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Studying the Integrated Stress Response in Tumor Progression and Drug Resistance

Nektaria-Maria Leli, B.Sc., MSc.

Molecular Biologist and Geneticist

DOCTORAL THESIS

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Abstract

Development and growth of a tumor as well as its ability to metastasize involves a complex relationship with the tissue microenvironment. A proliferating tumor encounters multiple stress conditions from the microenvironment such as hypoxia, lack of nutrients and acidosis. To cope with these conditions, cancer cells have co-opt elaborate cytoprotective mechanisms which provide them with distinct advantages to thrive. These mechanisms constitute a complex of homeostatic signaling pathways and are collectively known as the Integrated Stress Response (ISR). Thus, deciphering the signaling pathways which get activated in the tumor microenvironment has been paramount to develop new therapeutic strategies for treatment. The Unfolded Protein Response (UPR) (which is a more specialized form of ISR), is activated in response to unfolded proteins in the Endoplasmic Reticulum (ER) and involves translational and transcriptional activation of regulated signaling pathways designed to relieve cellular stress and attenuate cancer cell death. Intriguingly, in the case of unresolved or acute ER stress, the UPR can promote cell death. However, the mechanisms by which the UPR influences cell fate in the presence of ER stress are poorly understood. As a result, a comprehensive analysis to determine critical regulators of UPR is of utmost importance. To address the abovementioned question we have used a functional CRISPR mediated genetic knockout screen to determine novel regulators of UPR and to investigate the mechanisms by which these regulators control cellular fate following chronic ER stress. More specifically, we delivered a lentiviral genome-scale CRISPR-Cas9 knockout (GeCKO_V2) library to Sq20B cells (human squamous head and neck carcinoma) and A375 (human melanoma) cells. The library is targeting 18,080 genes with 64,751 unique guide sequences and enables both negative and positive selection screening, according to which sgRNAs are over-represented (pro-apoptotic genes), or underrepresented (pro-survival genes) following ER stress induced by thapsigargin and tunicamycin, known activators of the ER stress through distinct mechanisms. Our highest negative ranking gene candidates included Survivin/BIRC5, a well-studied protein which is overexpressed in tumor cells compared normal tissues. Survivin has been reported to act as an inhibitor of apoptosis; however, its main function is to promote proper regulation of mitosis and

cytokinesis as part of the Chromosomal Passenger Complex. Our results show that genetic knock down or chemical inhibition of Survivin led to sensitization to ER stress. Additionally, its ablation caused formation of micronuclei and multinucleated cells due to failure of cytokinesis and as a result promotion of aneuploidy and chromosomal instability. Intriguingly, Survivin-deficient cells had also an expanded ER and activated UPR. We hypothesize that these cells that have significantly increased DNA content which imposes higher transcriptional and translational demands on the cells, thereby causing ER expansion in order to support them. This, in turn, creates a reliance of these cells on a functioning UPR, whose inhibition results in cell death. Our results demonstrate that polyploidy, and perhaps more generally, genomic instability represents cellular stresses during which a hyperactive UPR may be required for survival. Thereby this study reveals new vulnerabilities for therapeutic intervention in malignancy as well as new therapeutic schemes by combinational targeting of Survivin and inhibition of UPR activation.

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PART 1

INTRODUCTION

1. INTRODUCTION

1.1 The Endoplasmic Reticulum

One of the largest organelles in eukaryotic cells is the endoplasmic reticulum (ER), a network of branching interconnected tubules through an enclosed space called the ER lumen, which is separated from the surrounding cytosol by a single intracellular lipid bilayer, the ER membrane. The ER membrane serves as a boundary between the cytosol and the ER lumen and regulates the passage of molecules between these two compartments (Fagone et al., 2009). The ER plays a major role in protein synthesis, folding, and structural conformation and also in the Ca^{+2} storage in the cell. More specifically, all proteins destined for the ER, plasma membrane, Golgi apparatus, or lysosomes are translated on ER membrane bound ribosomes and then injected into the ER lumen. In a similar way, most proteins that are secreted from the cell begin their journey in the ER. Proteins targeted to the ER have an N-terminal signal sequence that directs them to the ER membrane while they are still being synthesized on ribosomes. These proteins are being translated while moving through the translocon complex and the signal sequence is removed by a protease once translation of the polypeptide is completed. Once in the ER lumen, proteins are folded into their 3D shapes and can undergo various post-translational modifications like glycosylation or disulfide bond formation. These processes are catalyzed by enzymes that exist in the ER, such as chaperones, glycosylating enzymes, and oxidoreductases. Some of the most important proteins involved in maintaining ER homoeostasis are Glucose Regulated Protein 78 (GRP78, aka BiP), Calreticulin, Calnexin and Protein Disulfide Isomerase (PDI). To favor this enzymatic processes, the ER environment has a much higher calcium concentration and a more oxidizing redox potential compared to the cytosol which is important for all the catalysis events (Anelli et al., 2008). Chaperones bind to proteins and aid in their proper folding and achieving their final native 3D conformation. Thus, these enzymatic processes ensure that secretory proteins are properly folded, modified, and assembled into multiprotein complexes in the ER, before they traffic further downstream in the secretory pathway (Yadav et al., 2014). However, the success rate for proper folding is still low (<20%). Because proteins of the secretory pathway often mediate important signaling roles,

incompletely folded forms are not tolerated by the cell and instead are disposed of by stringent quality control systems, through a process called ER-associated degradation (ERAD). Unfolded proteins are moved to the cytosol for subsequent ubiquitylation and degradation by the 26S proteasome (McCracken et al., 2003). Moreover, folding efficiency for an individual protein can be further compromised by the presence of a mutation in the gene that encodes it. For example, specific mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause problems in its folding within the ER, leading to depletion of this essential ion channel that normally transports chloride across epithelial tissues and finally to cystic fibrosis (Rowe et al., 1990-2001).

1.2. The Unfolded Protein response pathway

The proper functioning of the ER is important in maintaining cellular homeostasis, which ensures that only correctly assembled proteins are allowed leave the ER. Many cells like the pancreatic β cells that produce insulin or the B lymphocytes of the immune system that produce antibodies, are in a constant need of protein production and lead to increased ER demands. In addition to that, a variety of intrinsic stimuli like oncogenes (Tameire et al., 2019) and also extrinsic disturbances, such as changes in Ca^{2+} concentration, hypoxia, glucose deprivation, or oxidative stress, will lead to an increased amount of unfolded or misfolded proteins in the ER, a condition termed as ER stress. To maintain protein homeostasis and ensure that protein folding capacity is in balance with protein synthesis and bioenergetics demands, cells constantly monitor the state of protein folding in the cell. In case of imbalance between client proteins and chaperones in the ER, or when conditions are such that favor unfolding (e.g., hypoxia), (REFS) reestablishment of homeostasis can be achieved by the activation of a series of finely regulated signaling pathways called the Unfolded Protein Response (UPR) (Corazzari et al., 2017) (Tameire et al., 2015). The UPR is a highly conserved pathway among mammals and yeast and is initiated by three ER transmembrane proteins: the protein kinase (PKR)-like ER kinase (PERK) pathway, the inositol-requiring protein-1 (IRE1) pathway and the activating transcription factor-6 (ATF6) pathway (Walter and Ron, 2011). All three proteins act as direct or indirect sensors of misfolded proteins by interacting with BiP in their inactive state. Upon concentration of misfolded proteins

in the ER, BiP dissociates from the complex to act as a chaperon facilitating folding, thus activating the three UPR braches. Each of the proteins oligomerizes and activates the downstream activities of the pathway. The overall goal of these adaptive responses is to activate signaling pathways that either help the cell relieve the stress and survive when the stress is acute and mild, or lead the cell to apoptosis when the signal is chronic and severe. The activation of its pathway is described below (**Figure 1.1**).

1.3. PERK arm of the UPR

During ER stress, PERK dissociates from Grp78 and gets autophosphorylated and oligomerized to an active state. P-PERK phosphorylates the elongation initiation factor a (eIF2α) at Ser51 (Liu et al., 2000) (Bertolotti et al., 2000). P-EIF2a inhibits global translation and favors the translation of mRNAs with short open reading frames in their 5'-untranslated regions, such as the Transcription Activation Factor 4 (ATF4) (Rozpedek et al., 2016). ATF4 is a transcription factor expressed selectively under ER stress conditions and is responsible for the regulation of genes like chaperones and autophagy promoting genes. One of the targets of ATF4 is the Growth Arrest and DNA Damage gene 34 (GADD34), a protein phosphatase cofactor responsible for the dephosphorylation of eIF2a and the reversion of global translation inhibition through this negative feedback loop (Yi et al., 2016. Another important target gene of ATF4 is the transcription factor C/EBP homologous protein (CHOP aka GADD153). Its main role is controlling the genes involved with apoptosis. CHOP and GADD34 are expressed when UPR can no longer rescue the cell form ER stress and switches to a more pro-apoptotic character, which can explain the uncertainty in whether the PERK branch plays a pro-survival or pro-apoptotic role.

In addition to eIF2a, P-PERK phosphorylates also the transcription factor NF-E2-related factor-2 (NRF2),which controls the antioxidant response pathway and promotes redox homeostasis during ER stress (Del Vechio et al., 2014) (Cullinana etr al., 2003) (Cullinana etr al., 2004). More recently, PERK has been shown to participate in the activation of the transcription factor FOXO, in a PERK dependent way in order to potentiate insulin resistance during the onset of type 2 diabetes (Zhang et al., 2013). To sum up, PERK in cancer has been contributing to activation of adaptive pathways rather than to cancer cell death, which is also supported by the

fact that pharmacological inhibition of PERK attenuates tumor growth in xenograft models (Axten et al., 2012) (Atkins et al., 2013). Furthermore, the PERK/ATF4 axis is important in the metastatic process through regulation of antioxidant response as well as autophagy and prevents apoptosis of cells undergoing extracellular matrix detachment, a process tightly linked to metastasis. Moreover, PERK/eIF2α/ATF4 axis-mediated expression of miRNA-211 promotes survival during ER stress by repressing CHOP and thus its pro-apoptotic character (Scott et al., 2015).

1.4. IRE1 arm of the UPR

The second arm of the UPR involves IRE1, which uses unconventional mRNA splicing to elicit the UPR signal. When the ER is under stress, IRE1 dissociates from Grp78 and auto-transactivates itself through its kinase activity domain, thus activating its RNAase domain activity. IRE1 then removes a 26 mucleotides long intron from X-box binding protein 1 (XBP1) protein. This splicing gives rise to the active form of transcription factor XBP-s (Sha et al., 2010) (Korennykh et al., 2012). The RNAase domain of IRE1 can cut more RNAs, thus decreasing protein synthesis. XBP1-s translocates to the nucleus were it activates genes that confer to ER stress relief by chaperones production and proteins degradation (Calfon et al., 2002) (Yoshida et al., 2001) (Yamamoto et al., 2007). High levels of XBP1s are observed in many cancers like breast cancer, hepatocellular carcinoma, lymphoma and multiple myeloma. Targeting the nuclease activity of IRE1 by small molecule inhibitors can be a promising therapeutic strategy.

1.5. ATF6 arm of the UPR

The third arm of the UPR involves ATF6. During ER stress ATF6 is dissociated from Grp78 in the luminal domain, packaged into transport vesicles and delivered to the Golgi apparatus. There, two proteases, S1P and S2P cleave it to the water-soluble N-terminal domain and allow ATF6(N) to translocate into the nucleus and act as a transcription factor activating UPR target genes, such as BiP, protein disulfide isomerase (PDI), ER associated degradation (ERAD) and glucose-regulated protein 94 (Ye et al., 2000) (Ron and Walter, 2011). Although the role of ATF6 in tumorigenesis is not very well studied so far, there has been detected increased ATF6 levels in

patient tissues of hepatocellular carcinoma (HCC) as well as Hodgkin lymphoma.(Scott et al., 2015).

1.6. The ambiguous UPR

Collectively, the PERK, IRE1 and ATF6 pathways converge in a coordinated way as a homeostatic feedback loop to alleviate ER stress when the stress is acute or mild (**Figure 1.1**). To succeed this, all three pathways aim to block global translation, expand the ER and selectively activate the expression of genes that contribute to relieving the stress and increasing the folding capacity, such as chaperon proteins. If successful in reducing the amount of misfolded proteins, UPR is attenuated and the cells survives. However, if the stress is severe and prolonged and homeostasis cannot be reestablished, then the UPR will be sustained and transform into the terminal UPR that promotes cell death (Bravo et al., 2013) (Shore et al., 2011). Although the proapoptotic character of the UP is not yet fully understood molecularly, hyper activation of PERK and prolonged inhibition of translation can lead the cells to death. Additionally, PERK hyperactivation can upregulate CHOP transcription factor which inhibits expression of the gene that encodes for the antiapoptotic BCL-2 (McCullough et al., 2001) (Marciniak et al., 2004).

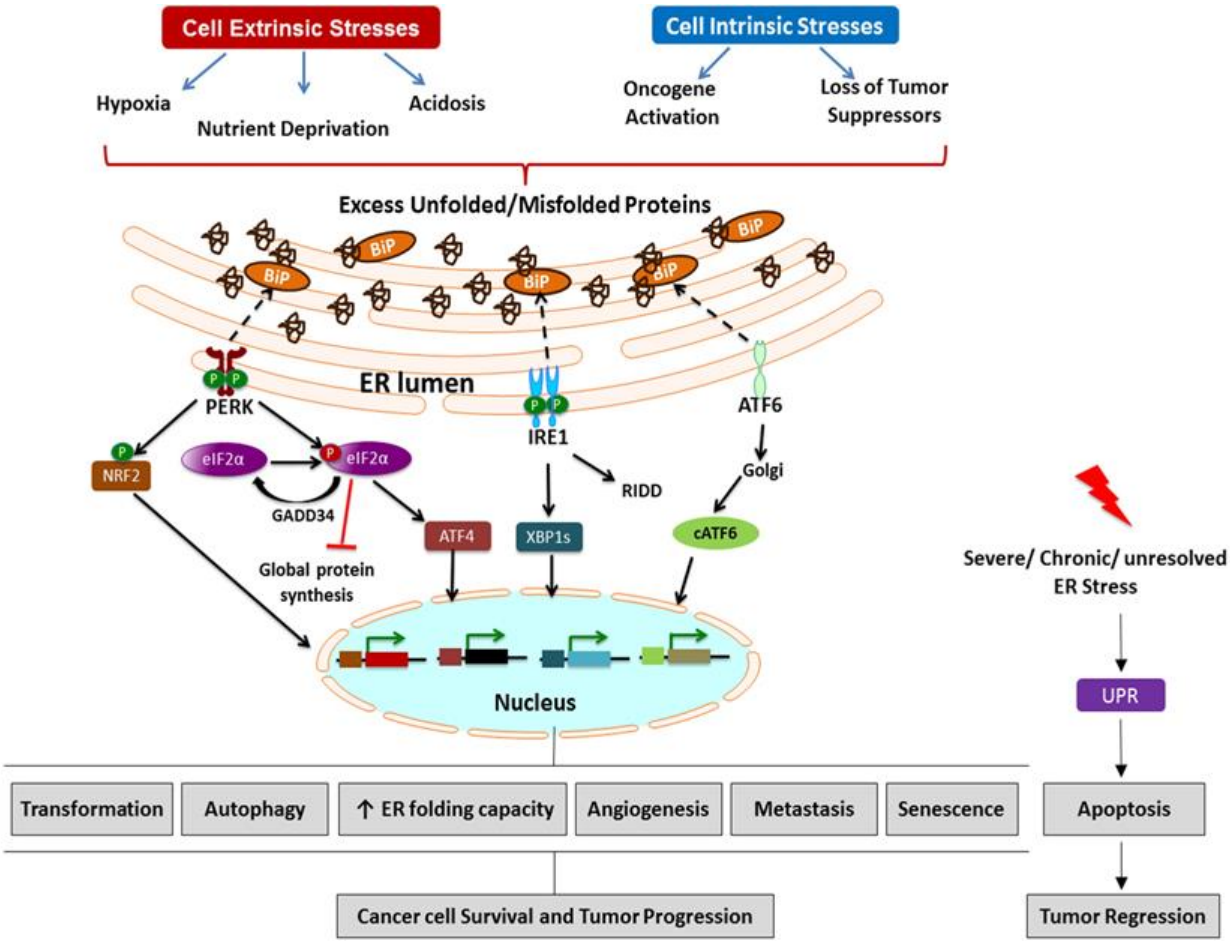


Figure 1.1. An overview of the Unfolded Protein Response pathway. Three transmembrane sensor proteins (PERK, IRE1 and ATF6) activate different signaling pathways upon accumulation of misfolded proteins in the ER. This activation occurs by dissociation with BiP protein chaperon that is in a complex with them when there are only folded proteins around. Upon activation, all of the three pathways converge in the activation of genes that aim to alleviate the stress, when it is acute and mild. However, when stress is severe and prolonged than UPR leads to apoptosis. This pathway is often hijacked by cancer cells that adopt it in order to overcome stressful conditions (Tameire et al., 2015).

1.7. UPR in disease and cancer

ER stress and sustained UPR have been documented in affected tissues in diabetes neurodegeneration, stroke, pulmonary fibrosis, viral infection, inflammatory disorders, heart disease and cancer (Oakes et al., 2015).

As mentioned above tumor cells often invade or metastasize into neighboring environments where unfavorable conditions, such as hypoxia, glucose deprivation, lactic acidosis, oxidative stress, and inadequate amino acid supplies cause erroneous protein folding in the ER. Many studies have shown prolonged activation of all three branches of the UPR in a wide range of human tumors, including glioblastoma (Gardner et al., 2011), multiple myeloma (Carrrasco et al., 2007), and carcinomas of the breast (Fernandez et al., 2000), stomach (Song et al., 2001), esophagus (Chen et al., 2002), and liver (Shuda et al., 2003). Genomic screens have identified rare somatic mutations in IRE1α in a small percentage of human solid tumors (Greenman et al., 2007). Another study showed that other components of the ER protein-folding machinery, most notably the chaperone BiP are overexpressed in cancer in a disease progression fashion (Pyrko et al., 2007) (Luo et al., 2013).

However, despite the evidence of ongoing ER stress and UPR activation in many forms of cancer, it is not fully clear weather these processes ultimately inhibit or promote tumor growth in patients, and need to be investigated further. On the one hand, most of the evidence supporting that the UPR supports tumor growth comes from xenograft studies in mice, in which genetically deleting one or more branches of the UPR or altering the expression of the ER chaperone BiP inhibits the in vivo growth of tumor cells (Amora et al., 1996) (Park et al., 2004) (Romero-Ramirez et al., 2004) (Austgen et al., 2012). For example, genetic deletion of IRE1α in a human glioma cell line resulted in reduced angiogenesis and decreased tumor growth when cells were injected into mice (Auf et al., 2010). On the other hand, the IRE1α signaling pathway has been found to induce a number of proangiogenic factors, such as Vascular Endothelial Growth Factor (VEGF), which may be a mechanism through which UPR can promote the growth of solid tumors (Ghosh et al., 2010). Collectively, these findings suggest that not only is UPR frequently activated in tumors, but it may be necessary for the survival and growth of the cancer cells under conditions that stress the ER.

A good example for understanding the ambiguous role of UPR in cancer is myeloma. Myeloma is a highly secretory tumor composed of malignant plasma cells and is a cancer for which the UPR is frequently mentioned as a potentially attractive target, because UPR is essential for plasma cell development. In mice, IRE1α is important for differentiation of B

lymphocytes into plasma cells (Reimold et al., 2001) (Zhang et al., 2005). Interestingly, up to 50% of primary myelomas show unusually high levels of XBP1s. Moreover, mice expressing the Xbp1-s spliced version in B lymphocytes develop a plasma cell malignancy resembling myeloma (Carrasco et al., 2007). There is also evidence to suggest that proteasome inhibition by Bortezomib (Velcade), which is approved by the US Food and Drug Administration (FDA) as a major therapy for myeloma, leads to myeloma cell death in part by preventing disposal of misfolded proteins through the ERAD pathway and thus triggering ER stress induced apoptosis (Voorhees et al., 2006). Based on these findings, several pharmacologic inhibitors of the IRE1α's RNase activity have recently been tested on human myeloma xenografts and were found to have a suppressive activity (Papandreou et al., 2011) (Mimura et al., 2012). However, the specificity and off-target effects of these pharmacological agents are not yet well understood.

Taken together, the above findings suggest an oncogenic role for XBP1s in the development of myeloma. However, there are studies that support the opposite. First, downregulation of XBP1s expression in myeloma correlates with resistance to bortezomib (Gu et al., 2012). Second, a study that did whole-exome sequencing of primary tumors from 38 myeloma patients led to the identification of XBP1 mutations in two of these patients (Chapman et al., 2011), which caused inactivation of XBP1s. Finally, it was recently reported that genetic knockdown of IRE1α or XBP1 in human myeloma cell lines is well tolerated and leads to bortezomib resistance (Leung-Hagestein et al., 2013), questioning the use of IRE1α inhibitors in this disease. Overally, the lessons from myeloma to date suggest that the effects of the UPR (or at least its IRE1a arm) on tumor development and maintenance are more complicated than originally anticipated.

