The Role of G3BPs in the Stress Response Pathway

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STATEMENT OF ORIGINALITY

The work presented in this thesis was undertaken at the N75 Eskitis Institute for Drug Discovery, formerly known as the N75 Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan (QLD), Australia, from February 2011 to September 2014 under the supervision of Dr Derek Kennedy.

The data presented in this thesis is, to the best of my knowledge and belief, original, except where acknowledge in the text, and has not been submitted, either in whole or in part, for a degree at this or any other university.

Adnan Naim
ACKNOWLEDGEMENTS

A journey is easier when you travel together. As an anonymous writer once stated, interdependence is certainly more valuable than independence. This thesis is the result of 3.5 years, during which I have been accompanied and supported by many people. It is pleasant that I have now the opportunity to express my gratitude for all of those people who stand out most notably in my mind as contributing to my thesis in one way or the other.

First, I would like to thank my supervisor, Dr Derek Kennedy, for giving me the opportunity to be a part of his laboratory as his graduate student. I would like to thank him for his supervision, guidance, and support throughout my PhD. I would also like to thank Prof. George Mellick, Deputy Director, Eskitis Institute for Drug Discovery and Prof. David Lambert, Dean (Research), Griffith Sciences for supervising me in completing the corrections in the thesis.

I would like to extend my gratitude to The Australian Government’s, Endeavour Awards Scholarship and Fellowship program for providing me with the prestigious Postgraduate Award, which assisted me to stay in Australia while I completed my PhD studies.

I would also like to thank Dr Anja Rockstroh for her analysis of the RIP-chip dataset, which set the platform for my PhD research work. I would also like to thank her for teaching me some of the important techniques that were required for my PhD research project and also for helping me in every possible manner throughout my research degree.

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During this journey of my PhD studies, I met several new people. There is a never-ending list of all the wonderful people who made my journey easy and comfortable, and I would like to thank all of them as well.

I would also like to thank the European Association for Cancer Research for providing me with a travel fellowship, because of which I was able to work in Prof. Juan Valcarcel’s laboratory at Centre for Genomic Regulation, Barcelona. I would like to thank Prof. Valcarcel for giving me this opportunity and also his team for teaching me some important techniques which were important for my research project.

Friendship requires no elaboration; still, I would like to quote few lines for Arun Singh, Dr Lokesh Kori, Dr Arpita Mishra and Faisal

“The rain may be falling hard outside,
But your smile makes it all alright.
I’m so glad that you’re my friend.
I know our friendship will never end”.

“If facts are the seeds that later produce knowledge and wisdom, then the emotions and the impressions of the senses are the fertile soil in which the seeds must grow,” said Rachel Carson. This is what I feel regarding Dr Suryakant Mishra, whom I met by chance but who is responsible for what I am today. I would like to thank him and his family for the moral support and guidance they have provided me with during these many years.

East or West, home is the best. For me, the best place is my home. Today, the happiest person to see me completing my PhD studies would have been my mother; unfortunately, she is no longer here to cherish this moment. Nevertheless, I would like to thank her and would like to say – that today, her dream has turned to reality. I feel short of words when I want to thank my father, as he is the one who has always inspired and motivated me, and advised me to keep moving patiently and with full determination until I accomplished my goal. I also want to thank my sisters and my niece and nephews for their love and support.
This thesis includes three published abstracts, which were co-authored with other researchers. My contribution to each co-authored abstract is outlined at the front of the relevant chapter.


**Chapter 4:** Naim, A., and Kennedy, D. (2013). G3BP1 interacts with NDUFB7 and regulates its expression at the translational level. *35th Lorne Genome Conference*, 80, Melbourne Australia.


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**PhD student:** Adnan Naim
**Date:** 01/02/2015

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**Corresponding Author and Supervisor:** Dr Derek Kennedy
**Date:** 01/02/2015
ABSTRACT

The ras-GTPase SH$_3$-domain Binding Proteins (G3BP) are a family of RNA-binding proteins that have been implicated in multiple cellular activities ranging from signal transduction to regulation of messenger RNA (mRNA). G3BPs were named after their interaction with the SH3 domain of Ras-GTPase-activating protein; however recent research did not find this interaction. All three members of the G3BPs family, G3BP1, G3BP2a and G3BP2b, share structural similarities with each other by having four distinct regions (1) the Nuclear Transporting Factor 2, (NTF2) domain at the N-terminal, (2) the acidic and proline-rich domain in the centre, (3) the RNA recognition motif (RRM) and (4) the arginine glycine (RGG)-rich region rich at the C-terminal. The presence of the NTF2 domain in its structure suggests G3BP might play a role in nucleocytoplasmic transportation, which was observed after serum stimulation where G3BP1 was translocated to the nucleus from the cytoplasm. The RNA recognition motif (RRM) region plays a vital role in its interaction with the target RNA. The RGG-rich box is a region rich in arginine and glycine residues, which plays a role assisting RRM in interactions with protein or RNA.

G3BP1 is found to be overexpressed in many cancers, including breast cancer, and head and neck tumours, as well as cell lines derived from human lung, prostrate, colon, thyroid and breast cancer. G3BPs have also been implicated in translational control within differentiating neurons, suggesting that G3BP may play several roles in controlling the translational fate of its cargo and that its role may be cell-specific. G3BP1 has also been found in β-integrin-induced adhesion complexes. This information highlights G3BPs as a dynamic protein that is involved in several biological functions.

The most remarkable feature of G3BP1 is its co-localisation in stress granules (SGs) which suggests its role in mRNA regulation. SGs are specialised cytoplasmic complexes where mRNA triage occurs after cellular stress such as oxidative stress. Under stress conditions, the fate of targeted mRNA is decided within SGs, where the transcripts are either degraded by movement to P bodies, stored or recycled for translation. The formation of SGs in a cell reprograms its major cellular activities in order to maintain the cellular homeostasis.
In tumours, the metabolism and function of mitochondria are often deregulated. To meet the increased energy requirements, cancer cells switch to enhanced glycolysis even in the presence of abundant oxygen, a process known as the Warburg Effect. Downregulation of β-F1-adenosine 5’-triphosphatase (ATPase) and deregulated mitochondrial function is considered as a hallmark of carcinogenesis and this feature distinguishes the mitochondria of a normal cell from that of a cancerous cell. β-F1-ATPase is a component of mitochondrial Complex V and has been shown to be directly involved in ATP production. Recently, human G3BP1 has been shown to interact with β-F1-ATPase mRNA and regulates its translation, which implies that G3BP1 might be involved in characterising the bio-energetics of the cancer cell. Complex I and complex V are components of the mitochondrial matrix oxidative phosphorylation system and together generate NADH, via adenosine 5’-triphosphate (ATP) from Complex I through to Complex V, to meet the energy requirements of the cell.

The research work conducted in this thesis utilised the results generated from a RNA immunoprecipitation-Chromatin immunoprecipitation (RIP)-Chip microarray that was performed to identify the putative targets of G3BP1. Analysis of the data identified several nuclear-encoded mitochondrial genes that were components of Complex I. After setting stringent threshold values for selecting the target genes of G3BP1, NADH dehydrogenase (ubiquinone) 1 β subcomplex subunit 7 (NDUFB7), NADH dehydrogenase [ubiquinone] 1 β subcomplex subunit 10 (NDUFB10) and NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1), were selected to study their downstream regulation. Based on the available information about G3BP1’s role in regulating the expression of its other target transcripts (c-myc, bart, ctnnb1, β-F1-ATPase and PMP22) it was hypothesised that G3BP1 might have a similar role in regulating the expression of NDUFB7, NDUFB10 and NDUFV1. It was hypothesised that G3BP1 might recruit all the three target genes to SGs during stress and may regulate their fate.

The functional role of G3BP1 in vivo for all the three transcripts during stressed and non-stressed conditions was explored by performing Luciferase reporter assays. The 5′ untranslated region (UTR), the coding region (CDS) and the 3′UTR of all the three mRNAs were sub-cloned onto the 3′ end of Luciferase in a reporter vector. In this way, if G3BP1 interacted with the cloned region, the genes would regulate the stability, longevity or translation of the chimeric Luciferase construct. The results of the Luciferase assay showed that a reporter containing the 3′UTR of all the three candidate genes was downregulated.
under stress conditions, suggesting that the 3′UTR might contain a *cis*-element where G3BP1 binds and regulates expression.

G3BP1 is also known to possess endoribonuclease activity and therefore the stability of the *Luciferase* mRNA in all the constructs with sub-cloned regions of the three candidate gene transcripts was studied by quantitative real time–polymerase chain reaction. Changes in the expression of endogenous NDUFB7, NDUFB10 and NDUFV1 at the transcriptional and translational level were also evaluated in separate experiments after knock-down of G3BP1. The results showed that endogenous mRNA expression was not regulated by the knock-down of G3BP1; however, NDUFV1 expression was downregulated at the translational level. These data were consistent with the results from the reporter assays, which showed that the expression and stability of the transcripts of the sub-cloned mRNA regions fused to *Luciferase* mRNA remained constant.

From the RIP-Chip dataset, which suggested that G3BP1 targets several nuclear-encoded mitochondrial Complex I proteins, it was hypothesized that G3BP1 might have some potential role in regulating Complex I activity. Similarly, from the report suggesting that G3BP1 regulates the expression of β-F1-ATPase mRNA, it was hypothesised that G3BP1 might have some role in regulating the energy shift that is observed in many cancer cells. Results from the experiments performed to validate these hypotheses suggested that G3BP1 does indeed play a pivotal role in regulating Complex I activity and generating glycolytic ATP.

In conclusion, data from the reporter assay suggested that G3BP1 might interact at the 3′UTR of *NDUFB7*, *NDUFB10* and *NDUFV1*, and regulate the expression of the reporter under stress. This is consistent with the results of the experiment where endogenous expression of the three candidate genes was studied under stress and normal conditions and significant downregulation in the expression of protein was observed. However, the expression of *NDUFB7* and *NDUFB10* did not change either at the transcriptional level or at the translational level when G3BP1 was knocked down. Only NDUFV1 protein expression was found to be downregulated. This suggests that G3BP1 might need some other co-factors or cellular proteins to regulate the expression of its target transcripts. Downregulation of *NDUFV1* when G3BP1 was knocked down suggests that G3BP1 regulates its expression irrespective of the stress response pathway, which means that it through the formation of SG.
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<tr>
<td>ADAR</td>
<td>Adenosine deaminase</td>
</tr>
<tr>
<td>AS</td>
<td>Alternative splicing</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich elements</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CDS</td>
<td>Coding region</td>
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<tr>
<td>2-DG</td>
<td>2-Deoxy-glucose</td>
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<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbeco-phosphate buffer saline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>eIF</td>
<td>Eukaryotic initiation factor</td>
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<tr>
<td>ELAV</td>
<td>embryonic lethal audio visual</td>
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<tr>
<td>G3BP</td>
<td>Ras-GTPase activating protein SH3 domain-binding protein</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GTP</td>
<td>Guanosine-5’-triphosphate</td>
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<tr>
<td>HIF</td>
<td>Hypoxia-induced factor</td>
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<tr>
<td>LB</td>
<td>Liquid broth</td>
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<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
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<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>mRNP</td>
<td>Messenger ribonucleoprotein complex</td>
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<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
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<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced</td>
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<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<td>NDUFB7</td>
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<tr>
<td>NDUFV1</td>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 1</td>
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<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation system</td>
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<tr>
<td>P-body</td>
<td>Processing body</td>
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<td>Abbreviation</td>
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<tr>
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</tr>
<tr>
<td>PABP</td>
<td>Poly(A) binding protein</td>
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<td>Polymerase chain reaction</td>
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<td>Pyruvate kinase M</td>
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<td>Poly(A)</td>
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<td>RNA-binding protein</td>
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<td>Stress granule</td>
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<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>ZBPs</td>
<td>Zipcode-binding proteins</td>
</tr>
</tbody>
</table>
CHAPTER 1
1. INTRODUCTION

1.1 RNA Biogenesis and Metabolism

Omnis cellula e cellula, a saying by Rudolf Virchow, means that every single cell has the capacity to give birth to another cell. It suggests that a cell has all the molecular ingredients and machinery available in the form of different organelles and other macro- and micro-molecules that are assigned a specific task in a temporally and spatially regulated manner to give rise to a new cell.

The central dogma of cell biology suggests that the normal flow of information in a cell is always uni-directional and proceeds from the DNA to RNA through the process of transcription and then after some necessary changes in the transcript, the information is decoded from messenger RNA (mRNA) to protein through the process of translation.

As soon as an mRNA molecule is generated, it has to go through several processes, which include splicing, capping, 3′ end formation, nuclear export and localisation to a pre-determined destination for its translation and, ultimately, degradation (Moore, 2005). All these steps in the life of an mRNA molecule are facilitated by RNA binding proteins (RBPs). The association of RBPs with the mRNA molecule leads to the formation of a dynamic complex called a ribonucleoprotein complex (RNP), which regulates all the steps of post-transcriptional regulation (Wahl et al., 2009).

An mRNA consists of a 5′ untranslated region (UTR), the coding region (CDS) and a 3′ untranslated region. Throughout the animal kingdom, the conserved sequences which regulate the expression of the particular transcript are found to be mainly located in the UTRs, which suggests the importance of these conserved sequences (Duret et al., 1993). However, CDS are also known to play a role in regulating the expression of an mRNA (Lee and Gorospe, 2011).

3′UTRs are known to carry regulatory nucleotide sequences that are responsible for the interaction between mRNA and the RBPs that regulate translation of the transcript (Szostak and Gebauer, 2013). AU-rich elements (AREs) are the main regulatory sequences which are present in the 3′UTRs and are the targets of the RBPs. 3′UTRs are known to have a
polyadenylation (poly(A)) tail which is added during the RNA processing in the nucleus. This poly(A) tail addition and removal regulates the expression of a transcript; for example, the longer the poly(A) tail, the more stable a transcript will be and *vice versa*. The CDS is known to have coding region determinants which regulate mRNA instability and translation (Ross, 1995, Lemm and Ross 2002).

**Post-transcriptional Gene Regulation:** Post-transcriptional gene regulation is a multi-step process, which includes capping, modification, editing, export, localisation, stabilisation, surveillance and controlled translation of the mRNA. The regulation of mRNA expression is mediated either by *cis*-acting elements, which generally reside in the 5′UTR, the 3′UTR and coding regions of the transcript or by *trans*-acting molecules, which are the RBPs (Glisovic et al., 2008). Unlike the situation in prokaryotic cells, where transcription and translation occur almost concurrently, in eukaryotic cells, these processes occur separately and in different parts of the cell. The process of transcription begins with the synthesis of a pre-mRNA, which occurs inside the nucleus. Later, a 7-methylguanosine ‘cap’ is added at the 5′ end of the newly synthesised pre-mRNA, which is considered to be essential for the translation of the transcript. The addition of a 5′ cap has also been reported to safeguard the transcript from exonucleases; as a result, it enhances the transcription. The 5′ cap is also known to have a role in the splicing of the transcript, and the addition of the poly(A) tail (Cowling, 2010). A pre-mRNA is composed of many introns and exons. The introns are spliced out but the exons are retained in an mRNA molecule. This mechanism of splicing the introns from the pre-mRNA is known as alternative splicing (AS), which is mediated by a complex of RNA and proteins, known as the spliceosome (Matlin et al., 2005). The spliceosome contains snRNPs, like U1, U2, U4, U5 and U6, which bind to the specific nucleotide at the 5′ and the 3′ end of the introns. These form the complex, which further performs the splicing activity and removes the introns from the pre-mRNA, leading to the formation of mRNA (Matlin et al., 2005). AS is an important mechanism involved in post-transcriptional gene regulation, which regulates the expression of the mRNA by splicing it at different locations, giving rise to the synthesis of different proteins (Cartegni et al., 2002; Black, 2003; Kim et al., 2006; Roy et al., 2013). All the processes involved in post-transcriptional gene expression are regulated by RBPs, which bind to their target ligands, leading to the formation of a complex called the messenger ribonucleoprotein (mRNP) complex (Hilleren et al., 2001; Kyburz et al., 2006; Millevoi et al., 2006; Rigo and Martinson, 2008).
1.2 RNA Binding Proteins

“This is the Night Mail crossing the border,
Bringing the cheque and the postal order,
Letters for the rich, letters for the poor,
The shop at the corner, the girl next door”.
W.H. Auden (Night Mail)

The eukaryotic genome is known to encode many RBPs. For example, in yeast, 5–8% of the genome encodes for proteins which are believed to be the RBPs by function. Similarly, 2% of the genome in Caenorhabditis elegans and Drosophila melanogaster encode RBPs (Keene, 2001; Lasko, 2000; Lee and Schedl, 2006).

RBPs in mammalian cells play a pivotal role in regulating the expression of their target mRNAs. This regulation of mRNA metabolism occurs with the interaction of RBPs via certain specific sequences which exist either at the 5′ or 3′UTR of their target transcripts. Based on their role in mRNA translation and turnover regulation, RBPs are also termed TTR RBPs (Pullman et al., 2007). In their study on heterogeneous nuclear ribonucleoproteins (hnRNPs), Burd and Dreyfuss (1994) reported that there are a variety of different RNA binding domains (RBDs) with a range of activities that exist in the different RBPs. A single RBD is capable of recognizing two to six nucleotides and can bind to that target sequence. Multiple copies of the binding domain can recognize larger and more complex target transcript and can increase the specificity and efficiency of the binding (Maris et al., 2005). Table 1.1 shows the best known RBD, their targets and examples of the RBPs (Chen and Varani, 2005; Lunde et al., 2007).

RBPs also contain some auxiliary domains that help RBDs to interact with the target transcripts and may facilitate the RBPs to interact with other proteins as well (Glisovic et al., 2008). These auxiliary domains are known to possess some vital biological activities such as intra-strand annealing of an RNA molecule, they were also found to be involved in protein–protein interactions (Biamonti and Riva, 1994). These auxiliary domains are also known to carry nuclear localization determinants. An example of this is the protein U1A, whose
auxiliary domain is considered to be responsible for its nuclear localisation (Kambach and Mattaj, 1992).

The binding of mRNA with the RBPs gives rise to an RNP complex (Rougemaille et al., 2008), which is formed inside the nucleus and contains the mRNA along with protein and several other components that play a role in the transportation of the mRNA from the nucleus to the cytoplasm (Smith et al., 2014). The RNP complex protects the mRNA molecule from degradation and it also helps in post-transcriptional processing of the mRNA molecule (Varani and Nagai, 1998, Nagai, 1996). Certain domains in RBPs are known to regulate the nucleocytoplasmic localization of the RBP, like the NTF2 domain of Ras-GTPase activating protein SH3-domain binding protein (G3BP1), which is considered to have a role in the nuclear localisation of G3BP1 (Tourriere et al, 2001; Vognsen et al., 2013).

RBPs are also known to carry some motifs that impart catalytic and protein–protein interaction properties to the RBP. For example, adenosine deaminases (ADARs) carry two or three double-stranded RBDs which possess a catalytic deaminase domain that edits the double-stranded nucleic acid substrates (Doyle and Jantsch, 2003). Drosha and Dicer are types of ribonuclease III enzyme which are another example where the double-stranded RBDs also possess RNase III domains that lead to the endonucleolytic degradation of primary and precursor-miRNA (Kishore et al., 2010; Han et al., 2006; Bernstein et al., 2001). Some RBDs play a crucial role in the interaction between two proteins, which can modify the RNP complexes (the interaction modifies the RNP complex) involved in the maturation, localisation, translation and stability of the target transcript. For example, splicing factors, hnRNPs, and serine- or arginine-rich proteins are known to support spliceosome assembly to detect exon–intron boundaries for splicing (Haynes and Iakoucheva, 2006). In *Drosophila*, the third RNA recognition motif (RRM) of embryonic lethal audio visual (ELAV) protein is considered to behave bi-functionally by interacting with the RNA and also with itself, similar to Human antigen R (Hu R) protein. However, the protein–protein interaction of ELAV seems to be dependent on the presence of RNA, which might be responsible for providing a conformational change in the protein to enhance the self-interaction (Toba and White, 2008; Sokolowski et al., 1999).

The spatial activity of RBPs can also be regulated by the presence of some sequence elements which can be responsible for their sub-cellular localisation (Kishore et al., 2010). For
example, Dyskerin carries a nuclear localisation signal, which is considered to be responsible for the nucleolar localisation of small nucleolar RNP (snoRNP) (Heiss et al., 1999). Fig. 1.1 describes some of the roles of RBPs in eukaryotes.

**Table 1.1** The best known RNA-binding domains, their structures and the nature of target RNA that they bind to, along with the examples of the RNA-binding proteins that contain the domains.

<table>
<thead>
<tr>
<th>RNA-Binding Domain</th>
<th>Structure of the RNA-Binding Domain</th>
<th>Type of Nucleic Acid Recognized</th>
<th>Examples of the RNA-Binding Proteins Containing the Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA Recognition Motif (RRM)</td>
<td>Bαββαβ barrel-like, with RNP1 and RNP2 motifs</td>
<td>ssRNA, ssDNA</td>
<td>hnRNP A1, PTB, HuD, Sx1, RBM3, G3BP</td>
</tr>
<tr>
<td>K-Homology</td>
<td>Three-stranded β-sheet packed against the α-helices</td>
<td>ssRNA, ssDNA</td>
<td>hnRNP K, FMR1</td>
</tr>
<tr>
<td>Cold Shock Domain</td>
<td>β-barrel structure (βββββ)</td>
<td>ssRNA, ssDNA</td>
<td>CspB, YB-1, UNR</td>
</tr>
<tr>
<td>dsRBD†</td>
<td>αββα fold</td>
<td>dsRNA</td>
<td>PKR, Staufen, RNAse III, Dicer, ADAR1</td>
</tr>
<tr>
<td>Zinc-finger</td>
<td>ββα structure</td>
<td>dsRNA</td>
<td>TFIIIA, WT1, Tra-1</td>
</tr>
<tr>
<td>Arginidine-rich</td>
<td>Alone, or in glycine or aromatic residue-rich domains</td>
<td>ssRNA, dsRNA</td>
<td>Tat, YB-1, hnRNP A1</td>
</tr>
<tr>
<td>RNA helicase</td>
<td>Domain 1 and Domain 2 with conserved motifs</td>
<td>dsRNA substrate</td>
<td>eIF4A, DHH1</td>
</tr>
<tr>
<td>PAZ (named after proteins PIWI, AGO and Zwille)</td>
<td>β-barrel reminiscent of OB-fold</td>
<td>dsRNA substrate</td>
<td>Dicer</td>
</tr>
<tr>
<td>PIWI (P-element induced wimpy testis)</td>
<td>RNase H core; five-stranded β-sheet surrounded by α-helices</td>
<td>dsRNA substrate</td>
<td>Argonaute (Ago)</td>
</tr>
<tr>
<td>SR (Serine/Arginine rich proteins)</td>
<td>Specific RNA hairpin loop structure. More than one RRM domain. The second RRM domain is called the RRM homologue</td>
<td>ssRNA</td>
<td>U2AF</td>
</tr>
<tr>
<td>RGG</td>
<td>Arginine-Glycine-Glycine</td>
<td>ssRNA</td>
<td>hnRNP proteins</td>
</tr>
</tbody>
</table>

† ss, single-stranded; ds, double-stranded.
Figure 1.1  The role of RNA binding proteins (RBPs) in eukaryotes
After the synthesis of the pre-mRNA molecule, it undergoes splicing for the removal of introns and the addition of a 3′ polyadenylation (poly(A) tail in order to form a mature mRNA. RBPs bind to the pre-mRNAs and mRNAs. The mRNAs bound with RBPs form messenger ribonucleoprotein complexes (mRNPs). The mRNPs exit the nucleus via the nuclear pore complex (NPC). In the cytoplasm, new cytoplasmic components are added to the mRNPs. The next step is mRNA surveillance. Nonsense-arbitrated degradation of the transcript takes place via the termination of the first translational round along with the ribosomes at the premature termination codon (PTC). The exon junction complex (EJC) binds to the mRNA during splicing and later on transported to the cytoplasm. The EJC blocks further translation and the generation of deleterious proteins. A functional mRNA has its 7-methylguanosine cap structure bound by eukaryotic initiation factor (eIF)4E and its polyadenylation tail bound by the poly(A) binding protein (PABP). Translation occurs via circularisation of mRNA and communication between the 5′ cap and the poly(A) tail. Figure taken from Richard et al. (2008) by permission.
1.3 Different Biological Activities where RBPs are Involved

RBPs have a versatile character, as they are involved in several biological activities, for example such as AS, mRNA transport, mRNA localisation, stability, translation and decay. They are also known to play a vital role in interconnecting the different biological processes (Hilleren et al., 2001; Kyburz et al., 2006; Millevoi et al., 2006; Rigo and Martinson, 2008). Some of the activities where RBPs are involved are explained below.

1.3.1. Alternative Splicing

Once a pre-mRNA is synthesised, it contains both introns and exons. During pre-mRNA splicing, exons are either retained in the mRNA or excised in various combinations to create a diverse array of mRNAs from one pre-mRNA. This process takes place in the nucleus and is called as alternative RNA splicing or AS (Mattaj, 1994; Nilsen and Graveley, 2010; Han et al., 2011). This mechanism regulates gene expression by generating different isoforms of a protein from a single gene. These isoforms may have a similar function or can act against each other. The best example to illustrate this is the generation of either pro- or anti-apoptotic isoforms from the genes that work in the apoptotic cell death pathways. Table 1.2 below describes the examples of some apoptotic factors and the changes in their activity after AS (David and Manley, 2010, Schwerk and Schulze-Osthoff, 2005).

