Determining the Role of the RNA-binding Protein, RasGAP—SH3 domain-Binding Protein, in the Mammalian Cellular Response to Ultraviolet Radiation

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Abstract

The RasGAP—SH3-domain Binding Protein (G3BP1) is an endoribonuclease that links Ras-signalling pathways and mRNA stability. Though implicated in many cellular roles, a well defined function for G3BP1 has yet to be determined. The aim of the following dissertation is to further implicate G3BP1 in mammalian cellular responses to environmental stress, most notably that of Ultraviolet Radiation. We observe G3BP1 transverse from the cytoplasm to the nucleus upon treatment of HeLa cells with apoptotic levels of UVC. Interestingly, forcing its nuclear accumulation in the absence of stress is sufficient to induce apoptotic characteristics. Preliminary observations suggest that plasma membrane signalling and the resultant activation of stress activated protein kinases, JNK and p38 MAPK, and CASPases might play a role in this process. Thus, we further elaborate G3BP1's role in cellular responses to stress and suggest a new function for the protein, that being the promotion of apoptosis.

Résumé

"RasGAP—SH3-domain Binding Protein (G3BP)" est un endoribonucléase qui associe la voie de signalisation de Ras et la stabilité d'ARN messager. On ne connaît pas une fonction bien défini pour cette protéine, même si c'est impliquée dans plusieurs rôles cellulaire. Le but de cette dissertation est d'appuyer l'implication du G3BP1 aux réponses cellulaire mammalien au stress environmental, particulièrement à la radiation ultraviolet. On observe le G3BP1 transverse du cytoplasm au noyau au traitement des cellules HeLa avec des doses UV apoptotique. La chose fascinante est quand on introduit s'accumulation du noyau en l'absence du stress est suffisant de provoquer les charactéristiques apoptotique. Les observations au premier degré suggèrent que les signalisations d'origine de membrane plasmique et l'activation resultant du JNK, p38 MAPK, et CASPases peuvent jouer un rôle en ce processus. Ainsi, on a élaboré le rôle du G3BP1 en réponse cellulaire au stress et suggère une nouvelle fonction pour la protéine : la promotion de l'apoptose.

Acknowledgements

For every end there is a beginning and the space between these two points would be nothing but a void if it were not for all of those we meet along the way and too simply label as friends, lovers, helpers, and yes, strangers. This is where I thank all those for filling that space. First and foremost I would like to devote this work to my late father, Hans Gerhard. Dad, I know one of your fondest desires was to see me go above and beyond in my university studies, an opportunity that was unfortunately not readily available to such a sharp mind. A certitude that I have gained from my studies in Biochemistry is solace from understanding that half of that chaotic assemblage of genetic matter that makes me, me, is you. So, congratulations Dad on your achievement, because half of this work is yours. And to my other half I accredit my strength, my determination; how does one express how powerful an effect a mother's pride for her son can have on a life, on a spirit? It can pull blood from all that is inanimate, terminate all that may be infinite, bring light from what was once thought to only be of darkness. Are we fools to merely ascribe such a force to love alone? What fond words are to be bestowed upon the two pendulums whom constantly sway in manners to counteract the negative forces in my life that try to pull me down? Margaret and Carol, from you I accredit my understanding of the fidelity in genuine acts of kindness. And to those who have the rare ability to fracture my hard exterior and expose my camouflaged, encased, anchored, and *copious,* reserves of sensitivity and emotion, I mark you as gifted, but it is I whom receives the gift of your friendship. Chrissie, Jules, Regina, Philippe, Laurie and the long list of other nuts I hold dear: life without you would be so stagnant, so frivolous.

Direct imaging has revealed that a positive feedback loop mechanism like no other co-localizes with room 904 and is composed of the Gallouzi laboratory members (Behrmann et al. 2007). Structure/function analysis has revealed that, above all others, Dr. Gallouzi saturates one's learning and the betterment of oneself by providing the opportunity to develop a form of creativity that only comes from discovering what was once unknown, the true exalted definition of *Sciencia*. The allotropic abilities of Eveline classify her as one that consistently provides order, confidence and skill in experimentation, and some of the most down-to-earth conversations I have had at McGill. Though the exact future role of Sami has yet to be self-determined, his display of a level of cognition and maturity far, far above the norm should assure him of stellar prospects. The tight association of Sergio to all laboratory members has proven helpful in ways ranging from excellent personal connections to the provision of experimental tools. Additionally, his most loved binding-partner, Michael, is characterized as having a smile that radiates stronger than the emissions from the Stratalinker, an energy source much welcomed in the lab. Though morphologically distinct, the functional significance of Kate and Rachid are homologous, primarily in their excellent skill of observation and their sharp ability to note potential problems and promptly redirect one back to a path with silver linings. Though an unfortunate short exposure time has limited my interactions with newer lab members, their provision of betterment is evident; Virginie by her skill and having a character one may confide in, Xian by his ability to catch my odd sense of humour, Olivier by his influential confidence, and Chris by his amazing ability to always abound with amusement. Helpful suggestions and endless smiles from Michel, Marie-Joelle, and Paola were worth their weight in gold. Adieux, and best wishes to all.

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Introduction

The known is finite, the unknown infinite; intellectually we stand on an islet in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land.

■ T.H. Huxley, 1887

Through the pursuit of years of research into the inner workings of life, we currently understand that the basis for the construction, maintenance, and replication of a cellular entity resides in the ability for biochemical reactions to occur in dynamic manners, orchestrated with an astounding degree of regulation and specificity. Remarkably, our present knowledge permits us to dissect the chemistry of life whereby defined physiological functions can be assigned to distinct, yet commonly interconnected reactions— bringing to light a web of characterized metabolic pathways. One class of a metabolic pathway is signal transduction cascades where extracellular stimuli is transformed into a biochemical currency of sequential and directed conformational changes of protein molecules, mediated primarily by posttranslational modifications, resulting in the conformed expression of genes. One prime example of such being the Ras-signal transduction cascade, a metabolic pathway of early evolutionary origin and currently heavily scrutinized for it is observed to mediate numerous integral cellular processes (Feig & Buchsbaum 2002).

The many faces of Ras-signal transduction cascades: from growth to death

Cellular responses to extracellular stimuli conveyed through Ras-signalling cascades, though numerous and varied, have a common beginning of the oligomerization and subsequent activation (by self-phosphorylation), of transmembrane plasma membrane Receptor Tyrosine Kinases (RTK). The activation of these RTKs is commonly by the binding of a complementary ligand, such as a growth factor. In response to RTKs, the membrane associated Ras GTP-binding protein is activated by guanine nucleotide

exchange factors (GEFs), which convert inactive GDP-bound Ras to the active GTP-bound form by such exchange of its bound nucleotide cofactor (reviewed in: Campbell et al 1998). Once activated, Ras confers activity to a myriad of metabolic pathways known as serine/threonine kinase cascades, which, simply put, sequentially phosphorylate and activate proteins with the net effect of appropriately altering cellular metabolism as dictated by the extracellular stimuli. One example of a Ras-induced kinase cascade is the Raf/MEK/ERK pathway seen to stimulate cellular proliferation. GTP-bound Ras associates with Raf, a serine/threonine kinase, resulting in activation of its kinase activity. Raf subsequently activates its down stream phosphorylation target, MEK (also known as mitogen activated protein kinase kinase) which mirrors the same process but on its substrate ERK (extracellular-signal-regulated kinase). Activated ERK subsequently translocates to the nucleus where it activates, by phosphorylation, specific transcription factors capable of inducing the transcription of genes that mediate promotion of the cell cycle and thus, cellular proliferation. To exemplify the versatility of Ras, the same protein key to proliferation is also key to inducing regulated cell death known as apoptosis (reviewed in: Kyriakis & Avruch 1996). Under apoptosis inducing conditions, such as excessive stress or inflammation, Ras results in the induction of kinase cascades leading to the activation of stress activated protein kinases like c-Jun kinase (JNK), resulting in the activation of transcription factors that promote the induction of apoptosis. Aside from the two examples provided, Ras signalling also mediates numerous biochemical processes ranging from cellular differentiation, tumourigenesis, to the *suppression* of apoptosis. This alone exemplifies the diversity and complexity that composes Ras-activated metabolic pathways. Logically, once Ras has succeeded in conveying extracellular signals to the cell, its activity is no longer needed whereby a class of effectors known as GTPase

activating proteins (GAPs) associate with Ras to promote the conversion of GTP to GDP, thus arresting the signalling process. Interestingly, evidence is emerging that like Ras, the GEFs and GAPs that regulate its activity may too induce signal transduction cascades. As one example of many, RasGAP has been shown to activate signalling pathways at the nexus of the serine/threonine kinase, Akt, independently of Ras (Yue et al 2004). Yue et al. (2004) observed that the N-terminal region of RasGAP could directly bind to Akt and enhance its kinase activity, which results in the activation of anti-apoptotic metabolic pathways. To summarize, Ras and its resultant signal transduction cascades mediate a multitude of metabolic pathways. Its effector proteins— like its silencer, RasGAP— may also serve as signalling molecules downstream of Ras, thus increasing the order of complexity and possible outcomes originating from extracellular stimuli.

To expand our understanding of the web of metabolic pathways influenced by Ras signalling and identify possible cellular members that might regulate the dizzying array of Ras-induced processes, the hunt was on to identify protein partners that associate with integral members in Ras-signal transduction cascades. Rationally, one integral member to place the spotlight on is RasGAP, mainly for its ability to inactivate Ras but also because of evidence that it too appears to be a signalling entity. Indeed, RasGAP did become the focus of such studies of one research team headed by Dr. Tocque (Parker et al 1996). They noted, as well as others (Koch et al 1991; Yue et al 2004), that proteins that contain SH3 domains, like RasGAP, are implicated in a diverse array of functions and thus hypothesized that this fact could only be orchestrated by numerous different protein binding partners. To clarify, SH3 domains are motifs commonly seen in protein molecules implicated in cellular signalling pathways (Mussachio et al 1992), such as in Ras signalling (Khosravi-Far & Der 1994). They serve to mediate specific protein-protein

interactions by their selective association with complementary proteins at proline-rich regions (Ren et al 1993). Therefore, their research endeavour commenced with isolating protein-binding partners of RasGAP, but to that of its SH3 domain specifically. Glutathione S-transferase (GST)-pull down experiments with the recombinant protein composed of the SH3 domain region of RasGAP served as the means to isolate such binding partners. Subsequent the incubation with lysates from exponentially growing ER22 cells, a Chinese hamster lung fibroblast cell line, one 68 kDa protein was predominantly isolated. They noted its specificity of binding to RasGAP, for its association with SH3 domains belonging to other proteins did not occur. Additionally, by co-immunoprecipitation analysis this interaction was shown to occur only when Ras was in an active, GTP-bound, state, which was subsequently confirmed by Gallouzi et al. (1998). This pioneering finding marked the beginning of two new fields of research. One, it represented the first instance that cellular proteins, for whatever reason, were seen to bind the SH3 domain of RasGAP. Two, it christened the known existence of a protein that was aptly named the RasGAP—SH3 domain-binding protein, or G3BP.

Characterization of a novel RNA-binding protein: structures and enzymatic functions

Upon its identification, Parker et al. (1996) set out to characterize this novel cellular unit. Firstly, partial peptide sequencing of purified G3BP allowed for identification of its mRNA transcript from a cDNA library, which corresponded to a single mRNA species of approximately 3.3 kb encoding for a 466-amino-acid protein with a predicted molecular mass of 52 kDa[*]. The chromosomal location of the corresponding gene is within the long arm of chromosome 5 (Kennedy et al 2001).