1.8. The Integrated Stress Response

The Integrated Stress Response (ISR) is an elaborate signaling pathway present in eukaryotic cells, which is activated in response to a wide range of physiological changes and different pathological conditions. These activating stimuli can be both extrinsic and intrinsic. Extrinsic stresses include factors like hypoxia, amino acid deprivation, glucose deprivation and viral infection. Intrinsic stresses involve the endoplasmic reticulum (ER) stress which is caused by the accumulation of unfolded or misfolded proteins in the ER, or oncogene activation (Tameire et

al., 2019). The common point where all these stresses converge is the activation of eIF2a by phosphorylation on Serine 51. The ISR is mediated by four Ser/Thr kinases which lead to the phosphorylation of eIF2a: PERK, General control non-derepressible 2 (GCN2), RNA-dependent protein kinase (PKR) and heme regulated inhibitor (HRI). Each of these kinases senses different stimuli experienced by the cells. The first one to evolve was GCN2 enabling eukaryotes to respond to amino acid deprivation, PKR responds to double stranded RNA (dsRNA) usually after viral infections and HRI is responsive to iron deficiency and other stressful stimuli like oxidation. Last but not least, PERK is already discussed above as it is the common denominator between the ISR and the UPR as it senses the efficacy of protein folding in the endoplasmic reticulum (ER) (**Figure 1.2**).

When eIF2a is phosphorylated, protein synthesis is blocked, serving a number of cytoprotective roles. During ER stress this slows down the rate of proteins entering the ER, thereby off-loading overburdened chaperones. In conditions of amino acid starvation or iron limitation it reduces the rate at which these nutrients are being consumed, and during viral infection blocking protein synthesis, blocks viral replication.

1.9. Consequences of the eIF2a phosphorylation

In general, phosphorylation of eIF2a by any of the aforementioned kinases leads to transient inhibition of global protein synthesis which helps to conserve amino acids but also energy through less use of adenosine 5-phosphate (ATP) under ER stress conditions. Additionally, there is preferential translation of select mRNAs containing a short upstream open reading frame (uORF) in their 5' untranslated region. Proper termination of the ISR signaling is essential, because it enables cells to reactivate protein synthesis and return to normal function. eIF2a dephosphorylation is mediated by the association of protein phosphatase (PP)1 and its catalytic subunit PP1c to either growth arrest and DNA-damage-inducible protein (GADD34), which is induced downstream of ISR signaling, or to constitutive repressor of eIF2a phosphorylation (CReP), which exists at basal levels even under unstressed conditions and helps to maintain low levels of phosphorylated eIF2a (Santos-Ribeiro et al., 2018).

1.10. ATF4 and CHOP

One of the well-studied effectors of ISR and who is selectively translated during ER stress is ATF4 which was mentioned above. ATF4 is a leucine zipper transcription factor and a member of the cAMP responsive element binding protein (CREB/ATF4) family of proteins that is ubiquitously expressed but whose translation is increased after phosphorylation of eIF2a (Ameri et al., 2008). ATF4 contains a transcriptional activation domain at the N-terminus and a DNA binding domain at the C-terminus. ATF4 mediates the expression of genes by binding to CCAAT-enhancer binding protein-activation factor (C/EBP-ATF) response elements (CARE) (Fawcett et al., 1999). ATF4 is an important regulator both of the UPR and the ISR signaling pathways that is why he can bind to to different monomeric transcription factors, forming heterodimers and activate or repress the expression of genes (Hai et al., 1991). ATF4 interacting partners are the ones that determine its transcriptional selectivity and help in the activation of expression of genes that aim to relieve ER stress and facilitate proper protein folding so that the cell survives the respective stress (Pakos-Zebrucka et al., 2016) (**Figure 1.2**).

CHOP is a non ER localized transcription factor that is induced by a variety of adverse physiological conditions including ER stress, although it does not participate to protein folding regulation. It is unclear whether CHOP induction during ER stress occurs only through the ER stress response element (ERSE) that is conserved in both CHOP and ER chaperon promoters, or through a separate regulatory pathway induced by different cellular stress conditions. It has been proposed that a C/EBP-ATF composite site exists in its promoter and it is responsible for its transcriptional activation by other cellular stress conditions (Ma et al., 2002).

CHOP expression is low under non-stressed conditions, but its expression increases in response to ER. More specifically, the activation of ATF4, which is induced by the PERK-mediated phosphorylation of eIF2α, is thought to play a dominant role in the induction of CHOP in response to ER stress (Harding et al., 2000). The overexpression of CHOP promotes apoptosis in several cell lines, whereas CHOP-deficient cells are resistant to ER stress-induced apoptosis (Kim et al., 2008) (Oyadomari et al., 2004). Therefore, CHOP plays an important role in the induction of apoptosis. $CHOP^{-/-}$ mice based studies have shown that CHOP-mediated apoptosis contributes to the pathogenesis of a number of ER stress-related diseases (Tabas et al., 2011).

However, exactly how CHOP mediates ER stress-induced apoptosis remains controversial. A possible explanation is the down-regulation of Bcl-2 and the induction of the BH3 only pro-apoptotic proteins Bim, Puma and Bax as well as DR5, a member of the death-receptor protein family (Kim et al., 2008) (Oyadomari et al., 2004) (Yamaguchi et al., 2004) (Schneider et al., 2000). Additionally, CHOP also induces the depletion of cellular glutathione and increases the production of reactive oxygen species in the ER (Kim et al., 2008) (Oyadomari et al., 2004). Moreover, CHOP transcriptionally induces ERO1α, which catalyzes the reoxidation of PDI, resulting in the production of hydrogen peroxide (Lu et al., 2004) (Gross et al., 2006). Thus, ERO1α may be possibly an important mediator of apoptosis downstream of CHOP. The cellular calcium signaling pathway has also been proposed to be implicated in ER stress-induced and CHOP-mediated apoptosis (Seimon et al., 2006). The CHOP-induced expression of ERO1α activates the ER calcium release channel IP3R1 (Li et al., 2009). Cytoplasmic calcium released from the ER triggers apoptosis by the activation of CaMKII, which in turn leads to activation of downstream apoptosis pathways. The ERO1α–IP3R1–CaMKII pathway may be a main axis in CHOP-mediated apoptosis.

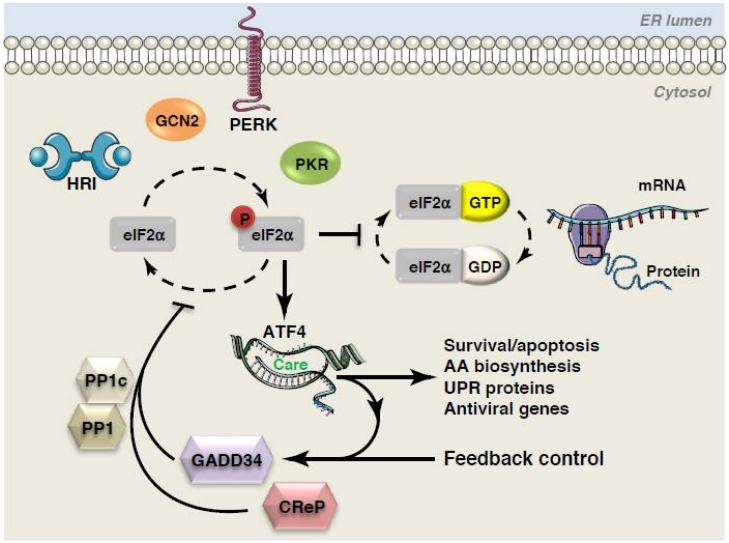


Figure 1.2. Overview of the ISR pathway. Four major kinases converge upon stressful stimuli in the phosphorylation of eIF2a and the activation of downstream effectors like ATF4 transcription factor (Santos-Ribeiro et al., 2018).

1.11. Apoptosis and its inhibitors

Death in the cellular world comes with a physiological process that called apoptosis. Apoptosis contributes to the development and maintenance of healthy cells and tissues. When the pathways involved in apoptosis are deregulated this can lead to many diseases such as cancer, immunodefficient and neurodegenerative disorders. Organisms have evolved two major ways to induce apoptosis, through extrinsic or intrinsic pathways that both converge in the activation of enzymes that are called caspases. There are the initiator caspases (caspase 2, 8, 9 and 10) and the effector Caspases (Caspase 3, 7 and 6). One of them upon stimulation of a proapoptotic signal will activate preforms of effector caspases by cleaving them. In turn, the effector caspases (aka death caspases) cleave protein substrates. This pathway can be inhibited by a family of proteins known as the Inhibitors of Apoptosis (IAP).

The Inhibitors of Apoptosis (IAP) family of proteins is a group of proteins that inhibit apoptosis. All the members contain one to three BIR (Baculovirus IAP Repeats) domains. BIR domains are approximately ~70 amino acids long and their name derives from the discovery of these apoptosis suppressors in the genomes of baculoviruses. Some of them contain also a RING domain (Really Interesting New Gene) and/or a CARD domain (Caspase Recruitment Domain). Proteins containing BIR domains have been identified in a wide range of eukaryotic species but membership in the IAP family of proteins requires both the presence of a BIR domain and the ability to suppress apoptosis. In fact, many of these BIR-containing proteins have not been tested in respect to apoptosis suppression. Structure–function studies have demonstrated that at least one BIR domain is required for the suppression of apoptosis, although other domains like RING seam to participate in the inhibition of apoptosis in cell context dependent manner, CARD domain does not seem to be essential at all.

In humans there are six IAP members of the IAP family: NAIP (*BIRC1*), c-IAP1/HIAP-2 (*BIRC2*), c-IAP2/HIAP-1 (*BIRC3*), XIAP/hILP (*BIRC4*), BIRC5 (Survivin) and BRUCE/Apollon(*BIRC6*). Out of them Survivin is the smallest (142 aa) and the only member of this group that has one BIR5 domain (**Figure 1.3**).

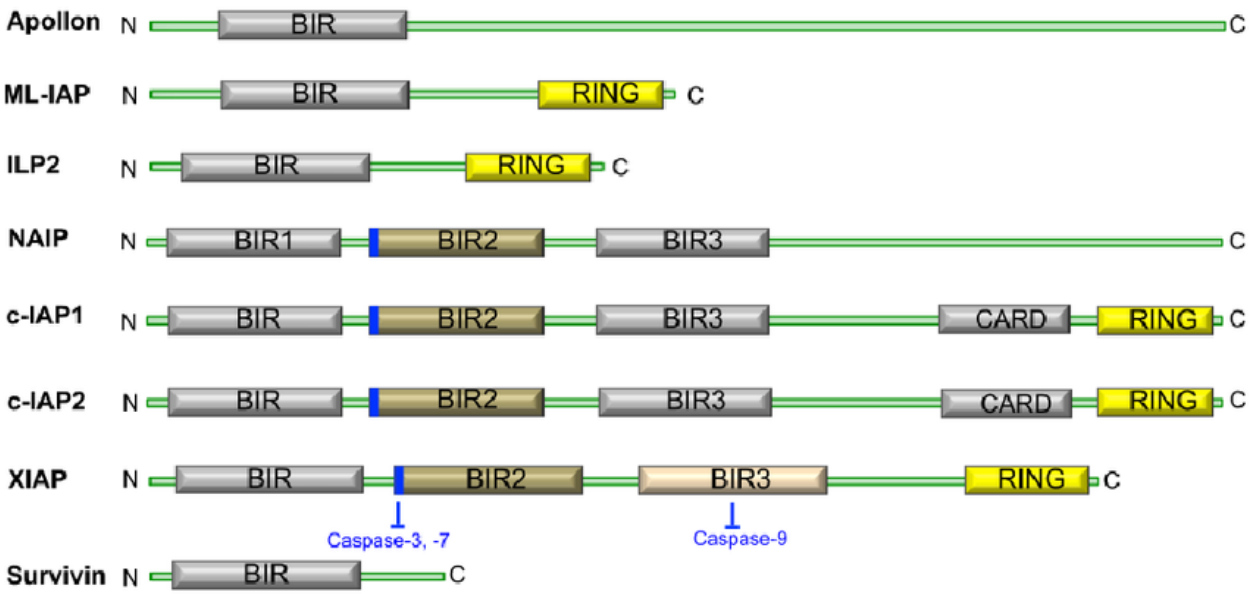


Figure 1.3. The structure of the Inhibitors of Apoptosis (IAPs) in mammals. There are 8 members in this family of inhibitors. All have a common feature the BIR domain which is responsible for their inhibitory role. Some proteins have more than one copy of this domain but Survivin only has one and is the smaller member of the family (15kDa). Some of the protein members contain also a CARD and/or a RING domain, that facilitate the formation of complexes with other proteins (CARD) or nucleic acids (RING) (Mobahat et al.m 2014).

1.12. Role of Survivin in apoptosis and cell cycle progression

As mentioned above Survivin was firstly identified as a member of the Inhibitors of apoptosis family. Its role in this inhibition though has been so far widely disputed. Survivin was reported to bind to caspases 3, 7 and 9 thus inhibiting their cleavage and subsequent apoptotic activity. However, there are studies that contradict this finding (Chandele et al., 2004). In coimmunoprecipitation assays Survivin precipitated with the active Caspase 3 and 7 but not their inactive forms (Wright et al., 2000). An in vitro study in ovarian cancer cells showed that the phosphorylation at Thr34 is important for its interaction with caspases 3,7 and 9 (Singh et al., 2013) and that mutation in this site in melanoma cells can cause cytochrome c release from the mitochondria and thus initiate the apoptotic pathway (Liu et al. 2004).

Survivin has a very important role in the regulation of the cell cycle. Early studies showed that BIRC5 promoter contains four copies of G_1 repressor elements that have been implicated in

controlling cell cycle periodicity in some G_2/M-regulated genes (Borbely et al., 2007). Moreover, reporter gene assays indicated that Survivin exhibits typical M phase inducible transactivation, suggesting that Survivin is a cell cycle regulated gene and raising the possibility that Survivin expression may be induced in dividing cells (Li et al., 1998). Later, it was discovered that Survivin is a member of the Chromosomal Passenger Complex (CPC).

1.13. Structure and Function of the Chromosomal Passenger Complex

The CPC complex coordinates chromosomal and cytoskeletal events of mitosis. The enzymatic core of this complex is AuroraB, a kinase that is guided through the mitotic cell by its companions, the inner centromere protein (INCENP), Borealin/Dasra-B and Survivin (**Figure 1.4**).

Members of the CPC complex (except for Survivin):

- **AuroraB** (*AURKB*): A Ser/Thr kinase family member. There are three Auroras. AuroraA functions at the mitotic spindle poles. AuroraB functions at the centromeres. AuroraC is like AuroraB but regulated meiosis and mitosis during early embryonic development. Together with Cyclin Dependent Kinases (CDKs) and Polo-Like Kinases (PLKs) the Auroras are master controlers of the cell cycle checkpoints.
- I**NCENP** (*INCENP*): A protein scaffold on which the CPC assembles. The N-terminus is required for the localization to the centromeres. Apart from binding with its complex companions it also binds to heterochromatin protein 1 (HP1) and this interaction is important for CPC localization. INCENP is regulated by AuroraB and CDK1-cyclinB complex, that control the entry and exit of mitosis. Phosphorylation of INCENP by CDK1 is important for the localization of PLK in the inner centromeres () but for its activation the phosphorylation of PLK1 from AuroraB is also required.
- **Borealin** (CDCA8): This protein binds to INCENP and Survivin with a 1:1:1 ratio. The central region of Borealin interacts with the EndoSomal Complex Required for Transport III (ESCRT-III) by directly binding to the Shrb and CHMP4C subunit. This interaction is very important since it is the connection with the final step of mitosis, since ESCRT-III regulates the separation of the two cells, a process known as abscission. Borealin is regulated by CDK1

phosphorylation, which leads to interaction of Borealin with Shugosin 1 (Sgo1) and Sgo2, which participate in the localization of CPC in the centromeres.

1.13.1 Structure of the CPC

The CPC comprises of two modules, a kinase module and a localization module. The localization unit is composed of Survivin, INCENP's amino terminus and Borealin which are associated in a three helix bundle. In this bundle the BIRC5 domain of Survivin interacts with the C-terminus of Borealin and both domains are needed for the localization of the CPC to the centromeres. Altogether, this module is required for the localization to the mitotic spindle to the midbody of anaphase. The kinase module is composed of AuroraB which is bound to the IN box of the INCENP C-terminus, and is responsible for the phosphorylation of substrates (**Figure 1.4**).

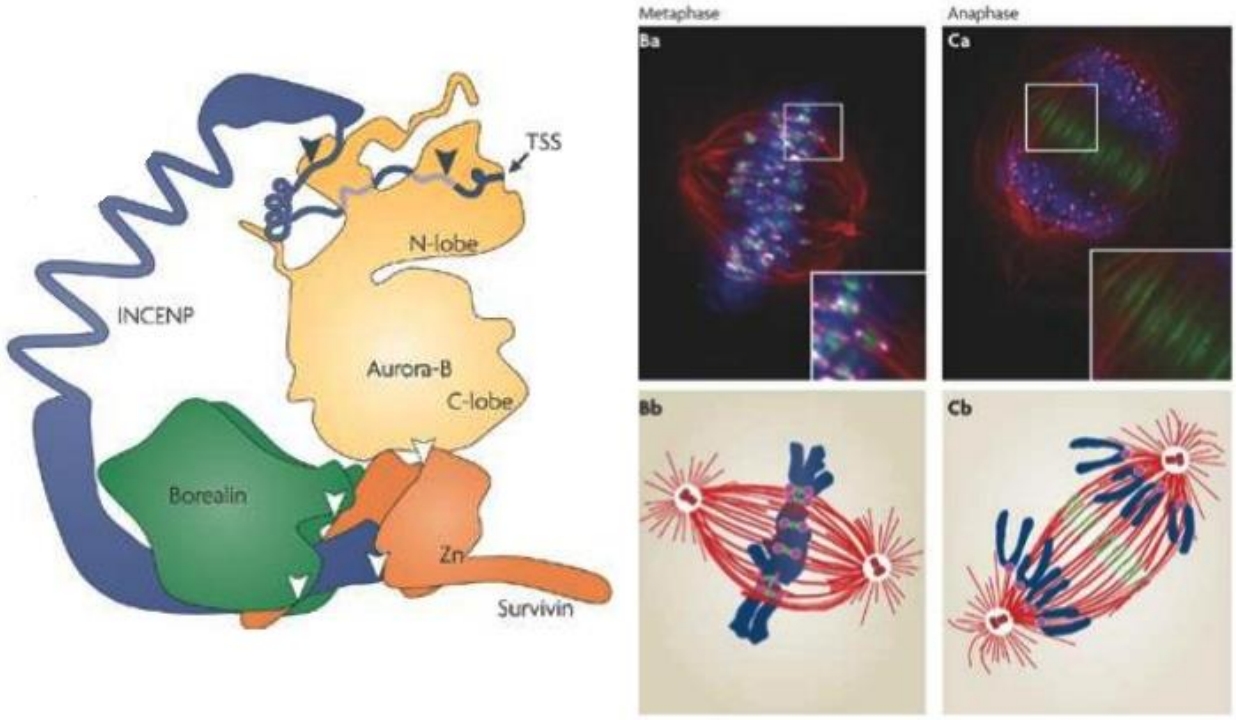


Figure 1.4. Graphic representation of the Chromosomal Passenger Complex (CPC). During metaphase CPC becomes abundant in the inner centromeres and in anaphase it relocalizes to the center of the mitotic spindle due to interaction failure with the centromeres (Carmena et al., 2012).

1.13.2. Function and localization of the CPC

Proper mitosis requires accurate nuclear division, followed by cytoplasmic partitioning into two daughter cells during cytokinesis. Nuclear division is monitored by the spindle assembly checkpoint (SAC), that prevents the onset of anaphase until all sister chromatids have attached properly to the mitotic spindle. Dynamic changes in CPC localization throughout mitosis are very important in order to ensure the spatially restricted phosphorylation of substrates that are involved in the major CPC functions. To begin, proper localization is important for chromosome condensation and interaction of chromosomes with Condensin, a multimeric protein that is essential for the maintenance of the mitotic chromosome architecture. Also, proper localization ensures the correction of erroneous kinetochore-microtubule attachments (Tanaka et al., 2002)(Lampson et al., 2004), the initiation of the spindle assembly checkpoint (SAC) a signaling cascade that is triggered by unattached kinetochores and delayes sister chromatid separation and cell cycle progression until all kinetochores attain bipolar microtubule attachments, and last but not least, cytokinesis, the separation of the mitotic cell in two.

To regulate these processes, the members of the CPC are localized at the inner centromere in prophase and metaphase, on the central spindle during anaphase and at the midbody during cytokinesis (Vagnarelli et al., 2004). Interference with Aurora B has been shown to cause failure of cytokinesis (Kaitna et al., 2000) (Severson et al., 2000) (Ditchfieldt al., 2003) (Hauf et al., 2003). Interference with the other CPC members has the same mitotic consequences as when interfering with AuroraB. This indicates that AuroraB functions are determined by its nuclear localization pattern and the complex members are equally important for the promotion of mitosis. INCENP is important for complex stability, since silencing its expression leads to decreased protein levels for AuroraB and Survivin (Vader et al., 2006). Although INCENP contains a highly conserved centromere-targeting domain, it is Survivin that serves as the determining factor in the CPC localization. INCENP by itself cannot concentrate in the centromeres during mitosis in the absence of Survivin but rather diffuses in the cytoplasm (Vader et al., 2006). Additionally, Borealin has only a facilitating role in the interaction between Survivin and INCENP, since it binds to Survivin cannot properly interact with INCENP without Borealin.

