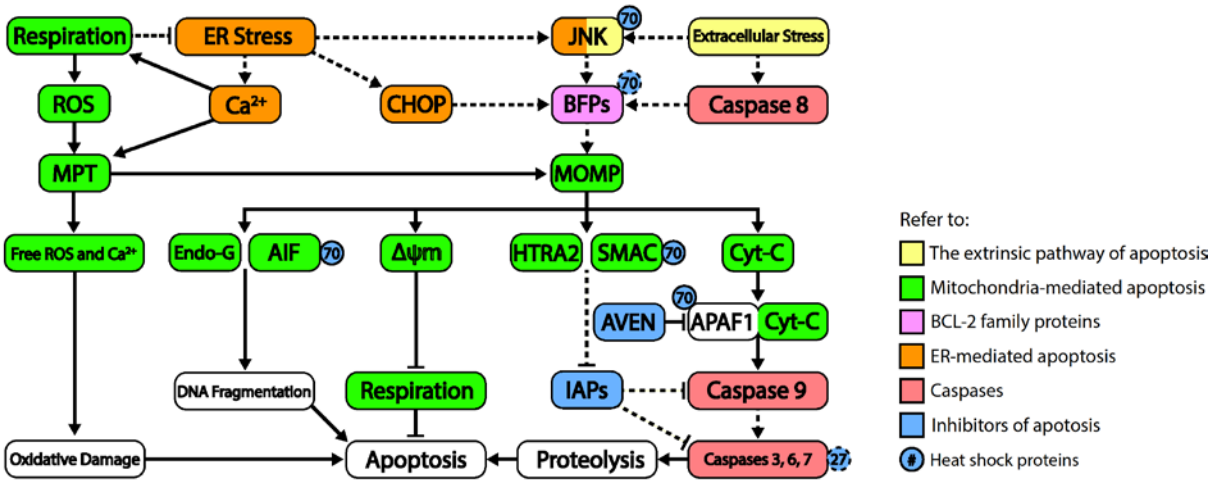

Figure 4: The BCL-2 Family Proteins (BFPs). Individual BFPs may interact with one or more members of another class through general mechanisms above. The annotated numbers (in brackets) mark the specific interactions of other relevant factors with individual BFPs.

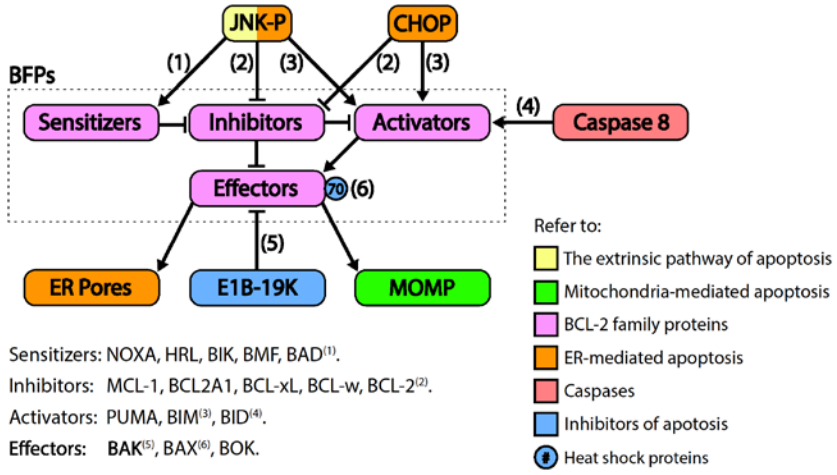

Figure 5: ER-mediated apoptosis. The mechanisms by which the endoplasmic reticulum regulates translation of, for example, recombinant proteins, can be grouped into control loops, with the build-up of stress signals eventually leading to escalation of the response and possibly even cell death. Dashed lines and borders indicate simplified or partial interactions (e.g. between families); refer to corresponding sections for further detail.