[*] G3BP1 is observed to have an electrophoretic mobility profile of a 68 kDA entity.

Second, comparison of its amino acid sequence with other proteins noted that G3BP contains several distinct protein motifs reminiscent of heterogeneous ribonucleoprotein particles (hnRNPs), a class of RNA-binding proteins of the RNP family (Figure A). The corresponding consensus sequences for these motifs were specifically RNP1 and RNP2 domains, and an RGG-rich box (arginine-glycine rich), a motif noted to confer cooperative binding of RNA to RNP domains (Kennedy et al 1996; Mayeda et al 1994). Scrutiny of G3BPs amino acid composition noted additional domains, such as a central acidic rich region that appears to mediate protein-protein interactions and is also characteristic of proteins of the RNP family (Burd & Dreyfuss 1994; Kennedy et al 2002; Parker et al 1996; Prigent et al 2000). Furthermore, the N-terminus shows high homology to the protein, Nuclear Transport Factor-2 (NTF2), a protein that serves to import proteins it associates with into the nucleus from the cytoplasm via the RanGDP/GTP gradient (Lodish et al 2001). In brief, upon association of NTF2 and its cargo to GTP-bound Ran, the complex translocates into the nucleus were exchange of GDP to GTP results in abolition of the complex (Lodish et al 2001; Ribbeck et al 1998). G3BP's similarities to NTF2 suggest that its cellular movement might too be mediated through a RanGDP/GTP gradient, but an inhibitor to the formation of this gradient, leptomycin b (Ullman & Powers 1997), was found to have no effect on G3BP's cellular localization (Prigent et al 2000) nor has Ran been observed to associate with this domain (Tourriere et al 2003). Despite this, some evidence has shown G3BP's NTF2-like domain still targets it to, and causes its association with, the nuclear envelope (Prigent et al 2000). Other observations have shown that this domain mediates specific protein interactions— similar observations of such have been reported with other RNA-binding proteins that contain NTF2-like

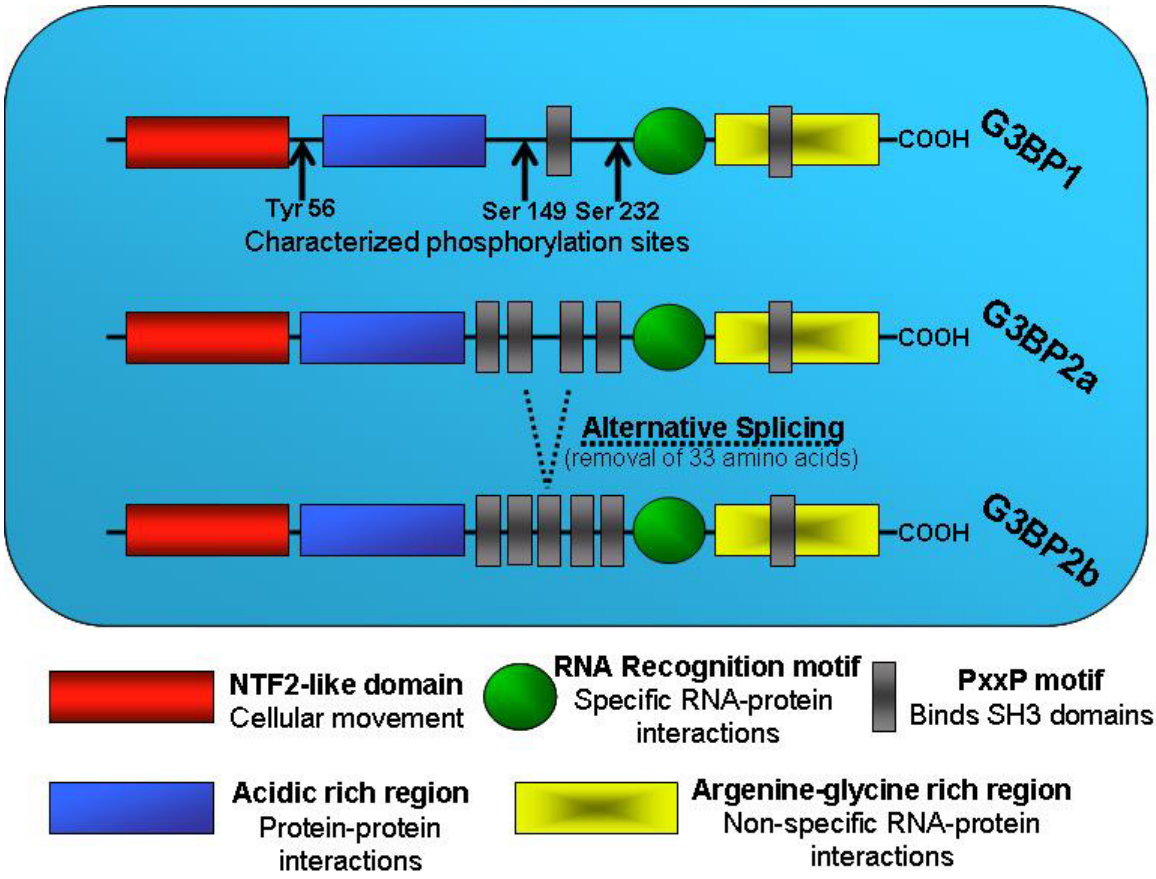


Figure A. Structural characteristics of G3BP proteins. In higher eukaryotes, there exists three G3BP protein isoforms that are highly homologous. While the 1 and 2 isoforms are produced from distinct genes, the 2a and the 33 amino acid shorter, 2b, isoform originate from alternative splicing of a common mRNA transcript. G3BP contains protein domains seen in RNA-binding proteins of the RNP family, namely an RNA recognition motif (RNP1 and RNP2 domains), an arginine-glycine rich region, and an acidic rich region. Additional domains include an NTF2-like domain, known to mediate the proteins' interaction with RasGAP, and a PxxP motif, a characteristic SH3-domain binding sequence. The only known post-translational modification of the protein is phosphorylation of G3BP1 on residues Y56, S149, and S232. Figure adapted from Irvine et al. (2004).

domains (for example, the protein, TAP (Suyama et al 2000)). One observation being the association with itself to form G3BP oligomers (Tourriere et al 2003). Remarkably, pull-down experiments with deletion mutants of G3BP have shown that it is this NTF2-like region that associates with RasGAP, which is unconventional for SH3-domain associations (Kennedy et al 2002). The presence of characteristic motifs that mediate interactions with SH3 domains is not surprising, that being the PXXP[†] sequence, but the fact that it is not responsible for G3BP's association with RasGAP, is (Kennedy et al 2002). Therefore, these SH3-domain binding motifs probably serve to associate G3BP with other SH3 domain-containing proteins other than RasGAP (Kennedy et al 2002), which has indeed been reported (Rahmouni et al 2005).

The G3BP family has since grown with identification of an isoform encoded by a separate gene on the long arm of chromosome 4 (Kennedy et al 2002) (Figure A). Additionally, the mRNA of this isoform, termed G3BP2 (therefore the initial isoform is termed G3BP1), is seen to encode two protein products produced from alternative splicing of the same transcript, thus demarcating a 2a and, the 33 amino acid shorter, 2b version (Kennedy et al 2002). All three isoforms are highly homologous, primarily differing in their central region and their number of SH3-domain binding motifs, where G3BP1 and 2 have 65% similarity in their amino acid sequences. Regions of high degree of conservation between isoforms, and amongst other species' orthologues, are, the NTF2-like domain used by all for their association with RasGAP (Kennedy et al 2002), and their RNA-binding region (Kennedy et al 1996).

The majority of investigations concerning G3BP proteins have focused on the G3BP1 isoform primarily. From here on, we will term G3BP1 as simply "G3BP", except

[†] PXXP: Proline, any amino acid, any amino acid, Proline sequence

where confusion between isoforms, or required specific classification, may arise.

G3BP is evolutionarily conserved

The foundation of G3BP appears to have an early evolutionary origin since orthologues of this RNA-binding protein exist in species ranging from yeast to mammals. Notable alterations of the protein have occurred through the eons. To begin, lower organisms supposedly contain only one form of G3BP. The yeast orthologue, Bre5 is described as having a significant, but very low, amount of sequence conservation to that of human G3BP1 (Pazman et al 2000), while the *Drosophila* orthologue, Rasputin (Rin), contains 40% amino acid similarity (Pazman et al 2000). The expansion of G3BP family members to be of 3 isoforms appears to have a late evolutionary origin for they have only been formally reported in mammals, though 3 isoforms have been suggested in Zebrafish (Irvine et al 2004). The homology between mammalian orthologues is extreme where the protein sequence between human and murine G3BP1 and G3BP2 are 94.4% and 98.5%, respectively (Kennedy et al 2002; Kennedy et al 1996). Once again, the NTF2-like and RNA-binding regions displays the greatest sequence conservation between species, while the central acidic region shows great divergence (Pazman et al 2000). This fact implicates that the conserved regions provide essential functions within the cell.

Break in the chain; G3BP makes link to mRNA stability: Following the classification of G3BP as an RNP family member, investigations identified a functional significance of its ability to associate with RNA, that being its ability to catalyze the cleavage of mRNA transcripts. Gallouzi et al. (1998) first identified that recombinant G3BP could effectively degrade the 3' untranslated region (3'-UTR) fragment of the mouse *c-myc* mRNA transcript. The cleavage products generated were dose dependant— high concentrations of G3BP would produce a series of distinct, small RNA fragments while lower

concentrations produced larger fragments. Additionally, Gallouzi et al. (1998) noted that the RNase activity of this enzyme appeared to be modulated by structural characteristics, which was subsequently linked to its phosphorylation status of serine residues. Indeed, prior phosphatase treatment of G3BP virtually abolishes its RNase activity. On a larger perspective, they observed that its RNase activity is inversely correlated with its association with RasGAP. Specifically, in serum starved cells— which are in a quiescent state where the G3BP-RasGAP complex is known to be abolished (Parker et al 1996)— both its phosphorylation state and RNase activity were seen as highest. These observations are to be seen as very significant. They represent the first known instance of an endoribonuclease that requires site-specific phosphorylation for its activity. In addition, G3BP represents a new family of signal transduction proteins which connect tyrosine kinase linked receptors to cellular RNA metabolism (Gallouzi et al 1998).

