‘Omics driven discoveries of gene targets for apoptosis attenuation in CHO cells

Orellana, Camila A.; Martínez, Verónica S.; MacDonald, Michael A.; Henry, Matthew N.; Gillard, Marianne; Gray, Peter P.; Nielsen, Lars K.; Mahler, Stephen; Marcellin, Esteban

Published in:
Biotechnology and Bioengineering

Link to article, DOI:
10.1002/bit.27548

Publication date:
2021

Document Version
Peer reviewed version

‘Omics driven discoveries of gene targets for apoptosis attenuation in CHO cells

Camila A. Orellana[1*], Verónica S. Martínez[2], Michael MacDonald[2], Matthew N. Henry[1], Marianne Gillard[1], Peter P. Gray[2], Lars K. Nielsen[2,3,4], Stephen Mahler[2] and Esteban Marcellin[2,3]

[1] Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD 4072, Australia

[2] ARC Training Centre for Biopharmaceutical Innovation (CBI), Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, Australia

[3] Metabolomics Australia, The University of Queensland, Brisbane, QLD 4072, Australia

[4] The Novo Nordisk Foundation Centre for Biosustainability, Technical University of Denmark, Kgs. Lyngby, Denmark

*Corresponding author: Dr Camila A. Orellana

Phone: +61 4 2470 4900, Email: c.orellana@uq.edu.au

Abstract

Chinese Hamster Ovary (CHO) cells are widely used in biopharmaceutical production. Improvements to cell lines and bioprocesses are constantly being explored. One of the major limitations of CHO cell culture is that the cells undergo apoptosis, leading to rapid cell death, which impedes reaching high recombinant protein titres. While several genetic engineering strategies have been successfully employed to reduce apoptosis, there is still room to further enhance CHO cell lines performance. 'Omics analysis is a powerful tool to better understand different phenotypes and for the identification of gene targets for engineering. Here, we present a comprehensive review of previous CHO 'omics studies that revealed changes in the expression of apoptosis-related genes. We highlight targets for genetic engineering that have reduced, or have the potential to reduce, apoptosis or to increase cell proliferation in CHO cells, with the final aim of increasing productivity.

Keywords: Apoptosis, biopharmaceutical production, Chinese hamster ovary cells, genetic engineering, 'omics.

Introduction

The biopharmaceutical industry is fast growing, with global sales of over US$188 billion in 2017 (Walsh, 2018). Major application areas include oncology and autoimmune/inflammatory disorders. Chinese hamster ovary cells (CHO) are the preferred production system for biopharmaceutical production due to their ability to perform post-translational modifications and their long term safety record and successful history of approval by regulatory bodies. These advantages are unlikely to change shortly (Walsh, 2018). As such, strategies for improving recombinant therapeutic protein titres are continuously being investigated with reports highlighting more than 100-fold increase in product titre, mainly due to media and process optimization (Datta, Linhardt, & Sharfstein, 2013; Wurm, 2004).

In contrast to the success in bioprocess optimization, cell line engineering for enhanced biopharmaceutical production has only delivered modest improvements in productivity per cell. One of the few exceptions is the targeting of the apoptosis pathway. Programmed cell death, or apoptosis, is a suicide response from cells exposed to different stresses (nutrient limitation, accumulation of toxic by-products, hypoxia, etc.) (Arden & Betenbaugh, 2004). Logically, apoptosis is an undesirable feature for biopharmaceutical production cell lines as it results in a decrease in viable cell density and shorter culture duration, ultimately resulting in lower product titres (Fussenegger & Bailey, 1998). There are two pathways involved in initiating apoptosis, the extrinsic pathway, which senses extracellular stress signals, and the intrinsic pathway (endoplasmic reticulum- (ER) and mitochondria-mediated pathways), which senses intracellular stress signals, both reviewed in detail elsewhere (Elmore, 2007; Henry et al., 2020).

Many of the proteins involved in apoptosis pathways have been overexpressed (anti-apoptotic proteins) or knocked-down (pro-apoptotic proteins) in CHO cells. Examples include the BCL-2 family proteins, caspases and heat shock proteins, with positive outcomes in delaying

apoptosis and consequently improving production, as reviewed previously (Dietmair, Nielsen, & Timmins, 2011; Henry et al., 2020).

Modern rational cell design relies on 'omics strategies for the identification of new gene targets (Lewis, Abu-Absi, Borys, & Li, 2016). Various 'omics contrast studies have been performed in CHO cells to define features for improved biopharmaceutical production, as reviewed elsewhere (Dietmair, Nielsen, & Timmins, 2012; Stolfa et al., 2018). These studies have revealed changes in gene/protein expression belonging to diverse biological functions including protein synthesis, folding and secretion, transcription, cytoskeleton, cell cycle and apoptosis. This review focuses on these 'omics datasets and describes apoptosis-related findings (Figure 1). Novel promising apoptosis gene targets for CHO cell engineering are highlighted (Table 1). This review is divided into studies that directly study apoptosis and the ones that focus on improving productivity.

Understanding apoptosis: growth phase comparisons

To better understand apoptosis, non-induced apoptotic response of CHO cells has been studied at the transcriptome (Wong, Wong, Lee, et al., 2006), proteome (Wei et al., 2011) and metabolome (Chong et al., 2011) levels. Transcriptional profiling of apoptotic pathways in batch and fed-batch cultures of CHO cells producing recombinant human IFN-γ was studied. It was found that apoptosis occurred predominantly via the extrinsic death receptor- and mitochondria-mediated signalling pathways rather than the ER-mediated signalling pathway (Wong, Wong, Lee, et al., 2006). The authors found key early apoptosis signalling genes and proposed them as targets to delay apoptosis onset. These include *FasL*, *Fadd*, *Bim*, and *Bak* in batch cultures and *Fadd*, *Bim*, *Bad*, *Bax*, *Alg-2*, and *Requiem* in fed-batch cultures (Wong, Wong, Lee, et al., 2006). The overexpression of *Fadd* and the knock-down of *Alg-2* and *Requiem* (Wong, Wong, Nissom, Heng, & Yap, 2006) and the simultaneous knockout of *Bak* and *Bax* (Cost et al., 2010) were later proven to delay apoptosis and increase product titre.

