Characterisation of the Response by MDA-MB-231 Breast Cancer Cells to Amino Acid Starvation

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Abstract

Breast cancer, like many other types of cancer, exhibit abnormal amino acid metabolism and dependency on external sources of specific amino acids. The specific amino acids cancer cells rely on to support their growth and survival may provide potential targets for treating breast cancer patients. Moreover, targeting the specific pathways involved in overcoming amino acid starvation is another potential breast cancer therapy. Starvation of specific amino acids results in a reduction of breast cancer cell proliferation and activation of apoptosis. However, the involvement of the Amino Acid Response (AAR) mechanism during amino acid starvation conditions is poorly understood in breast cancers. In this study, we investigated the response of the triple-negative breast cancer (TNBC) cell line - MDA-MB-231 - to glutamine and leucine starvation. We scored for the activation of the General Control Non-derepressible 2 (GCN2) protein and phosphorylation of alpha subunit of eukaryotic initiation factor 2 (eIF2α). We relate a higher activation and phosphorylation of GCN2 and eIF2α to a more severe AAR by MDA-MB-231 cells. In this study, we concluded that the MDA-MB-231 cells responded more strongly to glutamine starvation than leucine starvation by a greater activation of their AAR. Furthermore, the detection of the greater activation of apoptosis and a greater reduction in cell proliferation also suggests that glutamine supply is more critical than leucine to support MDA-MB-231 cell survival. We discuss the roles and consumption rates of each amino acid as potential causes for this difference in AAR to glutamine and leucine starvation by MDA-MB-231 cells.

Furthermore, we inhibited GCN2 via siRNA mediated knockdown, before imposing glutamine and leucine starvation and screened for activation of apoptosis and a change in cell proliferation. We found that knockdown of GCN2 sensitised the MDA-MB-231 cells to both leucine and glutamine starvation, with a greater reduction of cell proliferation and activation of apoptosis during glutamine starvation. Knockdown of GCN2 in unstressed MDA-MB-231 cells also led to a decrease in cell proliferation and increased apoptosis. In summary, these results suggest that, GCN2 appears to be important for MDA-MB-231 cell survival under unstressed conditions, and that GCN2 loss sensitises the cells to amino acid starvation, leading to a greater reduction in cell viability.

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Abbreviations

In addition to the Système international d' unites (SI), the following abbreviations are used:

AAR	Amino Acid Response
ALL	Acute Lymphoblastic Leukemia
APS	Ammonium Persulfate
ASCT2	Amino Acid transporter-2
ASNS	Asparagine Synthetase
ATF4	Activating Transcription Factor 4
DMEM	Dulbecco's Modified Eagle's Medium
DMEM-LM	Dulbecco's Modified Eagle's Limiting Medium
EAA	Essential Amino Acid
EDTA	Ethylene Diamine Tetra acetic Acid
EIF2AK4	Eukaryotic Translation Initiation Factor 2 Alpha Kinase 4
eIF2	Eukaryotic Translation Initiation Factor 2
eIF2α-P	Eukaryotic Translation Initiation Factor 2 - Phosphorylated alpha subunit
eIF2B	Eukaryotic Translation Initiation Factor 2B
FASN	Fatty Acid Synthase
FBS	Foetal Bovine Serum
GCN2	General Control Non-derepressible 2
GCN2-P	General Control Non-derepressible 2 Phosphorylated
GDH/H6PD	Glutamate Dehydrogenase
GDP	Guanosine Diphosphate
GLN	Glutamine
GLS1/GLS	Glutaminase 1
GTP	Guanosine Triphosphate
HER2	Human Epidermal growth factor Receptor 2
HRI	Heme-Regulated Inhibitor
HRP	Horseradish Peroxidase
ISR	Integrated Stress Response
Leu	Leucine

MEF	Mouse Embryonic Fibroblast
MEM	Modified Eagle's Medium
Met-tRNAi	Methionyl initiator tRNA
mRNA	Messenger ribonucleic acid
NEAA	Non-Essential Amino Acid
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PERK	PKR-like ER Kinase
PKR	Protein Kinase double-stranded RNA-dependent
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
siRNA	Small interfering RNA
siGNC2	Small interfering GCN2 RNA
shRNA	Short Hairpin RNA
siScr	Small interfering Scrambled RNA
SLC1A5	Solute Carrier Family 1, Member 5
SLC3A2	Solute Carrier Family 3, Member 2
SLC7A5	Solute Carrier Family 7, Member 5
SRB	Sulforhodamine B
SSP	Staurosporine
TBS	Tris-Buffered Saline
TBS-T	TBS-Tween
TEMED	N, N, N, N- Tetramethylethylenediamine
TNBC	Triple Negative Breast Cancer
tRNA	Transfer RNA
uORF	Upstream Open Reading Frame
VEGF	Vascular Endothelial Growth Factor

1.Introduction

1.1. Breast Cancer

In recent years there has been only a small decrease in the breast cancer mortality rate despite the development of new breast cancer therapies (Siegel, Miller, and Jemal 2019). Most breast cancer therapies are specific, only treating one type of breast cancer. This is due to breast cancers high genetic diversity (Stephens et al. 2012; Geck and Toker 2016). Breast cancers high genetic diversity leads to there being no generic therapeutic target for treating breast cancers and therefore, no single therapy is effective against all breast cancer types. The lack of a generic breast cancer therapy has led to the recent interest in identifying more general therapeutic targets - a property all types of breast cancer cells depend on - to treat breast cancer. These general therapeutic targets may also apply to more cancers than just breast cancer. One such generic “hallmark” property of cancers, including breast cancer, is their altered metabolism and increased proliferation rates (Hanahan & Weinberg 2011). By having an increased proliferation rate and altering their metabolism causes the cancerous cells to become dependent on the availability of specific nutrients such as amino acids and their mechanisms to obtain them.

1.2. Breast Cancer Amino Acid Metabolism

Targeting the supply of amino acids is becoming popular as a novel breast cancer therapy. Amino acids account for the majority of the cell mass of cancerous cells (Hosios et al. 2016). Cells utilise amino acids for a diverse array of processes necessary for cell growth, including; biosynthesis of proteins, nucleotides, lipids, and a source of carbon and nitrogen. Of the 20 amino acids, mammalian cells cannot synthesise nine which are considered as essential amino acids (EAA), in that they must come from an extracellular source. Whereas mammalian cells can synthesise the other 11 amino acids and are considered nonessential amino acids (NEAA). Cancer cells, including breast cancer, rely on external sources of not only the essential amino acids but also some

NEAA. The reliance on external NEAA's supports cancer cells increased proliferation rates by decreasing the amount of energy required by the *de novo* synthesis processes of NEAA (Hosios et al. 2016). Reliance on external sources of specific amino acids creates a "metabolic addiction" by which cancer cell proliferation and survival is reliant on certain NEAA's. This reliance on NEAA's causes these specific amino acids to be considered as conditionally essential, in that, although the cell can synthesise the amino acids, they rely on an external supply to meet their requirements.

Breast cancer's "metabolic addiction" to some conditionally essential NEAA is a unique cancer property. This "metabolic addiction" to NEAA, in theory, could be used to treat breast cancer by starving the cancerous cells of the conditionally essential NEAA, inhibiting cancer cell proliferation and inducing cancer cell death. Under conditionally essential NEAA starvation conditions, the non-cancerous cells will remain unaffected, as they do not rely on an external source of the NEAA. Thereby, having a generic cancer therapy which kills cancerous cells and does not affect normal cells. The observation of cancer's "metabolic addiction" to conditionally essential amino acids is not new. Acute lymphoblastic leukemia (ALL) is treated by the depletion of serum asparagine by ASNase (Hill et al. 1967). ALL cells cannot synthesise asparagine; therefore, the cells rely on an external source of asparagine. Thereby, the depletion of serum asparagine leads to the death of the ALL cells. The potential of conditionally essential NEAA to specific cancers has created interest in therapies which target the conditionally essential NEAA supply by; depleting serum amino acids, inhibition of transporters, or inhibition of proteins which aid in the supply of amino acids (Covini et al. 2012; Boroughs and DeBerardinis 2015; Altman, Stine, and Dang 2016; M.-S. Chen et al. 2017; Lukey, Katt, and Cerione 2017; Rajanala, Ringquist, and Cryns 2019). These therapies ultimately starve the cell of conditionally essential amino acids leading to cell death.

1.3. Targeting Breast Cancers' Amino Acid Metabolism as a Therapy

Amino acid starvation protocols are becoming promising for novel breast cancer therapies. Since the discovery in 1955 by Harry Eagle, we have known that the required serum concentrations for some amino acids are required to be significantly higher to culture cancerous cells than non-cancerous cells *in vitro (*Eagle *1955)*. Eagle noted the cancerous cell line, HeLa cells, require amino acid concentrations at a 3-fold higher concentration than mouse embryonic fibroblasts (MEF). Moreover, breast cancer patients show a tumour dependent change in blood serum amino acid concentrations (Poschke et al. 2013). This change in serum amino acid concentration is dependent on tumour type, with the possibility that the depleted amino acids are important for the specific tumour type's survival. Recent research by Hosios et al. 2016, determined the consumption rates of cancer cells for each amino acid, with the highest rates of consumption for glutamine, serine, asparagine, and leucine. Being the most consumed of all amino acids indicates each of these four amino acids as a possible target for breast cancer starvation therapies. Other recent research has found other amino acids such as cysteine also being a potential target for breast cancer amino acid starvation therapy (M.-S. Chen et al. 2017). In this study, we will focus on the non-essential amino acid glutamine and the essential amino acid leucine.

The most consumed amino acid in cultured cells lines is glutamine (Hosios et al. 2016). Glutamine is a major carbon source for nonessential amino acids and accounts for a significant proportion of nitrogen within cancerous cells (Hosios et al. 2016). Breast cancers increase the expression of a variety of glutamine metabolism-related proteins to increase their supply of glutamine; including glutaminase 1 (GLS1/GLS), glutamate dehydrogenase (GDH/H6PD), and amino acid transporter-2 (ASCT2/SLC1A5)(S. Kim et al. 2013). The deprivation of glutamine dramatically decreases breast cancer cell growth *in vitro* (Yuneva et al. 2007; Lampa et al. 2017; Parzych et al. 2019). The roles of glutamine, with its essentiality to cell growth, leads glutamine to be considered as a conditionally essential NEAA, with the potential as a therapeutic target. However, therapies - such as GLS inhibition - have been ineffective or significantly toxic *in vivo*

(Budczies et al. 2015). A greater understanding of how the molecular mechanisms by which the cancerous cells react to glutamine may aid in the development of a successful glutamine starvation therapy.

The fourth most consumed amino acid and the most consumed essential amino acid in cultured cell lines is leucine (Hosios et al. 2016). Leucine participates in hormonal secretion and a variety of intracellular signalling pathways such as the mTORC1 signalling pathway (Hara et al. 1998; Nair and Short 2005; Averous et al. 2016). The deprivation of leucine significantly reduces the growth of breast cancer cells *in vitro* and *in vivo*, without decreasing the growth of non-cancerous breast cells (Xiao et al. 2016). This study shows a possibility for leucine starvation as a potential amino acid starvation breast cancer therapy. However, leucine deprivation therapy still requires further research to determine the safety of a short and long-term leucine deprivation diet's effects in humans. Furthermore, a greater understanding of how cancerous cells react to leucine starvation would aid in the development and efficacy of a leucine deprivation treatment.

Other research for possible amino acid starvation therapies to treat breast cancer has focused on the amino acids cancerous cells consume the most such as serine and arginine (Chiaviello et al. 2012; Labuschagne et al. 2014; S. K. Kim, Jung, and Koo 2014; Qiu et al. 2014). Recent research is now targeting other amino acids; cysteine, and methionine, which also appear to be essential to cancer cell survival (M.-S. Chen et al. 2017; Mazor et al. 2018; van Geldermalsen et al. 2018; Rajanala, Ringquist, and Cryns 2019). However, there appears to be no consensus into which amino acid starvation condition is the best to inhibit breast cancer growth, or that breast cancer relies on the most. Moreover, there is no determination of the molecular mechanisms in response to each starvation condition. Thereby, the molecular response to each amino acid starvation condition remains poorly understood.

The current breast cancer amino acid starvation research either focuses on starvation of only one single amino acid or the combination of several amino acids starved at once.

Furthermore, this is usually completed without screening for the activation of the primary amino acid response mechanisms, focusing only on the final cell outcome of starvation - the change in cell proliferation or activation of cell death pathways (Xiao et al. 2016; Mazor et al. 2018; M.-S. Chen et al. 2017; Parzych et al. 2019). Thereby, we have information on how a single amino acid starvation or the combination of multiple amino acid starvation conditions affects cell fate, but no comparison between single amino acid starvation conditions. Moreover, for an unknown reason research has not yet compared single amino acid starvation conditions while screening for the primary cellular response mechanism to amino acid starvation, activation of General Control Non-derepressible 2 (GCN2/EIF2AK4) of the Integrated Stress Response (ISR) Pathway. Screening for this activation of the ISR/AAR will be valuable for identifying the most appropriate amino acid starvation therapy.

1.4. Integrated Stress Response (ISR)

Eukaryotic cells activate the ISR to maintain homeostasis under a range of stressors (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). During a variety of cellular stressors, one of four different protein kinases is activated to return cell homeostasis (Figure 1.1). **PERK** (**P**KR-like **ER K**inase) primarily responds to the accumulation of misfolded proteins, **PKR** (**P**rotein **K**inase Double-stranded **R**NA-dependent) is activated in response to viral infection, **HRI** (**H**eme-**R**egulated **I**nhibitor) regulates cellular heme concentrations, and **GCN2** (**G**eneral **C**ontrol **N**on-derepressible **2**) responds to cellular amino acid starvation. These four ISR protein kinases phosphorylate the single substrate, Ser-51 of the α subunit of **e**ukaryotic **i**nitiation **f**actor **2** (**eIF2α**). The phosphorylation of eIF2α leads to a global decrease in translation, while paradoxically increasing the activation of genes involved in restoring homeostasis, such as activating transcription factor 4 (ATF4). ATF4 upregulates expression of homeostasis restoring genes, such as amino acid transporters and biosynthesis enzymes (H. P. Harding et al. 2000; Heather P. Harding et al. 2003).

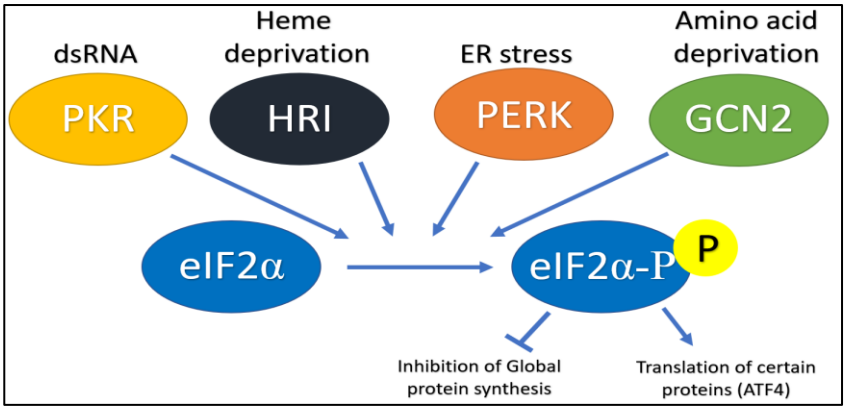

Figure 1.1 - The Integrated Stress Response pathway.

In response to stressors, four different kinases in mammals phosphorylate the eukaryotic translation initiation factor eIF2α. eIF2α phosphorylation leads to the inhibition of global protein synthesis, while paradoxically upregulating specific proteins, such as ATF4.

1.5. GCN2 and the Amino Acid Response Pathway (AAR)

In response to amino acid starvation, the protein kinase GCN2 is activated (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). GCN2 senses a deficiency in amino acids by the accumulation of uncharged tRNAs (Figure 1.2). The increased abundance of uncharged tRNAs activates GCN2 by stimulation of GCN2's kinase catalytic domain and autophosphorylation of GCN2. Phosphorylated GCN2 (GCN2-P), as with the other ISR kinases, phosphorylates Ser-51 amino acid of the alpha subunit of eIF2. Phosphorylation of eIF2α competitively inhibits eIF2B, slowing the recycling of the eukaryotic translation initiation factor 2 (eIF2) (Figure 1.2). eIF2 in a GTP-bound form delivers initiator methionyl tRNA (Met-tRNA$_i^{Met}$) to the ribosome, initiating translation. After the translation initiation, eIF2 is released in a GDP-bound form. eIF2-GDP is recycled by eIF2B to eIF2-GTP, allowing eIF2-GTP to deliver Met-tRNAi^{Met} to the ribosome again and initiating translation. The phosphorylation of eIF2α by GCN2 and subsequent competitive inhibition of the recycling of eIF2 reduces the global amount of translation. By reducing the amount of global translation, the cell reduces amino acid consumption allowing the cell to preserve amino acids when starved (Castilho et al. 2014). Furthermore, the reduction of translation via reduced eIF2 recycling

simultaneously allows for increased translation of specific mRNAs with unique upstream open reading frames (uORFs), such as those found in ATF4, which upregulate transcription of genes involved in nutrient uptake and synthesis. Overall, the reduction of global protein synthesis reduces the consumption of vital nutrients, while simultaneously upregulating genes involved in amino acid uptake to restore amino acid supply and overcome periods of amino acid starvation sensed by GCN2.

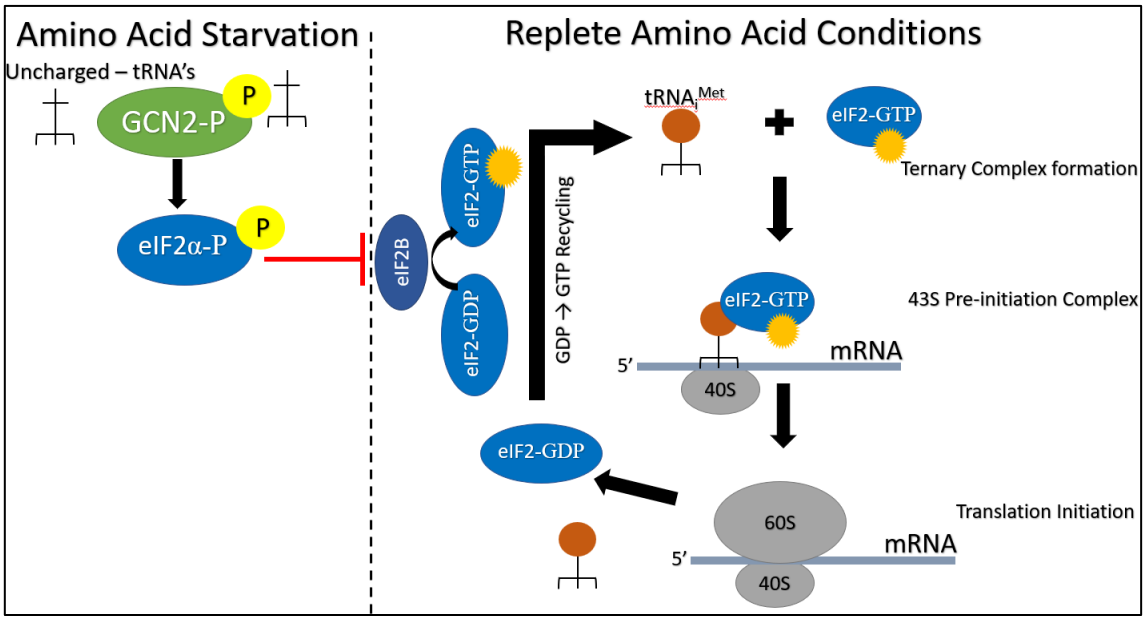

Figure 1.2 - The Amino Acid Response Pathway (AAR) and the effect on translation initiation.