Table 1.2 Changes in the function of few apoptotic factors as a consequence of alternative splicing (AS)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Cellular Activity</th>
<th>Functional Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas</td>
<td>Apoptosis</td>
<td>Altered solubility, dominant negative phenotype</td>
</tr>
<tr>
<td>Bcl-x</td>
<td>Antiapoptotic</td>
<td>Antagonistic functions during apoptosis</td>
</tr>
<tr>
<td>p53</td>
<td>Regulates apoptosis and the cell cycle</td>
<td>Altered apoptotic potential</td>
</tr>
</tbody>
</table>

In tumour biology, AS plays a key role by regulating various pathways involved in tumour cell metabolism, apoptosis, the cell cycle, metastasis and angiogenesis. Through AS, different proteins or isoforms of the same proteins are formed, which supports the growth requirements of the proliferating tumour cells (Venables, 2004; Ghigna et al., 2008; David and Manley, 2010).
Perturbed AS is also considered as a hallmark of cancer (Ladomery, 2013), as alteration in the process of AS can lead to the formation of isoforms of a protein, which can act against other isoforms of the same protein and can support cancer progression. For example, the Bcl-x pre-mRNA transcript after AS can give rise to two isoforms of the protein: Bcl-x(L), which is known to have anti-apoptotic characteristics, and Bcl-x(s), which is known to support cellular apoptosis (Revil et al., 2007; Mercatante et al., 2001). Caspase-2 and Fas receptor mRNA are two more examples which demonstrate how AS can give rise to different protein isoforms, which can help a cancer cell overcome apoptosis and escape from immune system recognition respectively (David and Manley, 2010).

Apart from regulating the expression of apoptotic proteins, AS also has a role in regulating the metabolism of a cancer cell (Bonomi et al., 2013). AS regulates cancer cell metabolism by controlling the expression of the pyruvate kinase M (PKM) gene. Due to AS, PKM can exist in two isoforms, PKM1 or PKM2. Many tumours uniformly express PKM2; this is considered a promoter of the Warburg Effect (discussed in detail below). The Warburg Effect describes a shift in the energy state of cancer cells from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, where the cells consume different intermediates that are produced as a by-product during the metabolism of glucose to synthesize the nucleotides, lipids and amino acids that are required by the new cancer cells along with the production of lactate as a by-product. Interestingly, the PKM1 isoform leads to a reduction in the production of lactate and also supports OXPHOS (David and Manley, 2010).

Similarly, Sam68, which is an RBP, regulates the expression of the proto-oncogenes Cyclin D1 and H-Ras. Through AS, the Cyclin D1 isoform, CyclinD1b, is found to be upregulated in breast and prostate cancers (Paronetto et al., 2010; Sette et al., 2011). From the examples mentioned above, it is clear how RBPs regulate the expression of mRNAs through the process of AS and can lead to several diseases (Tazi et al., 2009).

1.3.2 RNA Modification

mRNA modification is one of the events which occur during the process of post-transcriptional gene regulation. In this process, the modification of the nucleotide contents of an mRNA molecule takes place. The most commonly seen modification process is deamination, in which ADAR proteins catalyse the conversion of adenosine (A) to inosine (I) (Palladino et al., 2000; Valente and Nishikura, 2005). By accepting these changes in the
nucleotide content, the final mRNA molecule is translated differently to the unedited transcript, leading to related proteins with different functions. Such modifications are known to occur in both coding as well as non-coding regions of the mRNA transcript (Nishikura, 2006). Mostly, adenosine to inosine modifications are known to occur in the nervous system; any mutation in the Drosophila ADAR proteins leads to neuronal dysfunction (Ma et al., 2001).

1.3.3 mRNA Export

RBPs are also involved in the transport of mRNA from the nucleus, through the nuclear pore, reaching the cytoplasmic destination where the translation takes place (Carmody and Wente, 2009). For example, TAP (also called NXF1 protein) contains a non-canonical RNP-type RBD, which is rich in four leucine repeats, an NTF2-like domain which facilitates heterodimerization with p15 (also known as NXT1), and a ubiquitin-associated domain that mediates the interaction with nucleoporins (Herold et al., 2000). The TAP/NXF1:p15 heterodimer has been found to have a key role in the transport of mRNA in Saccharomyces cerevisiae and its overexpression in Xenopus laevis oocytes enhances mRNA transport, which is otherwise inefficiently transported (Grüter et al., 1998; Katahira et al., 1999; Santos-Rosa et al., 1998). Both TAP and p15 need adaptor proteins that enhance their affinity for the transcript and are also responsible for the actual transport of its target transcript (Glisovic et al., 2008).

1.3.4 mRNA Localisation

RBPs also localise target mRNAs to sub-cellular destinations in order to regulate gene expression spatially and temporally (Martin and Ephrussi, 2009). The process of mRNA localisation begins in the nucleus with its interaction with the RBP to its target sequence, which generally lies in the 3′UTR but can also exist in the 5′UTR of the mRNA as well. These target sequences are known as “localising elements” or “zipcodes” (Singer, 1993). The RBPs then recruit these transcripts from the nucleus to their sub-cellular destination and may also play a role in subsequent translation. For example, Staufen is an RBP which binds to dsRNAs and was found to be involved in localizing oskar 3′UTR in Drosophila oocytes. It also binds to stem-loop structures existing in the bicoid 3′UTR (St Johnston et al., 1991; Jansen, 2001). In addition, Staufen has also been found to have a role in localising CamKIIα mRNA to neuronal dendrites (Micklem et al., 2000; Ferrandon et al., 1994).
Zipcode-binding proteins (ZBPs) are other RBPs that bind to the zipcode sequences and localise the mRNAs. In *Gallus gallus domesticus* embryo fibroblasts, ZBP1 is a protein that binds to the β-actin mRNA. ZBP1 has also been found to be present in mammalian neurons binding to β-actin mRNA which localises them to growth cones in response to different environmental cues (Zhang et al., 2001; Lin and Holt, 2007).

In another example, *S. cerevisiae* ASH1 mRNA localises in the bud of the daughter cell through myosin (Myo4) and actin associations (Bobola, et al., 1996). This interaction of proteins with the mRNA is dependent on the presence of two more proteins, She2 and She3 (Bohl et al., 2000; Long et al., 2001).

1.3.5. mRNA translation and turnover

RBPs have an important role in target transcript stability and decay (Mitchell and Tollervey 2000). The RBPs bind to *cis*-regulatory elements, which exist in the 5′UTR or 3′UTR of the mRNA, are generally AU-rich and are also referred to as AREs (Barreau et al., 2005). They have been found to have “AUUUA” pentamer repeats. Because of this feature, the RBP that binds these elements are also named ARE-RBPs; however, recently, they have been named TTR-RBPs due to their role in turnover and translation regulation (Pullman et al., 2007). RBPs are involved in regulating the translation of the target transcript temporally and spatially manner (Siomi and Dreyfuss, 1997). For example, phosphorylation of ZBP1 is found to regulate the translation of β-actin mRNA. Translation occurs once the ZBP1-RNA complex reaches the destination, where protein kinase Src enhances translation after phosphorylating the tyrosine residue in ZBP1, which is necessary for mRNA binding. (Huttelmaier et al., 2005).

RBPs involved in degradation of mRNAs generally include tristetraprolin, AU-binding factor 1 (AUF1, also known as hnRNPD), K-homology splicing regulatory protein, and butyrate response factor 1, whereas RBPs such as ELAV or Hu proteins are generally found to have a role in mRNA stability (Pang et al., 1998; Mitchell and Tollervey 2000). AUF1 has been correlated with mRNA degradation, as it has been found to be a constituent of proteasome assembly (Schneider et al., 1999) which is known for the degradation of mRNA transcripts. The proteasome assembly targets the mRNA which contains premature termination codons, triggering nonsense-mediated decay, and also the transcripts which lack a stop codon, in order to avoid aberrant protein synthesis from such mRNA. Proteasomes are also known to
target AREs for binding and activating endonucleolytic degradation of the mRNA (Klauer and van Hoof, 2012). Other proteins like K-homology splicing regulatory protein and butyrate response factor 1 are known to associate with processing bodies (P-bodies), which have all the components required for mRNA degradation (Franks and Lykke-Andersen, 2008).

1.4 Cancer

Cancer is a heterogeneous disease where the abnormal or transformed cells start dividing in an uncontrolled and deregulated manner, giving rise to a tumour. Traditionally, researchers considered genetic mutations as the only cause of cancer, as they are found to exist in all the cancers (Modica-Napolitano and Singh, 2004). However, there are additional factors that contribute and possibly cause the progression of cancer; these include the tumour microenvironment and epigenetics. Mitochondrial dysfunction is also considered to be one of the major causes of cancer. In the 1920s, Otto Warburg noticed for the first time that cancer cells have enhanced glycolysis even when oxygen is present. This is known as the Warburg Effect (Warburg et al., 1927). He correlated this glycolytic shift in cancer cell metabolism to meet the energy requirements of the developing cancer with the dysfunction of mitochondria. This enhanced glycolysis also leads to the excess formation of lactic acid, which enhances the invasiveness of a tumour (Gatenby et al., 2006; López-Lazaro, 2007). In later stages of cancer development, the disrupted metabolism of cancer cells is considered to contribute to the migration of primary tumour cells to adjacent tissues, defined as metastasis.

Post-transcriptional gene regulation has been found to be involved in controlling cell proliferation, cellular differentiation, cell invasion, metastasis, apoptosis and angiogenesis, which can influence the initiation and the aetiology of a tumour (Kim et al., 2009; Silvera et al., 2010; Sette et al., 2011). Many RBPs have been found to be over-expressed in cancers, which directly co-relate the tumorigenesis to unregulated RNA metabolism (Sonenberg and Gingras, 1998; Sueoka et al., 1999). Table 1.3 explains different translation factors and their aberrations involved in human cancer aetiology. Spatial and temporal translational control plays a vital role in cancer development and progression by selectively translating specific mRNAs that are required for tumour cell survival and metastasis (Silvera et al., 2010). An increased rate of protein synthesis is required for aggressively multiplying tumour cells. The stability of a certain mRNA can lead to over-production of a protein, which could direct an uncontrolled cell division and can be a
hallmark of a particular cancer. For example, increased expression of Eukaryotic Initiation Factor 2α (eIF2α) is observed in benign and malignant melanomas as well as colon cancers (Rosenwald et al., 2003). Similarly, decreased expression of eIF3e is found in breast and lung carcinomas (Gillis and Lewis, 2013).

**Table 1.3** Role of translation factors and translation regulatory factor alterations in human cancer aetiology†

<table>
<thead>
<tr>
<th>Factor</th>
<th>Observed modification</th>
<th>Cancer association</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKR (eIF2α kinase)</td>
<td>Decreased expression</td>
<td>Progression from benign to malignant HNSCC, indolent papillary thyroid carcinomas compared with aggressive non-papillary form</td>
</tr>
<tr>
<td>PKR</td>
<td>Increased expression</td>
<td>Colon adenomas and carcinomas, melanoma lymph node metastases compared with less aggressive primary melanomas, aggressive IDC and high-grade viral HCC</td>
</tr>
<tr>
<td>eIF2α</td>
<td>Increased expression</td>
<td>Benign and malignant melanomas and colon cancers; progression of thyroid neoplasms, aggressive non-Hodgkin’s and Hodgkin’s lymphomas; associated with more aggressive brain cancers</td>
</tr>
<tr>
<td>eIF3a</td>
<td>Increased expression</td>
<td>Breast, cervical, lung oesophageal and stomach cancers</td>
</tr>
<tr>
<td>eIF3c</td>
<td>Increased expression</td>
<td>Testicular seminomas. Meningiomas in NF2 (eIF3c interacts with merlin)</td>
</tr>
<tr>
<td>eIF3h</td>
<td>Increased expression</td>
<td>High-grade prostate cancers and gene amplification in NSLC</td>
</tr>
<tr>
<td>eIF3f</td>
<td>Decreased expression</td>
<td>Pancreas, vulva, breast, ovary and small intestine tumors; gene loss in melanomas, but not in nevi</td>
</tr>
<tr>
<td>eIF3e</td>
<td>Decreased expression</td>
<td>Breast and lung carcinomas</td>
</tr>
<tr>
<td>EIF3e</td>
<td>Increased expression</td>
<td>Associated with PFS in tamoxifen-treated breast cancers</td>
</tr>
<tr>
<td>eIF5A2</td>
<td>Increased expression</td>
<td>Amplified with increased stage in ovarian cancer; associated with metastatic progression in colorectal carcinomas, associated with reduced PFS and RFS in bladder carcinomas, and poor prognosis following bladder excision</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Increased expression</td>
<td>Correlates with worse clinical outcome and decreased survival in breast, head and neck, colorectal, lung, prostate, bladder, skin and cervical cancers, and lymphomas; correlates with increased malignancy in meningiomas, glioblastomas and astrocytomas; associated with decreased survival in advanced prostate cancers; LAEC</td>
</tr>
<tr>
<td>Phosphoryr-eIF4E</td>
<td>Increased phosphorylation</td>
<td>In prostate cancer compared with normal tissue; correlates with anti-apoptotic gene expression in DLBCL and Burkitt’s lymphoma; associated with serious histological type and better survival in ovarian tumours; observed in most human cancers; associated with lower stage colorectal, lung and gastric cancers; no correlation with malignancy in meningiomas, glioblastomas and astrocytomas despite eIF4E</td>
</tr>
<tr>
<td></td>
<td>correlation</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>eIF4G</td>
<td>Increased expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Associated with decreased metastatic progression in LABC; associated with IBC and formation of metastatic cancer cell emboli; associated with increased CCND1 translation in squamous lung carcinoma</td>
<td></td>
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<tr>
<td>4E-BPs</td>
<td>Decreased expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduced survival in advanced prostate cancers</td>
<td></td>
</tr>
<tr>
<td>4E-BPs</td>
<td>Increased expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Associated with reduced tumour grade in breast cancers, reduced metastatic progression in LABC and with LAEC</td>
<td></td>
</tr>
<tr>
<td>Phosphor-4E-BP1</td>
<td>Increased phosphorylation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased survival in advanced prostate cancers; higher grade and reduced survival in breast cancers; poor differentiation and higher mitotic rates in ovarian tumours; LAEC</td>
<td></td>
</tr>
<tr>
<td>eIF4A</td>
<td>Increased expression</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Some primary HCCs and possibly some melanomas</td>
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</tbody>
</table>

‡ CCND1, cyclin D1; DLBCL, diffuse large B cell lymphoma; HNSCC, head and neck squamous cell carcinoma; HCC, hepatocellular carcinoma; IBC, inflammatory breast cancer; IDC, invasive ductal carcinoma; LAEC, locally advanced oesophageal cancer; NSCLC, non-small-cell lung cancer; NF2; neurofibromatosis type 2; PFS, progression-free survival; RFS, recurrence-free survival; †This table is reproduced with permission granted by Robert Schneider (Silvera et al., 2010)

### 1.5 Ras-GTPase Activating Protein SH3 Domain Binding Protein

Ras-guanosine-5’-triphosphatease (GTPase) activating protein SH3-domain binding proteins (G3BPs) are an evolutionarily conserved, small family of three proteins, G3BP1, G3BP2a and G3BP2b, derived from two distinct genes in humans residing on Chromosomes 5 and 4 respectively (Irvine et al., 2004). G3BP1 contains 465 amino acids and G3BP2 exists in two spliced isoforms (G3BP2a and G3BP2b), which have 482 and 449 amino acids respectively (Irvine et al., 2004). The N-terminus of G3BP (see Fig. 1.2) is characterised by the presence of a Nuclear Transport Factor 2-like domain, which is considered to be responsible for defining the nuclear destination of other proteins, including NTF2 itself (Smith et al., 1998). The central region of G3BPs contains a segment which is rich in acidic residues and proline-rich PXXP motifs. The C-terminal portion of the proteins are categorised by motifs that are involved in RNA binding and hence are known as RRMss and an arginine- and glycine-rich RGG box which helps RRMss to interact with the target RNA and also with other proteins (Irvine et al., 2004).
Figure 1.2  Predicted domain structure of human G3BP2a, 2b and 1
Image taken from Irvine et al. (2004); reproduced by permission.

G3BP1 and G3BP2 are considered as primarily cytoplasmic proteins (Parker et al., 1996), but both proteins are known to have the potential to move into the nucleus (Barnes et al., 2002; French et al., 2002; Gallouzi et al., 1998). However, there is a controversy about G3BP’s subcellular localisation, as Tourriere and co-workers observed the nuclear localisation of phosphorylated G3BP1 in non-proliferating mouse (Mus musculus) embryonic fibroblasts (Tourriere et al., 2001), whereas Parker and co-workers did not observe G3BP1 in the nucleus of epidermal growth factor-transformed fibroblasts throughout the cell cycle (Parker et al., 1996). Unlike G3BP1, the host lab found G3BP2 to be cytoplasmic in serum-starved non-proliferating fibroblasts and to move to the nucleus when stimulated with serum (French et al., 2002).

The expression of these proteins is highly tissue-specific and isoform-specific. G3BP1 is highly expressed in the lung, kidney and colon, whereas it is less abundant in the heart, liver and spleen. Both the isoforms of G3BP2 are known to be expressed in the lung, liver, kidney, stomach and colon, whereas the spleen is known to express only G3BP1 (Kennedy et al.,...
A G3BP1 knockout study performed on mice (*Mus musculus*) showed extensive neuronal cell death in neonates and retardation in the foetal growth (Zekri et al., 2005). Recently, G3BP1 has been found to have a role in neuronal synaptic plasticity and calcium homeostasis (Martin et al., 2013).

G3BP is known as a Ras-GTPase activating protein (GAP) SH3-domain-binding protein because of the interaction of its NTF2 domain with the SH3 domain of GAP (Kennedy et al., 2001). Contrary to this, the recent work by Annibaldi and co-workers suggest that there is no genuine interaction between rasGAP and G3BP1 (Annibaldi et al., 2011). Enhanced phosphorylation on several serine residues of G3BP1 in non-proliferating cells has been observed; however, during cellular proliferation, the pattern of phosphorylated residues changes (Gallouzi et al., 1998). In particular, dephosphorylation of Ser-149 in proliferating cells is found to be dependent on Ras-GAP, which also influences the subcellular localisation of G3BP1, where it moves to the nucleus (Gallouzi et al., 1998, Tourriere et al., 2001). In another report, Barnes and co-workers showed in breast cancer cells, stimulation of the epidermal growth factor receptor ligand heregulin induces the expression of G3BP1 and enhances its phosphorylation (Barnes et al., 2002).

G3BP possesses a versatile nature, as it has been found to influence mRNA metabolism and cell-cycle regulation. Fig. 1.3 explains the role of G3BP1 involved in the regulation of a transcript. The RBDs of G3BPs are believed to be the domains which are involved in signal-regulated mRNA metabolism. G3BP has also been implicated as having a role in several other signalling transduction pathways which are involved in cancer, including Ras signalling (Malumbres and Pellicer, 1998), NFkB signalling (Prigent et al., 2000) and the ubiquitin proteasome system (Soncini et al., 2001). G3BP was found to have a functional DNA and RNA helicase property (Costa et al., 1999). G3BP1 has also been found in the 3S3-induced integrin associated complexes. Work done by Meng and co-workers (2004) showed that G3BP1 was a component of the complex formed as a consequence of blocking the β1 integrin by using 3S3 antibody. This suggests that G3BP1 plays a role in regulating cellular adhesion mediated through β1 integrin (Meng et al., 2004).

Over-expression of G3BP1 and G3BP2 has been observed in various tumours and has been found to be associated with enhanced proliferation (French et al., 2002; Barnes et al., 2005, Guitard et al., 2001; Kociok et al., 1999). G3BP1 over-expression was detected in human
head and neck tumours and cell lines, and also in human lung, prostrate, colon, thyroid and breast cancer cell lines (Guitard et al., 2001; Barnes et al., 2005; French et al., 2002; Liu et al., 2001). Guitard et al. (2001) also showed that G3BP1 enhances S-phase entry in serum-depleted fibroblasts.

**Figure 1.3** Diagram showing how G3BP1 may regulate the translation of target transcripts by binding to the 3′UTR of the mRNA

Generally, an mRNA is translated in a circular way by the 5′–3′ communication through the interaction of the eIF4F complex (green) and poly(A) binding protein (PABP, yellow). When G3BP1 is involved, it binds to the 3′UTR (purple) of mRNA (black) and blocks the initiation of translation of the transcript. G3BP1 binding could also block the translational enhancing activity of the 3′UTR and prevent the interaction of the mRNA with the 43S ribosome, and hence block mRNA circularisation. (Willers and Cuezva, 2011).

Recently, G3BP1 has been identified as a component of stress granules (SGs) (Tourriere et al., 2003). When cells are exposed to adverse environmental conditions or different kinds of cellular stresses, they initiate responses to enhance their survival. One of the immediate responses is a reprogrammable regulation of mRNA translation to maintain cellular homeostasis to adapt to stress (Tourriere et al., 2001; Kedersha et al., 2005). Over-expression of G3BP1 has been shown to induce the formation of SGs. SGs are known as a site which decides the fate of mRNAs. G3BP1s are RNA-binding proteins and are recruited to these granules, and therefore could play some role in regulating the expression of target mRNA transcripts. The available information has facilitated in our understanding of the role of SGs as translation detention centres and G3BP’s role in response to cellular stress, but there is still only minimal information regarding the regulation of specific mRNA targets and how specific targets are selected by the components of the SGs, which needs to be explored in future. SGs are explained in more detail below.
Many RBPs are over-expressed in a wide range of cancers, providing early correlative evidence that disrupted RNA metabolism plays a role in tumorigenesis (Sonenberg and Gingras, 1998, Sueoka et al., 1999). It has been found that post-transcriptional mechanisms also facilitate the activation of selective and adaptive expression programs (Holcik and Sonenberg, 2005), which enhance tumour survival and therapy resistance (Gatenby and Gillies, 2004). The activity of RBPs may also influence metastasis (Yaniv and Yisraeli, 2002). G3BPs play a vital role in several biological pathways which are involved in cancer, including ras signalling (Tocque et al., 1997), c-myc mRNA turnover (Gallouzi et al., 1998, Tourriere et al., 2001), NF-kappaB signalling (Prigent et al., 2000) and the ubiquitin proteasome system (Soncini et al., 2001). Recently, G3BP1 was also found to have a role in regulating the expression of PMP22 mRNA, which affects the proliferation of breast cancer cells (Winslow et al., 2013). G3BP1 has been found to stabilize the tau mRNA, whereas it is known to degrade c-myc (Gallouzi et al., 1998), bart (Taniuchi et al., 2011) cttnb1 (Bikkavilli and Malbon, 2011), ATP5B (Ortega et al., 2010), IGF-11 and GAS5 mRNA (Zekri et al., 2005) through its endoribonuclease activity. G3BP proteins are known to promote cell growth, cell survival and proliferation, and may influence carcinogenesis. G3BP1 was reported to be over-expressed in human tumours (French et al., 2002; Barnes et al., 2005). G3BP1 was also shown to promote S-phase entry in serum-deprived cell tumour (Guitard et al., 2001). The accumulated data on G3BP1 implicates it in a role in cell cycle progression, which supports the notion that G3BPs are involved in tumour progression.

1.6 Stress Granules

When exposed to stressful environments (i.e. oxidative stress, high temperature, viral infection, hyperosmolarity, ultraviolet (UV) irradiation), eukaryotic cells are left with three options. One is to initiate an adaptive mechanism and respond to stress, repairing the damage caused by the stress and subsequently restoring normal cellular functions. Alternatively, the cell can activate the apoptotic mechanism. A third option is to initiate a stress response but if recovery is not achieved, then apoptosis is initiated. The path a cell selects depends on the extent of the stress. In the case of stress survival, the eukaryotic cell reprograms the translational mechanism and regulates the expression of a highly selective set of transcripts which supports the cell’s survival and maintains cellular homeostasis (Kedersha et al., 2013)

RNA granules were discovered in the cytoplasm of tomato (Solanum lycopersicum) cells exposed to heat shock, which were named SGs (Nover et al., 1983). SGs are cytoplasmic
granules that are rich in non-translating mRNPs, which consist of individual or pools of mRNA transcripts bound by proteins that regulate the post-transcriptional events of gene expression. These are the sites of mRNA triage that regulate mRNA stability and translation (Kedersha and Anderson, 2002). SGs are known to be formed by the stress-induced phosphorylation of the translation initiation factor eIF2α, leading to inhibition or delay of the onset of mRNA translation (Kedersha et al., 1999). The process of translation begins with the recruitment of small ribosomal subunits and translation initiation factors to the mRNA to form a 48S complex. In stressed cells, phosphorylation of eIF2α limits the availability of the eIF2–guanosine-5′-triphosphate (GTP)–tRNAMet ternary complex to initiate translation, which leads to the accumulation of a 48S pre-initiation complex (Kedersha et al., 1999; Kedersha and Anderson, 2002). Fig 1.4 explains the process of translation in the presence and absence of stress, how SGs are formed and the role played by G3BP1.

The fact that phosphorylated eIF2α is responsible for the formation of SGs was validated by the work done by McEwen and co-workers, showing that mutant MEFs which expressed the non-phosphorylated eIF2α only failed to assemble SGs in the presence of stress, whereas the phosphomimetic mutant of eIF2α was potent enough to induce SG assembly (McEwen et al., 2005). Contrary to this, SGs were also observed to assemble when eIF4A helicase function was blocked by drugs such as hippuristanol, which blocks eIF4A, and pateamine A, which inhibits the formation of the translation initiation complex. This shows that SGs can be formed even in the absence of eIF2α phosphorylation (Mazroui et al., 2006).