Tourriere et al. (2001) made further characterizations of G3BP's RNase activity through studies with its substrate, *c-myc* mRNA. First off, they demonstrated that recombinant G3BP could not cleave poly-rU, rG, rA, or rC homopolymers *in vitro*, suggesting that its target cleavage site is sequence specific. In comparison, cleavage of *c-myc* mRNA — in a metal-independent manner— occurred mainly at its 3'-end exclusively at cytosines followed by adenines, generating a 5' hydroxyl group (OH) at the cytosine. Though the C-terminal region of G3BP (which contains its RNA-binding motifs) is capable of binding RNA, only the full-length protein was capable of RNA cleavage. Tourriere et al. (2001) later derived a potential consensus binding sequence for G3BP by use of an *in vitro* genetic selection SELEX[‡] technique (Table 1). The

[‡] SELEX: systematic evolution of ligands by exponential enrichment

Table 1. The following genes contain a perfect match to the G3BP1 cleavage SELEX consensus sequence (ACCCAUACGCAG). The following genes were determined by a BLAST search on NCBI. Table was adapted from Tourriere et al. (2001). UTR: Untranslated region.

cDNAs that contain G3BP SELEX consensus sequence		
Species	**Encoded Protein**	**Position in mRNA Sequence**
Homo sapiens	Cornified envelope precursor	Coding sequence
Homo sapiens	KIAA0711	3' UTR
Homo sapiens	2,3-oxidosqualene-lanosterol cyclase	3' UTR
Homo sapiens	Cyclin-dependant kinase 9	Coding sequence
Homo sapiens	Lanosterol synthase	3' UTR
Homo sapiens	v-yes-1 Yamaguchi sarcoma virus-related oncogene homologue (LYN)	5' UTR
Homo sapiens	Carboxypeptidase M (CPM)	5' UTR
Homo sapiens	NF2/meningioma region of 22q12 (PK1.3)	Coding sequence
Homo sapiens	Solute carrier family 20 (phosphate transporter), member 1	5' UTR
Homo sapiens	Lyn tyrosine kinase	5' UTR
Homo sapiens	Gibbon ape leukemia receptor 1	5' UTR
Mus musculus	Goblin mRNA for Golgi-associated band 4.1-like protein	Coding sequence
Mus musculus	Interferon regulatory factor 7 (IrF7)	Coding sequence
Mus musculus	Integral membrane protein (Nramp2)	Coding sequence
Mus musculus	Midnolin	5' UTR
Mus musculus	Radixin	3' UTR
Mus musculus	Odorant receptor gene	Coding sequence
Rattus novegicus	Nerve growth factor receptor	Coding sequence

determined sequence was ACCCA(U/C)(A/C)(C/G)G(C/A)AG, whereby a BLAST search of this sequence in a genomic database identified 11 human mRNAs having a near or identical sequence match. Indeed, this SELEX prediction of G3BP substrates appears to hold truth within living systems. A subsequent publication demonstrated the binding and degradation of *cdk9* mRNA (Lypowy et al 2005), which was a predicted G3BP target that surfaced from the initial BLAST search (Tourriere et al 2001). To conclude, G3BP is a phosphorylation-dependant RNase that cleaves specific mRNA transcripts and provides the first known link between extracellular signalling and mRNA stability.

Twist and shout: Another enzymatic ability of G3BP is that it may act as a helicase, a function that is seen in numerous enzymes involved in cellular tasks like DNA replication, DNA repair, transcription, translation, and RNA splicing (Matson et al 1994; Matson & Kaiser-Rogers 1990; Sancar & Sancar 1988). Costa et al. (1999) embarked on the task of isolating protein fractions from HeLa cell nuclear extracts that possessed helicase activity. This resulted in the isolation of several non-canonical helicases, one being that of G3BP1. For clarification, the familiar 'canonical' helicases are described as containing a characteristic DEAD/H[§] amino acid motif (Ellis 1997). Use of an *in vitro* helicase assay demonstrated that G3BP's helicase activity is dependant on ATP and magnesium (N.B.: ATP was shown to directly bind to G3BP). This helicase activity was functional on DNA, RNA, DNA/RNA double helixes with relatively similar efficiencies.

Other defined specifics for this enzymatic activity exist. First, G3BP is most proficient at unwinding double helixes that have shortly unannealed ends, also described as "hanging tails" or fork-like structures. Second, the direction of unwinding is

[§] DEAD/H: signifies the amino acid sequence where D corresponds to aspartic acid, E to glutamic acid, A to alanine, and H to histidine.

unidirectional, being in the 5' to 3' direction. Third, this helicase is non-processive for its unwinding activity is inversely proportional to the length of the duplex. One should note that whether if this helicase ability has any significance *in vivo* is still unknown. Thus, G3BP can be classified as a non-canonical helicase that does the twist in an ATP and Mg^{+2} dependant beat. Whether or not it can shout has yet to be heard.

Cellular localization: the many places G3BP calls home

Cytoplasmic localization: Within proliferating cultured cell lines, G3BP1 is seen as a cytosolic protein (French et al 2002; Gallouzi et al 1998; Parker et al 1996) and remains so throughout the cell cycle (Parker et al 1996). Deeper analysis by Gallouzi et al. (1998) with centrifugation techniques demonstrated that in proliferating tissue, the G3BP—RasGAP complex is specifically localized to the plasma membrane.

Conversely to G3BP1 being cytoplasmic in proliferating NIH 3T3*RasGAP*$^{+/-}$ cells (Tourriere et al 2001), G3BP2 is observed to acquire a cytoplasmic localization during growth arrest (by serum starvation) in comparable cells (NIH 3T3) (French et al 2002). The effect of sera on G3BPs localization may have tissue specificity for GFP-tagged G3BP2 constructs are seen as cytoplasmic in proliferating HeLa cells (Prigent et al 2000), and G3BP1 remains cytosolic in serum starved ER22 cells (Parker et al 1996) and SKBR3 cells (Barnes et al 2002). Some evidence has implicated that the RNA-binding domain of G3BP promotes its cytoplasmic localization (Prigent et al 2000).

Nuclear localization: G3BP family members are nuclear entities under several, or certain, conditions. We first note that identification of G3BP as a helicase was determined through the examination of *nuclear* extracts.

Conversely to G3BP2 being nuclear in proliferating NIH 3T3 cells (French et al 2002), G3BP1 is observed to acquire a nuclear localization during growth arrest (by

serum starvation) in comparable cells (NIH 3T3*RasGAP*$^{+/-}$) (Tourriere et al 2001). Once again, the effect of sera on G3BP localization appears to be tissue specific for treatment of SKBR3 cells with the growth factor Heregulin promotes G3BP1's nuclear accumulation (Barnes et al 2002). Contrary to G3BP1, G3BP2 has been noted to be nuclear in several breast tumours, and in the healthy tissue that bordered the tumour (French et al 2002).

Granule structures: Within the cytoplasm, G3BP can be constrained within different granule structures constructed of proteins and RNA molecules. One such structure is the motile RNA-protein granule within neuronal cells, where such granules are observed to punctuate along the length of axons (Atlas et al 2004). Another structure is the stress granule, which normally form under conditions of stress such as elevated heat and oxidative conditions (Cande et al 2004; Hofmann et al 2006; Hua & Zhou 2004; Tourriere et al 2003). Interestingly, is has been observed that G3BP can promote the formation of stress granules in the absence of stress solely by its mere overexpression (Tourriere et al 2003). The 'Rationale' section of this thesis will re-address this topic.

To conclude, G3BP proteins can acquire distinct cellular locations, which implicate them as having spatially regulated specific functionally. It is interesting to note how conditions that promote the nuclear localization of the G3BP1 and 2 isoform are opposite (in reference to the effect of sera specifically), such that the cytoplasm and the nucleus appear to contain either isoform, but not both. This may imply that the isoforms have distinct cellular roles from one another, or, this mutual migration may serve as a mechanism to compensate for the absence of a specific isoform within a cellular locale.

Biochemical promiscuity: several integral processes associated with G3BP

To this date, G3BP has no well-defined physiological role in the cell. Yet, this RNA-binding protein has been observed to affiliate with numerous biochemical processes. Therefore, one may view the question, "what does G3BP do?" as better phrased as, "what does G3BP *not* do?".

Absence of G3BP1 results in neonatal lethality: Recently, the Tazi Laboratory successfully generated G3BP1 *knock-out* ($G3BP^{-/-}$) mice, whereby the homozygous progeny where phenotypically non-viable and had notable growth retardation (Zekri et al 2005). Additionally, the $G3BP^{-/-}$ neonates were unable to breathe, showed signs of cyanosis, and had elevated levels of cell death within the nervous system, notably of hippocampal, cortical, and internal capsule cells. Zekri et al. (2005) subsequently compared the proliferation levels and gene expression profiles between Mouse Embryonic Fibroblasts (MEFs) of $G3BP^{-/-}$ and wild type neonates. Though the morphology and cell cycle distribution of the cell types were similar, $G3BP^{-/-}$ MEFs had a 65% lower proliferation rate. Gene Chip analysis revealed that the absence of G3BP1 resulted in the several fold increase, and decrease, of numerous genes, whereby ones implicated in cell signalling and control of cellular proliferation were of the majority. The observed increased expression level of certain growth arrest genes partly divulged the mechanism for the reduced proliferation rate of $G3BP^{-/-}$ MEFs. One such gene being *GAS5* (a snoRNA expressed during growth arrest (Coccia et al 1992)), where its abundance was due to abolishing its endonucleolytic degradation by G3BP (Zekri et al 2005). Additionally, these results demonstrate that G3BP has gained in physiological necessity throughout evolution. In lower organisms, like *Drosophila*, the orthologous Rasputin-null organisms are viable (Pazman et al 2000). To conclude, G3BP is essential

for proper embryonic growth and development. Its ability to degrade specific RNA transcripts appears integral to regulating cellular proliferation.

Growth and development of certain tissues involves G3BP: To complement the observations of G3BP being essential in embryonic growth and development, studies have shown its involvement in the growth and differentiation of specific tissues. To link this discussion with the previous paragraph that addressed $G3BP^{-/-}$ mice having elevated neuronal cell death, we will now present evidence that implicates G3BP with the differentiation of neurons. Neuronal differentiation is a complex process orchestrated through many events, one in general being the transportation of key mRNA transcripts to their site of need in order to perform spatial specific translation. One such mRNA is that of *tau*, which is transported from the cell body of the neuron to regions of the axon by means of its formation into a motile RNA-protein granule (RNP) (Aronov et al 2002; Binder et al 1985; Schuman 1999). Atlas et al. (2004) sought out to characterize the protein entities that compose the *tau* RNP by performing GST-pulldown experiments with GST-HuD as the bait— HuD being a previously known protein member of the complex (Aronov et al 2002). They identified 4 proteins in the complex, two of them being G3BP1 and IGF-II mRNA binding protein 1 (IMP-1), which were studied in greater detail. First, they identified that the interactions between G3PB, HuD, and IMP-1 was RNase-sensitive showing that their associations are mediated by their binding with mRNA, and that of *tau* mRNA specifically. These proteins did not appear to compete for binding regions on the RNA. Second, they confirmed this protein assemblage exists in living systems by immunoprecipitations of extracts of embryonic mouse brain. Third, immunofluorescence assays showed the localization of the proteins in differentiated P19 cells, where their co-localization was most apparent in the cell body of the neuron.

Interesting observations surfaced when Westerns and immunoprecipitations, respectively, characterized the protein levels and the interactions between the proteins throughout the process of P19 differentiation. IMP-1 and G3BP were roughly evenly expressed throughout the 8 day process, IMP-1 being slightly higher on day 2, G3BP being maximal on day 4, while an HuD prominence was observed by day 4. Further observations showed that the order of magnitude of the interactions between the three proteins, on *tau* mRNA, changes during the process. G3BP and IMP-1 co-precipitated throughout differentiation but was maximal early on, while HuD and G3BP co-precipitated starting day 6. Atlas et al. (2004) conclude their observations with the statement that the early interaction between IMP-1 and G3BP likely serves to control the initial phases of *tau* mRNA RNP assembly and expression regulation in the cytoplasm. Recently, another study has observed that G3BP2a to also be a member within RNP complexes of actively translated mRNAs in neuronal synapses (Angenstein et al 2002). Thus, G3BP may mediate neuronal function and differentiation through the formation of RNP's, such as that which is seen with *tau* mRNA and others.