Translation initiation depends on the delivery and binding of $tRNA_i^{Met}$ and eIF2-GTP to the 40S ribosomal subunit. Along with mRNA, this builds the 43S pre-initiation complex. The pre-initiation complex scans the mRNA for the start codon. At the start codon, the 60S ribosomal subunit joins releasing the $tRNA_i^{Met}$ and the used eIF2-GDP protein. To reinitiate translation, the eIF2-GDP is recycled to eIF2-GTP, allowing another translation initiation process to begin. During amino acid starvation GCN2 is activated by autophosphorylation. The activated GCN2 phosphorylates the alpha subunit of the eIF2 molecule, competitively inhibiting recycling of eIF2-GDP to eIF2-GTP by eIF2B. This reduces the available eIF2-GTP molecules for translation initiation, thereby decreasing global protein synthesis.

1.6. The GCN2-eIF2α effector ATF4

GCN2 activation and subsequent eIF2α phosphorylation lead to the translational upregulation of specific effectors to overcome amino acid starvation (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). One of the most characterised effectors that GCN2 upregulates in response to amino acid starvation is the activating transcription factor 4 (ATF4) in mammals. ATF4 enhances the transcription of many genes involved in maintaining amino acid homeostasis by increasing the uptake or biosynthesis of amino acids. Mammalian ATF4 translation is dependent on two short 5' uORFs. During normal translation, under no cellular stress, translation begins at the first uORF. The ribosome proceeds to scan along the mRNA and reinitiates at the second uORF (uORF2). The second uORF terminates within the ATF4 gene and is out of frame to the ATF4 start codon. Thereby, the translation of ATF4 is not initiated. However, under stressed conditions, eIF2α is phosphorylated, and the cellular abundance of eIF2-GTP is low. This slows the recruitment of eIF2-GTP by the ribosome and slows the scanning of the ATF4 mRNA. The slowed scanning of the ATF4 mRNA leads the ribosome to reinitiate translation, not at uORF2 but instead the ATF4 start codon, which produces the functional ATF4. ATF4 then proceeds to transcriptionally activate many genes involved in overcoming the specific stressor. For example, Major categories of genes upregulated by ATF4 are amino acid transporters and biosynthesis enzymes; examples of upregulated amino acid transporters are; solute carrier family 1, member 5 (SLC1A5), solute carrier family 3, member 2 (SLC3A2), and solute carrier family 7, member 5 (SLC7A5) (Heather P. Harding et al. 2003). Examples of biosynthesis enzymes are; Fatty Acid Synthetase (FASN) and many aminoacyl tRNA synthetases (H. P. Harding et al. 2000; Xiao et al. 2016).

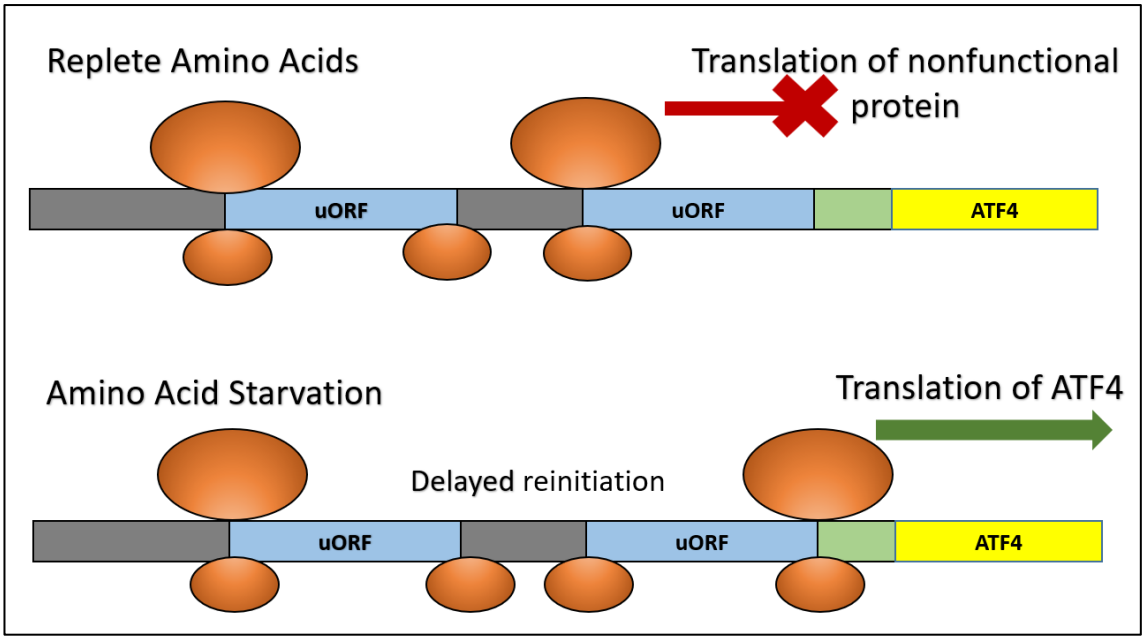

Figure 1.3 – Translation Initiation of ATF4 during Amino acid starvation.

During replete amino acid conditions, the ribosomes translate the two uORF's upstream of ATF4, resulting in the translation of a nonfunctional protein. Under amino acid starvation conditions, eIF2α is phosphorylated, leading to a delay in translation reinitiation. This delay results in reinitiation of translation at the start codon of ATF4, allowing translation of a functional ATF4 protein.

1.7. GCN2 promotes cancer cell survival

The tumour microenvironment is harsh, characterised by frequent limitations in the availability of nutrients. This environmental stressor is further compounded by the tumour cells increased demand for nutrients as compared with normal cells (DeBerardinis et al. 2008; Boroughs and DeBerardinis 2015). As the tumour grows and develops the cancerous cells' demand for nutrients increases while supply becomes limited. The limited supply is due to the unorganised structure of cancer cell growth and outgrowing their vasculature, leading to reduced blood and nutrient supply. To survive, the cells develop strategies to overcome the increased demand for nutrients. One strategy is for cancer cells to over-activate the amino acid response (AAR) pathway to increase the supply of nutrients. Overactivation of the AAR begins with increased and continuous activation of GCN2 within the cancer cell. GCN2 has been found to be overactivated in a variety of different cancer cell types such as colon, lung, and breast cancers (Jiangbin Ye et al. 2010). This suggests GCN2 over-activation is a common

mechanism for cancer cells to increase nutrient supply to overcome the harsh tumour microenvironment and support their increased proliferation rates.

Tumour cell survival is promoted by various processes downstream of GCN2-eIF2α-ATF4 pathway, with a reliance on GCN2 activation. Human head and neck squamous cell carcinoma, glioblastoma, and breast cancer cells were shown to over-activate the GCN2/ATF4 pathway to promote tumour growth and angiogenesis by upregulation of vascular endothelial growth factor (VEGF) (Y. Wang et al. 2013). The increased VEGF expression and subsequent blood vessel formation increases the overall blood supply and total nutrients to the cell. Thereby, by increasing the tumour blood supply via the GCN2/ATF4/VEGF pathway, gaining an increase in the overall supply of nutrients while also the ability to better overcome any amino acid starvation conditions. Furthermore, knockdown of GCN2 in human head and neck squamous xenografts reduced VEGF expression leading to the tumours containing fewer blood vessels as compared with the wildtype control (Y. Wang et al. 2013). Moreover, the xenografted tumours failed to gown during GCN2 knockdown. This suggests that GCN2 is required to support adequate tumour blood supply and tumour survival. Another effector of the GCN2/eIF2α/ATF4 pathway is the glutamine amino acid transporter alanine, serine, cysteine-preferring transporter 2 (ASCT2; SLC1A5) (van Geldermalsen et al. 2016). SLC1A5 expression is correlated with the expression of ATF4, aiding in the uptake of glutamine. Furthermore, many breast cancers - including Triple-Negative Breast Cancer - upregulate SLC1A5 and is usually a sign for poor prognosis (van Geldermalsen et al. 2016). The expression of ATF4/SLC1A5 pathway was shown to be essential for tumour survival. Overall, there are multiple lines of evidence that the overactivation of GCN2, leading to the overexpression of effectors involved in supply and synthesis of nutrients supports tumour formation and promotes cancer cell survival.

1.8. GCN2’s role in determining cancer cell fate

If the cell cannot overcome a stressor, the cell can abandon the attempt to restore cell homeostasis and instead, the ISR/AAR drives the activation of cell death pathways

(Pakos-Zebrucka et al. 2016). There are multiple types of cell death in which amino acid starvation induces, such as necrosis and apoptosis (Jiangbin Ye et al. 2010; Xiao et al. 2016; M.-S. Chen et al. 2017; Rajanala, Ringquist, and Cryns 2019). Apoptosis is an intrinsic cellular pathway which begins by cleavage of caspase proteins leading to their activation. The caspase family of proteins are proteases which selectively degrade proteins vital to cell function, ultimately degrading the entire cell. A crucial checkpoint in the caspase-apoptosis pathway is the cleavage and subsequent activation of Caspases-3 and 7. Once the Caspase-3/7 proteins have been cleaved; the cell cannot revert from undergoing apoptosis. The cleavage of Caspases-3/7 is a vital checkpoint which can be used to confirm the activation of apoptosis and determine the final cell fate. Apoptosis activation has been linked to being activated by multiple different proteins, including the GCN2-eIF2α pathway.

In response to amino acid starvation, the level of GCN2/eIF2α activation has been linked to the regulation of apoptosis by cancer cells (Liu et al. 2010; W. Chen et al. 2015; Xiao et al. 2016). The GCN2-eIF2α pathway has been proposed to act as a "switch" between restoring homeostasis at high phosphorylation levels - overcoming the stressor - or at low phosphorylation activating apoptosis (Liu et al. 2010). Various types of cancer cells activate apoptosis during times of amino acid starvation. Furthermore, when GCN2 was knocked down, the cells were found to increase the activation of apoptosis. More recent research has identified the activation of apoptosis in response to leucine by MDA-MB-231 and MCF-7 cells, whereas, cysteine starvation induces necrosis in MDA-MB-231 cells (Xiao et al. 2016; M.-S. Chen et al. 2017). Both of the recent studies did not progress into GCN2 knockdown experiments to determine any GCN2-eIF2α-apoptosis link. However, they provide support for the activation of cell death pathways in response to amino acid starvation.

1.9. GCN2 is critical for cancer cell survival

GCN2 is critical for cancer cell survival (Jiangbin Ye et al. 2010; Y. Wang et al. 2013). By the use of silencing GCN2 mRNA by siRNA or shRNA, reducing GCN2 protein

levels, multiple studies have shown a reduction in cancer cell growth or viability in response to GCN2 knockdown. In mice xenograft models, the human fibrosarcoma cell line HT1080 tumours failed to grow during knockdown of GCN2 in comparison to their wildtype controls (Jiangbin Ye et al. 2010). Moreover, GCN2 knockdown also reduced the tumour growth rate of the human head and neck squamous cell carcinoma cell line UM-SCC-22B in mouse xenografts (Y. Wang et al. 2013). Recently, A549 lung adenocarcinoma cells, treated with GCN2 knockdown, were shown to have an increased percentage of cell death in comparison to untreated samples (Parzych et al. 2019). These studies indicate that GCN2 is essential for some types of cancer cells to survive *in vivo* and *in vitro*. With the identification of GCN2 as a potential therapeutic target to treat cancer.

1.10. GCN2 inhibition as a possible anti-cancer therapy

Since GCN2 is identified as critical for tumour cell survival, there have been several recent attempts at the creation of a GCN2 inhibitor to treat cancer (Lough et al. 2018; Nakamura et al. 2018; Fujimoto et al. 2019). By targeting GCN2's kinase domain, each proposed compound removes the ability for GCN2 to phosphorylate eIF2α. Thereby inhibiting the cells ability to respond to amino acid starvation and sensitising the cancerous cells to the amino acid starvation condition leading to increased cell death. Thus far the proposed GCN2 inhibitors have demonstrated a decrease in a variety of cancer cell types in conjunction with ASNase treatment - depletion of serum asparagine (Lough et al. 2018; Nakamura et al. 2018; Fujimoto et al. 2019). Moreover, the sensitivity to ASNase and GCN2 inhibitor treatment was further increased in cells expressing low Asparagine synthetase (ASNS) expression. However, the GCN2 inhibitor compounds have poor selectivity or solubility, leaving further modifications required before progressing with further drug trials. Nonetheless, these studies illustrate the promise of GCN2 as a potential drug target for cancer. With their focus on ASNase treatments, they also leave the possibility that a GCN2 inhibitor could be more effective if used with other amino acid starvation conditions. Such as one that the cancers rely on GCN2 the most to overcome the starvation condition.

1.11. Hypothesis and aims of this research

Recent studies have begun investigating the effects of a specific amino acid starvation condition on breast cancer cells (Xiao et al. 2016; M.-S. Chen et al. 2017; Dilshara et al. 2017). While these studies show a reduction in cell viability in response to a specific amino acid starvation, none have completely investigated the activation of the amino acid response (AAR) pathway. Specifically, they did not compare GCN2 activation with eIF2α phosphorylation. This leaves a gap of knowledge of a potential difference in the activation level of AAR by breast cancer in response to a specific amino acid starvation condition. To investigate a specific amino acid AAR by breast cancer, we will use the model of the triple-negative breast cancer (TNBC) cell line MDA-MB-231. TNBC cells are negative for estrogen/progesterone receptors and HER2 - commonly used as targets to treat breast cancer - with a poor prognosis due to lack of treatment options. We will screen for GCN2 activation and eIF2α phosphorylation in response to leucine or glutamine starvation. From the GCN2 activation and eIF2α phosphorylation result, we will interpret the relative activation of the Amino Acid Response pathway by the MDA-MB-231 cells. We hypothesise that GCN2 activation and eIF2α phosphorylation will be more significant in response to leucine starvation in comparison with glutamine starvation. This difference is expected due to leucine being an essential amino acid, whereas glutamine is non-essential.

Amino acid starvation reduces cell proliferation and induces apoptosis (Jiangbin Ye et al. 2010; Xiao et al. 2016; M.-S. Chen et al. 2017; Rajanala, Ringquist, and Cryns 2019). Thereby, during the leucine or glutamine starvation conditions, we will also screen for any change in MDA-MB-231 cell proliferation and activation of apoptosis. We hypothesise similar to the AAR, there will be a more significant decrease in cell proliferation and activation of apoptosis in response to leucine starvation, in comparison to glutamine starvation, by MDA-MB-231 cells. This is again expected due to leucine being an essential amino acid, whereas, glutamine is a non-essential amino acid.

Several studies have begun investigating reducing GCN2 function during amino acid starvation as a potential method to fight cancer (Jiangbin Ye et al. 2010; Y. Wang et al.

2013; Nakamura et al. 2018; Lough et al. 2018; Rajanala, Ringquist, and Cryns 2019; Parzych et al. 2019). To our knowledge, there is no research comparing breast cancers GCN2 activation response during single amino acid starvation and effect during GCN2 inhibition. We predict an amino acid starvation condition which activates GCN2 more than others, would cause the cell to become more susceptible to GCN2 inhibition in addition to the amino acid starvation condition. To test this hypothesis, we compared the effect of glutamine and leucine starvation during GCN2 knockdown of MDA-MB-231 cell viability and their activation of apoptosis. Thereby, we can determine the susceptibility of MDA-MB-231 cells during GCN2 knockdown and the different amino acid starvation conditions. We can also relate these findings to the relative activation of the amino acid response pathway. Thus, allowing further comprehension of how the molecular cellular response to specific amino acid starvation conditions and inhibition of GCN2 affect cell fate.

1.12. Relevance

While there is evidence of the starvation of specific amino acids causing different amounts of decreased cancer cell proliferation, the molecular cell response remains unclear. In response to amino acid starvation, GCN2 becomes active and phosphorylates eIF2α during the Amino Acid Response. Nevertheless, for an unknown reason, no one has yet screened and compared for the activation of GCN2 by cancerous cells during the different amino acid starvation conditions. This study will allow the comparison of MDA-MB-231 cells amino acid response between glutamine or leucine starvation. The cells AAR are interpreted by the levels of active GCN2 and phosphorylated eIF2α in response to glutamine or leucine starvation. We will also be able to relate the amino acid response pathway activation and corresponding cell fate by determining the cell proliferation and apoptosis levels.

With the recent interest in GCN2 inhibitors (Lough et al. 2018; Nakamura et al. 2018; Fujimoto et al. 2019), this study will aid in determining what experimental conditions are the best to score for the AAR with relation to cell fate. Moreover, these results will help determine which amino acid starvation would be best to use in conjunction with a GCN2 inhibitor. By screening for the relative GCN2 activation levels between starvation conditions, we aim to determine if there is a link between the amount of GCN2 activation and the sensitivity to GCN2 knockdown by breast cancer cells. Due to the activation of GCN2 in many cancer types, our results may apply to other cancers than breast cancer.

2. Materials/Methods

2.1. Cell Culture

The human breast cancer cell line MDA-MB-231 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Thermo Fisher Scientific, #11965-092), supplemented with additives shown in Table 2.2.1. The cells were incubated in a controlled climate of 5% CO_2 at 37^{o}C. The MDA-MB-231 cells used for experiments were between 4-30 passages.

2.2. Amino Acid Starvation

MDA-MB-231 cells were seeded in a 6-well plate at 0.15 x 10^5 cells/well. The cells were incubated for 24-hours to allow for the cells to adhere before beginning any experiment. For each starvation condition, the culture was washed with 0.1M Phosphate Buffered Saline (PBS) (MERK, #P5368) and the medium was switched to DMEM lacking a specific amino acid (Table 2.2.1). The cells were starved for 0, 3, 6, 24, and 48-hours, after which they were harvested for protein detection.