SGs are believed to be composed of translational abortive mRNPs that have the small but not the large ribosomal subunits, along with translational initiation factors such as eIF4E, eIF3, eIF4A and eIFG. SGs have been found to contain many different kinds of RBPs, one of these being the G3BP (Tourriere et al., 2003).

Tourriere and co-workers showed that G3BP regulates the formation of SGs assembly (Tourriere et al., 2003). Furthermore, both G3BP1 and G3BP2 are known to form SGs (Matsuki et al., 2013). Alterations in the phosphorylation state of G3BP1 can affect SG formation. This was demonstrated when GFP-G3BP1 and a GFP-G3BP1 Ser149Glu phosphomimetic mutant (GFP-G3BP1 S149E) were found to be recruited to SGs, but the mutant was shown to have a reduced capacity to induce spontaneous SG formation (Tourriere et al., 2003). Similar to T-cell Internal Antigen-1 (TIA-1), over-expression of G3BP1 has the
potential to induce SG assembly (Tourriere et al., 2003). Recently, the sirtuin silent information regulator, SIRT6, one of the seven sirtuin homologues (SIRT1–SIRT7), was also found to regulate SG assembly formation in *C. elegans* and mammals (Jedrusik-Bode et al., 2013). Work done by Jedrusik-Bode and co-workers showed that G3BP knock-out MEFs were able to form SGs irrespective of sodium arsenite-induced stress. Similar to TIA-1, over-expression of G3BPs is known to form SGs; hence they are also called as SG-nucleating proteins (Tourriere et al., 2003). G3BP’s recruitment to SGs implicates G3BP in a role in the regulation of certain mRNAs under stress. G3BP’s *in vitro* endoribonuclease activity was reported to be phosphorylation-dependent and the same is true for its sub-cellular localisation through the formation of SGs. This might suggest that G3BP’s reported RNase activity may be linked to its localisation to the SG. However, this correlation has not been demonstrated *in vivo*.
The process of mRNA in the presence and absence of stress

The left-hand panel in green explains the normal process of translation. eIF2B enhances the charging of the eIF2–guanosine-5’-triphosphate (GTP)–tRNA\textsubscript{Met} ternary complex by exchanging guanosine diphosphate (GDP) for GTP. After the formation of the eIF2–GTP–tRNA\textsubscript{Met} ternary complex, a canonical 48S pre-initiation complex is formed at the 5′ end of capped transcripts and scanning of the codons begins. Upon recognition of the initiation codon by the anticodon of tRNA\textsubscript{Met}, eIF5 promotes GTP hydrolysis, and early initiation factors are displaced by the 60S ribosomal subunit. As additional ribosomes are added to the transcript, the mRNA is converted into polysomes. Under stress conditions (right), the phosphorylation of eIF2α converts eIF2 into a competitive antagonist of eIF2B, depleting the availability of eIF2, GTP or tRNA\textsubscript{Met}. Under these conditions, G3BP1 is included in a non-canonical, eIF2- or eIF5-deficient 48S pre-initiation complex which is translationally silent. G3BP1 self-aggregation then promotes the accumulation of these complexes at discrete cytoplasmic foci known as stress granules (Cougat, 2004). Blue square, eIF5; green triangle, eIF2 bound to GTP; yellow triangle, eIF2 bound to GDP; red triangle, phospho-eIF2 bound to GDP; yellow bubble, G3BP1/TIA-1. Image taken from Kedersha and Anderson (2002); reproduced by permission.
1.7 Mitochondria

Mitochondria are known as the “power generators” of the cell, as they synthesise adenosine triphosphate (ATP) molecules to meet the energy requirements of the cell in order to perform normal functions and sustain cell viability. At the same time, mitochondria are also known to regulate cell death by having a role in apoptosis or programmed cell death (Zamzami et al., 1996; Wang, 2001; Kroemer and Reed, 2000; Ott et al., 2007) and have also been found to support cell proliferation (Rustin, 2002). Eukaryotic mitochondria are double membranous organelles, consisting of an outer membrane and an inner membrane. The latter has many infolds, called cristae, which possess most of the enzymes of the complexes involved in the respiratory chain. One of these enzymes is ATP synthase. The space between the two membranes of mitochondria is known as the inter-membranous space and is responsible for maintaining the proton gradient which is responsible for ATP synthesis.

Cancer cell mitochondria are morphologically and functionally different from those of the normal cells as they perform enhanced aerobic glycolysis to meet the energy requirements of the uncontrolled proliferating cells (Gogvadze et al., 2008; Modica-Napolitano et al., 2007). Moreover, tumour cells show an exclusive metabolic reprogramming which makes them more susceptible to mitochondrial dysfunction than non-tumorigenic cells (Bellance et al., 2009).

Since the start of the 20th century, cancer cells have been studied for their deregulated energy metabolism, which is also known as the Warburg Effect. According to Otto Warburg, the enforced shift to aerobic glycolysis in cancer cells is to meet the increasing energy demand of the proliferating cells and is due to deregulated mitochondrial respiration, which could be a reason for the continuous proliferation of cancer cells (Warburg et al., 1927, Warburg, 1956b).

H+-ATP synthase is known to be a key factor for defining the bioenergetic activity of the cell, and β-F1ATPase, which is its catalytic subunit, is known to be downregulated in several cancer cells (Willers et al., 2010) and is considered to be a hallmark for cancer progression. Work done by Ortega and co-workers explained the role of G3BP1, in controlling the post-translational activity of β-F1-ATPase mRNA, suggesting that G3BP1 might be involved in the glycolytic shift that occurs in cancer (Ortega et al., 2010).
1.7.1 Mitochondrial Complex I

Mitochondria are the energy production units of the cell using the electron transport chain (ETC) as a means for the synthesis of ATP molecules, through the OXPHOS system. The entire respiratory chain consists of five complexes of enzymes which are involved in the transfer of electrons, creating the proton-motive gradient across the mitochondrial membrane which drives the synthesis of ATP (Sharma et al., 2009). Complex I is the largest complex and is the first enzyme in the mitochondrial respiratory chain (Walker, 1992). The initial characterisation of Complex I composition came from a study which isolated Complex I from bovine (Bos taurus) heart mitochondria (Hatefi et al., 1962). The details of the composition of this complex became clearer after the discovery of the mitochondrial genome sequence.

Complex I consists of approximately 41–45 subunits; out of these, only seven are encoded by the mitochondrial DNA (mtDNA) (Chomyn et al., 1985, 1986) and the rest are encoded in the nucleus (Sazanov et al., 2000; Carroll et al., 2002) and are transported to the mitochondria by a special system (Schatz, 1996). Complex I is involved in oxidising sugars and fats, and extracts the energy trapped in Nicotinamide adenine dinucleotide, reduced (NADH) through the translocation of electrons, which generates a difference in potential across the mitochondrial inner membrane. The difference in potential generated across the membranes is used to synthesise ATP via the Complex V mitochondrial enzyme ATP synthetase (Mitchell, 1961). Dysfunction of Complex I is one of the major causes of mitochondria-related diseases, including breast cancer (described below in detail) (Smeitink et al., 1998; Smeitink and van den Heuvel, 1999; Distelmaier et al., 2009).

1.7.2 Subunits of Complex I

The prokaryotic Complex I consists of 14 subunits which carry out all the required redox reactions taking place in the mitochondria. Eukaryotes have an additional 25–30 subunits, which are also called “accessory” subunits or “supernumerary” subunits, and may have some additional functions or regulatory units (Sharma et al., 2009), thereby making a total of 45 subunits in Complex I (Kmita and Zickermann, 2013). These accessory subunits are known to share sequence similarities with many cellular proteins (Clason et al., 2010).

Based on the sequence study of the 14 basic subunits which exist both in prokaryotic and eukaryotic Complex I, they can be classified into two general categories: hydrophobic and
non-hydrophobic. Out of the 45 subunits, seven subunits are found to be highly hydrophobic with the presence of 52–59 transmembrane helices that do not exist in the rest of the 38 subunits. All the subunits contain binding domains in order to interact with the redox prosthetic groups and the substrate, NADH (Clason et al., 2010). Table 1.4 describes all the subunits of Complex I.

Table 1.4  The structural constituents of mammalian complex I (Sharma et al., 2009).

<table>
<thead>
<tr>
<th>Complex I (45 subunits)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nuclear DNA Encoded Subunits</strong> (n = 38)</td>
</tr>
<tr>
<td>NDUFV1–3</td>
</tr>
<tr>
<td>NDUF51–8</td>
</tr>
<tr>
<td>NDUFA1–13</td>
</tr>
<tr>
<td>NDUFB1–11</td>
</tr>
<tr>
<td>NDUFB1</td>
</tr>
<tr>
<td>NDUFC1</td>
</tr>
<tr>
<td>NDUFC2</td>
</tr>
</tbody>
</table>

1.7.3. Complex I Structure

The information about the composition of Complex I, and its structural and functional organisation were obtained from B. taurus, bacteria and fungi (Grigorieff, 1998, 1999; Hatefi, 1985; Walker, 1992; Leonard et al., 1997; Weidner et al., 1993; Weiss et al., 1991). By using X-ray crystallography (Hunte et al., 2010) and electron microscopy (Clason et al., 2010; Radermacher et al., 2006), researchers have been able to elucidate the whole structure of Complex I. Table 1.5 shows molecular and genetic information about the human nuclear-encoded subunits of mitochondrial Complex I that are relevant to the research presented in this thesis.
Table 1.5  Molecular and genetic information of three, human, nuclear-encoded subunits of the mitochondrial Complex I.

<table>
<thead>
<tr>
<th>Species: Homo sapiens</th>
<th>Chromosomal Localisation</th>
<th>Protein Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDUFB7</td>
<td>Chromosome 19</td>
<td>18 kDa</td>
</tr>
<tr>
<td>NDUB10</td>
<td>Chromosome 16</td>
<td>21 kDa</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>Chromosome 11</td>
<td>51 kDa</td>
</tr>
</tbody>
</table>

The information about the structure of Complex I was first studied through electron microscopy on Neurospora crassa enzyme crystals and later on bovine cardiac muscle mitochondria to understand the structure of mammalian Complex I (Grigorieff, 1998). The study suggested that both structures are similar and exist as an L-shape with two distinct arms (Leonard et al., 1997): the hydrophobic arm and a hydrophilic arm. The hydrophobic arm possesses subunits which are encoded by mtDNA and resides in the inner mitochondrial membrane, whereas the hydrophilic peripheral arm resides in the matrix (Clason et al., 2010).

1.7.4 The Functional Role of Complex I

The functional roles of these accessory subunits have not been well described. However, some researchers have divided Complex I into three categories: the first one is the electron input module, which is also called the dehydrogenase module (N module), whose role is to accept electrons from NADH; the second one is the electron output module, also called the hydrogenase module (Q module), which transports electrons to ubiquinone; and the last one is the proton translocation module (P module), which pumps protons across the inner mitochondrial membrane (Sharma et al., 2009; Lazarou et al., 2009). The N and Q modules form parts of the matrix arm; however, the P module remains within the membrane arm (Grigorieff, 1998, 1999).

1.8 The Warburg Hypothesis

In 1927, Otto Warburg published a paper revealing that there are two phases in which normal cells transform to the cancerous stage. The first phase is that of irreversible damage to the process of respiration. The second phase starts when the process of respiration is taken over by fermentation (Warburg et al., 1927). Utilising the energy generated by fermentation, the differentiated cells transform into undifferentiated cells and grow vigorously into cancer cells. Cancer cells were observed to have enhanced glycolysis even in the presence of oxygen, which was termed the “Warburg Effect” (Warburg, 1956b) which was in contradiction to what Louis Pasteur observed in the late 19th century. Pasteur observed that
with a decrease in the amount of oxygen, ATP generation shifts from OXPHOS to glycolysis (López-Lázaro, 2008), which is also known as the “Pasteur Effect”. Lactic acid was found to be produced in excess as a consequence of the Warburg Effect, which was considered to be a reason for creating the acidic environment surrounding the tumour which correlates with an increase in tissue invasion (Gatenby et al., 2006; López-Lazaro, 2007).

In reality, the Warburg Effect meets both the vital requirements of a cancer cell in order to survive and multiply: bioenergetics and biosynthesis. Through aerobic glycolysis, utilising excess glucose, a cancer cell generates enough ATP for the survival and multiplication of tumour cells. The intermediates generated through the metabolic pathways serve as ingredients for the biosynthesis of new macro- and micromolecules for the cancer cells. As a by-product, excess production of lactic acid during these metabolic pathways creates an acidic environment surrounding the cancer cells, which assists the invasion of nearby tissues. In a tumour cell, the shift from OXPHOS to aerobic glycolysis serves the purpose of generating ATP as a substrate to the pentose phosphate pathway for nucleotide production and involves glutaminolysis to deliver carbon and nitrogen, which are required by dividing tumour cells (DeBerardinis et al., 2008a 2008b). The by-products of the glutamine metabolic pathway serve as the intermediates required for the synthesis of macromolecules (Fischer et al., 1998). The “glutaminolytic” shift observed in tumour cell metabolism is also considered to be a characteristic feature of tumour cells (Mazurek et al., 2005). The continuous activation of glycolysis in cancer cells makes it favourable to support tumour growth. Along with enhanced aerobic glycolysis and glutaminolysis, cancer cells are also found to have enhanced gluconeogenesis (Lundholm et al., 1982) and decreased fatty acid oxidation (Ockner et al., 1993).

However, the Warburg hypothesis has been disproved by many researchers, as they have found an enhanced glycolytic rate in cancer cells even when the mitochondria are normal, similar to non-cancerous cells. Therefore, mitochondrial dysfunction may not be the only cause for the glycolytic shift needed for energy production in cancer cells, as proposed by Warburg.

Recently, Smolkova and co-workers have proposed a “wave-like” gene regulation process for the suppression and restoration of the OXPHOS in cancer cells (Smolkova et al., 2011). According to Smolkova et al., during the process of transformation of a normal cell to a
tumour cell, a series of changes occurs in the form of “waves”, which alter the metabolic phenotype at every stage in cancer cells and this is controlled by reprogramming of gene expression. The very first wave is known as oncogene-mediated gene reprogramming, which starts from an unknown stimulus which activates oncogenes, making the cells partially glycolytic (the Warburg Effect). The next wave is initiated by hypoxia, generated by the highly proliferating cancer cells and leads to hypoxia-induced factor (HIF)-mediated reprogramming of metabolism. HIF activates many genes that play roles in glycolysis and includes genes which are glucose transporters (e.g. GLU1 and GLU3) (Lu et al., 2002, 2005; Shaw, 2006; Denko, 2008). HIF-1 activation enhances the transcription of many genes that support cancer progression, apoptosis resistance, invasiveness, metastasis and angiogenesis. For example, HIF-1 activates the signalling pathways of MAPK and PI3K, which enhance cell proliferation and survival. Similarly, HIF-1α and HIF-1β expression was found to be correlated with apoptosis and pro-apoptotic factors like caspase-3 and Fas (Ke and Costa, 2006; Semenza, 2003, 2006). The third wave is responsible for reprogramming tumour cell survival and selection of tumorigenic cells by enhancing “glutaminolysis”, which supports the Kreb cycle, involving succinate dehydrogenase which re-establishes OXPHOS. This third wave that causes gene reprogramming is an outcome of the activation of oncogenes, like dysregulated MYC and p53. Aglycemia and nutrient limitation could also be responsible for the activation of this third wave. The fourth wave consists of retrograde signalling from revitalized mitochondria, which corresponds to the gene reprogramming.

1.9 Role of Mitochondria in Cancer Progression

According to Otto Warburg, “Cancer, above all other diseases, has countless secondary causes. But even for cancer, there is only one prime cause, which is the replacement of the respiration of oxygen in normal cells by fermentation of sugar” (Warburg, 1956a). Irreversible damage to the OXPHOS was considered to be the major cause of the aerobic glycolytic shift (Warburg, 1956b) and also led to the downregulation of the observed ATP synthase, which was considered to be the hallmark of most cancers (Cuezva et al., 2002; Isidoro et al., 2005). Little is known about the cause of cancer cells shifting to aerobic glycolysis instead of OXPHOS which generates fewer ATP molecules, but some proposals have been put forward. These include: mitochondrial dysfunction, upregulation of the genes involved in the process of glycolysis, intermediates of the glycolytic pathway being used for the synthesis of the macro-molecules required by the new cancer cells and the downregulation of the respiration process because of the hypoxic condition (Ward and
Initially, mutations in the mtDNA-encoded respiratory genes were considered to be pro-tumorigenic (Ishikawa et al., 2008; Sharma et al., 2011) which led to failure of the mitochondrial OXPHOS and reduced production of the mitochondrial ATP (Brandon et al., 2006; Chatterjee et al., 2006).

Several reports suggest a major role of mitochondria as a hallmark for cancer (Kroemer 2006). For example, Zhou and co-workers observed that mutations in mtDNA-encoded Complex I Subunit 2 stimulates aerobic glycolysis, enhances reactive oxygen species generation and enhances tumorogenesis (Zhou et al., 2007). Stress granules are also known to inhibit cell apoptosis by enhancing the production of reactive oxygen species (Takahashi et al., 2013). In another example, downregulation of the β subunit of F1F0ATPase in liver, kidney and colon cancers as well as lung and breast cancer is also considered to be a hallmark of cancer progression. This may be correlated to changes in the morphology of cancer cell mitochondria which are typically small in size, and lack cristae and lack the β subunit of F1F0ATPase (Lopez-Rios et al., 2007).

1.10 NADH Dehydrogenase (Ubiquinone) 1 β Subcomplex Subunit 7

NADH dehydrogenase (ubiquinone) 1 β subcomplex subunit 7 (NDUFB7) is an enzyme of the multisubunit mitochondrial Complex I. NDUFB7 is a 16.8-kDa protein (See Appendix I for the gene sequence (Source: National Center for Biotechnology Information (NCBI)). (Smeitink and van den Heuvel, 1999). NDUFB7 localises to the mitochondrial inner membrane on the matrix side (Szklarczyk et al., 2011).

This subunit contains a conserved pattern of double cysteine residues, CX$_9$C, which is a characteristic of polypeptides transported across the intermembranous space by the Mia40 import system (Stojanovski et al., 2012).

The protein encoded by NDUFB7 has NADH dehydrogenase activity and oxidoreductase activity. This protein plays an active role in the electron transport chain of OXPHOS, which synthesises energy molecules (Triepels et al., 2000).

RNA immunoprecipitation (RIP) assays done in the host laboratory to screen for the mRNA targets of G3BP1 showed that NDUFB7 mRNA was the prime target transcript, interacting with G3BP1 irrespective of the treatment conditions. The interaction between G3BP1 and the
mRNA of NDUFB7 suggests an exciting possibility that G3BP1 regulates several mitochondrial responses in cancer cells.

1.11 NADH Dehydrogenase (Ubiquinone) Flavoprotein 1

NADH dehydrogenase (ubiquinone) flavoprotein 1 (NDUFV1) is a subunit of the NADH ubiquinone oxidoreductase Complex I, a large complex with at least 45 nuclear- and mitochondrial-encoded subunits that release electrons from NADH and channels them to ubiquinone (See Appendix III for the gene sequences (Source: NCBI)). NDUFV1 is a 51-kDa subunit that carries the NADH-binding site along with the flavin mononucleotide (Chen and Guillory, 1981) and a tetra-nuclear iron-sulphur cluster (Pilkington et al., 1991). The NDUFV1 gene is localised on Chromosome 11q13 (Spencer et al., 1992; Ali et al., 1993) and contains 10 exons, coding a 444-amino acid protein. The protein sequences are found to be highly conserved among humans and cattle (B. taurus) (Coo et al., 1999).

Cloning of the human mitochondrial NDUFV1 subunit by Schuelke et al. (1998) revealed that the 48-base pair 3’UTR of NDUFV1 has 100% antisense homology with the 5’UTR of the γ-interferon inducible protein precursor (Schuelke et al., 1998). The γ-interferon inducible protein might have some role in γ-interferon mediated immune reactions. Schuelke et al. also suggested that NDUFV1 mRNA might play a role as an antisense suppressor that restrains the translation of the γ-interferon inducible protein in tissues with high energy demand. This could explain the correlation observed between Complex I deficiency and inflammatory myopathy, which are reported to occur together. On the other hand, NDUFV1 mutations in C. elegans make them hypersensitive to oxidative stress (Grad and Lemire, 2004).

A recent publication (Santidrian et al., 2013) stated that deregulation of mitochondrial Complex I NADH dehydrogenase activity can profoundly enhance the aggressiveness of human breast cancer cells, whereas therapeutic normalisation of the nicotinamide adenine dinucleotide (NAD⁺):NADH ratio balance can inhibit metastasis and prevent disease progression, which implicates the role of NDUFV1 in cancer progression. The article also stated that knock-down of the subunit NDUFV1 downregulates Complex I activity by 92%.

1.12 NADH Dehydrogenase (Ubiquinone) 1 β Subcomplex Subunit 10

As limited literature is available on NADH dehydrogenase (ubiquinone) 1 β subcomplex subunit 10 (NDUBF10), very little is known about this gene. NDUBF10 is an accessory
subunit of Complex I residing in the inner membrane of the mitochondria. It is believed not to have any role in catalysis. See Appendix II for its gene sequence (Source: NCBI).

1.13 β-F1-ATPase

β-F1-ATPase is the mitochondrial Complex V β subunit of ATP synthase, which is responsible for the synthesis of ATP molecules, which are required to meet the energy requirements of the cell. It uses the proton gradient developed across the mitochondrial membrane by the electron transport chain to synthesise ATP (Boyer, 1993).

The ATP synthase enzyme consists of two parts: the F0 region, which is responsible for the translocation of the protons through the mitochondrial electron transport chain, and the main catalytic region for ATP synthesis, which is the F1 region. The process of ATP synthesis is complex and requires many conformational changes in both regions of ATP synthase. The F1 region of ATPase consist of five different subunits: α, β, γ, δ and ε. Out of the five subunits, only the β subunit takes part in the synthesis of ATP molecules (Leyva et al., 2003; Antes et al., 2003).

The expression of the β subunit of ATPase is considered to play a major role in cancer progression, as its downregulation is considered to be a hallmark of several cancers including breast cancer (Lin et al., 2008, Willers et al., 2010). In 1997, Izquierdo and Cuezva reported that the 3′UTR of the β-F1-ATPase mRNA bears a cis-acting regulatory element that plays a role in regulating its translation (Izquierdo and Cuezva, 1997). The downregulation of the bioenergetic requirements of a cancer cell is related to the inhibition of β-F1-ATPase translation (Formentini et al., 2010).

Recently (in 2010) it was found that G3BP1, which is over-expressed in many cancers, interacts with the β-F1-ATPase mRNA and is responsible for silencing its translation (Ortega et al., 2010) (see Appendix IV for its gene sequence (Source: NCBI)).

This work led us to hypothesise that the interaction of G3BP1 with the cis-element of β-F1-ATPase mRNA could be similar to its interactions with NDUFB7, NDUFB10 and NDUFV1. As all these are nuclear-encoded mitochondrial transcripts, G3BP1 might regulate the energy shift that is generally observed in many cancer cells.
1.14 RNA immunoprecipitation-Chromatin immunoprecipitation (RIP-Chip) Microarray

G3BP1 is known to be an RNA-binding protein as it bears an RRM. However, the transcriptome wide subset of mRNAs bound and regulated by G3BP1 has not been explored so far. A RIP-Chip assay was designed to identify the RNA ligands of G3BP1. MCF7 breast cancer cells over-expressing G3BP1 were used for this assay. The RIP-Chip experiment was performed in the host laboratory by a research fellow and not by the PhD student.

RIPs followed by microarray-based identification of the target transcripts (RIP-Chip) were performed using a protocol that was modified from the original methodology developed by Keene and co-workers (2006). MCF7 cells stably over-expressing human G3BP1 from a pFLAG-CMV4-hG3BP1 vector were either treated with 500 µM sodium arsenite for 30 min or were left untreated as a control. Approximately 5 million cells per sample were lysed in Immuno Precipitation (IP) lysis buffer and centrifuged at 10,000× g for 10 min at 4°C to remove cell debris. After pre-clearing the lysates with Protein A/G coupled beads (UltraLink Protein A/G, 53132, Pierce) for 1 hr at 4°C, 500 µg protein was incubated with 2.5 µg of monoclonal anti-G3BP1 antibody (611126, BD Biosciences) in IP wash buffer with freshly added protease inhibitors and RNAse inhibitors, and 250 µg bovine serum albumin (BSA) for 2 h at 4°C. Protein–antibody complexes were precipitated by adding 10 µL of settled beads and incubation for 1 hr at 4°C. The precipitate was collected by centrifugation at 2500× g for 1 min at 4°C and washed four times with the IP buffer. RNA was extracted from the precipitate using the RNEasy Mini Kit (74104, Qiagen) according to the manufacturer’s protocol, including the on-column DNase treatment step. Negative control immunoprecipitations were run in parallel without addition of an antibody and a sample resembling total cellular RNA content was extracted from the IP lysates. RNA quantity was determined on a NanoDrop Spectrophotometer (ND-1000, Agilent), and RNA quality and integrity were verified on a Bioanalyzer (Agilent). The success of the G3BP1 immunoprecipitation was verified via western blot using protein that was acetone-precipitated from the RNA column run as described in the RNEasy manual.

