The Role of Chikungunya nsP3 in Regulating G3BP Activity, Stress Granule Formation and Drug Efficacy

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Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no materials previously published or written by another person except where due reference is made in the thesis itself.

Xue Lu

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Publications Arising from This Thesis

In Preparation:

The role of Chikungunya nsP3 in regulating G3BP activity, Stress Granule formation and drug efficacy.

Abstract

Ras-GTPase activating protein SH3 domain binding proteins (G3BP) are a small family of RNA-binding proteins that have been implicated in regulating gene expression when a cell undergoes transformation or in response to external stimuli. Changes in the cellular expression of G3BPs are correlated to several cancers including head and neck, thyroid, colon, pancreatic and breast cancer. G3BPs are components of stress granules (SGs) and can regulate their formation. SGs are specialised ribonucleoprotein (RNP) particles that respond to cellular stresses, including chemotherapy, to triage mRNA with selected transcripts being degraded, stored or translated. The net result to the cell is a change in the gene expression which confers a survival response to the cell. This can include the upregulation of genes needed to survive external stresses and this can include the development of drug resistance in cells. Many viruses, including Chikungunya, target SG and dismantle the granules so that the cell cannot respond to the viral infection. Non-structural protein3 (nsP3), from the Chikungunya virus, has been shown to translocate G3BP from SG, and the inability of the cell to form SG is required for the virus to complete an infectious cycle. Interestingly, the formation of SG is correlated to drug-resistance and blocking their formation has been shown to reestablish the efficacy of the chemotherapeutic, bortezomib.

The present study explored the disruption of SG formation, caused by the interaction of nsP3 with G3BP, and as a result restored cytotoxicity of bortezomib and improved the selectivity of the bortezomib for cancer cell lines when compared to HEK cells. In addition, the interaction of nsP3 with G3BP also disrupted the activity of G3BP and led to the significantly downregulation of G3BP target genes NDUFV1 and IFITM3. These findings represent a tool for future study into strategies to block G3BP activities and disrupt SG formation in anti-cancer therapeutics.

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List of Abbreviations

ADP	Adenosine diphosphate
APS	Ammonium persulfate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CHIKV	Chikungunya virus
CMV	Cytomegalovirus
CTNNB1	Catenin beta-1
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate-Buffered saline
EGFP	Enhanced green fluorescent protein
eIF	Eukaryotic translation initiation factor
FBS	Fetal bovine serum
FMN	Flavin mononucleotide
G3BP	Ras-GTPase activating protein SH3 domain binding protein
GAP	GTPase activating
hnRNPs	Heterogeneous nuclear ribonucleoproteins
HRI	heme-regulated inhibitor kinase
IC ₅₀	Half maximal inhibitory concentration
IFITM	Interferon-induced transmembrane
IFNs	Interferons

ISGs	Interferon-stimulated genes
JAK	Janus-Kinase
LB	Luria broth
MAPK	Mitogen-activated protein kinase
MDR	Multiple drug resistance
mRNA	Messenger RNA
mRNPs	Messenger ribonucleoproteins
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced)
NDUFV1	NADH Dehydrogenase (ubiquinone) Flavoprotein 1
NF- κ B	Nuclear factor-kappaB
nsP3	Non-structural polyprotein 3
NTF2	Nuclear Transport Factor 2
OPN	Osteopontin
PCR	Polymerase chain reaction
PMP22	Peripheral myelin protein 22
PVDF	Polyvinylidene difluoride
RACK1	Receptor of activated protein C kinase 1
Ras-GAP	Ras GTPase activating protein
RGG	Arginine/Glycine rich
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RRM	RNA Recognition Motif
SART3	Squamous cell carcinoma antigen recognized by T-cells 3

SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG	Stress granules
STAT	Signal activator and transducer
TAE	Tris-acetate-EDTA
TBS	Tris Buffered Saline
TEMED	Tetramethyl ethylenediamine
TIA	T-Cell internal antigen
UTR	Untranslated regions
β -F1-ATPase	Mitochondrial H ⁺ -ATP synthase subunit beta

Chapter 1

Introduction

1.1 General introduction

Cancer is a heterogeneous disease where abnormal or transformed cells start dividing in an uncontrolled and deregulated manner with the potential to invade or spread to other parts of the body. Cancers are typically caused by genetic aberrations, such as mutations or gene silencing. However, other factors contribute to the progression of cancer, such as epigenetics and the tumour microenvironment (Verma et al., 2014). In the past decades, significant improvements have been achieved in the diagnosis and treatment of cancer. However, the occurrence and mortality rate of cancer continues to increase. By 2030, it has been predicted that over 20.3 million new cases will appear, compared with 12.7 million new cases in 2008 (Ferlay et al., 2015). Among the various types of cancer, breast cancer is the most frequent cancer in women and is the second most commonly diagnosed overall (Gallo et al., 2017). It has been reported that approximately 1.67 million new cases of breast cancer with about 500,000 deaths happened worldwide in 2012, and the occurrence continues to increase (Ferlay et al., 2015).

Current treatments for cancer patients include chemotherapy, radiation, hormone therapy, immunotherapy and surgery. Chemotherapy is one of the effective approaches for cancer treatment. However, depending on the drug and its regime, it may be toxic to normal cells and have contraindications. In addition, advanced stage tumours may contain a mixed population of cells containing drug-sensitive and drug-resistant cancer cells. The treatment of cancer with chemotherapeutic compound kills the drug-sensitive cells and leaves the drug-resistance cells alive. Moreover, the survival of these cells can lead to the development of multiple drug resistant (MDR) within the tumour. Treatment of patients with advanced cancers which have become multi-drug resistance is an enormous challenge because the doses required to kill resistant cancer cells will be toxic to normal cells, and the presence of MDR cells limits the application of chemotherapy (Yardley, 2013). Therefore, new approaches to overcome these situations are urgently needed for anti-cancer therapies.

An innovative approach to the treatment of MDR cells involves targeting stress granules (SG) during anti-cancer treatment. SGs are non-membranous cytoplasmic foci that contain non-translating messenger ribonucleoproteins (mRNPs) which aggregate in cells under stress conditions (Anderson and Kedersha, 2008). These stresses can

include heat stress, oxidative stress, viral replication and stresses associated with chemotherapeutic treatment. Ras-GTPase activating protein SH3 domain binding protein (G3BP) is considered to be a core protein involved in the formation of SGs and may play a role in the transport of mRNA to the granules in response to stress or signalling (Anderson and Kedersha, 2008), however, it should be noted that not all stress granules contain G3BPs and the protein composition of SG may be contextual depending on cell type and the source of stress (Smith et al., 2014b, Buchan and Parker, 2009). G3BPs have also been associated with cellular mechanisms leading to drug resistance. In brief, G3BPs have been implicated in changing the bias of gene expression within the cell to favour multi-drug resistant genes and to modulate the expression of those involved in cancer progression, which has been proven by the down-regulation of G3BP1 which suppressed MDA-MB-231 breast cancer cell invasion and metastasis. (Zhang et al., 2015, Ashikari et al., 2017, Takayama et al., 2018b, Takayama et al., 2018a, Zhang et al., 2017).

A recent study has shown that the non-structural polyprotein 3 (nsP3) from Chikungunya virus (CHIKV) can block the assembly of SGs by recruitment of G3BP out of SG and into cytoplasmic foci (Fros et al., 2012). Therefore, nsP3 has the capacity to disrupt SGs and thereby disrupt the balance of gene expression required by drug resistance cells to survive during treatment with chemotherapies. Furthermore, transfection of nsP3 into cancer cells and subsequent treatment with chemotherapies may represent a tool to explore therapies as an adjuvant therapy to be used alongside drugs such as Bortezomib. Bortezomib is a peptide boronate inhibitor of the 26S proteasome that is highly efficient for the treatment of haematological tumours (Richardson, 2004). Data from cell-based assays using Bortezomib in cytotoxic assays suggest that knockout of heme-regulated inhibitor kinase (HRI), which catalyses the phosphorylation of translation initiation factor eIF2 α , stops SGs formation and restores Bortezomib efficacy in cancer cells (Fournier et al., 2010). These data suggest that blocking SG formation may provide a pathway to combinational treatments to improve the efficacy of current FDA approved drugs. However, knockout of genes, such as HRI, in humans for the purpose of cancer therapy is not currently practicable. Therefore, new therapeutic approaches are required to reach similar endpoints as demonstrated by the HRI knockout and new drugs or strategies need to be explored.

1.2 Ras-GTPase activating protein SH3 domain binding protein (G3BP)

Ras-GTPase activating protein SH3 domain binding protein (G3BP) is a protein that is expressed in mammalian cells and plays important roles in biological systems. The family consists of three proteins G3BP1, G3BP2a and G3BP2b (a splice isoform of G3BP2a) which are encoded by distinct genes on human chromosomes 5 and 4 respectively (Irvine et al., 2004). G3BPs display differential expression in human tissues and although typically located in the cytoplasm, they also translocate to the nucleus (Kennedy et al., 2001, French et al., 2002).

G3BPs comprise of four domains (see Figure 1.1). Both G3BP1 and G3BP2 include a highly conserved domain which is the N-terminal Nuclear Transport Factor 2 (NTF2)-like domain. The NTF2-like domain facilitates the nuclear import of proteins through the nuclear pore complex and also mediates protein-protein interaction (Vognsen et al., 2013). The nuclear shuttling of the G3BP might be due to the function of NTF2. However, this domain is also responsible for RasGAP binding (Kennedy et al., 2001). The C-terminal of G3BP contains two motifs associated with RNA binding and these are the RNA Recognition Motif (RRM) and Arginine/Glycine rich (RGG) regions. The central region of G3BP constitutes a segment that is rich in proline-based SH3 domain-binding motifs (PxxP) as well as an acid-rich domain. PxxPs motifs are considered to be the minimal SH3 domain-binding consensus sequence that can bind with an SH3 domain (Irvine et al., 2004). The acid-rich domain of G3BP remains relatively uncharacterised and is believed to be involved in protein-protein interactions.

Figure 1.1 Predicted domain structure of human G3BP1, 2a and 2b

Image taken from Irvine et al. (2004) and reproduced by permission.

G3BP1 was first identified by its interaction with the SH3 domain of the Ras GTPase activating protein (Ras-GAP) (Parker et al., 1996). At the time this was believed to be modulated by G3BP's SH3 domain binding motifs, however, it was later revealed that the Ras-GAP and G3BP binding was facilitated via the NTF2-like domain (Kennedy et al., 2001). Ras-GAP acts as a target downstream of Ras which is a small GTPase that is regulated by GTPase activating proteins (GAP) in the Ras signalling cascade. The SH3 domain of Ras-GAP is essential for the downstream signal transduction of Ras in a MAPK-independent manner (Leblanc et al., 1999) and for the cytoskeletal reorganization, cell adhesion and the induction of gene expression in a Ras-independent manner (Leblanc et al., 1998). Due to the interaction with SH3 domain of Ras-GAP, G3BP was originally proposed to act as a downstream effector of the Ras signalling pathway which could link external signals to RNA metabolism and DNA replication in a Ras-GTP-dependent manner (Costa et al., 1999, Barnes et al., 2002). However, contrary to these speculations, a recent study by Annibaldi and co-workers indicate that there is no genuine interaction between G3BP1 and Ras-GAP (Annibaldi et al., 2011) and the literature remains unclear regarding the significance of these apparently contradictory findings.

The phosphorylation status of G3BP during the transition from quiescent to dividing cells suggests that Ras-GAP may have a role in cell cycle or be regulated by it. It has been reported that G3BP is phosphorylated at Ser149 in quiescent cells. This phosphopeptide is absent in Ras-GAP^{-/-} fibroblasts regardless of its proliferation state, indicating that cell-cycle regulated phosphorylation of Ser149 is RasGAP dependent (Tourrière et al., 2001). G3BP phosphorylation on Ser149 affects a number of protein functions, including RNase activity and stress granule assembly (Tourrière et al., 2001, Tourrière et al., 2003). G3BP has been associated with s-phase entry in tumour cells providing a link with cellular proliferation (Guitard et al., 2001). Overexpression of both G3BP1 and G3BP2 have been observed in various tumors and has been found to be associated with enhanced proliferation of several cancers including head and neck, thyroid, colon, pancreatic and breast cancer (Barnes et al., 2002, Guitard et al., 2001, French et al., 2002, Taniuchi et al., 2011).

G3BPs have also been shown to play a critical role in several biological pathways that lead to cancer development, including the Ras signalling pathway (Leblanc et al., 1999), *c-MYC* mRNA turnover (Tourrière et al., 2001), NF-κB signalling pathway (Prigent et al., 2000) and the ubiquitin proteasome system (Soncini et al., 2001). Recently, G3BP1 has been found to regulate the expression of PMP22 mRNA, which influences cell proliferation of breast cancer (Winslow et al., 2013). The activity of G3BPs appear to be regulated by one or more signalling pathways and G3BPs transduce these signals by regulating the stability or translation of its target transcripts. The result of this regulation in normal cells appears to resist environmental stress, but in tumours, this activity also appears to protect cancer cells as well. These activities will be addressed in the following sections.

Targeting G3BPs has shown to reduce cancer development, for example, depletion of G3BP1 inhibits metastasis of lung cancer in a mouse model (Somasekharan et al., 2015), and knock-down of G3BP1 can reduce the mesenchymal phenotype of MDA-MB-231 (Zhang et al., 2015, Dou et al., 2016). Therefore, it is not surprising that peptides and compounds have been used to target G3BP with a view that this strategy may provide a basis for cancer therapies (Oi et al., 2015, Shim et al., 2010, Zhang et al., 2014, Zhang et al., 2012). There are significant evidences suggesting that G3BPs have a role in cancer progression and this activity is presumably facilitated by its regulation of mRNA.

However, relatively few RNA targets of G3BPs have been identified. Known transcript targets of G3BP2 include SART3 (Gupta et al., 2017), while targets for G3BP1 include *c-MYC* (Gallouzi et al., 1998), Tau (Atlas et al., 2004), CTNNB1 (Bikkavilli and Malbon, 2011), PMP22 (Winslow et al., 2013), and β -F1-ATPase (Álvaro D. Ortega, 2010). Both G3BP1 and 2 target the interferon induced transmembrane proteins (IFITM1, IFITM2 and IFITM3, publication in submission). A growing body of evidence suggests that G3BPs promote cell growth, cell survival and proliferation, as well as cell cycle progression and tumor progression, making the regulation of G3BP activities an interesting target for anti-cancer therapies.

1.3 RNA Regulation

RNA plays a central role in the pathway from DNA to proteins, known as the central dogma of cell biology. It suggests that the normal flow of information in a cell is unidirectional and proceeds from DNA to RNA via the process of transcription. After that, the information is decoded from message RNA (mRNA) to protein through the translation process. All three major classes of RNA (mRNA, tRNA, and rRNA) are synthesised by transcription of the appropriate genes and are involved in protein synthesis. RNA regulation is crucial for transcription and degradation of molecules, and it involves all the regulatory steps affecting RNA including transcription, post-transcriptional modifications, and translation.

Post-transcriptional gene regulation has been reported to participate in the control of cell proliferation, cellular differentiation, cell invasion, apoptosis and angiogenesis (Mee Young et al., 2009). Moreover, translational initiation represents the main end-point of mRNA, nevertheless, this is also one of the most deregulated steps in cancer (Nasr and Pelletier, 2012). Translational control plays a crucial role in cancer development and progression through selectively translating mRNAs which are essential for tumour cells' survival and metastasis (Silvera et al., 2010).

Most nascent transcripts are processed and exported to the cytoplasm for immediate translation while other transcripts are sequestered to translationally silent structures in the cytoplasm. Some of these structures contain both 40S and 60S ribosomal subunits, while others are stored in quiescent RNA granules containing both 40S and 60S

ribosomal subunits. Specific extra-cellular stimuli are required to facilitate the active translation of mRNA stored in these RNA granules (Anderson and Kedersha, 2006). Conversely, the active translation of transcripts can be diverted into a dormant state in response to the environmental stress, and the translation of mRNAs encoding housekeeping proteins is aborted during this process. Under stress conditions, translational active mRNAs are shifted to translational inactive sites for mRNA storage, which is also referred to as stress granules (SGs). Selective recruitment of specific mRNA into SGs regulates their stability and translation (Anderson and Kedersha, 2006).

1.4 RNA binding proteins (RBPs)

RNA binding proteins (RBPs) have been described as regulatory components that interact with RNA. The binding of mRNA with the RBPs gives rise to a ribonucleoprotein (RNP) complex which are important in the regulation of translation but have also been implicated in other cellular activities such as DNA replication, histone gene expression and regulation of transcription and translation (Glisovic et al., 2008). The RNP complex protects the mRNA molecule from degradation and it is also involved in post-transcriptional processing of the mRNA transcripts (Glisovic et al., 2008). Certain domains in RBPs are known to regulate the nucleocytoplasmic localization of RBP such as the NTF2 domain of G3BP1, which is considered to have a role in the nuclear localization of G3BP1 (Vognsen et al., 2013).