On the other hand, when studying an IgG producer CHO cell line during prolonged batch cultivation, flow cytometry analyses of various activated caspases demonstrated that the onset of apoptosis was primarily through the mitochondria and/or ER pathways (intrinsic), rather than the extrinsic apoptotic pathway (Wei et al., 2011). Proteomics analysis did not identify differentially expressed proteins that directly modulate apoptosis, although only 62 proteins were identified using differential gel electrophoresis (DIGE). Major changes involved in the regulation of stress response and energy metabolism were hypothesised to help cope with the stress of nutrient depletion (Wei et al., 2011).

In fed-batch cultures, Chong et al. found a good correlation between some metabolites (nucleotides/nucleosides and amino acid derivatives) and caspases-3 and -7 activity (Chong et al., 2011). These metabolites were then added to CHO cell cultures in new media. Oxidized glutathione, adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP) and dimethylarginine were shown to induce apoptosis. Following these results, the authors suggested targets for cell line engineering, which included the knock-down of ATP and adenosine receptors, overexpression of γ-glutamylcysteine synthetase (GCLC/GCLM) and glutathione synthetase (GSS) to prevent glutathione depletion, and overexpression of dimethylarginine dimethylaminohydrolase (DDAH) to convert dimethylarginine into citrulline (Chong et al., 2011). *Gclm* overexpression was later proven to increase mAb titre in CHO cells by 70% (Orellana, Marcellin, Gray, & Nielsen, 2017). Overexpression of *Ddah1* (anti-apoptotic) in human prostate cancer cell lines promoted cell proliferation by 28-50% by degrading the endogenous nitric oxide synthase inhibitor, asymmetric dimethylarginine, as well as increasing nitric oxide production (Reddy et al., 2018), looking promising as a target for CHO cell engineering (Table 1 and Figure 2).

Hernandez Bort et al. also characterized CHO mRNA and miRNA expression patterns during lag, exponential, and stationary phases (Hernandez Bort et al., 2012). The authors found miRNAs up-regulated during the exponential growth phase and/or down-regulated in the stationary phase exhibiting functions linked to cell proliferation, cell cycle and apoptosis. Ten miRNAs were predicted to target mRNAs up-regulated during stationary phase and whose transcription levels negatively correlate to their mRNA target levels. From these target mRNAs, 12 were related to programmed cell death, suggesting that the down-regulation of miRNAs in late batch culture could be related to cell death. The miRNAs that target these apoptosis-associated genes were *miR15a*, *miR-16, miR-17*, *miR-27a* and *miR-30d*. The authors also suggested that the down-regulation of the *miR-17-92* cluster in stationary phase could be involved in growth arrest (Hernandez Bort et al., 2012). The *miR-17*, *miR-92a* and *miR-17–92a* clusters were later overexpressed in CHO cells, and only *miR-17* overexpression enhanced growth and specific productivity, increasing EpoFc titre by 3-fold (Jadhav et al., 2014).

Carlage et al. analysed the proteome of CHO cells overexpressing the anti-apoptotic gene *Bcl-xL* during exponential and stationary phases and found that transglutaminase 2 (TGM2, pro- and anti-apoptotic) and clusterin (CLU, pro- and anti-apoptotic) were up-regulated in the stationary phase (Carlage et al., 2012). TGM2 catalyses a Ca^{2+}-dependent crosslinking of lysine and glutamine residues and also binds GTP (Fesus & Szondy, 2005). *Tgm2* downregulation in U937 (Oliverio, Amendola, Rodolfo, Spinedi, & Piacentini, 1999) and human neuroblastoma cells decreased apoptosis, while it induced apoptosis in meningioma cells (Huang et al., 2014). CLU is a glycoprotein implicated in several biological processes, including cell adhesion and apoptosis. Overexpression of *Clu* in a mouse neuroblastoma cell line (B103) inhibited H_2O_2-induced apoptosis (You, Ji, & Kwon, 2003) and its downregulation in HL-60 acute myeloid leukaemia cells triggered apoptosis and inhibited cell proliferation (Wang, Liu, Wang, Cai, & Zhang, 2015). However, its overexpression in both malignant and non-malignant prostate

epithelial cells induced apoptosis (Scaltriti et al., 2004). Even though contradictory results were observed for different cell lines, it is worthwhile testing TGM2 and CLU as targets for CHO cell engineering as they do have a strong effect in apoptosis (Table 1 and Figure 2).

Templeton et al. used ^{13}C fluxomics to understand central metabolism in cells overexpressing the engineered anti-apoptotic gene Bcl-2Δ (Templeton et al., 2014). The authors found that the reduced lactate accumulation observed in the engineered cell line was due to the redirection of pyruvate toward mitochondrial oxidation during the lactate-producing phase and an increase in lactate uptake rate during the lactate-consuming phase. These changes in flux were associated with the increase in biomass yield, peak viable cell density and integrated viable cell density observed in the Bcl-2Δ overexpressing cell line.

Overall, transcriptomics strategies have provided the greatest information about apoptosis, highlighting genes and mi-RNAs that have presented larger effects in enhancing CHO cell culture. However, it would be interesting to repeat the proteomics approach applying newer technologies such as tandem mass spectrometry, which nowadays is able to provide quantitative information for over 5,000 proteins in CHO cells.

Improving productivity: culture condition comparison

Several studies have compared CHO cell lines under culture conditions that enhance specific productivity (q_p) in which apoptosis-related genes are differentially expressed with respect to controls. In most of these studies, the growth rate and integral viable cell density decreased when modifications were applied to the culture conditions, such as higher osmolarity, lower temperature shift or sodium butyrate addition. Nonetheless, the final titre remained similar or increased compared to the control, thus increasing q_p. Shen et al. found three apoptotic transcripts differentially expressed under hyperosmotic stress which repressed cell growth: *Malt1* down-regulated and *Ddit4* and *Rassf5* up-regulated (Shen et al., 2010), while Lee et al. did not find any apoptotic proteins de-regulated from the 54 identified proteins (Lee, Kim,

Kim, & Lee, 2003) using two-dimensional gel electrophoresis. Ras association domain family member 5 (RASSF5) acts as a tumour suppressor, and it is downregulated in various cancer cell lines. Downregulation of *Rassf5* looks promising as a target for CHO cells (Table 1 and Figure 2) as its overexpression in human osteosarcoma cell lines suppressed cell proliferation and induced apoptosis (Zhou et al., 2014).