One publication of interest has shown that the Drosophila homologue of G3BP, Rasputin (Rin), is required as an effector in Ras-signalling for proper eye development. Development and differentiation of the *Drosophila* eye is dependant on Ras-signalling (Simon et al 1991) and has proven to be a model system to identify novel components and effectors involved in Ras metabolic pathways (Freeman 1997; Wasserman et al 1995). Pazman et al. (2000) used such a model system to search for additional protein members in Ras pathways. One isolated mutant strain of fly displayed defects in: a) photoreceptor recruitment, and differentiation, and b) ommatidial polarity, such that the eyes have a roughened appearance. Characterization of the mutant strain classified the effect to be due

to mutations only in *rin*— this being confirmed by reversing the mutant phenotype upon re-introducing the full-length cDNA for the gene. Curiously, overexpression of *rin* produced the same eye abnormalities. The authors proclaim that this loss- and equivalent gain-of-function polarity phenotype phenomena is similar to those produced by other genes that function in tissue polarity (Strutt et al 1997). To further characterize Rin's metabolic place in Ras signalling, Pazman et al. (2000) analyzed genetic interactions and complementation between Rin and Ras pathway components. In brief, their observations showed that Rin functions early in Ras signalling pathways at the level of the Ras protein or above. This analysis also noted that Rin appears to play a positive role in signalling downstream of RhoA, another metabolic signal transduction pathway. The main conclusions established from the observations of Rin being required for *Drosophila* eye development is that it represents a clear role in early Ras-signal transduction cascades. The authors speculate that this may be due to modulating the activity of the *Drosophila* homologue of RasGAP. The fact that *rin* was found to interact genetically with RhoA signalling was seen as surprising since that pathway was not observed to have common members to that of Ras pathways (Pazman et al 2000). It appears that research tied to Rin has identified a protein link between Ras and Rho signal transduction cascades.

Cellular growth is not equivalent to proliferation, but rather signifies an increase in cell size and mass. It involves the increase in the production of total cellular proteins, and such, their corresponding mRNA's. The method by which total mRNA synthesis is regulated during cell growth is unknown but Abdellatif et al. (1998) noted that overexpression of RasGAP produces an upregulation of mRNA synthesis. A subsequent study from the same laboratory sought out to further delineate this pathway using a model system for cell growth: cardiac myocyte cells (Lypowy et al 2005). This study

demonstrated that RasGAP's interaction with Filamin-C (FLN-C), a protein that cross links actin filaments, is necessary for the upregulation of mRNA synthesis. Disruption of the interaction, or knock-down of either protein, resulted in significant diminution of marker mRNA levels and inhibited myocyte growth. The mechanism as to how RasGAP—FLN-C might regulate gene expression was unknown but it was hypothesized that this interaction may regulate the function of a protein involved in mRNA expression. Since G3BP1 was a known RNA-binding protein that associates with RasGAP, it became scrutinized it their studies. Remarkably, disruption of the RasGAP—FLN-C interaction, which did not disrupt the RasGAP—G3BP interaction, decreased G3BP's ability to associate with mRNA. The mRNA of *cdk7* was specifically analyzed whereby its promoted release from G3BP, through disruption of the RasGAP—FLN-C interaction, *decreased* its levels by 70%. This observation is significant for two reasons. One, it is the first incident that describes G3BP conferring a stabilizing effect on RNA. Two, Cyclin dependent kinase 7 (Cdk7) is a kinase that modulates the activity of RNA polymerase II, the polymerase responsible for mRNA transcription (Abdellatif et al 1998; Lypowy et al 2005). Thus, the stabilization of *cdk 7* by G3BP in a FLN-C dependant manner unifies the observations between the up-regulation of mRNA levels during cell growth and its dependence on the RasGAP—FLN-C association. To conclude, recent work has implicated G3BP as a player in the process of myocyte growth, curiously, by its apparent ability to *stabilize* the mRNA message encoding for Cdk7 or modulate key mRNA levels in general.

Regulation of genetic expression of self and by self: Information regarding the regulation of the expression of G3BPs is sparse and fragmented. Several studies have demonstrated the overexpression of all G3BP isoforms in proliferating cells, most notably

in cancer tissue, the significance of which will be described in a later section. In brief, G3BP1 is observed to be overexpressed in a variety of human tumours in a manner that is independent of the tissue source for the tumour (Barnes et al 2002; French et al 2002; Guitard et al 2001) and in proliferating retinal pigment epithelial cells (Kociok et al 1999). Likewise, the G3BP2 isoform is observed to be in abundance in a variety of breast tumours specifically, while corresponding healthy breast tissue contains virtually none (French et al 2002). The expression of G3BP does appear to show tissue specificity. Initial Northern blot analysis of human adult and fetal tissues demonstrated that G3BP1 mRNA appears to be ubiquitous but varying in amounts whereby its mRNA is observed to be low in some tissues, like spleen and brain, while very high in skeletal muscle (Parker et al 1996). Additional studies assessing the protein levels of G3BP in adult murine tissues by Western blotting show variability in the expression of all three isoforms (Kennedy et al 2001). Several tissues contain all three isoforms— like lung, colon, and kidney— while others had one or two. For example, spleen, muscle, and small intestine only contain G3BP1, G3BP2a, and G3BP2b, respectively. This tissue specific expression of G3BP alludes to the fact that the isoforms have specific functions in certain tissues and their biochemical roles may be distinct from one another. What is most interesting is to note the contradictions, like that of human *G3BP1* mRNA being highly expressed in muscle (Parker et al 1996) while the protein is nonexistent in mouse muscle (Kennedy et al 2001). Does this discrepancy provide us with an example that separates man from beast, or does it suggest that the means for the regulation of the protein expression of G3BP is far more complex than merely the transcription of its corresponding gene? Without a doubt, gene expression is known to be regulated via numerous means beyond that of transcription, such as by mRNA transport, mRNA stability, and at the level of

translation (Gallouzi & Steitz 2001; Mazroui et al 2006; Xu et al 2005). How, and if, these regulatory processes are involved in G3BP protein expression has yet to be determined, but evidence exists that G3BP is regulated on several levels.

Two examples in the literature demonstrate that extracellular signalling can regulate G3BP expression. It was observed that treatment of a breast cancer cell line with the growth factor, Heregulin, induced the expression of *G3BP1* mRNA which resulted in its increased protein levels (Barnes et al 2002). The other example of extracellular control of G3BP was observed by its protein upregulation in response to mitogenic stimulation of primary human T cells (Huang et al 2005). One should note that G3BP expression by extracellular signalling might be tissue specific and stimuli specific. Some studies did not show increased G3BP expression upon addition of serum to serum starved cells (Gallouzi et al 1998; Parker et al 1996) or epidermal growth factor (Parker et al 1996) with fibroblast cell lines.

Another study has demonstrated that G3BP1 protein levels are regulated by the tumour suppressor, PTEN (Huang et al 2005). By use of a PTEN-null leukemia cell line, the expression profiles of 800 proteins were analyzed by a high-throughput immunoblotting approach before and after reintroducing PTEN levels. They observed that G3BP protein levels decreased by three fold upon re-introduction of this tumour suppressor. It is unknown whether the effect of PTEN on G3BP expression is a direct or indirect effect, but the researchers of this study showed by real-time RT-PCR that the rate of *G3BP* gene transcription was independent of the presence of PTEN. PTEN has been noted to regulate cellular activity by numerous means, the most notable being through its phosphatase activity that specifically hydrolyzes, and thus inactivates, the potent second messenger, phosphatidylinositol-3,4,5-triphosphate (PIP_3) (Myers et al 1998). To

determine if the decrease in protein levels of G3BP by PTEN was due to its ability to inhibit signalling through PIP_3, Huang et al. (2005) employed the use of a phosphatase-dead PTEN (C124S, cannot hydrolyze PIP_3) and the inhibitors of the formation of PIP_3, wortmanin and LY294002. Indeed, use of these tools mimicked the observations seen with the absence of PTEN, thus deducing that G3BP protein expression controlled by PIP_3-dependant signalling is likely. Binding of PIP_3 modulates the activity of several proteins, most notably the serine/threonine kinase, Akt, which is known to indirectly modulate gene expression transcriptionally (Downward 2004), translationally (Richardson et al 2004), and post-translationally (Plas & Thompson 2003). Thus, Huang et al. (2005) conclude their observations by stating G3BP protein expression is likely controlled through the PIP_3/Akt nexus, presumably at the translational or post-translational level.

Another study observed that the transcription of G3BP was down-regulated in cells obtained from patients afflicted with Fragile X syndrome, the most commonly inherited form of mental retardation (Zhong et al 1999). The vast majority, but not all, of fragile X cases contain deleterious mutations in the *FMR1* gene, resulting in a non-functional form of FMRP (Fu et al 1991). The authors of the study state that their observations provide evidence for *G3BP* gene regulation by FMRP. A mechanism as to how FMRP might regulate G3BP expression was not investigated. An assumption may be that this regulation is at the post-transcriptional level since FMRP is a RNA-binding protein that is involved in processes of mRNA processing, transport, and translation. One should note that Fragile X may be caused by other genetic abnormalities (Hoogeveen et al 2002), so the observation of reduced levels of G3BP may be due to a more general attribute of the syndrome than just inactivation of FMRP. A deeper discussion concerning

G3BP and Fragile X syndrome is presented in the section: *Does G3BP have a role in the mechanism of disease?*.

Now let us change the focus of our discussion from the topic of genetic regulation *of* G3BP to that of genetic regulation *by* this RNA-binding protein. To begin, G3BP plays a role in gene expression at the level of transcription. Katsanfanas et al. (2004) observed that intermediate stage transcription of the vaccinia virus genome is complemented by G3BP and its association with the protein p137. Purification of HeLa cell extracts and subsequently using those in *in vitro* transcription assays first identified these transcriptional activities. The fraction with the highest activity was subjected to HPLC and mass spectroscopy for protein identification, revealing the presence of G3BP and p137. Repeating the *in vitro* transcription assays with recombinant forms of both proteins confirmed the specificity of this interaction. Katsanfanas et al. (2004) subsequently proved that G3BP and p137 exist as a heterodimer and both are able to induce transcription. The authors point the finger at G3BP's helicase activity to explain its involvement in this process. Plus, they noted that its requirement may be part of a mechanism to selectively produce virions in growing cells— which serve as best hosts— for it is in proliferating tissues that G3BP is most highly expressed (Guitard et al 2001; Rahmouni et al 2005). It appears that G3BP's function as a transcription regulator may be broader in scope than in viral replication. It has been previously shown in one case that when G3BP acquires a nuclear localization, by Heregulin stimulation, it co-localized specifically with acetylated histone H3 (Barnes et al 2002). Acetylated histone H3 is a well documented marker of sites of active transcription (Kruhlak et al 2001).