Table 2.2.1 - Dulbecco’s Modified Eagle’s Medium Formulations

Ingredient	Concentration	Source	Product Number
Additives to all Medium			
Penicillin/Streptomycin	1% V/V	Gibco Thermo Fisher Scientific	15140-122
Foetal Bovine Serum	10% V/V	Gibco Thermo Fisher Scientific	10270106
Glutamine Starvation Medium			
Dulbecco’s Modified Eagle’s Medium (DMEM), high glucose, pyruvate, no glutamine	89%	Gibco Thermo Fisher Scientific	10313039
Glutamine Replaced Medium - As Above with additive			
L-Glutamine (200 mM)	4mM	Gibco Thermo Fisher Scientific	25030081
Leucine Starvation Medium			
Dulbecco's Modified Eagle's Limiting Medium (DMEM-LM)	89%	Gibco Thermo Fisher Scientific	30030
L-Methionine	0.2mM	Gibco Thermo Fisher Scientific	A37733IN
Leucine Replaced Medium - As above with additive			
L-Leucine	0.80mM	Gibco Thermo Fisher Scientific	A37732IN

2.3. Harvesting cells

At the time of harvesting, the medium was removed, and cells were washed with PBS. Denaturing protein sample buffer was adapted from Silva, Castilho, and Sattlegger 2018 to create our Cell Lysis Solution. The Cell Lysis Solution was added to the wells with mechanical removal of the adhered cells. The resulting lysed sample was stored in a 1.5ml microfuge tube at -20ºC or -80ºC for extended periods. Frozen protein lysates were further denatured via mixing heating block for 10mins at 90ºC, at 1500 rpm before Western Blotting.

Cell Lysis Solution

0.625M Tris-HCl pH 6.8; 10% glycerol; 3% SDS; 0.5 mM EDTA; 5% (v/v) 2-mercaptoethanol; 0.1% (w/v) bromophenol blue

2.4. Western Blot

Each sample was subjected to 4-20% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE gel was made from mixing equal amounts of the 4% and 20% acrylamide stock solution with 10% APS and 1% TEMED. The solution was then cast in gel plates and left to solidify for 1-2hours. Once the gel was solidified, the samples were loaded onto the gel with electrophoresis carried out in a Bio-Rad Criterion Vertical Electrophoresis Cell and Protein Running Buffer. Once the proteins of the loaded samples were sufficiently separated – detected via the Invitrogen BenchMark™ Protein Ladder (Thermo-Fisher Scientific, #LTS10747012) – electrophoresis was stopped.

The proteins were then transferred to a 0.45µM nitrocellulose membrane (Bio-Rad, #1620115) via the wet transfer method. The wet transfer method was carried out by sandwiching the gel and the nitrocellulose membrane in the Bio-Rad Criterion™ Blotter and Transfer Buffer. After transfer, the nitrocellulose membrane was then subjected to immunoblotting using antibodies against each specific protein (Table 2.4.1). The primary antibodies were detected by secondary antibodies conjugated to horseradish peroxidase (Table 2.4.2). The blotted proteins were then detected using a

chemiluminescence detection solution (Pierce^{TM} ECL Plus Western Blotting Substrate, Thermo Fisher Scientific, #32132) with the Bio-Rad Chemidoc touch Imaging system.

Table 2.4.1 - Primary antibodies and their dilutions

Antibody	Dilution	Secondary Antibody	Incubation Time	Source	Product Number
GCN2-Phosphorylated (T899)	1:1000	Rabbit	15 Hours	Sigma-Aldrich	# AB75836
GCN2-Total	1:1000	Mouse	15 Hours	Sigma-Aldrich	#SAB1306336
eIF2α-Phosphorylated (S51)	1:1000	Rabbit	15 Hours	Sigma-Aldrich	#SAB4504388
eIF2α-Total	1:1000	Mouse	15 Hours	Sigma-Aldrich	#SAB4500
α-Tubulin	1:1000	Rabbit	2 Hours	Sapphire Bioscience	#GTX110717

Table 2.4.2 - Secondary antibodies and their dilutions

Secondary Antibody	Dilution	Incubation Time	Source	Product Number
Rabbit	1:50,000	1 Hour	Pierce	#31460
Mouse	1:10,000	1 Hour	Merk	#31460

SDS-PAGE Gel

<u>4% Acrylamide stock solution (100mL)</u>

40% Acrylamide 10mL

1.5M Tris-HCL pH8.8 25m10% SDS 1mL

; H_20 (MQ) 64mL

<u>20% Acrylamide stock solution (100mL)</u>

40% Acrylamide	50mL
1.5M Tris-HCL pH8.8	25mL
10% SDS	1mL
H_20 (MQ)	24mL

Protein Running Buffer (2.5L)

10% SDS	25 mL
10X Tris-glycine	250 mL
H_20 (MQ)	2225mL

10x Tris-glycine (1L)

Tris	30.3g
Glycine	144g

10x TBS pH 7.4 (1L)

Tris	24g
NaCl	80g
HCl	pH to 7.4

1.5M Tris pH8.8 (1L)

Tris	18.171g

Transfer Buffer (1L)

Methanol	200mL
10x Tris-glycine	100mL
H_20 (MQ)	700mL

2.5. Quantification of western signals

Western signals were quantified by Bio-Rad Image Lab™ Software 6.0.1. Each phosphorylated protein signal was normalised to 0-hour controls. The phosphorylation: total protein ratio is used to determine relative protein activation.

2.6. siRNA Oligonucleotides

Three different siRNA oligonucleotides were synthesised. Two are predicted to target GCN2 mRNA, and one siRNA oligonucleotide predicted to target GFP mRNA for an off-target control. The siRNA oligonucleotides were used to knockdown GCN2 mRNA. Oligonucleotides will not be provided in this report on the supervisor's request for confidentiality of proprietary information.

2.7. Small Interfering RNA (siRNA)-mediated gene-specific knockdown

MDA-MB-231 cells were seeded at 4 x 10^5 cells/well (70-90% confluence) in a 6-well plate. Cells were left to adhere for 24-hours. Lipofectamine 3000 Reagent (Invitrogen™, Thermo Scientific-Technologies, L3000001) and 30 pM of siRNA DNA combined in Opti-MEM (Gibco ™, Thermo Scientific-Technologies, #31985088) were incubated for 10-mins at room temperature. The siRNA-reagent mixture was then added to each seeded cell well and incubated for 24, 48, 96, and 148-hours for the initial protein knockdown efficacy trial, or 24-hours before the beginning of other experiments.

2.8. Proliferation Assay

Cell Proliferation was measured using the sulforhodamine B (SRB, Abcam, ab235935) assay, measuring total protein content. To screen for cell proliferation MDA-MB-231 cells were seeded in a 96 well Plate at 5,000 cells/well. Cells were left for 24-hours to adhere to the well. After 24-hours adherence, the cells were washed with PBS, and the medium was replaced with complete DMEM. The cells undergoing siRNA knockdown were treated as above with siRNA-reagent mixture (Chapter 2.7). After 24hours to ensure efficient GCN2 protein knockdown, each well was washed with PBS and Medium was replaced with; complete DMEM, with or without FBS, Glutamine/ Leucine starvation medium, and with or without FBS for the positive control (Table 2.2.1).

After 0, 3, 6, 24, 48, and 96-hours, the cells were fixed for 1 hour at 4 °C with 100 mg/mL trichloroacetic acid. Cells were subsequently stained with SRB solution for 15mins and then washed repeatedly with 10 mg/mL acetic acid. The SRB protein-bound dye was dissolved in 10 mM Tris base solution for 10mins. An absorbance reading at 560 nm was obtained via the microtiter plate reader (FLUOstar Optima).

2.9. Cell Viability Assay

The cell viability of MDA-MB-231 cells was determined by the trypan blue assay. The MDA-MB-231 cells were seeded at 1.0×10^{5} cells/well in a 6-well plate. Cells were left to adhere for 24-hours. MDA-MB-231 cells undergoing GCN2 silencing were treated as per Chapter 2.7. At the beginning of the experiment, cells were washed with 0.1M Phosphate Buffered Saline (PBS) pH7.4 (MERK, #P5368) and the medium was switched to DMEM with or without glutamine of leucine (Table 2.2.1). For the positive control, the cells were incubated in DMEM without FBS.

After 24 and 48-hours, the medium was removed from each well and the cells were washed with 0.1M Phosphate Buffered Saline (PBS) (MERK, #P5368). Adhered cells were then detached using 100µl 0.5% Gibco Trypsin-EDTA (Gibco Thermo Fisher Scientific, #15400054). After 5 minutes, the trypsin was deactivated with 300µl DMEM. From the suspended cell solution, 50µl was incubated with 50µl of 0.4% Trypan Blue solution (Gibco Thermo Fisher Scientific, #15250061). Immediately after incubation with the Trypan Blue solution, cells were placed on a hemocytometer slide, and percentage of blue-stained was determined by the Invitrogen Countess™ II FL Automated Cell Counter (Gibco Thermo Fisher Scientific, AMQAF1000). The percentage of dead blue-stained cells was used to infer MDA-MB-231 cell viability.

2.10. Apoptosis Assay

Cell apoptosis was determined by the percentage of positive fluorescent cells after incubation with the CellEvent™ Caspase-3/7 Green Detection Reagent. The MDA-MB-231 cells were seeded at 1.0 x 10^5 cells/well in a 6-well plate. Cells were left to adhere for 24-hours. MDA-MB-231 cells undergoing GCN2 silencing were as per Chapter 2.7. At the beginning of the experiment, cells were washed with 0.1M Phosphate Buffered Saline (PBS) (MERK, #P5368) and the medium was switched to DMEM with or without glutamine of leucine (Table 2.2.1). For the positive control, each well was treated with 1µM of Staurosporine (Fisher Scientific, #62996-74-1).

After 24 and 48 hours, the medium was removed from each well and the cells were washed with 0.1M Phosphate Buffered Saline (PBS) (MERK, #P5368). Adhered cells were then detached using 100µl 0.5% Gibco Trypsin-EDTA (Gibco Thermo Fisher Scientific, #15400054). After 5 minutes, the trypsin was deactivated with 300µl DMEM. From the suspended cell solution, 100µl was aliquoted into a 500µl Eppendorf tube and spun at 1000G for 5mins. The supernatant was removed, and cells were resuspended in 100µl of 2mM CellEvent™ Caspase-3/7 Green Detection Reagent (Thermo Fisher Scientific, #C10723), 0.1M Phosphate Buffered Saline (PBS) (MERK, #P5368), and 5% (v/v) foetal bovine serum (1:10, Gibco Thermo Fisher Scientific, #10270106) and incubated at 37^{o}C for 30 minutes. After 30 minutes incubation with the CellEvent reagent, cells were placed on a hemocytometer slide and % of positive

fluorescent cells were determined by the Invitrogen Countess™ II FL Automated Cell Counter (Gibco Thermo Fisher Scientific, AMQAF1000).

CellEvent™ Reagent mix

CellEvent™ Caspase-3/7 Green Detection Reagent	2mM
Phosphate Buffered Saline (pH 7.4)	0.1M
Foetal bovine serum	5%

2.11. Statistics

Data are presented as the mean ± Standard Error Mean (SEM). GraphPad Prism 8.2.1 was used for statistical analyses. Two-Tailed Students-Paired T-test was used to indicate the significance of results where applicable with a p-value of 0.05.

3. Results

3.1. Characterisation of the Amino Acid Response Pathway in MDA-MB-231 cells.

In times of amino acid deprivation, eukaryotic cells activate the amino acid response pathway to overcome amino acid starvation and restore cellular homeostasis. Initially, the cellular response begins with activation of General Control Non-depressible protein (GCN2). GCN2 activation is initiated by the detection of uncharged tRNA ($tRNA^{deacyl}$). The $tRNA^{deacyl}$ binds to GCN2. GCN2 becomes autophosphorylated into an active state. Active GCN2 subsequently phosphorylates serine-51 of the Eukaryotic Translation Initiation Factor 2α (eIF2α). The phosphorylated eIF2α alters the translational profile of the cell by reducing overall global protein synthesis, conserving amino acids, while simultaneously increasing translation of specific mRNA's involved in the recovery of amino acid starvation.

While previous studies have identified the ultimate cellular fate of breast cancer cells during amino acid starvation or screened for the starvation response via eIF2α phosphorylation or ATF4 upregulation, the initial starvation response - GCN2 activation – is poorly understood. With the recent development of GCN2 inhibitors and knockdown studies used in conjunction with amino acid starvation, the unknown relative activation levels of activated GCN2 in response to specific amino acid starvation conditions is crucial information that should be explored. If the GCN2 response differs between cells and type of amino acid starved, the ultimate cell fate of the cell may change as well when GCN2 is inhibited. For example, the removal or inactivation of GCN2 may sensitise the cell to a specific amino acid starvation condition leading to a greater reduction in proliferation.

In this work, we aim to characterise the Amino Acid Response (AAR) pathway of MDA-MB-231 cells in response to glutamine and leucine starvation. We also relate any

potential activation of the cell death pathway apoptosis to each starvation condition. Furthermore, we knock down the GCN2 protein of the AAR pathway and determine effects on MDA-MB-231 cells proliferation, and activation of apoptosis in response to glutamine or leucine starvation.

3.1.1. GCN2 and eIF2α are phosphorylated more in response to glutamine starvation than leucine starvation.

First, we aimed to characterise the starvation response of the MDA-MB-231 cell line to the starvation of glutamine or leucine amino acids. For this, we scored for the phosphorylation of the General Control Non-depressible (GCN2) and Eukaryotic Translation Initiation Factor 2α (eIF2α). To score for GCN2 and eIF2α phosphorylation levels, we seeded MDA-MB-231 cells in a 6-well plate dish in complete DMEM. The cells were left for 24-hours to adhere to the surface of the well. Amino acid starvation was initiated by removing the seeding medium and washing the cells with Phosphate-Buffered Saline (PBS). After washing, the medium was replaced with either DMEM missing a specific amino acid or DMEM with the missing amino acid supplemented. After 0, 3, 6, 24, and 48-hours of incubation, the cells were harvested using the cell lysis solution described in the methods. The generated cell extracts were subjected to SDS-PAGE gel electrophoresis to separate proteins by size from each sample. The separated proteins were then transferred from the SDS-PAGE gel, via the wet transfer method, to a nitrocellulose membrane, and incubated with antibodies to phosphorylated GCN2 (GCN2-P), total GCN2, phosphorylated eIF2α (eIF2α-P), and total eIF2α. The antibodies were visualised on the membrane using horseradish peroxidase (HRP) linked secondary antibody and a chemiluminescence substrate solution. The relative band intensity was used to infer the amount of the specific phosphorylated or total protein present at each time point.

To determine the relative phosphorylation levels of each protein, we scored for GCN2-P and eIF2α-P and the total amount of each protein. Next, we determined the level of protein phosphorylation by the relative ratio between phosphorylated and total protein. The ratio between the phosphorylated: total protein ratio is used to control for fluctuations of protein amount between samples. Each timed sample was further normalised to their respective 0-hour complete medium control, consistent for each western blot. This allows the comparison between timed samples and different western blots. Overall, we compared the relative GCN2 and eIF2α phosphorylation levels during starvation of glutamine or leucine for 0, 3, 6, 24, and 48 hours.

The amino acid response pathway proteins GCN2 and eIF2α are activated in response to amino acid starvation (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). Thereby, in response to each amino acid starvation, we expected to see an increase in GCN2 phosphorylation and subsequent eIF2α phosphorylation, relative to the complete medium controls. An increase in GCN2 phosphorylation - relative to control samples - would suggest that GCN2 is activated more and the cell is responding to the starvation. The level of cellular response to starvation is determined by the amount of phosphorylated GCN2 and eIF2α, with a more significant amount of phosphorylated GCN2/eIF2α suggesting a greater cellular response. Furthermore, as leucine is an essential amino acid compared to glutamine being non-essential, we would expect to observe a greater AAR response - increased GCN2 activation and eIF2α phosphorylation - to leucine starvation in comparison to glutamine starvation.

3.1.2. Glutamine starvation leads to significant GCN2 activation and eIF2α phosphorylation

GCN2 phosphorylation was increased 1.7-fold - relative to the complete medium controls - after 3 hours of glutamine starvation with a further significant 6.96-fold increase after 6 hours (Figure 3.1.1). GCN2 phosphorylation was still increased after 24 hours, but this was not significant and lower when compared to the 6-hour GCN2

phosphorylation level, 4.26-fold difference. Finally, GCN2 phosphorylation further decreased after 48-hours of glutamine starvation, with a similar 1.7-fold increased phosphorylation level relative to the control and the 3-hour samples. There was an unexpected increase in GCN2 phosphorylation in the unstarved samples 3 hours after the medium change. The initial increase in GCN2 phosphorylation suggested activation of GCN2 after 3 hours in response to glutamine starvation. The GCN2 activation response appeared to be sustained for at least 6 hours but decreases after 24 hours of glutamine starvation. GCN2 activation normalisation begun to occur after 48 hours. Moreover, the increase in GCN2 phosphorylation after 3 hours in the complete medium samples suggest some activation of GCN2 after undergoing the PBS washing and medium switching process.

MDA-MB-231 cells increased eIF2α phosphorylation in response to glutamine starvation. After 3 hours of glutamine starvation, eIF2α phosphorylation increased 1.8-fold relative to the complete medium controls (Figure 3.1.2). Our largest observed increased phosphorylation occurred after 6 hours of starvation with a 2.4-fold increase - this pattern was similar to that of GCN2 phosphorylation (Figure 3.1.1). After 24 hours of glutamine starvation the relative eIF2α phosphorylation level was still increased two-fold when compared to control, but lower than the 6-hour phosphorylation, similar to the GCN2 phosphorylation. Finally, after 48 hours of glutamine starvation, the eIF2α phosphorylation had returned to levels below the unstarved control and similar to the unstarved control. The increased eIF2α phosphorylation suggests a clear cellular response to glutamine starvation after 3 hours. This response is maintained up to 24 hours of glutamine starvation, with a decrease to a similar phosphorylation level when compared to the complete medium response after 48 hours.

Overall, these results suggest that the cellular amino acid response (AAR) pathway is activated in response to glutamine starvation by MDA-MB-231 cells. GCN2 is activated and eIF2α phosphorylated within 3 hours in response to glutamine starvation. As expected, activated GCN2 correlated with an increased eIF2α phosphorylation. After 24 hours of glutamine starvation, the GCN2 and eIF2α phosphorylation decreased,

suggesting that the AAR response is decreased and the MDA-MB-231 cells are either overcoming the starvation or abandoning the stress response and potentially activating alternative response mechanisms, with the possibility of activating cell death pathways.

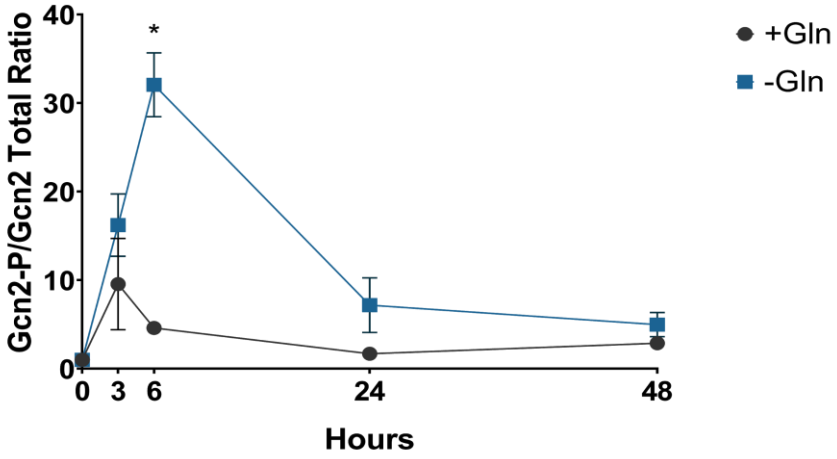

Figure 3.1.1 - MDA-MB-231 cells increase GCN2 phosphorylation in a time-dependent manner in response to glutamine starvation.