For the microarrays, 50 ng of RNA were processed with the Illumina TotalPrep RNA Amplification Kit (AMIL 1791, Ambion/Applied Biosystems) and 750 ng of labelled cRNA per sample were used to hybridise an Illumina HumanHT-12 v1 Whole-Genome Gene
Expression BeadChip. Raw data processing including quantile normalisation, fold change calculations and statistical analysis was done using GeneSpringGX11 software (Agilent Technologies). Array probes that showed an enrichment of more than 1.5-fold between the G3BP1 IP sample and the negative control IP, as well as ≥1.5-fold enrichment of the G3BP1 IP sample over the total RNA with a P-value of <0.05 were considered as target transcripts bound by G3BP1. Array probes showing a fold change of more than 1.5-fold between the arsenite-treated and untreated total RNA samples with a P-value of <0.05 were considered as differentially expressed. Gene Ontology analysis was carried out using the online tools GOrilla and DAVID (cbl-gorilla.cs.technion.ac.il and https://david.ncifcrf.gov Feb-2012). Functional and pathway analysis was executed with the licensed software Ingenuity Pathway Analysis.

Samples of stressed and unstressed MCF7 cells, including 81 putative binding targets of G3BP1 were identified in comparison to 156 bound transcripts in cells treated with sodium arsenite to induce oxidative stress. Out of the 156 putative targets, 26 transcripts appeared to be bound regardless of the treatment condition and 130 appeared to be specific to the stress condition. An analysis of the 156 bound transcripts from the total mRNA isolated from stressed versus non-stressed cells showed that only one of the target mRNAs changed expression levels significantly.

Thus the observed difference in the profile of G3BP1-bound transcripts between untreated and stressed cells was not an artefact arising from changes in the overall expression levels of the putative G3BP1 targets within the cells. Functional and gene ontology analyses using Ingenuity and Gorilla software tools, revealed a significant enrichment of the transcripts encoding mitochondrial proteins within the subset of G3BP1-bound target RNAs. This suggests that G3BP1 binds to RNA ligands that encode mitochondrial proteins. This outcome sets a foundation step for the PhD thesis, where several chapters were designed to study the regulation of NDUFB7, NDUFB10 and NDUFV1.
1.15 Outline of this Thesis

This research work was based on the available information about deregulated post-transcriptional gene regulation and the role of RBPs in tumorigenesis, and also about the glycolytic energy shift observed in many cancer cells. The research work aimed to explore the potential of G3BP1 in binding and regulating the nuclear-encoded mitochondrial Complex I mRNA transcripts, which have a role in the electron transport chain and play a role in generating the energy molecule, ATP. Research work published in 2010 showed that G3BP1 interacts with the 3′UTR of β-F1-ATPase mRNA and regulates its translation, which suggests that G3BP1, being a component of SGs, might regulate the fate of this mRNA and might also have a role in controlling the energy shift observed in many cancer cells.

The host laboratory conducted a RIP-Chip assay using a stable MCF7–pFLAG-CMV4-hG3BP1 construct over-expressing G3BP1 cells in order to find novel target genes for G3BP1 under stress and normal conditions. The RIP-Chip data were analysed using Ingenuity software, and the Gorilla tool was used to explore the potential cellular role of the target genes. Based on the dataset from the RIP-Chip assay, three target transcripts, NDUFB7, NDUFB10 and NDUFV1, were selected. All these three target transcripts were nuclear-encoded mitochondrial proteins and were subunits of the mitochondrial Complex I proteins. Complex I proteins play a major role in the mitochondrial electron transport chain, which is a part of mitochondrial OXPHOS. Therefore, the overarching aim of this thesis was to explore the potential of G3BP1 to bind to its target transcripts (NDUFB7, NDUFB10 and NDUFV1). Additionally, the research also aimed to study the role of G3BP1 in regulating the expression of these transcripts under different environmental conditions (i.e. oxidative stress induced by sodium arsenite vs control). The following is an outline of each of the chapters of this thesis and how they contribute to the overall aim of the work.

Chapter 3 describes how to optimise the stress conditions for the MCF7 mammalian breast cancer cell line using sodium arsenite. The formation and quantification of SGs were studied in this chapter. Optimisation of the sodium arsenite concentration was done with the aim of having a negligible effect on cell viability and cell morphology. In the same chapter, the co-localisation of G3BP1 with TIA-1, another SG marker protein was studied under stress and normal conditions.
Chapter 4 describes the experiments performed to study the endogenous expression of the three candidate gene transcripts, *NDUFB7*, *NDUFB10* and *NDUFV1*, under stress and normal conditions at the transcriptional level by performing quantitative real-time polymerase chain reaction (qRT-PCR) and at the translational level by performing western blotting. *Actin* was used as a housekeeping gene to normalise target gene expression levels. G3BP1 is known to interact with the target elements that generally reside in the 3′UTR of the transcript and regulate translation (for example: c-myc, cttnb1 and β-F1-ATPase). Therefore, reporter assays were performed with the cloned UTRs of all three candidate target transcripts in order to study the functional role of G3BP1 *in vivo*.

Chapter 5 describes the experiments designed to study the endogenous expression of NDUFB7, NDUFB10 and NDUFV1 at the transcriptional level and also at the translational level after knock-down of *G3BP1* by using siRNAs.

Chapter 6 describes experiments designed to explore whether G3BP1 has a role as a master regulator of the energy shift which is observed in many cancer cells. G3BP1’s role in regulating Complex I activity was also studied in this chapter.

The design of this thesis is presented in the form of a flow chart, which is shown in Fig. 1.5.
Figure 1.5  Flowchart describing the outline of the work presented in this thesis, in the form of different chapters
† qRT-PCR, quantitative real-time polymerase chain reaction
2. MATERIALS AND METHODS

This chapter describes the materials and methodology used in this thesis for performing different experiments. All the tools and techniques have been classified as per the research area where the specific techniques are used.

2.1 Molecular Biology Tools and Techniques

2.1.1 Standard Solutions and Media

The following media were used in this study:

- **Liquid broth (LB) medium**: Total volume: 1 L
  - 10 g bacto-tryptone
  - 5 g bacto-yeast extract
  - 10 g NaCl

- **LB agar**: Total volume: 1 L
  - 15 g bacto-agar per litre LB

- **1× tris[hydroxymethyl]amino-methane (Tris)-acetate ethylene diamine tetraacetic acid (EDTA)**:
  - 40 mM Tris-acetate (pH 8.3),
  - 1 mM diaminoethane tetra-acetic acid,
  - disodium salt (EDTA)

- **1× Tris-EDTA**: 10 mM Tris-HCL (pH 8.0), 1 mM EDTA

2.1.2 Plasmid Vector

The pGL3 promoter vector E176A (Promega) was used for generating the reporter constructs used in the reporter assay and the *Luciferase* mRNA stability assay. Appendix V shows the plasmid map of the pGL3 promoter vector.

2.1.3 Primers

All primers were synthesised by Geneworks (Australia). Table 2.1 shows a list of all primers used for cloning and generating reporter constructs for this thesis.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Region and Direction of Primer</th>
<th>Primer Sequence†</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDUFB7</td>
<td>5′UTR forward</td>
<td>gatctagaCTGACTGAGGGGTCAGTGGTTC</td>
</tr>
<tr>
<td>NDUFB7</td>
<td>5′UTR reverse</td>
<td>gatctagaGGCTGACTGAGGGTCCAGATCC</td>
</tr>
<tr>
<td>NDUFB7</td>
<td>CDS forward</td>
<td>gatctagaATGGGGGCCACCTGGTCCG</td>
</tr>
<tr>
<td>NDUFB7</td>
<td>CDS reverse</td>
<td>gatctagaCTACAGGGCACCTTGGGGTCA</td>
</tr>
<tr>
<td>NDUFB7</td>
<td>3′UTR forward</td>
<td>cttctagaGGGGGTGCAACCCCTCCTGCCTTcatagactc</td>
</tr>
<tr>
<td>NDUFB7</td>
<td>3′UTR reverse</td>
<td>gatctagaAGGGGGCTGAAGGCTTTATTGACTGATCCATAGGGGTCCTGAGC</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>5′UTR forward</td>
<td>ctgtctagagaAGCCCGGCGCGGCCAGGACTCC</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>5′UTR reverse</td>
<td>ctgtctagaGGCCGGCGGGGCGCGGACTCC</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>CDS forward</td>
<td>ctgtctagaATGCCGGACAGCAGGGACAG</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>CDS reverse</td>
<td>ctgtctagaTCAGAGGGGTCAGCGCGGC</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>3′UTR forward</td>
<td>ctgtctagagaAGCCCGGCGCGGCCAGGACTCC</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>3′UTR reverse</td>
<td>ctgtctagagaAGCCCGGCGCGGCCAGGACTCC</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>5′UTR forward</td>
<td>ctgtctagaGAAGGGGCAGGACTCC</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>5′UTR reverse</td>
<td>ctgtctagaCGCGCGGGGCCAGGACTCC</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>CDS forward</td>
<td>ctgtctagaATGCTGGGCAACCGGCGGC</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>CDS reverse</td>
<td>ctgtctagaTCAGAGGGGTCAGCGCGGC</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>3′UTR forward</td>
<td>ctgtctagagaCACCACCTGGCGACTGAGGCTGATTATGAGGGCTGAGGACAGC</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>3′UTR reverse</td>
<td>ctgtctagagaCACCACCTGGCGACTGAGGCTGATTATGAGGGCTGAGGACAGC</td>
</tr>
<tr>
<td>Firefly Luciferase</td>
<td>Forward</td>
<td>GGTCTTACCGGAAAAACTCGACG</td>
</tr>
<tr>
<td>Firefly Luciferase</td>
<td>Reverse</td>
<td>GCATTCTAGTTGGTGGTGTC</td>
</tr>
</tbody>
</table>

† Lower case plus underlined text represents restriction sites that were added to the primer sequences (e.g. tctaga – XbaI); lower case letters represent the additional nucleotides that were added to the primers for the ease of restriction digestion.
2.1.4 Polymerase Chain Reaction

Polymerase chain reaction (PCR) amplification was performed in 25–50 µL reactions under the following conditions, unless otherwise specified: 2.5 mM MgCl₂, 1× standard PCR buffer, 125 µM deoxynucleotides (dNTPs) (Fermentas), 20 ng plasmid DNA or 100 ng cDNA as appropriate, 1 U of Taq DNA polymerase (New England Biolabs), 20 µM forward primer and 20 µM reverse primer. Reactions were performed in a DNA Engine thermal cycler (MJ Research). Cycling conditions were as follows: denaturation of DNA at 95°C for 1 minute, annealing at 65°C for 1 minute (or as specified elsewhere) and extension at 72°C for 1 minute per 1 kb. PCR using plasmid DNA as the template was performed for 30 cycles, whereas PCR using cDNA as the template was performed for 35 cycles.

PCR products were separated on 2% agarose (Bio-Rad) gel and purified with NucleoSpin Gel and a PCR clean-up kit (740609.50, Macherey Nagel).

2.1.5 Competent Cells

Competent cells used in this research work were Escherichia coli strain JM109 (Promega). The cells were aliquoted per use and stored at -80°C for later use. The cells were cultured under standard conditions (Sambrook et al., 1989) in LB medium or on LB agar plates containing 50 µg/mL of ampicillin, as the bacteria possess the ampicillin resistance gene.

2.1.6 Plasmid DNA Preparation

Extraction of plasmid DNA from the culture for genetic manipulation and sequencing was prepared by using the NucleoSpin Plasmid (740588.50, Macherey Nagel) kit and the protocol was followed as per the instructions supplied by the manufacturer. Plasmid DNA preparations for mammalian cell transfections were prepared using the NucleoBond Xtra Midiprep (Macherey Nagel-740410.10) kit following the manufacturer’s instructions.

2.1.7 Restriction Endonuclease Digestion

Restriction endonucleases were obtained from New England Biolabs. Restriction digestion was carried out under the standard conditions recommended by the manufacturer. For this step, 0.5 µg of DNA was incubated with 1–5 units of enzyme in a final volume of 20 µL at
37°C for at least 2 h. Appropriate buffer solutions and BSA was used as per the manufacturer’s protocol.

2.1.8 Agarose Gel Electrophoresis

Electrophoresis of digested DNA was carried out at room temperature using 0.8–1% (weight/volume) agarose (Bio-Rad) gels with appropriate molecular weight markers in order to determine the success of the enzyme digestion and to estimate the fragment lengths. The molecular weight markers used were 1-kb Plus molecular markers (10787-018, Invitrogen).

Gels were run at 85–100 V for 30–100 min in an electrophoresis tank (Bio-Rad) containing 1× Tris-acetate buffer. One percent agarose (Bio-Rad) gels were prepared by adding 0.5 g of agarose to 50 mL of 1× Tris-acetate buffer, and the solution was boiled in a microwave until the agarose was completely dissolved. After the solution was cooled to about 50°C, ethidium bromide (Bio-Rad) was added to the agarose gel at a concentration of 0.5 µg/mL, allowing DNA bands to be visualised using the Gel Logic 200 Imaging System (Kodak). After that the gel was poured. Once the gel had solidified, the wells were loaded with the DNA samples containing a loading buffer.

2.1.9 Ligation of the Restriction Fragments into Vector DNA and Transformation

Insertion of the restriction fragments into plasmid DNA was used to create new constructs. The plasmid used in this research work was pGL3 (Promega), which is a reporter vector. The ligation mixture consisted of the digested insertion DNA and vector in a 3:1 ratio, 2 µL of the ligation buffer, 1 µL of T4 DNA ligase and water to reach a final volume of 10 µL. This mixture was left for a minimum of 2 h at room temperature. Plasmids were transformed into competent bacteria using the method described by the suppliers of the competent cells (Promega). Briefly, 10 µL of the ligation mix was added to 20 µL of competent cells and placed on ice for 10 min. The cells were then heat-shocked by incubation in a water bath at 42°C for 45 s, followed by a 2-min incubation on ice. Next, 500 µL of LB was added to the cells and the cells were placed in a shaking incubator for at least 1 h to recover. Cells were plated on LB agar plates containing the ampicillin (SIGMA) antibiotic and incubated overnight at 37°C.
2.1.10 Sequence Analysis

All DNA constructs generated during this research were sequenced in order to confirm the presence of the insert in the clone and to eliminate the possibility of mutations being generated during the cloning procedures. DNA sequencing was performed using the Applied Biosystems 3130x1 Genetic Analyser at Griffith University’s DNA sequencing facility, and the reagents used in the reactions were those supplied in the sequencing reaction kit and were used according to the manufacturer’s instructions. Sequencing results were analysed using the BioEdit software (Tom Hall Ibis Biosciences; www.mbio.ncsu.edu/bioedit/page2.html).

2.1.11 Quantitative real time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen), following the instructions provided in the manual, and cDNA was prepared using the Qiagen QuantiTect Kit (205310), using random and oligo-dT primers. For qRT-PCR, cDNA was added to SYBR Green Master Mix (QT605 – SMPS-110E Quantace SensiMix, Bioline) and run in a Rotor Gene 6000 (Corbett). The cycling program was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in triplicate and the results were normalised to the level of Actin. Table 2.2 shows the primers used for the qRT-PCR. The data were analysed using Linreg software (Ruijter et al., 2009) based on the ΔΔCt method (Pfaffl et al., 2001).

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Region and Direction of Primer</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDUFB7</td>
<td>Forward</td>
<td>ttccccgaacgcaaggagcg</td>
</tr>
<tr>
<td>NDUFB7</td>
<td>Reverse</td>
<td>gccaactctgccgctttcttc</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>Forward</td>
<td>ctgggacaaggatgtgtacc</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>Reverse</td>
<td>gtagtaatacctggttttgc</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>Forward</td>
<td>acctccatttggctgctgaa</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>Reverse</td>
<td>ctcacaacccgagtcttggat</td>
</tr>
<tr>
<td>Actin</td>
<td>Forward</td>
<td>caccatggcaatgagggttc</td>
</tr>
<tr>
<td>Actin</td>
<td>Reverse</td>
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</tr>
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<td>G3BP1</td>
<td>Forward</td>
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</tr>
<tr>
<td>G3BP1</td>
<td>Reverse</td>
<td>tggacggggctgtgaagctg</td>
</tr>
</tbody>
</table>
2.2 Protein Chemistry Tools

2.2.1 Standard Solutions

The standard solutions used in this work for protein chemistry analysis are as shown in Table 2.3.

Table 2.3 Composition of solutions used for protein chemistry analysis

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbeco-phosphate buffer saline (pH 7.4)</td>
<td>137 mM NaCl, 2.6 mM KCl, 10.1 mM Na₂HPO₄</td>
</tr>
<tr>
<td>Tris buffer saline (TBS)</td>
<td>20 mM Tris base (pH to 7.4 with HCl), 137 mM NaCl</td>
</tr>
<tr>
<td>TBS-Tween</td>
<td>0.1% Tween-20 in TBS</td>
</tr>
<tr>
<td>1× Tris-glycine</td>
<td>25 mM Tris base, 250 mM glycine, 0.1% SDS† in water</td>
</tr>
<tr>
<td>5× SDS sample buffer:</td>
<td>0.313 M TrisHCl (pH 6.8), 10% SDS, 0.05% bromophenol blue, 50% glycerol</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>3 % BSA or 5% milk powder in TBS-Tween</td>
</tr>
</tbody>
</table>

† SDS, sodium dodecyl sulphate; BSA, bovine serum albumin

2.2.2 Total Cell Lysate Preparation

The cells were cultured in flasks 75 cm² dimension and were incubated until 80–85% confluent. Once the cells were confluent, cells were treated with media supplemented with an optimal concentration of NaAsO₂ (SIGMA) (15 μM) to induce stress and were incubated for specific time periods as per the experiment’s requirements. After a set time interval, cells were trypsinised (GIBCO) and harvested. The cell pellet was lysed in a radioimmunoprecipitation assay buffer (SIGMA) supplemented with a cocktail of protease inhibitor (SIGMA) and Phenylmethanesulfonyl fluoride PMSF (SIGMA). The cells were lysed using a 25G needle (BD Injection Products) and were incubated at -80°C for 15 min. After 15 min cell lysate was thawed and centrifuged for 20 min at 14,000 x g. The supernatant was collected into fresh tubes and the protein concentrations of the total cell lysates were determined using the Bio-Rad DC protein assay kit.
2.2.3 Protein concentration estimation

The assay was performed as per the manufacturer’s instructions. In brief, the samples were diluted 1:15 and kept on ice and 5 μL of each sample dilution was used for measuring the concentration. Reagent A (an alkaline copper tartrate solution), Reagent B (a dilute folin reagent) and Reagent S were provided with the assay kit. Reagents S and A were mixed at a ratio of 1:50 to each other and 25 μL was added per sample. Next, 200 μL of Reagent B was added to each sample, mixed properly and incubated 15 min at room temperature, and then absorbance was measured at 750 nm using a plate reader (Bio-Tek). Sample absorbance was plotted against the BSA standards and concentrations were calculated.

2.2.4 Western Blot Analysis

Proteins were separated on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and were transferred to a Polyvinylidene difluoride (PVDF) (Millipore) membrane. The equal loading of protein samples and proper transfer of protein to the membrane was assessed via Ponceau S (SIGMA) staining. Five percent milk powder in Tris buffer saline–Tween was used as a blocking solution to block the membrane in order to avoid any non-specific protein antibody binding. Actin was used as a loading control. The membranes were incubated overnight at 4°C with primary antibodies (i.e. anti-G3BP1 (1:500), anti-NDUFB7 (1:150), anti-NDUFB10 (1:150), anti-NDUFV1 (1:150) and anti-actin (1:1000)) diluted in the blocking solution. The membranes were washed three times for 10 min each and then incubated with Horse Radish-Perioxidase HRP-conjugated secondary antibodies for 1 h at room temperature. The membranes were then washed three times for 10 min each and the antigen–antibody reaction was detected using enhanced chemiluminescence detection with the Millipore Immobilon kit (WBKLS0500), following the manufacturer’s instructions, followed by exposure and imaging of the membranes using a Versadoc imaging station (Bio-Rad).

2.2.5 Densitometry Analysis:

The western blot data were analysed with Quantity One software (Bio-Rad) to study the intensity of the bands of the target gene and were normalised against Actin as a loading control. A volume rectangle tool box was created around the band of the target protein and the loading control (i.e. Actin). The volume analysis report was generated to show the values for specific protein expression densities. The density values of the target protein were
normalised to the density value of the loading control, actin. These values were compared for the target samples with the control samples. All experiments were performed using at least 3 biological replicates.

The antibodies used in this research work were as follows:

- Anti-G3BP1 (611126, BD Bioscience, Australia)
- Anti-NDUFB7 (ab55531, Abcam, Australia)
- Anti-NDUFB10 (ab70206, Abcam)
- Anti-NDUFV1 (ab55535, Abcam)
- Anti-ACTIN (5060, SIGMA)
- Anti-TIA Polyclonal (ab40693, Abcam)

2.3 Animal Cell Culture

2.3.1 Cell Lines and Culture Conditions

Wild-type MCF7 mammalian breast cancer cells (MCF7-wt) were used in this research, which were collected from the liquid N stock maintained at the host laboratory. All cell cultures were grown in Dulbecco’s Modified Eagle’s Medium (11320, Life Technologies) supplemented with 10% foetal bovine serum (GIBCO) and 1× penicillin streptomycin (GIBCO). Cells were maintained in 5% CO₂ at 37°C in a humidified incubator (SANYO). Cells were harvested by trypsin (GIBCO) digestion as per the manufacturer’s instructions.

2.3.2 Transient Transfection

Cells were plated at a density of 2 × 10⁵ per well in a six-well plate and incubated for 24 h. Cells were transfected using Lipofectamine2000 (Invitrogen), following the manufacturer’s instructions. This technique was used for the transfection of reporter constructs and siRNAs for G3BP1 and the control.

2.4 siRNAs Used to Knock Down G3BP1

Customised Dharmacon (www.dharmacon.gelifesciences.com) siRNAs were used to knock down the target gene, G3BP1, along with non-targeting siRNAs as a control. Table 2.4 shows the details of the different siRNAs used in the present thesis work.
### Table 2.4 siRNAs used in the research work

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>siRNA</th>
<th>Target Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>G3BP1</td>
<td>siGENOME SMART pool siRNA D-012099-05 G3BP1</td>
<td>GAAGGCGACCGACGAGAUA</td>
</tr>
<tr>
<td></td>
<td>siGENOME SMART pool siRNA D-012099-18 G3BP1</td>
<td>GUGCGAGAAACAACGAAUAA</td>
</tr>
<tr>
<td></td>
<td>siGENOME SMART pool siRNA D-012099-04 G3BP1</td>
<td>UGACAUGGAAGAAACUUA</td>
</tr>
<tr>
<td></td>
<td>siGENOME SMART pool siRNA D-012099-01 G3BP1</td>
<td>UAACAGUGGUGGGAAUUA</td>
</tr>
<tr>
<td>Non-Targeting Control</td>
<td>siGENOME non-targeting siRNA</td>
<td>UAAGGCUAUGAAGAGAUAC</td>
</tr>
</tbody>
</table>

#### 2.5 Luciferase Reporter Assay

For the reporter assay, the Promega Luciferase reporter assay kit with Reporter Lysis Buffer was used (E4030, Promega). The kit was provided with a Luciferase assay substrate (E151A) in lyophilised form, which was reconstituted using the Luciferase assay buffer (E152A). The transiently transfected cells were lysed using the 1× reporter lysis buffer, which was reconstituted from a stock 5× reporter lysis buffer solution. Nunc white plates (Nunc 136101) were used in the assay. In total, 20 μL of the cell lysate and 100 μL of the reconstituted Luciferase assay substrate was added in each well of the plate and luminescence values were calculated by reading them in a plate reader (Bio-Tek).
3. STRESS GRANULE FORMATION

3.1 Introduction

Eukaryotic cells, when exposed to environmental insult in the form of oxidative stress, high temperature, viral infection, hyperosmolarity or UV irradiation, are left with three options. One is to initiate an adaptive mechanism and respond to the stress, repairing the damage caused by the stress and subsequently restoring normal cellular function. Alternatively, the cell can activate apoptosis. A third option is to initiate a stress response but if recovery is not achieved, then apoptosis is initiated. The path a cell selects depends on the extent of the stress. In the case of stress survival, the eukaryotic cell reprograms the translational mechanism and regulates the expression of a highly selective set of transcripts that support the cell’s survival (Holcik and Sonenberg, 2005; Spriggs et al., 2010; Liu and Qian, 2014; Kedersha et al., 2013).

RNA granules were discovered in the cytoplasm of tomato cells exposed to heat shock, which were named SGs (Nover et al., 1983). SGs are cytoplasmic granules that are rich in non-translating mRNPs, which consist of individual or pools of mRNA transcripts bound by proteins that regulate the post-transcriptional events of gene expression. SGs are the sites of mRNA triage, regulating mRNA stability and translation (Kedersha and Anderson, 2002; Anderson and Kedersha, 2002). However, a recent report suggests that cells recovering from cold shock restore translation long before the disassembly of SGs. This current finding challenges the traditional concept of SGs as translational “detention centres” (Hofmann et al., 2012; Kedersha et al., 2013), raising the possibility that SGs may have roles that have not so far been described in the literature, such as a centre to sequester proteins involved in the regulation of RNA but not directly binding to them. Traditionally, G3BP1 is considered as an essential component of SG and this chapter was designed to confirm its presence in the MCF7 cells used throughout this thesis.

The process of translation begins with the recruitment of small ribosomal subunit and translation initiation factors to the mRNA to form a 48S complex. In stressed cells, phosphorylation of eIF2α limits the availability of the eIF2–GTP–tRNAMet ternary complex to initiate the translation, leading to the accumulation of a 48S pre-initiation complex (Kedersha and Anderson, 2002; Kedersha et al., 1999). The stress-induced phosphorylation
of the translation initiation factor eIF2α, which is mediated by various stress-activated kinases (Kedersha et al., 2013), leads to the inhibition or delay of mRNA translation initiation (Kedersha et al., 1999). Table 3.1 summarises the different kinds of kinase and the types of stress which activates them (Kedersha et al., 2013).