G3BP may also serve to regulate gene expression post-transcriptionally. As previously described, one of the first identified enzymatic qualities of this RNA-binding

protein was its ability to act as a phosphorylation-dependant RNase that may degrade specific mRNA transcripts. Evidence exists that its protein-binding partner, RasGAP, may modulate this activity. Tourriere et al. (2001) observed that though its RNA-binding ability is not hampered by the absence of RasGAP, G3BP's RNase activity appears to be absent in RasGAP$^{-/-}$ fibroblast cells, as monitored by the observed increased stability of its marker substrate, *c-myc* mRNA. This observation is strengthened by the fact that G3BP's phosphorylation status, key to its RNase activity (Gallouzi et al 1998), was also seen to differ between RasGAP$^{-/-}$ and RasGAP$^{+/-}$ cells. Specifically stated by Tourriere et al. (2001), it is observed that, "RasGAP$^{-/-}$ cells harbour a G3BP isoform in which Serine-149 is not phosphorylated". Additionally, knock-down of RasGAP, or inhibition of its interaction with G3BP, was shown in another work to inhibit the degradation of another G3BP target mRNA, that of *cdk9* (Lypowy et al 2005).

Some evidence exist that G3BP is not solely bent on destruction but may also regulate gene expression post-transcriptionally by conferring a protective influence on mRNA. Firstly, Lypowy et al. (2005) noted that G3BP, also in a RasGAP-association dependant manner, was able to bind and stabilize *cdk7* mRNA. Secondly, the previously mentioned binding of G3BP to *tau* mRNA was not related to its degradation, but rather stabilization (Atlas et al 2004). It is interesting to note that unlike the degraded *c-myc* and *cdk9* transcripts, the stabilized *cdk7* and *tau* transcripts do not contain the SELEX predicted consensus sequence for G3BP cleavage substrates (Lypowy et al 2005; Tourriere et al 2001). Thirdly, Zekri et al. (2005) noted that the expression of roughly an equal number of mRNA transcripts fall, as well as rise, in G3BP$^{-/-}$ MEFs; but whether or not this is directly due to G3BP is unknown. To conclude, G3BP appears to regulate

genetic expression post-transcriptionally, where its association with RasGAP is highly implicated with this function.

G3BP may regulate protein degradation: G3BP appears to regulate protein expression by potentially influencing a protein's targeting for degradation, as implicated through an observed protein-binding partner. Protein degradation is orchestrated via numerous means, one being the proteolytic degradation by the proteosome of substrates tagged with polymers of the peptide, ubiquitin (Hershko & Ciechanover 1998; Soncini et al 2001). The ubiquitination of proteins can be a reversible process mediated by Ubiquitin C-terminal Hydrolases and Ubiquitin Specific Proteases (USPs) (Ciechanover et al 2000). Through the use of a yeast two-hybrid system, Soncini et al. (2001) noted an interaction between the amino terminus of the ubiquitin specific protease, USP10, and G3BP. I*n vitro* binding assays and immunoprecipitation experiments done with lysates from a human osteosarcoma cell line confirmed the association between these entities. This interaction appears to be specific to USP10 since G3BP was not observed to associate with other USP's used as controls. This interaction was seen not to be influenced by the presence of serum, as is observed with G3BP's association with RasGAP (i.e.: diminished association between G3BP and RasGAP during serum starvation (Gallouzi et al 1998)). Soncini et al. (2001) subsequently sought out to identify the functional significance of the G3BP-USP10 partnership by use of an *in vitro* de-ubiquitination assay. Interestingly, the presence of recombinant G3BP strongly inhibited USP10's ability to de-ubiquitinate its substrate, marking it as a negative regulator of USP10. It appears that G3BP's role as a regulator in ubiquitination is of early evolutionary origin for the yeast homologues for G3BP and USP10, Bre5 and Ubp3 respectively, were also shown to associate within a de-ubiquitinating complex (Cohen et al 2003a). To conclude, it appears that G3BP might

regulate protein expression by indirectly regulating the ubiquitination status of certain proteins.

Control of NF-κB signalling linked to G3BP: Though few in number, one publication on the G3BP2 isoform has expanded the potential cellular roles for G3BP proteins to include regulation of key metabolic signalling pathways. NF-κB is a transcription factor that has been immensely scrutinized for it is known to play a major role in directly inducing expression of genes involved in immune, inflammatory, anti-apoptotic, and stress, responses (Baldwin 1996; Devary et al 1993; Marok et al 1996; May & Ghosh 1998). Within an unstimulated cell, NF-κB is retained in the cytoplasm in an inactive form by tight association with its inhibitor protein, IκB (Henkel et al 1992). Upon stimulus, activated kinases phosphorylate IκB, resulting in its release of NF-κB and its degradation by the ubiquitin-proteosome pathway (DiDonato et al 1997; Scherer et al 1995). Once released, NF-κB translocates into the nucleus and activates transcription of pertinent genes, including that of IκB. IκB can shuttle between the cytoplasm and the nucleus, removing NF-κB from the nucleus and returning it to its cytoplasmic resting place along the way (Arenzana-Seisdedos et al 1995; Sun et al 1993).

The mechanism behind the cytoplasmic-nuclear shuttling and cytoplasmic retention of IκB and the IκB— NF-κB complex is ill defined. This process has been explained partly by research performed by Prigent et al. (2000) on HeLa cells. Through their mutational analyses, they were able to define amino acid sequences of IκB that are responsible for its cytoplasmic retention. To identify proteins that interact with— and thus might regulate— the defined Cytoplasmic Retention Motif (CRM) of IκB, researchers performed transient transfections and subsequent immunoprecipitations with the motif. G3BP2 did co-immunoprecipitated with this entity. This interaction was

confirmed by *in vitro* binding assays with recombinant GST-IκB, and *in vivo* by observed co-immunoprecipitation of an IκB/NFκB/G3BP2 complex. Mutational analysis and subsequent co-immunoprecipitation studies identified the region of G3BP2 responsible for its interaction with the CRM of IκB. Deletions in the central acidic rich region prevented complex formation while deletions in the RNA-binding and NTF2-like domain did not. To complete their study, Prigent et al. (2000) identified that the function for the formation of this complex serves to retain IκB in the cytoplasm, as shown by fluorescence imaging of GFP-tagged G3BP2. In brief, overexpression of G3BP2 by transient transfections resulted in titrating out IκB from the nucleus, which did not occur with IκB containing deletions in its CRM. In summary, one of the sole publications on G3BP2 identified its role in retaining IκB and the IκB— NF-κB complex in the cytoplasm during a resting cell state. This function may be key to regulating aspects of NF-κB signalling.

T-cell activation augmented by G3BP: Through its ability to associate with the protein tyrosine kinase, Csk, G3BP appears to have a role in immune responses. T-cells, or thymus-derived lymphocytes, are entities of the immune system that respond to antigens, by means of them binding to antigen specific surface receptors, with the eventual production of antibodies against the antigen (Pelczar et al 1993). The mechanism behind resultant signal transduction cascades from T-cell-antigen receptor activation (or simply "T-cell activation") has been intensely investigated, such that it is known that protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP) play crucial roles (Mustelin et al 2002a; Mustelin et al 2002b). One such PTK is Lck, which becomes activated by posttranslational modifications upon T-cell receptor activation (TCR) and subsequently induces numerous signal transduction cascades (Rahmouni et al 2005). Since PTKs like Lck play central roles in T-cell activation, Rahmouni et al. (2005)

hypothesized that they must be tightly regulated and thus wished to define mechanisms by which this is done. It was previously shown that the activity of Lck could be inhibited via phosphorylation of its amino acid residue tyrosine 505 (Y505) by the PTK, Csk (Bergman et al 1992). How the interaction between these two PTKs was controlled became the focus of investigation.

Rahmouni et al. (2005) commenced their studies by identifying proteins that complex with Csk in Jurkat T cells by pull down experiments with recombinant GST-Csk. MALDI-TOF spectrometry identified that the main protein isolated from the analysis to be that of G3BP. Co-immunoprecipitations confirmed this interaction, but only in *activated* T-cells, signifying that the interaction was inducible. They employed immunoblotting techniques to investigate whether if the phosphorylation status of Lck on Y505 could be influenced by G3BP. Indeed, overexpression of GFP-G3BP did decrease the phosphorylation of this protein. Observations defined that the basis for the mechanism by which this occurs is through G3BP's ability to isolate spatially Csk. Immunofluorescence imaging demonstrated sequestration that the G3BP—Csk complex in the cytoplasm, away from Lck that resides at lipid rafts in the plasma membrane. Csk's sequestration from Lck was not apparent in non-activated T-cells. Additionally, knock-down of G3BP by RNA interference had the converse effect as its overexpression on Lck Y505 phosphorylation. G3BP's positive role in T-cell activation was further exemplified by observing reduced expression of TCR responsive genes in activated T-cells with knocked-down G3BP— the opposite being observed with G3BP's overexpression. As previously stated, the G3BP—Csk complex is inducible. This may be explained by Rahmouni et al.'s (2005) observation that G3BP became phosphorylated upon TCR activation, specifically on tyrosine residue 56. To conclude, it appears that G3BP serves

as a key regulator in T-cell activation through inducible sequestration of the inhibitory protein, Csk, from its substrate Lck.

As final summation, G3BP's 'promiscuity' in cellular biochemistry allows one to believe that it is obviously a multifunctional protein, which has evolved a variety of physiological roles (summarized in Table 2).

Does G3BP have a role in the mechanism of disease?

Interesting avenues of research have emerged concerning G3BP family members from their observed implication in specific maladies. As well as identifying G3BP as a potential therapeutic target, its liaison with a diseased state provides clues to its physiological role in the cell.

Cancer and tumourigenesis: The association of G3BP proteins in the process of tumourigenesis is particularly noteworthy (Figure B). Northern and Western blot analysis of dozens of human cancer cell lines, examples being HeLa (a commonly studied endometrial carcinoma) and H1299 (a $p53^{-/-}$ lung carcinoma), showed that G3BP was upregulated in virtually all samples assessed, in comparison to controls. This overabundance appeared to be independent of the site of origin of the tumour or its stage of development (Guitard et al 2001). This overexpression is dramatic, being five to ten-folds higher than healthy tissue. To determine what possible ramifications vast abundance of G3BP may have, Guitard et al. (2001) subsequently transfected NIH 3T3 cells with G3BP and assessed their proliferation via bromodeoxy uridine incorporation assays. These cells markedly entered S-phase of the cell cycle in a manner that was dose dependant on the quantity of the *G3BP* plasmid transfected. Remarkably, twin transfections with a truncated version of G3BP, one that does not contain its RNA-binding domain, had no effect on cell cycle progression and could act as a dominant-

Table 2.