MDA-MB-231 cells were incubated in control (+Gln) medium or glutamine deficient medium (-Gln) for the time indicated, followed by western blotting using antibodies against the phosphorylated and total GCN2 protein (Figure 6.1-2). The data shown is the ratio between the phosphorylated GCN2 and the total Gcn2, normalised to the 0-hour +Gln control. Values shown are the means ±Standard Error Mean (n=4). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

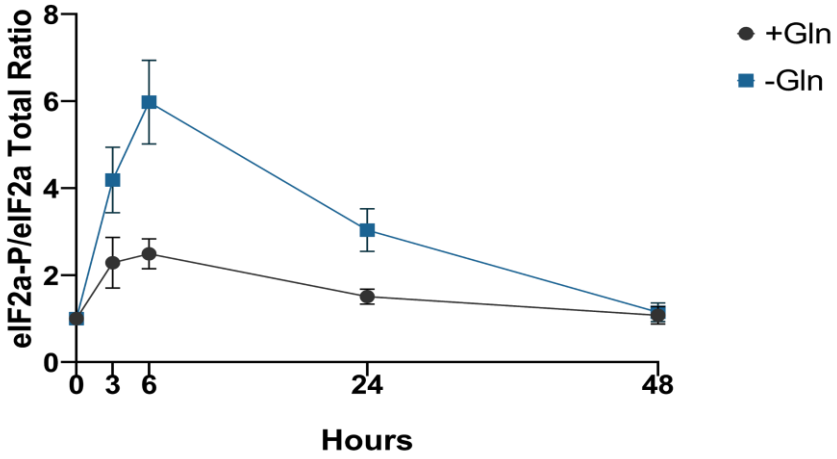

Figure 3.1.2 - MDA-MB-231 cells increase eIF2α phosphorylation in a time-dependent manner in response to glutamine starvation.

MDA-MB-231 cells were incubated in control (+Gln) medium or glutamine deficient medium (-Gln) for the time indicated, followed by western blotting using antibodies against the phosphorylated and total eIF2α protein (Figure 6.1-2). The data shown is the ratio between the phosphorylated eIF2α and the total eIF2α, normalised to the 0-hour +Gln control. Values shown are the means ±Standard Error Mean (n=4). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05)

3.1.3. Leucine starvation leads to a modest increase of GCN2 and eIF2α phosphorylation

In order to score for the effect of leucine starvation on GCN2 activation and eIF2α phosphorylation, the same experiment was done as above (Chapter 3.1.1-2), with the medium lacking leucine instead of glutamine. During the leucine starvation, we observed a moderate increase in GCN2 phosphorylation by MDA-MB-231 cells (Figure 3.1.3). The GCN2 phosphorylation level was significantly increased at all but the 6-hour time point during leucine starvation compared to the unstarved controls and the 0-hour unstarved control. After 3 and 6 hours of leucine starvation, GCN2 phosphorylation was increased by 1.6 and 1.5-fold, respectively. After 24 of leucine starvation, we observed an increase in GCN2 phosphorylation by 3.4-fold. GCN2 phosphorylation was further increased after 48 hours of starvation. However, a concurrent increase in GCN2 phosphorylation was observed at 24 and 48 hours in the controls. The concurrent increase of GCN2 phosphorylation during starvation as well as the unstressed samples suggest that the cells could have utilised all of the supplemented leucine or be responding to another unknown stressor and not leucine starvation. The modest increase in GCN2 phosphorylation suggests the MDA-MB-231 cells are initially activating GCN2 in response to leucine starvation. However, after 24 and 48 hours, another stressor could also be activating GCN2.

MDA-MB-231 cells, in response to leucine starvation, were observed to be modestly increase eIF2α phosphorylation (Figure 3.1.4). After 6 hours of leucine starvation, we observed the greatest increase of 2.2-fold of eIF2α phosphorylation when compared to all other time points. However, the variability between repeats causes this observation questionable. After 48 hours of leucine starvation, the eIF2α phosphorylation levels had decreased to the same level as the complete medium controls and similar to the 0-hour control phosphorylation level.

Overall, these results suggest that in response to leucine starvation, MDA-MB-231 cells moderately activate GCN2. However, the pattern of increased GCN2 phosphorylation levels throughout the leucine starvation did not correlate with the pattern of eIF2α phosphorylation, unlike glutamine starvation. This raises the possibility of a different eIF2α-kinase being involved in activation of eIF2α phosphorylation instead of, or addition to, GCN2 in response to leucine starvation. Therefore, in response to leucine starvation, it is unclear whether the cell is ultimately activating the AAR pathway.

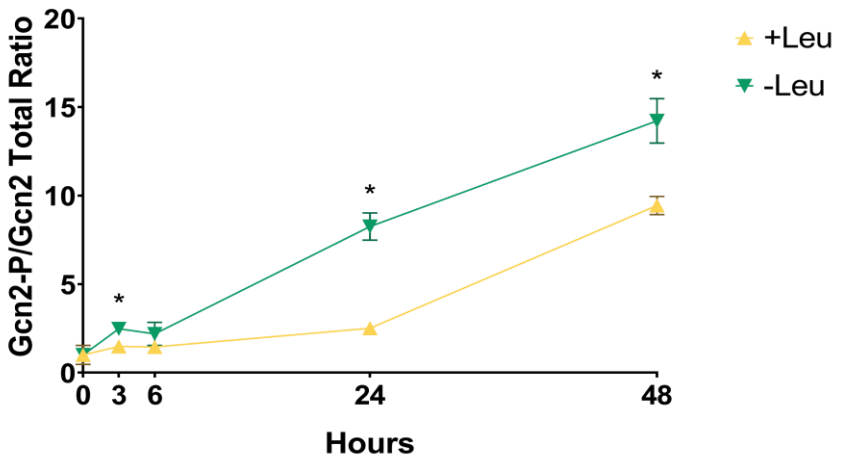

Figure 3.1.3 - MDA-MB-231 cells increase GCN2 phosphorylation in a time-dependent manner in response to Leucine starvation.

MDA-MB-231 cells were incubated in control (+Leu) medium or leucine deficient medium (-Gln) for the time indicated, followed by western blotting using antibodies against the phosphorylated and total GCN2 protein (Figure 6.3-4). The data shown is the ratio between the phosphorylated GCN2 and the total Gcn2, normalised to the 0-hour +Leu control. Values shown are the means ±Standard Error Mean (n=4). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05)

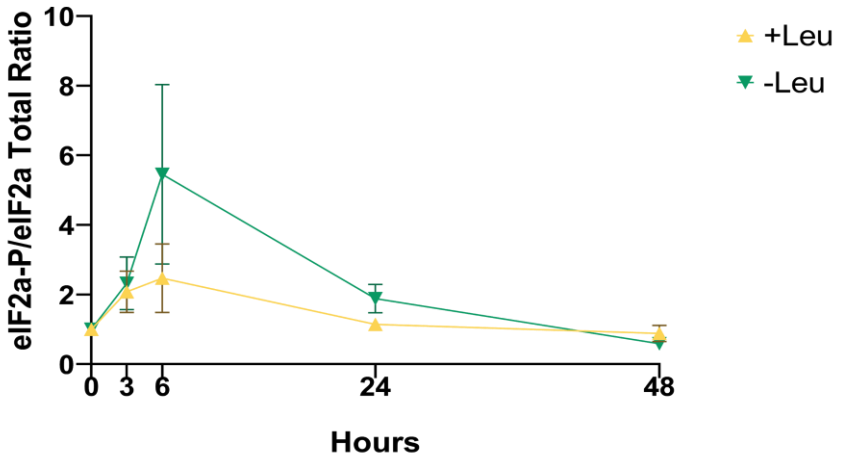

Figure 3.1.4 - MDA-MB-231 cells increase eIF2α phosphorylation in a time-dependent manner in response to glutamine starvation.

MDA-MB-231 cells were incubated in control (+Leu) medium or leucine deficient medium (-Leu) for the time indicated, followed by western blotting using antibodies against the phosphorylated and total eIF2α protein (Figure 6.3-4). The data shown is the ratio between the phosphorylated eIF2α and the total eIF2α, normalised to the 0-hour +Leu control. Values shown are the means ±Standard Error Mean (n=4). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05)

3.1.4. GCN2 and eIF2α phosphorylation are increased more during glutamine starvation than leucine starvation.

Once we had determined the GCN2 and eIF2α phosphorylation levels in MDA-MB-231 cells responding to glutamine and leucine starvation, we next compared the pattern of responses between the different amino acid starvation conditions. The experiments done above were completed simultaneously, allowing a comparison between all media conditions. We expected to see a greater activation of GCN2 and eIF2α phosphorylation in response to leucine starvation than glutamine starvation. This was expected due to leucine being an essential amino acid, whereas, glutamine is a non-essential amino acid.

Glutamine starvation led to a 6.5-fold higher GCN2 phosphorylation response after 3 hours in comparison to leucine starvation. This difference between the GCN2 phosphorylation response further increased after 6 hours starvation with a 14.6-fold difference (Figure 3.1.5). After 24 and 48 hours of starvation, GCN2 phosphorylation was reduced during glutamine starvation - similar to the controls - but it was increased during leucine starvation. However, in the leucine control samples also had elevated GCN2 phosphorylation after 24 and 48 hours. This comparison suggests a stronger initial activation of GCN2 in response to glutamine starvation than leucine starvation. However, after 48 hours of each amino acid starvation, GCN2 activation appears to be higher in response to leucine starvation.

Similar to GCN2 phosphorylation, we observed a greater increase in eIF2α phosphorylation in response to glutamine starvation than leucine starvation by MDA-MB-231 cells (Figure 3.1.6). However, different from GCN2 phosphorylation, we observed a similar trend of eIF2α phosphorylation over time between both starvation conditions. Both starvation conditions initially led to an increase in eIF2α phosphorylation. eIF2α phosphorylation decreased over time and returned to a similar phosphorylation level after 48 hours compared to the 0-hour sample.

Overall, these results suggest that MDA-MB-231 cells activate GCN2 more in response to glutamine than leucine starvation. This difference in GCN2 activation response is transmitted downstream of the AAR pathway to eIF2α phosphorylation, with greater eIF2α phosphorylation in response to glutamine starvation. The trend in eIF2α phosphorylation during each starvation condition was similar. Thereby, suggesting during glutamine starvation GCN2 is concurrent with eIF2α phosphorylation. Whereas, during leucine starvation, GCN2 activation does not necessarily correlate with eIF2α phosphorylation. These results suggest that GCN2 may not be initially activated in response to leucine starvation. However, in comparison, GCN2 is initially activated in response to glutamine starvation, leading to activation of the Amino Acid Response pathway.

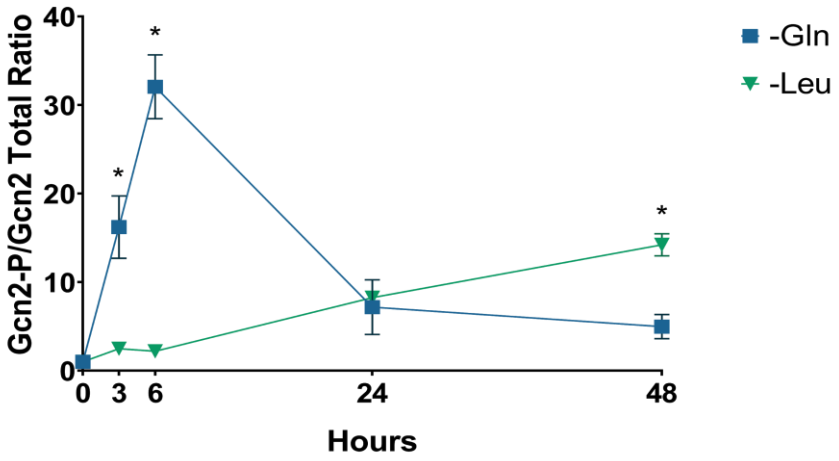

Figure 3.1.5 - MDA-MB-231 cells increase GCN2 phosphorylation in response to glutamine starvation more than leucine starvation.

MDA-MB-231 cells were incubated in control (+Gln/+Leu) medium - not shown - or glutamine/leucine deficient medium (-Gln/-Leu) for the time indicated, followed by western blotting and use of antibodies against the phosphorylated and total GCN2 protein (Figure 6.1-4). The data shown is the ratio between the phosphorylated GCN2 and total Gcn2, normalised to each 0-hour +Gln or +Leu control. Values shown are the means ±Standard Error Mean (n=4).

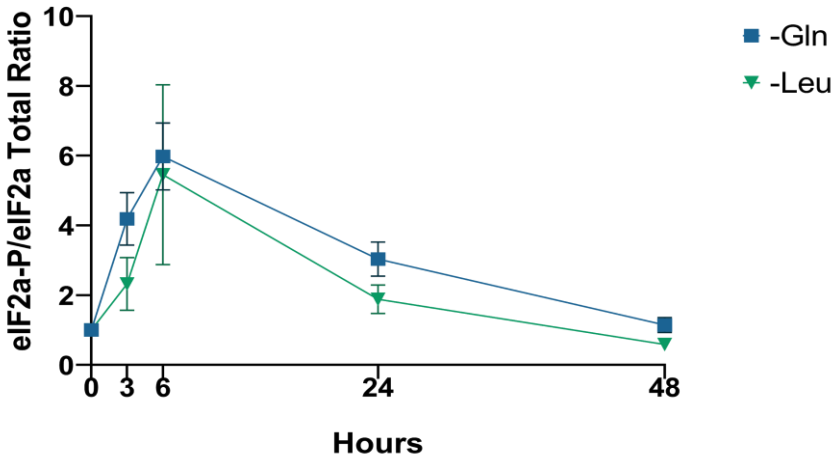

Figure 3.1.6 - MDA-MB-231 cells eIF2α phosphorylation is not significantly different in response to glutamine starvation or leucine starvation.

MDA-MB-231 cells were incubated in control (+Gln/+Leu) medium - not shown - or glutamine/leucine deficient medium (-Gln/-Leu) for the time indicated, followed by western blotting and use of antibodies against the phosphorylated and total eIF2α protein (Figure 6.1-4). The data shown is the ratio between the phosphorylated eIF2α and total eIF2α, normalised to each 0-hour +Gln or +Leu control. Values shown are the means ±Standard Error Mean (n=4).

3.1.5. The effect of glutamine and leucine starvation on the proliferation of MDA-MB-231 cells

Given that MDA-MB-231 cells respond differently to specific amino acid starvation, we next asked whether cell proliferation also differed between starvation conditions. As leucine is an essential amino acid, whereas glutamine is non-essential, we would expect to see a more significant decrease in cell proliferation in response to leucine starvation. To determine changes in proliferation, the SRB assay was used. For the SRB assay, MDA-MB-231 cells were seeded into a 96-well plate. These cells were left for 24 hours in replete DMEM to adhere to the well surface. After adherence, the seeding medium was removed, and the cells washed with PBS to remove any leftover medium. The medium was then replaced with DMEM lacking or supplemented with glutamine or leucine. After 0, 3, 6, 24 and 48 hours of additional growth, the cells were fixed with the SRB fixation solution. After fixation, the cells were subjected to the SRB dye assay. The SRB assay relies on the stoichiometric binding of the SRB dye to proteins under mildly acidic conditions. The SRB dye is then extracted using a basic solution, and the extracted dye absorbance is used to infer the number of cells present. Once the dye absorbance was calculated, we used the cells grown in complete medium absorbance readings as a 100% cell growth control to which the cells grown under starvation conditions were compared too.

In response to glutamine starvation, we observed a decrease in MDA-MB-231 cell proliferation (Figure 3.1.7 A & B). Cell proliferation was initially increased in the glutamine starvation medium. After 6 hours of glutamine starvation, cell proliferation significantly decreased to 62% of the complete medium control sample. This significant decrease was maintained after 24 and 48 hours of glutamine starvation. In comparison, leucine starvation did not significantly decrease MD-MB-231 cell proliferation (Figure 3.1.7 A & B). After 6 hours of leucine starvation, cell proliferation was decreased to 85% of the complete medium control. However, this result was not found to be

significant. Cell proliferation during leucine starvation remained at a similar percentage decrease, similar to the glutamine starvation decrease.

Overall, we observed a decrease in MDA-MB-231 cell proliferation during both glutamine and leucine starvation. This suggested that both glutamine and leucine are essential to support MDA-MB-231 cell proliferation. However, glutamine starvation led to a greater and significant decrease in cell proliferation when compared with leucine. The more significant decrease in cell proliferation in response to glutamine starvation suggests MDA-MB-231 cells rely more on glutamine than leucine to support cell proliferation.