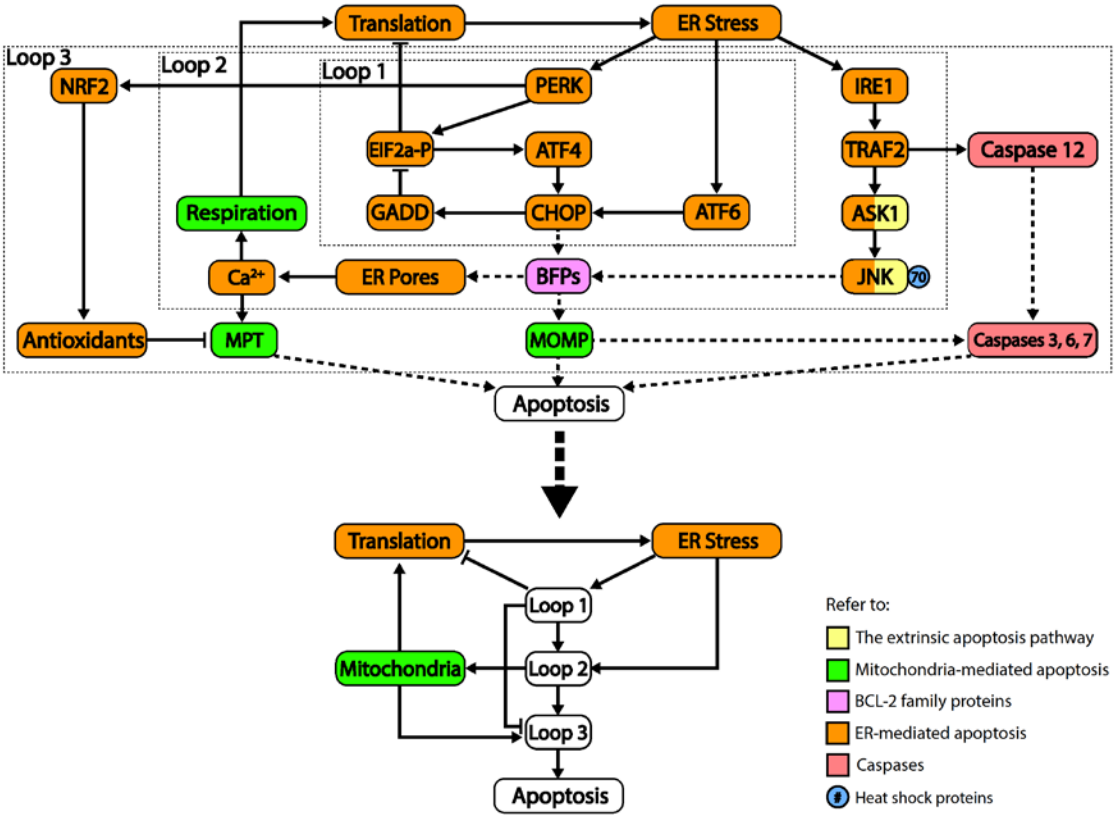

Figure 6 (part 1 of 2): Summary of results of anti-apoptosis genetic engineering approaches in CHO cells up to date. Fuller details of each finding are listed in tables S1-S7 along with additional information on the metrics used define and quantify parameters of culture performance relevant to apoptosis.

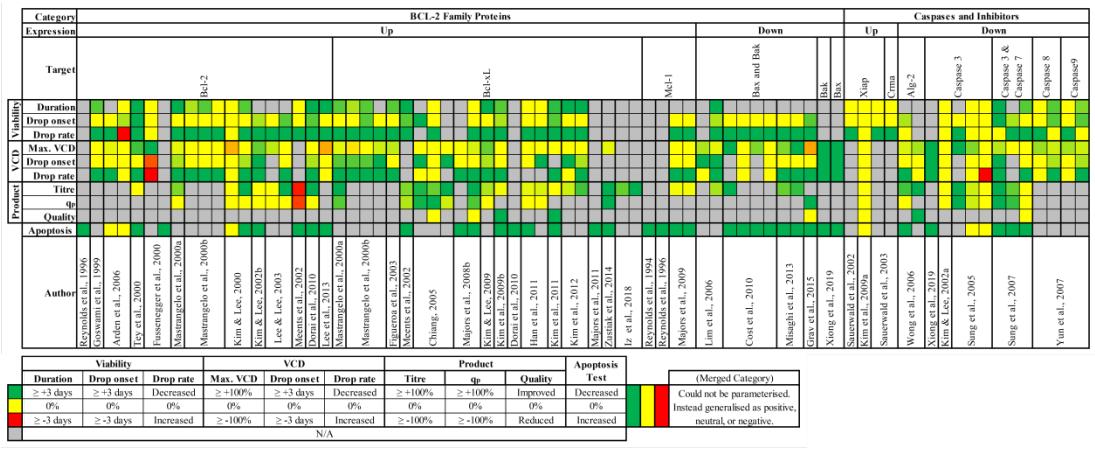

Figure 6 (part 2 of 2): Summary of results of anti-apoptosis genetic engineering approaches in CHO cells up to date. Fuller details of each finding are listed in tables S1-S7 along with additional information on the metrics used define and quantify parameters of culture performance relevant to apoptosis.