To sum up, Survivin expression is regulated so that it peaks in G2/M phase and rapidly declines in G1 phase. Survivin is dynamically localized at different regions on the chromosomes during the cell cycle as part of the CPC (Jeyaprakash et al., 2007). During mitosis Survivin localizes to the mitotic spindle where it interacts with tubulin microtubules and regulated the proper separation of sister chromatids. In G2 phase, Survivin accumulates in the centromeres and then it starts to diffuse to the chromosomes and in the prophase and metaphase it becomes abundant in the inner centromeres. In anaphase Survivin relocalizes to the center of the mitotic spindle due to interaction failure with the centromeres. During cytokinesis, Survivin is concentrated at the midbodies (Beardmore et al., 2004). Moreover, Survivin as mentioned above is phosphorylated by CDK1 a key kinase for the cell cycle regulation. Supportive evidence of Immmunoprecipitation experiments showed that Survivin interacts with CDK1 during mitosis (Chandele et al., 2004).

1.14. Survivin: Expression and Role in Cancer

1.14.1. Regulation of Survivin expression

Survivin is regulated both in the transcriptional and translational level. As a cell cycle regulated protein, Survivin has a cell cycle-dependent element (CDE) and also a cell cycle homology region (CHR) at its promoter. There are also many miRNAs that have been identified to regulate Survivin expression by binding to the 3-untranslated region (UTR). Two well studied miRNAs with respect to Survivin are miR-34a and miR-203. The first one, miR-34a, regulates Survivin directly and indirectly. For direct regulation the 3-UTR of Survivin contains a unique region for its binding, and indirectly it can repress the upstream activators or transcriptional factors of Survivin, thereby leading to decreased Survivin expression. The second one, miR-203, is targeting Survivin mRNA which significantly contributes to prostate cancer and metastasis (Huang at al., 2015). Survivin can also be post-translationally regulated by phosphorylation and polyubiquitination which directly affect its activity. The most important phosphorylation site is at Thr34 by Cell Division Cycle protein 2 [CDC2, aka cyclin dependent kinase 1 (CDK1)]. Mitotic phosphorylation at this site can promote its stability in metaphase. As far as ubiquitination is concerned, the ubiquitin-proteasome pathway can lead to Survivin degradation in a cell cycle-

dependent manner. Additionally, in insulinoma INS-1 cells, dephosphorylation on Ser20 translocates Survivin from mitochondria to cytoplasm to inhibit caspase cleavage (Dohi et al., 2004) (Dohi et al., 2007), and in cervical and breast cancer cell lines, Survivin acetylation on Lys129 by CREB-binding Protein increases its nuclear localization (Wang et al., 2010).

Furthermore, Survivin is implicated in many major signaling pathways. The tumor suppressor p53 inhibits cell cycle progression and induces apoptosis. Since Survivin overexpression and loss of wild-type p53 expression/function occur in most cancers, many studies have already aimed to find the link between those two molecules. It is shown that wild type p53 and not mutant is shown to transcriptionally repress Survivin expression (Mirza et al., 2002). Another group showed that there are two p53 binding sites into BIRC5 promoter but neither of these is potent to suppress Survivin expression (Ulason et al., 2009). However, other studies showed that in cells that overexpress Survivin there is reduced p53 levels due to enhanced p53 degradation resulting from Survivin-mediated inhibition of Mdm2 cleavage by caspases (Wang et al., 2004). Another cancer associated is Notch. Many studies showed that overexpression of Survivin can lead to Notch signaling activation (Chen et al., 2011) and also that Notch 1 signaling pathway can vice versa increase Survivin expression(Lee et al., 2008). Moreover, STAT3, an oncogene activated in many cancer types regulates the transcriptional activation of many genes including Survivin (Carpenter et al., 2014), while TGFβ has been recognized as a negative regulator of Survivin at the transcriptional level through a mechanism dependent on Smad2, Smad3 and Smad4 (Song et al., 2013).

1.14.2. Survivin expression in normal tissues and malignancy

Survivin expression is restricted in adult tissues. It is developmentally regulated and has been reported to be low in most terminally differentiated tissues except for CD34+ hematopoietic stem cells, placental and basal cells of the colonic epithelium and thymus. It can only be detected by northern blotting only in human and mouse healthy adult tissues (Ambrosini et al., 1997). Immunohistochemical analysis and in situ hybridization studies have shown Survivin expression in many apoptosis-regulated fetal tissues. In mouse embryos ubiquitous distribution of Survivin was detected at embryonic day E11.5, whereas at E15–E21, Survivin expression was

deteriorated to only a few locations, suggesting that expression of Survivin in embryonic life may contribute to tissue homeostasis and differentiation, and later becomes quiescent during adulthood. However, it is still unclear whether Survivin has a developmental role or it is just detected due to the rapid cell division in fetal tissues (Adida et al. 1998). Despite its undetected levels recent reports suggest roles for Survivin in normal cells (Fukuda et al., 2006) including T-cells (Xing et al., 2004), hematopoietic progenitor cells (Fukuda et al., 2001), vascular endothelial cells (Mesri et al., 2001), liver cells (Deguchi et al., 2002), gastrointestinal tract mucosa (Chiou et al., 2003), erythroid cells (Gurbuxani et al., 2005) and polymorphonuclear cells (Altznauer et al., 2004).

Survivin seems to be selectively expressed in transformed cells and in most human cancers, like lung (Monzo et al., 1999), pancreas (Satoh et al., 2001), breast (Tanaka et al., 2000), colon carcinomas (Kawasaki et al., 1998), brain tumors (Chakravarti et al, 2002), neuroblastomas (Adida et al., 1998; Islam et al., 2000), melanomas (Altieri et al., 1999) and many other types of blood cancers. Survivin expression can alter in cancer cells in comparison to normal cells through various mechanisms. In some cancers like neuroblastoma there is an amplification of the chromosomal locus where BIRC5 gene is located (17q25) (Islam et al., 2000). Additionally, the exons of Survivin can be demethylated (Hattori et., 2001) or its expression can be increased by upstream signaling pathways like PI3K and mTOR pathways (Vaira et al., 2007).

It is important to note that the role of Survivin in cancer biology extends beyond the inhibition of apoptosis. As mentioned above, Survivin is implicated in the regulation of the mitotic spindle checkpoint, from kinetochore to spindle assembly, so its overexpression in cancer cells may allow them to continue through cell division despite spindle defects, thus contributing in the aneuploeidy phenomenon detected in cancer. Additionally, Survivin may have an important role in the formation of solid tumors because it is highly expressed in endothelial cells during the remodeling and proliferating phase of angiogenesis (Tiran et al., 1999) (Harfouche et al., 2002).

1.14.3. Survivin in chemoresistance and radioresistance

Early data indicated that Survivin causes resistance to chemotherapy agents in cancer cells (Giodini et al., 2002). In this early studies, overexpression of Survivin caused inhibition of apoptosis induced by taxol agents (paclitaxel) in Hela cells and also in various human ovarian and prostate cell lines (Zhang et al., 2005) (Zaffaroni et al., 2002). Interestingly, it was also shown that taxol induced cell cycle arrest leads to increase in the expression of Survivin (O'Connor et al., 2002). Survivin was also suggested to induce resistance to flutamide anti-ansrogen therapy in prostate cancer (Zhang et al., 2005). Furthermore, many studies have shown that Survivin expression in cancer cells suppresses radiation induced cytotoxicity. High expression of Survivin has been reported to cause radioresistance in squamous cell carcinomas (Khan et al., 2010), pancreatic cancer (Asanuma et al., 2000) (Asanuma et al., 2002) and rectal cancer (Roedel et al., 2005).

1.14.4. Survivin as a diagnostic & prognostic marker

In the last few years Survivin has emerged as a potential early predictor of malignancies. In a study of oral cancerous lesions, 33% of precancerous lesions that had no malignant progression and 94% of precancerous lesions that evolved in full squamous cell carcinomas showd expression of Survivin. Tumors that evolved from these precancerous lesions preserved 100% of their Survivin positivity (Lo Muzio et al., 2003). Additionally, in bladder cancer, a disease with high recurrence rates, urinal detection of Survivin is used as a marker to spot both newly diagnosed but also recurrent malignancies (Shariat et al., 2004).

Increased Survivin expression (RNA or protein level) in cancer patients is in general a non-favorable prognostic marker and is associated in some cases with high risk of recurrence, lymph node invasion and metastasis. It also seems to correspond with higher malignant grades and it is usually correlated with decreased survival in different cancer types like hepatocellular carcinoma (Ye et al., 2007), esophageal cancer (Rosato et al., 2006), glioblastoma (Shirai et al., 2009), lung cancer (Mohamed et al., 2009), B-cell non-Hodgkin's lymphoma and breast cancer (Brennan et al., 2008) (Yamasita et al., 2007). Using Survivin as a general prognostic marker still

needs to be fatherly verified and combined with the assessment deriving from other prognosis markers.

1.14.5. Survivin as a therapeutic target

The aberrant high expression of Survivin in cancer cells, coupled with its very low expression in most normal tissues, makes Survivin an attractive anticancer target. Several experimental therapeutic strategies that target Survivin have been developed over the years. Developing drugs that target Survivin might initially seam difficult because Survivin is not an enzyme with a targetable enzymatic cleft, nor it is a cell surface protein. Nonetheless, approaches to deplete its expression have already been tested.

Antisense oligonucleotides (AO) are short single stranded RNA or DNA sequences that are complimentary to a specific RNA area and hybridize to the target strand thus suppressing the expression of the particular protein. The first attempt to create such a therapeutic approach was done in melanoma cells in vitro and then it was shown to inhibit Survivin in various tumor cell lines like lung, sarcoma, lymphoma, thyroid and head and neck (Cao et al., 2004) (Sharma et al., 2005) (Du et al., 2005) (Fuessel et al., 2004) (Ansell et al., 2004). Additionally, downregulation of Survivin with AO enhanced sensitivity in cytotoxic agents like TRAIL, cisplatin, imatinib, taxol and etoposide (Azyhata et al., 2006) (Fisker et al., 2007). In vivo testing of AO however was not equally successful. The only AO based drug targeting Survivin that is currently in phase II clinical trials is LY2181308 (ISIS 23722, Eli Lilly and Co and ISIS Pharmaceuticals Inc.) for hepatocellular carcinoma (ID: NCT00415155). It is administered in combination with docetaxel vs docetaxel alone in patients with non-small cell lung carcinoma (ID: NCT01107444).

Other ways to achieve molecular targeting of Survivin have also been tested. Small interfering RNAs (siRNAs) are short double stranded sequences that inhibit gene expression. Many preclinical studies downregulated Survivin this way showing also subsequent sensitization to vincristine (Jiang et al., 2006) doxorubicin, TNF-a targeting (Huynh et al., 2006) and radiation (Kappler et al., 2005). Furthermore, ribozymes, endonoucleolytic enzymes that cleave RNA have been used to target BIRC5 mRNA. Some of them decreased Survivin expression but not

sufficiently and some showed sensitization to cisplatin (Pennati et al., 2002), topotecal (Pennati et al., 2004) and radiation (Pennati et al., 2003).

Another therapeutic approach was based on the interaction of Survivin with the molecular chaperone Hsp90 which is believed to stabilize the protein. To target this interaction Shepherdin, a cell-permeable peptidomimetic, was designed and modeled on the binding interface between Hsp90 and the Survivin. Shepherdin makes contacts with the ATP pocket of Hsp90, destabilizes its client proteins, and induces massive death of tumor cells by apoptotic and nonapoptotic mechanisms. Interestingly, it did not affect the viability of normal cells, which made it an even more interesting drug candidate. Systemic administration of Shepherdin in vivo is well tolerated, and inhibits human tumor growth in mice without toxicity (Plescia et al., 2005). Shepherdin is still in a preclinical phase.

Sepantronium bromide aka YM155 (Astellas Pharma) is a small imidazolium-based molecule inhibitor that was selected through a high throughput screening assay with a Survivin promoter luciferase assay (Nakahara et al., 2007). It binds to Survivin promoter inhibiting the transcription of mRNA and it has been tested successfully in many cell lines. It has shown an antiproliferative activity in many tumor preclinical models more notably breast, prostate, ovarian, non-small cell lung carcinoma, melanoma, non-Hodgins lymphoma and leukemia. YM1155 has been in phase II clinical trials for solid tumors and non-small cell lung carcinoma (ID: NCT01100931) but the results have been rather disappointing. Another inhibitor that was developed for targeting Survivin is Terameprocol aka EM-1421 (Erimos Pharmaceuticals), which was under phase I clinical trials for refractory solid tumors (ID:NCT00664586) and in phase II clinical trials for leukemia (ID:NCT00664677) but was terminated due to funding reasons. In summary, all inhibitors exhibited a favorable safety profile but failed to demonstrate an improvement in response rate (Kelly et al. 2013) (Erba et al., 2013). Thus they could be used refined and be used in combination with other conventional therapies.

Another therapeutic approach is immunotherapy. Early reports using immunotherapy for targeting Survivin showed that cytotoxic CD8+ T lymphocytes had significant cytolytic activity against Survivn-specific epitopes both *in vitro* and *in vivo*. Thus, Survivin-based anticancer vaccines emerged targeting lung cancer, lymphoma, neuroblastoma, pancreatic and prostate

cancers (Song et al., 2008) (Zhu et al.2007) (Fuessel et al., 2006). These have been successfully tested in mouse models with no significant toxicities observed. There are currently ongoing Survivin-based vaccines in clinical trials including: against multiple myeloma autologous hematopoietic cell transplant (HCT) (ID: NCT02851056), multiple myeloma (ID: NCT03762291) and the better studied SurVaxM against glioblastoma in combination with Temozolomide (ID: NCT02455557). The most recent development based on Survivin is a long synthetic peptide (LSP)-based vaccine that targets the tumor antigen SVX and demonstrated high therapeutic efficacy against four different established murine tumor models. It generated both specific cytotoxic CD8+ and multifunctional Th1 CD4+ T cell response, and after the tumors were eradicated it protected against re-challenging by triggering memory T cell response (Onodi et al., 2018).

Gene therapy approaches that target Survivin are also promising and two main approaches have been developed. One is the activation of apoptosis in tumor cells transfected with Survivin containing vectors (Pennati et al., 2007) and the other involves the expression of cytotoxic genes driven by the promoter of Survivin (Song et al., 2013) (Bates et al., 2013).

1.15. Aim of Study

One of the unique traits of the UPR is its dubious response to ER stress. When the stress is acute and mild then the UPR adopts a prosurvival role, whereas if the stress is chronic or severe the UPR can switch to a pro-apoptotic character guiding the cells to death. Key mediators in this ambiguous response are the levels of ATF4 and CHOP, two transcription factors with opposite roles towards the fate of a cell in distress. However, the line that separates whose expression predominates is not yet well defined and the equilibrium rules are not yet well understood.

ER stress often promotes unfavorable microenvironment conditions such as low pH and hypoxia that often result from an accumulation of misfolded proteins. Normally, these conditions would lead to apoptosis in case UPR is not able to reestablish homeostasis, but cancer cells can hijack the UPR pathway and use it as a protective mechanism in order to circumvent the environmental stress, enhance the ER protein-folding capacity and promote ER protein homeostasis, thus sustaining the tumor's rapid growth and survival.

The mechanisms involved in the overcoming of ER stress in cancer and tumor progression are not yet fully understood. Our primary aim was to elucidate the mechanisms involved in this process, so we hypothesized that utilizing a genome wide functional KO CRISPR screen will help us identify new mediators of the UPR that regulate cell fate after ER stress exposure. Additionally, we aimed to decipher the role of these potential regulators in the context of ER stress and inquire if and how these candidates can be used as new targets for novel therapeutic approaches.

PART 2

Materials and Methods

2. Materials and Methods

2.1. Reagents

Reagents used in this study were purchased as follows: YM155 (Sigma-Aldrich, Cat#57462), Thapsigargin (Sigma-Aldrich, Cat#T9033), Tunicamycin (Sigma-Aldrich, Cat#T7765), B-IO9 (TOCRIS, Cat#6009).

2.2. Plasmids & other constructs

The following plasmids used in this study are commercially available: GeCKO-V2 backbone (Addgene, Cat#52961) (**Figure 2.1**), pLJM1-EGFP (Addgene, Cat#19319) (**Figure 2.2**), psPAX2 (Addgene, Cat#12260) (**Figure 2.3**), pVSVG (Addgene, Cat#14888) (**Figure 2.4**). For silencing Survivin expression we used a smart pool siRNA mix (Dharmacon, Cat# L-003459-00-0005) and an inducible TRIPZ i-shRNA (Dharmacon, Cat#RHS4740-EG332) (**Figure 2.5**). Transfection was performed with RNAimax (Thermo-Fisher Scientific, Cat#13778030) and Lipofectamine 2000 (Thermo-Fisher Scientific, Cat#11668019) respectively, as per manufacturer's protocol. For silencing INCENP a smart pool siRNA mix (Dharmacon, Cat# L-006823-02-0005) was used.

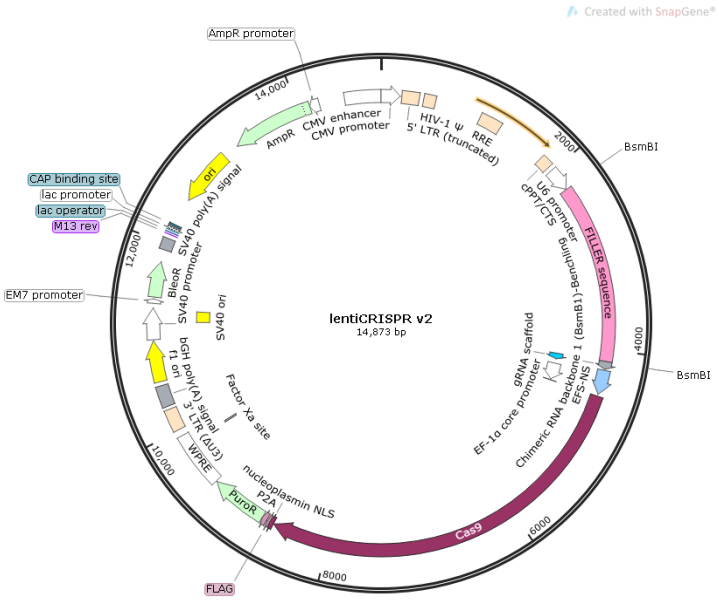


Figure 2.1. Schematic representation of the structure of the GeCKO_V2 backbone lentiviral plasmid. This is the backbone of the one vector system used in the screening libraries. The Cas9 open reading frame (ORF) is portrayed with plum color and the non-targeting filler sequence is portrayed with pink. The filler sequence is removed when digested with BsmBI restriction enzyme that flanks the filler sequence (indicated in the picture above), and is replaced with the gene specific sequence that makes the sgRNA

unique. The plasmid has a puromycin resistance gene for mammalian selection and an ampicillin resistance gene for bacterial selection (both portrayed in green). The other components pictured above are used for the expression of the ORF and the proper packaging of the lentivirus. There is also a FLAF sequence tag to facilitate the tacking of successful infection. Schematic created by SnapGene software.

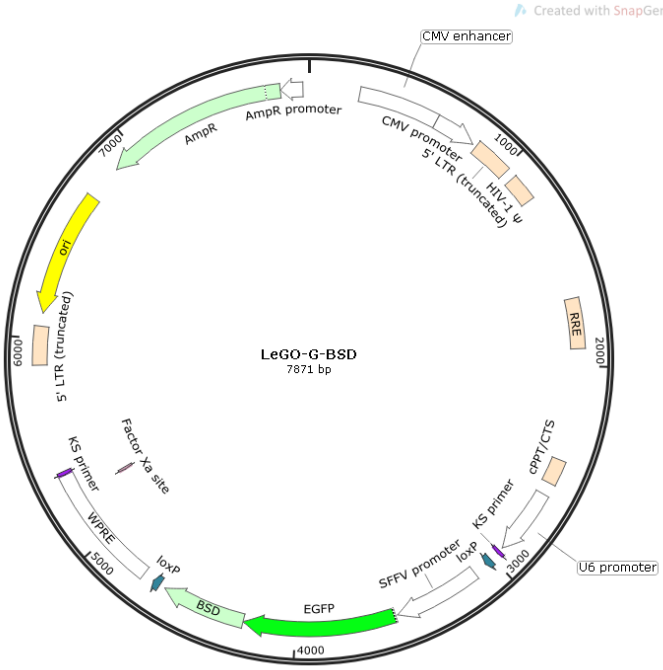


Figure 2.2. Schematic representation of the structure of the LeGO-G/BSD-EGFP lentiviral plasmid. This plasmid was used to create a GFP expressing cell line for the control experiment described in Figure 3.1 testing the efficacy of the GeCGO_V2 system. The EGFP ORF is portrayed in green and it is under the regulation of a U6 promoter. The other components pictured above are used for the expression of the ORF and the proper packaging of the lentivirus. There is also a FLAF sequence tag to facilitate the tacking of successful infection. Schematic created by SnapGene software.

Figure 2.3. Schematic representation of the structure of the psPax2 lentivirus packaging plasmid. This plasmid carried the viral enzymatic elements that are needed for the proper packaging of a viral particle (gag and pol proteins portrayed in plum color). They derive from an inactivated form of the HIV virus. The Rev Response Element (RRE) of HIV-1 allows for Rev-dependent mRNA export from the nucleus to the cytoplasm and enhances titer by increasing packaging efficiency of a full length viral genome. It is used in every transfection in 293T cells that aims in the production of a virus

along with pVSVG and the lentiviral plasmid of interest.