Under sodium butyrate treatment Yee et al. found that ten transcripts involved in apoptosis were differentially expressed: *Pdcd4* (down-regulated), pro-apoptotic factor *Bnip2* (up-regulated), anti-apoptotic factors *Aip5, Pasg* and *Mcl1* (up-regulated) and *Prkdc*, *Pdcd6ip*, *Tde1*, *Cul2* and *Cul3* (up-regulated) (Yee, de Leon Gatti, Philp, Yap, & Hu, 2008). Helicase, lymphoid-specific (PASG or HELLS) is a member of the SNF2 family of chromatin remodelling proteins, and high levels have been found in tumour cells (von Eyss et al., 2012). *Pasg* deletion in murine neural stem/progenitor cells reduced cell proliferation and increased apoptosis (Y. Han et al., 2017) and its overexpression could be tested in CHO cells (Table 1 and Figure 2). Birzele et al. showed that the pro-apoptotic gene tumour protein p53 inducible nuclear protein 1 (*Tp53inp1*) was up-regulated (Birzele et al., 2010) while De Leon et al. commented on an alteration of apoptosis-related genes but did not mention the gene names (De Leon Gatti, Wlaschin, Nissom, Yap, & Hu, 2007). Again, using two-dimensional electrophoresis, a subsequent investigation was unable to find apoptotic proteins to be de-regulated in the 28 proteins identified (Baik, Joo, Kim, & Lee, 2008). The knockdown of the tumour suppressor *Tp53inp1* in hepatocellular carcinoma cells (MHCC97L) increased the ability of cells to form tumours in the liver (Ng et al., 2017) and it could be used as a target to increase cell proliferation (Table 1 and Figure 2).

In studies involving a temperature shift to mild hypothermia, the anti-apoptosis related transcript *Tpt1* was down-regulated (Yee, Gerdtzen, & Hu, 2009), the anti-apoptotic HSPA8 protein was up-regulated (Baik et al., 2006), importin-α was down-regulated and LGALS1

protein was up-regulated (Kumar, Gammell, Meleady, Henry, & Clynes, 2008) after the temperature shift. Overexpression of tumour protein, translationally-controlled 1 (*Tpt1*) prevented HeLa cells and U2OS cells from undergoing etoposide-induced apoptosis (Li, Zhang, & Fujise, 2001) and its downregulation in human glioma cell lines (U251) inhibited cell proliferation and induced apoptosis (Jin et al., 2015). These results indicate that it is worthwhile testing *Tpt1* in CHO cells (Table 1 and Figure 2).

These proposed genes, if engineered in CHO cells, are likely to improve or restore the reduced growth seen in cultures with operational conditions such as hyperosmolarity, temperature shift or addition of sodium butyrate. However, it is unclear whether the engineered cells will keep the higher specific productivity observed under these conditions.

Improving productivity: CHO cell lines comparison

Other authors have compared CHO cell lines with different specific productivities (q_p), and again apoptosis-related genes have been found to be differentially regulated. Chen et al. found the negative apoptosis regulators *Madd, Birc2* and *Birc3,* the positive apoptosis regulator *Nod1* and the apoptotic genes *Fastkd1* and *Rnf216* down-regulated in the CHO-DG44 high producer cell line (Chen et al., 2017). Orellana et al. found 90 apoptosis regulator transcripts up-regulated in the CHO-K1 high producer cell line, including the negative regulators *Clu*, *Gclm*, *Hsp27 (Hspb1)*, *Hsp70 (Hspa1a)*, *Nod2, Xiap* and *Birc3*, the positive regulators *Anxa1* and *Nod1*, and the *Lgals1* gene (Orellana et al., 2018). Discrepancies between both studies may arise from differences in the cell lines, product, media and culture conditions used. Annexin A1 (ANXA1) is involved in diverse biological functions such as in inflammatory pathways, cell proliferation and regulation of apoptosis, amongst others (Lim & Pervaiz, 2007). Overexpression of *Anxa1* induced apoptosis in prostate cancer cells (Hsiang, Tunoda, Whang, Tyson, & Ornstein, 2006), while its downregulation partially inhibited TRAIL (tumour necrosis factor-related apoptosis-inducing ligand)-induced apoptosis in follicular

undifferentiated thyroid carcinoma cells (Petrella et al., 2005). However, the overexpression of *Anxa1* also promoted proliferation of oesophageal squamous cell carcinoma cells (G. Han et al., 2017) and both, overexpression and knockdown strategies could be tested in CHO cells (Table 1 and Figure 2).

Orellana et al. also found Galectin 1 (LGALS1) protein up-regulated (Orellana et al., 2015) in the CHO-K1 high producer clone while three other proteomics studies found that LGALS1 was down-regulated in the CHO-DG44 and CHO-DXB11 high producer clones (Carlage et al., 2009; Meleady et al., 2008; Nissom et al., 2006). LGALS1 is a member of the family of β-galactoside binding proteins and is known to activate apoptosis in T cells (Kovacs-Solyom et al., 2010; Perillo, Pace, Seilhamer, & Baum, 1995) and colorectal cancer cells (Satelli & Rao, 2011), and its downregulation seems a promising strategy for CHO genetic engineering. However, LGALS1 is also known to be upregulated in several cancer cells, promoting tumour cell growth (Astorgues-Xerri et al., 2014), so its downregulation might inhibit cell proliferation and survival, and its overexpression should also be tested (Table 1 and Figure 2).

Nissom et al. also found *Bcl10* pro-apoptosis transcript down-regulated in the CHO-DXB11 high producer clone (Nissom et al., 2006), while Meleady et al. showed that the apoptosis inhibitor HSP27 was up-regulated in the CHO-DXB11 high producer clone (Meleady et al., 2008), which has already been shown to reduce apoptosis and increase titre when overexpressed (Tan et al., 2015).

Finally, a ^{13}C fluxomics analysis showed that the CHO-K1 high producing clones produce less lactate, have an elevated citric acid cycle flux and direct a greater amount of pyruvate toward mitochondrial metabolism compared to low- or non-producing cell lines (Templeton et al., 2017). Furthermore, clones with apoptosis-resistant features (Bcl-2Δ overexpression) simultaneously increased antibody production and glucose metabolism (hexokinase and phosphofructokinase fluxes).

Relevant genes that have shown to alter apoptosis and or proliferation have been discussed here and coluld be targets for CHO cell culture enhancement in the future.