Summary of Implicated Functions of G3BP Proteins		
Implicated function or associated biochemical pathway	**Associated observations or proposed mechanism**	**Reference**
Effector in Ras-signalling pathways	• Binds RasGAP • Linked to Ras pathways in drosophila	(Gallouzi et al 1998; Parker et al 1996; Pazman et al 2000)
Post-transcriptional regulation of mRNA expression (degradation)	• Phosphorylation-dependant RNase • Cleavage of specific transcripts	(Gallouzi et al 1998; Lypowy et al 2005; Tourriere et al 2001; Zekri et al 2005)
Post-transcriptional regulation of mRNA expression (stabilization)	• Association with *tau* and *cdk7* has stabilizing effect	(Atlas et al 2004; Lypowy et al 2005)
Helicase activity, non-canonical helicase	• Unwinds DNA, RNA, DNA/RNA double helices	(Costa et al 1999)
Tissue development and differentiation	• Axon development of P19 cells and neuronal death in G3BP-null neonates • Down-regulated in Fragile-X syndrome • Eye development in *Drosophila* • Over-expression results in de-differentiation	(Atlas et al 2004; Kociok et al 1999; Pazman et al 2000; Zekri et al 2005; Zhong et al 1999)
Neonatal development and cellular proliferation	• Severe growth retardation in G3BP-null neonates	(Zekri et al 2005)
Cellular growth	• Growth of cardiac myocyte cells	(Lypowy et al 2005)
Pathogenic cellular proliferation	• Over-expression in numerous tumour types • Linked to Proliferative Vitreoretinopathy • Linked to 5q-syndrome • Expression regulated by tumour suppressor, PTEN	(Barnes et al 2002; Boultwood et al 2002; French et al 2002; Guitard et al 2001; Huang et al 2005; Kociok et al 1999)
Regulation of transcription	• Complements vaccinia virus transcription • Associates with acetylated histones	(Barnes et al 2002; Katsafanas & Moss 2004)
Regulation of protein degradation	• Inhibits activity of USP10 • Binds Ubp3 in yeast	(Cohen et al 2003a; Soncini et al 2001)
Role in Nf-kB signalling	• Binds IκB and IκB- NF-κB complex, sequesters it in cytoplasm	(Prigent et al 2000)
T-cell activation	• Regulates interaction between Csk and Lck	(Rahmouni et al 2005)

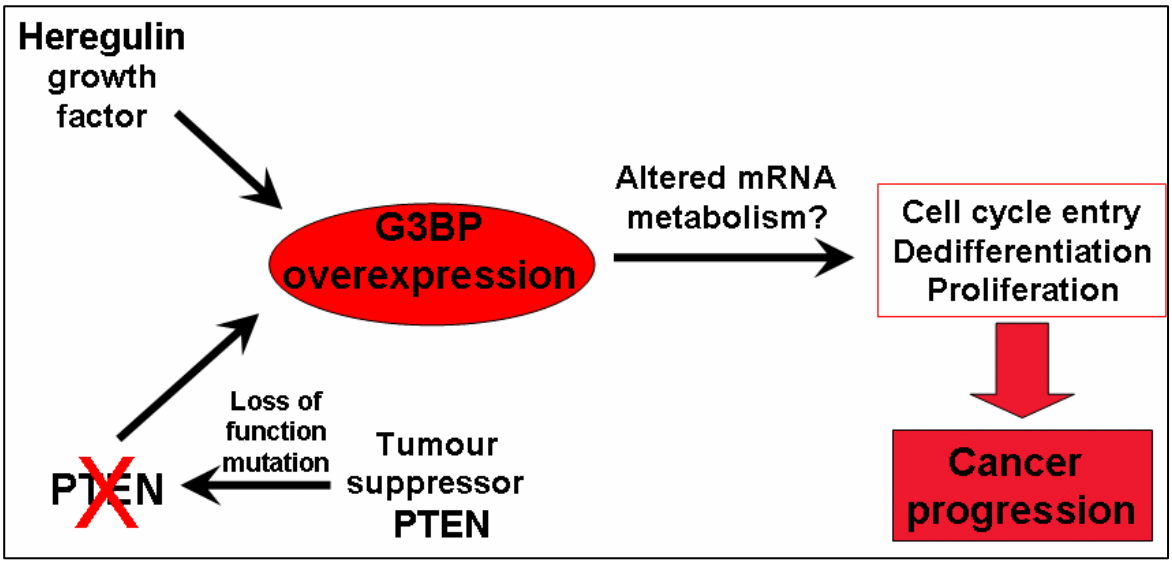


Figure B. G3BP and its link to cancer biology. G3BP proteins are dramatically overexpressed in numerous tumour types. The mechanism for the deregulation of G3BP protein levels is unclear. One possibility is that loss of function mutations of the tumour suppressor, PTEN, contributes to this process. Additionally, treatment of breast cancer cells with the growth factor, Heregulin, can increase cellular levels of the protein. Elevated G3BP levels might promote cancer progression. For one, G3BP's overexpression promotes cell cycle entry and proliferation, which appears coupled to its associated perturbation of mRNA metabolism. Furthermore, its overexpression may also participate in the dedifferentiation of tissue, which is characteristic of tumours.

negative repressor to the pro-proliferative effect of full length G3BP. G3BP's promotion of entry into the cell cycle appears to be a general trait since its specific overexpression was also observed in a separate investigation to increase the rate of cell cycle entry in primary human T cells (Huang et al 2005). A subsequent study assessed the expression profile of all G3BP isoforms in human breast cancers, specifically (French et al 2002). Along with G3BP1 overexpression, this study observed that the G3BP2 isoform was abnormally high in 88% of breast tumours assayed by immunohistological techniques. Once again, there was no observed correlation between G3BP overexpression and the origin of the tumour. Interestingly, while G3BP1 was primarily cytoplasmic in breast cancer cells, the majority of breast tumours overexpressing G3BP2 had this protein prominently in the nucleus. A complementing study provided further insight into these findings, where it was shown that G3BP expression was induced by Heregulin treatment of human breast tumours (Barnes et al 2002). Heregulin serves as a growth factor ligand for human epidermal growth factor receptors, which are seen to be overexpressed in breast tumours and other malignancies (Kumar et al 2000). Thus, the Heregulin-induced promotion of breast cancer cell growth may be due in part to the subsequent promoted expression of G3BP (Barnes et al 2002).

Proliferative Vitreoretinopathy: The overexpression of G3BP is also associated with the disease of Proliferative Vitreoretinopathy (PVR), which is a primary cause for failure of retinal reattachment surgery marked by abnormal dedifferentiation and proliferation of eye tissue. Using a model system for PVR (proliferative retinal pigment epithelial cell lines), changes in gene expression as the tissue mimicked a diseased state were assessed by PCR-based differential expressed mRNA analysis (Kociok et al 1999). Both the mRNA and protein levels of G3BP were observed to be upregulated in a proliferation-

dependant manner where the authors additionally suggest that this may play a role in the process of dedifferentiation (Kociok et al 1999). One should note that cancer cells are characterized as having acquired traits of dedifferentiated tissue (Bodey et al 2005), whereby the authors that characterized G3BP levels in human tumours also proclaimed that the overexpression of G3BP is likely to promote this process (Guitard et al 2001).

It is also interesting to note that, as mentioned previously, *G3BP* expression was observed to be down regulated by the tumour suppressor PTEN; a tumour suppressor that is mutated in numerous cancers and is implicated in pathways of proliferation and regulation of cell growth (Huang et al 2005). Additionally, restoration of PTEN in PTEN-null cells is seen to substantially reduce their rate of proliferation (Huang et al 2005). A proposition is that the observations of PTEN and G3BP in cancer are in and of the same thing. Therefore, a potential pathway to tumourigenesis appears to be 1) loss of function mutations in *PTEN*, which results in 2) releasing the inhibition of *G3BP* expression resulting in overexpression, generating 3) cellular proliferation and deregulated promotion of the cell cycle, possibly through its endonucleolytic degradation of growth arrest genes like *GAS5,* as previously described (Zekri et al 2005). To summarize, G3BP abundance manifests a diseased state by encouraging cellular proliferation apparently by promoting advancement of the cell cycle. This appears to be associated with its ability to associate with RNA.

5q-syndrome: 5q-syndrome is a myelodysplastic syndrome that causes refractory anemia, hypolobulated megakaryocytes, and an increased incidence of acute myeloid leukemia. Those afflicted all possess a deletion within the long arm of chromosome 5. One study sought out to characterize the most commonly deleted region of the chromosome from 16 patients, through Fluorescence In-Situ Hybridization. A region of 1.5 megabases

extending from 5q13 to 5q33 was narrowed down (Boultwood et al 2002). This region is observed to contain 16 novel and 24 characterized genes, G3BP1 being of the latter. Through comparison of expression levels with corresponding healthy tissue, 33 of the aforementioned genes were considered candidates, whereby the authors proclaim G3BP to be of primary interest, curiously without giving any explanation as to why (Boultwood et al 2002). It is interesting to note that this is the first incident where the possible underexpression of G3BP1 is associated with a higher incidence of cancer. Aside from the 5q-syndrome, other classes of deletions and abnormalities within, or proximal to, the *G3BP1* gene have previously been linked to myeloid leukemias (Horrigan et al 1999).

Fragile X syndrome: It was previously noted above that a reduction of G3BP mRNA levels occurs in the absence of the RNA-binding protein, FMR (FMRP), such as is the case in Fragile X syndrome. Though the syndrome, being the most common inherited mental retardation disorder, is known to be due primarily to the inactivation of the fragile X gene, *FMR1*, the details behind the causative effect of this inactivation are unknown (Verheij et al 1993). By the use of an RNA differential display assay, researchers identified differentially expressed transcripts in lymphoblasts obtained from fragile X syndrome patients (Zhong et al 1999). Strangely, this study only detected one mRNA to be of lower levels in comparison to controls— that of *G3BP* (by about 10 fold) — though FMRP is stated to bind a relatively large population (about 4%) of brain mRNAs[**]. *G3BP* mRNA levels were confirmed to be of low abundance in these cell lines by quantitative RT-PCR but the mechanism as to why remains unknown. Zhong et al. (1999) hypothesize that since FMRP plays a role in transcription, RNA transport, and translation, aberrations

[**] It is not stated why lymphoblasts were used instead of brain tissue but one may assume that this is due to the ethical implications of obtaining brain tissue, instead of blood, from living patients.

in any of these processes may directly, or indirectly, affect the expression of *G3BP* mRNA. As an aside note, Fragile X syndrome is associated with mutations in genes other than FMR1, such as Fragile X Related protein 2 (FXR2) (Hoogeveen et al 2002). FXR2 was another protein besides G3BP that was observed to decrease upon reintroduction of PTEN in the previously discussed study by Huang et al. (2005). If FMRP and FXR2 behave similarly, could a decrease in FXR2 also be a means to affect negatively the expression of G3BP, as observed with FMRP? An interesting question that should be addressed in future research endeavours. Regardless, linking G3BP to mental retardation is palatable. For one, G3BP$^{-/-}$ mice were observed to have excessive cell death in neuronal tissue specifically (Zekri et al 2005), and G3BP has been shown to help orchestrate the process of neuronal differentiation (Atlas et al 2004). To conclude, it appears evident that a decline in G3BP within neuronal tissues is detrimental to proper brain development and function.

Summary

In attempts to further our understanding of the regulation and mechanisms of signal transduction cascades that are at the nexus of Ras, the search for novel protein members within these pathways went underway. One result from these endeavours was the identification of the evolutionarily conserved G3BP RNA-binding proteins. These proteins appear to bridge extracellular signalling, from their ability to associate with RasGAP, with mRNA stability, since they function as phosphorylation-dependant RNases. Though the literature concerning these new members of the ribonucleoprotein (RNP)-family is diverse, a well-defined functional role for these proteins has remained elusive. Accumulative observations demonstrate that they are essential for proper embryological development by means pertinent to the proliferation, growth, and

development of tissues— most notably that of neuronal tissues. Preliminary observations show that G3BP may be of research interest in numerous fields due to their apparent multifunctional nature. G3BPs help mediate such diverse processes as T-cell activation, NFκ-B signalling, genetic expression at the transcriptional and posttranscriptional level, and protein degradation. It appears that G3BP proteins are becoming strongly associated with the biology of cancer and other nefarious forms of cellular proliferation, thus providing much impetus to gain further understanding of the functionality and regulation of these proteins. With the implication of G3BP proteins in an array of physiological roles after only a decade since their identification, it appears that most prominent research findings concerning them are yet to come. It is here that we point out an important aspect of G3BP that was purposefully masked in this introduction, that being it appears to play an important role in cellular responses to environmental stress. Our discussion and present research endeavour will now focus on the topic of G3BP1 and its cellular localization within a stressed cellular state. Our overarching goal is to contribute to gaining further insight on this enigmatic cellular entity and general methods by which our bodies heal at the cellular, and molecular, level.