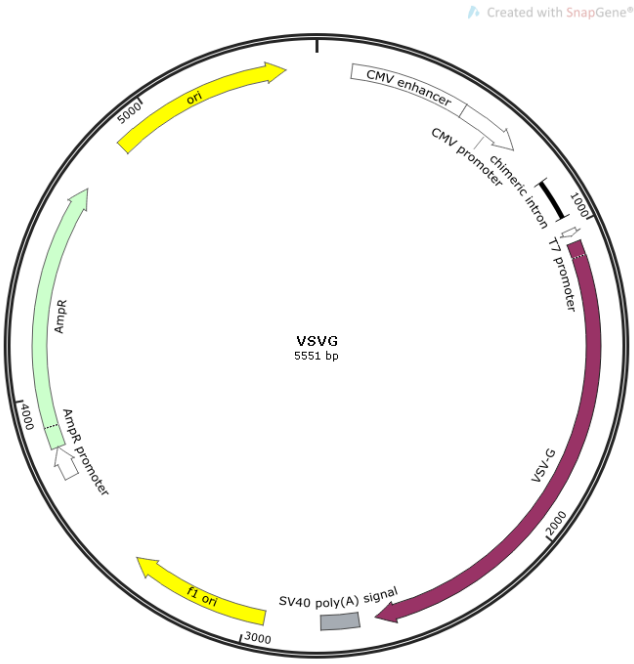


Figure 2.4. Schematic representation of the structure of the pVSVG (Vesicular Stomatitis Virus G glycoprotein) lentivirus packaging plasmid. This plasmid carries the VSVG glycoprotein (portrayed in plum color) under a CMV promoter. VSVG is important for the formation of the viral envelope because in can enclose plasmid DNA. They derive from an inactivated form of the HIV virus. The f1 ori is the bacteriophage origin of replication element; arrow indicates direction of (+) strand synthesis. It is used in every transfection in 293T cells that aims in the production of a virus along with psPax2 and the lentiviral plasmid of interest.

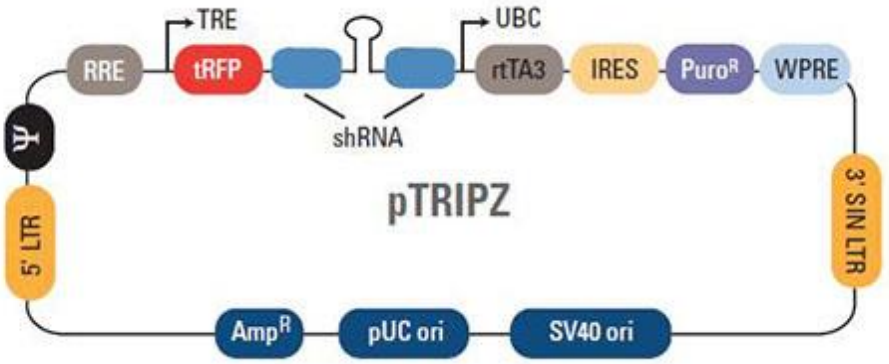


Figure 2.5. Schematic representation of the structure of the pTRIPZ plasmid. This plasmid is carrying the doxycycline inducible shRNA specific for *BIRC5* gene (blue features). It has a tetracycline inducible promoter (TRE) and a turbo RFP reporter for visual tracking of the transduction and shRNA expression. rtTA3 is the reverse tetracycline transactivator 3 for tetracycline dependent induction of the TRE promoter. There is an Internal Ribosomal Entry Site (IRES) element that allows the expression of rtTA3 and puromycine resistance mammalian selection gene in a single transcript. The other components pictured above are used for the proper packaging of the lentivirus. The full sequence of this construct is not available because it is under restrictions of patent protection.

2.3. Bacterial Transformations and strains

For the growing of plasmids we used competent E. Coli bacteria DH5A (Thermo-Fischer Scientific, Cat# 12034013) and for the cloning of the CRISPR/Cas9 K.O. clones we used the competent Stbl3 bacteria (Thermo-Fischer Scientific, Cat#C737303). The transformation of the bacteria was made through the heat shock technique. 0.5ul of plasmid was put in 50ul of competent bacteria and incubated on ice for 30 min. Then it was incubated for 45 sec at 42 ^{o} C and then incubated on ice for 10 min. Then 300ul of LB broth was added for recovery and the plasmids were left shaking at 200rpm at 37 ^{o} C for 30min. The bacteria were then plated in 100mm^2 LB agarose dishes that were precoated with antibiotic (specific for the antibiotic resistance gene that each plasmid carries) and were left overnight (O/N) at 37 ^{o} C under rotation in the bacteria incubator. The antibiotics used for culturing cells are ampicillin (Sigma, Cat#) and kanamycin (Sigma, Cat#) and they were both used in a final concentration of 100ug/ml.

2.4. Mini and Maxi preps

For growing and purifying a stock of bacteria, the procedure was continued from the last step of the instructions above. A random colony was picked by a sterilized tip from the agar plate. The colony was put in a small bacteria incubation tube, containing 5ml LB broth enriched with the antibiotic for which the enclosed plasmid is resistant to, and incubated it for 6h at 37 ^{o} C under rotation 250rpm. For proceeding with plasmid purification a large bacterial culture was made in which 500ul of the small one were pipeted in 500ml of LB broth with antibiotic. The large culture was incubated O/N at at 37 ^{o} C under rotation 250rpm. The next day the bacteria cells were pelleted by centrifugation in 3000xg for 30min. The bacteria pellet was used for plasmid purification utilizing an Endofree Plasmid Maxi Kit (Qiagen, Cat# 12362).

For growing a plasmid after cloning (as done with the CRISPR KO plasmids) all colonies were picked from the agar plates and grown in small liquid 5ml LB broth cultures O/N at 37 ^{o} C under rotation. The next day minipreps were prepared to isolate plasmid in a small scale by using Plasmid Mini Kit (Qiagen, Cat# 27104). The plasmids were then sent for Sanger sequencing

in the UPenn sequencing facility. The primers used for sequencing of the area around the sgRNA are listed in **Table 2.2**.

2.5. Lentivirus production

Lentivirus was produced in the packaging cell line HEK 293T, by co-transfecting the lentiviral vector and packaging plasmids. Targeting construct (shRNA carrier vector or expression vector) and packaging plasmids psPax2 and pVSVG were co-transfected into 293T cells by lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. Lentiviral supernatant was collected 48-72 hours post transfection, spinned at 1000xg at 4^{o} C for 5min and passed through a 0.45 uM filter (Millex-HV, Cat#SLHV033PS). Then the supernatant was used to infect target cells by being directly added to their culture medium. The target cells were incubated with the viral supernatant for 48h and then medium was refreshed and selection with antibiotics took place.

2.6. CRISPR/Cas9 GeCKO_V2 library description

The library used in this study was the GeCKO-V2 (Genome-scale CRISPR Knock-Out_Version 2) which was designed by the Zhang lab of the Broad Institute and was commercially available through Addgene (Cat#1000000048). The library is a one vector system, meaning that the gene coding for the Cas9 enzyme and the sgRNA are part of the same vector. The library was human species specific and was delivered in two half-libraries (A & B), each containing 3sgRNAs for each gene (total 6 sgRNAs for each gene). Each half-library contains 1000 internal control sgRNAs (non-targeting) and libA also targets miRNAs. For the screen both half libraries were mixed and used for transfection in order to achieve maximum representation of every sgRNA and every gene. The exact numbers describing the library's characteristics are listed in **Table 2.1**.

2.7. Library Production

The GeCKO library was diluted to 50 ng/uL in water and it was transformed into competent bacteria though electroporation. The electrocompetent cells used were Lucigen E. cloni 10G (Elite, Cat# 60052- 1). More specifically, 2 uL of 50 ng/uL GeCKO library were added to 25 uL of electrocompetent cells with an efficiency of ≥109 cfu/ug and were pulsed with 6

electroporations at 120mV. Cells recovered in 975 uL Lucigen provided SOC medium and transfered to a loosely capped tube with an additional 1 mL of SOC and rotated at 250 rpm for 1 hour at 37^{o} C. All transformations were pooled together to a final volume of 8 ml in SOC medium. Out of it 10 ul were diluted in 1ml of recovery medium and 20 ul of this were plated in a prewarmed ampicillin coated petri dish. This is a 40000-fold dilution of the full transformation and will enable the estimation of the transformation efficacy in order to ensure that full library representation is preserved. The rest of the pooled electroporated cells are plated in 36 prewarmed ampicillin plates by spreading 400 ul per dish. All plates grew inverted overnight at 37^{o} C and next day the transformation efficiency was calculated. This was done by counting the number of colonies on the control plate and multiplying this number by 40000 for the total number of colonies on all plates. If the number of colonies is less than 6.5 x 10^{7}, then the representation is insufficient and proceeding with the screen is not recommended since no equal representation of sgRNAs can be achieved. This efficiency is equivalent to 1,000 colonies per lentiCRISPR construct in the GeCKO library. Since representation was adequate, colonies were harvested by pipetting 500ul per 10 cm petri dish and scraping the colonies off with a cell spreader. The liquid plus scraped colonies were transferred into a tube and the procedure was repeated a second time with additional 5-10 ml in order to ensure all colonies was collected. The mix was spinned down at 3500xg for 30 min at 4^{o} C to pellet the bacteria and then discard the supernatant. The bacterial pellet was weighted and the weight of the tube was subtracted for future calculations. Bacteria were “Maxi-preped” for downstream virus production and future amplification as mentioned above. A sufficient number of maxi preps ware performed as to not overload a column. Lentiviruses were produced as described in the section above.

2.8. Cell Culture

A375 human malignant melanoma (CRL-1619), HT1080 human fibrosarcoma (CCL-121), RKO human colon carcinoma (CRL-2577), DLD-1 human colorectal adenocarcinoma (CCL-221), MCF7 human mammary gland epithelial adenocarcinoma, MCF10A normal immortalized were obtained from ATCC (Manassas, VA). SQ20B human head and neck carcinoma cells were kindly donated from Dr. Thomas Erwin lab (Weichselbaum et al., 1986). All cell lines were cultured in

Dulbecco's Modified Eagle's Medium (Gibco, Cat#11965084) supplemented with 10% fetal bovine serum (SFB, Cat#F6178), 100 U/mL penicillin & 100 µg/ml streptomycin sulfate (Gibco, Cat#15140122). These cell lines were cultured at 37°C in a humidified incubator at 5% CO_2. The cell lines used in these in vivo studies were validated by American Type Culture Collection. All of the cell lines were determined to be negative in a mycoplasma test and authenticated using STR fingerprinting provided by the UPenn Cell Center.

For the maintenance and experimental culturing the cells were grown in 10cm^2 dishes with 10ml DMEM. Once they became 80% confluent the cells were washed with 10ml PBS (Gibco, Cat#) and then were detached and separated to single cells by adding 1ml of Trypsin (Gibco, Cat#). Then they were counted on a Neubauer cell chamber and plated according to the needs of every experiment. All experiments aimed for Western Blot analysis were done in 10cm^2 dishes and the ones aimed for clonogenic survival assays in 60cm^2 dishes. For storing purposes the cells were washed, trypsined, pelleted at 1500rpm for 5min and then resuspended in freezing medium [5% DMSO (Sigma-Aldrich, Cat#472301) in FBS]. Then they were stored in a liquid nitrogen tank in freezing vials.

For treating the cells throughout this study the drug treatment times are: Sepantronium bromide aka YM155 (10nM for 24h), doxycycline (4ug/ul for 24 or 48h), thapsigargin and tunicamycin (500nM for various time points). The time points vary for the purpose of each experiment and are specified in the caption of each figure, in the results chapter.

2.9. Generation of CRISPR/Cas9 KO cell lines

Sequences targeting EIF2A3K and XBP1 were designed and cloned within the GeCKO-V2 lentiviral vector according to the protocol instructions from the manufacturer (Shalem et al., 2014). A375, SQ20B and HT1080 cell lines were transduced with the 20ug sgRNA specific GeCKO-V2 lentiviral vectors using Lipofectamine 2000 per manufacturer's instructions. Sequences targeting these genes were designed and were cloned in. Puromycin selection medium was added 48 h after transfection and maintained for 5 days. HT1080 cells were selected in 3 µg/ml puromycin and SQ20B in 5 ug/ml. The cell pool was expanded and clonally diluted. Genomic modifications of individual clones were confirmed by Sanger sequencing

(Upenn sequencing facility) and a PCR and WB screen was used to confirm the deletion of the gene. As a control, we used a GeCKO-V2 empty vector plasmid that did not undergo CRISPR–Cas9-mediated gene editing, but maintained the filler non-targeting sequence. Primers used for the introduction of the sgRNA into the GeCKO_V2 backbone are listed in supplementary Table 1.

2.10. Immunoblotting

Cells were harvested by scrapping them off the plates and pelleting them at 1000xg at 4^{o} C for 5 min. Whole cell lysates were prepared by resuspension of the pellet in RIPA buffer (Thermo Scientific/Pierce, Cat#89900) supplemented with 10 uM protease (Roche, Cat#11836153001) and 10uM phosphatase inhibitors (Sigma-Aldrich, Cat#p5726, Cat#p0044) and DTT (5uM), followed by 15 min incubation in -80^{o} C, sonication (6 pulses at 40% duty circle) and keeping the supernatant after a final centrifugation (10000xg, 15min, 4^{o} C). For obtaining cytoplasmic and nuclear fractions BioVision nuclear cytoplasmic fractionation kit (Cat#K266) was utilized. Protein concentrations were determined by DC protein assay (BioRad). Equal protein of each sample (80-100ug) was loaded and separated in SDS-PAGE (BioRad-Criterion precast gels) and transferred onto 0.45 micron polyvinylidene difluoride (PVDF) membranes. Membranes were blocked for avoiding non-specific binding with 5% milk in TBS-T (20 mM Tris-Base, 150 mM NaCl, 0.1% Tween-20). Proteins of interests were incubated overnight with primary antibody in blocking solution, washed with TBST 3 times and the next day incubated with secondary antibody for 2h, washed again 3 times with TBST and visualized with Pierce ECL Wester Blotting substrate (Cat#32106) according to the provided protocol.

2.11. Antibodies

The primary antibodies used here are as follows: rabbit polyclonal anti-Survivin (CST, Cat#71G4B7E), rabbit polyclonal anti-Cleaved PARP (CST, Cat#9541), rabbit polyclonal anti-PERK (CST, Cat#3192), rabbit polyclonal anti-XBP1-s (CST, Cat#12782) rabbit polyclonal anti-β-tubulin (CST, Cat#2146), rabbit anti-Cleaved Caspase3 (CST, Cat# 9661), rabbit anti-eIF2α (CST, Cat# 9722), mouse anti-β-actin (Sigma-Aldrich, Cat#A5441, AC-15), mouse anti-RNA POL II (Active Motif, Cat# 39097). Rabbit anti-P-PERK T982 was provided by Eli Lilly. The following secondary

antibodies were used: Horseradish peroxidase-conjugated goat anti-rabbit IgG (Thermo Scientific, Cat#31460) and goat anti-mouse (Thermo Scientific, Cat#31430). All membranes were incubated with primary antibodies in a 1:1000 ratio and with primary antibody in a 1:500 ratio. Both antibodies were diluted in blocking solution. Incubation times are as mentioned in above.

2.12. Clonogenic Survival Assay

The clonogenic assay was performed as previously described (Ye et al., 2010). Briefly, same number of cells (usually 500, 1000, 5000 and 10000 cells) from each treatment were suspended in DMEM complete medium, and plated in 60 mm^2 dishes (1000 cells/dish). Cells were incubated at 37 ^{o} C for 10 days without moving them or changing the medium so that colonies are formed. The colonies were stained with 0.4% crystal violet in 20% ethanol and quantified in ImageJ software.

2.13. RNA isolation, reverse transcription and qPCR

Total RNA was isolated using TRIzol (Life Technologies, Cat#15596018) according to manufacturer's instructions. Complementary DNA (cDNA) was synthesized using AMV-RT Reverse Transcriptase (Promega, Cat#M5101) in the presence of RNase inhibitor (Promega, N2111), with 2 µg of purified RNA. The qPCR reaction was set up using Power SYBR green PCR master mix (Applied Biosystems, Cat#4367659) and the amplification curves were monitored in a Quant Studio Real Time PCR machine (Thermo Fischer). For each reaction 500ng of RNA was used and 0.3 uM of each primer in a total volume of 20ul. All measurements were carried out in triplicates and 18S was used as internal standard control for standard curve calculation and quantification. QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems) was used for data analysis. Primers used for qPCR are listed in **Table 2.4.** The pPCR program used is listed in **Table 2.5**.

2.14. Flow Cytometry

Cells were trypsinized, count and diluted to a concentration of 5 x 10^6 per ml in PBS with 2% BSA. Cell apoptosis was assessed by staining with eBioscience™ Annexin V Apoptosis Detection

Kit FITC (Thermo Scientific, Cat#88-8005-74) according to manufacturer's instructions. Cells were subjected to to flow cytometry on a FACSCanto flow cytometer using BD FACSDiva software (BD Biosciences). Data were analyzed using FlowJo version X (Tree Star Inc).

2.15. Flow Microscopy

Cells were trypsinized, count and diluted to a concentration of $5x10^6$ per ml in PBS with 2% BSA. Then they were stained with Vybrant Cell cycle Violet (Thermo Scientific, Cat#V35003) for staining of the nucleus and with anti PDI SelectFX™ Alexa Fluor™ 488 Endoplasmic Reticulum Labeling Kit for fixed cells (Thermo Scientific, Cat#S34200) according to manufacturer's instructions. Cells were subjected to Imagestream analysis to an AMNIS imagestream XMark II. Images were acquired on a 2 camera, 12 channel ImageStreamX (Amnis Corporation, Seattle, WA) utilizing 405, 488658, and 785 nm lasers, 60X magnification, using Amnis' INSPIRE data acquisition software. Brightfield was collected in Channels 1 and 9 at an intensity of 800, SSC was collected in Channel 6 at a 785nm power of ~2 mW, Vyrant Violet was detected in Channel 2 (405 nm filter) at a 405 nm laser power of 10 mW, PDI was detected in Channel 3 (532nm filter). Data were analyzed using Amnis Imagestream analysis software.

2.16. Cell Staining

Cells aimed to be used for imaging techniques were cultured in 2-well chamber slides (Lab-Tek, Cat#154526). 50000 cells were plated in each chamber, and after the treatment with a small molecule inhibitor or with Doxycycline (used to knock down Survivin) the cells were washed with PBS and fixed in 4% PFA (paraformaldehyde) for 20 min at 37° C. The ER was visualized by using the SelectFX™ Alexa Fluor™ 488 Endoplasmic Reticulum Labeling Kit for fixed cells (Thermo Scientific, Cat#S34200) according to the manufacturer's protocol. The nucleus was stained last by treatment with Hoechst dye (Thermofischer, Cat#33342) for 15 min at RT in 1:2000 dilution. Slides were washed in PBS, and mounted in Vectashield medium (Vector Laboratories H-1000, Burlingame, CA). All microscopy images were obtained by a Leica X-CITE series 120 microscope and analyzed in ZEN blue version suite.

2.17. Statistics

GraphPad Prism 7 and Excel 2017 were used for statistical analysis. Error bars indicate mean ± S.D. or mean ± SEM (as indicated in Figure legends) and statistical significance was determined by unpaired, two tailed student's t-test. A p value less than 0.05 was considered statistically significant.