Conclusions

'Omics approaches have contributed to the understanding of apoptosis in CHO cells and have led to the identification of propitious targets for rational genetic engineering. Differential expression analysis helps pinpoint non-trivial gene targets, which otherwise would be difficult to identify. In CHO cells, various apoptosis-associated genes were found to be de-regulated in phenotypes of interest, such as increased productivity. Specifically, apoptosis regulators whose functions in apoptosis are not yet completely understood, appear as differentially expressed. The overexpression or downregulation of these targets have successfully reduced apoptosis or increased cell proliferation in various cancer cells, and are consequently promising targets for CHO cell engineering. Some of these gene targets have shown contradictory results for different cancer cells. Therefore both overexpression and downregulation should be tested.

Most of the CHO 'omics studies to date have been simple contrasts with a single type of 'omics, analysed using standard t-test statistics. The depth and power of 'omics studies can be increased by using a multi-omics approach combing transcriptomics, proteomics, metabolomics, and fluxomics and exploiting network topology when analysing the data. Furthermore, to best exploit the continuous creation of 'omics datasets in CHO cells, uniform conditions across a broad set of cell lines should be considered to find universal gene targets. This review shows that there is still room for improvements of CHO cell lines for biopharmaceutical production and the use of 'omics studies for gene target elucidation offers unprecedented opportunities to positively impact final product titre.

Acknowledgement

LKN was supported by Novo Nordisk Foundation grants NNF10CC1016517 and NNF14OC0009473.

Abbreviations

Aip5 (Api5), apoptosis inhibitor 5; **Alg-2,** ALG2 alpha-1,3/1,6-mannosyltransferase; **Anxa1,** annexin A1; **Bad,** BCL2 associated agonist of cell death; **Bak,** BCL2 homologous antagonist killer; **Bax,** BCL2 associated X protein; **Bcl10,** BCL10 immune signaling adaptor; **Bim (Bcl2l11),** BCL2 like 11; **Birc2,** Baculoviral IAP repeat-containing 2; **Birc3,** Baculoviral IAP repeat-containing 3; **Bnip2,** BCL2 interacting protein 2; **CHO,** Chinese hamster ovary; **Clu,** clusterin; **Cul2,** cullin 2; **Cul3,** cullin 3; **Ddah,** dimethylarginine dimethylaminohydrolase; **Ddit4,** DNA-damage-inducible transcript 4; **EpoFc,** erythropoietin fusion protein; **ER,** endoplasmic reticulum; **Fadd,** Fas-associated death domain; **FasL (Faslg),** Fas ligand; **Fastkd1,** FAST kinase domains 1; **Gclc,** glutamate-cysteine ligase catalytic subunit; **Gclm,** glutamate-cysteine ligase modifier subunit; **Gss,** glutathione synthetase; **Hsp27 (Hspb1),** heat shock protein family B (small) member 1; **Hsp70 (Hspa1a),** heat shock protein family A (Hsp70) member 1A; **Hspa8,** heat shock protein family A (Hsp70) member 8; **IFN-γ,** interferon gamma; **Lgals1,** galectin 1; **Madd,** MAP-kinase activating death domain; **Malt1,** MALT1 paracaspase; **Mcl1,** MCL1 apoptosis regulator, BCL2 family member; **Nod1,** nucleotide-binding oligomerization domain containing 1**; Nod2,** nucleotide-binding oligomerization domain containing 2; **Pasg (Hells),** helicase, lymphoid specific; **Pdcd4,** programmed cell death 4; **Pdcd6ip,** programmed cell death 6 interacting protein; **Prkdc,** protein kinase, DNA activated, catalytic polypeptide; **Rassf5,** Ras association domain family member 5; **Rnf216,** ring finger protein 216; **Tde1 (Serinc3),** serine incorporator 3; **Tgm2,** transglutaminase 2; **Tp53inp1,** tumor protein p53 inducible nuclear protein 1**; Tpt1,** tumor protein, translationally-controlled 1; **Xiap,** X-linked inhibitor of apoptosis.

References

Abroun, S., Otsuyama, K., Shamsasenjan, K., Islam, A., Amin, J., Iqbal, M. S., . . . Kawano, M. M. (2008). Galectin-1 supports the survival of CD45RA(-) primary myeloma cells in vitro. *Br J Haematol, 142*(5), 754-765. doi:10.1111/j.1365-2141.2008.07252.x

Arden, N., & Betenbaugh, M. J. (2004). Life and death in mammalian cell culture: strategies for apoptosis inhibition. *Trends Biotechnol, 22*(4), 174-180. doi:10.1016/j.tibtech.2004.02.004

Astorgues-Xerri, L., Riveiro, M. E., Tijeras-Raballand, A., Serova, M., Neuzillet, C., Albert, S., . . . Faivre, S. (2014). Unraveling galectin-1 as a novel therapeutic target for cancer. *Cancer Treat Rev, 40*(2), 307-319. doi:10.1016/j.ctrv.2013.07.007

Baik, J. Y., Joo, E. J., Kim, Y. H., & Lee, G. M. (2008). Limitations to the comparative proteomic analysis of thrombopoietin producing Chinese hamster ovary cells treated with sodium butyrate. *J Biotechnol, 133*(4), 461-468. doi:10.1016/j.jbiotec.2007.11.008

Baik, J. Y., Lee, M. S., An, S. R., Yoon, S. K., Joo, E. J., Kim, Y. H., . . . Lee, G. M. (2006). Initial transcriptome and proteome analyses of low culture temperature-induced expression in CHO cells producing erythropoietin. *Biotechnol Bioeng, 93*(2), 361-371. doi:10.1002/bit.20717

Birzele, F., Schaub, J., Rust, W., Clemens, C., Baum, P., Kaufmann, H., . . . Hildebrandt, T. (2010). Into the unknown: expression profiling without genome sequence information in CHO by next generation sequencing. *Nucleic Acids Res, 38*(12), 3999-4010. doi:10.1093/nar/gkq116

Carlage, T., Hincapie, M., Zang, L., Lyubarskaya, Y., Madden, H., Mhatre, R., & Hancock, W. S. (2009). Proteomic profiling of a high-producing Chinese hamster ovary cell culture. *Anal Chem, 81*(17), 7357-7362. doi:10.1021/ac900792z

Carlage, T., Kshirsagar, R., Zang, L., Janakiraman, V., Hincapie, M., Lyubarskaya, Y., . . . Hancock, W. S. (2012). Analysis of dynamic changes in the proteome of a Bcl-XL overexpressing Chinese hamster ovary cell culture during exponential and stationary phases. *Biotechnol Prog, 28*(3), 814-823. doi:10.1002/btpr.1534