RBPs have an important role in target transcript stability and decay (Mitchell and Tollervey, 2000). RNA-binding protein components of RNPs usually associate with the 5' or 3' UTR of transcripts to alter mRNA stability and translation rates (Bolognani and Perrone-Bizzozero Nora, 2007). RBPs can target the translation initiation step by coupling translation to mRNA localization (Glisovic et al., 2008). For instance, zip code binding protein 1 (ZBP1) binds to the 3' UTR of β -actin mRNA and increases the translocation of its transcript target to actin-rich protrusions in primary fibroblasts and neurons. Translation only happens if the ZBP1-RNA complex reaches its destination at the periphery of the cell, where protein kinase Src increases translation after phosphorylating the tyrosine residue in ZBP1 (Stöhr et al., 2006).

Many RBPs have been found to be overexpressed in cancer cells. Since each RBP is associated with many target mRNAs which affect various biological processes,

deregulation of RBP affects every step of cancer development, including cell proliferation, evasion of apoptosis and activating metastasis (Hong, 2017). The main function of RBPs in cancer development is sustaining cell proliferation by suppressing or enhancing expression levels of negative or positive regulators respectively (Hong, 2017). RBPs also enhance cancer progression by stimulating the expression of genes involved in the invasion and metastasis of cancer (Vikesaa et al., 2006) and some of these genes are the targets of G3BPs including PMP22, a target of G3BP1 or SART3, a target of G3BP2.

1.5 Stress granules

When exposed to a stressful environment, such as oxidative stress, high temperature, viral infection, hypoxia and ultraviolet irradiation, stress responses in eukaryotic cells usually arrest translation initiation or specific transcripts, typically those considered as housekeeping genes, and this leads to the formation of cytoplasmic protein-RNA complexes that referred to stress granules (SGs). SGs are non-membranous cytoplasmic foci that contain non-translating messenger ribonucleoproteins (mRNPs) which aggregate in cells under stress conditions (Anderson and Kedersha, 2008, Takahashi et al., 2013, White et al., 2007). Studies have shown that mRNA and many ribosomal proteins are components of SGs, including eIF4E, eIF4G, eIF4A, eIF4B, eIF2, eIF3, TIA, G3BP, HuR and TTP (Kedersha et al., 1999, Buchan and Parker, 2009). SGs provide cells with a mechanism to modulate gene expression during stress so that non-essential genes can be down-regulated and those required to survive the stress remain stable (Anderson and Kedersha, 2009). SG formation leads to the inhibition or delay of the onset of mRNA translation (Kedersha et al., 1999). Therefore, it has been considered as a mechanism to protect RNAs from harmful conditions (Anderson and Kedersha, 2009). However, the SGs also triage transport of mRNAs to P bodies to facilitate selective degradation of specific transcripts (Kedersha et al., 2005, Buchan and Parker, 2009). P bodies are another cytoplasmic RNP granule which contains nontranslating mRNA, translation repressors and some mRNA degradation machinery. They are simultaneously assembled in cells when exposed to stress environment (Buchan and Parker, 2009). To date, P bodies have been demonstrated to play a fundamental role in general mRNA decay, induced mRNA silencing and translation repression (Decker and Parker, 2012). Together, SGs and P bodies provide a dynamic

cycle, wherein mRNPs can move between polysomes, with implications for the control of mRNA stability, storage or translation (Buchan and Parker, 2009, Decker and Parker, 2012).

The formation of SGs in mammalian cells involves phosphorylation of eukaryotic translation initiation factor eIF2 α , which is usually catalysed by HRI (heme-regulated initiation factor 2 α kinase) (Anderson et al., 2015, Tourriere et al., 2003). This phosphorylation leads to the block of translation initiation and polysome disassembly by reducing the availability and formation of a ternary complex (Kim et al., 2005).

SGs can also form through the self-aggregation of either TIA proteins or G3BP (Kedersha et al., 1999). G3BP has been shown to regulate the formation of SGs assembly, and overexpression of G3BP1 has the potential to induce SGs formation (Tourriere et al., 2003). The recruitment of G3BP into the SGs depends on the NTF2-like domain and its RNA binding domains (Reineke et al., 2015, Tourriere et al., 2003). Previous studies have shown that the knockout of G3BP1 inhibits SGs formation in response to several stressors (Takahashi et al., 2013, Reineke et al., 2015, Ghisolfi et al., 2012). Furthermore, the overexpression of both TIA1 and G3BP is able to induce SG assembly and, therefore, they are also referred to as SG-nucleating proteins (Tourriere et al., 2003).

SGs also seem to be involved in cancer cell proliferation and chemotherapeutic treatment (Anderson et al., 2015). SG formation has been reported to negatively regulate the JNK MAPK (SAPK) apoptotic response and RACK1. RACK1 is the scaffold protein functioning as a mediator between cellular responses, and it is sequestered into SGs after exposure to stress conditions, and subsequently, inhibits apoptosis by suppressing stress-responsive MAPK pathways (Arimoto et al., 2008). In addition, some chemotherapeutic drugs promote SGs formation such as sorafenib (Adjibade et al., 2015) and Bortezomib (Anderson et al., 2015, Fournier et al., 2010). The induction of SGs by chemotherapeutic agents has also been shown to contribute to the resistance of chemotherapeutic-mediated apoptosis (Kaehler et al., 2014, Martins et al., 2010, Fournier et al., 2010).

1.6 Non-structural polyprotein 3 (nsP3) from Chikungunya virus

Chikungunya virus (CHIKV) is a member of the alphavirus genus that causes debilitating disease in humans with symptoms including joint pain and fever (Schwartz and Albert, 2010, Weaver and Lecuit, 2015). CHIKV is endemic in Africa, India and Southeast Asia, and has recently re-emerged in the Indian Ocean islands, Singapore and Italy. It is transmitted by the Asian tiger mosquito, *Aedes albopictus*. (Malet et al., 2009, Powers and Logue, 2007, Thiboutot et al., 2010). CHIKV has a single-stranded RNA genome that encodes two classes of polyproteins (*i.e.* non-structural and structural polyproteins). The structural proteins are translated from a subgenomic RNA, which is transcribed during infection from the viral 26S promoter. The non-structural polyprotein (nsP) is cleaved into 4 mature non-structural proteins which are nsP1, 2, 3 and 4. nsP1 to 4 are necessary for viral replication, however, the essential role of nsP3 in alphavirus RNA replication still remain enigmatic (Frolova et al., 2010, Kumar et al., 2015, Rathore et al., 2014). It has been shown that within the viral replication complex, nsP1 is involved in capping of RNA and nsP2 has protease and helicase activity which causes host shut-off and inhibits the unfolded protein response and interferon-induced JAK-STAT signaling (Fros et al., 2015). The nsP4 protein harbours the viral RNA-dependent RNA polymerase activity, and nsP3 is an essential cofactor of this enzyme (Rubach et al., 2009, Kumar et al., 2015).

nsP3 comprises three regions including an N-terminal macro domain which is a binding site for the ADP-ribose ligand, an essential zinc-binding site in the central regions, and a C-terminal hypervariable domain that is highly variable among different CHIKV strains (Thaa et al., 2015, Fros et al., 2015). In addition, nsP3 has been shown to block the assembly of stress granules by the recruitment of G3BP into cytoplasmic foci and this activity appears to be dependent on an SH3 domain-binding motif within nsP3 (Fros et al., 2012). The disruption of the normal cellular activities of G3BP by nsP3 is yet to be assessed but some targets of G3BP have been identified and could provide surrogate markers to assess the changes of activity associated with the disruption caused by nsP3. Thus, nsP3 has the capacity to disrupt SGs and transfection of nsP3 into cancer cells and subsequent treatment with chemotherapies may represent a tool to explore SG-disrupting therapies to be used in conjunction with drugs such as Bortezomib.

1.7 Mitochondria

Mitochondria are the energy source of the cell and play a critical role in ATP production and host a number of biochemical reactions including catabolic and anabolic metabolism, signalling and generation of reactive oxygen species (ROS) (Zamzami et al., 1996, Rossignol et al., 2004). At the same time, mitochondria are also known to regulate the cell death by playing a role in apoptosis and have also been found to support cell proliferation (Zamzami et al., 1996, Rustin, 2002). Eukaryotic mitochondria are surrounded by an outer membrane and a sophisticated inner complex, which contains many infolds referred to as cristae. Cristae possess most of the enzyme complexes involved in the respiratory chain and ATP synthesis. The space between the two membranes of mitochondria is known as the intermembrane space that is responsible for maintaining the proton gradient for ATP synthesis (Nunnari and Suomalainen, 2012).

Mitochondria are different in cancer cells in terms of morphology and function when compared to normal cells (Carew and Huang, 2002). According to the Warburg Effect, cancer cells prefer metabolizing glucose by glycolysis over the traditional oxidative phosphorylation and this changed metabolism occurs even in the presence of abundant oxygen, which leads to the downregulation of metabolism and function of mitochondria (Courtney et al., 2015, Warburg, 1956). Moreover, cancer cells show an exclusive metabolic reprogramming which makes them more susceptible to mitochondrial dysfunction than normal cells (Bellance et al., 2009).

The mitochondrial oxidative phosphorylation system is composed of five essential enzyme complexes which are involved in the transfer of electrons via redox reactions, creating the proton-motive gradient across the mitochondrial membrane and drives the synthesis of ATP. These five complexes are referred to as NADH ubiquinone oxidoreductase (complex I), succinate dehydrogenase (complex II), coenzyme Q cytochrome c - oxidoreductase (complex III), cytochrome c oxidase (complex IV), and ATP synthase (complex V) (Sharma et al., 2009). Complex I is the largest complex and is the first enzyme in the mitochondrial respiratory chain. It oxidases NADH and uses two electrons to reduce ubiquinone to ubiquinol. The ubiquinol subsequently transfers electrons to reduce molecular oxygen to water at complex IV, which generates proton-motive force across the inner mitochondrial membrane at complex I, II and IV.

Complex V subsequently produces ATP from ADP by this proton-motive force. The entire process constitutes oxidative phosphorylation and plays a central role in energy metabolism.

Complex V is responsible for ATP synthesis and hydrolysis. It is the site of the final step in oxidative phosphorylation that uses the proton gradient across the inner mitochondrial membrane for the production of ATP. H⁺-ATP synthesis in complex V is known to be a critical factor for the bioenergetic activity of cells. The catalytic subunit of H⁺-ATP (β -F1-ATPase) has shown to be downregulated in several cancer cells and is a hallmark for cancer progression (Willers et al., 2010). Data from Ortega et al. indicated that G3BP regulates mitochondrial H⁺-ATP synthase subunit beta (ATP5B, β -F1-ATPase) (Álvaro D. Ortega, 2010). Since G3BP is known to downregulate the expression of mitochondrial β -F1-ATPase, it suggests the role of G3BP in the energy generation pathway (Álvaro D. Ortega, 2010, Willers et al., 2010).

1.8 The Warburg Effect

The term ‘Warburg effect’ was first postulated by Otto Heinrich Warburg and is referred to as aerobic glycolysis. Otto Warburg hypothesized that cancer and tumour growth are supported by the fact that tumour cells mainly generate energy by glycolysis which is a non-oxidative breakdown of glucose (Warburg, 1956). Glucose is the main source of energy and carbon for mammalian cells. It provides not only ATP but also metabolites for various anabolic pathways (Vander Heiden et al., 2009). In the presence of oxygen, most normal cells typically metabolize glucose to carbon dioxide by oxidation of glycolytic pyruvate in the mitochondrial tricarboxylic acid cycle. Most pyruvate enters mitochondrial oxidative metabolism for efficient energy generation (i.e. ATP) and high levels of ATP attenuate glycolysis through feedback inhibition (Lu et al., 2015). It is only under anaerobic conditions that differentiated cells produce large amounts of lactate and undergo aerobic glycolysis (Vander Heiden et al., 2009). By contrast, most cancer cells show certain alterations in glucose metabolism. Proliferating and cancer cells exhibit an increased uptake of glucose and increased rate of glycolysis which generate more intermediate glycolytic metabolites and large amounts of ATP from glycolysis even in the presence of sufficient oxygen (Lu et al., 2015). Furthermore, this process predominantly increases lactic acid fermentation of cancer cells (Ngo et al.,

2015). Warburg hypothesized that existing mitochondrial dysfunction in oxidative phosphorylation pathways may be responsible for this phenomenon (Chen et al., 2015).

The Warburg effect has been contested by many researchers and there is controversy over the hypothesis. Many studies have been conducted and the researchers have found an enhanced glycolytic rate in cancer cells even when the mitochondrion is normal, which is similar to non-cancerous cells. Therefore, the mitochondrial dysfunction may not be the only reason for the glycolytic shift required for energy production in cancer cells.

The major characteristics described in the Warburg effect make it a potential target for developing therapeutics for cancer treatment. For instance, inhibiting glucose transport should result in a shortage of glucose supply to cancer cells, which would slow down general cancer metabolism and make cancer cells stop growing and potentially undergo apoptosis (Chen et al., 2015). In addition, drugs that target the regulation of glycolysis are also in development, such as 2-deoxy-glucose, a hexokinase inhibitor competing with glucose, which is widely studied as a potential anti-cancer drug (Raez et al., 2013).

1.9 NADH Dehydrogenase (ubiquinone) Flavoprotein 1 (NDUFV1)

NADH Dehydrogenase (ubiquinone) Flavoprotein 1 (NDUFV1) is a subunit of the NADH ubiquinone oxidoreductase complex I. NDUFV1 is a 51-kDa subunit that carries the NADH binding site along with the Flavin mononucleotide (FMN) and a tetra-nuclear iron-sulphur cluster (Pilkington et al., 1991). NDUFV1 gene is localised on Chromosome 11q13 and contains 10 exons, coding a 444 amino acid protein (Ali et al., 1993). The protein sequences are identified to be highly conserved among human and bovine (de Coo et al., 1999).

It has been found that the mutations in NDUFV1 genes are related to mitochondrial complex I deficiency, which leads to the myopathies, encephalomyopathies and neurodegenerative disorders such as Parkinson's disease and Leigh syndrome (Vilain et al., 2011). In addition, a recent study has also shown that the downregulation of mitochondrial complex I NDUFV1 can profoundly enhance the aggressiveness of human breast cancer cells, while therapeutic normalization of the NAD⁺/NADH

balance can inhibit metastasis and prevent disease progression (Santidrian et al., 2013). It indicates the potential role of NDUFV1 in cancer progression.

1.10 β -F1-ATPase

β -F1-ATPase is the mitochondrial complex V beta subunit of ATP synthase. Mitochondrial ATP synthase is responsible for the ATP synthesis and utilizing a proton electrochemical gradient across the inner membrane during oxidative phosphorylation (Boyer, 1993). The ATP synthase is composed of two regions: the F1 region, which is the major catalytic core for ATP synthesis, and the F0 region that is responsible for the translocation of the protons through the mitochondrial electron transport chain. The catalytic portion of mitochondrial ATP synthase (i.e. F1 region) consists of five different subunits which are α , β , γ , δ , and ϵ . Among all five different subunits, the β subunit is the catalytic site and is the only subunit that participates in the synthesis of ATP molecules (Leyva et al., 2009).

The expression of β -F1-ATPase is considered to play a crucial role in cancer progression. The downregulation of the bio-energetic requirements of cancer cells is related to the inhibition of β -F1-ATPase translation. The study by Willers and the co-workers has shown that the downregulation of β -F1-ATPase is a hallmark of most human carcinomas including lung, breast and colon cancer (Willers and Cuezva, 2011).

1.11 Interferon-induced transmembrane (IFITM)

Interferons (IFNs) are a group of signalling proteins that are released by host cells to inhibit the infection and invasion of several pathogens, including viruses, bacteria, and parasites as well as tumour cells by activating the innate immune system. Spontaneous innate immune sensing of cancers that leads to adaptive immune responses is dependent on host type I IFN production and signalling (Thomas F. Gajewski, 2015). The innate immune system has a dual role in cancer development in both tumour initiation and progression. Innate immune cells can aid malignant transformation and tumour outgrowth, however, in contradiction to this, can also prevent tumour progression (Catharina Hagerling, 2015). A recent study suggests that targeting interferon responses

sensitizes aromatase inhibitor resistant breast cancer cells (AI-resistant cells) to estrogen induced cell death. It has been shown that in AI-resistant breast cancer cells several interferon-stimulated genes (ISGs) including IFITM1, PLSCR1 and STAT 1 are constitutively overexpressed and their knockdown inhibited the cell's ability to proliferate, invade and migrate (Hye Joung Choi, 2015).

Type I interferon responses also provide the first line of defence against viral infections by inducing the expression of several antiviral proteins which inhibit viral infections by establishing the antiviral state (van Boxel-Dezaire et al., 2006). They belong to the group of cytokines, which induce the expression of a broad number of the interferon-stimulated genes (ISGs) to trigger the protective defence mechanism of the immune system that inhibits viral infection (Yu et al., 2015). Antiviral defences are very important in restricting a viral infection prior to the induction of the adaptive immune response (Iwasaki and Medzhitov, 2015). Interferon-stimulated genes (ISGs) are an integral component of innate immunity and recent evidence would suggest that G3BP1 and G3BP2 also have an important role in innate immunity and are targeted by various viruses to allow replication and to spread infection (Galan et al., 2017, Scholte et al., 2015, Cristea et al., 2006, White et al., 2007, Panas et al., 2012). Among the ISGs with antiviral activity, the interferon-induced transmembrane (IFITM) proteins are specialized in their activity. The human IFITM genes locate on a 26 kb region on chromosome 11 and have four members: IFITM1, IFITM2, IFITM3 and IFITM5 (Bailey et al., 2014). The expression of all four human IFITM proteins is induced robustly by IFNs with the exception of IFITM5. Nevertheless, the expression of IFITM3 is the most strongly induced by IFNs, while the others are less responsive to type I IFN treatment (Diamond and Farzan, 2012). Recent studies identified possible roles of IFITM proteins in carcinogenesis. It seems that IFITM genes have the ability to inhibit viral entry which is possibly achieved by modulating the fluidity of cellular membranes (Smith et al., 2014c). In addition, IFITM1 is identified as a key player in both the invasion and carcinogenesis in patients with glioma (Zhao et al., 2013) and this may be related to regulating fluidity of cellular membranes. Furthermore, IFITM2 is critical in the regulation of p53 independent pro-apoptotic gene in cancer apoptosis pathway (Daniel-Carmi et al., 2009).