Table 2.1. Characteristics of GeCKO_V2 human specific library

Lib Characteristics	**GeCKO_V2 human library**
Number of genes targeted	19052
Targetign constructs per gene	6 per gene (3 in LibA, 3 in LibB)
Number of miRNA targeting	1864
Targeting constructs per miRNA	4 per miRNA
Control (non-targeting) sgRNAs	1000
Total sgRNA constructs	122417 (65386 in LibA, 58031 in LibB)
Viral plasmid vectors	Single & dual vector: lentiCRISPR_V2 & lentiGuide-Puro

Table 2.2. List of primers used for the sequencing of the GeCKO_V2 backbone to verify the introduction of a gene locus specific sgRNA

Gene	Primer	Sequence 5'→3'
hU6-F	One way	GAGGGCCTATTTCCCATGATT
LKO.1	One way	GACTATCATATGCTTACCGT

Table 2.3. List of primers used for generation of CRISPR/Cas9 K.O. cell lines

Gene	Primer	Sequence 5'→3'
EIF2AK3 (PERK)	Forward	CACCGCCATTTCGTCACTATCCCAT
	Reverse	AAACATGGGATAGTGACGAAATGGC

Table 2.4. List of qPCR primers

Gene	Primer	Sequence 5'→3'
h18sRNA	Forward	CAATTACAGGGCCTCGAAAG
	Reverse	AAACGGCTACCACATCCAAG
hBIRC5	Forward	GAGGAGACAGAATAGAGTGATAG
	Reverse	GAGCTGCTGCCTCCAAAGAA
hATF4	Forward	CCCTTCACCTTCTTACAACCT
	Reverse	TGCCCAGCTCTAAACTAAAGGA
hCHOP	Forward	GGAGGAGCCAGAACCAGCAG
	Reverse	TTCTCTTCAGCTAGCTGTGCCA
hASNS	Forward	GGAAGACAGCCCCGATTTACT
	Reverse	AGCACGAACTGTTGTAATGTCA
hATF3	Forward	CTCGGGGTGTCCATCACA
	Reverse	TCTTCTTGTTTCGGCACTTTG
hXBP1-t	Forward	CAAATGCCCTTCCCCAGAGCC
	Reverse	AATGGCTTCCAGCTTGGCTGAT
hXBP1-s	Forward	CCGCAGCAGGTGCAGG
	Reverse	GAGTCAATACCGCCAGAATCCA
hEDEM1	Forward	TCCTTAAAGGGGAAGCGAGCC
	Reverse	AGCGCTCGCCATTGCATGGT
hTRIB3	Forward	GGCCTTATATCCTTTTGGAACGA
	Reverse	CGCTGGCAGGGTACACCTT
hERN1	Forward	AGAGAAGCAGCAGACTTTGTC
	Reverse	GTTTTGGTGTCGTACATGGTGA
hHERPDu1	Forward	CGTTGGGTGGTTTCCATTTA
	Reverse	TGGTTGGGGTCTTCAGTTTC
hHGRP78	Forward	CCAAGAGAGGGTTCTTGAATCTCG
	Reverse	ATGGGCCAGCCTGGATATACAACA
hP58IPK	Forward	GAGGTTTGTGTTGGGATGCAG
	Reverse	GCTCTTCAGCTGACTCAATCAG
hEro1L	Forward	CCATTAGTGCTGCCAACCAGTA
	Reverse	ATCTGCATCAGCATCACGGTC
heIF6	Forward	CCGACCAGGTGCTAGTAGGAA
	Reverse	CAGAAGGCACACCAGTGATTC
hERdj4	Forward	TGGTGGTTCCAGTAGACAAAGG
	Reverse	CTTCGTTGAGTGACAGTCCTGC
hATF5	Forward	ACCTTCTTTCTTCAGCCGA
	Reverse	GAGTTTCCCATAGTCTACGA
hATF6A	Forward	GAGTCATCGCGTCTCTCCAC

	Reverse	GGCCTCAGAGTTGACGGAAG

Table 2.5. Amplification protocol used for qPCR analysis

Stage	Steps	Temperatures	Speed/Time
Hold Stage	Step 1	25 ° C→95 ° C	1.9 ° C/sec
		95 ° C	25 sec
PCR stage (40 cycles)	Step 1		1.9 ° C/sec
		95 ° C	1 sec
	Step 2		1.6 ° C/sec
		60 ° C	20 sec
Melt Curve Stage	Step 1		1.9 ° C/sec
		95 ° C	15 sec
	Step 2		1.6 ° C/sec
		60 ° C	1 min
	Step 3		0.05 ° C/sec
		95 ° C	15 sec

PART 3

RESULTS

3. RESULTS

3.1. Performing large-scale CRISPR KO screening in vitro

To perform a CRISPR-based genome wide screen we used the GeCKO_V2 library (Shalem et al., 2014). In this system, the gene encoding for the Cas9 enzyme and the sgRNA are part of a single viral vector system. The cancer cell models we used were the human A375 melanoma and the SQ20B squamous head and neck carcinoma cell lines. For our studies we used thapsigargin (TG) and tunicamycin (TN), both of which are widely used ER stress agents. Thapsigargin is a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and tunicamycin is an inhibitor on N-glycosylation. We performed 3 screens: the first was done in SQ20B cells with TG as the ER stressor, the second one in A375 with TG and the third one in A375 but with TN. Before performing the screens, we tested the efficacy of the GeCKO_V2 system, by depleting the EGFP gene expression by CRISPR/Cas9 targeting in SQ20B cells which already overexpress constitutively GFP. The results showed full depletion of the GFP signal (**Figure 3.1**).

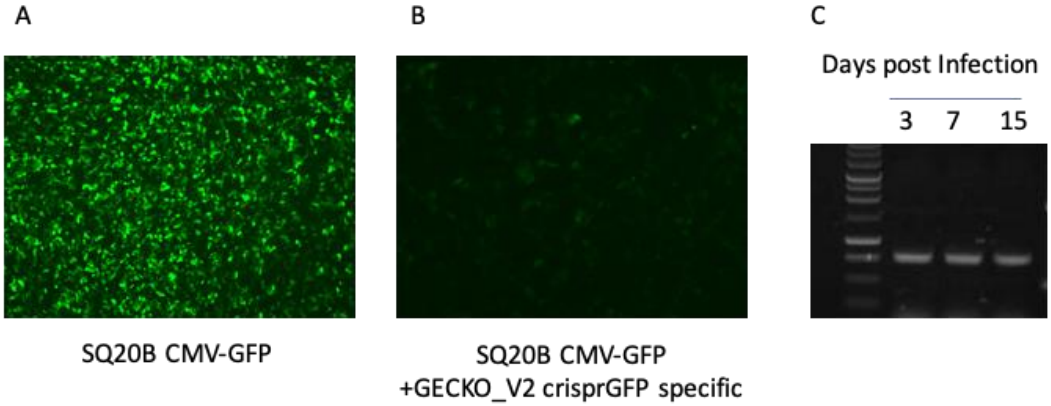


Figure 3.1. Efficacy of the GeCKO_V2 system. Testing the efficacy of the GeCKO_V2 system, by depleting the EGFP gene expression by CRISPR/Cas9 targeting in SQ20B cells. GeCKO_V2 CRISPR control experiment. SQ20B cells were transfected with a CMV-GFP lentiviral vector. After stably expressing GFP (**A**), the cells were transfected with the GeCKO_V2 plasmid which was expressing sgRNAs targeting specifically GFP. GFP expression was ablated in almost all cells (**B**). PCR showing the expression of Cas9 enzyme days post infection (**C**)

To maintain adequate sgRNA coverage for each expressed gene, we infected 16 x 10^7 cells at ~0.3 multiplicity of infection (MOI). More specifically, the cells were distributed in 6 well plates each containing 2 x 10^6 cells and 5ul of the library. The transfection was performed by spinfection at 2000rpm for 2 h. After 24h we started selection with 0.75 ug/ml puromycin and after a week the surviving cells were randomly divided into 9 batches, each containing 3 x 10^7 cells. Of these, one sample was harvested as day 0 (untreated) and others were maintained in culture and split in two groups. One group was treated with 500nM of TG or TN for 24 h and the other with the same concentration of DMSO as a vehicle control. Next day almost 50% of the population in the experimental group was dead so we allowed the cells to grow for 72 h in fresh medium. Then we repeated the stress application with 500nM of TG or TN for 24 h in order to increase the resistant to ER stress population. After 72 h of growing all viable resistant remaining cells were collected and their DNA was isolated and amplified by PCR. A second PCR step enabled the addition of sample-specific sequence barcodes and Illumina technology based adaptors in order to facilitate the next step, which was NGS analysis in an HiSEQ2500 sequencer (**Figure 3.2**).

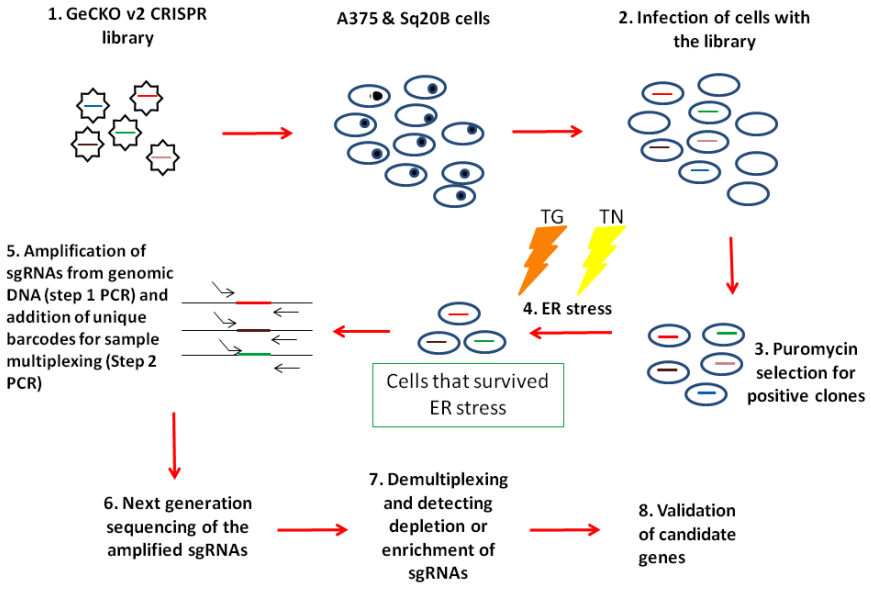
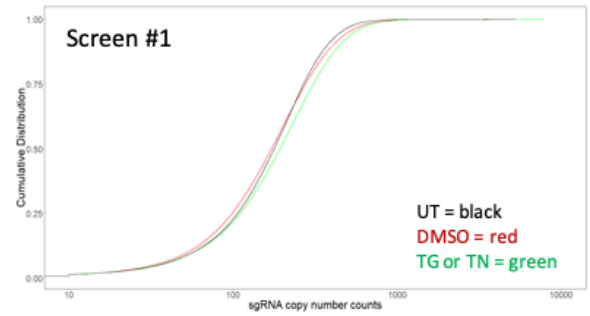
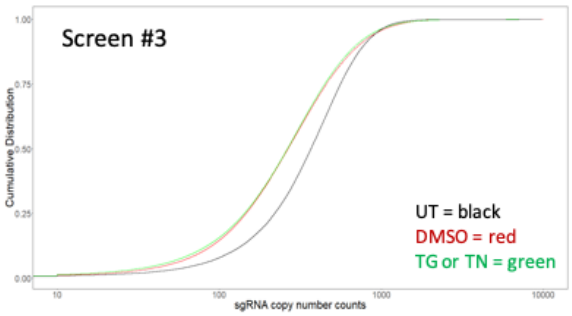


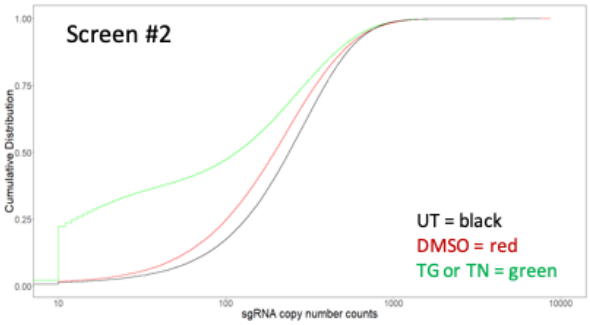
Figure 3.2. Schematic experimental layout describing the basic steps of the screens. Cells were transfected with the GeCKO_V2 lentiviral library and the positive clones were selected with puromycin and expanded. ER stress was applied by Thapsigargin (TG) in SQ20B cells and by Tunicamycin (TN) in the A375 cells and the surviving population went through a second round of ER stress exposure in order to increase the resistant population. After that DNA from the cells was collected and amplified to be analyzed through Next Generation Sequencing (NGS). Bioinformatics-based analysis of the NGS resulted in a ranking of genes based on the overall representation of the sgRNAs.

The relative abundance of each sgRNA was assessed by targeted amplification and deep sequencing and data analysis was performed by using previously established analytic tools (Li et al., 2014). The sgRNA distribution analyses showed that the majority of the sgRNAs were detectable both in control and in experimental cells (**Figure 3.3**).

Our experimental model is based to sgRNA abundance at the end of the screen analysis. If an sgRNA is overrepresented in the experimental (TG/TN treated) population in comparison to the control, then the gene it corresponds to, must have a proapoptotic role in response to ER stress, because the cells that harbored this sgRNA survived the stress. Vice versa, if a sgRNA is underrepresented in the experimental group in comparison to the control, then the gene it corresponds to must have a prosurvival role because the cells that incorporated the sgRNA (and inhibited expression of the corresponding gene) did not survive. At the same time, the library is designed to target each gene six times. So the number that represents how many sgRNAs out of six were detected for every gene is also an important parameter for the scoring system of the genes.



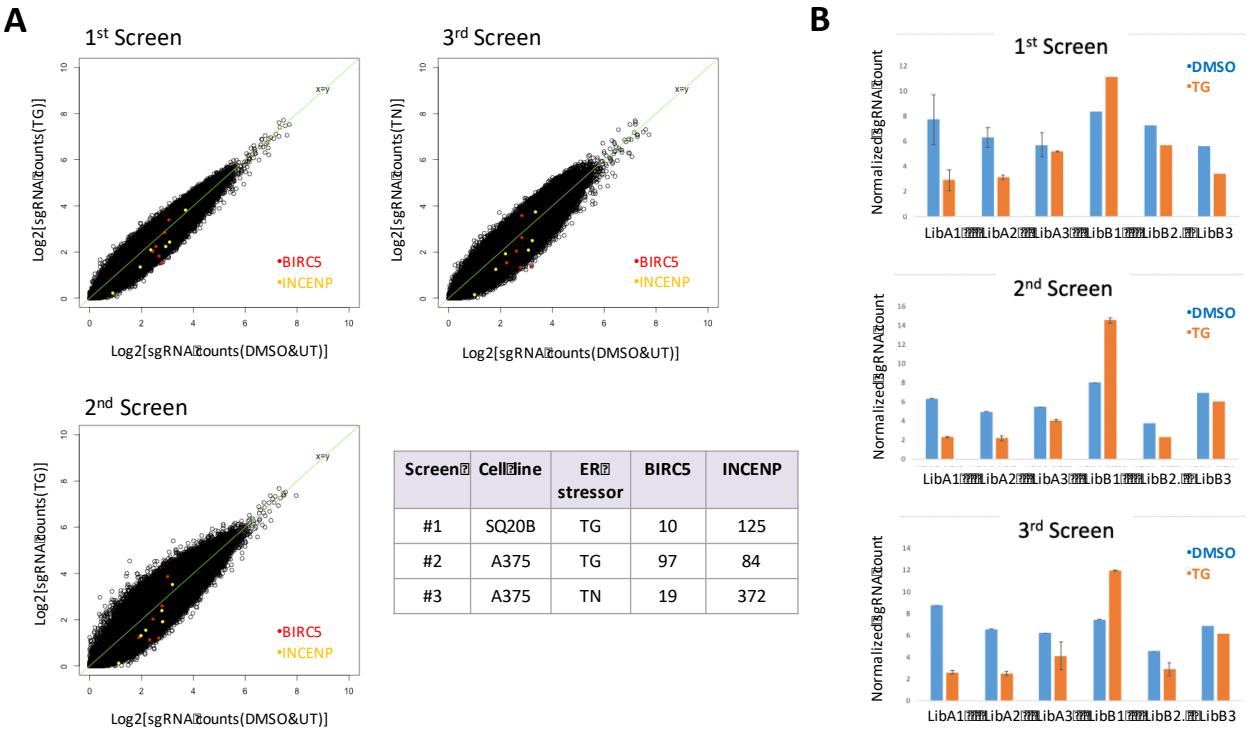




Screen	Cell line	ER stressor	UT	DMSO	TG	TN
#1	SQ20B	TG	99.23%	99.24%	99.22%	N/A
#2	A375	TG	99.22%	99.25%	97.39%	N/A
#3	A375	TN	99.25%	99.28%	N/A	99.21%

Figure 3.3. **Distribution plots of sgRNA representation across all three screens**. Table indicates the assigned numbers for each screen and the percentage of the sgRNAs detected in each treatment. UT stands for Untreated cells.

For this reason, all the genes that were screened in this library were ranked according to both their deep sequencing reads count but also the number of the sgRNAs for every gene. One of the most interesting targets in the negative selection scale was BIRC5 which encodes for the protein Survivin. BIRC5 was ranked 10th in the first screen, 97th in the second and 19th in the third screen (**Figure 3.4A**), and five out of six sgRNAs that target it were underrepresented in comparison to the controls in all three screens (**Figure 3.4B**). As mentioned in the introduction, Survivin was identified as an inhibitor of apoptosis but it is more important for its role as a mitotic spindle regulator as part of the CPC complex. Another member of the CPC complex, INCENP was also highly ranked (this will be discussed later). Survivin is an interesting protein in respect to each wide spectrum of roles in the cell but also because it is highly expressed only in cancer cells and undetectable in healthy tissues, a trait that makes it an attractive pharmacological target. However, Survivin has never been implicated with ER stress or responsive to it mechanisms like the UPR. For that reason, we seeked to identify this connection and its importance to deciding cell fate under ER stressful conditions.



Screen	Cell line	ER stressor	BIRC5	INCENP
#1	SQ20B	TG	10	125
#2	A375	TG	97	84
#3	A375	TN	19	372

Figure 3.4. Gene ranking following CRISPR/Cas9 based screening. (**A**) Scatter plot illustrating the comparison of sgRNA read abundance between treatment (TG or TN) vs control (untreated and DMSO). Within the graph data points of sgRNA for BIRC5 are colored in red and for INCENP are colored in yellow. The table lists the negative ranking positions of BIRC5 and INCENP in all

three screens. (B)sgRNA against BIRC5 representation in all three screens. Five out of six sgRNAs were underrepresented as compared to the DMSO control.

3.2. Genetic Knock Down of Survivin causes sensitization to ER stress

Survivin was negatively ranked in all our screens, something that indicated a possible prosurvival role in respect to ER stress. To study the role of Survivin in ER stress we knocked down its expression using a doxycycline inducible shRNA (i-shBIRC5). A375 and SQ20B cells underwent ER stress induced by TG or TN in the presence and absence of Survivin. Both cell lines were much more sensitive to ER stressed when Survivin was knocked down as shown by the cleavage of PAPR, a well-established apoptosis marker by western Blotting (**Figure 3.5 A, B**). Additional data from Annexin V staining and clonogenic survival assay supported these results (**Figure 3.5 C, D**). Interestingly, treatment with TG or TN itself caused decrease in the protein levels of Survivin, suggesting that its expression is negatively regulated by ER stress, and possibly eIF2a phosphorylation. The precise mechanism for this remains to be examined. To verify that this sensitization effect that we observed was not due to off-target effects of the shRNA, we performed a rescue experiment by overexpressing the mouse homologue of Survivin in our cells. As we hypothesized, the sensitizing effect of TG or TN upon Survivin ablation was reversed once mSurvivin was expressed as seen by Cl-PARP levels and also the respective clonogenic survival assay (**Figure 3.5 E, F**). We then questioned whether this sensitizing effect is specific to ER stress or whether it applies also to other types of stresses. To test that, we applied ionizing (IR) and ultraviolet (UV) radiation to A375 i-shBIRC5 cells in the presence or absence of Survivin and we saw no difference in apoptosis (**Figure 3.6 A, B**) or overall survival (**Figure 3.6 C, D**) between the two groups, indicating that the cytoprotective effect of Survivin that we observe, is specific to ER stress and not to other stresses.

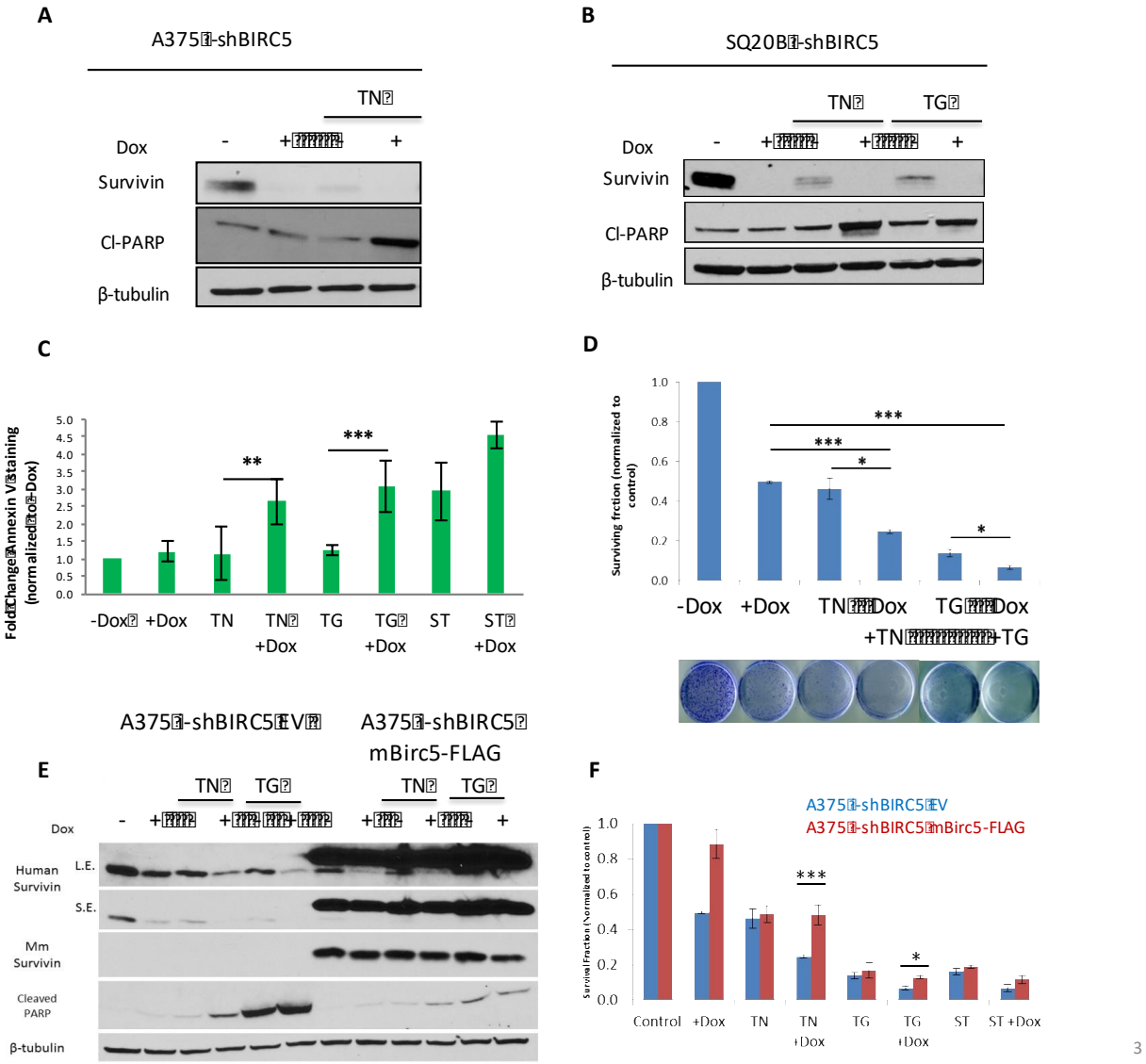


Figure 3.5. Genetic knockdown of *BIRC5* (Survivin) increases sensitivity to ER stress. (**A**) A375 and (**B**) SQ20B cells were stably transfected with a doxycyclin inducible shRNA against Survivin (i-si*BIRC5*). Pretreatment with 2 ug/ml Doxycyclin (Dox) was followed by Thapsigargin (TG) and Tunicamycin (TN) for 24 h. Knockdown of *BIRC5* and levels of apoptosis marker Cleaved PARP (Cl-PARP) were verified by western blot analysis. (**C**) Cell death was measured by Annexin V staining and (D) cell survival was measured by clonogenic assay. Data are presented as mean ± SEM, **p<0.01. (**E**) Overexpression of mSurvivin prevents sensitization to ER stress caused by knock down of endogenous Survivin. A375 i-sh*BIRC5* cells were transfected with a vector expressing the mouse homologue of Survivin (mBirc5). Cells were pretreated with 2 ug/ml Dox for 48 h and then with TG and TN for 24 h. Western blot analysis was performed for measuring apoptosis markers (**F**). Cell survival was measured by and clonogenic assay. Data are presented as mean ± SEM, **p<0.01;.