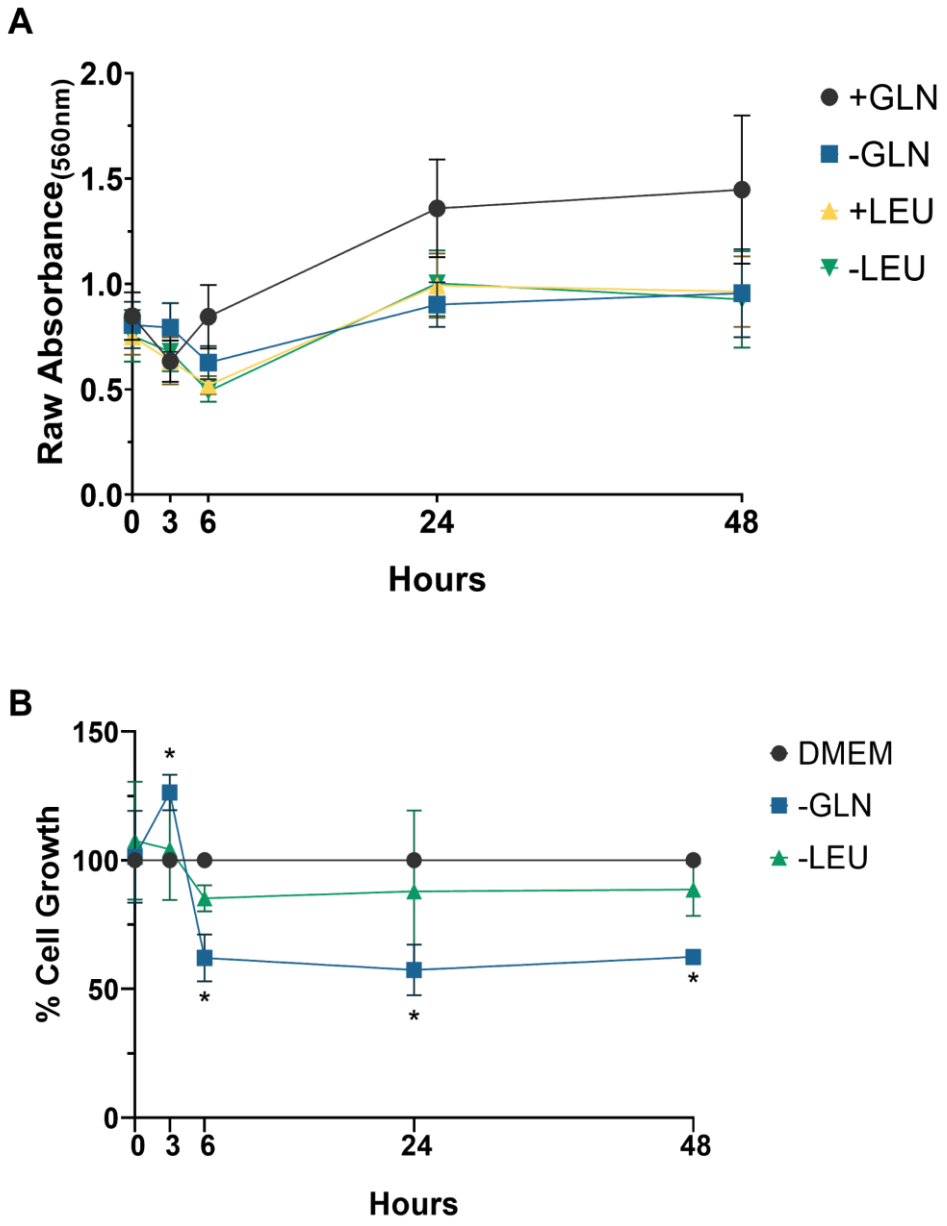

Figure 3.1.7 - Glutamine starvation reduced the rate of MDA-MB-231 cell proliferation more than leucine starvation.

MDA-MB-231 cells were plated in a 96-well plate and incubated in complete DMEM for 24 hours. After 24 hours, the medium was changed to control (+Gln/+Leu) medium or glutamine/leucine deficient media (-Gln/-Leu). Cell proliferation was determined by the SRB assay. The data shown is the % of inferred cell growth normalised to the complete medium control of the respective incubation time. 100% would mean the cell mass is the same as those grown during the same incubation time but in replete DMEM. **A)** Raw absorbance values form the SRB Assay. **B)** % cell growth values determined from the raw absorbance values. Values shown are the means ±SEMs (n=2-3). Statistical analysis was performed between the starvation conditions (-GLN/LEU) and the complete media controls (DMEM - +GLN/+LEU) using the Two-Tailed Student T-Test (*P<0.05).

3.1.6. MDA-MB-231 cells activate cell apoptosis during glutamine and leucine starvation

After 24 hours of either glutamine or leucine starvation, we observed a decrease in eIF2α phosphorylation levels (Chapter 3.1.1-4). This suggested that the cells were either overcoming the starvation conditions, activating an alternative mechanism. Our findings of decreased proliferation rates in response to glutamine and leucine starvation supported a hypothesis of the MDA-MB-231 cells abandoning the survival response and instead activate a cell death pathway (Chapter 3.1.5). Therefore, we investigated if MDA-MB-231 cells activated the apoptosis pathway. To determine if the cells were activating cell apoptosis, we utilised the CellEvent™ Caspase-3/7 Green Detection Reagent. This reagent is a four-amino acid peptide, consisting of; Aspartic acid, Glutamic acid, Valine, and Aspartic acid (DEVD), that is conjugated to a nucleic acid-binding dye. The DEVD peptide sequence is cleaved by the activated apoptosis enzymes Caspase-3 & 7, which releases the conjugated dye. The released dye is then able to bind to DNA and produces a fluorogenic response that can be detected under a fluorescence microscope. An increased amount of positive fluorescent cells would suggest that more cells have initiated their apoptosis pathway and are undergoing cell death.

To score for apoptosis, the MDA-MB-231 cells were seeded in 6-well plates and left to adhere for 24-hours in complete DMEM. At the beginning of the experiment, the medium was removed, and the cells were washed with PBS solution. After PBS washing the medium was replaced with DMEM lacking glutamine or leucine. For the negative control, the medium was replaced with DMEM supplemented with glutamine or leucine. For the positive control, cells were grown in different medium as above with the addition of 1µM Staurosporine, a known apoptosis inducer (McKeague, Wilson, and Nelson 2003). After 24- and 48-hours incubation, we removed the medium from the cells and detached the cells via 0.5% Trypsin-EDTA. Once cells were detached, a cell suspension mix was created by the addition of DMEM. The cell suspension mix was aliquoted and spun down, with the supernatant removed to yield only the cell pellet.

This cell pellet was resuspended in CellEvent™ Caspase-3/7 Green Detection Reagent - described in materials and methods. After 30-mins incubation with the CellEvent reagent, the cells were visualised for green fluorescence using the Countess™ II FL Automated Cell Counter. Positive fluorescent cells would indicate that apoptosis was activated within those cells.

Glutamine starvation was associated with an increase in the presence of fluorescent MDA-MB-231 cells (Figure 3.1.8). However, this increase was not found to be significant. The percentage of fluorescent cells decreased after 48 hours. However, the amount was still higher compared to the unstarved control. During unstarved conditions, there were some fluorescent cells observed, suggesting that under replete amino acid conditions, some cells may activate apoptosis. These results suggest that MDA-MB-231 cells in response to glutamine starvation cannot overcome the starvation condition and activate apoptosis after 24 and 48 hours.

Leucine starvation was associated with a significant increase in the activation of cell apoptosis by MDA-MB-231 cells (Figure 3.1.9). Similar to glutamine starvation, we observed the presence of a small percentage of cells under no amino acid starvation. During leucine starvation conditions, we observed a significant increase in fluorescent cells after 24 hours of starvation. After 48 hours there was a similar increase in fluorescent cells, but less than the 24 hour time point. These results suggest that similar to glutamine starvation, MDA-MB-231 cells are activating apoptosis in response to leucine starvation.

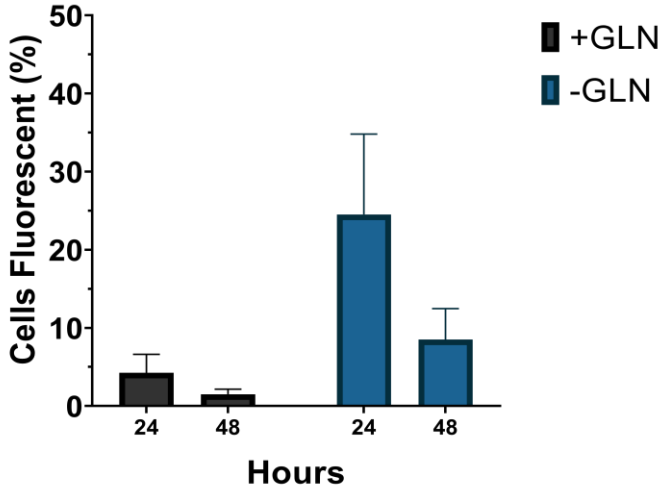

Figure 3.1.8 - Glutamine starvation induces apoptosis in MDA-MB-231 cells.

MDA-MB-231 cells incubated in control (+Gln) medium or glutamine/leucine deficient medium (-Gln) for 24 and 48 hours, followed by incubation with CellEvent™ Caspase-3/7 Green Detection Reagent. After CellEvent Reagent incubation, the cells were observed under a fluorescent microscope. The data shown is the % of positive fluorescent cells. Values shown are the means ±SEMs (n=2). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

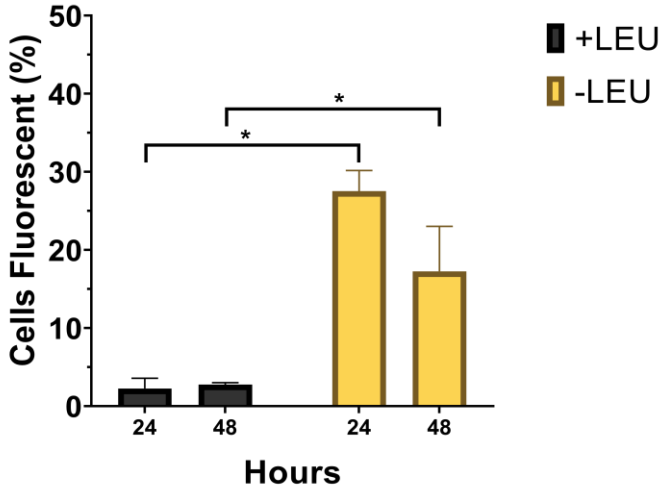

Figure 3.1.9 - Leucine starvation significantly induces apoptosis in MDA-MB-231 cells.

MDA-MB-231 cells incubated in control (+Leu) medium or glutamine/leucine deficient medium (-Leu) for 24 and 48 hours, followed by incubation with CellEvent™ Caspase-3/7 Green Detection Reagent. After CellEvent Reagent incubation, the cells were observed under a fluorescent microscope. The data shown is the % of positive fluorescent cells. Values shown are the means ±SEMs (n=2). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

3.2. Knockdown of GCN2 reduced MDA-MB-231 cell viability during replete and amino acid starved conditions.

GCN2 of the Amino Acid Response pathway (AAR) is activated to overcome amino acid starvation (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). However, if GCN2 is inhibited, we questioned how the MDA-MB-231 cells would respond during replete and starvation of amino acids. We tested whether MDA-MB-231 cells depend on GCN2 for proliferation, viability, and apoptosis during unstressed and amino acid starvation. To inhibit GCN2, we used two siRNA complexes predicted to knockdown *GCN2* mRNA. The siRNA complexes degrade *GCN2* mRNA, thereby preventing its translation and the cellular GCN2 protein levels. We first tested whether GCN2 knockdown was achieved to a sufficient extent before investigating cell proliferation, viability, and apoptosis. Once sufficient GCN2 knockdown was observed, we used the SRB Assay and the Trypan-Blue exclusion assay to infer cell proliferation. Using our two siRNA complexes individually and combined, we determined the efficacy of each GCN2 siRNA knockdown complex relative to a siRNA scrambled control.

3.2.1. GCN2 knockdown efficacy in MDA-MB-231 Cells

To knockdown GCN2, MDA-MB-231 cells were seeded in 6-well plates and incubated in complete DMEM and left for 24 hours to adhere. After 24 hours, we added our siRNA complexes and reagents, described in the methods, to our MDA-MB-231 cells. After siRNA treatment, the cells were incubated for 24 hours. After siRNA treatment, the cells for day one samples were harvested using our cell lysis solution described in our methods. Cells undergoing longer incubation times had their medium removed and were washed with PBS solution. The medium was replaced with complete DMEM,

without adding any extra siRNA complex. These cells were left for 2, 4, and 6 days post siRNA treatment and then harvested using the cell lysis solution described in methods.

The generated cell extracts were subjected to SDS-PAGE gel electrophoresis to separate the proteins by size from each sample. The separated proteins were transferred from the SDS-PAGE gel, via the wet transfer method, to a nitrocellulose membrane, and incubated with antibodies to Total GCN2 and α-Tubulin. The antibodies were visualised on the membrane using the HRP linked secondary antibody and a chemiluminescence substrate solution. The relative band intensity was used to infer the amount of the total protein present at each time point. We expect to observe a decrease in total GCN2 protein in the cell extracts which have undergone siGCN2 treatment and no GCN2 decrease in our siScr control.

As expected, in MDA-MB-231 cells, at all-time points, the total GCN2 protein levels were reduced by either of the two siRNA complexes used, and in particular when a combination of both was used (Figure 3.2.1). siGCN2-2 reduced GCN2 protein levels initially after 1 and 2 days. Subsequently, after 4 and 6 days, siGCN2-2 treated cells had an increased total GCN2 protein level, but still lower than the siScr control. The siGCN2-4 complex also reduced GCN2 protein levels at a similar efficacy over six days. However, this reduction was not as considerable as the siGCN2-2 complex. The mix of both siGCN2-2 and siGCN2-4 led to the most considerable GCN2 protein reduction. With the lowest GCN2 total protein over all six days. The off-target siRNA - siScr control did not appear to knockdown GCN2 protein levels. In the siScr control, there is a reduction of total GCN2 protein in the cells after 4 and 6 days. This reduction is most likely due to the relative levels of GCN2:α-Tubulin being skewed due to the increased numbers of cells after six days of incubation. Thereby, this data suggests our siRNA-complexes can knockdown the GCN2 protein from one to at least six days. Overall, the mix of both siGCN2-2 and GCN2-4 gave the highest GCN2 knockdown efficacy.

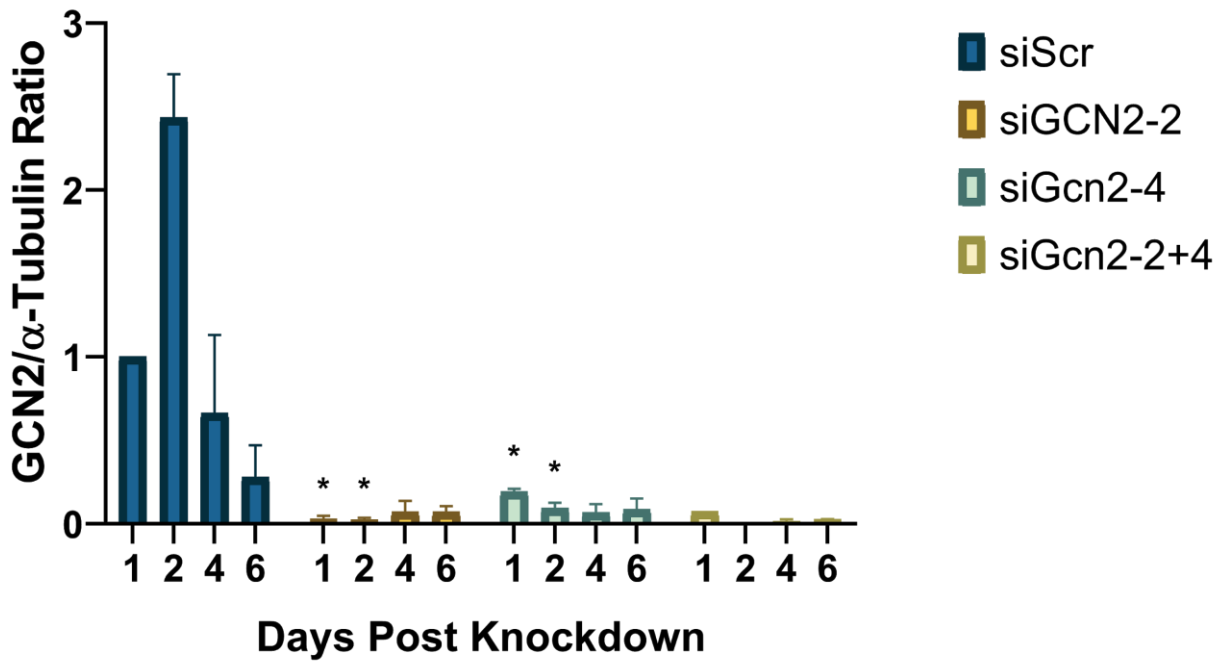

Figure 3.2.1 - siRNA mediated knockdown of GCN2 in MDA-MB-231 cells.

MDA-MB-231 cells incubated with a scrambled control siRNA (siScr) or a siRNA predicted to silence GCN2 mRNA; siGCN2-2, siGCN2-4, or both combined siGCN2-2+4.GCN2 knockdown efficacy was determined via western blotting, with the detection of the total GCN2 protein and α-Tubulin (Figure 6.5). The data shown is the ratio of the total GCN2: α-Tubulin protein, normalised to the 0-hour siScr control. Values shown are the means ±SEMs (n=2). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

3.2.2. SRB Assay was unsuitable for determining MDA-MB-231 cell proliferation during GCN2 knockdown and amino acid starvation.

After we had successfully established siRNA mediated knockdown of GCN2 protein levels, we next wished to determine the effects, if any, of GCN2 knockdown on MDA-MB-231 cell proliferation rates. First, we seeded the cells in a 96-well plate in complete DMEM. After 24 hours to allow the cells to adhere, the siRNA complexes were added to the wells undergoing siRNA treatment. After 24 hours of siRNA treatment, the medium was removed, and cells were washed with PBS solution. Post washing, new DMEM with supplemented or without glutamine or leucine was added to the wells. Subsequently after 0, 3, 6, 24, and 48 hours of starvation we attempted to use the SRB Assay - as explained above in section 3.1.2 - to determine any change in MDA-MB-231 cell proliferation during GCN2 knockdown and glutamine or leucine starvation. Unfortunately, there were no consistent results to be determined (Figures 3.2.2 - 4). We observed significant variations between repetitive experiments and experimental replicates. This led to observations in most of the silenced GCN2 samples of a decrease in SRB absorbance, relative to the siScr control, followed by an increase in SRB absorbance and then again, a decrease in SRB absorbance (Figures 3.2.2-4). Moreover, the significant variations between experiments led to large error bars and all non-significant observations of a difference in knockdown GCN2 SRB absorbance, relative to the siScr control.

Unlike our previous SRB assay where only the medium was changed to either a complete amino acid profile or glutamine or leucine starvation medium, this experiment included the initial siRNA knockdown portion before starvation. The siRNA knockdown compound can be cytotoxic to cells, causing the death to a portion of the cells (T. Wang et al. 2018). We hypothesised that the addition of siRNA led to sometimes more or less death of cells at the beginning of the starvation experiment. This change in initial cell number, from siRNA death, and sensitivity of the SRB Assay, led to the significant variations and inconsistent observations of this SRB Assay-GCN2

knockdown experiments in comparison to our previous SRB Assays. Therefore, we looked for alternative methods to determine MDA-MB-231 cell proliferation when GCN2 is knocked down during amino acid starvation.

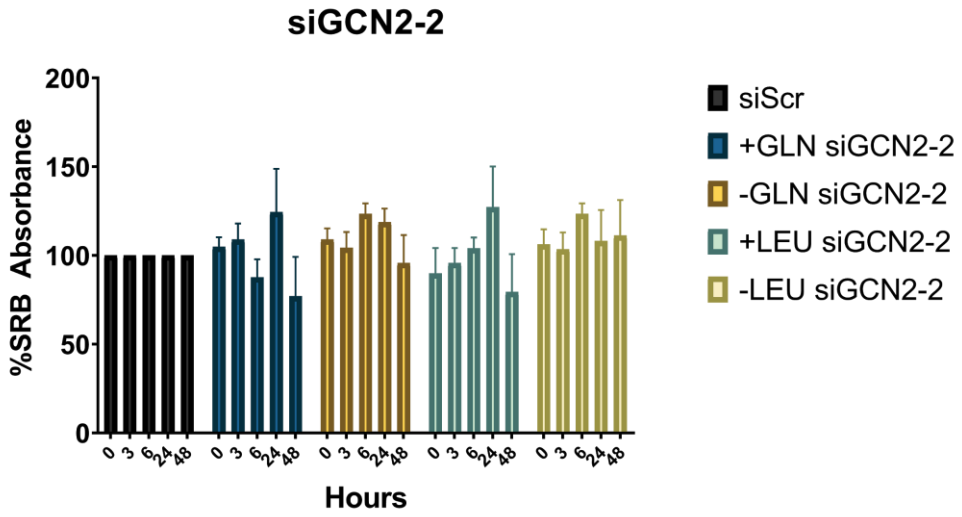

Figure 3.2.2 - The SRB Assay was unable to detect changes in MDA-MB-231 cell proliferation when incubated with siGCN2-2.