Table 3.1 Different kinds of stress-activated kinases and their role (Kedersha et al., 2013)

<table>
<thead>
<tr>
<th>Kinase</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRI (heme-regulated initiation factor 2α kinase)</td>
<td>Recognises redox stress produced by sodium arsenite</td>
</tr>
<tr>
<td>PKR (protein kinase RNA-activated)</td>
<td>A double-stranded RNA-dependent kinase activated by viral infection, heat and UV irradiation</td>
</tr>
<tr>
<td>PERK (PKR-like endoplasmic reticulum (ER) kinase)</td>
<td>An ER protein sensor activated by unfolded proteins in the ER lumen</td>
</tr>
<tr>
<td>GCN2 (general control non-derepressible 2)</td>
<td>A protein that monitors amino acid levels and is activated by amino acid deprivation</td>
</tr>
</tbody>
</table>

The fact that phosphorylated eIF2α is responsible for the formation of SGs was validated by McEwen et al. (2005). In their work, it was shown that mutant MEFs expressing the non-phosphorylated eIF2α failed to assemble SGs in the presence of stress, whereas the phosphomimetic mutant of eIF2α was capable of inducing SG assembly (McEwen et al., 2005). However, SGs were also observed to assemble when eIF4A helicase function was blocked by using drugs such as hippuristanol, which blocks eIF4A and pateamine A, which inhibits formation of the translation initiation complex, which, in turn, suggests that SGs can be formed even in the absence of eIF2α phosphorylation (Mazroui et al., 2006).

SGs are believed to be composed of translational abortive mRNPs, which contain the small but not the large ribosomal subunits, along with translational initiation factors such as eIF4E, eIF3, eIF4A and eIFG. SGs contain many different kinds of RNBPs that regulate the expression of their target transcripts; one of them is G3BP (Tourriere et al., 2003).

Tourriere et al. (2003) showed that G3BP regulates the formation of SG assembly. G3BP’s *in vitro* endoribonuclease activity was reported to be phosphorylation-dependent and the same was found for its sub-cellular localisation through the formation of SGs. This suggests that
G3BPs RNase activity may be linked to its localisation to the SG. However, this correlation has not been demonstrated in vivo. Alteration in the phosphorylation state of G3BP1 can affect SG formation. This was demonstrated when GFP-G3BP1 and a GFP-G3BP1 Ser149Glu phosphomimetic mutant (GFP-G3BP1 S149E) were both found to be recruited to SGs. However, the mutant showed a reduced capacity to induce spontaneous SG formation (Tourriere et al., 2003). Similar to TIA-1, over-expression of G3BP1 has the potential to induce SG assembly and hence it is also known as SG-nucleating protein. However, the work done by Jedrusik-Bode et al., (2013) showed that G3BP knockout MEFs can also form SGs, suggesting that G3BP is not an essential component for SG formation. Recent work done by Matsuki et al. (2013) confirmed that G3BP1 and G3BP2 both contribute to the formation of SG assembly. In stress conditions, G3BP’s recruitment to SG assembly suggests that G3BP has a role in the regulation of its target mRNAs.

G3BPs are considered to be primarily cytoplasmic proteins (Parker et al., 1996), but both proteins, G3BP1 and G3BP2, are known to possess the capacity to enter the nucleus (French et al., 2002; Barnes et al., 2002; Costa et al., 1999). However, there is a controversy about the subcellular localisation of G3BP as Tourriere et al., (2001) observed the nuclear localisation of phosphorylated G3BP1 in quiescent MEFs, whereas Parker et al. (1996) did not observe G3BP1 in the nucleus of epidermal growth factor-transformed fibroblasts at any stage of the cell cycle. One of the G3BP2 isoforms is known to shuttle between the nucleus and the cytoplasm in a cell cycle dependent manner. In contrast to G3BP1, French and co-workers (2002) found G3BP2 to be exclusively cytoplasmic in quiescent fibroblasts and to rapidly enter the nucleus upon serum stimulation.

This chapter aimed to study the localisation of G3BP1 under stress and normal conditions in MCF7 cells. Cells that formed SGs were also quantified. Stress conditions were induced by using sodium arsenite, which causes oxidative stress and leads cells to form SGs. The sodium arsenite concentration was titrated (15 μM–1 mM) and the cells were observed under the microscope for cell death and changes in the cell morphology, compared with the non-stressed cells as control.
3.2 Materials and Methods

3.2.1 Cell Culture
Mammalian MCF7-wt breast cancer cells were used for this research. The cells were cultured as described in Chapter 2, Section 2.3.1.

3.2.2 Immunofluorescence Microscopy
Immunofluorescence microscopy on cultured MCF7-wt cells was performed in order to study the localisation of G3BP1. The MCF7-wt cells were cultured and seeded at a density of 1 × 10^6 cells per 22-mm coverslip in six-well plates, and were incubated for 24–48 h to obtain 60–80% confluence. Stress was induced by replacing the media with sodium arsenite-supplemented media (15 μM–1 mM) and the cells were incubated for 12–48 h or as otherwise indicated. After treatment, coverslips were briefly rinsed in Dulbeco-phosphate buffer saline (DPBS) and were fixed for 30 min in 4% paraformaldehyde in DPBS. After fixing, the cells were rinsed in DPBS three times for 3 min each. After that, cells were permeablised using 1% Triton X-100 (SIGMA) in DPBS for 5 min. Subsequently, cells were washed and incubated in a blocking buffer (1% BSA in DPBS) for 1 h before the application of primary antibodies. Primary antibodies (anti-G3BP1, 1:200; anti-TIA, 1:200) were diluted in blocking buffer and the cells were incubated for 1 h at room temperature. After 1 h of incubation, cells were washed three times for 3 min each in DPBS and were incubated for 1 h in secondary antibodies (Alexa Fluor 488, 1:1000; Alexa Fluor 546, 1:1000). After 1 h of incubation, cells were washed three times for 3 min each in DPBS and the nucleus was stained using 4’,6-diamidino-2-phenylindole (DAPI) for 5 min, followed by two washes for 3 min each. Coverslips were mounted, left overnight to dry, and later visualised and the images captured using a confocal microscope (Floview FV1000, Olympus).

3.3 Results

3.3.1 Recruitment of G3BP1 to SG
The cellular localisation of G3BP1 was evaluated under normal conditions and under oxidative stress induced by sodium arsenite (1 mM for 30 min). Fig. 3.1 shows the images for the endogenous G3BP1 under normal (Panel A) and stress conditions (Panel B). The results confirmed that under normal conditions, endogenous G3BP1 remains localised in the
cytoplasm, whereas during stress, endogenous G3BP1 was recruited to the cytoplasmic granular bodies known as SGs.

![Image of recruitment of G3BP1 to stress granules (SGs)](image)

**Figure 3.1 Recruitment of G3BP1 to stress granules (SGs)**
Panel A shows the endogenous G3BP1 in the cytoplasm under normal conditions (green, left), DAPI (blue, centre) and merged (right). Panel B shows the recruitment of endogenous G3BP1 (Green, left), to cytoplasmic granular bodies after 30 min of stress from NaAsO$_2$ at 1 mM concentration. White arrows show the endogenous G3BP1 in the cytoplasm during normal conditions and the yellow arrows indicate recruitment of G3BP1 to SGs under stress. Scale bar, 10 µm (white bar).

### 3.3.2 Co-localisation of G3BP1 and TIA-1 to SGs

In order to confirm the localisation of G3BP1 to SGs, immunofluorescence microscopy was performed using double staining for G3BP1 and TIA-1, which is another SG marker protein (Kedersha et al., 1999). Fig. 3.2 F, shows the co-localisation of G3BP1 and TIA-1 to SGs upon induction of stress, which is consistent with the work done by Ortega et al. (2010).
Figure 3.2 Co-localisation of G3BP1 and TIA-1 to stress granules (SGs)
Panel A shows the endogenous expression of G3BP1 in the cytoplasm under normal conditions (green, left), Panel B shows the endogenous expression of TIA-1 (red, centre) under normal conditions and Panel C shows the merged image, where G3BP1 and TIA-1 overlap each other. Nuclei were stained with DAPI, which is shown in blue. Panel D shows the recruitment of G3BP1 to SGs, marked with white arrows, Panel E shows the recruitment of TIA-1 to SGs, marked with white arrows, and Panel F shows the merged image of co-localised G3BP1 and TIA-1 to SGs. Panels G, H and I show close-ups of the area highlighted by yellow boxes in Panels D, E and F, respectively. Scale bar, 10 µm (white bar).

3.3.3 Optimisation of Sodium Arsenite Concentration for Inducing stress in MCF7-wt Cells

Additional experiments were designed to optimise the concentration of sodium arsenite for SG formation, with minimal effects on cell viability and morphology at different time intervals. Titration of sodium arsenite ranged from 15 μM to 1 mM and its effects on cell morphology were studied by observation under a light microscope (Flouview FV1000,
Olympus) and comparing the stressed cells with non-stressed cells as controls. The data collected showed that concentrations of sodium arsenite ranging from 125 μM to 1 mM were too high and lethal for the cells; this was observed at 12 h, 24 h, 36 h and 48 h of incubation under these conditions (data not shown). The results of treating cells with 15–62.5 μM sodium arsenite-supplemented media are shown in Figs 3.3–3.5, along with non-stressed cells (Fig. 3.6). The analysis suggested that treatment with 15 μM sodium arsenite has negligible effect on the cell viability and morphology compared to all the other concentrations; hence, 15 μM was chosen for the subsequent experiments.

![Figure 3.3](image)

Figure 3.3  Cells observed after treatment with 15 μM sodium arsenite for different time intervals
Panel A, 12 h after treatment; Panel B, 24 h after treatment; Panel C, 36 h after treatment; Panel D, 48 h after treatment. No significant changes in cell survival and morphology were observed. Scale bar, 10 μm (white bar).
Figure 3.4  Cells observed after treatment with 31.25 μM sodium arsenite at different time intervals
Panel A, 12 h after treatment; Panel B, 24 h after treatment; Panel C, 36 h after treatment; Panel D, 48 h after treatment. Cell survivability decreased with incubation time and there was also a change in the morphology of the cells. Scale bar, 10 μm (white bar).
Figure 3.5  Cells observed after treatment with 62.5 μM sodium arsenite at different time intervals
Panel A, 12 h after treatment; Panel B, 24 h after treatment; Panel C, 36 h after treatment; Panel D, 48 h after treatment. Cell survival decreased with incubation time. Scale bar, 10 μm (white bar).
Figure 3.6  Control cells observed at different time intervals
Panel A, 12 h after treatment, Panel B, 24 h after treatment, Panel C, 36 h after treatment; Panel D, 48 h after treatment. Scale bar, 10 µm (white bar).
3.3.4 Optimisation of the Incubation Time to Maximise the Number of Cells Responding to Stress

A previously determined optimal sodium arsenite concentration of 15 μM was used to determine the optimum time point required to achieve the maximum number of cells forming SGs in culture (see Section 3.3.3). These conditions were considered to be the most appropriate in which to study the changes in the gene expression that may be involved with SG activity. The experiment was performed using immunofluorescence microscopy. MCF7-wt cells were treated with 15 μM sodium arsenite for 12, 24, 36 and 48 h and stained with G3BP1 and a secondary antibody conjugated with Alexa Fluor 488. The optimum time for the maximum number of cells was 24 h after the stress treatment (Fig. 3.7B) to respond to the stress by forming SGs. After 24 h, cells started to recover from stress, as observed by a decrease in the SG count (Fig. 3.7C,D). Cells forming SGs were quantified via microscopy through five randomly chosen fields of view and this was represented in the form of a bar diagram (Fig. 3.8).

Figure 3.7 Stress granule (SG) formation at 15 μM sodium arsenite after 12, 24, 36 and 48 h after stress treatment
Panel A shows the formation of SGs 12 h after stress treatment: G3BP1 (green, right), DAPI (blue, centre) and merged (left). Panel B shows the formation of SGs 24 h after stress treatment, G3BP1 (green, right), DAPI (blue, centre) and merged (left). Panel C shows the
formation of SGs 36 h after stress treatment, G3BP1 (green, right), DAPI (blue, centre) and merged (left). Panel D shows the formation of SGs 48 h after stress treatment, G3BP1 (green, right), DAPI (blue, centre) and merged (left).

**Figure 3.8 Stress granule (SG) quantification**
The graph displays the percentage of cells that formed SGs at different time intervals after treatment with 15 μM sodium arsenite. The period 24 h after the stress treatment showed a statistically significant increase in the number of cells that formed SGs. The cells with SGs were counted in five different randomly selected views under the Flview FV1000 (Olympus) confocal microscope. Data are from three independent experiments. Student’s t-test was performed; *, P ≤ 0.05.

3.3.5 Cell Viability Assay
MCF7-wt cells were cultured and treated with different concentrations of sodium arsenite (15 μM–1 mM) for 12, 24, 36 and 48 h. The viability of cells was calculated using the trypan blue assay at each time interval. Concentrations of 1 mM–125 μM of sodium arsenite were too toxic for the cells to survive; hence, the data were collected for cell viability after treatment with 15 μM (Fig. 3.9), 31.25 μM (Fig. 3.10) and 62.5 μM (Fig. 3.11) sodium arsenite for 12–48 h.
Figure 3.9  Cell viability (%) after treatment of cells with 15 μM sodium arsenite
The graph shows the viability of cells at different time points after treatment with 15 μM sodium arsenite (12, 24, 36 and 48 h) compared with the control. There was no significant change in cell viability.

Figure 3.10  Cell viability (%) after treatment of cells with 31.25 μM sodium arsenite
The graph shows the viability of cells at different time points after treatment with 31.25 μM sodium arsenite (12, 24, 36 and 48 h) compared with the control. Cell viability was reduced with an increase in incubation time.
Figure 3.11  Cell viability (%) after treatment of cells with 62.5 μM sodium arsenite
The graph shows the viability of cells at different time points after treatment with 62.5 μM sodium arsenite (12, 24, 36 and 48 h) compared with the control. There was a significant decrease in cell viability with incubation time.

3.6. Discussion

Tourriere and co-workers (2003) found that cytoplasmic G3BP recruited to SGs when the cells were exposed to stress induced by sodium arsenite (Tourriere et al., 2003). SGs are translationally silent cytoplasmic granular bodies and are known as triage centres for mRNAs (Anderson and Kedersha, 2002; Kedersha and Anderson, 2002). Tourriere and co-workers associated G3BP’s recruitment to SG assembly with its role in deciding the fate of mRNA. Through this chapter, the localisation of G3BP1 to SGs was studied by immunofluorescence microscopy after inducing stress by using 1 mM sodium arsenite for 30 min. The data (Fig. 3.1A) suggest that the endogenous G3BP1 remained in the cytoplasm under non-stressed conditions, whereas after the induction of oxidative stress, G3BP1 was recruited to cytoplasmic granular bodies (Fig. 3.1B).

G3BP1, primarily a cytoplasmic protein which is over-expressed in several cancers, including breast cancer, has been found to be localised in the nucleus as well (Barnes et al., 2002, French et al., 2002), but in the immunofluorescence microscopy experiments performed in this chapter, G3BP1’s localisation in the nucleus was not observed, irrespective of the treatment conditions.
Kedersha et al. (1999) showed that when cultured cells were treated with sodium arsenite, RBPs such as TIA-1 or TIA-1-related (TIAR), were recruited to SGs. However, Tourriere and co-workers (2003) reported that sodium arsenite-induced SGs lack TIA-1 but have G3BP; therefore, an experiment was designed to study G3BP1’s co-localisation with TIA-1 in order to validate the previously reported information. The data (Fig. 3.2) show that after treating the cells with sodium arsenite, both G3BP1 and TIA-1 moved to SGs, and an overlap of both proteins was observed (Fig 3.2F, I), which is consistent with the work done by Kedersha et al. (2005).

In Fig. 3.2D–F, both G3BP1 and TIA-1 were found to exist and overlap in some small and many large SGs. This discrimination of the overlapping of G3BP1 with TIA-1 in large SGs has not been reported before. However, Nadezhdina and co-workers (2010) have reported about the role of microtubules in regulating SGs mobility and dynamics. They reported the diffusive movement of SGs and the co-localisation of SGs with microtubules. They also mentioned the merging and separation of SGs, which occur in a microtubule-dependent manner. Kolobova and co-workers reported the absence of large SG granules from nocodazole–arsenite-treated cells, and the presence of several small RNP-containing particles that could be classified as premature SGs (Kolobova et al., 2009). The reason for the enlargement of SG has not yet been clearly reported. However, due to their size, the larger SGs can recruit many regulatory proteins that can regulate the cells’ response to stress.

Half an hour of stress, which was used to study G3BP1’s localisation to SGs, was not long enough to measure G3BP1’s mRNA metabolic activity for its target transcripts. In order to study the effect of G3BP1 on the regulation of its target transcripts, a longer period of stress treatment was required, but at a non-lethal concentration, which should provide enough stress to the cells that they form SGs. Optimisation of the sodium arsenite concentration was performed from 15 μM to 1 mM, starting from 12 h after treatment to 48 h. Cells were observed under a light microscope (Floview FV1000, Olympus) after every 12 h of incubation to detect any morphological changes and also cell survival. This study found that 125 μM–1 mM concentrations of sodium arsenite were found to be lethal for the cells. However, at concentrations ranging from 62.5 μM to 31.25 μM, cells were able to survive. However, due to oxidative stress, their morphology was changed (Fig. 3.4 and 3.5) compared to the non-stressed controls (Fig. 3.6). Sodium arsenite at a concentration of 15 μM was
found to be the optimum concentration, as negligible change in the cell morphology (Fig. 3.3) was observed compared to the control (Fig. 3.6).

Finally, an experiment was designed to find the optimum time for the cells to respond to the oxidative stress induced by 15 μM sodium arsenite by the formation of SGs. The response to stress was studied by quantifying the number of cells that formed SGs at different time intervals after stress treatment. An optimum time period of 24 h of stress incubation was selected, as a significant increase in SGs formation was observed at this point (Fig. 3.7B), which could be sufficient to see any post-transcriptional regulation mediated by G3BP1. Recovery of cells from the stress was observed as a decrease in the number of cells forming SGs (Fig. 3.7CD); this data were presented as a bar diagram (Fig. 3.8).

Data from the cell viability assay suggested that 62.5 μM was too lethal for the cells to cope with the stress and there was a significant decrease in cell viability (Fig. 3.9). Fig. 3.10 suggests that a 31.25-μM concentration of sodium arsenite was also lethal for cells, as there was a significant decrease in cell numbers with incubation time and, as per Fig 3.4, there was a change in cell morphology as well. Fig. 3.11 showed no decrease in cell viability, which suggests that 15 μM of sodium arsenite had the least effect on cell survival and morphology compared to other two concentrations; therefore, 15 μM sodium arsenite was used for future experiments in this thesis.

The data from the experiments performed in this chapter suggest that G3BP1, which is a cytoplasmic protein, is recruited to cytoplasmic granular bodies upon induction of oxidative stress using sodium arsenite. In addition, G3BP1 co-localises with TIA-1, which is another SG marker protein, and confirms the recruitment of G3BP1 to SGs. From the other experiments in this chapter, it was found that 24 h after treatment with 15 μM sodium arsenite was the optimum concentration and incubation time for the cells to respond to stress and to study any post-transcriptional regulation mediated by G3BP1. Therefore, 24 h of treatment with 15 μM sodium arsenite was selected for further studies, which are discussed in the following chapters.
CHAPTER 4
ABSTRACT ARISING FROM CHAPTER 4


Student’s Contribution: Introduction of the hypothesis for exploring the potential of G3BP1 to be used as a potential therapeutic target was proposed by the student based on the literature review and the start of the PhD project in 2011. Furthermore, the idea of studying the role of G3BP1 in controlling the expression of NDUFB7, NDUFB10 and NDUFV1 under stress conditions was proposed by the student.


Student’s Contribution: In order to work on the hypothesis that G3BP1 regulates the expression of NDUFB7, the necessary experiments were designed and performed by the student. The experiments were culture of MCF7-wt breast cancer cells, treatment of cultured cells with sodium arsenite to induce oxidative stress, and harvesting and extraction of proteins to perform western blotting to study the expression of NDUFB7 under stress and unstressed conditions. The reporter assay was designed by the student, which included cloning of the 5′UTR, CDS and 3′UTR of NDUFB7 into the reporter vector and transient transfection and then performing the reporter assay. Data generated from the reporter assay were analysed by the student. The abstract submitted to the conference was written by the student.

PUBLICATION ARISING FROM CHAPTER 4


Student Contribution: In Chapter 4, the student designed the experiments, such as the reporter assay, by cloning the 5′UTR, CDS and 3′UTR of NDUFV1 into the reporter vector and transient transfection in the MCF7-wt cells, and then performing the reporter assay. The data generated from the experiments were analysed by the student and the manuscript was written by the student.

PhD student: Adnan Naim Date: 01/02/2015

Corresponding Author and Supervisor: Dr Derek Kennedy
Date: 01/02/2015
4. STUDY OF THE REGULATION OF NDUFB7, NDUFB10 AND NDUFV1 UNDER OXIDATIVE STRESS AT THE TRANSCRIPTIONAL AND TRANSLATIONAL LEVEL

4.1 Introduction

RBPs play a key role in controlling the expression of mRNAs through post-transcriptional gene regulation. In order to maintain cellular homeostasis during adverse environmental conditions, a cell redirects its cellular translation machinery. Reprogramming mRNA translation is a rapid and efficient mechanism to maintain various vital cellular activities during stress conditions (Liu and Qian, 2014; Yamasaki and Anderson, 2008). A global shutdown to translation, which is mediated through the phosphorylation of eIF2α is a common response to cellular stress, as it leads to inhibition of the translation at its very first step (i.e. initiation), hence regulating the expression of genes during stress (Sheikh and Forance, 1999; Baird and Wek, 2012; Proud, 2005). Several RBPs like TIA-1/TIAR and G3BPs are known to self-aggregate as a response to stress and move to cytoplasmic granular bodies, called SGs, which are known to contain stalled mRNP complexes and to triage mRNAs (Yamasaki and Anderson, 2008; Keene, 2007). RBPs interact with their target transcripts and form RNP complexes, and regulate their stability and translation during stress. This regulation is mediated through interactions with cis-acting elements, which reside in the 5’/3’UTR or CDS of the transcript, and control the expression of the transcript (Moore, 2005).

4.1.1 Regulation Mediated by the 5’UTR

The secondary structures of a transcript are also known to have a regulatory role in translating certain mRNAs, as these structures might serve as the binding sites for several regulatory proteins that could obstruct the scanning of the 40S subunit and hence translation (Araujo et al., 2012). For example erythroid 5-aminolevulinate synthase and ferritin mRNAs contain iron-responsive elements in their 5’UTR, which serve as binding sites for iron response proteins, which inhibit translation (Melefors et al., 1993).
4.1.2 Regulation Mediated by the 3′UTR

Generally, 3′UTR destabilising elements are characterised by the presence of AU-rich regions, which are also known as AREs, and may contain one or more AUUUA pentamers. The 3′UTR is known to contain several regulatory elements that may influence transcript stability and/or translation efficiency. For example, the 3′UTR of 15-lipoxygenase (LOX) mRNA contains a pyrimidine-rich domain that is the interaction site for RBPs; similarly, the 3′UTR of several human cytokines, such as tumour necrosis factor-α, interleukin-1β, interferon-gamma, are rich in AU, which serve as binding sites for RBPs and are known to inhibit translation (Day and Taite, 1998; Rodriguez-Pascual, et al., 2000).

4.1.3 Regulation Mediated by 5′–3′ Interactions

Intermolecular communication between the 5′UTR and the 3′UTR of a transcript also plays a regulatory role in its efficient translation. The 3′ poly(A) tail and the 5′ cap structure interact and form a closed loop that enhances translation initiation. This may be due to an interaction of eIF4G with eIF4E and PABP (Sachs and Buratowski, 1997).

G3BP1 is known to have endoribonuclease activity (Tourriere et al., 2003) and is also a component of SG assembly. This activity might be responsible for the role of G3BP1 in regulating the fate of its target mRNA. The stability of the target transcripts rely on certain specific cis-acting elements possessed by either the 5′UTR or the 3′UTR of an mRNA (Tourriere et al., 2001). Gallouzi et al. (1998) showed that the C-terminal RRM region of G3BP interacts with the target transcript and cleaves between CA dinucleotides. c-myc mRNA is known to interact with G3BP1 through its 3′UTR and its degradation by G3BP1 has been found to occur in a similar fashion (Tourriere et al., 2001).

The intent of this chapter was to study the role of G3BP1 in regulating the expression of all three candidate genes (NDUFB7, NDUFB10 and NDUFV1), both at the transcript level and at the translational level, during stress. As mentioned in the introduction, G3BP1 is known to regulate the expression of its target transcripts (c-myc, ctbnn1, β-F1-ATPase mRNA) by interacting with the 3′UTR; therefore, it was hypothesised that G3BP1 might interact with the 3′UTR of NDUFB7, NDUFB10 and NDUFV1 in a similar fashion, and regulate their translation. In order to explore the functional role of G3BP1 in regulating the translation of its target genes in vivo, a reporter assay was designed. Different regions of the target transcripts
were sub-cloned into a reporter vector and firefly *Luciferase* expression was studied by performing the reporter assay. Changes in the expression of *Luciferase* would suggest that the region cloned into the reporter construct is likely to be the target region of a particular transcript that interacts with G3BP1 and regulates its translation.