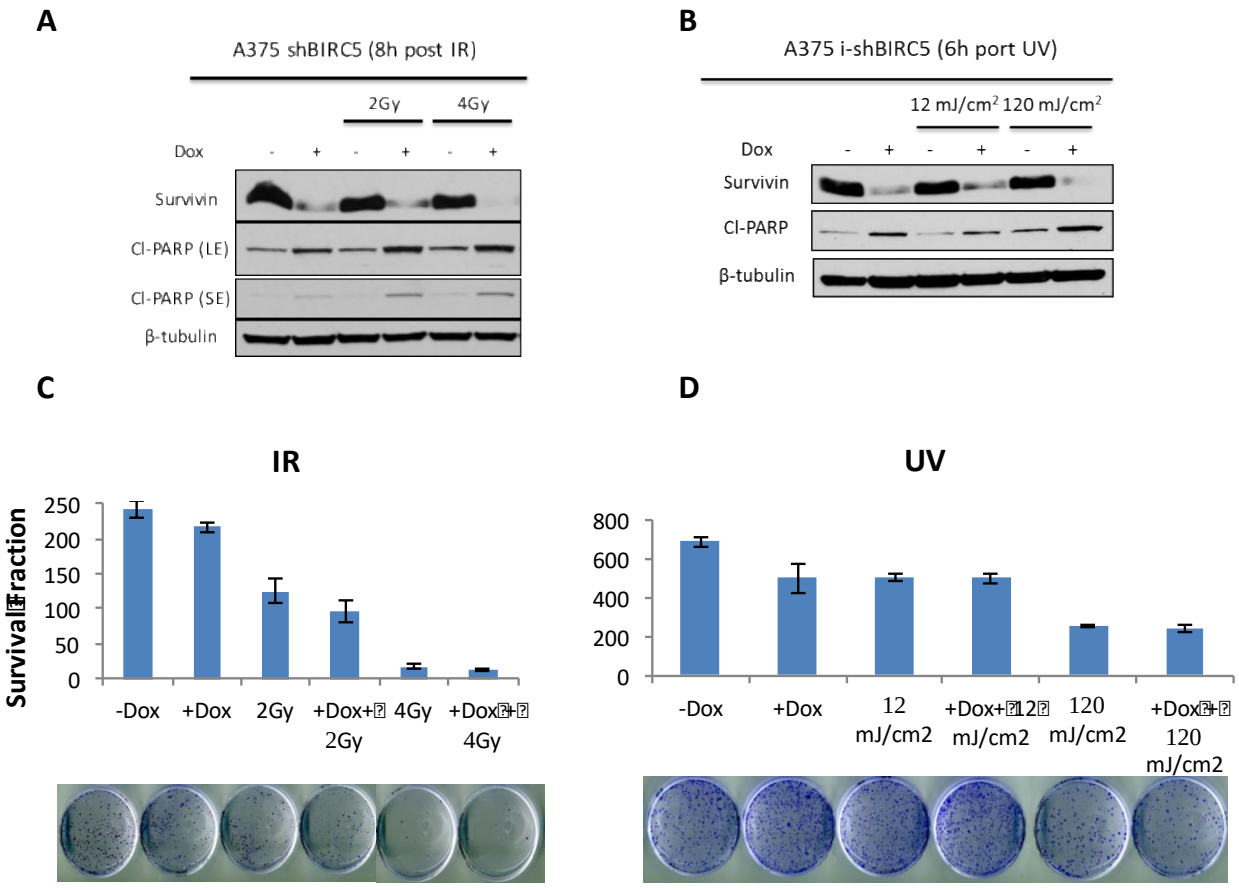


Figure 3.6. Genetic knockdown of BIRC5 (Survivin) does not increase sensitivity to IR or UV irradiation. A375cells were stably transfected with a doxycyclin inducible shRNA against Survivin (i-siBIRC5). Pretreatment with 2 ug/ml Dox was followed by treatment with IR (**A, C**) and UV (**B, D**) irradiation. Knockdown of BIRC5 and levels of apoptosis marker Cleaved PARP (Cl-PARP) were verified by western blot analysis (**A, B**). Cell survival was measured by clonogenic assay (**C**) and Annexin V staining (**D**). Data are presented as mean ± SEM, **p<0.01.

3.3. Chemical inhibition of Survivin causes sensitization to ER stress

Next we examined if this sensitization to ER stress is happening when we inhibit Survivin expression with the small molecule sepantronium bromide (aka YM155). YM155 has been used in clinical trials for non-small cell lung carcinoma and also for solid tumors. Our results showed that YM155 produced similar results as genetic inhibition of Survivin following ER stress. in both

SQ20B and A375 cells (**Figure 3.7 A, B**). These data were corroborated by the respective clonogenic survival assays (**Figure 3.7 C, D**).

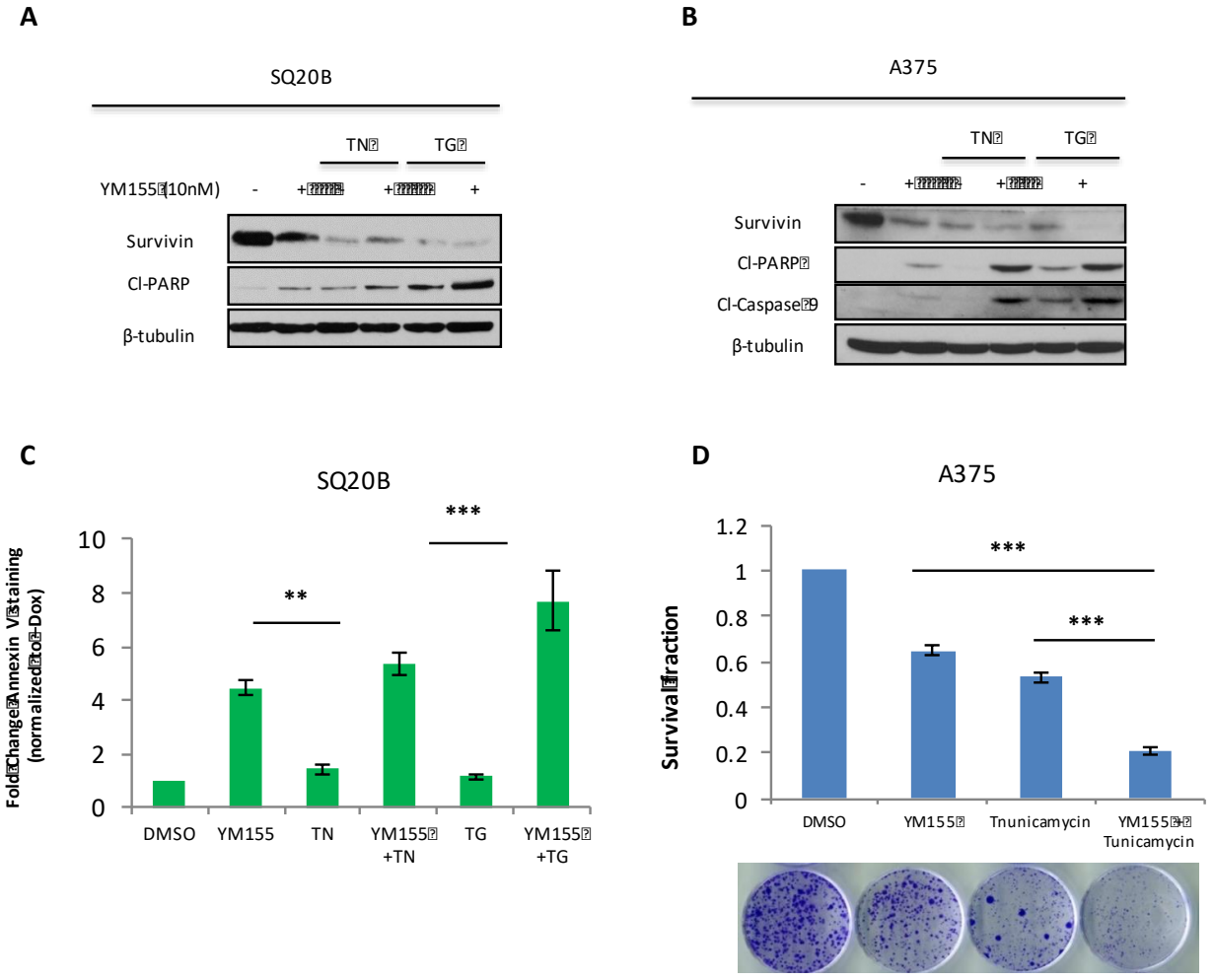


Figure 3.7. Chemical inhibition of Survivin causes sensitization to ER stress. (A) SQ20B and (**B**) cells were pretreated with 10 nm of YM155 (Survivin Inhibitor) for 24 h before 0.5 µM Tg was added to the cells for 24 h. Cells were treated with Tg YM155 as controls. Western blot analysis was performed for measuring apoptosis markers. (**C**) Apoptosis was measured by Annexin staining. Samples were normalized to DMSO treated cells. (**D**) Cell survival was measured by clonogenic assay. Data are presented as mean ± SEM, **p<0.01.

3.4. Ablation of Survivin results in distinct morphological changes that include multinucleation and ER expansion due to failure of cytokinesis

During Survivin ablation experiments a distinct phenotype was observed when studying the cells under light microscopy. The cells lacking Survivin appeared larger in size, stressed and an expanded ER phenotype (**Figure 3.8**).

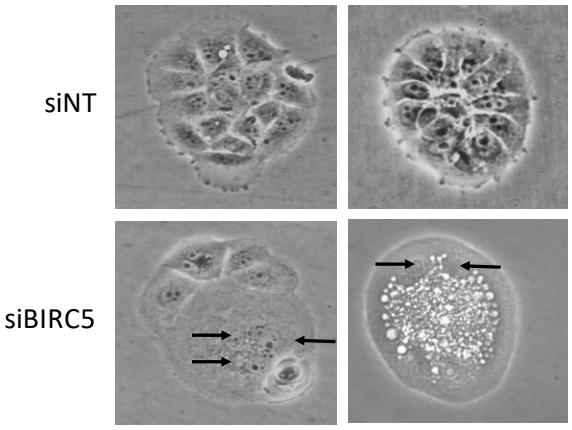


Figure 3.8. Knockdown of Survivin expression causes multinucleation and ER stress. SQ20B cells were transfected with siRNA against Survivin. The cells showed distinct phenotypic changes including ER expansion and multinucleation. 48h after transfection pictures were taken at an upright light microscope. Arrows are pointing at the nuclei. Brightfield. 40x magnification, scale bar 10 uM

Immunofluorescence and confocal laser microscopy demonstrated that either chemical inhibition or genetic knockdown of Survivin caused ER expansion as depicted by anti-PDI staining (an ER marker). Additionally, many cells were multinucleated and some of them harbored micronuclei around the main nucleus, as revealed by Hoechst staining (**Figure 3.9 A, B**). To further support these data, we performed single cell flow microscopy with an Imagestream analyser in SQ20B cells after inhibiting Survivin expression with YM155. The results demonstrated that the cells that lack Survivin expression become larger in size, have a distended ER and exhibit higher DNA content (**Figure 3.9 C**). More specifically, the quantitation of the cell and ER area showed that the increased area of the experimental cells is mainly occupied by the extended ER (**Figure 3.9 D, E**). Moreover, the cells that have the bigger size (hence bigger ER) are the ones displaying the higher DNA content as depicted by the yellow population in the scatter plots (**Figure 3.9 D**). Fluorescence microscopy results were repeated and validated in seven cell lines in total including HT1080 human fibrosarcoma, A375 human melanoma, HeLa human cervical carcinoma, RKO human colon adenocarcinoma and MCF7 breast adenocarcinoma, using both by chemical inhibition of Survivin (**Figure 3.10 B**) and i-shBIRC5 in the HT1080 cells (**Figure 3.10 A, B**). Collectively, the data indicate that following

Survivin ablation, cells experience failure of cytokinesis, since there are cells that are large and harbor more than one nucleus.

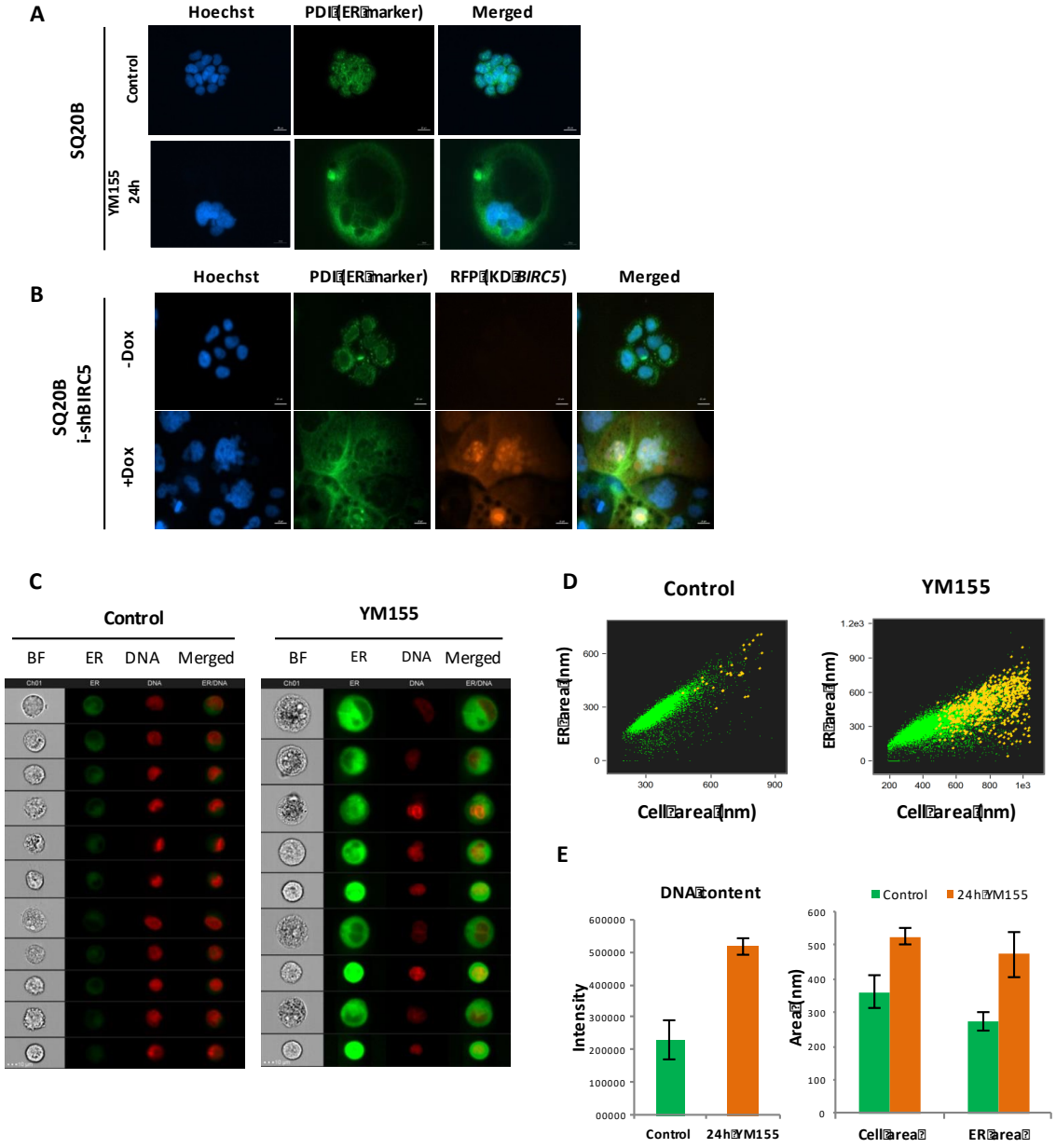


Figure 3.9. Ablation of Survivin results in ER expansion and multinucleation. (**A**) SQ20B cells were treated with 10 nM YM155 for 24 h and (B) SQ20B i-shBIRC5 cells were treated with 2 ug/ml Dox for 48 h. Both groups were visualized by staining with Hoechst (nuclei) and PDI (ER marker). The i-shBIRC5 cells are tagged with a Doxycycline inducible RFP marker. 40x magnification, scale bar 20 uM. (**C**) Chemical inhibition of Survivin in HT1080 cells results in ER expansion and DNA content increase. Single cell Imagestream analysis of cells treated with YM155 for 24 h. After treatment cells were stained with Vibrant Violet (DNA) and PDI (ER marker) and visualized by flow microscopy. (**D, E**) 40x magnification, scale bar 10 uM . Cell and

ER area were measured before and after treatment. Cells that had increased DNA content are represented in yellow (**B**). Data are presented as mean ± SEM, **p<0.01.

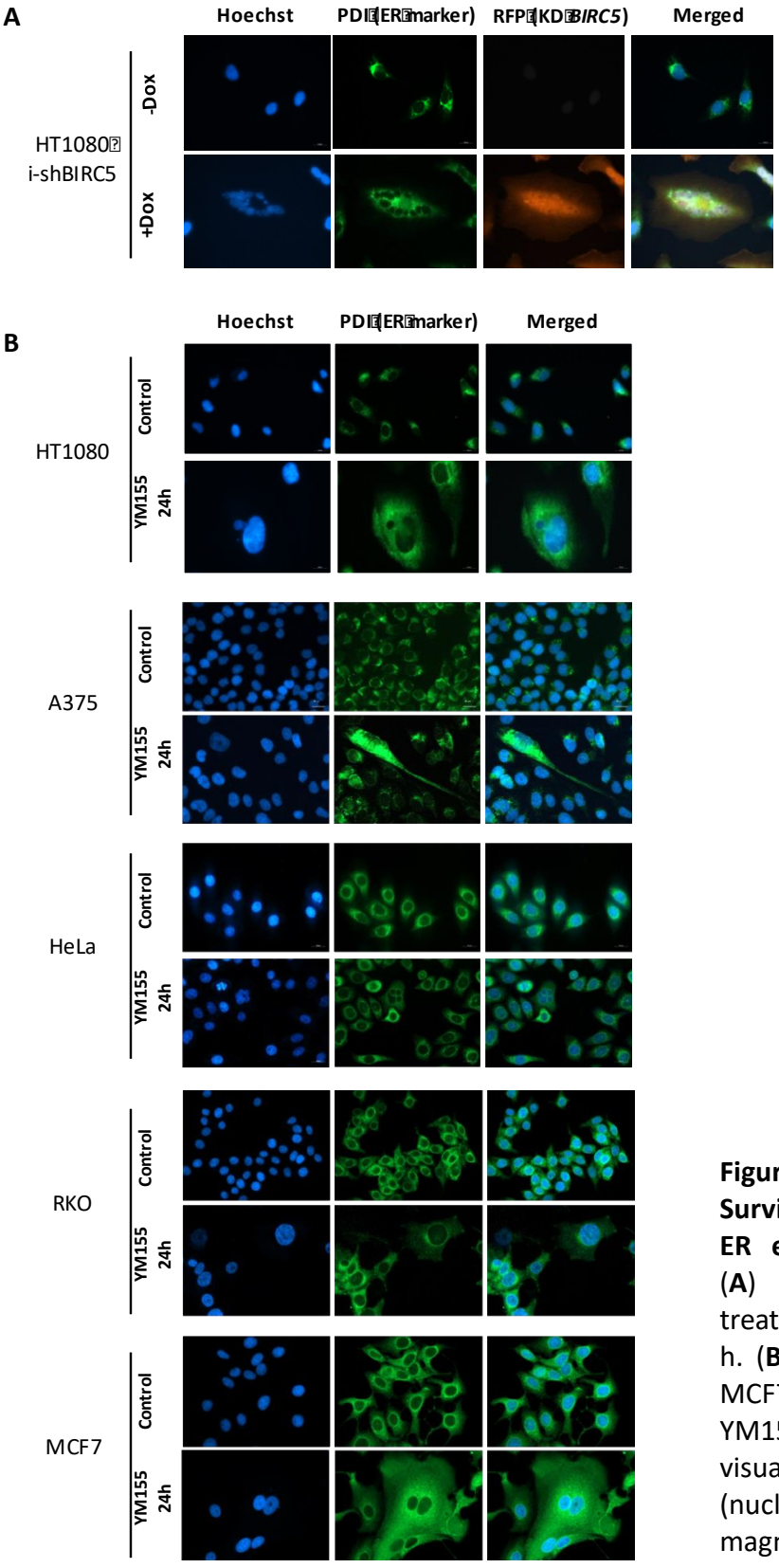


Figure 3.10. Ablation inhibition of Survivin in various cell lines results in ER expansion and multinucleation. (**A**) HT1080 i-shBIRC5 cells were treated with 2 ug/ml Doxycyclin for 48 h. (**B**) HT1080, A375, HeLa, RKO and MCF7 cells were treated with 10nM YM155 for 24 h. Both groups were visualized by staining with Hoechst (nuclei) and PDI (ER marker). 40x magnification, scale bar 20 uM.