MDA-MB-231 cells incubated with a scrambled control siRNA (siScr) or siGCN2-2 to knockdown GCN2 protein within the cells. Cells were further subjected to incubation in complete medium (+Gln/+Leu) or glutamine or leucine amino acid starvation medium (-Gln or -Leu). Cell proliferation was determined by the SRB assay. The data shown is the % of SRB absorbance of the siGCN2-2 samples relative to the siScr control. Values shown are the means ±SEMs (n=3). Statistical analysis was performed using the two-Tailed Student T-Test (*P<0.05).

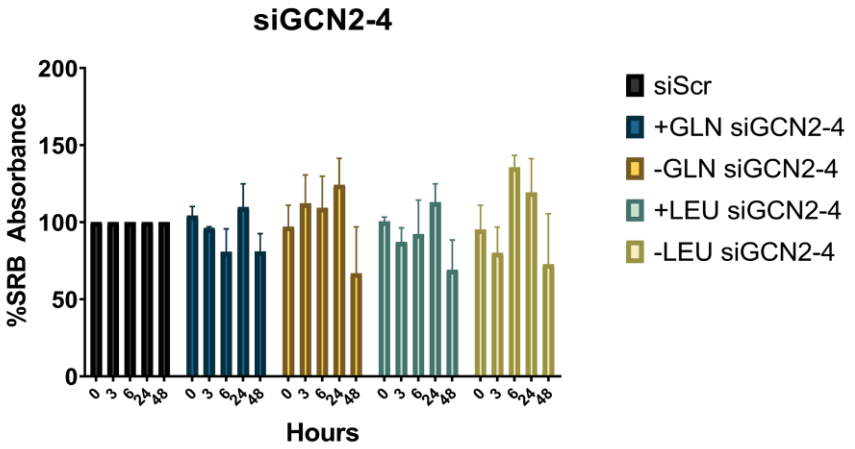

Figure 3.2.3 - The SRB Assay was unable to detect changes in MDA-MB-231 cell proliferation when incubated with siGCN2-2 siGCN2-4.

MDA-MB-231 cells incubated with a scrambled control siRNA (siScr) or siGCN2-4 to knockdown GCN2 protein within the cells. Cells were further subjected to incubation in complete medium (+Gln/+Leu) or glutamine or leucine amino acid starvation medium (-Gln or -Leu). Cell proliferation was determined by the SRB assay. The data shown is the % of SRB absorbance of the siGCN2-4 samples relative to the siScr control. Values shown are the means ±SEMs (n=3). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

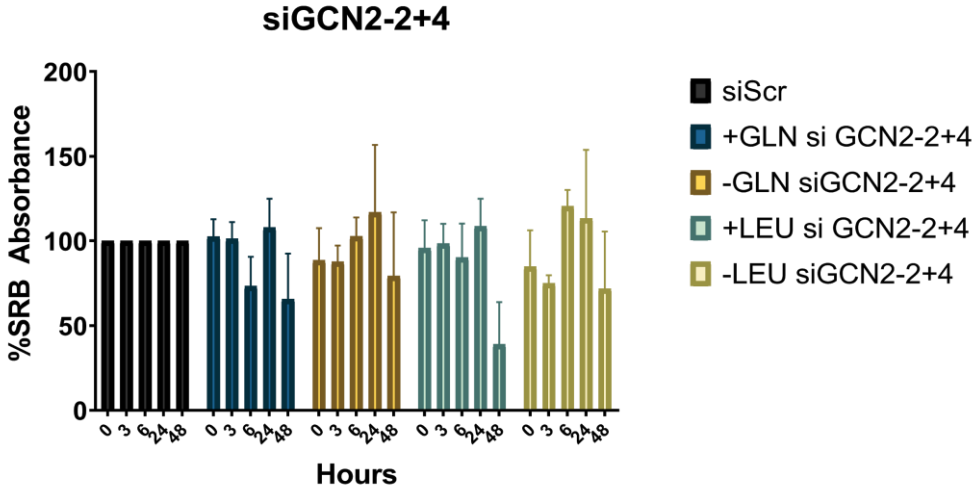

Figure 3.2.4 - The SRB Assay was unable to detect changes in MDA-MB-231 cell proliferation when incubated with siGCN2-2 siGCN2-2+4.

MDA-MB-231 cells incubated with a scrambled control siRNA (siScr) or siGCN2-2+4 to knockdown GCN2 protein within the cells. Cells were further subjected to incubation in complete medium (+Gln/+Leu) or glutamine or leucine amino acid starvation medium (-Gln or -Leu). Cell proliferation was determined by the SRB assay. The data shown is the % of SRB absorbance of the siGCN2-2+4 samples relative to the siScr control. Values shown are the means ±SEMs (n=3). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

3.2.3. Silencing GCN2 decreases cell viability during replete and amino acid starvation conditions.

Our SRB assay experiment was unable to determine any change in MDA-MB-231 cell proliferation during GCN2 knockdown and amino acid starvation conditions. Therefore, we utilised the Trypan Blue Exclusion Assay to determine cell viability. Trypan blue is a dye which passes through the cell membranes of dead cells, staining the cells blue. The membranes of live cells remain intact and exclude the dye, and therefore, remaining transparent. Thus, the viability of the cells can be determined as the ratio between the blue-stained “dead” cells and the transparent live cells. For these experiments, only the mix of the siGCN2-2 and the siGCN2-4 compound was used, shortened to siGCN2. As the control, we incubated the cells in medium lacking Fetal Bovine serum - essential for cell survival (van der Valk et al. 2010). We expect to observe a more significant number of dead cells - lower cell viability - in the GCN2 knockdown cells with an even greater decrease when the GCN2 knockdown cells undergo amino acid starvation.

To screen for changes in MDA-MB-231 cell viability, the cells were seeded in replete DMEM, and left for 24 hours to adhere to each well. After adherence, the cells undergoing siRNA treatment were incubated with each siRNA complex for 24 hours. Post siRNA treatment the medium, from each sample was removed, and the cells were washed with PBS solution. After washing the medium was replaced with the amino acid deficient DMEM or DMEM with replaced amino acids. The cells were further incubated for 24 or 48 hours. At each endpoint, the cells were detached and suspended using Trypsin-EDTA. Once the cells were suspended, the excess Trypsin-EDTA was deactivated using DMEM, creating a cell suspension solution. Trypan-blue solution was added to the cell suspension solution at a 50:50 mix and immediately visualised using the Countess™ II FL Automated Cell Counter to determine the cell viability.

We found that knockdown of GCN2 was associated with a reduction in MDA-MB-231 cell viability during replete amino acid conditions in our glutamine medium after 24 and 48 hours (Figure 3.2.5 A). There was a slight increase in cell viability after 48 hours in both our replete control siScr cells. This same phenomenon was also observed with the siGCN2 treated cells (Figure 3.2.5 B). Under glutamine starvation, GCN2 knockdown

dramatically reduced cell viability. After 24 hours of glutamine starvation, the viability of the siGCN2 cells was slightly reduced. After 48 hours of glutamine starvation, the GCN2 knockdown cells viability was observed to be at 0% in 4 replicates of 2 independent experiments. The reduction to 0% cell viability after 48 hours of glutamine starvation and siGCN2 treatment was significantly different in comparison the cells treated with scrambled siRNA. Together, these results suggest that GCN2 knockdown leads to a reduction in MDA-MB-231 cell viability under replete conditions, while under glutamine starvation, no cells were viable. This further suggests that GCN2 knockdown sensitises the cells to glutamine starvation, leading to a reduction in cell viability, ultimately leading to cell death.

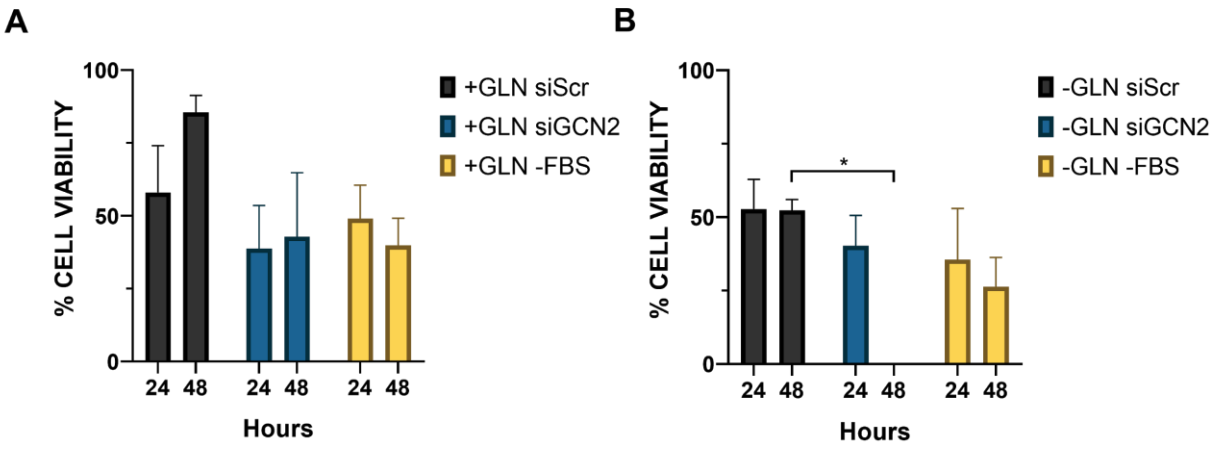

Figure 3.2.5 - GCN2 knockdown further reduced MDA-MB-231 cell viability during glutamine starvation.

MDA-MB-231 cells incubated with a scrambled control siRNA (siScr), siGCN2-2+4 to knockdown GCN2 protein within the cells, or without FBS as a positive control. (A) Cells were incubated in complete medium (+Gln). (B) Cells were incubated in glutamine deficient medium (-Gln). Cell viability was determined by the Trypan-Blue Assay. Values shown are the means ±SEMs (n=2). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

Our cells incubated in leucine replaced medium and treated with siGCN2 were observed to have a delayed decrease in cell viability, occurring after 48 hours (Figure 3.2.6 A). Incubation in leucine starvation medium was observed to have the same delayed decrease trend (Figure 3.2.6 B). These differences were too small to indicate any significance and a larger sample size would be required to validate any significance in the reduction of cell viability in our leucine medium samples. Similar to our glutamine medium, these results suggest that GCN2 knockdown reduces MDA-MB-231 cell viability during replete amino acid conditions. However, in comparison with our glutamine results, there was no reduction in cell viability after 48 hours of siGCN2 treatment in our leucine replaced medium. This suggests some unknown differences between each replete DMEM. Furthermore, the leucine starvation with knockdown of GCN2 results suggests, GCN2 knockdown sensitises the cells to leucine starvation, leading to a greater reduction in cell viability and increased cell death.

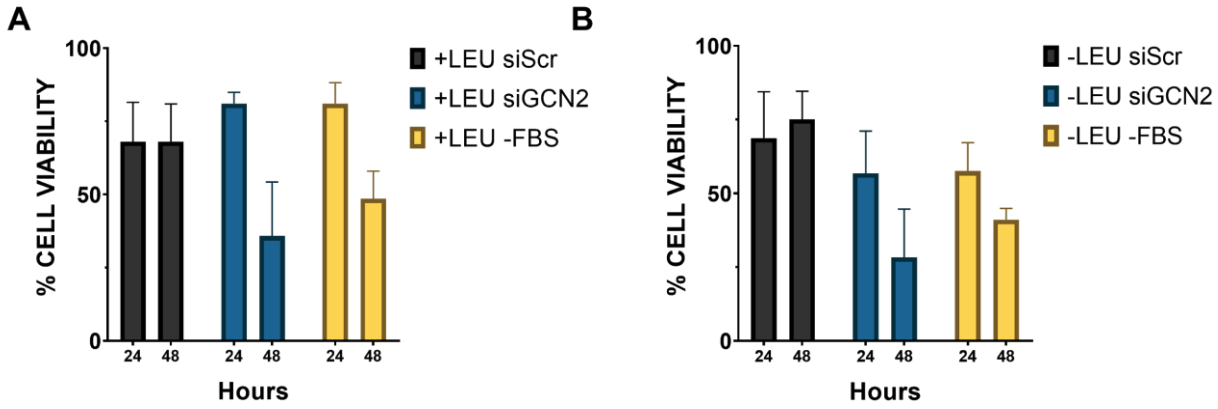

Figure 3.2.6 - GCN2 knockdown further reduced MDA-MB-231 cell viability during leucine starvation.

MDA-MB-231 cells incubated with a scrambled control siRNA (siScr), siGCN2-2+4 to knockdown GCN2 protein within the cells, or without FBS as a positive control. (A) Cells were incubated in complete medium (+Leu). (B) Cells were incubated in leucine deficient medium (-Leu). Cell viability was determined by the Trypan-Blue Assay. Values shown are the means ±SEMs (n=2). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

3.3. Silencing GCN2 in MDA-MB-231 cells leads to increased activation of apoptosis in response to amino acid starvation

In chapter 3.1, we observed a decrease in cell proliferation under both glutamine and leucine deprivation conditions. Furthermore, we observed activation of apoptosis during glutamine or leucine starvation. These results suggested that MDA-MB-231 cells when starved of each of these amino acids, the cells cannot overcome the starvation condition and activate apoptosis leading to cell death. In chapter 3.2, we observed a greater decrease in MDA-MB-231 cell viability during replete and glutamine or leucine starvation conditions when GCN2 was knocked down. These results suggested that when GCN2 is inhibited in MDA-MB-231 cells, the cells cannot overcome the starvation conditions sufficiently and instead activate apoptosis, leading to cell death. Moreover, GCN2 knockdown led to a greater reduction in cell viability, which may suggest that the cells were more sensitised to both glutamine and leucine starvation. Therefore, we tested whether MDA-MB-231 cells activated apoptosis when GCN2 was silenced. Furthermore, we wished to determine if silencing GCN2 led to an increased number of cells activating apoptosis during both amino acid starvation conditions. We expected to observe a higher number of cells activating apoptosis when GCN2 is silenced, with greater apoptosis during amino acid starvation.

To determine cell apoptosis, we used the CellEvent™ Caspase-3/7 Green Detection Reagent, as described in chapter 3.1.5. MDA-MB-231 cells were seeded in 6-well plates in complete DMEM and left for 24-hours to adhere to the well bottom. After 24-hours, the cells were subjected to GCN2 silencing via the addition of the siGCN2-2+4 complex and left again for 24 hours for GCN2 knockdown to occur. Post GCN2 knockdown, we removed the DMEM and washed the cells with PBS solution. After washing the cells, we replaced the medium with DMEM glutamine or leucine deficient medium, or complete DMEM with the amino acids supplemented. After 24- or 48-hours incubation, we removed the medium from the cells and detached the cells via 0.5% Trypsin-EDTA. Once cells were detached, a cell suspension mix was created by the addition of DMEM.

The cell suspension mix was aliquoted and spun down, with the supernatant removed to create a cell pellet. This cell pellet was resuspended in CellEvent™ Caspase-3/7 Green Detection Reagent - described in materials and methods. After 30-mins incubation with the CellEvent reagent, the cells were visualised for the presence of green fluorescence using the Countess™ II FL Automated Cell Counter. The positive fluorescent cells indicated that apoptosis is activated within those cells.

Under replete amino acid conditions, the % of fluorescent siGCN2 cells was found to be increased after 24 hours and more so after 48 hours (Figure 3.3.1 A & C). These results suggest that GCN2 is required for MDA-MB-231 cell survival even during replete conditions. Under glutamine starvation, the % of siGCN2 cells undergoing apoptosis was found to be dramatically increased after 24-hours, although since the standard error was large at this point it was not possible to gauge whether this change was significant (Figure 3.3.1 B). After 48 hours of glutamine starvation, the % of siGCN2 cells undergoing apoptosis was low. In comparison, during leucine starvation, the % of siGCN2 cells undergoing apoptosis was lower than the siScr control cells (Figure 3.3.1 D).

Overall, these results together suggest that GCN2 is critical for MDA-MB-231 cell survival. GCN2 knockdown leads to MDA-MB-231 cells being unable to survive and subsequently activate apoptosis. Furthermore, these results also suggest that knockdown of GCN2 sensitised the cells to both leucine and glutamine amino acid starvation. Without the ability to activate GCN2, MDA-MB-231 cells are more likely to activate apoptosis. Thereby leading to an increase in cell viability as observed in chapter 3.2.3.

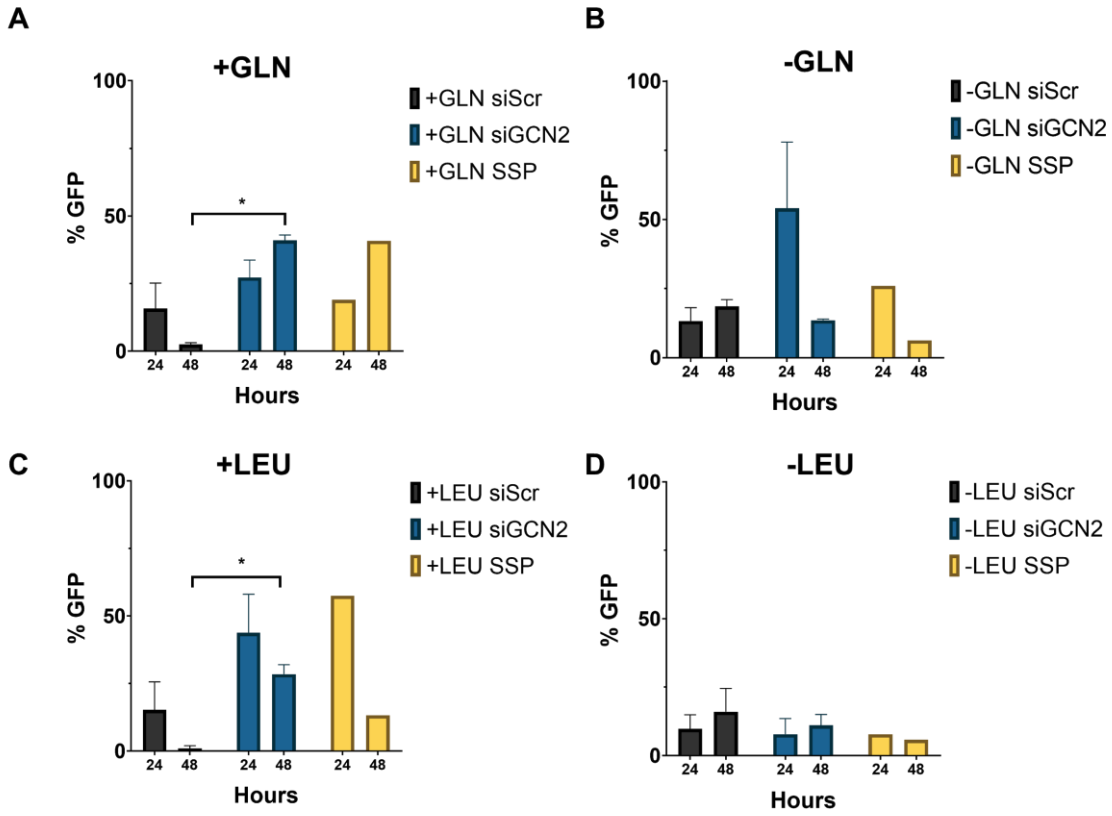

Figure 3.3.1 - GCN2 silencing increases cell apoptosis during glutamine starvation.