Chen, K., Li, D., Li, H., Li, B., Li, J., Huang, L., . . . Fang, J. (2017). Genetic analysis of heterogeneous sub-clones in recombinant Chinese hamster ovary cells. *Appl Microbiol Biotechnol, 101*(14), 5785-5797. doi:10.1007/s00253-017-8331-4

Chong, W. P., Yusufi, F. N., Lee, D. Y., Reddy, S. G., Wong, N. S., Heng, C. K., . . . Ho, Y. S. (2011). Metabolomics-based identification of apoptosis-inducing metabolites in recombinant fed-batch CHO culture media. *J Biotechnol, 151*(2), 218-224. doi:10.1016/j.jbiotec.2010.12.010

Cost, G. J., Freyvert, Y., Vafiadis, A., Santiago, Y., Miller, J. C., Rebar, E., . . . Gregory, P. D. (2010). BAK and BAX deletion using zinc-finger nucleases yields apoptosis-resistant CHO cells. *Biotechnol Bioeng, 105*(2), 330-340. doi:10.1002/bit.22541

Datta, P., Linhardt, R. J., & Sharfstein, S. T. (2013). An 'omics approach towards CHO cell engineering. *Biotechnology and Bioengineering, 110*(5), 1255-1271. doi:Doi 10.1002/Bit.24841

De Gennaro Colonna, V., Bianchi, M., Pascale, V., Ferrario, P., Morelli, F., Pascale, W., . . . Turiel, M. (2009). Asymmetric dimethylarginine (ADMA): an endogenous inhibitor of nitric oxide synthase and a novel cardiovascular risk molecule. *Med Sci Monit, 15*(4), RA91-101.

De Leon Gatti, M., Wlaschin, K. F., Nissom, P. M., Yap, M., & Hu, W. S. (2007). Comparative transcriptional analysis of mouse hybridoma and recombinant Chinese

hamster ovary cells undergoing butyrate treatment. *J Biosci Bioeng, 103*(1), 82-91. doi:10.1263/jbb.103.82

Dietmair, S., Nielsen, L. K., & Timmins, N. E. (2011). Engineering a mammalian super producer. *Journal of Chemical Technology and Biotechnology, 86*(7), 905-914. doi:Doi 10.1002/Jctb.2576

Dietmair, S., Nielsen, L. K., & Timmins, N. E. (2012). Mammalian cells as biopharmaceutical production hosts in the age of omics. *Biotechnology Journal, 7*(1), 75-89. doi:DOI 10.1002/biot.201100369

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicol Pathol, 35*(4), 495-516. doi:10.1080/01926230701320337

Fesus, L., & Szondy, Z. (2005). Transglutaminase 2 in the balance of cell death and survival. *FEBS Lett, 579*(15), 3297-3302. doi:10.1016/j.febslet.2005.03.063

Fussenegger, M., & Bailey, J. E. (1998). Molecular regulation of cell-cycle progression and apoptosis in mammalian cells: implications for biotechnology. *Biotechnol Prog, 14*(6), 807-833. doi:10.1021/bp9800891

Han, G., Lu, K., Huang, J., Ye, J., Dai, S., Ye, Y., & Zhang, L. (2017). Effect of Annexin A1 gene on the proliferation and invasion of esophageal squamous cell carcinoma cells and its regulatory mechanisms. *Int J Mol Med, 39*(2), 357-363. doi:10.3892/ijmm.2016.2840

Han, Y., Ren, J., Lee, E., Xu, X., Yu, W., & Muegge, K. (2017). Lsh/HELLS regulates self-renewal/proliferation of neural stem/progenitor cells. *Sci Rep, 7*(1), 1136. doi:10.1038/s41598-017-00804-6

Henry, M. N., MacDonald, M. A., Orellana, C. A., Gray, P. P., Gillard, M., Baker, K., . . . Martinez, V. S. (2020). Attenuating apoptosis in Chinese hamster ovary cells for improved biopharmaceutical production. *Biotechnol Bioeng, 117*(4), 1187-1203. doi:10.1002/bit.27269

Hernandez Bort, J. A., Hackl, M., Hoflmayer, H., Jadhav, V., Harreither, E., Kumar, N., . . . Borth, N. (2012). Dynamic mRNA and miRNA profiling of CHO-K1 suspension cell cultures. *Biotechnol J, 7*(4), 500-515. doi:10.1002/biot.201100143

Hsiang, C. H., Tunoda, T., Whang, Y. E., Tyson, D. R., & Ornstein, D. K. (2006). The impact of altered annexin I protein levels on apoptosis and signal transduction pathways in prostate cancer cells. *Prostate, 66*(13), 1413-1424. doi:10.1002/pros.20457

Huang, Y. C., Wei, K. C., Chang, C. N., Chen, P. Y., Hsu, P. W., Chen, C. P., . . . Yeh, T. H. (2014). Transglutaminase 2 expression is increased as a function of malignancy grade and negatively regulates cell growth in meningioma. *PLoS One, 9*(9), e108228. doi:10.1371/journal.pone.0108228

Jadhav, V., Hackl, M., Klanert, G., Hernandez Bort, J. A., Kunert, R., Grillari, J., & Borth, N. (2014). Stable overexpression of miR-17 enhances recombinant protein production of CHO cells. *J Biotechnol, 175*, 38-44. doi:10.1016/j.jbiotec.2014.01.032

Jin, H., Zhang, X., Su, J., Teng, Y., Ren, H., & Yang, L. (2015). RNA interference-mediated knockdown of translationally controlled tumor protein induces apoptosis, and inhibits growth and invasion in glioma cells. *Mol Med Rep, 12*(5), 6617-6625. doi:10.3892/mmr.2015.4280

Kim, Y. M., Kim, T. H., Seol, D. W., Talanian, R. V., & Billiar, T. R. (1998). Nitric oxide suppression of apoptosis occurs in association with an inhibition of Bcl-2 cleavage and cytochrome c release. *J Biol Chem, 273*(47), 31437-31441. doi:10.1074/jbc.273.47.31437

Kovacs-Solyom, F., Blasko, A., Fajka-Boja, R., Katona, R. L., Vegh, L., Novak, J., . . . Monostori, E. (2010). Mechanism of tumor cell-induced T-cell apoptosis mediated by galectin-1. *Immunol Lett, 127*(2), 108-118. doi:10.1016/j.imlet.2009.10.003