4.2 Materials and Methods

4.2.1 Cell Culture and Preliminary Work

Mammalian MCF7-wt breast cancer cells were used for the research described in this chapter. The cells were cultured as described in Chapter 2, Section 2.3.1. All cells were stressed for 24 h using 15 μM sodium arsenite as determined in Chapter 3 or as otherwise stated in the text. Western blotting was performed as described in Chapter 2, Section 2.2.4. Densitometry was performed as described in Chapter 2, Section 2.2.5, and qRT-PCR was performed as described in Chapter 2, Section 2.1.11.

The *in-vivo* functional role of G3BP1 was studied by performing *Luciferase* reporter assays. Different reporter constructs were generated for all three candidate genes, *NDUFB7*, *NDUFB10* and *NDUFV1*. The design of the reporter constructs is as described below and the design of the reporter assay is explained in Fig. 4.1 as a flowchart diagram.

4.2.2. Plasmid Construction

To identify the region of interaction between G3BP1 and the mRNA of the candidate target genes (*NDUFB7*, *NDUFB10* and *NDUFV1*), various reporter constructs were designed. To prepare constructs, harbouring the 5′UTR, CDS or 3′UTR of the three target mRNAs, specific primers were synthesised with additional XbaI cloning sites flanking the amplicons. All amplicons were amplified from MCF7-wt cDNA. The XbaI site downstream of the *Luciferase* gene in the pGL3 vector (E176A Promega) (see appendix V for a detailed vector map) was used to insert the amplified PCR products directly 3′ of the *Luciferase* translation stop codon but 5′ to the poly(A) site. The chimeric transcripts from these cloned constructs contained the *Luciferase* coding sequence and any one of the three target mRNA regions of interest, respectively, as shown in Fig 4.1. Changes in the expression level of the luciferase protein from these chimeric transcripts were attributed to the introduced *NDUFB7*, *NDUFB10* and *NDUFV1* regions, which might affect transcript stability and/or translation.
Constructs were amplified in the *E. coli* competent cell strain JM109, and plasmids from positive clones were purified, sequenced and used to transfect MCF7-wt cells. The generated reporter constructs were transiently transfected into MCF7-wt cells by following the method described in Chapter 2, Section 2.3.2 and stress was induced for 24 h, 4 h after transfection. Cells were harvested 24 h after stress treatment and the reporter assay was performed as described in Chapter 2, Section 2.5.
Figure 4.1 Flow chart of the reporter assay to study the functional role of G3BP1 in vivo

1. Primers were designed (blue arrows) to amplify the 5’UTR, CDS and 3’UTR of all the three candidate genes and digested with XbaI. 2. Digested PCR products or amplicons were cloned into the XbaI site (circled red colour) of the pGL3 reporter vector. 3. Chimeric Luciferase reporter constructs bearing specific sub-cloned regions of the three candidate genes were transiently transfected in MCF7-wt cells and were stressed using 15 μM sodium arsenite for 24 h. 4. Cells were harvested and reporter assays were performed. 4a. In the event
that there is no interaction between G3BP1 and any of the sub-cloned regions, the stressed sample will not show any difference in the expression of Luciferase compared to the non-stressed sample. 4b. In the event that G3BP1 interacted with any sub-cloned region of the transcripts and regulated the transcript stability or longevity of Luciferase mRNA, this will be detected by performing qRT-PCR using Luciferase primers. 4c. In the event that G3BP1 interacted with the sub-cloned regions of the transcript and regulated translation, a change (increase or decrease) in Luciferase expression would be observed. Luciferase expression has been represented in yellow (3 and 4). Luciferase mRNA stability has been represented by fragmentation of the Luciferase gene (4b) and a decrease in Luciferase expression has been represented by pale yellow (4c).

4.3 Results

4.3.1 Sodium Arsenite-Induced Stress Changed the Expression of Endogenous NDUFB7, NDUFB10 and NDUFV1

Endogenous protein expression of NDUFB7, NDUFB10 and NDUFV1 was studied by immunoblotting analysis using total cell lysate from MCF7-wt cells after stress was induced by 15 μM sodium arsenite for different time periods. Endogenous mRNA expression of NDUFB7, NDUFB10 and NDUFV1 under stress conditions was studied by performing qRT-PCR using specific primers (see Table 2.2 for primer details). All the data presented in the following figures are from a minimum of three independent experiments and a Student’s t-test was performed to determine if any changes in the mRNA expression and protein expression were significantly different.

Fig. 4.2A shows the immunoblot results for endogenous NDUFB7 expression under non-stressed and stressed conditions for the different time periods as described. The data from Chapter 3 suggested that 24 h of incubation after stress treatment was optimal and this was validated in these experiments; therefore, the densitometry assay was performed to quantify NDUFB7 protein expression (Fig. 4.2B) 24 h after stress treatment with 15 μM sodium arsenite. The immunoblot results also shows the expression of Actin, which was used as a loading control to normalise the expression of the target gene during the analysis.

Fig. 4.3 shows the endogenous expression of NDUFB7 mRNA after 24 h of stress induced by 15 μM sodium arsenite compared with the non-stressed sample.
Figure 4.2  Endogenous NDUF7 expression after 24 h of stress
Panel A shows the western blot results for the expression of NDUF7 under stress using 15 μM sodium arsenite and under non-stressed conditions from the whole-cell lysates of MCF7-wt cells. Panel B shows the densitometry data performed on the immunoblot results for the expression of endogenous NDUF7 after 24 h of stress compared to the non-stressed conditions. The data present three independent experiments. **, $P \leq 0.01$. 
Figure 4.3   Endogenous NDUFB7 mRNA expression after 24 h of stress

The graph represents the expression of endogenous NDUFB7 mRNA in the whole-cell lysates of MCF7-wt cells after 24 h of stress induced by sodium arsenite at a concentration of 15 μM compared with the non-stressed sample. The data present three independent experiments. *, \( P \leq 0.05 \).

Fig. 4.4A shows the immunoblot results for endogenous NDUFB10 expression under non-stressed and stressed conditions for the different time periods as described in the figure. Densitometry was performed to quantify NDUFB10 protein expression (Fig. 4.4B) 24 h after stress treatment with 15 μM sodium arsenite. The immunoblot results also show the expression of Actin, which was used as a loading control to normalise the expression of the target gene.

Fig. 4.5 shows the endogenous expression of NDUFB10 mRNA after 24 h of stress induced by 15 μM sodium arsenite compared with the non-stressed sample.
Figure 4.4  Endogenous *NDUFB10* expression after 24 h of stress
Panel A shows the western blot analysis for the expression of *NDUFB10* under stress using 15 μM sodium arsenite and under non-stressed conditions from the whole-cell lysates of MCF7-wt cells. Panel B shows the densitometry data performed on the immunoblot results for the expression of endogenous *NDUFB10* after 24 h of stress compared to non-stressed samples. The data present three independent experiments. No statistical difference was observed.
Figure 4.5  Endogenous *NDUFB10* mRNA expression after 24 h of stress

The graph represents the expression of endogenous *NDUFB10* mRNA in the whole-cell lysates of MCF7-wt cells after 24 h of stress induced by sodium arsenite at a concentration of 15 μM compared with the non-stressed sample. The data present three independent experiments. **, *P* ≤ 0.01.

Fig. 4.6A shows the immunoblot results for endogenous *NDUFV1* expression under stressed and non-stressed conditions for the different time periods as described in the figure. Densitometry was performed to quantify NDUFV1 protein expression (Fig. 4.6B) 24 h after stress treatment with 15 μM sodium arsenite. The immunoblot results also show the expression of Actin, which was used as a loading control to normalise the expression of the target gene.

Fig. 4.7 shows the endogenous expression of *NDUFV1* mRNA after 24 h of stress induced by 15 μM sodium arsenite compared with the non-stressed sample.
**Figure 4.6** Endogenous *NDUFV1* expression after 24 h of stress
Panel A shows the western blot results for the expression of *NDUFV1* under stress using 15 μM sodium arsenite and under non-stressed conditions from the whole-cell lysates of MCF7-wt cells. Panel B shows the densitometry data performed on the immunoblot results for the expression of endogenous *NDUFV1* after 24 h of stress compared to the non-stressed cells. The data presents three independent experiments. *, *P* ≤ 0.05.

**Figure 4.7** Endogenous *NDUFV1* mRNA expression after 24 h of stress
The graph represents the expression of endogenous *NDUFV1* mRNA in the whole-cell lysates of MCF7-wt cells after 24 h of stress induced by sodium arsenite at a concentration of
15 μM as compared with the non-stressed sample. The data present three independent experiments. **, $P \leq 0.01$.

### 4.3.2 Reporter Assay to Study the Functional Role of G3BP1 in Vivo

The 5'UTR, CDS and 3'UTR of all the three candidate genes (*NDUFB7*, *NDUFB10* and *NDUFV1*) were sub-cloned in the reporter vector (Appendix V). Constructs with specific cloned regions were transiently transfected and stress was induced using 15 μM sodium arsenite for 24 h; later, cells were harvested and the reporter assay was performed. Initially, *Renilla* luciferase was used as a control vector to normalise the firefly luminescence data. However, *Renilla* luciferase expression was sensitive to sodium arsenite and therefore total protein concentration was used to normalise the firefly luciferase expression values (Schagat et al., 2007). Figs 4.8–4.10 show the relative expression of firefly *Luciferase* for the constructs with the specific cloned regions of *NDUFB7*.

![Figure 4.8 NDUF7 5'UTR Luciferase reporter assay](image)

The graph represents the relative expression of firefly luciferase from the construct with the cloned 5'UTR of *NDUFB7* and the intact reporter vector under stress and non-stressed conditions. The data represent three independent experiments. There was no statistically significant difference in the expression of *Luciferase* in the cloned 5'UTR of *NDUFB7* under either condition.
Figure 4.9  **NDUFB7** coding region (CDS) **Luciferase** reporter assay
The graph represents the relative expression of firefly *Luciferase* for the construct with the cloned CDS of *NDUFB7* and the intact reporter vector under stress and non-stressed conditions. The data represent three independent experiments. There was no statistically significant difference in the expression of luciferase in the cloned CDS of **NDUFB7** under any condition.

Figure 4.10  **NDUFB7** 3′UTR **Luciferase** reporter assay
The graph represents the relative expression of firefly *Luciferase* for the construct with the cloned 3′UTR of **NDUFB7** and the intact reporter vector under stress and non-stressed conditions. The data represent three independent experiments. There was a statistically significant downregulation in the expression of *Luciferase* in the cloned 3′UTR of **NDUFB7** under stress conditions compared to the non-stressed conditions. *, $P \leq 0.05$.

Figs 4.11–4.13 show the relative expression of firefly luciferase for the constructs with the specific cloned regions of **NDUFB10**.
Figure 4.11  *NDUFB10* 5'UTR *Luciferase reporter assay*

The graph represents the relative expression of firefly luciferase for the construct with the cloned 5'UTR of *NDUFB10* and the intact reporter vector under stress and non-stressed conditions. There was no statistically significant difference in the expression of *Luciferase* in the cloned 5'UTR of *NDUFB10* under stressed and non-stressed conditions. The data present three independent experiments.

Figure 4.12  *NDUFB10* coding region (CDS) *Luciferase reporter assay*

The graph represents the relative expression of firefly luciferase for the construct with the cloned CDS of *NDUFB10* and the intact reporter vector under stress and non-stressed conditions. There was no statistical significant difference in the expression of luciferase for the construct with cloned CDS of *NDUFB10* under stress and non-stressed conditions. The data present three independent experiments.
Figure 4.13  NDUFB10 3'UTR Luciferase reporter assay
The graph represents the relative expression of firefly Luciferase for the construct with the cloned 3'UTR of NDUFB10 and the intact reporter vector under stress and non-stressed conditions. Statistically significant downregulation in the expression of luciferase was observed for the construct bearing the cloned 3'UTR of NDUFB10 under stress compared to non-stressed conditions. The data presents three independent experiments. *, P ≤ 0.05.

Figs 4.14–4.16 show the relative expression of firefly Luciferase for the constructs with the specific cloned regions of NDUFV1.

Figure 4.14  NDUFV1 5'UTR Luciferase reporter assay
The graph represents the relative expression of firefly Luciferase for the construct with the cloned 5'UTR of NDUFV1 and the intact reporter vector under stress and non-stressed conditions. There was no statistically significant difference in the expression of luciferase for the construct with the cloned 5'UTR of NDUFV1 under stressed and non-stressed conditions. The data present three independent experiments.
**Figure 4.15  NDUFV1 coding region (CDS) Luciferase reporter assay**
The graph represents the relative expression of firefly *Luciferase* for the construct with the cloned CDS of *NDUFV1* and the intact reporter vector under stress and non-stressed conditions. There was no statistically significant difference in the expression of *Luciferase* for the construct with the cloned CDS under stress and non-stressed conditions. The data present three independent experiments.

**Figure 4.16  NDUFV1 3'UTR Luciferase reporter assay**
The graph represents the relative expression of firefly *Luciferase* for the construct with the cloned 3'UTR of *NDUFV1* and the intact reporter vector under stress and non-stressed conditions. There was a statistically significant downregulation in the expression of *Luciferase* for the construct with the cloned 3'UTR of *NDUFV1* under stress conditions compared to the non-stressed conditions. The data present three independent experiments. **, \( P \leq 0.01 \).
4.3.3 Luciferase mRNA Stability in the Reporter Constructs of NDUFB7, NDUFB10 and NDUFV1 under Sodium Arsenite-Induced Stress

The stability of the chimeric Luciferase reporter transcripts was studied for each of the constructs that were used in the reporter assay (described in Section 4.2.5). The reporter constructs with the cloned 5′UTR, CDS and 3′UTR of NDUFB7, NDUFB10 and NDUFV1 were transiently transfected in MCF7-wt cells incubated in 15 μM sodium arsenite-supplemented media to induce stress for 24 h. Total RNA was extracted and qRT-PCR was performed as described in Section 2.1.11 using Luciferase primers (see Table 2.2 for the primer sequences). Figs 4.17–4.19 show the relative expression of Luciferase mRNA under stress and non-stressed conditions for the different constructs of NDUFB7, along with the intact pGL3 reporter vector as a control. Figs 4.21–4.22 show the relative expression of Luciferase mRNA under stress and non-stressed conditions for the different constructs of NDUFB10 along with the intact pGL3 reporter vector as a control. Figs 4.23–4.25 show the relative expression of Luciferase mRNA under stress and non-stressed conditions for the different constructs of NDUFV1 along with the intact pGL3 reporter vector as a control.

![Figure 4.17](image_url)

**Figure 4.17  Chimeric Luciferase–5′UTR NDUFB7 mRNA expression**
The graph shows the relative expression of Luciferase mRNA for the reporter construct bearing the 5′UTR of NDUFB7 under stressed and non-stressed conditions. There was no statistically significant difference in the expression of Luciferase mRNA under stress compared to the non-stressed conditions. The intact reporter vector was used as a control. Student’s t-test was performed. The data present three independent experiments.
Figure 4.18  Chimeric Luciferase–coding region (CDS) NDUFB7 mRNA expression
The graph shows the relative expression of Luciferase mRNA for the reporter construct bearing the CDS of NDUFB7 under stress and non-stressed conditions. There was no statistically significant difference in the expression of Luciferase mRNA under the stress conditions compared to non-stressed conditions. The intact reporter vector was used as a control. Student’s t-test was performed. The data present three independent experiments.

Figure 4.19  Chimeric Luciferase–3’UTR NDUFB7 mRNA expression
The graph shows the relative expression of Luciferase mRNA for the reporter construct bearing the 3’UTR of NDUFB7 under stress and non-stressed conditions. There was no statistically significant difference in the expression of Luciferase mRNA under stressed conditions compared to non-stressed conditions. The intact reporter vector was used as a control. Student’s t-test was performed. The data present three independent experiments.
Figure 4.20  Chimeric Luciferase–5′UTR NDUF10 mRNA expression
The graph shows the relative expression of Luciferase mRNA for the reporter construct bearing the 5′UTR of NDUF10 under stress and non-stressed conditions. There was no statistically significant difference in the expression of Luciferase mRNA under stressed conditions compared to non-stressed conditions. The intact reporter vector was used as a control. Student’s t-test was performed. The data present three independent experiments.

Figure 4.21  Chimeric Luciferase–Coding region (CDS) NDUF10 mRNA expression
The graph shows the relative expression of Luciferase mRNA for the reporter construct bearing the CDS of NDUF10 under stress and non-stressed conditions. There was no statistically significant difference in the expression of Luciferase mRNA under stress conditions compared to non-stressed conditions. The intact reporter vector was used as a control. Student’s t-test was performed. The data present three independent experiments.
Figure 4.22  Chimeric Luciferase–3'UTR NDUFB10 mRNA expression
The graph shows the relative expression of Luciferase mRNA for the reporter construct bearing the 3'UTR of NDUFB10 under stress and non-stressed conditions. There was no statistically significant difference in the expression of Luciferase mRNA under stressed conditions compared to the non-stressed conditions. The intact reporter vector was used as a control. Student’s t-test was performed. The data present three independent experiments.

Figure 4.23  Chimeric Luciferase–5'UTR NDUFV1 mRNA expression
The graph shows the relative expression of Luciferase mRNA for the reporter construct bearing the 5'UTR of NDUFV1 under stress and non-stressed conditions. There was no statistically significant difference in the expression of Luciferase mRNA under stress conditions compared to non-stressed conditions. The intact reporter vector was used as a control. Student’s t-test was performed. The data present three independent experiments.
Figure 4.24  Chimeric Luciferase–coding region (CDS) *NDUFV1* mRNA expression
The graph shows the relative expression of *Luciferase* mRNA for the reporter construct bearing the CDS of *NDUFV1* under stress and non-stressed conditions. There was no statistically significant difference in the expression of *Luciferase* mRNA under stress conditions compared to the non-stressed conditions. The intact reporter vector was used as a control. Student’s *t*-test was performed. The data present three independent experiments.

Figure 4.25  Chimeric Luciferase–3'UTR *NDUFV1* mRNA expression
The graph shows the relative expression of *Luciferase* mRNA for the reporter construct bearing the 3’UTR of *NDUFV1* under stress and non-stressed conditions. There was no statistically significant difference in the expression of *Luciferase* mRNA under stress conditions compared to non-stressed conditions. The intact reporter vector was used as a control. Student’s *t*-test was performed. The data present three independent experiments.
4.4 Discussion
As a consequence of adverse environmental conditions, a eukaryotic cell experiences a global shutdown of its translational machinery, and reprograms its cellular and metabolic activities with selective translation of mRNA transcripts which are essential for maintaining cellular homeostasis (Sheikh and Forance, 1999; Shenton et al., 2006; Liu and Qian, 2014). Because most cellular activities are mediated by proteins, translation of vital transcripts is regulated as a priority for overcoming cellular stress (Spriggs et al., 2010). One of the mechanisms involved in reprogramming mRNA translation is to transfer essential mRNA transcripts either for continuing translation or for storage via cytoplasmic granular bodies known as SGs, where they are stored temporarily until the environmental conditions return to normal or else they are transferred to processing bodies (P-bodies) for degradation in case the cell does not need that mRNA to be translated (Anderson and Kedersha, 2006; Keene, 2007; Moore, 2005). This chapter was designed to understand the regulation of the three candidate transcripts of G3BP1 (NDUFB7, NDUFB10 and NDUFV1) under oxidative stress conditions and also to explore G3BP1’s functional activity under stress conditions in vivo.

4.4.1 Oxidative Stress Caused by Sodium Arsenite Regulates the Expression of NDUFB7, NDUFB10 and NDUFV1
Table 4.1 summarises the data from the experiments described in Section 4.3.1, where endogenous expression, both at the transcriptional level and at the translational level of the three candidate genes (NDUFB7, NDUFB10 and NDUFV1) was studied under oxidative stress conditions induced by sodium arsenite. The data summarised in Table 4.1 suggest that with the induction of stress, MCF7-wt cells reprogram the gene expression machinery, where the expression of NDUFB7 and NDUFV1 was downregulated significantly both at the transcriptional level and at the translational level. The western blot data possibly show a moderate effect of stress on the translation of all three target proteins.

The endogenous NDUFB10 expression was found to be downregulated only at the transcriptional level and not at the translational level. This could be due to the better stability of the NDUFB10 protein compared to the other two proteins, NDUFB7 and NDUFV1. These findings were in agreement with the hypothesis that G3BP1 might regulate the expression of NDUFB7, NDUFB10 and NDUFV1 under stress conditions by recruiting them to SGs. In contrast, from the western blot results for NDUFB10 (Fig. 4.4), 36 h of treatment with
sodium arsenite show an enhanced protein band as compared to 24 h, which might be the recovery response of NDUF10 from the stress treatment.

Table 4.1 Changes in the expression of NDUF7, NDUF10 and NDUF1 under oxidative stress conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>Oxidative Stress Induced by Sodium Arsenite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target gene</td>
<td>Endogenous mRNA expression</td>
</tr>
<tr>
<td>NDUF7</td>
<td>Downregulated</td>
</tr>
<tr>
<td>NDUF10</td>
<td>Downregulated</td>
</tr>
<tr>
<td>NDUF1</td>
<td>Downregulated</td>
</tr>
</tbody>
</table>

The downregulation in the expression of endogenous NDUF7, NDUF10 and NDUF1 at the transcriptional level under stress conditions could be due to G3BP1’s endoribonuclease activity, which has been found to regulate the stability of several transcripts such as c-myc (Gallouzi et al., 1998), bart (Taniuchi et al., 2011), cttnb1 (Bikkavilli and Malbon, 2012), β-F1-ATPase (Ortega et al., 2010), IGF-II, GAS5 (Zekri et al., 2005), TAU (Atlas et al., 2004, 2007) and PMP22 (Winslow et al., 2013). Another possibility for the downregulation of the endogenous mRNA of these candidate genes could be their recruitment to cytoplasmic P-bodies, which are considered to regulate mRNA degradation (Parker and Sheth, 2007). P-bodies are also known to form as a consequence of stress and they have been found to exist in close vicinity to SGs. Both SGs and P-bodies share several components, although some components are unique to SGs and P-bodies respectively (Kedersha and Anderson, 2007; Buchan and Parker, 2009; Decker and Parker, 2012). P-bodies are considered to regulate the expression and stability of a mRNA transcript, as they are known to contain all the necessary components that are involved in the degradation of a transcript (Decker and Parker, 2012).

Table 4.2 summarises the data from the experiments presented in Sections 4.3.3 and 4.3.2, where changes in the expression of Luciferase at the transcript level and also at the translational level for the different reporter constructs bearing the 5’UTR, CDS and 3’UTR of NDUF7, NDUF10 and NDUF1 under the stress conditions was studied.
Table 4.2  Summary of the reporter assay and Luciferase mRNA stability assay performed to study the functional role of G3BP1 in vivo for the three candidate genes NDUFB7, NDUFB10 and NDUFV1 under oxidative stress conditions

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Reporter Construct Bearing the 5′UTR of the Target Transcript</th>
<th>Reporter Construct Bearing the Coding Region of the Target Transcript</th>
<th>Reporter Construct Bearing the 3′UTR of the Target Transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Luciferase mRNA expression</td>
<td>Luciferase Protein expression</td>
<td>Luciferase mRNA expression</td>
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<td></td>
<td>Luciferase mRNA expression</td>
<td>Luciferase Protein expression</td>
<td>Luciferase Protein expression</td>
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<tr>
<td></td>
<td>Luciferase mRNA expression</td>
<td>Luciferase Protein expression</td>
<td>Luciferase mRNA expression</td>
</tr>
<tr>
<td>NDUFB7</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>NDUFB10</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>NDUFV1</td>
<td>No change</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

4.4.2 The 3′UTR of NDUFB7, NDUFB10 and NDUFV1 might Contain Target Cis-Regions where G3BP1 Binds to Regulate Translation

Post-transcriptional gene regulation is mediated through the interactions of trans-factors like RBPs, with the cis-elements contained in their target transcripts. Ortega et al. (2010) reported that G3BP1 interacts with the β-F1-ATPase mRNA and regulates its translation. They performed RNA-bridged trimolecular fluorescence complementation assays to confirm the interaction of G3BP1 with the 3′UTR of β-F1-ATPase mRNA. The experiments described by Ortega et al. (2010) were repeated during the research performed for this thesis but neither the data for the 3′UTR of β-F1-ATPase mRNA, the control experiments nor any of the target genes described here could be replicated in a fashion similar to the data presented by Ortega et al. (2010). However, based on the available information, a hypothesis was generated that G3BP1 might interact with the 3′UTR of all the three candidate genes (NDUFB7, NDUFB10 and NDUFV1) and may recruit them to SGs under stress conditions, where it might regulate their translation or stability. The functional role of G3BP1 in vivo for its three candidate transcripts was studied by designing a reporter assay. An explanation of the concept of reporter assays has been provided in a flowchart (Fig. 4.1). The experimental model suggests that if there was an interaction between G3BP1 and a particular region of a transcript, then the expression of Luciferase will be regulated for that particular clone under stress conditions and if there was no interaction, the expression would remain unaltered irrespective of the treatment condition.

The summarised data from the reporter assay (Table 4.2) suggest that the clone bearing the 5′UTR and CDS of all the three candidate transcripts does not show any significant change in
the expression of Luciferase. However, the clone bearing the 3’UTR of all three candidate transcripts showed a significant decrease in the expression of Luciferase.