3.5. Sensitization to ER stress, ER expansion and multinucleation by Survivin ablation are cancer cell-specific

As mentioned before, Survivin expression is high in cancer cells, while its expression in normal cells and tissues is usually too low to be detected by immunoblotting. To examine weather our observations so far are cancer specific and can be used for therapeutic targeting without affecting normal cells, we tested if normal immortalized cells respond the same way upon Survivin ablation. Indeed, genetic knockdown of Survivin in normal immortalized MCF10A breast cells did not show any sensitization to ER stress (**Figure 3.11 A**). However, when the phenotype of these cells was observed when Survivin expression was inhibited both by chemical and genetic means, the cells displayed the multi-nuclear phenotype (**Figure 3.11 B, C**). This suggests that although Survivin is required to protect against multi-nucleation (potentially due to its activity as a mitotic spindle protein), its absence results in sensitivity to ER stress only in fully transformed cells. Although the precise mechanism is not clear at this point, this may be due to the fact that that normal or immortalized cells, maintain slower rates of proliferation and DNA replication compared to cancer cells that have a shorter cell cycle. Hence, failure of cytokinesis induced by Survivin ablation can be observed due to slow division.

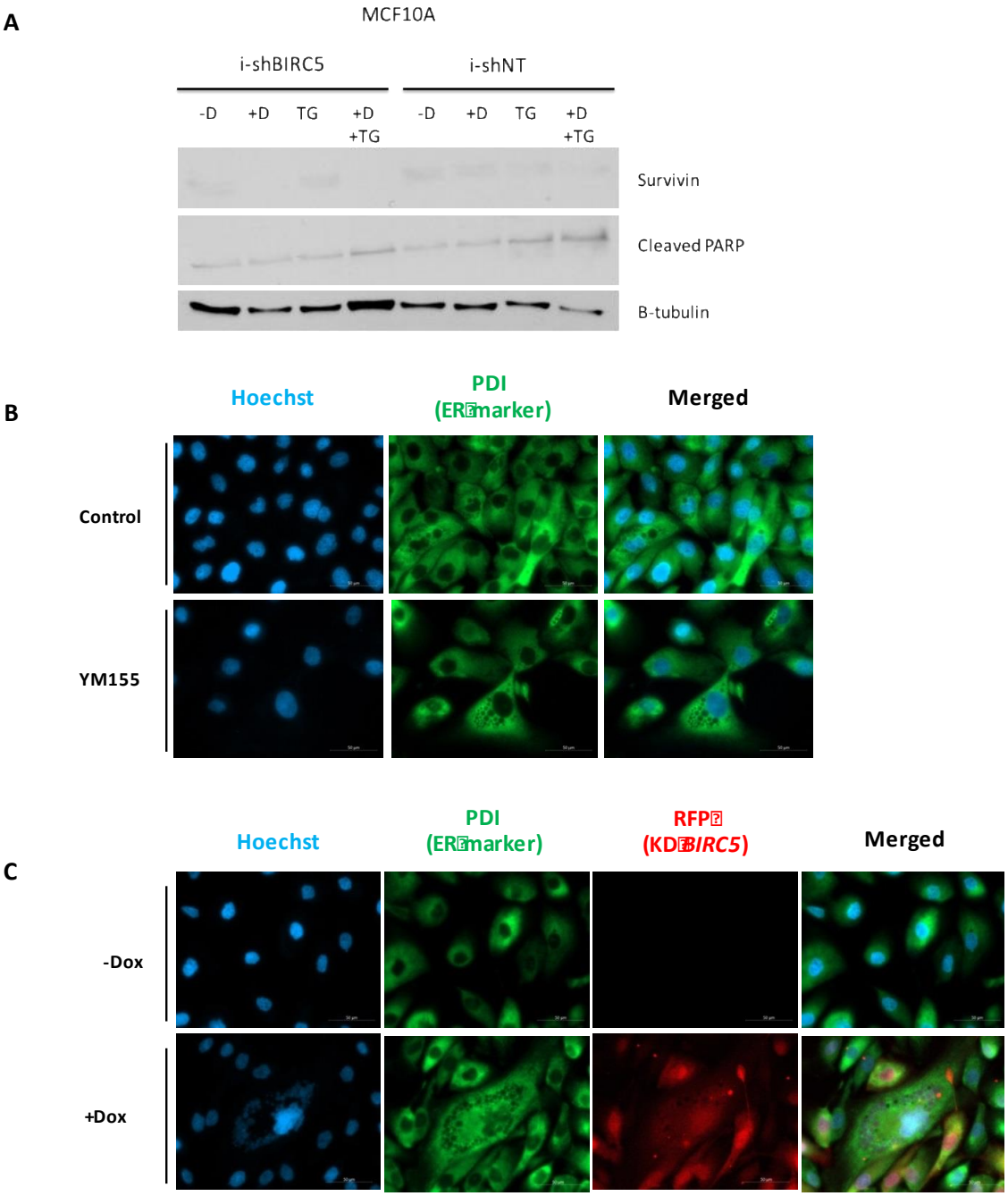


Figure 3.11. Ablation of Survivin in normal immortalized cell lines causes phenotypic alterations but no sensitization to ER stress. (**A**) MCF10A cells were stably transfected with a doxycyclin inducible shRNA against Survivin (i-si*BIRC5*). Pretreatment with 2 ug/ml Dox was followed by Thapsigargin (TG) and Tunicamycin (TN) for 24 h. Knockdown of *BIRC5* and levels of apoptosis marker Cleaved PARP (Cl-PARP) were verified by western blot analysis. (**B**) MCF10A and (**C**) MCF10A i-shBIRC5 cells were treated with 10nM Ym155 inhibitors for 24 h and 2 ug/ml Dox respectively. Both groups were visualized by staining with Hoechst (nuclei) and PDI (ER marker). 40x magnification, scale bar 20 uM.

3.6. Survivin ablation induces ER stress and UPR activation

Next, we wanted to validate that the phenotype we observed represented ER stress, and to validate that we firstly assessed the transcriptional activation of UPR specific genes. After 24h of Survivin inhibition, the levels of the transcriptional factors ATF4, CHOP and ATF3 (all downstream targets of the PERK arm of the UPR) were upregulated along with several other genes that relieve ER stress (**Figure 3.12 A**). Moreover, the protein levels of the respective proteins were also higher in the absence of Survivin (**Figure 3.12 B**). To further support these data, we utilized two reporter constructs. The first one is an ATF4-UTR-GFP reporter, in which GFP expression is activated when ATF4 mRNA is translated, and the other one is a CHOP-mCherry promoter reporter in which mCherry is under the transcriptional regulation of the CHOP promoter. SQ20B cells were transfected with the first construct and silencing of Survivin expression by siRNA led to GFP expression indicating that ATF4 is expressed when Survivin expression is inhibited (**Figure 3.12 C**). Additionally, A375 i-shBIRC5 cells were transfected with the ATF4-UTR GFP contract and A375 cells with the CHOP-mCherry one. Upon ablation of Survivin expression by Doxycycline administration or YM155 inhibitor, the cells increased expression of the GFP and mCherry fluorescent proteins respectively (**Figure 3.13 A, B**). These results further validate the notion that Survivin ablation causes ER stress that leads to subsequent UPR activation.

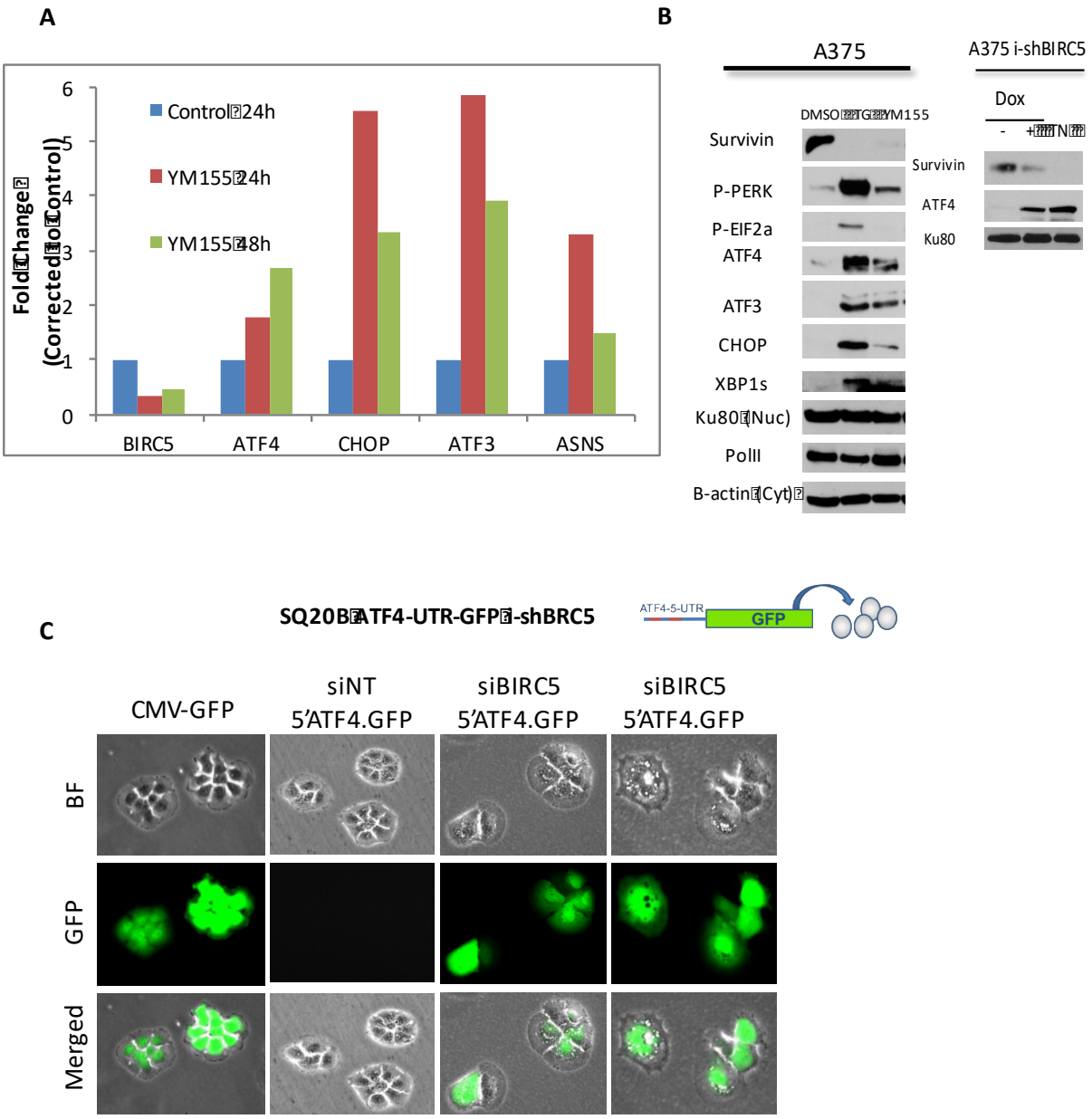


Figure 3.12. Ablation of Survivin induces UPR. (**A**) A375 cells were treated with 10nM or YM155 inhibitor for 24h and 48h respectively. mRNA expression of UPR target genes was detected. Normalized to untreated. (**B**) A375 cells and A375 i-shBIRC5 cells were treated with 10 nM YM155 for 24h and 2 ug/ml Dox for 48h respectively and expression of UPR pathway components was detected by WB. (**C**) SQ20B cells were stably transfected with the reporter 5'ATF4-UTR-GFP (CMV-5'UTR ATF4). Then they were treated with siNT or si*BIRC5* respectively. GFP fluorescence was measured by microscopy. CMV-GFP was used as an experimental control. Significant increase in GFP levels were detected in si*BIRC5* transfected cells as compared to siNT transfected SQ20B cells suggesting increase in ER stress following loss of Survivin.

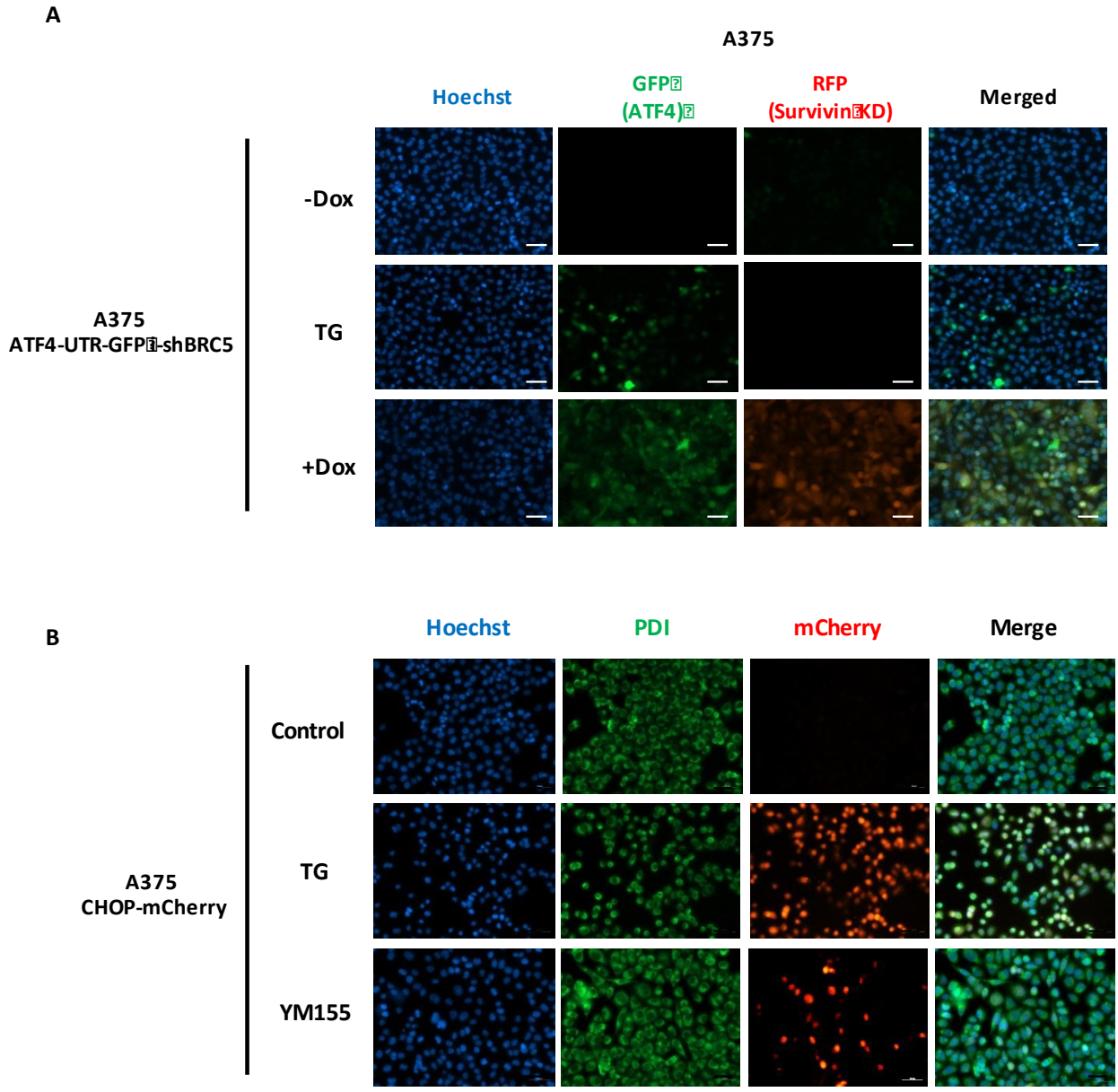


Figure 3.13. Ablation of Survivin induces UPR. (**A**) A375 i-shBIRC5 cells were stably transfected with the reporter 5'ATF4-UTR-GFP (CMV-5'UTR ATF4). Then they were treated with 2 ug/ml Dox. GFP fluorescence was measured by microscopy. Thapsigargin 500 nM for 12 h was used as an experimental control. Significant increase in GFP levels were detected in Dox treated cells as compared to untreated suggesting increase in ER stress following loss of Survivin. (**B**) A375 cells were transfected with promoter reporter CHOP-mCherry and treated with 10nM of YM155 for 24 h (**C**). Significant increase in mCherry levels were detected in YM155 treated cells as compared to untreated suggesting increase in ER stress following lchemical inhibition of Survivin.

3.7. PERK assumes a prosurvival role in the ER stress created by Survivin ablation

We next set out to understand which the role of UPR upon ablation of Survivin is. We chose to focus on the most prominent and well-studied out of the three pathways, the PERK arm of the UPR. To answer that question, we created an SQ20B PERK knockout cell line by utilizing CRISPR/Cas9 technology. We selected two clones (#12 and #34) that completely lacked expression of PERK and were sensitive to ER stress induced by TG, and also a control empty vector cell line (EV) (**Figure 3.14**). Using these two PERK KO cell lines, Survivin expression was inhibited by siRNA in both PERK KO and EV control cells. The results showed that both PERK KO clones were more sensitive to TG treatments compared to the EV ones, indicating that PERK undertakes a prosurvival role in response to ER stress elicited by lack of Survivin as shown by the levels of Cl-PARP and by clonogenic survival assay (**Figure 3.14 A, B**). To further validate this observation we silenced Survivin expression in SQ20B cells by siRNA and inhibited PERK phosphorylation 36h after siBIRC5 transfection. The agent we used for inhibiting PERK phosphorylation is the LY4 small molecule inhibitor that binds to the ATP pocket of PERK thus creating an inactivating allosteric conformation. Our results showed that Survivin ablation-induced cell death levels, as indicated by the levels of Cleaved PARP and Cleaved Caspase 7, were higher when there is no PERK activation due to LY4 treatment (**Figure 3.14 C**). These two experiments collectively indicate that the PERK arm of the UPR is activated to protect the cell, help it relieve the stress caused by Survivin ablation and survive.

Figure 3.14. **PERK assumes a prosurvival role in response to the ER stress induced by Survivin ablation.** (**A**) (A) SQ20B crisprPERK cell lines were created by using the GeCKO-v2 plasmid-based KO system and Survivin expression was silenced by siRNA specific transfection. Western blot analysis was performed for measuring apoptosis markers. (**B**) Cell survival was measured by and clonogenic assay. (C) SQ20B cells were transfected with siBIRC5 and 36h later with LY4 (1uM for 12h) Western blot analysis was performed for measuring Survivin apoptosis markers expression.

3.8. Ablation of proteins regulating the cell cycle leads to ER stress sensitization, ER expansion and multinucleation

Gene ontology (GO) analysis of the top 200 depleted genes form the CRISPR genomic analysis included categories such as cell cycle and cytokinesis which include Survivin and INCENP (**Figure 3.15**). Therefore, we inquired whether all the observations regarding the role of surviving in cell fate were specific to this protein or extended to other proteins with a similar role in the cell. We first went back over our screen ranking data and noticed that INCENP, a scaffold protein that holds the CPC complex together, was featured at a relatively high position in the negative ranking scale. (125^{th} in the first screen, 84^{th} in the second and 372^{th} in the third (**Figure 3.4 A**). Five out of six of the sgRNAs targeting INCENP were underrepresented in all three screens (**Figure 3.15 A**). Scatter plot analysis of the expression of sgRNAs in all three screens show the NGS read counts of treated cells vs controls and the placement of BICR5 and INCENP specific sgRNAs in the negative axis of the plot (**Figure 3.4 B**). To test if ablating INCENP demonstrate similar results to inhibition of surviving expression, we silenced INCENP expression by siRNA and tested for sensitization to ER stress. Similar to the surviving data, silencing of INCENP resulted in similar phenotypic alteration during ER stress as surviving ablation (**Figure 3.15 B**). Furthermore, inhibition of Chk1 kinase which is mitotic spindle checkpoint regulator and essential for the function of CPC, or inhibition of Aurora B kinase, the kinase module of the CPC complex, also led to ER expansion and multinucleation similar to that observed in the aforementioned surviving ablation studies (**Figure 3.15 C, D**). These results demonstrate that perturbation of the cell cycle and disorganization of the mitotic spindle, induced not only by Survivin, but also by its functional partners, can lead to aneuploidy and more generally genomic instability, which represents a form of ER stress.