Kumar, N., Gammell, P., Meleady, P., Henry, M., & Clynes, M. (2008). Differential protein expression following low temperature culture of suspension CHO-K1 cells. *BMC Biotechnol, 8*, 42. doi:10.1186/1472-6750-8-42

Lee, M. S., Kim, K. W., Kim, Y. H., & Lee, G. M. (2003). Proteome analysis of antibody-expressing CHO cells in response to hyperosmotic pressure. *Biotechnol Prog, 19*(6), 1734-1741. doi:10.1021/bp034093a

Lewis, A. M., Abu-Absi, N. R., Borys, M. C., & Li, Z. J. (2016). The use of 'Omics technology to rationally improve industrial mammalian cell line performance. *Biotechnol Bioeng, 113*(1), 26-38. doi:10.1002/bit.25673

Li, F., Zhang, D., & Fujise, K. (2001). Characterization of fortilin, a novel antiapoptotic protein. *J Biol Chem, 276*(50), 47542-47549. doi:10.1074/jbc.M108954200

Liao, T. J., Jang, H., Tsai, C. J., Fushman, D., & Nussinov, R. (2017). The dynamic mechanism of RASSF5 and MST kinase activation by Ras. *Phys Chem Chem Phys, 19*(9), 6470-6480. doi:10.1039/c6cp08596b

Lim, L. H., & Pervaiz, S. (2007). Annexin 1: the new face of an old molecule. *FASEB J, 21*(4), 968-975. doi:10.1096/fj.06-7464rev

Mann, A. P., Verma, A., Sethi, G., Manavathi, B., Wang, H., Fok, J. Y., . . . Mehta, K. (2006). Overexpression of tissue transglutaminase leads to constitutive activation of nuclear factor-kappaB in cancer cells: delineation of a novel pathway. *Cancer Res, 66*(17), 8788-8795. doi:10.1158/0008-5472.CAN-06-1457

Meleady, P., Henry, M., Gammell, P., Doolan, P., Sinacore, M., Melville, M., . . . Clynes, M. (2008). Proteomic profiling of CHO cells with enhanced rhBMP-2 productivity following co-expression of PACEsol. *Proteomics, 8*(13), 2611-2624. doi:10.1002/pmic.200700854

Mishra, S., & Murphy, L. J. (2006). The p53 oncoprotein is a substrate for tissue transglutaminase kinase activity. *Biochem Biophys Res Commun, 339*(2), 726-730. doi:10.1016/j.bbrc.2005.11.071

Nagano-Ito, M., & Ichikawa, S. (2012). Biological effects of Mammalian translationally controlled tumor protein (TCTP) on cell death, proliferation, and tumorigenesis. *Biochem Res Int, 2012*, 204960. doi:10.1155/2012/204960

Ng, K. Y., Chan, L. H., Chai, S., Tong, M., Guan, X. Y., Lee, N. P., . . . Ma, S. (2017). TP53INP1 Downregulation Activates a p73-Dependent DUSP10/ERK Signaling Pathway to Promote Metastasis of Hepatocellular Carcinoma. *Cancer Res, 77*(17), 4602-4612. doi:10.1158/0008-5472.CAN-16-3456

Nissom, P. M., Sanny, A., Kok, Y. J., Hiang, Y. T., Chuah, S. H., Shing, T. K., . . . Philp, R. (2006). Transcriptome and proteome profiling to understanding the biology of high productivity CHO cells. *Molecular Biotechnology, 34*(2), 125-140. doi:10.1385/mb:34:2:125

Oliverio, S., Amendola, A., Rodolfo, C., Spinedi, A., & Piacentini, M. (1999). Inhibition of "tissue" transglutaminase increases cell survival by preventing apoptosis. *J Biol Chem, 274*(48), 34123-34128. doi:10.1074/jbc.274.48.34123

Orellana, C. A., Marcellin, E., Gray, P. P., & Nielsen, L. K. (2017). Overexpression of the regulatory subunit of glutamate-cysteine ligase enhances monoclonal antibody production in CHO cells. *Biotechnol Bioeng, 114*(8), 1825-1836. doi:10.1002/bit.26316

Orellana, C. A., Marcellin, E., Palfreyman, R. W., Munro, T. P., Gray, P. P., & Nielsen, L. K. (2018). RNA-Seq Highlights High Clonal Variation in Monoclonal Antibody Producing CHO Cells. *Biotechnol J, 13*(3), e1700231. doi:10.1002/biot.201700231

Orellana, C. A., Marcellin, E., Schulz, B. L., Nouwens, A. S., Gray, P. P., & Nielsen, L. K. (2015). High-antibody-producing Chinese hamster ovary cells up-regulate

intracellular protein transport and glutathione synthesis. *J Proteome Res, 14*(2), 609-618. doi:10.1021/pr501027c

Peng, M., Deng, J., Zhou, S., Tao, T., Su, Q., Yang, X., & Yang, X. (2019). The role of Clusterin in cancer metastasis. *Cancer Manag Res, 11*, 2405-2414. doi:10.2147/CMAR.S196273

Perillo, N. L., Pace, K. E., Seilhamer, J. J., & Baum, L. G. (1995). Apoptosis of T cells mediated by galectin-1. *Nature, 378*(6558), 736-739. doi:10.1038/378736a0

Petrella, A., Festa, M., Ercolino, S. F., Zerilli, M., Stassi, G., Solito, E., & Parente, L. (2005). Induction of annexin-1 during TRAIL-induced apoptosis in thyroid carcinoma cells. *Cell Death Differ, 12*(10), 1358-1360. doi:10.1038/sj.cdd.4401645

Rabinovich, G. A., Alonso, C. R., Sotomayor, C. E., Durand, S., Bocco, J. L., & Riera, C. M. (2000). Molecular mechanisms implicated in galectin-1-induced apoptosis: activation of the AP-1 transcription factor and downregulation of Bcl-2. *Cell Death Differ, 7*(8), 747-753. doi:10.1038/sj.cdd.4400708

Reddy, K. R. K., Dasari, C., Duscharla, D., Supriya, B., Ram, N. S., Surekha, M. V., . . . Ummanni, R. (2018). Dimethylarginine dimethylaminohydrolase-1 (DDAH1) is frequently upregulated in prostate cancer, and its overexpression conveys tumor growth and angiogenesis by metabolizing asymmetric dimethylarginine (ADMA). *Angiogenesis, 21*(1), 79-94. doi:10.1007/s10456-017-9587-0