4.4.3 Luciferase mRNA Stability Remained Unchanged Irrespective of the Treatment Condition

G3BP1 has been found to be involved in the post-transcriptional regulation of several transcripts such as c-myc (Gallouzi et al., 1998), bart (Taniuchi et al., 2011), ctnnb1 (Bikkavilli and Malbon, 2012), β-F1-ATPase (Ortega et al., 2010), PMP22 (Winslow et al., 2013), IGF-II, GAS5 (Zekri et al., 2005) and TAU (Atlas et al., 2004). The initial data investigating the stability of endogenous NDUFB7, NDUFB10 and NDUF7 suggested that these transcripts might also be downregulated under conditions that caused the formation of SG (Figs 4.4–4.7). However, the downregulation of endogenous transcripts could also be under the control of several endogenous pathways, including transcriptional regulation. In order to study the effect of G3BP1 on the stability of a target transcript, Luciferase mRNA stability was measured from the same reporter constructs which bear the 5’UTR, CDS and the 3’UTR of NDUFB7, NDUFB10 and NDUFV1. No significant difference was observed in the expression of Luciferase mRNA, irrespective of the treatment conditions in any of the reporter constructs. The data from the Luciferase mRNA stability assay suggest that, despite the fact that G3BP1 might interact with the 3’UTR of NDUFB7, NDUFB10 and NDUFV1, and downregulate the expression of luciferase protein under stress conditions, it does not affect the stability or longevity of Luciferase mRNA under stress conditions. This finding is consistent with the structure of G3BP1, which does not contain any recognised or documented endoribonuclease domain that could be held responsible for regulating the stability of its target transcripts.

In conclusion, the induction of stress in the MCF7-wt cells reprogrammed the transcription and translation of NDUFB7 and NDUFV1, whereas NDUFB10’s transcript expression was downregulated but its protein expression remained unchanged (see Table 4.1). This outcome suggests that NDUFB10 protein might be a stable protein with a half-life that is longer than the experimental parameters used here. From the reporter assays, it appears that the 3’UTR of NDUFB7, NDUFB10 and NDUFV1 might be the target region for G3BP1 interactions. This was demonstrated by the regulation of Luciferase expression (Table 4.2). The data suggest that regulation of the expression of the chimeric Luciferase reporters is regulated at the level
of protein expression and not at the level of mRNA stability, because induced stress did not influence the stability of the chimeric *Luciferase* mRNA.

It is important to understand that all these experiments were performed in a whole-cell system, where other cellular proteins might be regulating the activity of G3BP1. Therefore, it is important to examine whether G3BP1 has a direct and independent role in regulating the expression of its target genes or whether this is a cumulative effect caused by some of its partners in the complex or by other unidentified mechanisms. These issues are explored in the next set of experiments in the following chapter, where G3BP1 knock-down studies were performed using the target gene (*G3BP1*) and non-targeting or scrambled siRNAs.
CHAPTER 5
ABSTRACT ARISING FROM CHAPTER 5


Student Contribution: The main concept of this abstract was proposed by the student from the outcome of the experimental work of this thesis performed in Chapter 5. The idea presented in the Abstract, namely that G3BP1 recruits to the cytoplasmic granular bodies called SGs under oxidative stress, which could modulate orphan RNA, was the main outcome of Chapter 5 of the thesis.

PUBLICATION ARISING FROM CHAPTER 5


Student Contribution: The independent role of G3BP1 in regulating the expression of NDUFV1 was studied in Chapter 5 of the PhD thesis. A G3BP1 knock-down experiment was performed and data generated from the experiments were analysed by the student. The manuscript was written by the student.

PhD student: Adnan Naim Date: 01/02/2015

Corresponding Author and Supervisor: Dr Derek Kennedy Date: 01/02/2015
5. THE INDEPENDENT ROLE OF G3BP1 IN REGULATING THE TRANSCRIPT STABILITY AND TRANSLATION OF NDUFB7, NDUFB10 AND NDUFV1

5.1. Introduction

Post-transcriptional gene regulation is considered to be a rapid mechanism for a cell to control the cellular proteome under adverse environmental conditions (Thomas and Lieberman, 2013). This can include induced stresses, such as those mentioned in previous chapters. However, adverse conditions also include changes in the cellular microenvironment, which includes the stresses associated with cell transformation during cancer progression (Wurth, 2012). RBPs proteins are considered to play a fundamental role in post-transcriptional gene regulation, as they have been found to regulate mRNA metabolism including, translation, stability and longevity during stress conditions. The regulation of a transcript’s expression is generally mediated through the interaction of trans-factors (e.g., RBPs) to the cis-elements, which are generally found at the 5'UTR or 3'UTR (Wilkie et al., 2003) and even sometimes in the CDS of a transcript (Lee and Gorospe, 2011).

Alterations in the stability of an mRNA transcript could regulate the expression of a gene rapidly and efficiently. Several RBPs, such as HuR, TIA-1/TIAR, hnRNP and tristetraprolin, are involved in regulating the translation and turnover of a transcript and therefore, they are also known as turnover and translation regulator RBPs (Kim et al., 2013; Abdelmohsen, 2012; Abdelmohsen and Gorospe, 2012; Abdelmohsen and Gorospe, 2010; Abdelmohsen et al., 2008; Pullmann et al., 2007; Aroca et al., 2011). Hu-proteins, which are ubiquitously expressed, are found to increase the stability of their target transcript, thus enhancing its translation. Hu-proteins are known to bind at the AREs, which typically reside at the 3'UTR of its target mRNA and regulate its expression (Brennan and Steitz, 2000; Abdelmohsen and Gorospe, 2010). In contrast, tristetraprolin, which is also found to interact with AREs in its target transcripts, regulates message expression by enhancing the degradation of the transcript. Similarly, TIA-1 or TIAR is found to interact at the AU-rich regions in a target transcript and downregulate their translation. As a consequence of cellular stress, these proteins are known to suppress translation of their target transcripts by recruiting an mRNP to
SGs (Kedersha and Anderson, 2002), which are known as temporary detention stations for mRNP cargo before it translates the message.

The presence of RRM and RGG domains in G3BP1’s structure suggests that G3BP1 is an RBP (Irvine et al., 2004). Furthermore, it has been shown that G3BP1 is a component of SG assembly (Tourriere et al., 2003), which suggests that it has a role in regulating the expression of its target transcripts under different environmental conditions, similar to the abovementioned example of TIA-1/TIAR. However, it is unlikely that the activity of G3BP1 is facilitated by binding to an ARE, since its reported recognition element is not an ARE (Gallouzi et al., 1998). Recent work done by Winslow et al. (2013) suggest that G3BP1 regulates the expression of PMP22, through which it controls cellular proliferation. In addition, G3BP1 has also been found to regulate several other transcripts such as c-myc, bart, ctnnb1 and β-F1-ATPase, where it has been found to interact at the 3′UTR of the transcript (Gallouzi et al., 1998; Tourriere et al., 2003; Bikkavilli and Malbon, 2011; Ortega et al., 2010). G3BP1 has been found to downregulate the expression of all its target transcripts except for TAU mRNA (Atlas et al., 2004, 2007).

It has been suggested that G3BP has RNase activity, as it is found to cleave the 3′UTR of mouse c-myc mRNA in vitro (Gallouzi et al., 1998); however, its endoribonuclease activity depends on the phosphorylation of serine 149 (Tourriere et al., 2001). This characteristic of G3BP connects the Ras signalling pathway to the turnover of its target mRNA. Supporting evidence for this role was provided by Lypowy (2005) who also showed that G3BP1 regulates several transcripts in a RasGAP-dependent manner. In his research work, Lypowy observed the release of cdk7 and cdk9 mRNA from G3BP after knock-down of RasGAP, and subsequently found a decrease and a significant increase in the translation of Cdk7 and Cdk9 transcripts respectively. However, the work done by Annibaldi et al. (2011) suggested that there is no interaction between G3BP1 and the SH3 domain of RasGAP, which still leaves some ambiguity regarding the function of G3BP1 in regulating the expression of its target transcripts.

Recent work by Matsuki et al. (2013) has reported that G3BP1 and G3BP2 both contribute to the formation of SGs and this adds to the evidence that G3BP1 might regulate the expression of its target transcript under stress conditions. However, Matsuki and co-workers also reported that G3BP2 was found to exist as a hetero-multimer in association with G3BP1, which indicates that G3BP1 might interact with other cellular proteins under different
environmental conditions that could regulate its activity and hence the regulation of a mRNA in an mRNP. Once again, research has suggested that G3BPs have conflicting roles in different cell lines and it is possible that its binding partners may regulate its activity. Therefore, there is a need to understand the cellular role of G3BP. One way to address this could be through the knock-down of G3BP’s expression.

The data from the previous chapter suggest that the 3’UTR of NDUFB7, NDUFB10 and NDUFV1 could be the regions of interaction where G3BP1 binds and further regulates their expression under stress. However, as those experiments were performed in a whole-cell system, it is therefore difficult to conclude that G3BP1 alone regulates the expression of the three candidate genes. It is possible that some other cellular proteins or stress-activated proteins regulated the expression of the endogenous candidate genes in co-ordination with G3BP1; a candidate for this might include G3BP2. Therefore, this chapter was designed to study the direct and independent role of G3BP1 in regulating the expression of NDUFB7, NDUFB10 and NDUFV1 by performing G3BP1 knock-down studies.

5.2 Materials and Methods

5.2.1 Cell Culture and Preliminary Work

Mammalian MCF7-wt breast cancer cells were used for the research described in this chapter. The cells were cultured as described in Chapter 2, Section 2.3.1. As described in Chapter 2, Section 2.3.2, MCF7-wt cells were transiently transfected with siRNAs for G3BP1 with scrambled siRNAs as a control (see Table 2.3 for siRNA details). Immunoblotting for G3BP1, NDUFB7, NDUFB10 and NDUFV1 proteins was performed as described in Chapter 2, Section 2.2.4., and qRT-PCR was performed to study the endogenous expression of G3BP1, NDUFB7, NDUFB10 and NDUFV1 mRNA as described in Chapter 2, Section 2.2.5.

5.3 Results

5.3.1 Endogenous Protein Expression of NDUFB7, NDUFB10 and NDUFV1 after G3BP1 Knock-down

The expression of all the three candidate genes (identified by the host laboratory as described in the introduction and initially characterised in Chapter 4) was studied after knock-down of G3BP1 in the MCF7-wt cells by using the siRNA GENOME SMART pool for G3BP1 (see
Chapter 2, Table 2.3). Knock-down of G3BP1 was confirmed at the transcript level by performing qRT-PCR (Fig. 5.1) using primers for G3BP1 (see Table 2.2). Knock-down of G3BP1 protein was assessed by western blot using anti-G3BP1 antibody (see Fig. 5.2A), on which densitometry was performed (Fig. 5.2B). After 72 h of treatment with siRNAs, the cells were harvested, total cellular extracts were prepared and immunoblot studies were performed using antibodies against NDUFB7, NDUFB10 and NDUFV1. The experiments were performed three times and the data shown in all the figures (Figs 5.3–5.5) are representative of all the experiments.

Out of the three target genes, only the expression of NDUFV1 was found to be significantly downregulated (Fig. 5.5) at the translational level after knock-down of G3BP1 and the remaining two genes, NDUFB7 and NDUFB10, did not show any change in their expression (Fig. 5.3 and Fig. 5.4).

![Figure 5.1](image)

**Figure 5.1** qRT-PCR to confirm the knock-down of G3BP1 after 72 h of treatment with siRNAs

The graph shows the significant downregulation in the expression of G3BP1 mRNA after 72 h of treatment with the siRNA for G3BP1 compared with the scrambled siRNAs. Actin was used as a housekeeping gene. n = 3; *, P ≤ 0.5.
Figure 5.2  Western blot analysis to confirm the knock-down of G3BP1 protein
Panel A shows the immunoblot results for the expression of G3BP1 after 72 h of treatment with G3BP1 siRNAs compared with the scrambled siRNAs as a control. Actin was used as a loading control. Panel B shows the relative expression of G3BP1 as a bar graph after densitometry analysis of the immunoblot results. Statistically significant downregulation in the expression of G3BP1 protein was confirmed. n = 3; *, P ≤ 0.5.
Figure 5.3  Relative expression of NDUFB7 protein after knock-down of G3BP1
Panel A shows the immunoblot results of the endogenous expression of NDUFB7 after knock-down of G3BP1. Panel B shows a bar graph of the densitometry performed on the immunoblot results to evaluate the expression levels of NDUFB7. There was no statistically significant difference between the relative expression of NDUFB7 protein after knock-down of G3BP1 and the control cells. $n = 3$. 

![Immunoblot and bar graph showing relative expression of NDUFB7](image)

**Figure 5.3** Relative expression of NDUFB7 protein after knock-down of G3BP1
Panel A shows the immunoblot results of the endogenous expression of NDUFB7 after knock-down of G3BP1. Panel B shows a bar graph of the densitometry performed on the immunoblot results to evaluate the expression levels of NDUFB7. There was no statistically significant difference between the relative expression of NDUFB7 protein after knock-down of G3BP1 and the control cells. $n = 3$. 

![Immunoblot and bar graph showing relative expression of NDUFB7](image)
Figure 5.4 Relative expression of NDUFB10 protein after knock-down of G3BP1
Panel A shows the immunoblot results of the endogenous expression of NDUFB10 after knock-down of G3BP1. Panel B shows a bar graph of the densitometry performed on the immunoblot results to evaluate the expression levels. There was no statistically significant difference between the relative expression of NDUFB10 protein after knock-down of G3BP1 and control cells. \( n = 3 \).
5.3.2 Endogenous Expression of NDUFB7, NDUFB10 and NDUFV1 mRNA after Knock-down of G3BP1

After treating the MCF7-wt cells with siRNAs for G3BP1 and scrambled siRNA for 72 h, total RNA was isolated and mRNA expression was studied by performing qRT-PCR as described in Chapter 2, Section 2.2.5 using specific primers for NDUFB7, NDUFB10 and NDUFV1 (see Table 2.2 for the primer sequences). Actin was used as a housekeeping gene for normalisation. Figs 5.6–5.8 show graphs representing the relative endogenous expression of NDUFB7, NDUFB10 and NDUFV1 mRNA after knock-down of G3BP1 compared with the control.
Figure 5.6  Relative expression of *NDUFB7* mRNA after knock-down of *G3BP1*
qRT-PCR was performed in order to study the expression of *NDUFB7* mRNA. There was no statistically significant difference in the endogenous expression of *NDUFB7* mRNA from *G3BP1* knock-down cells compared with the control. *n* = 3; Student’s *t*-test was performed.

Figure 5.7  Relative expression of *NDUFB10* mRNA after knock-down of *G3BP1*
qRT-PCR was performed in order to study the endogenous expression of *NDUFB10* mRNA. There was no statistically significant difference in the endogenous expression of *NDUFB10* mRNA from *G3BP1* knock-down cells compared with the control. *n* = 3; Student’s *t*-test was performed.
Figure 5.8  Relative expression of \textit{NDUFV1} mRNA after knock-down of \textit{G3BP1}

qRT-PCR was performed in order to study the endogenous expression of \textit{NDUFV1} mRNA. There was no statistically significant difference in the endogenous expression of \textit{NDUFV1} mRNA from \textit{G3BP1} knock-down cells compared with the control. $n = 3$; Student’s $t$-test was performed.

5.4 Discussion

G3BPs are a family of RBPs and it has been reported that they have a role in regulating the stability or longevity of some of their putative target transcripts. G3BP1 is known to regulate the stability of \textit{c-myc}, \textit{bart} and \textit{ctnnb1}, and also it regulates the expression of \textit{β-F1-ATPase} by interacting with the 3'UTR (Tourriere et al., 2001). Recently, G3BP1 has been found to regulate cellular proliferation in breast cancer cells through regulating the expression of \textit{PMP22} mRNA (Winslow et al., 2013). This chapter was designed to study the potential role of G3BP1 in regulating the expression of \textit{NDUFB7}, \textit{NDUFB10} and \textit{NDUFV1}. Therefore, it was hypothesised that G3BP1 might regulate the transcript stability or translation of \textit{NDUFB7}, \textit{NDUFB10} and \textit{NDUFV1}.

5.4.1 G3BP1 Significantly Downregulates the Expression of Endogenous NDUFV1 at the Translational Level, but not NDUFB7 or NDUFB10

In Chapter 4, significant downregulation in the expression of \textit{NDUFV1} at the translational level was observed after sodium arsenite-induced stress. The observed regulation could have been the cumulative effect of several pathways or as a result of other proteins within mRNPs, including G3BP1. The \textit{G3BP1} knock-down experiment was designed to study the role of G3BP1 in regulating the expression of \textit{NDUFV1}. Surprisingly, the data from this chapter (see
Fig. 5.5) suggest that \textit{NDUFV1} is downregulated when \textit{G3BP1} is knocked down, which could be because \textit{G3BP1} regulates \textit{NDUFV1} expression, independent of a stress response pathway, or because in the absence of \textit{G3BP1}, the mRNP does not reach the destined place where it’s translation should take place.

No significant changes in the protein expression of \textit{NDUFB7} (Fig. 5.3) and \textit{NDUFB10} (Fig. 5.4) were observed after knock-down of \textit{G3BP1}, which suggests that \textit{G3BP1} does not regulate the translation or stability of these proteins under normal environmental conditions, and may need some other co-factors to regulate their expression during stress conditions, as was observed in Chapter 4.

\textbf{5.4.2 \textit{G3BP1 does not Regulate the Stability of the Endogenous mRNA Levels of NDUFB7, NDUFB10 and NDUFV1}}

Figs 5.6–5.8 show the data from the experiments where \textit{G3BP1} expression was knocked down using siRNAs to study its downstream effects on the expression of all the three target transcripts (\textit{NDUFB7, NDUFB10} and \textit{NDUFV1}). No significant differences in the expression of endogenous mRNA of \textit{NDUFB7, NDUFB10} and \textit{NDUFV1} were observed after knock-down of \textit{G3BP1} compared to the control. This suggests that \textit{G3BP1} does not regulate the stability or longevity of any of the three transcripts of the candidate genes under normal conditions.

In conclusion, the findings of this chapter suggest that \textit{G3BP1} does not regulate the transcript stability of any of the three candidate genes under favourable environmental conditions. In addition, \textit{G3BP1} was not found to have a role in regulating the stability of \textit{NDUFB7} and \textit{NDUFB10} proteins, whereas it does cause downregulation of \textit{NDUFV1}’s expression after knock-down. Recent findings by Santidrian et al. (2013) suggested that knock-down of \textit{NDUFV1} downregulates the activity of Complex I by 92\% and the work done by Ortega and co-workers (2010) suggests that \textit{G3BP1} is involved in cancer cell bioenergetics.

Taken together, the data from the host laboratory RIP-Chip experiment suggests that \textit{G3BP1} targets some of the Complex I transcripts. The findings of this chapter set a platform for designing the next chapter under the hypothesis that \textit{G3BP1} might play a potential role in regulating the energy shift in MCF7 cancer cells, which may be regulated through Complex I activity.
CHAPTER 6
PUBLICATION ARISING FROM CHAPTER 6


Student Contribution: Part of the manuscript includes the outcome of experimental work performed in Chapter 6. The experiments were the Complex I assay and the ATP assay. The experiments and the data generated from the experiments of Chapter 6 were performed by the student.

PhD student: Adnan Naim Date: 01/02/2015

Corresponding Author and Supervisor: Dr Derek Kennedy Date: 01/02/2015
6. THE ROLE OF G3BP1 IN MITOCHONDRIAL COMPLEX I AND ENERGY REGULATION

6.1 Introduction

6.1.1 Mitochondrial Dysfunction in Tumour Cells

Mitochondria are primarily responsible for the synthesis of ATP through OXPHOS to meet the energy demands of the cells. They are also known to have an important role in apoptosis or programmed cell death (Zamzami et al., 1996). Mitochondria in cancer cells are different in morphology and function from normal cells, including differences in metabolism, molecular composition and mitochondrial DNA composition (Carew and Huang, 2002). Enhanced production of NAD\(^+\), NADH and nicotinamide adenine dinucleotide phosphate (reduced) has been found in tumour cells, which could alter the metabolic activities of a cell (Santidrian et al., 2013). This could lead to mitochondrial dysfunction, which, as an outcome, could inhibit OXPHOS and cause a shift towards glycolysis to generate ATP to meet the excessive energy demands of the uncontrolled proliferation seen in some cancer cells. A recent study by Santidriam et al. (2013) suggested that changes in NAD\(^+\)/NADH levels, which are controlled by mitochondrial Complex I activity, can regulate the invasiveness of mammalian breast cancer cells.

(mtDNA mutations in genes encoding Complex I subunits are found in malignancies, including breast cancer. Mutations in the nuclear genes encoding mitochondrial proteins that are involved in OXPHOS are considered to be a cause of mitochondrial dysfunction in cancer cells (Ohta, 2006). This suggests that defects in OXPHOS might contribute to tumorigenesis.

A variety of tumour cells are found to have an altered bioenergetics mechanism, as they shift towards the glycolytic pathway for energy production rather than using OXPHOS, even when oxygen concentrations are not limiting. This phenomenon was first discovered by Otto Warburg and is also known as the “Warburg Effect” in honour of how he was the first to correlate mitochondrial dysfunction with cancer progression (Warburg et al., 1927).
Several other metabolic alterations have been found in cancer cells compared to normal cells, for example, enhanced gluconeogenesis (Moreira et al., 2013), an increased rate of lactic acid generation and decreased rates of pyruvate oxidation (Chatterjee et al., 2006). All these features support the growth and invasiveness of cancer cells.

The specific reasons for this metabolic drift in cancer cells are still not clear. However, several mechanisms, including mitochondrial genetic regulation and overexpression of the enzymes involved in glycolysis, have been proposed (Moreno-Sanchez et al., 2007, 2009), some of which have been summarised in Table 6.1.

**Table 6.1 Several proposed mechanisms for enhanced glycolytic shift in the cancer cells.**

<table>
<thead>
<tr>
<th>Molecular mechanism involved in the enhanced glycolysis of human fast-growing tumors</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Overexpression of selected HIF-1α glycolytic argnet enzymes and glucose transporters</td>
<td></td>
</tr>
<tr>
<td>GLUT1</td>
<td>Bone (HOS), brain (HS683, H4, A-172), breast (MDA-MB-231, MDA-MB-435, MCF-7, T47D), Cervical (HeLa), choriocarcinoma (BeWo, JEG-3, Jar), colorectal (Caco-2, WiDr), epidermoid (A431), pancreatic, renal (786-0), skin (HTB 63), squamous (UM-SCC 22A, SCC-25) carcinomas</td>
</tr>
<tr>
<td>GLUT3</td>
<td>Breast (MDA-MB-231, T47D, ZR-75), choriocarcinoma (BeWo, JEG-3, Jar), Ischikawa endometrial cells, thyroid (papillary, follicular); lung, ovarian, and gastric carcinomas (biopsies)</td>
</tr>
<tr>
<td>HKI</td>
<td>Colorectal (SW480, SW620) carcinoma</td>
</tr>
<tr>
<td>HKII</td>
<td>Ependymoma, astrocytoma, glioma; pancreatic and cholangiocellular carcinoma (biopsies); cervical (HeLa), hepatocellular (HepG2) and alveolar (A549) carcinomas; stomach (AGS), and epidermoid (A431) cancers</td>
</tr>
<tr>
<td>PFK-L</td>
<td>HL-60, KG-1, K-562 myeloid leukaemia, MOLT-4 leukaemia, lymphoma, HeLa and KB carcinoma, glioma</td>
</tr>
<tr>
<td>ALD-A and C, PGK-1, PGAM-B, ENO-α, LDHA</td>
<td>Colorectal (SW480, SW620) carcinoma, hepatocellular carcinoma, squamous cell lung and pancreatic carcinomas and lung adenocarcinomas (biopsies)</td>
</tr>
<tr>
<td>2. Low number of mitochondria or mitochondrial DNA content per cell</td>
<td>Ovarian cancer, breast cancer, renal cell carcinoma</td>
</tr>
<tr>
<td>3. Low expression and activity of OXPHOS enzymes and transporters</td>
<td></td>
</tr>
</tbody>
</table>
Table reproduced with permission from Moreno-Sanchez et al. (2009).

### 6.1.2 G3BP1 and its Mitochondrial Targets

G3BP1 is known to interact with β-F1-ATPase mRNA and to regulate its expression (Ortega et al., 2010). β-F1-ATPase is a mitochondrial Complex V subunit, which is involved in the synthesis of ATP molecules, via OXPHOS. Downregulation of β-F1-ATPase expression is considered to be hallmark of several human cancers, and this characteristic might be connected with the metabolic shift of enhanced glycolysis, which is observed in many cancer cells (Willers et al., 2010). G3BP1 interacts with the catalytic subunit of the mitochondrial H^+ -ATP synthase (β-F1-ATPase mRNA), suggesting its role in the energy generation pathway (Ortega et al., 2010).

G3BP1 is also a constituent of SG assembly, and its interaction with the target transcripts might regulate the fate of mRNAs under different environmental conditions that could regulate the activity of a particular cellular component. The dataset generated from the RIP-Chip assay (see Chapter 1) suggests that several mitochondrial genes are targets for G3BP1, which could imply that G3BP1 has a regulatory effect for mitochondrial genes and that this might influence the energy metabolism of cancer cells. This chapter was conceptualised around the findings by Ortega et al. (2010), where it was reported that G3BP1 regulates the expression of β-F1-ATPase mRNA. Ortega and co-workers also suggested that in the regulation of β-F1-ATPase expression, G3BP1 might act as a master regulator in the energy shift that is observed in several cancers. Therefore, in this chapter, experiments were designed to study whether G3BP1 has any role in energy generation and regulating Complex I activity.
6.2 Materials and Methods

6.2.1 Mitochondrial Complex I Activity

Complex I enzyme activity microplate assays were performed using a kit from Abcam (ab109721). The kit is used to measure a diaphorase-type enzyme activity. Diaphorase refers to cytochrome b5 reductase, NADH dehydrogenase and nicotinamide adenine dinucleotide phosphate (reduced) dehydrogenase enzymes that are involved in regulating Complex I activity. The enzyme was immunocaptured in the wells of the 96-well plate and the enzyme activity was measured following the oxidation of NADH to NAD+ and the simultaneous reduction of colorimetric dye, which gives rise to enhanced absorbance at 450 nm. Complex I activity is regulated by the amount of enzyme and by post-translational phosphorylation at important regulatory residues. Cell metabolic activity regulates these two key factors.