MDA-MB-231 cells incubated with a scrambled control siRNA (siScr) or siGCN2-2+4 to knockdown GCN2 protein within the cells, or in parallel were incubated with Staurosporine (SSP) as a positive control. (A) Cells were incubated in complete medium (+Gln). (B) Cells were incubated in glutamine deficient medium (-Gln). (C) Cells were incubated in complete medium (+Leu). (D) Cells were incubated in leucine deficient medium (-Leu). Cell apoptosis was determined by the CellEvent™ Caspase-3/7 Green Detection assay. Values shown are the means ±SEMs (n=2). Statistical analysis was performed using the Two-Tailed Student T-Test (*P<0.05).

4. Discussion

The dysregulation of metabolism is a hallmark of cancerous cells. Cancerous cells, including breast cancers, alter their metabolism creating a reliance on specific nutrients and metabolic pathways. Targeting this reliance on individual nutrients and metabolic pathways are becoming promising as a cancer therapy. One promising therapy is to target the supply of specific amino acids that breast cancer cells have become reliant on (Kung, Marks, and Chi 2011; Xiao et al. 2016; Hosios et al. 2016; M.-S. Chen et al. 2017; Mazor et al. 2018). However, thus far, there is no consensus to which amino acid starvation condition would be the best to starve breast cancers. Furthermore, there is no comparison yet between the cellular response between amino acid starvation conditions. By finding different amino acids that cancer cells are reliant on to support their growth and proliferation, there is hope that the new starvation protocols would halt cancer proliferation and growth, leading to the development of a novel cancer therapy.

Moreover, another promising cancer target is the Amino Acid Response pathway (AAR). The AAR pathway is activated by cells to overcome amino acid starvation conditions and restore homeostasis (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). Cancerous cells have been shown to over-activate and rely on the GCN2 protein involved in the AAR pathway for cell survival not only during amino acid starvation but also, replete amino acid conditions (J. Ye et al. 2010). Overactivation of GCN2 and the AAR pathway is thought to support cancer cell growth and survival by increasing amino acid biosynthesis and transport pathways, increasing the supply of amino acids. In non-cancerous cells, the AAR is only activated in response to times of amino acid deprivation leading to activation of amino acid biosynthesis and transporter pathways to overcome the starvation condition, restoring cell homeostasis (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). If the cell cannot overcome the amino acid starvation, the cell switches from restoring homeostasis to activating cell death pathways (Pakos-Zebrucka et al. 2016). By targeting GCN2 and the AAR pathway, there is hope to reduce cancer cell survival or to sensitise the cancerous cells to an amino acid starvation condition.

In this study, we aimed to compare the AAR response, as judged by the activation and phosphorylation of GCN2 and eIF2α in MDA-MB-231 cells during glutamine or leucine starvation. Next, we tested the effect of each starvation condition on cell proliferation and if cell apoptosis was activated. We further studied MDA-MB-231 cell viability during glutamine or leucine starvation when GCN2 was knocked down in the cell. Finally, we screened for the activation of apoptosis by MDA-MB-231 when GCN2 was knocked down during glutamine or leucine starvation. This allowed us to compare the effects of GCN2 inhibition and amino acid starvation on the fate of MDA-MB-231 cells.

4.1. Characterisation of MDA-MB-231 cells AAR to glutamine and leucine starvation

In response to the deprivation of amino acids, cells respond by activation of the Amino Acid Response (AAR) pathway (Castilho et al. 2014). The AAR pathway is activated to overcome the starvation condition and restore homeostasis. We hypothesised that MDA-MB-231 cells would activate the AAR more in response to leucine starvation than glutamine starvation. We observed that in response to glutamine starvation, MDA-MB-231 cells activated the AAR pathway. Glutamine starvation activated GCN2 with a concurrent increase in eIF2α phosphorylation. In response to leucine starvation, MDA-MB-231 cells appear to activate the AAR. However, unlike glutamine starvation, GCN2 activation is delayed and lower in comparison. During leucine starvation, eIF2α phosphorylation occurs in a similar trend to glutamine starvation. When comparing the response to glutamine or leucine starvation, there was a greater response to glutamine starvation. This result suggests a greater AAR to glutamine starvation than leucine starvation. Thereby, we reject our initial hypothesis of leucine starvation leading to a greater AAR by MDA-MB-231 cells compared to glutamine starvation. Possible causes for this difference in AAR could be due to; the altered metabolic pathways of cancer cells, the different roles of each amino acid, the consumption rates and cellular requirements of each amino acid, or a possible GCN2 independent mechanism for responding to leucine starvation.

We observed a greater activation of GCN2 and subsequent eIF2α phosphorylation in response to glutamine starvation than leucine starvation. This suggests a greater AAR by MDA-MB-231 cells in response to glutamine starvation than leucine starvation. A possible explanation for this difference could be the different roles each amino acid is involved in for cell function. Glutamine is involved not only in protein synthesis, but is major a carbon and nitrogen source for use in the synthesis of other amino acids, while also being involved in the replenishment of intermediates within the TCA cycle, a major cell pathway (DeBerardinis et al. 2007; Yuneva et al. 2007; Kung, Marks, and Chi 2011)(Hosios et al. 2016)(DeBerardinis et al. 2007; Yuneva et al. 2007; Kung, Marks, and Chi 2011). These roles for glutamine places an essential demand for extracellular glutamine, to the point where deprivation of glutamine consistently leads to cell death of many types of cancer, including MDA-MB-231 cells (Eagle 1955; Yuneva et al. 2007; Dilshara et al. 2017; Parzych et al. 2019). Whereas, leucine - in conjunction with protein synthesis - is mainly involved in intracellular signalling, such as the mTORC1 sinalling pathway (Hara et al. 1998; Nair and Short 2005; Averous et al. 2016). Similar to glutamine, leucine is also essential to MDA-MB-231 cell survival - although not as comprehensively researched (Xiao et al. 2016). However, cancer cell survival has not been compared between glutamine and leucine starvation conditions. Thereby, this difference of roles between glutamine and leucine in cell function could account for our observed difference in the AAR. Moreover, the difference in roles and AAR implies the greater importance of glutamine by the cells, more so than leucine.

Amino acids are consumed in vastly different amounts by cancerous cells (Hosios et al. 2016). This difference in amino acid consumption rates could account for the difference observed in the AAR by MDA-MB-231 cells to glutamine or leucine starvation. Of all of the 20 amino acids, glutamine is the most consumed amino acid by cancer cells, with leucine the fourth (Hosios et al. 2016). To increase the consumption of glutamine and other various amino acids, breast cancer cells, upregulate glutamine transporters (Bhutia et al. 2015; Cha, Kim, and Koo 2018). In breast cancers, the glutamine amino acid transporters SLC1A5 and SLC6A14 are upregulated (Karunakaran and Ramachandran 2011; S. Kim et al. 2013; van Geldermalsen et al. 2016). Upregulation of amino acid transporters by cancer cells allows these cells to increase the influx and consumption of glutamine by the cell. Increasing the influx of glutamine increases a major source of

carbon and nitrogen cancerous cells use to grow and supports cancers excessive proliferation rate (van Geldermalsen et al. 2016). However, the increased influx and reliance on an extracellular source of glutamine could leave the cell vulnerable when starved. This starvation would, in turn, lead to activation of the AAR mechanism in an attempt to overcome the amino acid starvation. Thereby, due to the increased consumption rate of glutamine, in comparison to leucine, the MDA-MB-231 cells could initiate a greater AAR to glutamine than leucine starvation.

During leucine starvation, we observed an increase in eIF2α phosphorylation at a time when there was low GCN2 activation. A reason for this observation may be that initially, MDA-MB-231 cells activate a different eIF2α-kinase, other than GCN2, in response to leucine starvation. There is mounting evidence that multiple eIF2α-kinases can cooperate to overcome a single stressor in cancerous cells (Hamanaka et al. 2005; Liu et al. 2010; Lehman, Ryeom, and Koumenis 2015; Wanders et al. 2016; Khan et al. 2019). Of the eIF2kinases, PERK can respond to amino acid starvation to compensate for the loss of GCN2 in cancerous cells. For example, Soft Tissue Sarcomas compensated for the loss of GCN2 with the activation of PERK to maintain the stress response (Lehman, Ryeom, and Koumenis 2015). Moreover, in mouse studies, PERK fully compensated for a complete deletion of GCN2 within the mice and soft tissue sarcomas (Lehman, Ryeom, and Koumenis 2015). The authors mention that due to GCN2 being absent from the beginning of tumour formation, the tumour could have adjusted mechanisms to compensate from the GCN2 loss and may not be clinically relevant. However, this adds compelling evidence for the possibility of a cooperative role between eIF2α-kinases during a single stressor. Comparing the activation of the other eIF2α kinases will help address if MDA-MB-231 cells activate a GCN2 independent response to leucine starvation.

Overall, we show greater activation of the amino acid response (AAR) pathway to glutamine starvation in comparison to leucine starvation by MDA-MB-231 cells. This result suggests that MDA-MB-231 exhibit an amino acid starvation dependent GCN2 activation response - in that GCN2 activation is different depending on the amino acid starved. Moreover, the difference in GCN2 activation was conferred onto subsequent eIF2α phosphorylation, suggesting a specific level of AAR to each amino acid. Possible explanations for the differing AAR's could be due to the roles or the consumption rates

of each amino acid. Trialling other single amino acid starvation conditions will add further evidence to support this link between GCN2 activation and amino acid requirement. Furthermore, there is the potential for moonlighting of other eIF2α-kinases in response to amino acid starvation. Future studies should look to characterise the responses of other eIF2α-kinases to each amino acid starvation. Lastly, to determine which amino acid starvation therapy would be best to treat breast cancer would require the testing of multiple other amino acid starvation conditions. Overall, our results demonstrate that MDA-MB-231 GCN2 activation and subsequent AAR is amino acid-dependent, with a more significant response to glutamine starvation than leucine starvation.

4.2. MDA-MB-231 cell proliferation is reduced more to glutamine than leucine starvation.

In cases of amino acid deprivation, the cell will attempt to overcome the starvation condition by activating the AAR pathway to restore cell homeostasis. However, if the starvation stressor is prolonged or too severe, the cell will halt proliferation and drive the activation of cell death pathways (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). In Chapter 3.1.3, we observed a decrease in eIF2α phosphorylation in both amino acid starvation conditions after 24 hours to a similar level as the unstarved controls. This result suggested that the MDA-MB-231 cells had either overcome the starvation condition and restored cellular homeostasis or the cells had abandoned overcoming starvation and stopped proliferating. We initially hypothesised that leucine – being an EAA – would cause a greater decrease in MDA-MB-232 cell proliferation than the NEAA glutamine. Therefore, we screened for any change in MDA-MB-231 proliferation in response to glutamine or leucine starvation.

In chapter 3.1.4, we observed a greater decrease in cell proliferation during glutamine starvation than leucine starvation. Thereby, we rejected our initial hypothesis. Furthermore, this suggests that in response to both glutamine and leucine starvation, MDA-MB-231 cells cannot restore cellular homeostasis by activation of the AAR.

Therefore, the AAR is reduced, leading to a decrease in cell proliferation. Moreover, with a greater decrease in response to glutamine starvation than leucine starvation suggest that glutamine is more essential to support MDA-MB-231 cell proliferation than leucine.

Our results are consistent with previous research where proliferation rates of cancerous cells are dependent on amino acid starvation conditions, with glutamine starvation significantly decreasing cancer cell proliferation (Yuneva et al. 2007; Sheen et al. 2011; Kung, Marks, and Chi 2011; M.-S. Chen et al. 2017; Dilshara et al. 2017). However, our insignificant decrease in MDA-MB-231 cell proliferation during leucine starvation is inconsistent with a study by Xiao et al. 2016. Xiao et al. observed a significant decrease in MDA-MB-231 cell proliferation *in vivo* during leucine starvation. To determine cell proliferation, Xiao et al. utilised manual cell counting and expression of PCNA - a proliferation marker - to infer cell proliferation. Whereas, we used the SRB assay to infer cell proliferation. The SRB assay relies on the binding of the SRB dye to cell protein and subsequent release and absorbance to infer the number of cells. The difference in the methods used to determine cell proliferation could account for the difference in results.

To our knowledge, we are the first to look at phosphorylation of both GCN2 and eIF2α in response to specific amino acid starvation conditions, with relation to the proliferation rates of MDA-MB-231 cells. Overall, our results, along with previous findings, add further evidence that MDA-MB-231 cells require both glutamine and leucine to support proliferation. Furthermore, our results suggest that glutamine is more essential to MDA-MB-231 cells, leading to a greater decrease in cell proliferation as compared to leucine.

4.3. MDA-MB-231 cells activate cell apoptosis after prolonged exposure to glutamine and leucine starvation

After exposure to severe or prolonged stressors, if the cell cannot restore homeostasis, it will switch from cell survival and drive the activation of cell death pathways (Pakos-Zebrucka et al. 2016). During prolonged or severe amino acid starvation cancerous cells can activate cell apoptosis (Yuneva et al. 2007; J. Ye et al. 2010; Xiao et al. 2016; Dilshara et al. 2017). Thereby, we hypothesised that in response to both leucine or glutamine starvation, MDA-MB-232 cells Results the present study showed activation of cell apoptosis in response to both glutamine and leucine starvation. The activation of apoptosis was higher in response to glutamine starvation than leucine starvation.

In previous studies, cancer cells activate apoptosis in response to the starvation of a combination of NEAA (J. Ye et al. 2010). However, this response is not always consistent throughout all cancer types. In Triple-Negative Breast Cancer (TNBC) cells the activation of a cell death pathway was found to be dependent on the amino acid starved. Leucine and glutamine starvation individually activate apoptosis in TNBC (Xiao et al. 2016; Dilshara et al. 2017). Whereas, cysteine starvation-induced necrosis in TNBC (M.-S. Chen et al. 2017). Furthermore, amino acid starvation has not always been found to activate apoptosis in breast cancer. In disagreement with our study and the previous studies, (Mazor et al. 2018) found no activation of apoptosis during starvation of either; histidine, leucine, arginine, or methionine. However, their short 12-hour amino acid starvation times were most likely insufficient for detection of apoptosis. In comparison, our study and the previous studies used a starvation time of at least 24-hours before detecting apoptosis. Thereby, there is a greater consensus that apoptosis is induced after prolonged amino acid starvation.

In the present study, we found that both glutamine and leucine starvation-induced apoptosis in MDA-MB-231 cells. MDA-MB-231 cells have greater activation of apoptosis in response to leucine than glutamine starvation. Our results are consistent

with previous findings that TNBC activates apoptosis in response to prolonged leucine starvation (Xiao et al. 2016). In addition to leucine starvation-induced apoptosis, we further demonstrated that glutamine starvation also induced apoptosis in MDA-MB-231 cells. The glutamine starvation leads to greater activation of apoptosis than leucine starvation.

4.4. Silencing of GCN2 reduces MDA-MB-231 cell viability and sensitises the cells to glutamine and leucine starvation.

The Amino Acid Response pathway (AAR) is essential for cellular survival and restoration of homeostasis in response to amino acid starvation. The severe or prolonged amino acid starvation can drive the activation of cell death pathways (Castilho et al. 2014; Pakos-Zebrucka et al. 2016). The integral action of the AAR pathway is the activation of GCN2 and subsequent phosphorylation of eIF2α at serine 51. The phosphorylation of eIF2α alters the translational profile of the cell, inhibiting global translation while concurrently increasing the translation of mRNA involved in cellular adaptation to the amino acid starvation. Inhibition of GCN2 is predicted to interfere with the cells' ability to perceive and respond to the starvation of an amino acid by phosphorylating eIF2α. With the inability to effectively phosphorylate eIF2α, the cell would not be able to effectively alter their translational profile to conserve amino acids and upregulate amino acid synthesis pathways. Since the amino acid remains absent in the medium, without the cell able to overcome the starvation, the cell is predicted to halt proliferation and activate cell death pathways sooner. Moreover, cancer cells which over-activate GCN2 to support cell proliferation would be further susceptible to GCN2 inhibition, leading to a greater reduction in cell viability during amino acid starvation and potentially replete amino acid conditions. Thereby, we hypothesised that GCN2 knockdown would lead to a reduction in MDA-MB-232 cell viability, with a further decrease in cell viability when combined with amino acid starvation.

In response to glutamine and leucine starvation, we have shown that MDA-MB-231 cells activate GCN2 (Chapter 3.1.1-3). After 24 hours of each starvation condition, MDA-MB-231 cells abandon overcoming the deprivation and instead halt proliferation and activate apoptosis (Chapter 3.1.4-5). In chapter 3.2.2 our results showed the cells were sensitised to both glutamine and leucine starvation, with a greater reduction in cell viability to glutamine starvation. This result suggests that GCN2 is essential to MDA-MB-231 cell survival. Moreover, cell viability was reduced during replete amino acid

conditions, suggesting that GCN2 is essential to MDA-MB-231 cell survival. Thereby, confirming our initial hypothesis of GCN2 being critical to MDA-MB-231 cell viability and GCN2 knockdown sensitises the cell to amino acid starvation.

Our results are consistent with previous observations that GCN2 promotes cancer cells survival during amino acid starvation (J. Ye et al. 2010; Y. Wang et al. 2013; Xiao et al. 2016; Parzych et al. 2019). With the inhibition of GCN2 in the MDA-MB-231 cells, there is a possibility that they are unable to increase the expression of amino acid starvation response effectors. Thereby limiting their ability to increase amino acid transporters and synthesise amino acids used for proliferation. Previous studies have shown GCN2 knockdown to reduce vascular endothelial growth factor (VEGF) leading to decreased tumour blood vessel formation and tumour size (Y. Wang et al. 2013), and Fatty Acid Synthase (FASN) leading to reduced proliferation (Xiao et al. 2016). Without the ability to effectively activate AAR effectors to increase amino acid biosynthesis enzymes and transporters, the ability for the MDA-MB-231 cells to proliferate is reduced. Furthermore, the GCN2 knockdown removes the possibility for the cells to activate survival mechanisms, leaving the cells to be unable to overcome amino acid starvation effectively, thereby leading to MDA-MB-231 cells to become sensitised to amino acid starvation during GCN2 knockdown.