IFITM3, also known as 1-8U was initially identified in a cDNA screen from INF-treated neuroblastoma cell (Zhao et al., 2013). It is transcribed in most tissues and is

highly interferon-inducible. A recent study by Everitt and co-workers has shown that IFITM3 expression acts as an essential barrier to influenza A virus in vivo and in vitro (Everitt et al., 2012). In addition, some studies indicated that IFITM3 plays a critical role in inhibiting tumour development. Work by El-Tanani and co-workers found that IFITM3 reduces osteopontin (OPN) mRNA expression and inhibits OPN which mediates anchorage-independent growth, cell adhesion and cell invasion in breast cancer cells (El-Tanani et al., 2009). IFITM3 expression has also been found to be up-regulated in gastric cancer and colorectal tumours etc. Furthermore, a study by Zhao and co-workers indicates that the knockdown of IFITM3 reduces the cell proliferation and migration, as well as it promoted apoptosis in glioma cells (Zhao et al., 2013). Therefore, IFITM3 could be a potential target in anti-cancer therapies.

G3BP1 and G3BP2 play an important role in the translational regulation of IFITM₁₋₃ proteins mainly by interacting with their 3'-UTRs but G3BPs' role in the regulation of phosphorylation of MAP/ERK pathway might also play a role in the regulation of IFITM₁₋₃ (Umber and Kennedy, 2018). Therefore, the preliminary aim of this work was to explore the role of G3BPs in translational control of the IFITM3 gene as a surrogate marker for disrupted G3BPs activity. Two other targets of G3BPs, β -F1-ATPase (Álvaro D. Ortega, 2010) and NDUFV1 (unpublished data) were also used as surrogate makers to determine changed biological activity of G3BPs. We hypothesised that nsP3 can interact with G3BPs translocating it to nsP3 foci and this would change the biological activities of G3BPs which can be measured by the translational control of IFITM3, NDUFV1 and potentially β -F1-ATPase.

1.12 Chemotherapeutic agent Bortezomib

Among different types of chemotherapeutic agents the FDA-approved drug, Bortezomib (Velcade®), is a peptide boronate inhibitor of the 26S proteasome that is highly efficient for the treatment of haematological tumours (Richardson, 2004). It is the first therapeutic proteasome inhibitor that was applied to humans (Adams, 2004). Proteasomes are large protein complexes that responsible for the degradation of unwanted proteins which are tagged by polyubiquitin chains for recognition (Gu and Enenkel, 2014). They are located in the nucleus and the cytoplasm of eukaryotes. These regulatory proteins that are eliminated through this process are fundamental to the

control of cell-cycle progression, tumour growth and cell apoptosis (Adams, 2004, Adams et al., 1999). In addition, the proteasome is also essential for activation of transcription factor nuclear factor-kappaB (NF- κ B) by degradation of its inhibitory protein, I κ B (Adams et al., 1999). NF- κ B is a crucial modulator of promoting growth in cancer cells and reduces the effectiveness of anticancer therapy (Adams, 2003, Richardson, 2004).

Due to the critical roles of the proteasome in cell process regulation, proteasome inhibitors are investigated as potential anti-cancer therapies (Teicher and Tomaszewski, 2015). Proteasome inhibitors are drugs that block the action of proteasome and induce apoptosis in cancer cells (Dou and Zonder, 2014). One mechanism of the function of proteasome inhibitors is related to the blockage of NF- κ B activity, which makes cells more susceptible to apoptosis (Adams and Kauffman, 2004, Patel et al., 2000).

In addition to its other activities, the proteasome inhibitor Bortezomib has been shown to induce the assembly of SGs effectively due to its cytotoxicity (Anderson et al., 2015). It triggers the phosphorylation of eIF2 α by activating heme-regulated inhibitor kinase (HRI), and subsequently promoting SG formation (Fournier et al., 2010, Anderson et al., 2015). Data from cell-based assays using Bortezomib in cytotoxic assays suggest that knockout of HRI, which catalyses the phosphorylation of translation initiation factor eIF2 α , stops SGs formation and restores Bortezomib efficacy in cancer cells (Fournier et al., 2010). These data suggest that blocking SGs formation may provide a pathway to combinational treatments to improve the efficacy of current FDA approved drugs. However, knockout of genes, such as HRI, in humans for the purposes of cancer therapy is not currently practicable. Therefore, new therapeutic approaches are required to reach similar endpoints as demonstrated by the HRI knockout and new drugs or strategies need to be explored. Strategies that block SG formation appear to be one pathway that may be appropriate for use, in conjunction with developed cancer therapies.

1.13 Rationale and Project aims

1.13.1 Hypotheses

- 1) nsP3 from Chikungunya virus blocks SG assembly by recruitment of G3BP into cytoplasmic foci and restores the cytotoxicity of anti-cancer drug Bortezomib.
- 2) nsP3 interacts with G3BP and change the activities of G3BP in the translational control of mitochondrial genes, NDUFV1 and β -F1-ATPase as well as IFITM₁₋₃ genes.

1.13.2 Aims

- 1) To determine if nsP3 protein, from Chikungunya virus, can translocate G3BP from SGs. (Chapter 3)
- 2) To determine if the nsP3 protein can influence the translational regulation of G3BP target genes (ATPase, NDUFV1, IFITM3). (Chapter 4)
- 3) To determine if the nsP3 protein can interact with G3BP in heterogeneous nuclear ribonucleoproteins (hnRNPs) particles and restore cytotoxicity of anti-cancer drug Bortezomib by translocating G3BP from SG. (Chapter 5)

1.13.3 Significance

Data from cell-based assays using Bortezomib in cytotoxic assays suggests that knockout of heme-regulated inhibitor kinase (HRI), which catalyses the phosphorylation of translation initiation factor eIF2 α , stops SGs formation and restores Bortezomib efficacy in cancer cells (Fournier et al., 2010). These data suggest that blocking SG formation may provide a pathway to combinational treatments to improve the efficacy of current FDA approved drugs. However, knockout of genes, such as HRI, in humans for the purposes of cancer therapy is not currently practicable. Therefore, new therapeutic approaches are required to reach similar endpoints as those demonstrated by the HRI knockout and new drugs or strategies need to be explored. Strategies that block SG formation appear to be one pathway that may be appropriate for use in conjunction with cancer therapies. As mentioned previously, nsP3 has the capacity to disrupt SG and transfection of nsP3 into cancer cells and subsequent

treatment with chemotherapies may represent a tool to explore adjuvant or combinational therapies with drugs such as Bortezomib.

The significance of this work is that if treatment of cells with nsP3 can restore Bortezomib activity, it will serve as a proof of concept for further research into strategies to block G3BP activities in anti-cancer therapeutics. In addition, the host lab has determined that mitochondrial complex I NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1) and interferon-induced transmembrane (IFITM3) (paper in submission) can also be regulated by G3BP. Therefore, this work explores the role of G3BP in the translational control of the NDUFV1, β -F1-ATPase, and ITIFM₁₋₃ genes as a surrogate marker for disrupted G3BP activity. We hypothesised that nsP3 can interact with G3BP by translocating it to nsP3 foci and this would change the biological activities of G3BP which can be measured by the translational control of the NDUFV1, β -F1-ATPas, and ITIFM₁₋₃ genes. The aim of the research was focussed on disrupting G3BP activity to determine if this will serve as a tool to explore the role of G3BP in the progression of cancer and its potential role in the Warburg effect.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1. Plasmid Vector

The empty pEGFP-N1 vector and the vector containing the CHIKV nsP3 insert plasmid (pEGFP-nsP3) were provided by Margit Mutso from the laboratory of Suresh Mahalingam (Griffith University). The restriction enzymes utilized for the insertion of the nsP3 sequence into the vector were BamHI and XhoI. Appendix 1 shows the plasmid map of the pEGFP-N1 vector.

2.1.2. Primers

Table 2.1 List of primers used to perform research work presented in this thesis

Gene name	Primer direction	Primer sequence (5' - 3')	Application
CMV	Forward	CGCAAATGGGCGGTAGGCGTG	Sequencing PCR
EGFP_N	Reverse	CGTCGCCGTCCAGCTCGACCAG	Sequencing PCR
NDUFV1	Forward	ACCTCATTTGGCTCGCTGAA	RT-qPCR
NDUFV1	Reverse	CTCAAACCCGATGTCTTGAT	RT-qPCR
IFITM3	Forward	GCTGATCTTCCAGGGCTATGG	RT-qPCR
IFITM3	Reverse	GCGAGGAATGGAAGTTGGAGT	RT-qPCR
β -Actin	Forward	CACCATTGGCAATGAGCGGTTC	RT-qPCR
β -Actin	Reverse	AGGTCTTTGCGGATGTCCACGT	RT-qPCR

2.1.3. Chemicals and Reagents

Table 2.2 List of chemical and reagents

Reagents	Supplier
Alamar Blue	Thermo Fisher Scientific
BSA	Sigma Aldrich
DAPI	Life Technologies
DMSO	Sigma Aldrich
DPBS (1×)	Life Technologies
Ethanol / Methanol	Chem Supply
Kanamycin	Chem Supply
Lipofectamine 2000	Invitrogen (Cat # 11668-019)
Paraformaldehyde	Scharlau
Protease Cocktail Inhibitor	Thermo Fisher (Cat # 87786)
RIPA Buffer	Sigma Aldrich (Cat # R0278)
RNase	Sigma Aldrich
SDS	Riedel-de Haën
Sodium arsenite	Chem Supply
TEMED	Sigma Aldrich (Cat # T9281)
Triton X-100	Sigma Aldrich (Cat # T9284)
Tween 20	Chem Supply (Cat # TL020)

2.1.4. Solutions

Table 2.3 List of solutions

Solution	Composition	Application
Luria broth (LB) medium	10 g/L Tryptone 5 g/L Yeast Extract 10 g/L NaCl	Plasmid transformation
LB agar	15 g/L Agar 10 g/L Tryptone 5 g/L Yeast Extract 10 g/L NaCl	Plasmid transformation
TAE buffer	40 mM Tris-Cl 20 mM acetic acid 1 mM EDTA, pH 8.0	Agarose gel Electrophoresis
SDS-PAGE 5×loading buffer	5% (w/v) SDS 40% (v/v) glycerol 250 mM Tris-Cl, pH 6.8 50 mM DTT	SDS-PAGE
5×SDS-PAGE running buffer	25 mM Tris 192 mM Glycine 0.1% SDS	SDS-PAGE
Western blot transfer buffer	25 mM Tris 192 mM Glycine 20% (v/v) Methanol	Western blot
TBS (pH 7.5)	50 mM Tris-Cl 300 mM NaCl	Western blot
TBS/Tween (pH 8.0)	50 mM Tris-Cl 300 mM NaCl 0.05% (v/v) Tween 20	Western blot
Blocking buffer	50 mM Tris-Cl 300 mM NaCl 5% (w/v) skim milk	Western blot

2.1.5. Antibodies

Table 2.4 List of antibodies

Antibody	Description	Dilution used	Use	Source
G3BP1	Anti-G3BP1 monoclonal antibody produced in mouse	1:200	IF	Abcam Cat # ab56574
TIA1	Anti-TIA1 monoclonal antibody produced in rabbit	1:200	IF	Abcam Cat # ab40693
Goat anti-mouse IgG (H+L)	Secondary antibody against mouse Alexa Fluor 546	1:1000	IF	Invitrogen Cat # 870965
Goat anti-rabbit IgG (H+L)	Secondary antibody against rabbit Texas Red	1:1000	IF	ThermoFisher Cat # 1526650
NDUFV1	Anti-NDUFV1 polyclonal antibody produced in rabbit	1:1000	WB	ABclonal Cat # A13333
β -F1-ATPase	Anti- β -F1-ATPase polyclonal antibody produced in rabbit	1:1000	WB	ABclonal Cat # A5769
IFITM3	Anti-IFITM3 polyclonal antibody produced in rabbit	1:2000	WB	GeneTex Cat # GTX115407
β -Actin	Anti- β -Actin polyclonal antibody produced in rabbit	1:2000	WB	GeneTex Cat # GTX109639
Goat anti-rabbit IgG-HRP conjugated	Secondary antibody against rabbit produced in goat	1:2000	WB	LI-COR Cat # C5061701

IF = Immunofluorescence WB = Western blot

2.2 Methods

2.2.1. General Cell Culture

2.2.1.1. Cell Lines and Culture Conditions

Vero (African green monkey kidney cells), MCF-7 (mammalian breast cancer cells), T-47D (mammalian breast cancer cells), HEK293 (human embryonic kidney cells) and HeLa (mammalian cervical cancer cells) were originally obtained from ATCC (USA) and stored in liquid nitrogen or maintained in the host laboratory. These cells were cultured in Dulbecco's Modified Eagle's Medium DMEM/F12 (1:1) (GIBCO Life Technologies) supplemented with 10% fetal bovine serum (FBS) (GIBCO Life Technologies) in the host laboratory. 5×Trypsin (GIBCO) was applied to harvest Vero cells and HeLa cells and 1×Trypsin was used to harvest MCF7, T47D and HEK293 cells. All cell cultures were maintained in 5% CO₂ at 37°C in a humidified incubator.

2.2.1.2. Transient Transfection

500ng of pEGFP-nsP3 plasmid or empty pEGFP-N1 vector were transfected into Vero cells at a density of 2×10^5 cells per well in a 6-well plate using Lipofectamine 2000 following the manufacturer's instructions. Cells were then incubated at 37°C for 48 hours before oxidative stress treatment or incubated at 37°C for 72 hours before harvested for cell lysate preparation or RNA extraction.

2.2.2 Plasmid Preparation

2.2.2.1. Plasmid Transformation

Competent bacterial cells used in this research were *Escherichia coli* strain JM109 (Promega Corporation). Heat shock transformation was performed using the empty pEGFP-N1 vector or the pEGFP-nsP3 plasmid as per the instruction supplied by the manufacturer. The culture was then plated onto LB agar plates containing 100ug/ml of kanamycin. Plates were incubated for 12-16 hours at 37°C incubator.

2.2.2.2. Miniprep of Colonies

Colonies were picked with pipette tips transferred to LB medium with 100ug/ml of kanamycin and cultured at 37°C at 225rpm overnight. Subsequently, plasmid was extracted from the overnight grown cultures using the NucleoSpin Plasmid kit (Promega Corporation) as instructed by the manufacturer. The concentration of miniprep products was measured at 260nm to 280nm by NanoDrop ND-1000 (ThermoFisher Scientific). The ratio OD260/OD280 provided an estimation of the purity. Plasmids were only used if the ratio was approximately 1.8 for a DNA.

2.2.2.3. Restriction Endonuclease Digestion

The restriction enzymes and buffers are all provided by New England Biolabs (NEB). Endonuclease digestion of plasmid DNA was carried out for 1-2 hours using 1 unit of enzyme to digest 1µg of DNA at 37°C following manufacturer's instruction. The efficiency of digestion was analyzed via agarose gel electrophoresis.

2.2.2.4. Agarose Gel Electrophoresis

Samples were diluted in loading buffer and loaded onto 1% agarose gel containing 1µg/ml of ethidium bromide in 1% TAE buffer at 100V for 30-40 minutes. DNA samples were then visualized by UV transillumination, using GEL Logic 200 imaging system (Kodak).

2.2.2.5. Big Dye Sequencing PCR

20µl sequencing PCR reaction mixtures contained 1×BigDye sequencing buffer, 3.2 pmol of CMV forward primer or EGFP_N reverse primer, 4µl of Big Dye Terminator v3.1 cycle mix and 500ng of the template. The conditions for PCR-based sequencing comprised of 1 minute at 96°C for the initial denaturation, followed by 30 cycles of reaction at 10 seconds for 96°C denaturation, 5 seconds for annealing at 50°C and then 4 minutes at 60°C was used for extension (Table 2.5). Ethanol precipitation was conducted to purify the sequencing products as per the manufacturer's instruction. The

precipitated reactions were then sequenced at Griffith University DNA Sequencing Facility at Nathan campus.

Table 2.5 Sequencing PCR program for amplification

Cycle step	Temperature	Time	Cycles
Initial denaturation	96°C	1 min	1
Denaturation	96°C	10 secs	30
Annealing	50°C	5 secs	
Extension	60°C	4 mins	
Final extension	72°C	4 mins	1

2.2.3. Stress Treatment

Oxidative stress was induced in transfected Vero cells by replacing the DMEM medium with 0.5mM sodium arsenite supplemented medium for 1 hour in the 37°C incubator before fixation.