Rationale

To what purpose should I trouble myself in searching out the secrets of the stars, having death or slavery continually before my eyes?

■ A question put to Pythagoras by Anaximenes (c. 600 B.C.), according to Montaigne

G3BP is implicated in numerous biochemical processes, though a well-defined role for the protein has yet to be defined. Thus, we seek to shed light onto the functionality of this protein through further investigation of key biochemical phenomena it has been associated with in previous research. One such phenomena being its connection to cellular responses to environmental stress (Gallouzi et al 1998; Parker et al 1996; Tourriere et al 2003).

Environmental stress is an umbrella term that signifies externally originating agents or circumstances that are toxic to living systems. They include biological agents, like viruses, physical agents, like heat and radiation, chemical agents, like poisons and strong oxidizers, and nutrient deprivation, such as serum starvation. A fundamental necessity of life is to maintain the integrity of one's tissues and to protect cells from damage accruing from one's environmental surroundings. Therefore, biochemical mechanisms have evolved to thwart the stress of daily existence that either serve to correct the damage or mediate regulated death if the cell is irreparable.

Regulated cell death, or apoptosis, is a biochemically orchestrated collapse of a cell that occurs through the induction of specific metabolic pathways with resultant morphological characteristics of cellular shrinkage (pyknosis), nuclear condensation and ruffling (karyorrhexis), and the eventual fragmentation of the cell into vesicle-like apoptotic bodies (reviewed in: Hacker 2000). Though this morphological transformation appears destructive, the plasma membrane of the apoptotic cell, and subsequent vesicles,

maintains integrity. Distinct from that of apoptosis, if a stress is particularly violent (example being severe blunt force), cells may die via necrosis, which signifies the physical phenomena of cellular rupturing.

Though hardly representative of the numerous metabolic pathways that execute apoptosis, major ones that will be pertinent to the results presented in this thesis include the induction of CASPases and Stress Activated Protein Kinases (SAPKs). SAPKs are a collection of protein members of the extracellular signal-regulated protein kinases (ERKs), which are integral to signal transduction cascades activated under conditions of stress and inflammation. Two prime examples being p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinases (JNK) (reviewed in Kyriakis & Avruch 1996). Unlike the majority of kinase cascades activated by *mitogenic* stimuli, these proteins become active by signals from inflammatory cytokines and stress-produced molecular markers. As an example, ultraviolet radiation can induce the hydrolysis of the plasma membrane component, sphingomyelin. A latter product of this being ceramide which can act as a second messenger that selectively activates JNK (Hannun 1994; Verheij et al 1996). The activation of SAPKs can promote apoptosis and cell cycle arrest by subsequent activation of transcription factors such as AP-1, NF-κB, c-JUN, and p53 (Devary et al 1992; Devary et al 1993; Hibi et al 1993; Ko & Prives 1996; Levine 1997)[††]. These factors induce the transcription of genes that will orchestrate the cellular response to stress. The p53 protein, also described as the 'cellular gatekeeper' (Levine 1997), is of particular interest. Virtually any environmental stress will activate this transcription factor by post-translational modifications (Ko & Prives 1996; Levine 1997;

[††] SAPKs target, and thus regulate, proteins additional to transcription factors that are essential members to stress responses.

Takekawa et al 2000). The degree of its activation will hold the balance of whether a cell will arrest its growth and repair stress-induced damage or self destruct via apoptosis. Loss of this cellular gatekeeper attenuates a cell's ability to respond aptly to stress and induce apoptosis, and such, is strongly attributed to carcinogenesis.

Aside from alterations in gene expression, endogenous proteins that can induce immediate changes in cellular metabolism upon their activation also orchestrate apoptosis. One pinnacle class of such proteins are the cysteine proteases (CASPases). CASPases are present in the cell as inactive zymogens, whereby apoptotic signals causes their proteolysis and resultant activation. Within the active state, these enzymes are of proteolytic function that are observed to have substrate specificity (Nicholson & Thornberry 1997). Once active, these proteases in effect shutdown cellular metabolism by their targeted attack of metabolic pathways that are essential to cellular viability. For example, CASPases cleave proteins integral to aerobic respiration, focal adhesion and components of the cytoskeleton, and initiate nuclear condensation and disintegration (Hacker 2000).

Contrary to death, the cell may transiently arrest its proliferation and attempt to protect itself or heal at the molecular level. One known example of such a situation is the formation of cytoplasmic punctuate bodies termed stress granules (SG), which are believed to confer a protective element under stress (reviewed in: Anderson & Kedersha 2002; Kedersha & Anderson 2002). SGs are dynamic bodies known to be an assemblage of mRNA transcripts and RNA-binding proteins like HuR, TIA, TIAR, eukaryotic initiation factors 3, 4G, and 4E (Kedersha & Anderson 2002; Mazroui et al 2006). Evidence seems to indicate that they serve as storage centres for RNA transcripts in an arrested state of translation. It appears that these granules sequester constitutively

expressed mRNA messages so that the translation of stress-induced mRNA transcripts will be favoured (Anderson & Kedersha 2002). Common stresses known to induce SG formation include mild UV, elevated heat, and the oxidative property of arsenite.

The following observations, previously characterized in the literature, prompted us to delve into deciphering G3BP's place in the scheme of a stressed environment. For one, G3BP is one of several protein entities recruited to the aforementioned stress granules. Tourriere and colleagues (2003) noted that G3BP appears to play a scaffolding role in the assembly of SG's where the protein oligomerizes through self-interactions with its NTF2-like domain. Additionally, a surprising attribute of G3BP is that it may be sequestered in stress granules by its mere overexpression, in the complete absence of stress (Tourriere et al 2003). Furthermore, its recruitment to SGs appears to be inducible, or at least regulated, through posttranslational modification of the protein. This posttranslational modification is in reference to the absence of phosphorylation of serine residue 149 (Hofmann et al 2006; Tourriere et al 2003). Moreover, the observations by Tourriere and colleagues (2003) strengthened perceptions that G3BP's cellular role is strongly associated with Ras-signalling. They noted that G3BP's ability to assemble SGs was faster in cells that have constitutively active Ras-signalling. To conclude, G3BP is linked to stress responses by its ability to have an assemblage role in the formation of SGs. Posttranslational modification of the protein appears to regulate this recruitment, which is possibly orchestrated through Ras-signal transduction cascades.

Some of the earliest research on G3BP demonstrated that it changes its subcellular localization upon treatment with the stress of serum starvation, where it becomes enriched in the nucleus (Gallouzi et al 1998; Tourriere et al 2001). On top of its relation to stress granule formation, phosphorylation at the serine residue 149 also relates to G3BP's

nuclear localization upon serum starvation, being phosphorylated in this situation. Interestingly, as with SG formation, Ras-signalling pathways appear to regulate its change in its subcellular localization by serum deprivation. More specifically, G3BP's phosphorylation on serine residue 149 is dependant on the presence of its binding partner, RasGAP (Tourriere et al 2001). It is unknown why G3BP translocates to the nucleus during serum starvation, but this is likely associated with an activation of its enzymatic activity. Phosphorylation of serine residue 149 induces G3BP's ability to act as an RNase where it may then serve to degrade specific mRNAs, mRNAs like *c-myc*, which encodes for a pro-proliferative protein whose impeded expression would be logical under serum deprivation (Gallouzi et al 1998; Tourriere et al 2001). To conclude, G3BP is linked to stress responses by its change in subcellular localization under serum starvation. Posttranslational modification of the protein appears to regulate it nuclear localization, where its binding partner, RasGAP, plays a role in this process. The stress-induced phosphorylation of G3BP apparently serves to activate its enzymatic function as an RNase.

As a final note on posttranslational modifications of G3BP and stress, we *stress* the fact that its serine 149 residue is located within a Casein Kinase II (CK2) consensus site (Meggio et al 1994; Tourriere et al 2001). Whether or not this serine residue is indeed a target for this kinase has yet to be determined, but knowledge exists concerning CK2 and its induced activity by stress. For example, is observed to be active upon treatment with ionizing radiation and DNA-damaging agents and serves to moderate the progression of the cell cycle and apoptosis by phosphorylating key regulators like p53 (Olsten & Litchfield 2004; Yamane & Kinsella 2005a; 2005b). Since G3BP contains a

consensus site for a stress-activated kinase, it may likely be a stress-targeted substrate for CK2.

Our current research project aims to examine the role G3BP plays in cellular responses to environmental stress. Current knowledge indicates that this RNA-binding protein changes its subcellular localization under certain stresses, where it attains a higher structural organization by self-oligomerizing as it assembles into stress granules and accumulates in the nucleus as an active RNase under serum starvation. These changes in structure, enzymatic activity, and subcellular localization implicate it as having specific, inducible functions during stress. G3BP's stress-induced changes in subcellular localization appear influenced by Ras-signalling, a rational association since Ras-signalling does regulate many stress-induced metabolic pathways (ex.: apoptosis, cell cycle arrest, stress granule formation, others) (Feig & Buchsbaum 2002; Tourriere et al 2003). Overall, it seems logical that this RNA-binding protein may be a key player in stress-responses since a multitude of other RNA-binding proteins alter their subcellular localization, and function, under stressful conditions. Aside from the aforementioned list of members that compose stress granules, RNA-binding proteins involved in the regulation of mRNA transport and stability, like HuR (Gallouzi et al 2001; Gallouzi et al 2000), and translation, like proteins bound to the 28S rRNA of ribosomes (Iordanov et al 1998), are cases in point that show the diverse range of RNA-binding members linked to stress responses. There are many additional examples, but the main message conveyed here is that preliminary findings prognosticate that G3BP resembles other RNA-binding proteins from the perspective of directing cellular responses to environmental stress. We seek to affirm these preliminary findings.

We succeeded in further implicating G3BP1 as a key player in mammalian cellular responses to environmental stress by observing changes in its subcellular localization through immunofluorescence techniques (Figure C). Initial investigations with novel stresses absent in the current literature pertaining to G3BP showed that osmotic stress and low exposures to UVC promoted the protein's recruitment to stress granules. Remarkably, elevated UVC exposures induced its rapid accumulation within the nucleus where it remained more than a day after the stress. Our preliminary findings suggest that stress–activated protein kinases and CASPases are linked to this nuclear accumulation. Lastly, we hypothesize that G3BP's nuclear localization under elevated levels of UVC serves to promote apoptosis.