Overall, GCN2 knockdown led to a greater decrease in MDA-MB-231 cell proliferation during glutamine than leucine starvation. Furthermore, during replete amino acid conditions, GCN2 knockdown further decreased MDA-MB-231 cell proliferation, suggesting that MDA-MB-231 cells are reliant on GCN2 for survival. To adequately characterise the GCN2 knockdown effect on proliferation, future studies should screen for the phosphorylation of eIF2α during GCN2 knockdown and amino acid starvation. Additionally, downstream effectors of the GCN2/eIF2α pathway could be screened for, allowing a greater determination of why the cell has reduced proliferation. These recommendations would aid in determining effective GCN2 knockdown and which cellular responses could lead to the reduction in cell proliferation.

4.5. Silencing of GCN2 promotes activation of apoptosis by MDA-MB-231

In the previous Chapters 3.1.5, we showed the activation of apoptosis in response to prolonged glutamine or leucine starvation. Apoptosis and other cell death pathways have previously been found to be affected by the GCN2-eIF2α pathway (Liu et al. 2010; J. Ye et al. 2010; Muaddi et al. 2010; Xiao et al. 2016; M.-S. Chen et al. 2017). Moreover, it has been proposed that GCN2-eIF2α phosphorylation serves as a "switch" that determines cell fate in response to stressors (Liu et al. 2010). With a higher level of GCN2 activation and eIF2α phosphorylation promoting cell survival and a lower GCN2 activation and eIF2α phosphorylation level driving cell apoptosis. Therefore, we screened if the activation of apoptosis by MDA-MB-231 cells was altered in response during GCN2 knockdown and glutamine or leucine starvation.

In this study, we demonstrated that the knockdown of GCN2 led to the greater activation of apoptosis in unstarved and glutamine starved MDA-MB-231 cells. Leucine starvation led to a slight decrease in apoptosis activation during GCN2 knockdown. However, with a similar activation of apoptosis to our positive staurosporine control suggests leucine starvation with additional GCN2 silencing could be so severe that the majority of the cells had already undergone apoptosis and the sensitivity of our assay was unable to detect this. Our results suggest that when GCN2 is knockdown, MDA-MB-231 cells are more likely to activate cell apoptosis. This increase of apoptosis could be due to an inability of the MDA-MB-231 cell to effectively phosphorylate eIF2α to overcome the amino acid starvation - since GCN2 is no longer present.

GCN2 inhibition in mammalian cells is linked with the greater activation of apoptosis. Our results further support the findings of GCN2 inhibition leading to an increase of apoptosis. (Liu et al. 2010) found that inhibition of GCN2 in MEF cells during hypoxia led to greater activation apoptosis and proposed the GCN2-eIF2α phosphorylation to cell survival during stressors. (J. Ye et al. 2010), further demonstrated the removal of GCN2 in MEF cells led to the greater induction of apoptosis in response to glutamine deprivation. These results add to the evidence supporting that the inhibition of GCN2,

potentially leading to a lower eIF2α phosphorylation level, drives the cell to activate apoptosis. However, this hypothesis cannot be confirmed without screening for GCN2 and eIF2α phosphorylation. Furthermore, Xiao et al. 2016 found activation of apoptosis by TNBC in response to leucine starvation but did not progress into GCN2 silencing experiments. These results suggest that breast cancer cells activate apoptosis in response to a specific amino acid starvation, but were unable to determine if the activation of apoptosis was, in part, due to the proposed GCN2-eIF2α phosphorylation "switch".

Our study shows that MDA-MB-231 cells activate apoptosis during prolonged starvation of the amino acids glutamine or leucine. Our results are consistent with the previous studies, supporting the possibility of a GCN2-eIF2α phosphorylation cell survival "switch" mechanism, where the level of GCN2 activation is linked to the activation of apoptosis (Figure 4.5.1). During GCN2 knockdown, the activation of apoptosis increased. Thereby, the silencing of GCN2 - expected to decrease eIF2α phosphorylation - led to greater activation of apoptosis. Future work would be to screen for relative GCN2-eIF2α phosphorylation during our GCN2 silencing-apoptosis experiments to confirm if the GCN2 silence led to lower levels of GCN2-eIF2α phosphorylation and increased apoptosis. Moreover, our study supports the evidence for the feasibility of targeting GCN2 for novel cancer therapy.

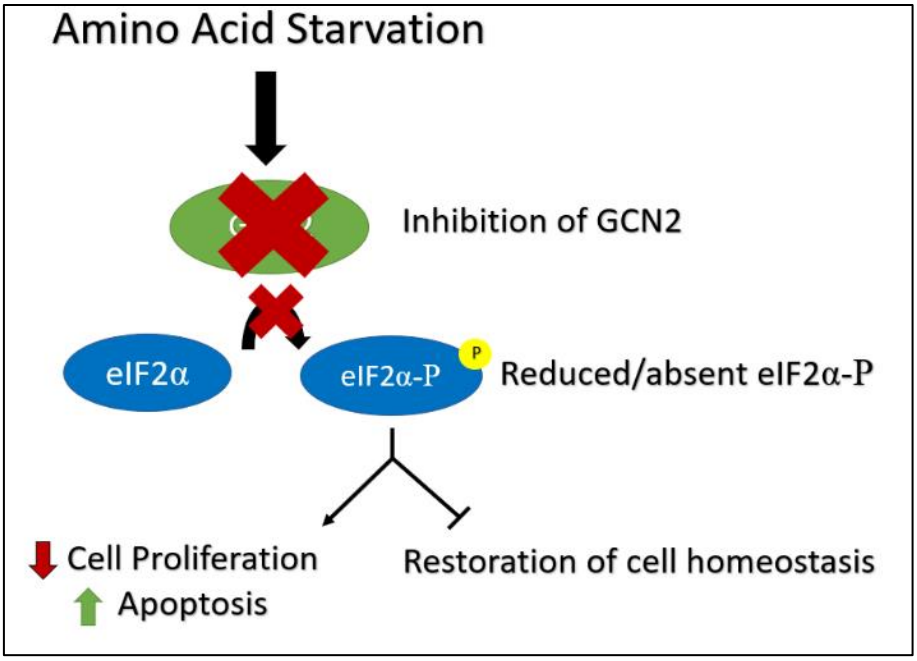

Figure 4.5.1 – Model of GCN2 inhibition effects on cell fate during amino acid starvation.

Upon amino acid starvation, GCN2 phosphorylated eIF2α to alter the cells translational profile and restored cell homeostasis. During GCN2 inhibition, eIF2α is not effectively phosphorylated, leaving the cell unable to restore cell homeostasis effectively. Thereby, the increases the activation of apoptosis along with a decrease in cell proliferation.

4.6. Conclusions and Future perspectives

The results of the current study suggest a specific activation level of the Amino Acid Response pathway. The activation level of the AAR is dependent on the amino acid starvation condition experienced by the MDA-MB-231 cells. Glutamine compared to leucine starvation causes a greater starvation response by increasing GCN2 phosphorylation. MDA-MB-231 proliferation rates also decreased more during glutamine starvation than leucine starvation. This difference in starvation response could be due to the role each amino acid plays in cellular function, the potentially the consumption rates of amino acids, or another eIF2α-kinase affecting the response to leucine starvation. Furthermore, both glutamine and leucine starvation led to the activation of apoptosis. We observed a greater increase of apoptosis in response to glutamine starvation. Thereby, we conclude that glutamine is required more than leucine to support MDA-MB-231 cell survival. In a search for a new effective amino acid starvation cancer therapy, it appears that glutamine starvation would be more applicable than leucine starvation.

To increase the power of our study, a panel of breast cancer cell lines should be starved of glutamine and leucine amino acids with the determination of their respective AAR activation. The screening of AAR activation of multiple cell lines will help determine if there is a typical response to glutamine and leucine starvation between breast cancer cells. The panel of breast cancer cell lines should also be starved of more amino acids, such as asparagine, serine, or guanine. Starvation of more amino acids would allow a greater comparison between AAR and amino acid starvation. Moreover, a more substantial amount of amino acid starvation conditions would aid in the determination of which amino acid would be best to use to develop a therapy to treat breast cancers.

Our GCN2 knockdown results suggest that MDA-MB-231 cells are reliant on GCN2 for survival. GCN2 silencing reduced MDA-MB-231 cell survival under replete conditions and further sensitised the MDA-MB-231 cells to glutamine and leucine starvation. The silencing of GCN2 also led to an increase in cell apoptosis. Thereby our results add further support for a GCN2-eIF2α phosphorylation cell survival “switch” mechanism. However, we would be required to further screen for the relative GCN2-eIF2α

phosphorylation during our GCN2 knockdown experiments to confirm this. Moreover, these results provide evidence for GCN2 being a target for a novel cancer therapy.

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6. Appendix

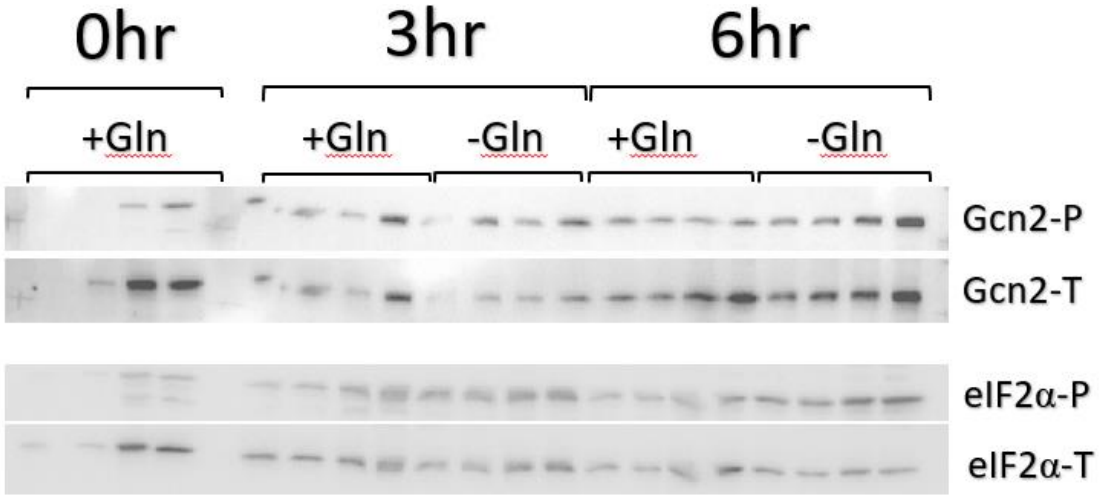

Figure 6.1 – Western Blot of GCN2 and eIF2α phosphorylated and total proteins of MDA-MB-231 cells during glutamine starvation.

MDA-MB-231 cells were incubated in control (+Gln) medium or glutamine deficient medium (-Gln) for the time indicated, followed by western blotting using antibodies against the phosphorylated and total GCN2 protein. The western blot shown is of 4 independent samples.

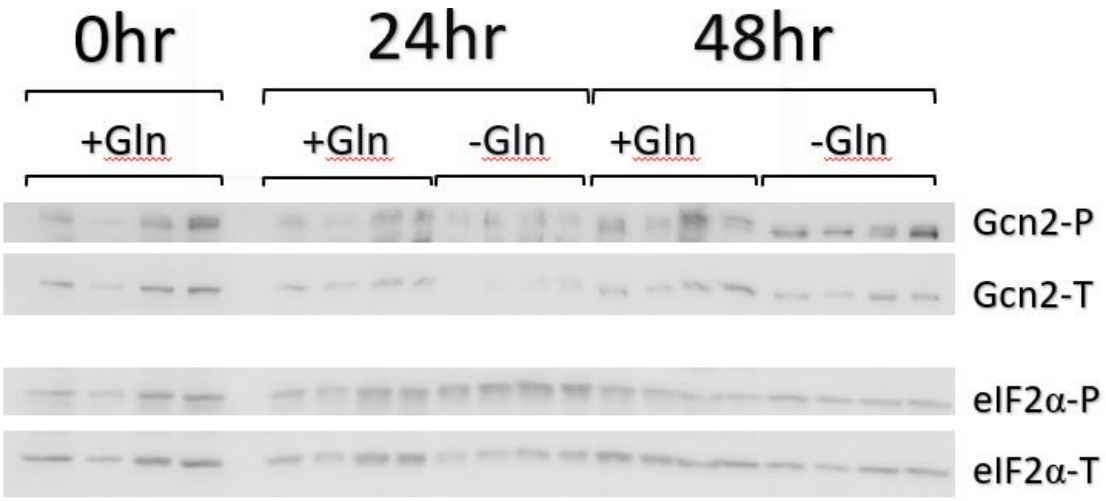

Figure 6.2 – Western Blot of GCN2 and eIF2α phosphorylated and total proteins of MDA-MB-231 cells during glutamine starvation.

MDA-MB-231 cells were incubated in control (+Gln) medium or glutamine deficient medium (-Gln) for the time indicated, followed by western blotting using antibodies against the phosphorylated and total GCN2 protein. The western blot shown is of 4 independent samples.

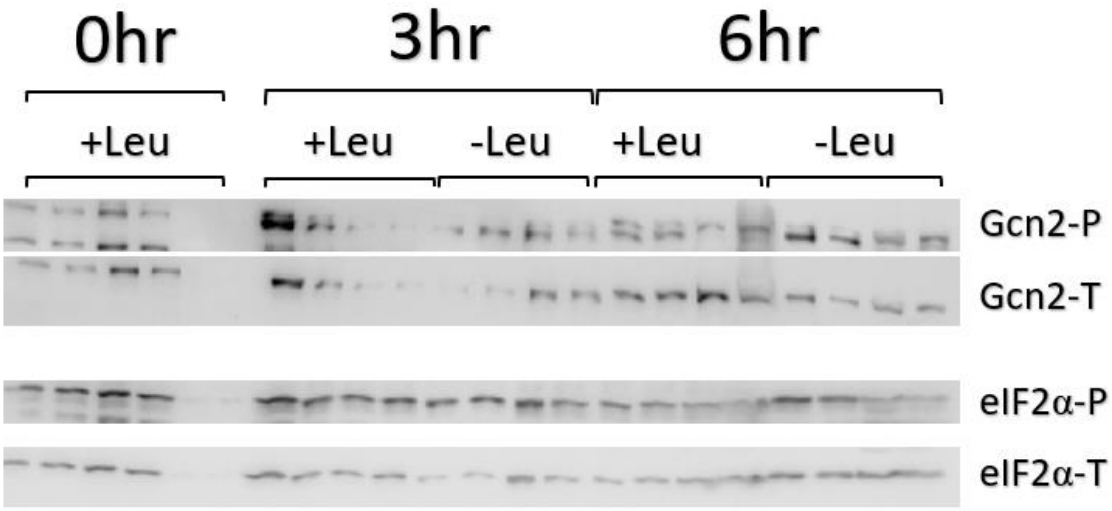

Figure 6.3 – Western Blot of GCN2 and eIF2α phosphorylated and total proteins of MDA-MB-231 cells during leucine starvation.

MDA-MB-231 cells were incubated in control (+Leu) medium or leucine deficient medium (-Leu) for the time indicated, followed by western blotting using antibodies against the phosphorylated and total GCN2 protein. The western blot shown is of 4 independent samples.

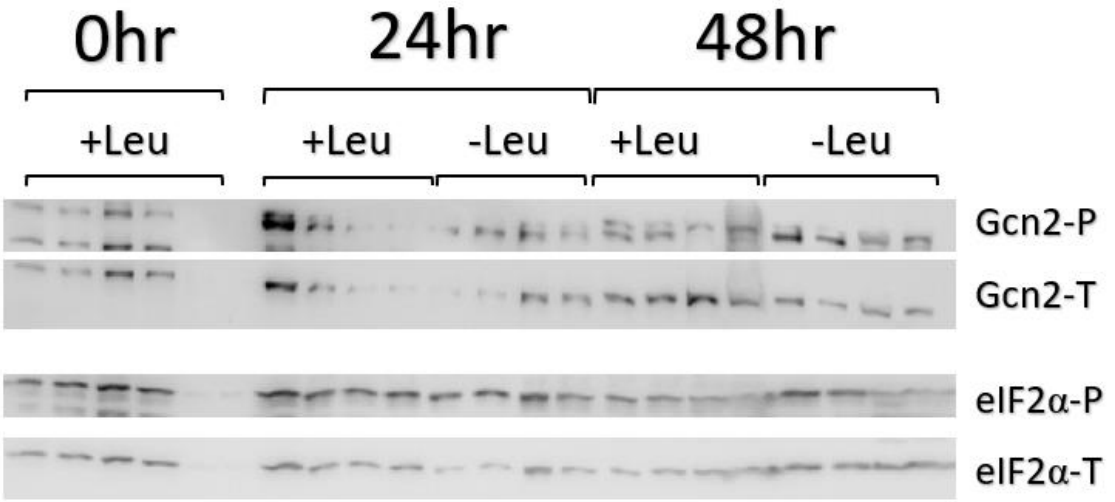

Figure 6.4 – Western Blot of GCN2 and eIF2α phosphorylated and total proteins of MDA-MB-231 cells during leucine starvation.

MDA-MB-231 cells were incubated in control (+Leu) medium or leucine deficient medium (-Leu) for the time indicated, followed by western blotting using antibodies against the phosphorylated and total GCN2 protein. The western blot shown is of 4 independent samples.

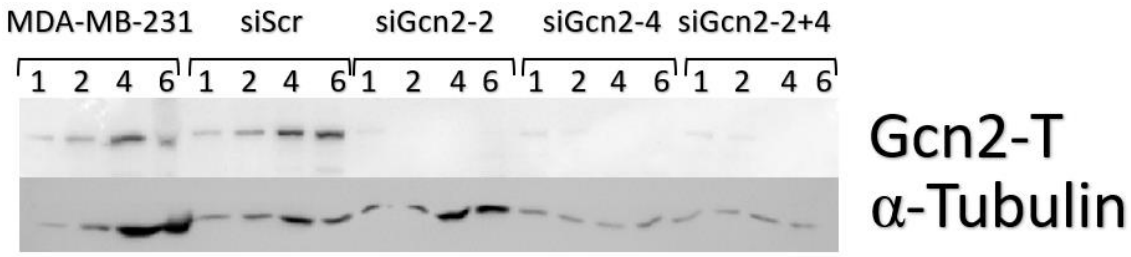

Figure 6.5 – Representative western blot of total GCN2 and α-tubulin proteins during siRNA GCN2 knockdown in MDA-MB-231 cells.

MDA-MB-231 cells incubated with a scrambled control siRNA (siScr) or a siRNA predicted to silence GCN2 mRNA; siGCN2-2, siGCN2-4, or both combined siGCN2-2+4.GCN2 knockdown efficacy was determined via western blotting, with the detection of the total GCN2 protein and α-Tubulin.