2.2.4. Immunofluorescence Microscopy

The Vero cells with transfected nsP3 plasmid or the empty vector were fixed with 4% paraformaldehyde in DPBS for 30 minutes, followed by permeabilization in 1% Triton X-100 for 15 minutes. Subsequently the cells were incubated in blocking buffer (2% BSA in DPBS) for 1 hour and afterwards washed 3 times with DPBS. Subsequently, primary antibodies for G3BP1 and TIA1 (see Table 2.4) were applied for 1 hour at room temperature respectively. After incubation, cells were washed 3 times with DPBS before being incubated with secondary antibodies against the primary antibodies. After washing 3 times with DPBS, the nuclei were stained with DAPI for 5 minutes and then rinsed in DPBS. The images of localisation of nsP3 with G3BP and TIA were visualised and captured by confocal microscope (Olympus FV1000).

2.2.5. Protein Manipulation

2.2.5.1. Cell Lysate Preparation

Vero cells transfected with nsP3 plasmid or empty vector were harvested with 5×Trypsin when the cells were observed to reached 80-85% confluence in a 6 well plate. Then the cell pellets were lysed in cold RIPA buffer supplemented with protease inhibitors for 15 minutes. Subsequently, the cell lysate was centrifuged for 20 minutes at 14800rpm. The supernatant was then collected and stored at -80°C for analysis.

2.2.5.2. Protein concentration estimation

A DC Bio-Rad assay was used to estimate the concentration of samples following the instructions of the manufacturer. The absorbance was then measured at 750nm by a plate reader (Bio-Tek). The concentration of the samples was calculated using a standard concentration curve of BSA.

2.2.5.3. Western blot

Protein samples were separated on a 10% or 15% SDS-PAGE (Recipes in Table 2.6) for 90-120 minutes and then the separating gel was transferred onto 0.45 µm hydrophobic polyvinylidene fluoride (PVDF) (Merck Millipore Cat # IPVH00010) membranes at 100 V for 90 minutes in the presence of western blot transfer buffer (Table 2.3) at 4 °C . The membranes were blocked in a 5% blocking buffer (Table 2.3) for 1 hour. The membranes were subsequently incubated with primary antibodies against NDUFV1, β-F1-ATPase and IFITM 3 (see table 2.4) at 4°C overnight, β-actin was used for the loading control. After washing 3 times with TBST, the membranes were then incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature. The antigen-antibody reaction was detected by using an Enhance Chemiluminescence detection kit (ECL) as per the instructions from the Millipore Immobilon kit (WBKLS0500). The images of the membrane were visualized using a VersaDoc (Bio-Rad) imaging station.

Table 2.6 Recipes for SDS-PAGE gels

Stacking Gel Recipe (5 ml)	Volume	
Distilled water	3 ml	
30% Acrylamide (BioRad)	0.67 ml	
0.5 M Tris-HCl, pH 6.8	1.25 ml	
10% SDS	50 μ l	
10% APS	50 μ l	
TEMED	5 μ l	
Resolving Gel Recipe (16 ml)	Volume (10%)	Volume (15%)
Distilled water	6.3 ml	3.7 ml
30% Acrylamide (BioRad)	5.33 ml	8 ml
1.5 M Tris-HCl, pH 8.8	4 ml	4 ml
10% SDS	160 μ l	160 μ l
10% APS	160 μ l	160 μ l
TEMED	6 μ l	6 μ l

2.2.6. RNA Manipulation

2.2.6.1. RNA Extraction

Transfected Vero cells were trypsinized after reaching 80-85% confluency using 5 $\times$ Trypsin and harvested. The cell pellet was collected, and the total RNA was extracted using a RNeasy Mini Kit (Qiagen Cat # 74104) as per manufacturer's instructions. Subsequently, the qualification of RNA was performed by using a NanoDrop ND 1000 spectrophotometer and RNA samples were stored at -80°C until used.

2.2.6.2. Real-Time Quantitative PCR

For real-time quantitative PCR (RT-qPCR), the Luna Universal One-Step RT-qPCR Kit (E3005S) was employed and the samples were run in a Corbett Rotor Gene 6000 machine. The cycling programs comprised of 10 minutes at 55°C for cDNA synthesis, followed by 2 minutes at 95°C for polymerase activation. Then 40 cycles of 10 seconds at 95°C, 5 seconds at 60°C and 5 seconds at 72°C was applied (Table 2.7). Each experiment contained technical repeats (n=3) and was performed in three independent biological assays and the results were normalized to the level of actin. Table 2.1 shows the primers used for RT-qPCR. The data was analyzed by LinReg software based on the delta-delta Ct method.

Table 2.7 RT-qPCR program for mRNA expression studies

Cycle step	Temperature	Time	Cycles
Reverse transcription	55°C	10 mins	1
Initial denaturation	95°C	2 min	1
Denaturation	96°C	10 secs	40
Annealing	60°C	5 secs	
Extension	72°C	5 secs	
Melt curve	60-95°C		1

2.2.7 Cell Density Assay

Four cell lines were utilised for cytotoxicity determination which were HeLa, HEK293, MCF7 and T47D. Then cells reached 80-90% confluence, they were harvested using trypsin and counted by a Trypan Blue exclusion method following the instructions of the manufacturer (Thermo Fisher). Cells were seeded in a 384-well plate with concentration ranging from 50 to 2400 cells per well and allowed to grow for 72 hours in a 37°C incubator. The plates were read with an EnVision TM 2104 Multilabel Reader

(PerkinElmer, USA) after 4 hours incubation of 5 μ l of Alamar Blue (12.5 mg resazurin dissolved in 100 ml distilled water) was added to each well. The cell survival was then calculated and plotted using Graph Pad Prism 6.

2.2.8 IC₅₀ Determination

Stock Bortezomib drug solutions were prepared in 100% DMSO at 10mg/ml. Cells with optimal density were cultured in 384 well plates containing drug concentrations from 0.05nM to 80nM prepared by serial dilutions. Untreated cells and cells treated with 1% DMSO were applied as the negative or vehicle controls respectively. After 72 hours of incubation, 5 μ l of Alamar Blue were added to each well and the plates were incubated for another 4 hours. Then the plates were read with fluorescent and colorimetric plate reader. IC₅₀ values were calculated and plotted using the microplate reader software Graph Pad Prism 6 following the instruction of Prism 6 on IC₅₀ determination.

Chapter 3

**CHIKV nsP3 protein recruits G3BP into
cytoplasmic foci and blocks the assembly of
Stress Granules**

3.1 Introduction

Ras-GTPase activating protein SH3 domain binding proteins (G3BP) are a small family of three conserved RNA-binding proteins consisting of G3BP1, G3BP2a and its spliced isoform G3BP2b (Kennedy et al., 2001). G3BPs display differential expression in human tissues and although typically located in the cytoplasm, they also translocate to the nucleus (Kennedy et al., 2001, French et al., 2002, Costa et al., 1999). G3BP1 was first identified by its binding to ras-GTPase (Parker et al., 1996), but has subsequently been implicated in several biological systems (Irvine et al., 2004, Barnes et al., 2002, Prigent et al., 2000, Soncini et al., 2001). Changes in G3BP expression have been associated with several cancers including head and neck, thyroid, colon, pancreatic and breast cancer (Barnes et al., 2002, Guitard et al., 2001, French et al., 2002, Taniuchi et al., 2011), and several lines of evidences suggest that G3BPs regulate pathways leading to cancer development (Guitard et al., 2001, Wei et al., 2015, Zhang et al., 2015, Kim et al., 2007, Huang et al., 2005). Targeting G3BPs has shown to reduce cancer development, for example, depletion of G3BP1 inhibits metastasis of lung cancer in a mouse model (Somasekharan et al., 2015), and knock-down of G3BP1 can reduce the mesenchymal phenotype of MDA-MB-231 (Zhang et al., 2015, Dou et al., 2016). Therefore, it is not surprising that peptides and compounds have been used to target G3BP with a view that this strategy may provide a basis for cancer therapies (Oi et al., 2015, Shim et al., 2010, Zhang et al., 2014, Zhang et al., 2012). There is significant evidence suggesting that G3BPs have a role in cancer progression and this activity is presumably facilitated by its regulation of mRNA, however, relatively few RNA targets of G3BPs have been identified. Known transcript targets of G3BP2 include SART3 (Gupta et al., 2017), while targets for G3BP1 include *c-MYC* (Gallouzi et al., 1998), Tau (Atlas et al., 2004), CTNNB1 (Bikkavilli and Malbon, 2011), PMP22 (Winslow et al., 2013), and β -F1ATPase (Álvaro D. Ortega, 2010).

One of the biological functions of G3BPs is closely associated with formation and activity of stress granules (SG). SGs are non-membranous cytoplasmic foci that contain non-translating messenger ribonucleoproteins (mRNPs) which aggregate in cells under stress conditions such as arsenite, hypoxia, and heat stress (Anderson and Kedersha, 2008, Takahashi et al., 2013, White et al., 2007). SGs formation leads to the inhibition or delay of the onset of mRNA translation (Kedersha et al., 1999). Therefore, it has

been considered as a mechanism to protect RNAs from harmful conditions. However, the SGs also triage transport of mRNAs to P bodies to facilitate selective degradation of specific transcripts (Kedersha et al., 2005, Buchan and Parker, 2009). Studies have shown that mRNA and many ribosomal proteins are components of SGs, including eIF4E, eIF4G, eIF4A, eIF4B, eIF2, eIF3, TIA, G3BP, HuR and TTP (Kedersha et al., 1999, Buchan and Parker, 2009). SGs provide cells with a mechanism to modulate gene expression during stress so that non-essential genes can be down-regulated and those required to survive the stress remain stable (Anderson and Kedersha, 2009). The formation of SGs involves phosphorylation of eukaryotic translation initiation factor eIF2 α , which is usually catalysed by HRI (heme-regulated initiation factor 2 α kinase) (Anderson et al., 2015, Tourriere et al., 2003). This phosphorylation leads to the block of translation initiation and polysome disassembly by reducing the availability and formation of a ternary complex (Kim et al., 2005). SGs can also form through the self-aggregation of either TIA proteins or G3BPs (Kedersha et al., 1999). G3BP1 plays important roles in the assembly of SG, as proved by the fact that knockout of G3BP1 inhibits SGs formation in response to several stressors (Takahashi et al., 2013, Reineke et al., 2015, Ghisolfi et al., 2012). The recruitment of G3BP into the SGs depends on the NTF2-like domain and RNA binding domains (Reineke et al., 2015, Tourriere et al., 2003). Moreover, SGs also seem to be involved in cancer cell proliferation and chemotherapeutic treatment (Anderson et al., 2015). Some chemotherapeutic drugs promote SGs formation such as sorafenib (Adjibade et al., 2015) and Bortezomib (Anderson et al., 2015, Fournier et al., 2010). The induction of SGs by chemotherapeutic agents has also been shown to contribute to the resistance of chemotherapeutic-mediated apoptosis (Kaehler et al., 2014, Martins et al., 2010, Fournier et al., 2010).

Recent studies have shown that the nsP3 from CHIKV can block the assembly of SGs by recruitment of G3BP into cytoplasmic foci (Fros et al., 2012). Chikungunya virus is a member of the alphavirus genus that causes debilitating disease in human with symptoms including joint pain and fever (Schwartz and Albert, 2010, Weaver and Lecuit, 2015). CHIKV is endemic in Africa, India and Southeast Asia, and has recently re-emerged in the Indian Ocean islands, Singapore and Italy transmitted by the Asian tiger mosquito, *Aedes albopictus*. (Malet et al., 2009, Powers and Logue, 2007, Thiboutot et al., 2010). It has a single-stranded RNA genome that encodes two classes

of polyproteins (*i.e.* non-structural and structural polyproteins). The non-structural polyprotein (nsP) is cleaved into 4 mature non-structural proteins which are nsP1, 2, 3 and 4. nsP1 and 4 are necessary for viral replication, while the essential roles of nsP3 in alphavirus RNA replication still remain enigmatic (Frolova et al., 2010, Kumar et al., 2015, Rathore et al., 2014). It has been shown that within the viral replication complex, nsP4 harbours the viral RNA-dependent RNA polymerase activity, and nsP3 is an essential cofactor of this enzyme (Rubach et al., 2009, Kumar et al., 2015). In addition, nsP3 can block the assembly of stress granules by the recruitment of G3BP into cytoplasmic foci and this activity appears to be dependent on an SH3 domain-binding motif within nsP3 (Fros et al., 2012). The disruption of SGs by chikungunya may represent a mechanism through which the virus can disrupt a cell-based immune response against the virus. The disruption of the normal cellular activities of G3BP by nsP3 is yet to be assessed but some targets of G3BP have been identified and could provide surrogate markers to assess the changes of activity associated with the disruption caused by nsP3.

This chapter aimed to determine if nsP3 protein, from Chikungunya virus, can translocate G3BP into cytoplasmic foci and block the formation of SGs. The localisation of G3BP1 and TIA1 under stress and non-stress conditions were also quantified. TIA was used in these studies because it is an essential component of SG (Kedersha et al., 1999, Buchan and Parker, 2009) and previous studies show that its subcellular localisation is unaffected by the expression of the nsP3 protein (Panas et al., 2014). SGs are linked to the inhibition of cell apoptosis and the development of multidrug resistance genes in cancer cells. Therefore, the inhibition of SGs assembly represents a promising strategy to augment anti-cancer therapeutics in future. Stress granules were induced by using sodium arsenite, which causes oxidative stress and leads to the assembly of SGs. The optimal concentration of sodium arsenite and the time for stress treatment for these assays was determined to be 0.5mM of sodium arsenite for 1 hour. Vero cells were transfected with pEGFP-N1 empty vector or pEGFP-nsP3 plasmid by lipofectamine 2000 for 24 hours before stress treatment. The nsP3 transfected Vero cells were stained for G3BP1 and TIA1 and were then observed under a confocal microscope for SGs formation and localisation, compared with untreated Vero cells and Vero cells transfected with the empty vector as controls.

3.2 Results

3.2.1 Co-localisation of G3BP1 and TIA1 to stress granules

To characterise the formation of Stress Granules in Vero cells, they were evaluated under untreated conditions and oxidative stress conditions which were induced by 0.5 mM sodium arsenite for 1 hour. The cells were then stained for both G3BP1 and TIA1, which are both known as markers of SGs, to check their colocalization under non-stressed and stressed conditions. Figure 3.1 shows that G3BP1 and TIA1 remain colocalised in the cytoplasm under normal condition, and they were recruited to the cytoplasmic SGs after sodium arsenite treatment.

Figure 3.1 Co-localisation of G3BP1 and TIA1 to SGs. The colocalization of G3BP1 and TIA1 in Vero cells in untreated conditions or stressed with 0.5mM sodium arsenite for 1 hr. Red staining represents TIA1, green staining is for G3BP1 and cell counter stained with DAPI (blue) to detect nuclei. Staining shows the co-localisation of G3BP1 (green staining in the left panel) and TIA1 (red staining in the left panel) in SG as seen in the overlay (yellow staining in the right panel). Yellow arrows indicate the formation of SG. All photos are shown at 60x magnification and are typical examples of 3 biological repeats.

3.2.2 The interaction between G3BP and CHIKV nsP3 protein

To investigate the subcellular colocalization of G3BP and the CHIKV protein nsP3, Vero cells were transfected with pEGFP-N1 vector expressing a nsP3-GFP fusion protein (pEGFP-nsP3) for 48 hours. Subsequently, transfected Vero cells were stressed with 0.5 mM sodium arsenite for 1 hour before they were stained for G3BP1 to check their sub-cellular locations. Empty pEGFP-N1 vector transfected Vero cells were used as a negative control. As can be seen in Figure 3.2A, G3BP1 remains in the cytoplasm under normal conditions and the cells can form SGs normally after stress treatment in pEGFP-N1 vector transfected Vero cells, indicating that the transfection of the pEGFP-N1 vector did not affect the assembly of SGs. In contrast to Figure 3.2A, Figure 3.2B demonstrates the changes in SG formation in Vero cells transfected with nsP3. As shown in the images, the nsP3 protein colocalised with G3BP1 even under non-stressed conditions. It demonstrates that the nsP3 protein can interact with G3BP1 and form cytoplasmic foci under both normal and stressed conditions.

Figure 3.2 The interaction between G3BP1 and CHIKV nsP3 protein. (A). The negative control shows Vero cells transfected with pEGFP-N1 vector and stained for G3BP1 under non-stressed (upper untreated panels) and stressed conditions (lower AsNaO₂ treated panels). The green channel represents the GFP expressed from the empty vector pEGFP-N1, and red channel shows stain for G3BP1. (B). Vero cells were transfected with the nsP3 protein and stained for G3BP1 to check their colocalization. Images showed the formation of G3BP1-containing nsP3 foci. Staining shows the co-localisation of pEGFP-nsP3 expressed GFP (green staining in the centre panel) and G3BP1 (red staining in the left panel) in nsP3/G3BP1 foci as seen in the overlay (yellow staining in the right panel). White arrows indicate the formation of G3BP/nsP3 cytoplasmic foci. Note that these images were taken from cell transiently transfected by either pEGFP-N1 or pEGFP-nsP3 and therefore not all cells express GFP. The untransfected cells form the expected SG after AsNaO₂ as indicated by the red SG in the lower-right panel.