Satelli, A., & Rao, U. S. (2011). Galectin-1 is silenced by promoter hypermethylation and its re-expression induces apoptosis in human colorectal cancer cells. *Cancer Lett, 301*(1), 38-46. doi:10.1016/j.canlet.2010.10.027

Scaltriti, M., Bettuzzi, S., Sharrard, R. M., Caporali, A., Caccamo, A. E., & Maitland, N. J. (2004). Clusterin overexpression in both malignant and nonmalignant prostate epithelial cells induces cell cycle arrest and apoptosis. *Br J Cancer, 91*(10), 1842-1850. doi:10.1038/sj.bjc.6602193

Shahbazi, J., Lock, R., & Liu, T. (2013). Tumor Protein 53-Induced Nuclear Protein 1 Enhances p53 Function and Represses Tumorigenesis. *Front Genet, 4*, 80. doi:10.3389/fgene.2013.00080

Shen, D., Kiehl, T. R., Khattak, S. F., Li, Z. J., He, A., Kayne, P. S., . . . Sharfstein, S. T. (2010). Transcriptomic responses to sodium chloride-induced osmotic stress: a study of industrial fed-batch CHO cell cultures. *Biotechnol Prog, 26*(4), 1104-1115. doi:10.1002/btpr.398

Solito, E., Kamal, A., Russo-Marie, F., Buckingham, J. C., Marullo, S., & Perretti, M. (2003). A novel calcium-dependent proapoptotic effect of annexin 1 on human neutrophils. *FASEB J, 17*(11), 1544-1546. doi:10.1096/fj.02-0941fje

Stolfa, G., Smonskey, M. T., Boniface, R., Hachmann, A. B., Gulde, P., Joshi, A. D., . . . Campbell, A. (2018). CHO-Omics Review: The Impact of Current and Emerging Technologies on Chinese Hamster Ovary Based Bioproduction. *Biotechnol J, 13*(3), e1700227. doi:10.1002/biot.201700227

Sun, L. Q., Lee, D. W., Zhang, Q., Xiao, W., Raabe, E. H., Meeker, A., . . . Arceci, R. J. (2004). Growth retardation and premature aging phenotypes in mice with disruption of the SNF2-like gene, PASG. *Genes Dev, 18*(9), 1035-1046. doi:10.1101/gad.1176104

Tan, J. G., Lee, Y. Y., Wang, T., Yap, M. G., Tan, T. W., & Ng, S. K. (2015). Heat shock protein 27 overexpression in CHO cells modulates apoptosis pathways and delays activation of caspases to improve recombinant monoclonal antibody titre in fed-batch bioreactors. *Biotechnol J, 10*(5), 790-800. doi:10.1002/biot.201400764

Templeton, N., Lewis, A., Dorai, H., Qian, E. A., Campbell, M. P., Smith, K. D., . . . Young, J. D. (2014). The impact of anti-apoptotic gene Bcl-2 expression on CHO central metabolism. *Metab Eng, 25*, 92-102. doi:10.1016/j.ymben.2014.06.010

Templeton, N., Smith, K. D., McAtee-Pereira, A. G., Dorai, H., Betenbaugh, M. J., Lang, S. E., & Young, J. D. (2017). Application of (13)C flux analysis to identify high-productivity CHO metabolic phenotypes. *Metab Eng, 43*(Pt B), 218-225. doi:10.1016/j.ymben.2017.01.008

Verma, A., Guha, S., Wang, H., Fok, J. Y., Koul, D., Abbruzzese, J., & Mehta, K. (2008). Tissue transglutaminase regulates focal adhesion kinase/AKT activation by modulating PTEN expression in pancreatic cancer cells. *Clin Cancer Res, 14*(7), 1997-2005. doi:10.1158/1078-0432.CCR-07-1533

von Eyss, B., Maaskola, J., Memczak, S., Mollmann, K., Schuetz, A., Loddenkemper, C., . . . Ziebold, U. (2012). The SNF2-like helicase HELLS mediates E2F3-dependent transcription and cellular transformation. *EMBO J, 31*(4), 972-985. doi:10.1038/emboj.2011.451

Walsh, G. (2018). Biopharmaceutical benchmarks 2018. *Nat Biotechnol, 36*(12), 1136-1145. doi:10.1038/nbt.4305

Wang, X., Liu, R., Wang, Y., Cai, H., & Zhang, L. (2015). Effects of down-regulation of clusterin by small interference RNA on human acute myeloid leukemia cells. *Int J Clin Exp Med, 8*(11), 20925-20931.

Wei, Y. Y., Naderi, S., Meshram, M., Budman, H., Scharer, J. M., Ingalls, B. P., & McConkey, B. J. (2011). Proteomics analysis of chinese hamster ovary cells undergoing apoptosis during prolonged cultivation. *Cytotechnology, 63*(6), 663-677. doi:10.1007/s10616-011-9385-2

Wong, D. C., Wong, K. T., Lee, Y. Y., Morin, P. N., Heng, C. K., & Yap, M. G. (2006). Transcriptional profiling of apoptotic pathways in batch and fed-batch CHO cell cultures. *Biotechnol Bioeng, 94*(2), 373-382. doi:10.1002/bit.20872

Wong, D. C., Wong, K. T., Nissom, P. M., Heng, C. K., & Yap, M. G. (2006). Targeting early apoptotic genes in batch and fed-batch CHO cell cultures. *Biotechnol Bioeng, 95*(3), 350-361. doi:10.1002/bit.20871

Wurm, F. M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature Biotechnology, 22*(11), 1393-1398. doi:Doi 10.1038/Nbt1026

Yee, J. C., de Leon Gatti, M., Philp, R. J., Yap, M., & Hu, W. S. (2008). Genomic and proteomic exploration of CHO and hybridoma cells under sodium butyrate treatment. *Biotechnol Bioeng, 99*(5), 1186-1204. doi:10.1002/bit.21665

Yee, J. C., Gerdtzen, Z. P., & Hu, W. S. (2009). Comparative transcriptome analysis to unveil genes affecting recombinant protein productivity in mammalian cells. *Biotechnol Bioeng, 102*(1), 246-263. doi:10.1002/bit.22039