6.2.2 ATP Assay

ATP assays were performed using a luminescence-based ATPLite assay kit from Perkin Elmer (6016943). The principle involved in the ATPLite assay system is that the production of ATP is considered to be a hallmark for cellular metabolic activity. The assay worked on the generation of light caused by the reaction of ATP with added luciferase and D-luciferin. The chemical reaction is as follows:

\[ \text{ATP} + \text{D-luciferin} + \text{O}_2 \text{ LUCIFERASE oxyluciferin} + \text{Mg}^{2+} \text{ AMP} + \text{PPi} + \text{CO}_2 + \text{light} \]

The emission of light is directly proportional to the ATP concentration within certain values. The MCF7 cells were seeded at a density of 500 cells per well and then treated with scrambled siRNA and G3BP1 for 72 h. After 72 h cells were treated with oligomycin to block Complex V activity for the production of ATP through OXPHOS and then with 2-deoxy-glucose (2-DG) for 1 h to block glycolytic production of ATP.

Standards were prepared with the stock ATP solution provided with the kit. Simultaneously, cells were lysed using 50 µL of a cell lysis buffer (provided with the kit) and incubated for 5 min. Then 50 µL of luciferin substrate was added and incubated for 5 min on a shaker. Luminescence was measured using a plate reader (Biotek Synergy-2) and the values were plotted against the ATP standard.
6.3 Results

6.3.1 G3BP1 regulates Complex I Activity

Complex I enzyme activity was studied using the ab109721 kit (Abcam) in MCF7-wt cells after knocking down G3BP1 using siRNA (see Chapter 2, Section 2.4) along with control siRNA. Fig. 6.1 shows the significant downregulation in the activity of Complex I in the G3BP1 knock-down cells compared to the control, which suggested that G3BP1 plays a vital role in regulating Complex I activity. Complex I activity was also studied in the cells treated with lipofectamine as a vehicle-only control.

![Complex I Activity Chart](chart)

**Figure 6.1 Complex I assay**
MCF7 cells were treated with siRNAs for 72 h to knock down G3BP1 before Complex I assays were performed. \( n = 3; \) ***, \( P \leq 0.001.\)

6.3.2 G3BP1 regulates ATP generation

To understand the involvement of G3BP1 in regulating mitochondrial versus glycolytic ATP synthesis, an ATP assay was performed on MCF7-wt cells after knock-down of G3BP1 using siRNAs and scrambled or control siRNA. The cells were treated with 2-DG, for 5 h to block glycolysis. Additional treatment with oligomycin for 1 h was done to block Complex V, thereby inhibiting OXPHOS ATP. Total cellular ATP was calculated as the sum of glycolytic ATP and mitochondrial ATP. Glycolytic ATP was estimated by subtracting the 2-DG treated value from the untreated value as total cellular ATP, and mitochondrial ATP was calculated.
by subtracting the 2-DG and oligomycin-treated value from the 2-DG-treated values. The formula for calculating the mitochondrial and glycolytic ATP was followed as per the work done by Matsumoto and co-workers (2012). The ATPLite kit (Perkin Elmer) was used to perform this assay following the manufacturer’s instructions.

Fig. 6.2 shows the energy generated through glycolysis and mitochondrial OXPHOS from the cells treated with siRNAs targeting G3BP1 or with control siRNAs. A significant change in glycolytic ATP generation was observed after knock-down of G3BP1 in MCF7 cells.

Figure 6.2 ATP assay
Assessing the role of G3BP1 on ATP production was studied by treating MCF7 cells with siRNAs for 72 h. Glycolytic and mitochondrial ATP was measured by taking luminescence values after treating cells with 2-DG and oligomycin. n = 3; **, P ≤ 0.01.

6.4 Discussion
The results of Chapter 4 suggested that NDUFV1 was downregulated under stress conditions. This suggest that the MCF7 cells that experienced oxidative stress as a result of treatment with sodium arsenite could have caused NDUFV1 to be recruited to SG assemblies by binding to G3BP1, where its translation might have stalled. The results of Chapter 5 showed that knock-down of G3BP1 causes downregulation of NDUFV1, which, as per Santidrian and co-workers (2013) is a master controller of Complex I activity. The results from this chapter, where G3BP1 knock-down reduced Complex I activity, are consistent with the results of Chapter 5, where knock-down of G3BP1 downregulated NDUFV1 expression, which is the main subunit of Complex I activity (Santidrian et al., 2013).
This chapter was designed to explore the potential role of G3BP1 in regulating Complex I activity and also to determine if it regulates energy generation in MCF7 cancer cells. Complex I acts as a gatekeeper for mitochondrial OXPHOS and its activity is perturbed in several cancers (Calabrese et al., 2013; Sharma et al., 2011); NDUFV1, being downregulated after G3BP1 knock-down, could be involved in this regulation. The RIP-Chip dataset suggested that G3BP1 binds to some of the mitochondrial Complex I gene transcripts and a recent study suggested that G3BP1 is involved in regulating the expression of β-F1-ATPase mRNA (Ortega et al., 2010) which is a Complex V subunit.

In the Complex I assay, where the enzyme activity was measured via a colorimetric assay indicating the decrease in the absorbance values after knock-down of G3BP1, a significant downregulation in Complex I activity compared to the control cells was measured. The results of this chapter are consistent with the results of Chapter 4, where it was observed that the chimeric transcripts bearing the 3'UTRs of NDUFB7, NDUFB10 and NDUFV1 downregulates the expression of luciferase under stress conditions. This suggests that G3BP1, by regulating the expression of NDUFB7, NDUFB10 and NDUFV1, regulates the activity of Complex I. The results of this chapter are consistent with the work done by Santidriam and co-workers (2013), where knock-down of NDUFV1 was shown to reduce Complex I activity by 90% and thus supports the results presented in Chapter 5 of this study.

The Warburg Effect is considered to be a hallmark of cancer cell bioenergetics, where cells shift from OXPHOS to glycolysis for the generation of ATP, thus supporting the energy requirements of aggressively growing cancer cells. G3BP1 was found to regulate β-F1-ATPase mRNA expression, which plays a vital role in cellular energy production through the mitochondrial OXPHOS. In this chapter, G3BP1’s role in regulating cellular energy and its potential for supporting the Warburg Effect was studied by performing a luminescence-based ATP assay. The total cellular energy was sub-classified into glycolytic energy and mitochondrial energy. The cells treated with G3BP1 siRNAs were found to enhance glycolytic ATP production compared to the control cells; however, no change in mitochondrial ATP generation was observed. This suggests that by interacting with its mitochondrial Complex I ligands and regulating their expression, G3BP1 could act as a controller of the energy switch in MCF7 cancer cells.
In conclusion, the results from this chapter suggest that G3BP1 acts as a potential master regulator of mitochondrial Complex I activity and also in the generation of glycolytic ATP.
CHAPTER 7
ABSTRACT ARISING FROM CHAPTER 7


Student Contribution: Chapter 7, Fig 7.1 formed the concept used to draft this abstract, where the possible role of G3BP1 in regulating its target ligands was described. The concept of orphan RNA came from Chapter 7.

PUBLICATION ARISING FROM CHAPTER 7


Student Contribution: Chapter 7, entitled General Discussion and Future Direction, gave an insight that shaped the introduction and discussion part of the manuscript. Fig. 7.1 from the thesis provided a foundation for writing the manuscript.

PhD student: Adnan Naim Date: 01/02/2015

Corresponding Author and Supervisor: Dr Derek Kennedy Date: 01/02/2015
7. GENERAL DISCUSSION AND FUTURE DIRECTIONS

7.1 General Discussion

The work presented in this thesis was designed to explore the role of G3BP1 in the stress response pathway. The findings of this thesis reports, for the first time, evidence that G3BP1 is involved in pathways that may act in parallel to or irrespective of the stress-regulated pathway. More specifically, the data suggest a role for G3BP1 in regulating components of the mitochondrial OXPHOS pathway, namely Complexes I and V. After interpretation of the data, a model for the cellular activity of G3BP1 was proposed, which is explained in Fig. 7.1. The work was initiated by selecting putative RNA ligands for G3BP1 which were regulated during stress. Interrogation of the data from the RIP-Chip dataset revealed *NDUFB7*, *NDUFB10* and *NDUFV1* as the possible targets of G3BP1. All of these genes were nuclear-encoded mitochondrial Complex I proteins. Therefore, this information was used to design experiments culminating in the data presented in Chapter 6. This chapter describes the important role of G3BP1 in regulating Complex I activity and glycolytic ATP in MCF7 cancer cells.

Chapter 3 was designed to study the cellular localisation of G3BP1 under stress and non-stressed conditions. G3BP1’s localisation in SGs (Tourriere et al., 2003) was confirmed by staining with TIA-1, which is another SG marker (Kedersha et al., 1999). However, some different sizes of SGs were observed when the cells were double-stained with G3BP1 and TIA-1 under stress conditions. Differences in the sizes of SGs that overlap with G3BP1 and TIA-1 have not been reported earlier. However, the difference in sizes could play an important role in diffusive movement and merging of the SGs with microtubules, as reported by Nadezhdina et al. (2010). In future, the role of microtubules in SG movement could be explored, which could help in understanding the role of microtubules in regulating the size of SGs. Overall, this observation suggested that G3BP1 could transport its candidate genes (*NDUFB7*, *NDUFB10* and *NDUFV1*) to SGs.

Chapter 4 was designed to study the downstream changes in the expression of all the three target genes (*NDUFB7*, *NDUFB10* and *NDUFV1*) under stressed and non-stressed conditions
at the transcriptional and at the translational levels. The findings of this chapter suggest that MCF7 cells reprogram the translation machinery of three candidate genes under stress conditions, which was observed as changes in the expression of endogenous mRNA and protein.

It is reported that G3BP1 regulates the expression of its target transcripts by interacting at the 3’UTR (e.g. c-myc (Tourriere et al., 2001), β-F1-ATPase (Ortega et al., 2010) and ctnnb1 (Bikkavilli et al., 2011)). Therefore, a reporter assay was designed to study the functional role of G3BP1 in vivo on the regulation of expression of the three candidate genes, NDUFB7, NDUFB10 and NDUFV1. The different regions of the three target genes were sub-cloned in a reporter vector and the expression of the Luciferase reporter gene was studied. An intact reporter vector was used as a negative control and the results suggested that there was no change in Luciferase expression, irrespective of the stress treatment. The 5’UTR, CDS and 3’UTR of β-F1-ATPase mRNA was also sub-cloned in a Luciferase reporter vector to use it as a positive control as per Ortega et al. (2010). However, Ortega and co-workers used the β-galactosidase reporter system instead of the Luciferase reporter to demonstrate G3BP1’s interaction with the 3’UTR of β-F1-ATPase mRNA. The reporter assay data suggested that the changes observed in the expression of the Luciferase construct bearing the sub-cloned regions of β-F1-ATPase mRNA does not support the hypothesis proposed in the reported work by Ortega et al. (2010); therefore, those data were not included in analysis or for any other experiments in this thesis.

The data generated from the reporter assay suggested that the 3’UTR of NDUFB7, NDUFB10 and NDUFV1 could be the binding site for G3BP1 and, as a consequence of stress, the chimeric transcript moves to SGs and hence a significant downregulation in the expression of firefly Luciferase was observed. This regulation was observed at the translational level. In order to study the effect of stress on the stability of the Luciferase transcript, the same reporter constructs were used to study the expression of Luciferase mRNA. The data from the experiment did not show any significant changes in the level of Luciferase mRNA, which suggested that G3BP1 does not influence the stability or longevity of the Luciferase mRNA by interacting with any of the cloned regions of the three target genes, NDUFB7, NDUFB10 and NDUFV1, irrespective of the stress treatment.
Chapter 5 was designed to study if the findings of Chapter 4 were due to a direct role of G3BP1 or, alternatively, if the observed regulation of target gene translation could be caused by a combinatorial effect of other stress-regulated cellular proteins. No significant differences in the expression of endogenous transcripts of NDUFB7, NDFUB10 and NDUFV1 in the knock-down samples compared to the control were observed, which suggests that G3BP1 does not regulate the stability of these target transcripts. However, endogenous expression of NDUFV1 was found to be downregulated at the translational level after G3BP1 knock-down. This led us to conclude that G3BP1, besides being a component of SG assembly, regulates the expression of NDUFV1, independent of the stress response pathway. This is considered as a novel finding of this thesis. This supports the hypothesis proposed in Fig. 7.1 regarding G3BP1’s activity. In the experiments where G3BP1 was knocked down, the mRNP or the mRNA became an “orphan” and failed to reach its destination, and hence could not be translated.

In conclusion, the mystery about the promiscuous behaviour of G3BP1 remains unsolved. However, we are one step closer to understanding its role in cells. The current understanding of G3BP1’s versatile nature suggests that its activity could be cell-specific and stimulus-specific. Typically, G3BP1 is considered to be a SG-nucleating protein because its overexpression leads to the formation of SGs irrespective of stress. However, it would be unfair to designate G3BP1 as having a role solely in SG formation, as recent reports suggest that it is not an essential component of SGs. This is because the recent work by Jedrusik-Bode et al. (2013) showed that G3BP1 knock-down MEFs were found to form SGs in cells. Reports where NDUFV1 knock-down was found to downregulate Complex I activity by 92% (Santidrian et al., 2013) support the findings of Chapter 5 and Chapter 6, where G3BP1 was found to regulate the translation of NDUFV1, which, in turn, regulates Complex I activity. From the information available in the literature and the knowledge gathered from the experiments performed in this thesis, it is clear that G3BP1 is involved in various cellular activities; however, the pathways downstream from its targets are not clear yet. In short, G3BP1 could be considered as a “Jack of all trades but master of none”. Nevertheless, before giving a final judgement, there is a need to identify several new RNA ligands for G3BP1 and to pool them to study their regulation in different cells and under different environmental cues.
7.2 Future Directions

In order to validate the proposed model (Fig. 7.1), and to study the role of G3BP1 in regulating its target mRNA (i.e. \textit{NDUFV1}), a rescue experiment should be performed. This would be achieved by expressing the target gene (\textit{G3BP1}) in a manner that is opposite to the siRNAs used in the experiments described in this thesis. A rescue experiment can be performed in order to confirm the specificity of siRNA for the target gene (Anonymous, 2003). This experiment would provide conclusive evidence that the changes in the expression of \textit{NDUFV1} after knock-down of \textit{G3BP1} are due to siRNAs and are not an “off-target” effect. It is proposed that the rescue experiment would return the expression levels of \textit{NDUFV1} to endogenous levels.

The NTF2 domain of G3BP has been found to be responsible for its dimerisation. G3BP1 has also been found to interact with several other proteins, such as RasGAP, USP10 (Soncini et al., 2001), G3BP2 (Matsuki et al., 2013) and Caprin (Solomon et al., 2007). This suggests that G3BP1 might exist as a multi-protein complex sharing a common target ligand. In order to study G3BP1’s co-partners in a multi-protein complex mRNP, RNase-assisted RNA chromatography combined with a mass spectrometry approach could be applied (Michlewski and Caceres, 2010). As compared to other available techniques like RIP-Chip, Cross-Link Immuno Precipitation (CLIP) and Systematic Evolution of Ligands by Exponential Enrichment (SELEX), which are widely used to study different RBPs for a target ligand in an mRNP, RNase-assisted RNA chromatography combined with mass spectrometry is highly sensitive and specific for identifying all the RBPs targeting a specific transcript. In brief, the protocol anchors a target mRNA to a matrix; subsequently, the whole-cell lysate is incubated with the immobilised target under the hypothesis that the appropriate RNP components will assemble on the transcript to be interrogated. Non-specific proteins are washed from the complex and then a cocktail of RNases is used to release all the proteins bound to the target transcript, which makes the separation of proteins easy through chromatography or gel electrophoresis. Finally, individual proteins can be identified by mass spectroscopy.

Experiments like RNA-fluorescent \textit{in situ} hybridization and RNA electrophoretic mobility shift assay could also be designed, which may show the direct interaction of G3BP1 with its target ligand. These experiments, if performed, could support the outcome of the abovementioned experiment (RNase-assisted RNA chromatography).
The work described in this thesis started with a hypothesis that G3BP1 recruits mRNA to SG for triage, possibly for destruction. However, the data from this research showed an unprecedented role of G3BP1. The work presented here showed the role of G3BP1 in controlling mitochondrial function. It was assumed that the downregulation of NDUFV1 would occur by sequestration of its transcripts to SG and possible destruction of the mRNA in P-bodies, but the control is much more subtle than that, because the data suggest that the mRNA remains intact and it is unlikely that it is shuttled to the SG in the absence of G3BP1, as demonstrated by its knock-down. Here, for the first time, we propose (as described in Fig. 7.1) that loss of G3BP1 causes the formation of a pool of orphan mRNA with nowhere to go in the cell until its escort, G3BP1, returns and allows it to continue to its destination; in the case of NDUFV1 mRNA, this is possibly to the periphery of mitochondria, where it will be translated and imported to Complex I. But how does G3BP1 manage other target mRNA? Does it protect mRNAs throughout the entire system from becoming homeless orphans? These are questions that need to be addressed in future research work.
Figure 7.1 G3BP1, Jack of all trades, but master of none

1. With the synthesis of mRNA, G3BP1 shuttles into the nucleus from the cytoplasm (2) and forms an mRNP complex along with several other components (3). The mRNP complex is transported out of the nucleus (4) and some components leave the mRNA cargo, while others remain (5) associated within the mRNP. Under stress (5a), the mRNP moves into the stress granules (SGs), along with some other stress-activated proteins. SGs are considered to be “translation detention centres”. If the mRNA is not required to be translated as a consequence of stress, they are passed onto processing bodies (P-bodies) for degradation (6) and, under stress, mRNA translation is stalled (7). On the other hand, under non-stressed conditions, G3BP1 transports the cargo to its destination (i.e. mitochondria) (9) and hence its translation is regulated (e.g. NDUFV1). The letters X, Y, A and B were used for representation purpose of generic components of an mRNP as reported by Buchan and Parker (2009). X represents Caprin-1, Y represents G3BP2, A represents TIA-1 and B represents tristetaprolin.
REFERENCES
REFERENCES


G3BP associate with tau mRNA and HuD protein in differentiated P19 neuronal cells. *Journal of Neurochemistry*, 89, 613–626.


APPENDICES
APPENDIX I: NUCLEOTIDE SEQUENCE OF \textit{NDUFB7}

\textit{Homo sapiens} NADH dehydrogenase (ubiquinone) 1 \( \beta \) subcomplex 7 (\textit{NDUFB7}), mRNA, 18kDa

NCBI Reference Sequence: NM_004146.5

\begin{verbatim}
1 ctgactgagg ggtcagtggt tccgggtagg agctaggtga cctctggctg ctgcagggat
61 ctgcagcgac tgcagccatg ggggcccacc tggtccggcg ctacctgggc gatgcctcgg
121 tggagccgca cccccctgcag atgcnaacct tccccccgca ctacggcttc cccgaacgca
181 aagagccgca gatggtggcc acacagcagq agatgatgga cgqagcagctg agqctccagc
241 tgcgacgcct cagctccatcc gggctgtcga gatcagcttc gacagcttcc
301 sgccctttcc ggcctgcaag caggagggcc acgggctggg gaagtgggag ctcctcagcact
361 atgtgatgcct catgaaggag tttgagcggg agcggaggct gctccagcgg aagaagcggc
421 gggagaagaa gggagggaggag ggcggcagag
481 ctggcctgta gggggcacc ccccccaacct atggaccagt caatataaag ccttcagggc
541 cctcactgta aaaaaaaaa aaaaaaaaaa aaaa
\end{verbatim}

\textbf{CDS}: 78 – 491
APPENDIX II: NUCLEOTIDE SEQUENCE OF *NDUFV1*

*Homo sapiens* NADH dehydrogenase (ubiquinone) flavoprotein 1 (*NDUFV1*), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA, 51kDa

NCBI Reference Sequence: NM_007103.3

CDS: 154 - 1548
APPENDIX III: NUCLEOTIDE SEQUENCE OF \textit{NDUFB10} \\

\textit{Homo sapiens} NADH dehydrogenase (ubiquinone) 1 \(\beta\) subcomplex 10 (\textit{NDUFB10}), nuclear gene encoding mitochondrial protein, mRNA, 22kDa  
NCBI Reference Sequence: NM\_004548.2 

\begin{verbatim}
1 gcagccgccct tcggcgctct ctagcgccgc gcacccggccc cgcgggaccc gacggaggt
61 agaggccagg gcagccgcgc ggcgacgcca gctgccccgc gcgtgccgca cgcggacacag
121 ctaggcaag gatgtgtacc ctagcccccgg cgcgtgccgc gcgtgccgca ccaatcccat
181 cagctcatag atgaaacggt tcgcctcctg cgctgacca gaacgtcgcg tcgtgagaga
241 attttatag cgcagcgcag caaagacaag ctattactac taccaccgcg cggctgcccg
301 cagctgcagac atcactgtgac gaaagagaca ccgatcagtt ccgtgacttc gccgatcagct
361 gaaagccgag aggggctagc agcgcggcgt ttctggtgag ccggttcggc cgggacggct
421 cagctgcagac gcagctgttgg ccggtgcagc gcggttcggc cgggacggct
481 cagctgcagac gcagctgttgg ccggtgcagc gcggttcggc cgggacggct
541 ttctgctcg gcggccgttg cccacagcag gcgggctttg ccggttcggc cgggacggct
601 aagagctgccc gcgcgtctgcg cccctcgctg ccggttcggc cgggacggct
661 gtcctgatgag ttcctgaga ttaagccctc cccctgctgc cccctgctgc
721 aaaaaaaaa aaaaaaaaa a
\end{verbatim}

CDS: 110 - 628
APPENDIX IV: NUCLEOTIDE SEQUENCE OF \textit{B-F1-ATPASE}

\textit{Homo sapiens} ATP synthase, H+ transporting, mitochondrial F1 complex, \(\beta\) polypeptide (\textit{ATP5B}), nuclear gene encoding mitochondrial protein, mRNA

NCBI Reference Sequence: NM_001686.3

\begin{verbatim}
1 agttcaccca atggaacctgc ctaactgcagc gtaggcctcg cctcaacggc aggagagcag
61 gcggctgcgg ttgctgcagc cttcagtctc cacccggact acgccatgtt ggggtttgtg
121 ggtcgggtgg cccgctgctcc ggcctccggg gccttgcgga gactcacccc ttcagcgtcg
181 ctgcccccag ctcagcttct atcgccggcc gtcgccacgag cggccatcatc tggccaggac
241 atgcaagtgc aagccagagga cagccagactgt gttttgagg tgccgcagca tttggtgag
301 agcagcattaa ggactattgc ttaggatggt aczaggtgag cagaggagat gcttggtgact
361 ctggattctg ctggtg
cggattggcaa gactgtactg atcatggagt taatcaacaa tgtcgccaaa
gcccatggtg gttactctgt gtgggtgaga ggacccgtga aggcaatgat
ttataccatg aaatgattga atctggtgtt atcaacttaa aagatgccac ctctaaggta
gtataacatct ttcgcttcac ccaggctggt tcagaggtgt ctgcattatt
gcacatttc ttcgcttcac ccaggctggt tcagaggtgt ctgcattatt
ggctgtgtat atgctcaaat gaatagccaa ccttgggtct gcggccaggt gcgtcagact
961 gggctgactg tggctgaata ctctagagac caagaaggtc aagatgtact gcctatttatt
gagcattcatgc gccttctgctg tgggctatca gcctaccctg gcctacgaca
ttgctgagct ggtgcacgga atcctcttgg atccctgact
cgtatcatgg acacatctac acaagaggg atctatcacc tctgtacagg
tatatttattt attttcttgtcc
gagaccatca aagagttcata cctccctcag gatattcattg ccattctggg tattgatgaa
gttctcatgg aagacaagtt gacgggtcgg cggccaggg aaatacagc tttccccattt
cagccattcc aggtgattgaa ggtcttccca ggtcatatgg ggaggtggtt acccttgaag
1561 gagacattca aaggattcca gcagatattt ggaggcaaat atgacctact ccacgagaca
cgtctcttata tgtgggacc cattgaagaa gctgctgaa gctgctgaa
gagcttcatc caggtgaggt tctcttccct tgaacttgtct tctctcttgc ccttacccca
1741 aaagcttcca tttttcttg taggtgcacag aagagctctt attgaagata tatttctttct
gaacagatt taaggtttcc aataaatgg attacccccca gaaaaa aaaaaa
\end{verbatim}

\textbf{CDS}: 106 – 1695
APPENDIX V: PLASMID MAP OF THE PGL3 PROMOTER
VECTOR E176A (PROMEGA)

pGL3 Promoter Vector
5010bp

- Amp^r
- f1 ori
- ori
- Sal I 2202
- BamHI 2196
- Hpa 2094
- Xba I 1934
- SV40 late poly (A) signal
  For luc+ reporter
- Synthetic poly(A) signal/transcriptional
  Pause site (for background reduction)
- SV40 promoter
- HindIII 245
- Nco I 278

Luc+
APPENDIX VI: WESTERN BLOT FOR NDUFV1

Western blot for NDUFV1 after knock-down of G3BP1 in MCF7-wt cells for three different biological repeats.

- **G3BP1 (68 kDa)**
- **NDUFV1 (51 kDa)**
- **ACTIN (42 kDa)**

(siRNA G3BP1) (siRNA Scrambled)