3.2.3 The formation of CHIKV nsP3 and G3BP foci blocks the assembly of SGs

When treated with 0.5M sodium arsenite for 1hr, SGs were formed in Vero cells transfected with empty vector (pEGFP-N1), while cells transfected with the pEGFP-nsP3 expression vector reveals that G3BP1 and nsP3 colocalised under stressed and non-stressed conditions (Figure 3.2B). Since the G3BP-nsP3 foci are similar to SGs in terms of morphology, another SG marker TIA1 was applied to check the colocalization of G3BP1 with nsP3 and the influence of G3BP/nsP3 foci on the formation of SGs. After 1 hr stress with 0.5M sodium arsenite, SGs formed normally in the empty vector transfected Vero cells as shown in Figure 3.3A, which displays that the transfection of pEGFP-N1 vector harbours no influence on SGs assembly. Figure 3.3B (upper row-untreated) shows that nsP3 foci are formed in cells transfected with pEGFP-nsP3 when untreated. However, Figure 3.3B (lower row-AsNaO₂ treated) shows that SGs are formed after sodium arsenite treatment but only in cells that were not transfected by pEGFP-nsP3 (Figure 3.3B lower row-AsNaO₂ treated). Cells that were not transfected by pEGFP-nsP3 show the expected localisation of TIA1 into SG. The data demonstrates that the CHIKV nsP3 protein can recruit G3BP into cytoplasmic foci and block the formation of SGs. In addition, the mutually exclusive localisation between SGs (as shown by TIA1 positive stained SG) and the nsP3/G3BP foci also serves as a proof that the nsP3/G3BP cytoplasmic foci are not SGs.

Figure 3.3 The CHIKV nsP3 and G3BP foci blocks the assembly of SGs. (A). Negative control pEGFP-N1 vector transfected Vero cells were stained for TIA1. The red channel represents TIA1 staining. The negative control showed no influence of transfection of pEGFP-N1 vector on SGs formation. **(B).** Vero cells transfected with nsP3 were stained for TIA1 after 48h post-transfection. As expected the cells transfected with the pEGFP-nsP3 vector form nsP3 foci (white arrows) in both untreated (top row) and AsNaO₂ treated cells (bottom row), however TIA (red staining) localised to SG (yellow arrows) but did not co-localise with nsP3 in cell transfected with the pEGFP-nsP3 vector. All photos are shown at 60x magnification and are typical examples of 3 biological repeats.

3.3 Discussion

Tourriere and co-workers found that cytoplasmic G3BP was recruited to SGs when the cells are exposed to oxidative stress such as sodium arsenite (Tourriere et al., 2003). SGs provide cells with a mechanism to modulate gene expression during stress so that non-essential genes can be down-regulated and those required to survive the stress remain stable (Anderson and Kedersha, 2009). SGs formation also leads to the inhibition or delay of the onset of mRNA translation (Kedersha et al., 1999). This chapter evaluated and optimised the conditions required for the formation of SGs and the localization of G3BP1 to SGs by immunofluorescence microscopy with a view that these results will inform the parameters used in subsequent chapters to interrogate the effects that nsP3 has on SG formation and the downstream effects on translational control of target genes and drug sensitivity. Oxidative stress was induced in the Vero cells using 0.5mM sodium arsenite for 1 hour. Figure 3.1 showed that G3BP1 was localized homogeneously within the cytoplasm under normal condition and showed non-specific punctate staining. However, G3BPs were recruited to the cytoplasmic granular bodies, characterised as SGs, after sodium arsenite treatment.

In addition to G3BP, TIA1 and TIAR have also been found to recruit to SGs under oxidative stress treatment (Kedersha et al., 1999). Therefore, to confirm the structure of the SG and validate the previous studies, the work performed in this chapter also checked the colocalization of G3BP1 and TIA1. As shown in Figure 3.1, after the sodium arsenite stress treatment, both G3BP1 and TIA1 were recruited to the SGs, and the overlap of G3BP1 and TIA1 can be observed, confirming the colocalization of the two proteins in the SG. The data indicated that both G3BP1 and TIA1 are SGs markers and they can move to SGs under sodium arsenite stress treatment and this was used to confirm the formation of SG during transfection with nsP3.

The recent study by Fros and co-workers indicated that nsP3 from CHIKV can block the assembly of SGs by recruitment of G3BP into cytoplasmic foci (Fros et al., 2012). To validate the relationship between G3BP and CHIKV nsP3, this chapter applied immunofluorescence microscopy to investigate the interaction between endogenous G3BP1 and recombinant nsP3 in transfected Vero cells. Figure 3.2B demonstrated the colocalization of G3BP1 and CHIKV nsP3 under both normal and stressed (AsNaO₂ treated) conditions, which indicated that the nsP3 protein interacted with G3BP1 to form cytoplasmic nsP3/G3BP foci even without stress treatment. This only occurred in cells that have been transiently transfected with the pEGFP-nsP3 construct (shown by

the green EGFP staining in Figure 3.3B, centre panels). It also indicated that the nsP3/G3BP foci are different from arsenite-induced SGs. Figure 3.2A demonstrated that the transfection of the pEGFP-N1 vector (transiently transfected cells were identified by the homogeneous EGFP staining in the cytoplasm, Figure 3.2A centre panels) did not affect the assembly of SGs as the G3BP1 remained in cytoplasm under normal condition and formed SGs normally after sodium arsenite treatment.

The research performed by Fros and co-workers used G3BP1 and eIF3, which is another marker of SGs to check the influence of the nsP3 protein on the assembly of SGs. This chapter used TIA1 instead of eIF3 to determine the changes in SGs formation. The recruitment of TIA1 to SGs under sodium arsenite treatment was reported previously by Kedersha and colleagues (Kedersha et al., 1999) and the colocalization of G3BP1 and TIA1 was validated in this research in Figure 3.1. As shown in Figure 3.3B, it can be observed that the cells transfected with nsP3 did not show any TIA1 positive SGs. The presence of nsP3/G3BP foci resulted in the inability of the cell to form SGs after stress induced by arsenite treatment. It strongly suggested that CHIKV nsP3 can recruit G3BP into cytoplasmic nsP3/G3BP foci and block the formation of SGs. In addition, the mutually exclusive localisation of SGs and nsP3/G3BP foci also served as evidence that the nsP3/G3BP cytoplasmic foci are different from SGs, which is consistent with the work done by Fros et al. (2012).

In summary, the data presented in this chapter suggested that G3BP1 was recruited to cytoplasmic granular bodies known as SGs under the oxidative stress conditions induced by sodium arsenite. G3BP1 colocalized with TIA1, which is another SGs marker protein, and the recruitment of G3BP1 and TIA1 to SGs was confirmed. The data from this chapter found that the CHIKV nsP3 protein can interact with G3BP1 and form cytoplasmic foci under both normal (untreated) and stressed (AsNaO_2 treated) conditions. The formation of nsP3/G3BP foci blocked the assembly of SGs which was demonstrated by the absence of TIA1 positive SGs when nsP3/G3BP foci were present. This data also demonstrated that nsP3/G3BP foci was different from SGs. The consequences of the interaction between CHIKV nsP3 and G3BP on the role of G3BP in the regulation of target transcript translation and the potential application in the anti-cancer therapies will be discussed in the following chapters.

Chapter 4

The influence of CHIKV nsP3 protein on the translational regulation of G3BP target genes

4.1 Introduction

Regulation of gene expression is controlled by a myriad of mechanisms which can be grouped into one of three basic stages: transcription, translation and post-translational modification. The control of translation is regulated by elements contained within the 5' and 3' untranslated regions (UTR) of a transcript (Smith et al., 2014a). Proteins bind the regulatory elements with the purpose to initiate, enhance or down-regulate translation. Alternatively, proteins can bind to the regulatory elements to change the stability of a transcript with a result that increasing the half-life of a transcript may increase translation whereas decay of a transcript terminates the possibility for translation to occur. G3BPs are a family of RBPs which are involved in the regulation of the stability and translational efficiency of their target mRNAs and subsequently play an important role in various cellular pathways. The current literature presents two apparent contradictory functions in regards to the role that G3BP plays when binding to 3' UTR elements with some groups suggesting that G3BP causes the degradation of a transcript (Tourriere et al., 2001), while other data suggests that the role of G3BPs are to regulate translation (Ortega et al., 2010). Several targets have been reported for G3BPs including *c-MYC* (Gallouzi et al., 1998), Tau (Atlas et al., 2004), CTNNB1 (Bikkavilli and Malbon, 2011), PMP22 (Winslow et al., 2013), and β -F1-ATPase (Álvaro D. Ortega, 2010), and the data from the groups reporting these targets suggests that G3BPs can regulate both mRNA stability and translation.

Mitochondria are the energy source of the cell and play a critical role in ATP production and host a number of biochemical reactions including catabolic and anabolic metabolism, signalling, generation of reactive oxygen species (ROS) and apoptosis (Zamzami et al., 1996, Rossignol et al., 2004). Furthermore, mitochondria are different in cancer cells in terms of morphology and function from normal cells (Carew and Huang, 2002). According to the Warburg Effect, cancer cells prefer metabolizing glucose by glycolysis to oxidative phosphorylation even in the presence of abundant oxygen, which leads to the downregulation of metabolism and function of mitochondria (Courtney et al., 2015, Warburg, 1956).

β -F1-ATPase is the mitochondrial complex V beta subunit of ATP synthase. Mitochondrial ATP synthase is responsible for the ATP synthesis and utilizing a proton electrochemical gradient across the inner membrane during oxidative phosphorylation

(Boyer, 1993). The downregulation of the bio-energetic requirements of cancer cells is related to the inhibition of β -F1-ATPase translation. Studies by Willers and the co-workers showed that the downregulation of β -F1-ATPase is a hallmark of most human carcinomas including lung, breast and colon cancer (Willers and Cuezva, 2011). Data from Ortega *et al.* indicates that G3BP regulates mitochondrial H^+ -ATP synthase β -F1-ATPase by regulating the translation of β -F1-ATPase. The changes in ATP synthesis, possibly caused by the down-regulation of β -F1-ATPase, are considered to be a hallmark of several cancers (Álvaro D. Ortega, 2010, Willers et al., 2010) and suggests a role for G3BP in the energy generation pathway. In addition, the host lab has determined that another mitochondrial complex I protein, NADH dehydrogenase (ubiquinone) flavoprotein 1 NDUFV1 can also be regulated by G3BP (unpublished data).

NDUFV1 is a subunit of the NADH ubiquinone oxidoreductase complex I. A recent study has also shown that the downregulation of mitochondrial complex I NDUFV1 can profoundly enhance the aggressiveness of human breast cancer cells, while therapeutic normalization of the NAD^+ / $NADH$ balance can inhibit metastasis and prevent disease progression (Santidrian et al., 2013). It indicates the potential role of NDUFV1 in cancer progression.

The host laboratory has identified genes from the interferon-induced transmembrane (IFITM) family, namely IFITM1, IFITM2 and IFITM3 (IFITM₁₋₃) which can be translationally regulated by G3BP activity (manuscript in submission). Furthermore, some studies indicated that IFITM3 plays a critical role in inhibiting tumour development. Work by El-Tanani and co-workers found that IFITM3 reduces osteopontin (OPN) mRNA expression and inhibits overall OPN expression which mediates anchorage-independent growth, cell adhesion and cell invasion in breast cancer cells (El-Tanani et al., 2009). IFITM3 expression has also been found to be up-regulated in gastric cancer and colorectal tumours etc. Furthermore, a study by Zhao and co-workers indicates that the knockdown of IFITM3 reduces cell proliferation and migration, as well as promoted apoptosis in glioma cells (Zhao et al., 2013). Therefore, IFITM3 could be a potential target in anti-cancer therapies.

This chapter explores the role of G3BP in translational control of mitochondrial proteins β -F1-ATPase and NDUFV1 as well as the IFITM3 genes as a surrogate marker

for disrupted G3BP activity. We hypothesised that nsP3 can interact with G3BPs translocating them to nsP3-foci and this would change the biological activities of G3BPs which can be measured by the translational control of the β -F1-ATPase, NDUFV1 and IFITM3 genes. The aim of this chapter was focussed on disrupting G3BP activity to determine if this will serve as a tool to explore the role of G3BP in the progression of cancer and its potential role in the Warburg effect. To test this hypothesis, CHIKV nsP3 was transfected into cells so that it would change the biological activities of G3BP in the regulation of its target genes. This was assessed by performing immunoblots and RT-qPCR to evaluate expression levels of the β -F1-ATPase, NDUFV1 and IFITM3 genes at the transcript and protein levels.

4.2 Results

4.2.1 Expression of NDUFV1 in nsP3-transfected Vero cells at transcript and protein levels

After Vero cells were transfected with pEGFP-N1 and pEGFP-nsP3 plasmid for 72 hours, the cells were harvested and the proteins were extracted. Immunoblotting was performed to assess the influence of the nsP3 protein on the biological activity of G3BP by measuring the translational regulation of its target gene NDUFV1. All the data presented in the following figures were from three biologically independent experiments and each experiment contained technical triplicates. The immunoblot images presented the technical triplicates of each sample. The data were analysed by Student's t-test to determine if there were statistically significant differences in the mRNA and protein expression of the target genes.

As shown in Figure 4.1 A and B, it can be observed that the expression of NDUFV1 was downregulated by the transfection of the pEGFP-nsP3 construct which expresses a GFP-nsP3 fusion protein (which will be referred to as nsP3 for the rest of this chapter) into Vero cells compared to untreated cells or cells transfected with pEGFP-N1 vector. The expression of β -actin was used as a loading control to normalise the expression of the target genes during the analysis. The densitometry data demonstrated statistically significant downregulation of the G3BP target gene, NDUFV1, indicating that the interaction of CHIKV nsP3 with G3BP potentially has an influence on the biological activity of G3BP and moderate the expression of its target mitochondrial gene, NDUFV1.

To determine if the protein expression levels of NDUFV1 were regulated at the translational levels, the steady state expression of the transcripts must be determined so that the possibility of decreased transcription of the gene or degradation of the mRNA can be excluded as the cause of decreased NDUFV1 protein levels. To investigate if the levels of the NDUFV1 transcript had been altered by the transfection of nsP3 into Vero cells, RT-qPCR was conducted with NDUFV1 primers. There was no significant difference observed in the mRNA expression levels of NDUFV1 compared to control groups which indicate that the regulation of expression of NDUFV1 by G3BP was affected by the nsP3 protein at the translational level (Figure 4.1C).

Figure. 4.1 Expression of NDUFV1 in nsP3 transfected Vero cells at transcript and protein levels. **Panel A** shows the densitometry data from immunoblotting for expression of NDUFV1 after normalization. The student's t-test was applied and statistically significant downregulation in the expression of NDUFV1 protein was confirmed. $n=3$ (** $P<0.01$ *** $P<0.001$). **Panel B** shows the immunoblot for the expression of NDUFV1 after Vero cells were transfected with the nsP3 protein for 72 hours compared with untransfected Vero cells or Vero cells transfected with the pEGFP-N1 empty vector for 72 hours as controls. **Panel C** shows the NDUFV1 mRNA expression levels after Vero cells were transfected with the nsP3 protein for 72 hours. No significant differences in the expression levels of the NDUFV1 transcript were observed when comparing untransfected Vero cells and Vero cells transfected with the pEGFP-N1 vector for 72 hours as controls.

4.2.2 Expression of β -F1-ATPase in nsP3-transfected Vero cells at protein level

To investigate the influence of CHIKV nsP3 on the biological activity of G3BP in regulating its target gene, β -F1-ATPase, immunoblotting analysis was performed using total cell lysates extracted from Vero cells after transfection with the pEGFP-N1 and pEGFP-nsP3 plasmids for 72 hours. The expression of β -actin was measured as a loading control to normalise the expression of the target gene during the analysis. The data were analysed using the Student's t-test to determine if there were statistically significant differences in protein expression.

As shown in the Figure 4.2B, there was no obvious downregulation in the expression of β -F1-ATPase in Vero cells transfected with nsP3 compared to Vero cells transfected with the empty vector, however, compared to the untransfected cells the nsP3 transfected cells have a significant down regulation of β -F1-ATPase protein expression. The densitometry data in Figure 4.2A also confirms that there were no statistically significant differences in the expression of β -F1-ATPase when nsP3 transfected cells were compared with cells transfected with an empty vector. It is difficult to reconcile this data which will be discussed later in this chapter (see 4.3). There was no significant difference in the regulation in protein expression of β -F1-ATPase after nsP3 transfected in Vero cells when compared to cells transfected with the empty vector, therefore, regulation of β -F1-ATPase at the transcript level was not examined.

Figure 4.2 Expression of β -F1-ATPase in nsP3 transfected Vero cells at the protein level. Panel A shows the densitometry data from immunoblotting for expression of β -F1-ATPase after normalization. Student's t-test was applied, and statistically significant downregulation in the expression of β -F1-ATPase protein in nsP3 transfected cells was observed compared to untreated Vero cells, while there was no significant difference in expression of β -F1-ATPase between nsP3 transfected cells and empty vector transfected cells. $n=3$ (** $P<0.01$). **Panel B** shows the immunoblot for the expression of β -F1-ATPase after Vero cells were transfected with nsP3 protein for 72 hours compared with untreated Vero cells and Vero transfected with pEGFP-N1 empty vector for 72 hours as negative controls.