You, K. H., Ji, Y. M., & Kwon, O. Y. (2003). Clusterin overexpression is responsible for the anti-apoptosis effect in a mouse neuroblastoma cell line, B103. *Z Naturforsch C J Biosci, 58*(1-2), 148-151. doi:10.1515/znc-2003-1-226

Zhou, X. H., Yang, C. Q., Zhang, C. L., Gao, Y., Yuan, H. B., & Wang, C. (2014). RASSF5 inhibits growth and invasion and induces apoptosis in osteosarcoma cells through activation of MST1/LATS1 signaling. *Oncol Rep, 32*(4), 1505-1512. doi:10.3892/or.2014.3387

Figure legends

Figure 1. Workflow to find promising apoptosis targets for CHO cell engineering. (1) A comprehensive literature review of CHO omics studies was performed. (2) In each study, the list of differentially expressed transcripts or proteins was filtered for apoptosis-related genes. (3) The genes that have not been engineered in CHO cells were then searched for engineering strategies in other cell lines. (4) If positive outcomes were found in other cell lines, then the genes were listed as promising targets for CHO genetic engineering.

Figure 2. Overview of the role of the proposed protein targets on apoptosis/cell proliferation-related events. Many of these proteins have multifaceted functions, and only some of the proposed pathways in the literature are shown. Proteins targets in red are pro-apoptotic or induce growth arrest while protein targets in green are anti-apoptotic or induce proliferation. **(i)** DDAH1 degrades asymmetric dimethylarginine (ADMA) which inhibits nitric oxide synthase (NOS) and thus, nitric oxide (NO) generation (De Gennaro Colonna et al., 2009). NO inhibits apoptosis by preventing the cleavage of the apoptosis suppressor B cell lymphoma gene 2 (BCL-2) and mitochondrial cytochrome-c (CYT-C) release. When released, CYT-C forms a complex with apoptotic protease-activating factor 1 (APAF-1) and pro-caspase-9, activating caspase-9 and subsequently executioner caspase-3, leading to apoptosis (Kim, Kim, Seol, Talanian, & Billiar, 1998). **(ii)** TGM2 activates p53 by phosphorylating the serine 15 and 20, reducing the ability of p53 to interact with MDM2, thus causing cell cycle arrest and apoptosis (Mishra & Murphy, 2006). However, TGM2 also promotes degradation of the tumor suppressor phosphatase and tensin homolog (PTEN) and results in constitutive activation of focal adhesion kinase (FAK) - phosphatidylinositol 3-kinase (PI3K) - protein kinase B (AKT) cell survival signalling (Verma et al., 2008). Furthermore, TGM2 induces constitutive activation of NF-κB, increasing cell proliferation (Mann et al., 2006). **(iii)** CLU inhibits apoptosis through stabilizing the association between

KU70 and BCL-2 associated X protein (BAX), preventing mitochondrial cytochrome-c release. CLU also inhibits TNF-alpha-induced apoptosis by promoting TNF-alpha-mediated activation of nuclear factor kappa B (NF-κB) and BCL-2 overexpression. However, CLU may induce apoptosis by stabilizing inhibitory molecules (IκBs) that reduce NF-κB activity, thus up-regulating p53 protein (Peng et al., 2019). **(iv)** Epidermal growth factor receptor (EGFR) activates RAS when an epidermal growth factor (EGF) signal is received. RASSF5 associates with RAS-GTP and phosphorylates and activates mammalian sterile 20- like kinase 1/2 (MST1/2) in the Hippo pathway. MSTG1/2 phosphorylates large tumor suppressor 1/2 (LATS1/2) and Mps one binder (MOB1), leading to phosphorylation of Yes associated protein 1 (YAP1) and thus its degradation. If not phosphorylated, YAP1 enters the nucleus, recruits the TEA domain (TEAD) family of transcriptional factors inducing gene transcription that contribute to cell proliferation (Liao, Jang, Tsai, Fushman, & Nussinov, 2017). **(v)** TP53INP1 forms complexes with the protein kinase homeodomain-interacting protein kinase-2 (HIPK2) or protein kinase C δ (PKCδ), phosphorylating p53 at serine 46. This leads to p53-target gene transcription (p53AIP1, p21, PIG3, and BAX), induction of G1 cell cycle arrest and increase in p53-mediated apoptosis (Shahbazi, Lock, & Liu, 2013). **(vi)** TPT1 inhibits p53-dependent apoptosis by binding and destabilizing p53 and repressing its transcription. TPT1 also bind and stabilize antiapoptotic BCL-2 family proteins, MCL1 and BCL-XL, which suppress apoptosis by binding and inactivating the proapoptotic proteins. Moreover, TPT1 blocks the formation of proapoptotic BCL-2 family protein BAX homodimers, required for its apoptotic activity. It also inhibits apoptosis induced by Ca^{2+} influx by binding to Ca^{2+}. High Ca^{2+} intracellular level injure mitochondrial membranes leading to the release of CYT-C and apoptosis-inducing factor (AIF), resulting in apoptosis (Nagano-Ito & Ichikawa, 2012). **(vii)** ANXA1 induces the dephosphorylation of the member of the BCL-2 family, BAD, which can then translocate to the mitochondria, heterodimerize

with BCL-2 or BCL-XL and promote apoptosis. Furthermore, ANXA1 activates Ca^{2+} influxes in a concentration-dependent manner, leading to apoptosis (Solito et al., 2003). **(viii)** LGALS1 binds to β1-integrin increasing the phosphorylation of extracellular signal-regulated kinases (ERK), protein kinase B (AKT) and inhibitor of kappa B (IkB), inducing proliferation. The phosphorylation of IκB induces NF-κB activation suppressing the apoptotic pathways. However, LGALS1 also binds to a protein tyrosine phosphatase receptor type C (CD45RA) inducing growth arrest or inhibition of the progression of the cell cycle by inducing suppression of ERK phosphorylation (Abroun et al., 2008). LGALS1 also stimulates activating protein-1 (AP-1) and downregulates BCL-2 by inhibiting concanavalin A induction of BCL-2 protein, inducing apoptosis (Rabinovich et al., 2000). **(ix)** PASG is required to maintain proper DNA methylation and gene expression patterns that are required for normal growth and longevity. The specific role remains unclear, however, PASG knockout upregulates tumor suppressor genes such as $p16^{INK4a}$, $p19^{ARF}$, p53, and p21 and downregulates BMI-1 (a negative regulator of $p16^{INK4a}$) (Sun et al., 2004).