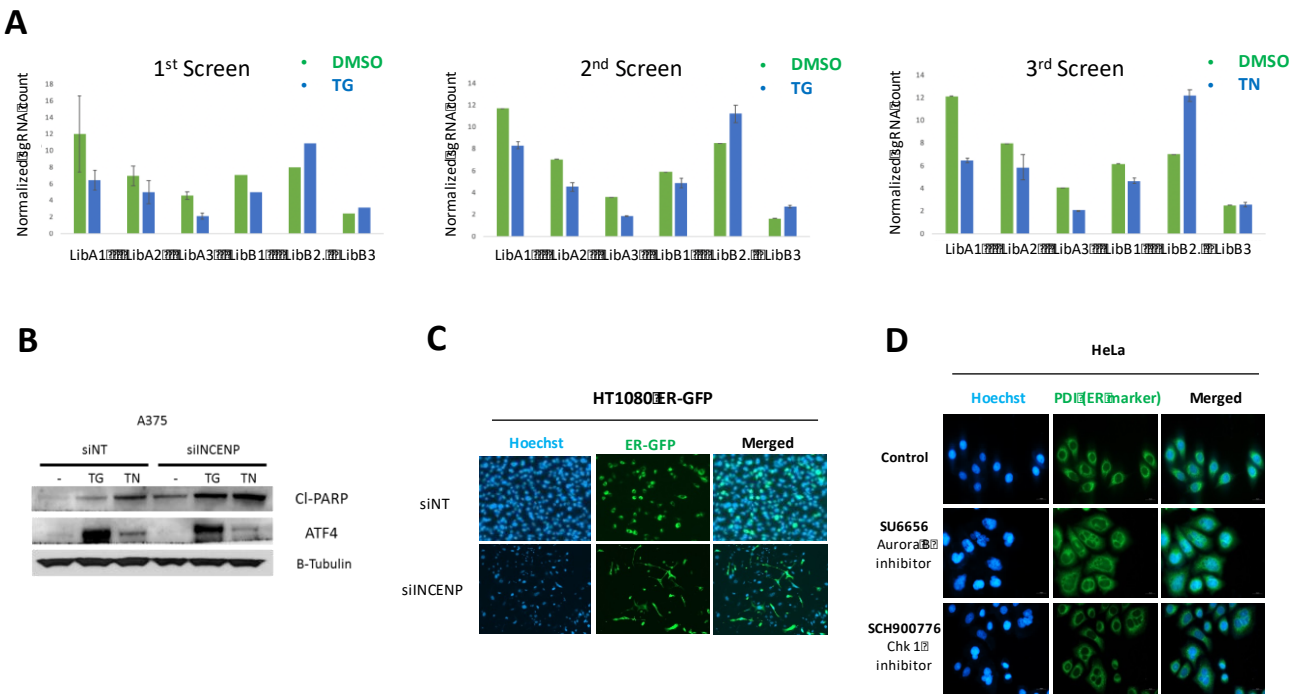


Figure 3.15. Negative ranking of the INCENP member of the CPC in all three screens (**A**) Five out of six sgRNAs from every screen were underrepresented in comparison to the controls. (**B**) Ablation of INCENP by siRNA leads to sensitization to ER stress. (**C**) HT1080 cells were transfected with a calmodulin-KDEL-GFP tagged expression vector and selected in G418 supplemented culture medium. Transfection of cells with fluorescence was measured by microscopy. INCENP expression was inhibited with siRNA transfection for 48 h. (**D**) Ablation of proteins that participate in the regulation of mitosis sensitizes cells to ER stress and led to an ER expansion and multinucleation. HeLa cells were treated with 10 ug/ml of SU6656 and SCH900776 inhibitor for 24 h. Cells were visualized by staining with Hoechst (nuclei) and PDI (ER marker). 40x magnification, scale bar 20 uM.

PART 4

DISCUSSION

4. DISCUSSION

4.1. Identifying novel mediators of the UPR

The UPR pathway is critical for survival in response to both extrinsic and intrinsic stresses that lead to accumulation of misfolded proteins and impair trafficking in the ER. UPR coordinates transcriptional induction, translational attenuation and ER associated protein degradation, allowing cells to adapt and survive the stress. Cancer cells can experience ER stress due to the increased metabolic and proliferating demands, and they highjack the UPR pathway in order to overcome these obstacles. Although one key outcome of the UPR is cellular adaptation to acute stress, chronic or unresolved stress can also result in the activation of apoptosis thereby preventing the propagation of damaged cells (Schroder et al., 2005). The mechanisms however that regulate this fine balance between life and death are not fully yet understood. Thus, the aim of this study was to identify new mediators of the UPR and confer to a better understanding of the parameters that govern it.

The CRISPR-mediated loss of function screen can robustly identify conditional or context dependent essential genes. Here, we utilized this tool in a vitro study in order to identify new gene targets whose deletion will positively or negatively affect cell fate in response to ER stress. Collectively, our results demonstrated the utility of CRISPR/Cas9 for conducting large-scale genetic screens in mammalian cells. This system is able to functionally target better non-transcribed elements, which are inaccessible to other silencing approaches such as RNAi, so it is more potent than the initially developed RNAi screening libraries and offers expanding possibilities. Although we limited our investigation to a cell fate readout-based phenotype, our approach can be applied to a much wider range of biological phenomena suiting the aim of the scientific question.

4.2. The role of Survivin in the context of ER stress

This functional knock out CRISPR-based approach identified some interesting genes whose depletion might contribute to the initiation of ER stress, most notably Survivin (BIRC5), who is a major regulator of the mitotic spindle and the promotion on M phase during cell cycle, as part

of the CPC complex. Our results so far indicated that ablation of Survivin can lead to sensitization to ER stress and also cause phenotypic changes that involve multinucleation and ER expansion. Lack of Survivin, caused ER stress to the cells and subsequent UPR activation that aims in protection of the cells from apoptosis through the PERK signaling arm of the UPR. Our hypothesis supports that ablating Survivin in the cell renders the CPC incapacitated and unable to properly organize the mitotic spindle. As a result, the cells go through an incorrect mitosis and become aneuploid due to chromosomal instability (CIN). The forming cells harbor multiple nuclei and micronuclei and are unable to go through cytokinesis. Consequently, these cells that cannot sufficiently divide in two daughter cells are loaded with excess DNA and expand their ER in order to accommodate the increased transcriptional and hence translational demands of the cell. All together, these processes lead to ER stress and activation of the UPR in order to protect the cell from dying (**Figure 4.1**). In further support of this model, another member of the CPC complex, the protein scaffold INCENP also exhibited the same effects upon ablation and induction of ER stress.

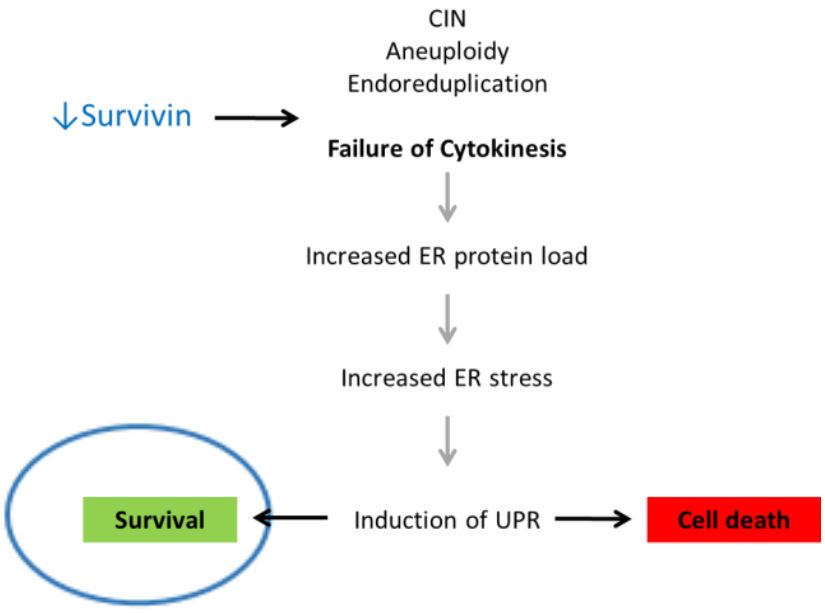


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Figure 4.1. The activation and role of UPR under Survivin induced ER stress.
This flow chart indicates the overall hypothesis model that explains the relation between Survivin and ER stress.

In support of our hypothesis, many studies showed that aneuploidy affects cell behavior in a gene dosage effect way, meaning that the concentration of the primary gene product is proportional to the copy numbers of a gene (Epstein et al., 1986). Because of unbalanced gene expression due to aneuploidy, cells may experience proteotoxic stress, broadly referring to the overburdening of cellular systems that maintain proper protein folding and homeostasis (Deshaies et al., 2014). Furthermore, the formation of multinucleated cells or cells that have large nuclei with micronuclei satellites is observed in cells with expanded ER, a phenomenon taking place when the UPR aims for homeostatic readjustment.

4.3. The role of polyploid giant cells in cancer

One of the most interesting findings in our study is the formation of giant multinucleated cells upon ablation of Survivin. Our aforementioned hypothesis in the context of giant aneuploid cells gives an interesting perspective to the role of Survivin in tumor progression. Tumors are complex systems that include heterogeneous cancer cells with markedly differing sizes and genomic contents. The majority of cancer cells within solid tumors is aneuploid (~90%), has alterations in chromosomal number, mostly gain of chromosomes and is usually not a multiple of the diploid component (2n) (Li et al., 2015). Cultures of both primary cancer cells from patients but also from established cell lines are aneuploidy and flow cytometry based techniques have been established in order to evaluate the number of aneuploid cells based on the size of the original tumor (Coward et al., 2014). Additionally, the spontaneous formation of giant polyploid cells has been described in many p53 mutant cell lines (Mirzayans et al., 2018). Importantly, giant tumor cells with increased nuclear content either stop proliferating or proliferate very slowly. That is why they usually cannot be recognized by a conventional colony formation assays. However, these cells are alive and can promote stemness through three identified ways: depolyploidization, nuclear bursting and horizontal transmission of sub-genome between cells (Diaz-Carballo et al., 2018).

During depolyploidization the polyploid/multinucleated giant cells undergo a ploidy cycle, which is regulated by key mediators of mitosis (like the CPC complex), meiosis, and self-renewal. Ultimately they result in the emergence of cells that have a near-diploid number of

chromosomes (known as “para-diploid” progeny) and exhibit mitotic propagation (Erenpreisa et al., 2013) (Erenpreisa et al., 2015). Nuclear bursting or budding is when giant cancer cells give birth to new cells via endoreduplication and then undergo budding like yeast does, to give rise to small daughter nuclei. These nuclei then acquire cytoplasm, split off from the giant mother cell, and separate. The new cells exhibit long-term proliferation potential. This process in cancer cells is known as neiosis (Solari et al., 1995) (Sundaram et al., 2004) (Niu et al., 2016). Last but not least, the multinucleated cancer cells can promote stemness of the surrounding cells via a sub-genome transmission. In this process, giant cells intracytoplasmically generate daughter cells that express high levels of cancer stem cell markers, which can then be transferred into surrounding cells via cytoplasmic “tunnels”, giving to the recipient cells stem cell properties (Diaz-Carballo et al., 2018). Taken together, it is of great significance to trace the cell fate of the giant cells formed after Survivin ablation. Our future experiments aim to track whether they are led to death or they remain alive, slowing down their processes and entering a senescent state, which will after time end by procreating new daughter cells with one of the aforementioned ways.

Giant cells and polyploidy are very important in the context of cancer because they are responsible for many of the characteristics of tumor growth. The proportion of polyploid/multinucleated giant cancer cells both *in vitro* and *in vivo* is shown to increase markedly under stressful conditions like hypoxia, and after treatment with radio and chemo therapeutic agents conferring to their resistance (Mirzayans et al., 2017) (Mourad et al., 2018) (Moore et al., 1998). There are many studies demonstrating that polyploid/multinucleated giant cells are more aggressive and metastatic than parental cells and are also responsible for cancer relapse, most notably in bone cancer (Klenke et al., 2011). As far as the latter case is concerned, a stage in cancer progression in which cell size is an indicative factor is cancer dormancy, a state in which cells cease dividing but survive in a quiescent mode, while waiting for appropriate environmental conditions, in order to begin proliferation again. A large body of evidence from studies with solid tumors and solid tumor-derived cell lines demonstrated that dormant cells remain viable and metabolically active for long times, sometimes weeks, post-treatment. This proliferation arrest is often accompanied by increased cell size, which can reflect stress-induced

premature senescence or the development of polyploid giant cells with either a highly enlarged nucleus or multiple nuclei. Altogether, these data show that increasing chromosome numbers provides a mechanism to generate tumor cells that cycle infrequently and a general resistance mechanism against cytotoxic chemotherapy treatments designed to target actively cycling cells (Donovan et al., 2014). Experiments in yeast and mouse embryonic fibroblasts (MEFs) have shown that increased transcription and translation caused by elevated genomic content causes cell cycle delays during G1 (Torres et al., 2007) (Rao et al., 2005) (Williams et al., 2008). A plausible hypothesis is that the larger polyploid tumor cells arrest during G0/G1 to allow for a sufficient growth to occur before committing to division, which is impeded due to the increased transcriptional and translational demands placed on polyploid tumor cells by their elevated and unbalanced chromosomal copy number.

4.4. Future directions

Survivin is an attractive target for the development of pharmacologic strategies, since it is highly expressed in most cancer cell types but is undetected to normal tissues. Thought there is a need to develop new inhibitors for Survivin, since the ones already tested in clinical trials have not demonstrated robust effects, the prosurvival role of UPR over the Survivin targeting induced ER stress can open a new therapeutic window. More specifically, combining inhibition of Survivin expression with targeting the PERK arm of the UPR can lead to increased cancer cell death, since UPR activation could be one of the contributing factors for the survival of dormant cells with proliferative potential. Currently there are several PERK kinase inhibitors available. The most well studied are GSK266414 and GSK2656157, and they have been recently reported to by non-specific, since they are potently binding to Receptor Interacting Ser/Thr Kinase 1 (RIPK1) (Rojas-Rivera et al., 2017). New generation inhibitors, such as HC4 (formerly known as LY4) have been published by us and others and appear to be more specific. Hence, development of both Survivin and PERK specific inhibitors could be an urgent need in cancer therapeutics.

Our future directions include delineating the role of UPR in response to Survivin ablation induced ER stress. More specifically, we shall focus on inhibiting Survivin expression while blocking the protective effect of UPR through inhibition of the PERK specific signaling cascade *in*

vivo and observe the effects on tumor growth and survival. Concurrently, tracing the fate of giant multinucleated cells formed after Survivin ablation will open a route towards a mechanistic understanding of the importance of polyploidy/multinucleated cells in cancer progression. Following these polyploid cells can help us understand if UPR is accountable for their survival and corroborates their entering in a dormant slow proliferating state that escapes cell death. Patient material deriving from the Survivin targeting-based clinical trials could be instrumental to study the activation of UPR and the potential formation of giant cells in the already treated harvester tissues.

4.5. Conclusions

In summary, this study aims to identify new mediators of the UPR signaling pathway and their role in the context of ER stress. A genome wide functional CRISPR/Cas9 based screen approach indicated Survivin, a "universal tumor antigen" as an attractive candidate. First, our studies showed that knocking down Survivin can sensitize cells to ER stress. Additionally, cells that lack Survivin expression become multinucleated and aneuploid, their ER expands. We attributed this to failure of cytokinesis due to the importance of Survivin in the CPC complex and the erroneous cell cycle and abscission completion. Last but not least, Survivin ablation caused ER stress and subsequent UPR activation and the PERK arm of the UPR signaling pathway assumed a prosurvival role in response to that stress. Our results so far demonstrate that cell cycle and cytokinesis failure can result in conditions conducive to ER stress and lead to the activation of a protecting UPR. Importantly, we have uncovered the therapeutic potential of targeting Survivin, thus providing a very high specificity treatment, combined with UPR inhibition in order to treat a broad range of tumors and prevent the survival of cells that can act as future cancer seeds.

6. Abstract in greek (Περίληψη)

Μελέτη του ρόλου της συγκλινόμενης απόκρισης στο στρες στην προαγωγή του καρκίνου και στην φαρμακευτική ανθεκτικότητα

Ταυτοποίηση νέων ρυθμιστών του στρες του ενδοπλασματικού δικτύου με τη χρήση λειτουργικής «βιβλιοθήκη» CRISPR/Cas9 σε επίπεδο γονιδιώματος

(«*Studying the Integrated stress response in tumor progression and drug resistance*»)

Ο πολλαπλασιασμός και η ανάπτυξη ενός όγκου καθώς και η μετάσταση σε άλλους ιστούς βασίζονται σε μία σύνθετη σχέση μεταξύ των καρκινικών κυττάρων και του μικροπεριβάλλοντος. Ένας αναπτυσσόμενος όγκος αντιμετωπίζει ποικίλα είδη στρες που προέρχονται από το μικροπεριβάλλον, όπως υποξία, έλλειψη θρεπτικών συστατικών και οξείδωση. Για να αντιμετωπίσουν αυτά τα στρεσογόνα ερεθίσματα και συνθήκες, τα καρκινικά κύτταρα μπορούν να οικειοποιηθούν φυσιολογικούς κυτταροπροστατευτικούς μηχανισμούς, όπως την πορεία απόκρισης στις μη-αναδιπλωμένες πρωτεΐνες (Unfolded Protein Response-UPR). Η UPR περιλαμβάνει την μεταγραφική και μεταφραστική ενεργοποίηση άλλων πορειών που στοχεύουν να καταπραΰνουν το στρες και να αναστείλουν τον κυτταρικό θάνατο όταν οι στρεσογόνοι παράγοντες είναι οξείς και μικρής διαρκείας. Αντίθετα, όταν το στρες είναι χρόνιο και έχει ισχυρές επιπτώσεις στο κύτταρο τότε η πορεία UPR θα στραφεί προς την ενεργοποίηση προαποπτωτικών μηχανισμών με σκοπό να οδηγήσει το κύτταρο σε θάνατο. Ωστόσο, οι μηχανισμοί που εμπλέκονται στο αν η UPR θα έχει προστατευτικό ή προαποπτωτικό ρόλο δεν είναι πλήρως κατανοητοί.

Για το σκοπό αυτό, ο στόχος της παρούσας ερευνητικής εργασίας ήταν η ταυτοποίηση νεών μορίων που ρυθμίζουν τους μηχανισμούς απόκρισης στο στρες του ενδοπλασματικού δικτύου (ΣΕΔ) και καθορίζουν την έκβαση των κυτταρικών αποκρίσεων. Η μεθοδολογία με την οποία προσεγγίστηκε αυτό το ερώτημα ήταν η διαλογή γονιδίων ως προς τον προστατευτικό ή προαποπτωτικό τους ρόλο σε σχέση με το ΣΕΔ, χρησιμοποιώντας σε επίπεδό γονιδιώματος μια

λειτουργική λεντΐική «βιβλιοθήκη» CRISPR/Cas9 (functional CRISPR-based genetic knockout screen). Πιο συγκεκριμένα, δύο καρκινικές κυτταρικές σειρές διαμολύνθηκαν με την βιβλιοθήκη ώστε από κάθε κύτταρο να αποσιωπηθεί η έκφραση ενός γονιδίου. Τα κύτταρα αυτά υποβλήθηκαν σε στρες του ενδοπλασματικού δικτύου και αυτά που επιβίωσαν χρησιμποποιήθηκαν για την ταυτοποίηση μικρών RNA-οδηγών (small guide RNAs-sgRNAs) που είναι συγκεκριμένα για κάθε γονιδιακό τόπο και οδηγούν το ένζυμο Cas9 να «κόψει» το DNA σε αυτό το σημείο. Αν ένα sgRNA αντιπροσωπεύεται στον τελικό κυτταρικό πληθυσμό σε μεγάλο αριθμό τότε το γονίδιο στο οποίο αντιστοιχεί είναι προαποπτωτικό, διότι τα κύτταρα στα οποία είχε απενεργοποιηθεί επιβίωσαν αντί να πεθάνουν. Αντίστοιχα, αν ένα γονίδιο έχει μικρό αριθμό αντιπροσωπευτικών sgRNA τότε έχει ρόλο προστατευτικό. Ένας από από τους πιο ενδιαφέροντες ρυθμιστές στην αρνητική κλίμακα κατάταξης με πιθανό προστατευτικό ρόλο έναντι του ΣΕΔ ήταν η Survivin (*BIRC5*), η οποία εκφράζεται μόνο στα καρκινικά κύτταρα και έχει χαρακτηριστεί αρχικά ως αναστολέας της απόπτωσης. Ωστόσο, ο βασικός της ρόλος είναι η οργάνωση της μιτωτικής ατράκτου και η προαγωγή του κυτταρικού κύκλου.

Η παρούσα ερευνητική εργασία κατέδειξε ότι η γενετική ή χημική αναστολή της έκφρασης της Survivin προκαλεί ευαισθησία στο ΣΕΔ, και οδηγεί στο σχηματισμό κυττάρων με εκτεταμένο ΕΔ και πολλαπλούς πυρήνες. Επιπλέον, η αναστολή της Survivin προκαλεί ΣΕΔ και συνεπαγόμενη ενεργοποίηση του UPR, το οποίο φέρεται να έχει προστατευτικό ρόλο έναντι στο στρες προκλινόμενο από την αναστολή της. Η υπόθεση μας βασίζεται στο ότι τα κύτταρα αυτά προβαίνουν σε λανθασμένη κυτταροκίνηση που οδηγει σε κύτταρα με ανευπλοειδία. Τα προκύπτοντα κύτταρα έχουν περισσότερο DNA, συνεπώς περισσότερες μεταγραφικές και μεταφραστικές απαιτήσεις και επεκτείνουν το ΕΔ για να ανταπεξέλθουν σε αυτές. Η UPR ενεργοποιείται ώστε να συμβάλει στη μείωση του στρες και την επιβίωση του κυττάρου. Τα αποτελέσματα αυτά υποδεικνύουν ότι η πολυπλοειδία και γενικότερα η γενετική αστάθεια αποτελούν από μόνα τους κυτταρικούς στρεσογόνους παράγοντες του ΕΔ για την αντιμετώπιση των οποίων απαιτείται η πορεία UPR. Παράλληλα, αναδεικνύονται νέες αδυναμίες των καρκινικών κυττάρων και πιθανοί στόχοι για το σχεδιασμό θεραπευτικών προσεγγίσεων που αποσκοπούν στην αποσιώπιση της έκφρασης της Survivin και την παράλληλη αναστολή της πορείας ενεργοποίησης της ΣΕΔ.

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