Statistically significant differences were observed between the expression of β -F1-ATPase in untransfected Vero cells and in Vero cells transfected with nsP3 plasmid (Figure 4.2A). Since the Lipofectamine 2000 reagent was used in the transient transfection step (described in 2.2.1), the cells might be affected by this reagent leading to downregulated expression of the target protein that was observed in the negative control group. Therefore, to investigate the influence of Lipofectamine in regulating the protein expression of β -F1-ATPase, Vero cells were treated with Lipofectamine 2000 for 72 hours, extracted and immunoblotting analysis was performed using the β -F1-ATPase antibody.

Figure 4.3A and B demonstrate that the addition of Lipofectamine 2000 had a minimal influence on the protein expression levels of β -F1-ATPase, and there were no significant differences observed in the data analyzed by densitometry, indicating that the transfection reagent does not significantly influence the expression of β -F1-ATPase. However, it should be noted that the trend to decrease expression of β -F1-ATPase by lipofectamine appears similar to the decrease seen in cells transfected with the empty vector.

Figure 4.3 Expression of β -F1-ATPase in mock transfected Vero cells at the protein level. Panel A shows the densitometry data from immunoblotting for the expression of β -F1-ATPase after normalization. A Student's t-test was applied, and no statistically significant downregulation in the expression of β -F1-ATPase protein was observed. n=3. **Panel B** shows the immunoblot for the expression of β -F1-ATPase after Vero cells that were treated with Lipofectamine 2000 reagent for 72 hours compared to untreated Vero cells as a control.

4.2.3 Expression of IFITM3 in nsP3 transfected Vero cells at the transcript and protein levels

To investigate the effect of CHIKV nsP3 on the translational regulation of the G3BP target gene, IFITM3, immunoblotting was performed with an anti-IFITM3 antibody. The expression of β -actin was measured as a loading control to normalise the expression of the target genes during the analysis.

From Figure 4.4A and B, the data demonstrates that the protein expression of IFITM3 was downregulated after the Vero cells were transfected with pEGFP-nsP3, compared to negative control groups which are untreated cells and Vero cells transfected with the empty pEGFP-N1 vector. The densitometry data shows that a statistically significant downregulation of IFITM3 occurred in the nsP3 transfected cells. This data indicates that the interaction between CHIKV nsP3 and G3BP might potentially decrease G3BP activity in the translational regulation of its target gene, IFITM3, leading to the downregulation of the expression of the IFITM3 protein.

Figure 4.4C evaluates the expression of IFITM3 mRNA level by conducting the RT-qPCR with IFITM3 primers and β -actin primers as a control. There is no significant difference in the expression levels of the IFITM3 transcript after cells were transfected with nsP3 as compared with control cells transfected with empty vector pEGFP-N1. The significant downregulation of IFITM3 protein in Vero cells transfected with nsP3 demonstrates the significant influence of CHIKV nsP3 on G3BP regulation of its target gene IFITM3 at the translational level.

Figure. 4.4 Expression of IFITM3 in nsP3 transfected Vero cells at the transcript and protein levels. **Panel A** shows the densitometry data from immunoblotting for expression of IFITM3 after normalization. A Student's t-test was applied, and statistically significant downregulation in the expression of the IFITM3 protein was confirmed. $n=3$ (** $P<0.01$). **Panel B** shows the immunoblot for the expression of IFITM3 after Vero cells were transfected with nsP3 protein for 72 hours compared with untransfected Vero cells and Vero cells transfected with the pEGFP-N1 empty vector for 72 hours as controls. **Panel C** shows the transcript levels of IFITM3 after Vero cells were transfected with nsP3 protein for 72 hours. No significant difference was observed when comparing untreated Vero cells, Vero cells transfected with pEGFP-N1 or Vero cells transfected with the pEGFP-nsP3 vector for 72 hours as controls.

4.3 Discussion

G3BPs are a family of RBPs which are involved in the regulation of stability and translational efficiency of their target transcripts. Several targets of G3BP1 have been reported including *c-MYC* (Gallouzi et al., 1998), *Tau* (Atlas et al., 2004), *CTNNB1* (Bikkavilli and Malbon, 2011), *PMP22* (Winslow et al., 2013), but these papers report that G3BPs can cause increased degradation of target transcripts or conversely cause the upregulation of proteins from the target transcript. In addition to these targets, recent data reports a role for G3BP1 in regulating the expression of β -F1-ATPase (Álvaro D. Ortega, 2010). Furthermore, work performed in the host laboratory has shown that G3BP can also regulate the translation of the nuclear encoded mitochondrial protein NDUFV1 (unpublished data) as well as the interferon-induced transmembrane IFITM₁₋₃ genes (manuscript in submission). Previous studies and those described in chapter 3 shows that there was an interaction between CHIKV nsP3 and G3BP as determined by G3BP's translocation to nsP3 foci and this concurs with data reported previously (Fros et al., 2012). This chapter was designed to study the biological consequences of the nsP3 interaction with G3BP and assessed if the interaction changed the cellular activities of G3BP in the translational control of two mitochondrial genes, NDUFV1 and β -F1-ATPase as well as the IFITM3 gene.

4.3.1 CHIKV nsP3 influences the activity of G3BP and significantly downregulates the expression of G3BP target genes NDUFV1 and IFITM3, at the translational level, but not β -F1-ATPase

The host laboratory has found that the knock-down of G3BP1 can significantly downregulate the protein expression of NDUFV1 (unpublished data), indicating that G3BP1 regulates the translation of this target transcript. Therefore, the expression of NDUFV1 has been utilised to determine the influence of CHIKV nsP3 on the translational regulation of G3BP target genes. In Figure 4.1A and B, it can be observed that the expression of NDUFV1 was significantly downregulated by the transfection of nsP3 into Vero cells compared to untreated cells or cells transfected with the empty pEGFP-N1 control vector. This data suggests that the interaction between CHIKV nsP3 and G3BP can affect the biological activity of G3BP and moderate the expression of its target gene, NDUFV1. The data shown in chapter 2 demonstrated that nsP3 can

translocate G3BP to cytoplasmic nsP3-containing foci and combined with the data provided in this chapter, it is highly suggestive that the interaction between nsP3 and G3BP blocks the usual biological activity of G3BP by forming the cytoplasmic nsP3/G3BP foci and therefore, G3BP cannot regulate the stability and translation of NDUFV1 which leads to the downregulation of NDUFV1 protein expression.

Similarly, another G3BP1 target gene, IFITM3, has been studied in the host laboratory (manuscript in submission). It has been found that the knock-down of G3BP1 results in a significant downregulation of IFITM3, suggesting that the regulation of G3BP is also important for the stability and translation of the transcript for this protein. This data suggests that G3BPs are required for the stability of the transcript and the translational efficiency of the NDUFV1 and IFITM3 transcripts and this data appears to contradict previous studies by Gallouzi and co-workers that show G3BP1 can degrade target transcripts such as *c-Myc* (Gallouzi et al., 1998). As shown in the Figure 4.3A and B, the protein expression of IFITM3 was significantly downregulated after Vero cells were transfected with pEGFP-nsP3, compared to the negative control groups. The data also confirms that the interaction between CHIKV nsP3 and G3BP can effectively decrease the G3BP activity in the translational regulation of its target transcript, IFITM3, leading to the downregulation of expression of the IFITM3 protein.

The data revealed that the expression of β -F1-ATPase was significantly down regulated by nsP3 when compared to untransfected cells, however, the data did not show significant down regulation when compared with cells transfected with the empty vector (Fig 4.2). G3BP1 has been reported to play a crucial role in the degradation of selective mRNA and potentially give rise to a functional differentiation between transcriptional and posttranscriptional controls (Tourrière et al., 2001). While work done by Ortega and co-workers suggests that G3BP1 interacts with β -F1-ATPase mRNA and inhibits its translation (Álvaro D. Ortega, 2010). Therefore, the expected result would be to see an increase in the expression of β -F1-ATPase after G3BP1 had been translocated to the nsP3 foci. However, if the expression of β -F1-ATPase was being expressed in a maximal steady state in the untransfected cells, it is conceivable that no potential increase in protein expression could be observed. One possibility is that the translocation of G3BPs to nsP3-foci could take a portion of target transcripts with it and sequester those transcripts from active sites of translation and this could, in part, explain the unexpected result. The experiments performed in this chapter differ

from knockdown or knockout experiments where G3BPs are depleted from cells. In the experiments reported in this work, the translocation of G3BPs to nsP3-foci did not remove G3BP from the cell and all the consequences of this have yet to be fully characterised.

4.3.1 CHIKV nsP3 does not influences the activity of G3BP in regulating the stability of mRNA levels of NDUFV1 and IFITM3

Figures 4.1C and 4.4C showed the data from experiments to determine if the interaction of nsP3 with G3BP in Vero cells could affect the stability of the NDUFV1 and IFITM3 transcripts. There were no significant differences observed in the levels of expression of NDUFV1 and IFITM3 transcripts after transfection with nsP3 compared to control groups (Figure 4.1C and 4.4C). This data indicated that transfection of nsP3 did not affect the stability of the two target transcripts by G3BP under these conditions.

In summary, work done by this chapter indicates that the interaction between CHIKV nsP3 with G3BP can affect the biological activity of G3BP and moderate the expression of its target genes NDUFV1 and IFITM3. The apparent mode of action appears to be that nsP3 translocates G3BPs into cytoplasmic nsP3/G3BP foci and blocks certain biological activities of G3BPs, causing the loss of function of G3BP in regulating the translation of its target genes, which leads to the significant downregulation of protein expression of target genes. The findings of this chapter serve as a tool for future study to explore the role of G3BP in the progression of cancer and its potential role in the Warburg effect.

Chapter 5

**Increased cytotoxicity of Bortezomib by
transfection of CHIKV nsP3 into T47D, MCF7,
HeLa and HEK293 cells**

5.1 Introduction

Bortezomib (Velcade®) is an FDA-approved chemotherapeutic which is a peptide boronate inhibitor of the 26S proteasome that is highly efficient for the treatment of hematological tumours (Richardson, 2004). It is the first therapeutic proteasome inhibitor that was applied to human treatment (Adams and Kauffman, 2004). Proteasomes are large protein complexes that are responsible for the degradation of unwanted proteins which are tagged by polyubiquitin chains for recognition (Gu and Enenkel, 2014). Proteins that are eliminated through this process are fundamental or regulatory proteins that include those used to control of cell-cycle progression, tumour growth and cell apoptosis (Adams and Kauffman, 2004, Adams et al., 1999). In addition, the proteasome is also essential for activation of transcription factor nuclear factor-kappaB (NF- κ B) by degradation of its inhibitory protein, I κ B (Adams et al., 1999). NF- κ B is a crucial modulator for promoting growth of cancer cells and reducing the effectiveness of anticancer therapies (Adams, 2003, Richardson, 2004). Due to the critical roles of the proteasome in cell process regulation, proteasome inhibitors were investigated as a potential target for anti-cancer therapy (Teicher and Tomaszewski, 2015). Proteasome inhibitors are drugs that block the action of the proteasome and induce apoptosis in cancer cells (Dou and Zonder, 2014). One mechanism of the function of proteasome inhibitors is related to the blockage of NF- κ B activity, which makes cells more susceptible to apoptosis (Patel et al., 2000, Adams and Kauffman, 2004). However, the proteasome inhibitor Bortezomib has been shown to induce the assembly of SGs effectively due to its cytotoxicity (Anderson et al., 2015). In addition, it triggers the phosphorylation of eIF2 α by activating heme-regulated inhibitor kinase (HRI), and subsequently promoting SGs formation (Fournier et al., 2010, Anderson et al., 2015).

Data from cell-based assays using Bortezomib in cytotoxic assays suggest that knockout of HRI, which catalyses the phosphorylation of translation initiation factor eIF2 α , stops SGs formation and restores Bortezomib efficacy in cancer cells (Fournier et al., 2010). These data suggest that blocking SG formation may provide a pathway to combinational treatments to improve the efficacy of current FDA approved drugs. However, knockout of genes, such as HRI, in humans for the purposes of cancer therapy is not currently practicable. Therefore, new therapeutic approaches are required to reach similar endpoints as demonstrated by the HRI knockout and new drugs or strategies

need to be explored. Strategies that block SG formation appear to be one pathway that may be appropriate for use with cancer therapies. As mentioned in chapter 3, nsP3 has the capacity to disrupt SGs. Therefore, transfection of nsP3 into cancer cells and subsequent treatment with chemotherapies could be a useful tool to validate the targeting of G3BP, or the formation of SG, as a target for future investigations into new chemotherapeutic strategies. It may represent a tool to explore therapies that could enhance the efficacy of drugs such as Bortezomib. The significance of this chapter is that if treatment of cells with nsP3 can enhance Bortezomib activity, it will serve as a proof of concept for further research into strategies to block G3BP activities in anti-cancer therapeutics.

This chapter aimed to determine if the nsP3 protein can interact with G3BP in heterogeneous nuclear ribonucleoproteins particles and restore cytotoxicity of the anti-cancer drug Bortezomib by translocating G3BP from SG. The HeLa, Hek293, T47D and MCF7 cell lines were utilised for Bortezomib cytotoxicity determination. Cells were cultured in 384-well plates containing drug concentrations ranging from 0.05nM to 80nM. The Alamar Blue assay was applied to determine the cell viability with fluorescent and colorimetric plate reader. Resazurin is the active ingredient of Alamar Blue reagent, it is a non-toxic, cell permeable compound which is blue in color and virtually non-fluorescent. Resazurin is reduced to resorufin upon entering cells, a compound that is red in color and highly fluorescent. Viable cells constantly convert resazurin to resorufin which leads to the increase in the overall fluorescence and also the change of the media color surrounding cells. An IC_{50} value represents the concentration of an inhibitor where the response is reduced by half. The aim of the research performed in this chapter was to determine if IC_{50} values for cytotoxicity of Bortezomib would be reduced in the presence of the nsP3 protein, a lower IC_{50} value would indicate that the drug causes 50% cytotoxicity at a lower concentration. This may have a potential for further applications in the discovery of strategies that can increase the efficacy of FDA approved drugs.

5.2 Results

5.2.1 Determination of optimal cell density in a 384-well plate

Cell growth can be inhibited by high cell density and this is attributed to the cell to cell contact. Conversely, low cell density may lead to the slow growth and might also affect cell responses to drug treatment. Therefore, a cell viability assay is required to determine the optimal cell density during the duration of the experiments performed, so that the cells do not reach inhibitory levels during the course of Bortezomib cytotoxicity determination. T47D, MCF7, HeLa and HEK293 were cultured as described in 2.2.1. Cells were seeded in a 384-well plate with concentrations ranging from 50 to 2400 cells per well and allowed to grow for 72 hours in a 37°C incubator. The Alamar Blue assay was applied, and growth was measured with a fluorescent and colorimetric plate reader. As shown in Figure 5.1, all four cells were kept in linear phase growth with 72 hours incubation, which is the optimal incubation time for Bortezomib cytotoxicity assay that has been determined in the host laboratory. According to the cell growth curve, 1600 cells per well was chosen to be the optimal cell density for T47D, MCF7, HeLa and HEK293 cells that grown in DMEM + 10% FBS media in a 384-well plate for 72 hours.

Figure 5.1 Determination of optimal cell density in a 384-well plate. Cell growth curves for T47D, MCF7, HeLa and HEK293 cells with concentrations ranging from 50 to 2400 cells per well in a 384-well plate and incubated for 72 hours. The graph shows cell proliferation on the Y axis represented by fluorescence from Alamar Blue assay. Error bars are generated from technical triplicates.

5.2.2 Transfection of the CHIKV nsP3 protein in HEK293, HeLa, T47D and MCF7 cells increases cytotoxicity of Bortezomib

To check if the CHIKV nsP3 protein can disrupt SGs and increase cytotoxicity of the anti-cancer drug Bortezomib by translocating G3BP from SGs, the IC_{50} of Bortezomib in T47D, MCF7, HeLa and HEK293 cells was determined with drug concentrations ranging from 0-80nM, as well as the corresponding value of the cells transfected with pEGFP-N1 or pEGFP-nsP3.

Figures 5.2 to 5.5 correspond to cell lines HEK293, HeLa, T47D and MCF7 respectively and they show the IC_{50} calculations for cells treated with the vehicle control (Panels A), cells transfected with pEGFP-N1 (panels B) and pEGFP-nsP3 (panels C). Comparing panels A of Figures 5.2-5.5 with Figure 5.2B-5.5B, it can be observed that the IC_{50} values of cells transfected with pEGFP-N1 and untreated cells had no significant differences, indicating the negligible influence of cell transfection with the empty pEGFP-N1 vector in all four cell lines.

When T47D cells transfected with nsP3 (*i.e.* pEGFP-nsP3), the IC_{50} of Bortezomib had a 22-fold decrease from 5.37nM to 0.24nM as shown in Figure 5.4C, which suggests that the translocation of G3BP from SG by nsP3 protein dramatically increased the cytotoxicity of the anti-cancer drug Bortezomib. Similarly, the IC_{50} curve in MCF7 showed a similar trend in the reduction of the IC_{50} concentration when MCF7 cells were transfected with nsP3 protein, from 13.27nM in mock cells to 1.86nM in nsP3 transfected cells, which is a 7-fold decrease (Figure 5.5C).

Nevertheless, as shown in the Figure 5.2C and 5.3C, it can be seen that the IC_{50} value of HEK293 cells and HeLa cells transfected with nsP3 protein had smaller reduction compared with cells transfected with the empty vector which corresponded to a 2.1-fold decrease for HEK293 cells and 1.8-fold decrease for HeLa cells respectively.

Figure 5.2 IC₅₀ curves of Bortezomib in HEK293 cells transfected with pEGFP-N1 or pEGFP-nsP3 plasmid. IC₅₀ curves for the Bortezomib drug in HEK293 cells which were treated with concentrations of Bortezomib ranging from 0-80nM. The curves represent 3 biological repeats and the curves shown here are representative of the three biological repeats. Error bars are generated from technical triplicates within each experiment. IC₅₀ values represent mean IC₅₀ value of biological triplicates ± SEM.

Figure 5.3 IC₅₀ curves of Bortezomib in HeLa cells transfected with pEGFP-N1 or pEGFP-nsP3 plasmid. IC₅₀ curves for Bortezomib drug in HeLa cells are treated with concentrations of Bortezomib ranging from 0-80nM. The curves represent 3 biological repeats. Error bars are generated from technical triplicates within each experiment. IC₅₀ values represent mean IC₅₀ value of biological triplicates ± SEM.

Figure 5.4 IC_{50} curves of Bortezomib in T47D cells transfected with pEGFP-N1 or pEGFP-nsP3 plasmid. IC_{50} curves for Bortezomib drug in T47D cells are treated with concentrations of Bortezomib ranging from 0-80nM. The curves represent 3 biological repeats. Error bars are generated from technical triplicates within each experiment. IC_{50} values represent mean IC_{50} value of biological triplicates $\pm$ SEM.

Figure 5.5 IC_{50} curves of Bortezomib in MCF7 cells transfected with pEGFP-N1 or pEGFP-nsP3 plasmid. IC_{50} curves for Bortezomib drug in MCF7 cells are treated with concentrations of Bortezomib ranging from 0-80nM. The curves represent 3 biological repeats. Error bars are generated from technical triplicates within each experiment. IC_{50} values represent mean IC_{50} value of biological triplicates $\pm$ SEM.

5.2.3 IC₅₀ values of Bortezomib in HEK293, HeLa, T47D and MCF7 cells

The data shown in Figures 5.2 to 5.5 show representative Bortezomib cytotoxicity curves (performed in biological triplicates with each repeat containing 3 technical repeats) for T47D, MCF7, HeLa and HEK293 transfected with pEGFP-N1 or pEGFP-nsP3, Table 5.1 shows the average of 3 biological repeats for these treatments. As shown in Table 5.1, all four cell lines transfected with pEGFP-nsP3 showed statistically significant decreases in the IC₅₀ values of Bortezomib compared to cells transfected with empty vector pEGFP-N1. Furthermore, mammalian breast cancer cell lines T47D and MCF7 showed a dramatically significant decrease of Bortezomib IC₅₀ value when transfected with pEGFP-nsP3, which was around a 22-fold decrease for T47D and a 7-fold reduction for MCF7 compared to cells transfected with empty vector (Table 5.1).

Table 5.1 IC₅₀ values of Bortezomib in HEK293, HeLa, T47D and MCF7 cells transfected with the nsP3 protein. Average IC₅₀ values for the Bortezomib drug in HEK293, HeLa, T47D and MCF7 cells which were treated with concentrations of Bortezomib ranging from 0-80nM. The values shown represent with mean IC₅₀ values of three biological repeats $\pm$ SEM. Cells transfected with nsP3 showed statistically significant decreases compared with the control group which were the cells transfected with the pEGFP-N1 vector. Student's t-test was applied. * represents P<0.05, ** indicates P<0.01 and **** means P<0.0001.

IC ₅₀ / nM Cell lines	Untreated cells	Cells transfected with pEGFP-N1	Cells transfected with pEGFP-nsP3
HEK293	2.40 $\pm$ 0.20	2.66 $\pm$ 0.22	1.12 $\pm$ 0.23 *
HeLa	4.56 $\pm$ 0.14	4.25 $\pm$ 0.09	2.45 $\pm$ 0.20 **
T47D	5.38 $\pm$ 0.19	5.17 $\pm$ 0.25	0.24 $\pm$ 0.01 ****
MCF7	13.27 $\pm$ 0.67	12.17 $\pm$ 0.73	1.86 $\pm$ 0.12 ****

5.3 Discussion

Bortezomib is the first proteasome inhibitor that is applied in anti-cancer therapeutics (Richardson, 2004). However, certain solid tumours of various histological origins show resistance to Bortezomib treatment. The mechanism of the resistance remains partly unknown, while the activation of stress response is considered to be the main reason (Fournier et al., 2010). Fournier and co-workers found that by knockout of the HRI gene, which catalyses the phosphorylation of translation initiation factor eIF2 α and leads to the formation of SGs, prevented the formation of bortezomib-induced SGs and restored drug efficacy (Fournier et al., 2010). However, knockout of genes, such as HRI, in humans for the purposes of cancer therapy is not currently practicable. Therefore, this chapter explores a new approach, other than the knockout of HRI, to block the formation of SGs and reach the similar endpoints.

As shown in the Figures 5.2C-5.5C, it can be observed that the transfection of CHIKV nsP3 protein into the four utilised cell lines (*i.e.* HEK293, HeLa, T47D and MCF7) can effectively increase the cytotoxicity of the Bortezomib drug (*i.e.* decrease the IC₅₀ value for the drug). All four cell lines showed a statistical significant decline in IC₅₀ values of Bortezomib when transfected with CHIKV nsP3 as compared to the negative control groups (Table 5.1). Moreover, transfection of nsP3 into cancer cells, especially breast cancer cells (T47D and MCF7), can dramatically enhance the cytotoxicity of Bortezomib, which is a 22-fold decrease of IC₅₀ for T47D and a 7-fold decrease for MCF7 cells (Table 5.1). The effect of cytotoxicity restoration was smaller in HEK293 and HeLa cells, which might be caused by the different activities of G3BP and less drug resistance in these two cell lines compared to breast cancer cell lines. Overall, it serves as a proof of concept for further research into strategies to block SGs assembly in anti-cancer therapeutics.

The IC₅₀ of Bortezomib in HEK293 and HeLa cells that were transfected with the nsP3 protein showed a minor difference, around a 2-fold decreases, compared with cells transfected with the empty vector. The assumption accounting for this situation is that the activities of G3BP in HEK293 and HeLa cells are different from the ones in breast cancer cells. Since G3BP has been found to be overexpressed in cancers including head and neck, thyroid, colon, pancreatic and breast cancer (Barnes et al., 2002, Guitard et al., 2001, French et al., 2002, Taniuchi et al., 2011), and the activity of nsP3 is to

translocate G3BP into cytoplasmic foci and block the assembly of SGs (Chapter 3). It can be assumed that CHIKV nsP3 has a greater biological effect on drug cytotoxicity when there is more G3BP activity in the cell. However, this requires further testing to be confirmed and would require a detailed quantification of G3BP expression levels in the cell lines as well as an analysis of all activities of G3BPs. Unfortunately, at the present time there is no assay to assess G3BP activity, so the initial studies would have to focus on G3BP expression levels.

The ability of nsP3 to enhance the Bortezomib cytotoxicity in MCF7 cells is slightly different from T47D cells. Both T47D and MCF7 are mammalian breast cancer cells, indicating that the activity of G3BP in these two cell lines could be similar. However, as shown in Table 5.1, the transfection of nsP3 into T47D showed a 22-fold decrease of IC_{50} , while in MCF7, it only had a 7-fold decline. One possible explanation for the differences is that MCF7 have different resistance to Bortezomib drug due to differences in signalling pathways that regulate G3BP activity. The host laboratory has already determined that expression of HRI is different in these cell lines (Kim et al., manuscript in submission) and this can influence stress granule formation. Furthermore, a previous study reported that MCF7 cells were resistant to Bortezomib treatment and that 80nM of Bortezomib could not reach the IC_{50} growth inhibition dose in MCF7 cells (Hideshima et al., 2014). The mechanism of action responsible for the significant decrease in the sensitivity to Bortezomib in MCF7 cell line remains unclear. However, it is possible that the resistance to Bortezomib is also regulated by pathways other than SGs assembly. If this were the case it could explain why the action of nsP3 in blocking SGs formation in MCF7 cells is less effective than in T47D cells.

The data presented here showed that the Bortezomib IC_{50} value in T47D cells was higher than that of HEK293 cells (Table 5.1). However, after their transfection with pEGFP-nsP3, the IC_{50} value in T47D breast cancer cells was lower than in normal human cell line HEK293. It can be proposed that the transfection of CHIKV nsP3 may empower the selectivity of Bortezomib drug in breast cancer treatment. Nevertheless, further experiments are required for verification.

Chapter 6

General Discussion and Future Directions

6.1 General Discussion

SGs are non-membranous cytoplasmic foci that contain mRNPs which aggregate in cells under stress conditions (Anderson and Kedersha, 2008, Takahashi et al., 2013, White et al., 2007). SGs appear to be involved in cancer cell proliferation and chemotherapeutic treatment (Anderson et al., 2015). Therefore, targeting SGs has been considered as a target for anti-cancer treatment. G3BP is a core protein involved in the assembly of SGs and may play a role in the transport of mRNA to the granules in response to stress or signalling (Anderson and Kedersha, 2008). SG formation has been implicated in changing the bias of gene expression within the cell to favour multi-drug resistant genes (ABCB1 gene) and to modulate the expression of those involved in cancer progression (Zhang et al., 2015, Ashikari et al., 2017, Takayama et al., 2018b, Takayama et al., 2018a, Zhang et al., 2017). CHIKV nsP3 has been reported to block the assembly of SGs by recruitment of G3BP into cytoplasmic foci (Fros et al., 2012). Therefore, it has the capacity to disrupt SGs and thereby disrupt the balance of gene expression required by drug resistance cells to survive treatment with chemotherapies. Moreover, SG also appears to regulate gene expression that assists cells to compensate for the hostile microenvironment created during the development and progression of cancer (Anderson et al., 2015) and therefore, disruption of SG during early development of cancer may also be an appropriate strategy to reduce cancer growth.

Transfection of nsP3 into cancer cells and subsequent treatment with chemotherapies may represent a tool to explore therapies to be used in combination with drugs such as Bortezomib. Data from previous cell-based assays using Bortezomib in cytotoxic assays suggest that knockout of HRI, which catalyses the phosphorylation of translation initiation factor eIF2 α , stops SGs formation and restores Bortezomib efficacy in cancer cells (Fournier et al., 2010). These data suggest that blocking SG formation may provide a pathway for combinational treatments to improve the efficacy of current FDA approved drugs. However, knockout of genes, such as HRI, in humans for the purposes of cancer therapy is not currently practicable. Therefore, new therapeutic approaches are required to reach similar endpoints, as demonstrated by the HRI knockout, and new drugs or strategies need to be explored.

The work presented in this thesis was designed to explore the role of dissociating Stress Granules, by transfection of nsP3 into cancer cells, in conjunction with the treatment of

cells with anti-cancer chemotherapies (*i.e.* Bortezomib). Transfection with nsP3 was designed to disrupt the biological activities of G3BP via the interaction between nsP3 and G3BP. This was assessed visually by fluorescent microscopy. The effect on the biological activity was assessed by measuring the expression of the mitochondrial protein NDUFV1 and the IFITM3 genes which have previously been shown to be translationally regulated by G3BPs (Umber and Kennedy, 2018). The hypothesis being explored is that if the biological activity of G3BP can be disrupted in cells the survival of cancer cells during treatment with chemotherapeutics would be compromised. The findings of this thesis represent a tool that can be used to explore adjuvant, or combinational therapies, with drugs such as Bortezomib, and it also serves as a tool for future study to explore the role of G3BP in the progression of cancer and its potential role in the Warburg effect. More specifically, the data shows that nsP3 can translocate G3BP into nsP3/G3BP foci and block the assembly of SGs, which significantly increases the cytotoxicity of the anti-cancer drug Bortezomib. The transfection of nsP3 may also empower the selectivity of Bortezomib drug in breast cancer treatment. In addition, the interaction between nsP3 and G3BP has been found to change the biological activity of G3BP and moderate the protein expression of its target genes NDUFV1 and IFITM3.

Chapter 3 was designed to study if the nsP3 protein, from Chikungunya virus, can translocate G3BP into cytoplasmic foci and block the formation of SGs. This was assessed by visualising the cellular localisation of G3BP1 and TIA1 under stress and non-stress conditions. Both G3BP1 and TIA1 are SGs markers and they both move to SGs under sodium arsenite stress treatment which is consistent with previous studies (Tourriere et al., 2003, Kedersha et al., 1999). In addition, it has been found that the CHIKV nsP3 protein can interact with G3BP1 and form cytoplasmic foci under both normal (untreated) and stressed (AsNaO_2 treated) conditions. The formation of nsP3/G3BP foci blocks the assembly of SGs, as reported by Fros and co-workers (Fros et al., 2012). Overall, this observation suggests that nsP3 can translocate G3BP into cytoplasmic nsP3/G3BP foci and block the assembly of SGs.

Chapter 4 was designed to explore the role of G3BP in translational control of nuclear encoded mitochondrial NDUFV1 and the IFITM3 gene as a surrogate marker for disrupted G3BP activity. It has been reported that G3BP1 regulates the expression of its target β -F1-ATPase (Álvaro D. Ortega, 2010) and the expression of this gene was

assessed. The host laboratory had previously determined that mitochondrial complex I protein, NDUFV1 (unpublished data) as well as the IFITM₁₋₃ genes (Umber and Kennedy, in submission 2018) can be regulated by G3BP activity. After transfection with CHIKV nsP3, the protein expression of NDUFV1 and IFITM3 was observed to be significantly downregulated compared to control groups. These results demonstrated that the interaction between nsP3 and G3BP blocked the biological activity of G3BP by forming the cytoplasmic nsP3/G3BP foci, and therefore, G3BP cannot regulate the stability and translation of the NDUFV1 and IFITM3 genes. There was no significant difference observed in the expression levels of NDUFV1 and IFITM3 mRNA after transfection with nsP3 compared to control groups, indicating that transfection of nsP3 does not affect the stability of these two transcripts. However, the protein expression of NDUFV1 and IFITM3 were significantly decreased after transfection with nsP3, suggesting that the interaction of G3BP1 and nsP3 regulates the translational control of these proteins. This was interpreted as a surrogate marker for the activity of G3BP indicating that the biological activity of G3BP was reduced when it interacted with nsP3. Interestingly, the protein expression of another mitochondrial gene, β -F1-ATPase, was not downregulated after transfection of nsP3 and this reflects an opposing, but not unexpected, role for the interaction between G3BP1 and β -F1-ATPase mRNA. Previous research by Ortega and co-workers showed that G3BP inhibits the translation of β -F1-ATPase (Álvaro D. Ortega, 2010). Therefore, the sequestration of G3BP by nsP3 to foci within cells was speculated to release the inhibitor role of G3BP on the translation of β -F1-ATPase, however, this was not observed in the experiments and may mean that G3BP takes some of its mRNA cargoes to the nsP3 foci. Overall the results from the experiments reported in this thesis support a model in which G3BP1 is required to maintain translation of NDUFV1 and IFITM3 but inhibits the translation of β -F1-ATPase. These findings represent a tool for future study into strategies to block G3BP activities in anti-cancer therapeutics.

Chapter 5 aimed to determine if the nsP3 protein can interact with G3BP in heterogeneous nuclear ribonucleoproteins particles referred to as nsP3 foci and restore the cytotoxicity of the anti-cancer drug Bortezomib by translocating G3BP away from SG. The transfection of CHIKV nsP3 protein into the four utilised cell lines (*i.e.* HEK293, HeLa, T47D and MCF7) can significantly increase the cytotoxicity of the Bortezomib drug compared to control groups. Moreover, due to the overexpression of

G3BP in various tumours (Barnes et al., 2002, Guitard et al., 2001, French et al., 2002, Taniuchi et al., 2011), the transfection of nsP3 protein into cancer cells, especially breast cancer cells (T47D and MCF7), has been shown to dramatically enhance the cytotoxicity of Bortezomib compared to normal cell lines. The data presented in Chapter 5 shows a 22-fold decrease in the IC₅₀ for T47D and a 7-fold decrease for MCF7 cells. The different responses by the two breast cancer cell lines (*i.e.* 22-fold decrease in T47D *versus* 7-fold decrease in MCF7) may be related to the resistance of MCF7 cells to Bortezomib treatment that has been reported previously (Hideshima et al., 2014). The mechanism of action responsible for the significant decrease in the sensitivity to Bortezomib in MCF7 cell lines remains unclear. However, it is possible that the resistance to Bortezomib is also regulated by pathways other than SG assembly. If this were the case it could explain why the action of nsP3 in blocking SGs formation in MCF7 cells is less effective than in T47D cells. In the future, more chemotherapy drugs should be assessed to determine if drug resistance is related to SGs assembly and if the transfection of nsP3 can effectively restore the drug cytotoxicity. In addition, more cell lines, especially cancer cell lines, should be tested in response to chemotherapy treatment with co-transfection of CHIKV nsP3 to explore the wider application.

The data presented in chapter 5 shows that the IC₅₀ of Bortezomib in T47D cells was higher than that of HEK293 cells (Table 5.1). However, after transfection with nsP3, the IC₅₀ value in T47D breast cancer cells was lower than that in the normal human cell line HEK293. It is possible that the transfection of CHIKV nsP3 may empower a significant selectivity of Bortezomib during treatment of breast cancer. The *in vitro* data presented in this research indicates that the drug, Bortezomib, selectively kills breast cancer cells (compared to HEK cells) at a lower IC₅₀ when those cells are transfected with nsP3. The significant increase in selectivity was unexpected and further experiments with more cancer cell lines and chemotherapy compounds should be performed to validate this finding as it has significant potential for future combinational drug treatment regimes.

In conclusion, this project explores a new approach to target SGs disassembly in combinational studies and chemotherapy drug resistance. Despite the fact that targeting transfection of CHIKV nsP3 into human cells during cancer therapy remains to be purely speculative, it provides a new insight in the discovery of adjuvant or

combinational therapies that can significantly increase the efficacy of FDA approved drugs. In addition, our knowledge of the role of G3BP has been enhanced by this project. Typically, G3BP is considered to be an SG-nucleating protein and blocking the activity of G3BP can effectively inhibit SGs assembly. Moreover, the inhibition of G3BP activity leads to the downregulation of its target proteins, indicating that G3BP is required for the stability and translation of the nuclear encoded mitochondrial NDUFV1 and IFITM3 proteins. The downregulation of NDUFV1 provides insight into the potential role of G3BP in regulating the activity of mitochondrial complex I during the Warburg effect (Santidrian et al., 2013). The data presented showing that G3BP1 can regulate the translation of the IFITM3 gene sheds light on how G3BPs might have a role in regulating the innate immune response to external stimuli. From the available literature and the knowledge gathered from experiments performed in this thesis, it is clear that G3BPs are involved in various cellular activities including SGs assembly. The results suggest that blocking the activity of G3BP and the inhibition of SG formation is a promising strategy to enhance drug efficacy and may even result in improved drug selectivity in anti-cancer therapies. The results obtained from this research has provided evidence that targeting G3BPs may offer a new strategy for developing anti-cancer therapies, however, further experiments are required to validate the improved drug efficacy and selectivity in breast cancers.

6.2 Future Directions

To investigate the preliminary finding which suggests a significant improvement of drug selectivity after cells were transfected with CHIKV nsP3, more FDA approved drugs should be tested using the systems described here. It should be noted that Bortezomib was used in the preliminary experiments described here and this was selected based on the data and experimental design used by Fournier and colleagues (Fournier et al., 2010), however, Bortezomib is a proteasome inhibitor and one mode of action is the phosphorylation of eIF2 α which can also cause SG assembly. Therefore, additional drugs that work via different pathways need to be assessed, these could include MEK inhibitors (*i.e.* sorafenib) or estrogen receptor blockers such as tamoxifen. Preliminary data from the host laboratory (not reported here) suggests that the efficacy of other drugs can be improved using strategies that block SG formation (Kim *et.al.*, in submission).

In addition to using more FDA approved drugs, more cancer cell lines, including cells derived from cancers other than breast cancer, should also be incorporated into future studies to determine if the findings based on T47D and MCF7 cells can be replicated in other breast cancer derived cell lines as well as non-breast cancer cell models.

The data presented in this thesis suggests that blocking the activity of G3BP or the inhibition of SGs assembly are promising strategies to enhance drug efficacy in anti-cancer therapies. However, is it unrealistic with current technology to specifically transfect nsP3 into cancer cells of a patient, therefore other approaches are necessary to explore additional mechanism that could reach similar endpoints as transfection with nsP3. For example, the screening of new compounds with a potential role in blocking the assembly of SGs could reveal new compounds that can be applied in adjuvant or combinational therapies with current FDA approved drugs to enhance drug efficacy or to improve cancer cell selectivity during chemotherapy.

Ultimately, one of the future steps in this line of research is to perform *in vivo* research. First, a vector would be required that can deliver nsP3 to a cancer mouse model to check cell survival after systemic delivery of nsP3 into cells and this would be essential for further *in vivo* studies. The current nsP3 protein is 57 kDa and consists of three domains (Mathur et al., 2016). Therefore, it will be helpful to determine the functional domain of nsP3 which binds to G3BP with a view to identify a smaller

peptide could be used for *in vivo* experiments. To facilitate *in vivo* studies, it would be more feasible to use a cancer explant into humanised mice (*i.e.* T47D cells injected into the fat pad of mice). This strategy would allow the establishment of cell lines that are stably transfected with the minimal nsP3 construct under the control of an inducible promoter (*i.e.* Tet-on promoter). Once a tumour was established in the mouse, tetracycline can be used to induce the expression of the minimal nsP3 construct in the cancer cells and the mouse could be treated with the selected chemotherapeutic (*i.e.* Bortezomib). These would be run in conjunction with control cohorts in which nsP3 was not induced and another cohort in which the minimal nsP3 construct was expressed but not in conjunction with chemotherapeutic treatment. If the experiment was successful a significant reduction of tumour size would be seen in mice expressing the minimal nsP3 protein in combination with the chemotherapeutic treatment when compared to the other experimental cohorts.

In conclusion, the experiments performed in this thesis have provided evidence that blocking G3BP activity or blocking SG formation may be a valid strategy for improving drug efficacy during combinational drug treatment, however, the data is preliminary and requires further validation.

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Appendixes

Appendix I: Plasmid Map of pEGFP-N1 Vector

Image taken from Addgene vector database website (cat # 6085-1)

Appendix II: Sequencing of pEGFP-nsP3 plasmid

Sequencing trace and Blast result with CMV forward primer

Chikungunya virus isolate Com5 non-structural polyprotein gene, complete cds; and structural polyprotein gene, partial cds

Sequence ID: KP702297.1 Length: 11171 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1849 bits(1001)	0.0	1042/1061(98%)	11/1061(1%)	Plus/Plus
Query 67	GATG-GGACCGTCGTACCGGGTAAACGCATGGACATCGCGAAGAACGAAGAARAGKCG	125		
Sbjct 4071	GATGTGCACCGTCGTACCGGGTAAACGCATGGACATCGCGAAGAACGATGAAGAGTGCG	4130		
Query 126	TAGTCAACGCCGCTAACCCCTCGCGGGTTACCGGGTGGCGGTGTTTGCAAGGCAGTATACA	185		
Sbjct 4131	TAGTCAACGCCGCTAACCCCTCGCGGGTTACCGGGTGGCGGTGTTTGCAAGGCAGTATACA	4190		
Query 186	AAAAATGGCCGGAGTCCTTTAAGAACAGTGCAACACCGAGTGGGAACCGCAAAACAGTTA	245		
Sbjct 4191	AAAAATGGCCGGAGTCCTTTAAGAACAGTGCAACACCGAGTGGGAACCGCAAAACAGTTA	4250		
Query 246	TGTGCGGTACGTATCCAGTAATCCACGCTGTTGGACCAAACCTCTCTAATTATTCGGAGT	305		
Sbjct 4251	TGTGCGGTACGTATCCAGTAATCCACGCTGTTGGACCAAACCTCTCTAATTATTCGGAGT	4310		
Query 306	CTGAAGGGGACCGGAATTGGCAGCTGCCTATCGAGAAGTCGCAAGGAAGTAAGTACAGGC	365		
Sbjct 4311	CTGAAGGGGACCGGAATTGGCAGCTGCCTATCGAGAAGTCGCAAGGAAGTAAGTACAGGC	4370		
Query 366	TGGGAGTAAATAGTGTAGCTATACCTCTCCTCTCCACAGGTGTATACTCAGGAGGGAAAG	425		
Sbjct 4371	TGGGAGTAAATAGTGTAGCTATACCTCTCCTCTCCACAGGTGTATACTCAGGAGGGAAAG	4430		
Query 426	ACAGGYTGACCCAGTCACTGAACACCTCTTTACAGCCATGGACTCGACGGATGCAGACG	485		
Sbjct 4431	ACAGGCTGACCCAGTCACTGAACACCTCTTTACAGCCATGGACTCGACGGATGCAGACG	4490		
Query 486	TGGTCATCTACTGCCGCGACAAAGAATGGGAGAAGAAAATATCTGAGGCCATACAGATGC	545		
Sbjct 4491	TGGTCATCTACTGCCGCGACAAAGAATGGGAGAAGAAAATATCTGAGGCCATACAGATGC	4550		
Query 546	GGACCCAAGTAGAGCTGCTGGATGAGCACATCTCCATAGACTGCGATATTGTTTCGCGTGC	605		
Sbjct 4551	GGACCCAAGTAGAGCTGCTGGATGAGCACATCTCCATAGACTGCGATATTGTTTCGCGTGC	4610		
Query 606	ACCCTGACAGCAGCTTGGCAGGCAGAAAAGGATACAGCACCACGGAAGGCGCACTGTACT	665		
Sbjct 4611	ACCCTGACAGCAGCTTGGCAGGCAGAAAAGGATACAGCACCACGGAAGGCGCACTGTACT	4670		
Query 666	CATATCTAGAAGGGACCCGTTTTTCATCAGACGGCTGTGGATATGGCGGAGATACATACTA	725		
Sbjct 4671	CATATCTAGAAGGGACCCGTTTTTCATCAGACGGCTGTGGATATGGCGGAGATACATACTA	4730		
Query 726	TGTGGCCAAAGCAAACAGAGGCCAATGAGCAAGTCTGCCTATATGCCCTGGGGGAAAGTA	785		
Sbjct 4731	TGTGGCCAAAGCAAACAGAGGCCAATGAGCAAGTCTGCCTATATGCCCTGGGGGAAAGTA	4790		
Query 786	TTGAATCGATCAGGCAGACATGCCCGGTGGATGATGCAGACGCATCATCTCCCCCAAAA	845		
Sbjct 4791	TTGAATCGATCAGGCAGAAATGCCCGGTGGATGATGCAGACGCATCATCTCCCCCAAAA	4850		
Query 846	CTGTCCCGTGCCTTTGCCGTTACGCTATGACTCCAGAACGCGTCACCCGGCTTCGCATGA	905		
Sbjct 4851	CTGTCCCGTGCCTTTGCCGTTACGCTATGACTCCAGAACGCGTCACCCGGCTTCGCATGA	4910		
Query 906	ACCACGTCACAAGCATAATTGTGTGTTCTTCGTTTCCCTCCCAAAGTAC-AAATAGAAG	964		
Sbjct 4911	ACCACGTCACAAGCATAATTGTGTGTTCTTCGTTTCCCTCCCAAAGTACAAAATAGAAG	4970		
Query 965	GAGTGCAAAAAGTCAAATGCTCTAAGGTAATGCTATTTGACCAC-ACGTGCCATCGCGCG	1023		
Sbjct 4971	GAGTGCAAAAAGTCAAATGCTCTAAGGTAATGCTATTTGACCACACGTGCCATCGCGCG	5030		
Query 1024	TA-GTCCA-GGG-ATATAGATCTT-CCAGGAGTCTGCACAGAAG-CGAGTAC-ATCACGT	1077		
Sbjct 5031	TAAGTCCAAGGGAATATAGATCTTCCAGGAGTCTGCACAGGAGGCGAGTACAATCACGT	5090		
Query 1078	CACTGACGCATAGTC-ATTCGACCTAAGCGTTGATGGGCGA 1117			
Sbjct 5091	CACTGACGCATAGTCAATTGACCTAAGCGTTGATGG-CGA 5130			

Sequencing trace and Blast result with EGPF_N reverse primer

Chikungunya virus gene for non-structural polyprotein, gene for non-structural polyprotein short and long variant and gene for structural polyprotein, genomic RNA, strain 06-049

Sequence ID: AM258994.1 Length: 11601 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
1816 bits(983)	0.0	1006/1016(99%)	7/1016(0%)	Plus/Minus
Query 70	GCCCTGTCTAGTCTTAAC	CGTCGTCCGTGCTGAGCACGTGGACCA	GTGCTGTCTGTC	129
Sbjct 5607	GCCCTGTCTAGTCATAACT	CGTCGTCCGTGCTGAGCACGTGGACCA	GTGCTGTCTGTC	5548
Query 130	AAGTCATCCACTTCTCCT	GGTAAGAAGTCTCCGAAAGTTAGTAGCTCAGAA	GACAAGCTT	189
Sbjct 5547	AAGTCATCCACTTCTCCT	GGTAAGAAGTCTCCGAAAGTTAGTAGCTCAGAA	GACAAGCTT	5488
Query 190	TCGATTTCTCCTTCGTT	GAAATGTAATGGGGAACGTCTCGCTTGATGCTCCG		249
Sbjct 5487	TCGATTTCTCCTTCGTT	GAAATGTAATGGGGAACGTCTCGCTTGATGCTCCG		5428
Query 250	AAGGAGATCGGCGGATGATT	CGGTTCCGTGGCGGTACTCGGTGGTGCCTGAAGAGACATT		309
Sbjct 5427	AAGGAGATCGGCGGATGATT	CGGTTCCGTGGCGGTACTCGGTGGTGCCTGAAGAGACATT		5368
Query 310	GCTGTGTACGCGTCTCCGCTGTTTCTTGTACGACCGGACACAGCTCTGCCCTAAAGAAT			369
Sbjct 5367	GCTGTGTACGCGTCTCCGCTGTTTCTTGTACGACCGGACACAGCTCTGCCCTAAAGAAT			5308
Query 370	CGGACGCTAGCCATGGGTGTTATATCCCTTCTCTCTCGTCACATGTCACAGTCAGGTTT			429
Sbjct 5307	CGGACGCTAGCCATGGGTGTTATATCCCTTCTCTCTCGTCACATGTCACAGTCAGGTTT			5248
Query 430	CTCCCTCGCCTTCTTCTGGGCGGCGGACAGGTACGGTGCTAATTACCCAATCAGACACG			489
Sbjct 5247	CTCCCTCGCCTTCTTCTGGGCGGCGGACAGGTACGGTGCTAATTACCCAATCAGACACG			5188
Query 490	GCCGCAAGGTTTCCGGTTGTGGATGGCAGCGTGTGTGCGCCCGTCTAGTGCTGGT			549
Sbjct 5187	GCCGCAAGGTTTCCGGTTGTGGATGGCAGCGTGTGTGCGCCCGTCTAGTGCTGGT			5128
Query 550	TCTAGGGCTGGGCGTCAGCATCCAGGTCTGACGGGACGGGAGTATCTCGCCATCAACG			609
Sbjct 5127	TCTAGGGCTGGGCGTCAGCATCCAGGTCTGACGGGACGGGAGTATCTCGCCATCAACG			5068
Query 610	CTTAGGTCGAATTGACTATGCGTCAGTGACGTGATTGTACTCGCCTCCTGTGCAGACTCC			669
Sbjct 5067	CTTAGGTCGAATTGACTATGCGTCAGTGACGTGATTGTACTCGCCTCCTGTGCAGACTCC			5008
Query 670	TGGGAAGATCTATATCCCTTGGACTTACGCGCGATGGCACGTTGTGGTCAAATAGCATT			729
Sbjct 5007	TGGGAAGATCTATATCCCTTGGACTTACGCGCGATGGCACGTTGTGGTCAAATAGCATT			4948
Query 730	ACCTTAGAGCATTTGACTTTTTGCACTCCTTCTATTTGTACTTTGGGAGGGGAAACGAA			789
Sbjct 4947	ACCTTAGAGCATTTGACTTTTTGCACTCCTTCTATTTGTACTTTGGGAGGGGAAACGAA			4888
Query 790	GAACACACAATTATGCTTGTGACGTGGTTCATGCGAAGCCGGGTGACGCGTTCTGGAGTC			849
Sbjct 4887	GAACACACAATTATGCTTGTGACGTGGTTCATGCGAAGCCGGGTGACGCGTTCTGGAGTC			4828
Query 850	ATAGCGTAACGGCAAAGGCACGGGACAGTTTTT	ggggggAGATGATGCGTCTGCATCATC		909
Sbjct 4827	ATAGCGTAACGGCAAAGGCACGGGACAGTTTTT	gggggg-AGATGATGCGTCTGCATCATC		4769
Query 910	CACCGGGCATTTCTGCCTGATCGATTCAATACTTTCCCCAGG-CATATAGGCAGACTTG			968
Sbjct 4768	CACCGGGCATTTCTGCCTGATCGATTCAATACTTTCCCCAGG-CATATAGGCAGACTTG			4709
Query 969	CTCATTGGCCTCTGTT-GCTTTGGCCACATAGTATGTATCTCCGCCATATCCACAGC-GT			1026
Sbjct 4708	CTCATTGGCCTCTGTTGCTTTGGCCACATAGTATGTATCTCCGCCATATCCACAGCCGT			4649
Query 1027	CTGATGAAAACGGGTCCTTCTAGATATGAGTACAGTGCGC-T-C-GTGGTGCTGT			1079
Sbjct 4648	CTGATGAAAACGGGTCCTTCTAGATATGAGTACAGTGCGCCTTCCGTGGTGCTGT			4593