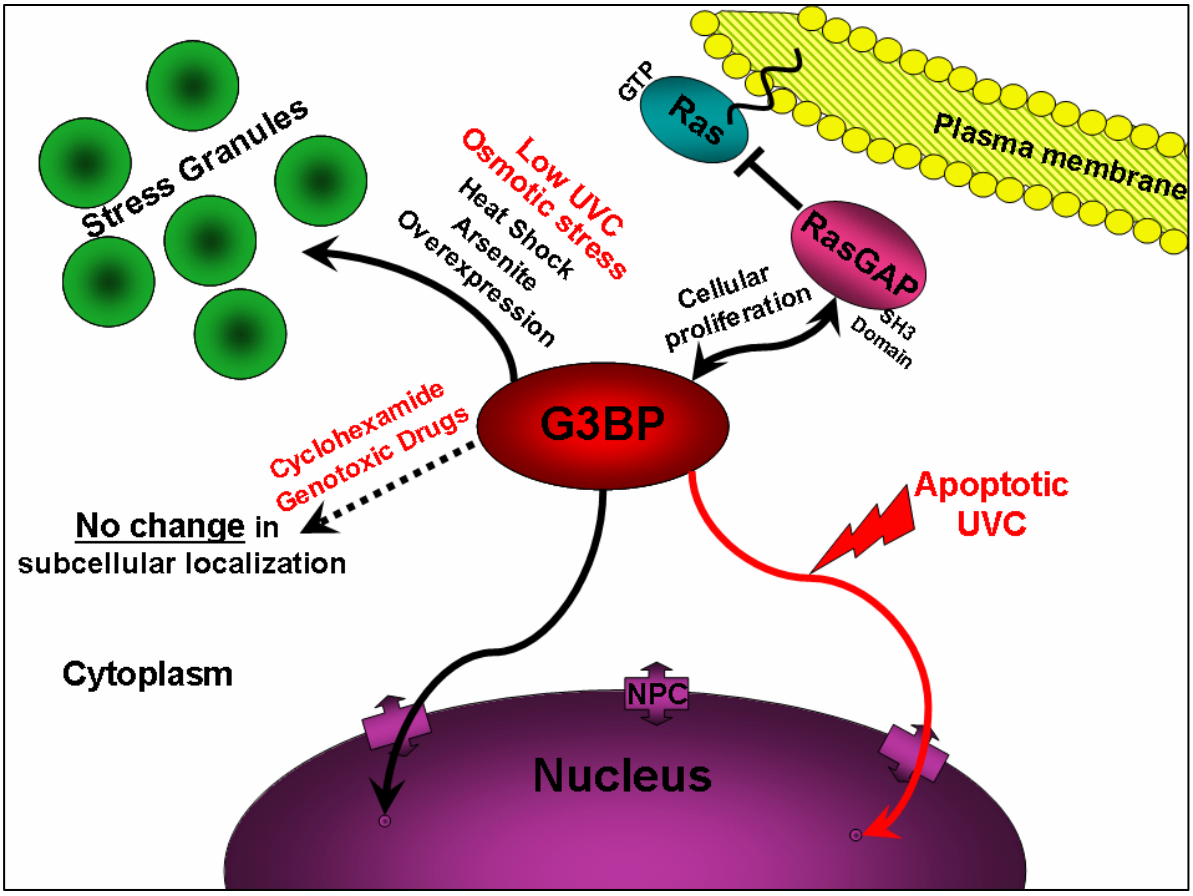


Figure C. G3BP changes its subcellular localization under stress. In its resting state, it resides in the cytoplasm. During cellular proliferation, a significant proportion of G3BP associates with RasGAP proximal to the plasma membrane. Under the stresses of elevated heat and arsenite treatment, G3BP assembles into cytoplasmic stress granules. G3BP's mere overexpression can induce the formation of stress granules. Under conditions of serum starvation, it accumulates in the nucleus. Highlighted in red are observations made during this thesis project. We observed that osmotic stress and low doses of ultraviolet radiation also sequester G3BP in stress granules. Treatment of cells with apoptotic levels of ultraviolet radiation induces G3BP's nuclear accumulation. Not all stresses cause changes in G3BP's subcellular localization: treatment of cells with toxins like cyclohexamide (data not shown) and genotoxic drugs did not induce a change in G3BP's localization. UVC: ultraviolet raiation C, NPC: Nuclear pore complex.

Results

Changes in G3BP's subcellular localization observed with novel stresses

To commence our investigations of G3BP's role in stress responses we reproduced observations reported previously with our model cell line, HeLa, and subsequently tested novel stresses that were absent in the current literature pertaining to G3BP. The localization of proteins of interest, namely G3BP and the RNA-binding protein HuR, were visualized by immunofluorescence (Figure 1). As expected, treatment of the cells with the oxidant stressor arsenite (0.5 mM for 30 minutes), elevated levels of heat (45^{o}C for 30 minutes, data not shown, for an example see Figure 4), and mere overexpression (data not shown) recruited G3BP to cytoplasmic stress granules (SGs). Confirmation that these interspersed bodies were in fact SGs was made through G3BP's observed colocalization with HuR, known to be recruited to SGs under such stresses (Mazroui et al 2006; Tourriere et al 2003). When we experimented with the novel osmotic stress via 0.5 M sorbitol treatment for 25 minutes, we observed a similar formation of SGs. Indeed, the SGs in this condition appear smaller and more dispersed, which we account to the short time frame of applying the stress before fixation of the cells for analysis. Our most interesting observations arose from testing a broad range of Ultraviolet Radiation C (UVC) levels, treatments varying in duration from seconds to minutes. A low UVC dose of $3x10^{-3}$ J/cm^2 (2 second exposure) was found to produce SG's when visualized 6 hours after the stress, whereby the cells are noted to be robust and viable in appearance. Remarkably, an elevated dose of 0.72 J/cm^2 UVC (3 minute exposure) resulted in the rapid accumulation of G3BP in the nucleus as observed by its colocalization with nuclear

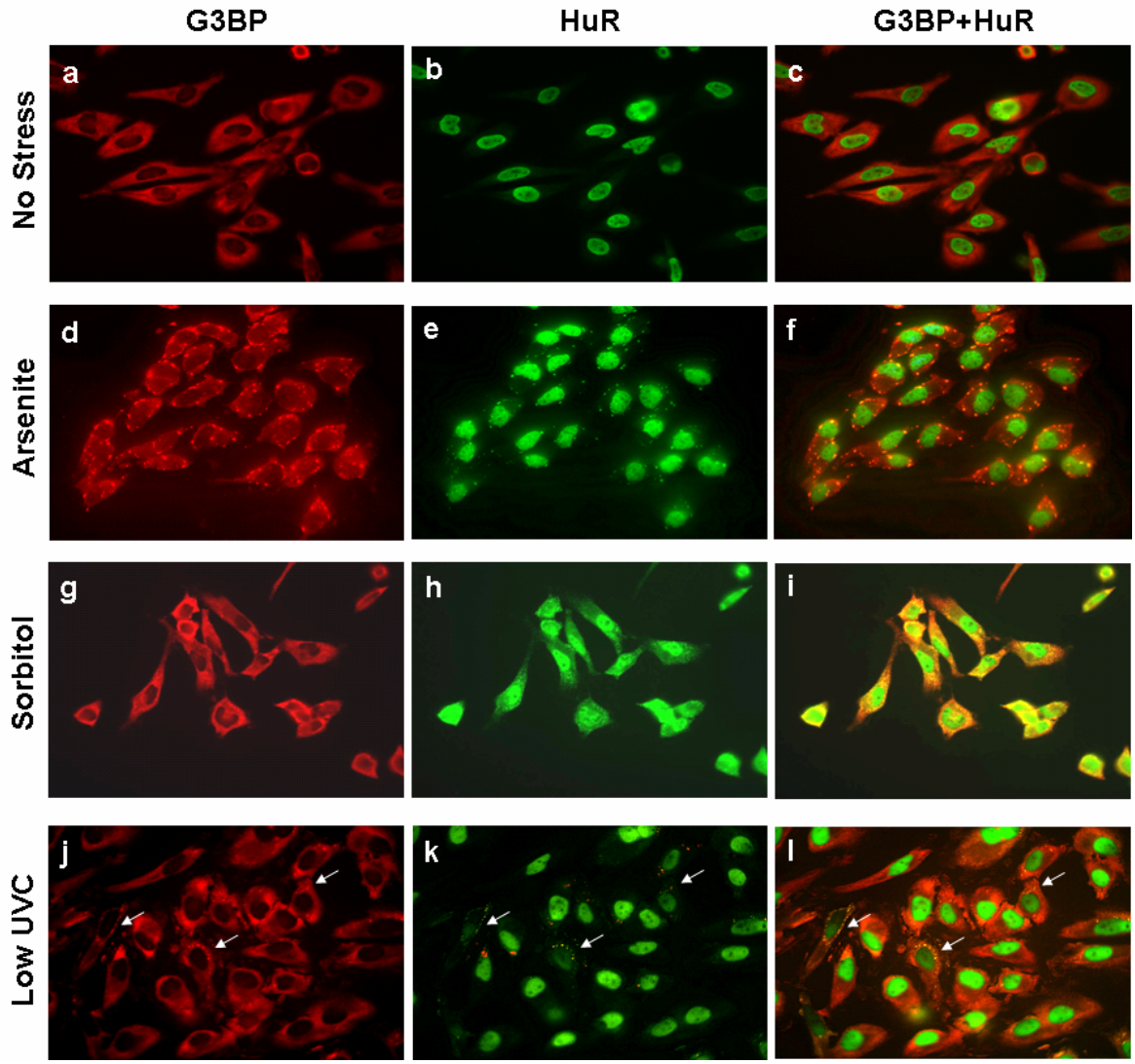


Figure 1. Oxidative, osmotic, and low UV stress recruit G3BP to cytoplasmic stress granules. Non-stressed cells display G3BP primarily in the cytoplasm (panel a) while the RNA-binding protein, HuR, is primarily in the nucleus (panel b). The two proteins do not co-localize in the absence of stress (panel c). As previously described, oxidative stress from arsenite treatment (0.5mM, 30 minutes) recruits G3BP to cytoplasmic stress granules (panel d), demonstrated by its co-localization with HuR (panel f, orange overlay). When treating cells with osmotic stress from sorbitol treatment (0.5M, 25 minutes) or low doses of UV ($3x10^{-3}$ J/cm^2, recovery 6 hours), cytoplasmic stress granules are observed as well (panels g and I, j and l, respectively). The stress granules for the sorbitol treatment are small and finely dispersed due to the short duration of applying the stress before fixation, but co-localization with HuR is evident (panel i).

DNA (DAPI staining), which appeared maximal one hour subsequent the stress (Figure 2). Nuclear localization of G3BP was confirmed by confocal microscopy of the samples presented in Figure 2, but the images obtained were of low quality due to inexperience with the microscope and will not be shown.

Interestingly, the kinetics of this nuclear localization mimic those of Barnes et al. (2002) whereby Heregulin-induced G3BP nuclear localization was observed one hour post-Heregulin treatment. Conversely to the low UVC treatments, these cells are overtly different in morphology in comparison to the non-stressed control; most notably they exhibit a decreased cytoplasmic and nuclear volume reminiscent of apoptotic cells (Hacker 2000). To conclude, different doses of the same UVC stress re-localized G3BP to different subcellular locales. It is noted in the literature that a cellular response to stress is dose dependent (Adler et al 1996b; Latonen et al 2001), which is confirmed by these observations.

SG formation through osmotic or low UV stress has been previously documented (Kedersha et al 1999). Thus, due to the gross cellular morphological changes and rapid nuclear accumulation of a primarily cytoplasmic entity, the observations obtained with elevated UVC doses were seen as most novel and prompted us to investigate these conditions further.

Characterization of G3BP's nuclear localization upon elevated UVC treatment

Further investigations sought out at identifying whether or not the observed nuclear accumulation was in fact occurring during the induction of apoptosis and whether the observations might be of a more general biochemical phenomenon. To affirm our belief that our UVC treatment was resulting in apoptotic cells specifically, and not necrotic cells, we performed two tests (Figure 3). The initial analysis was performed

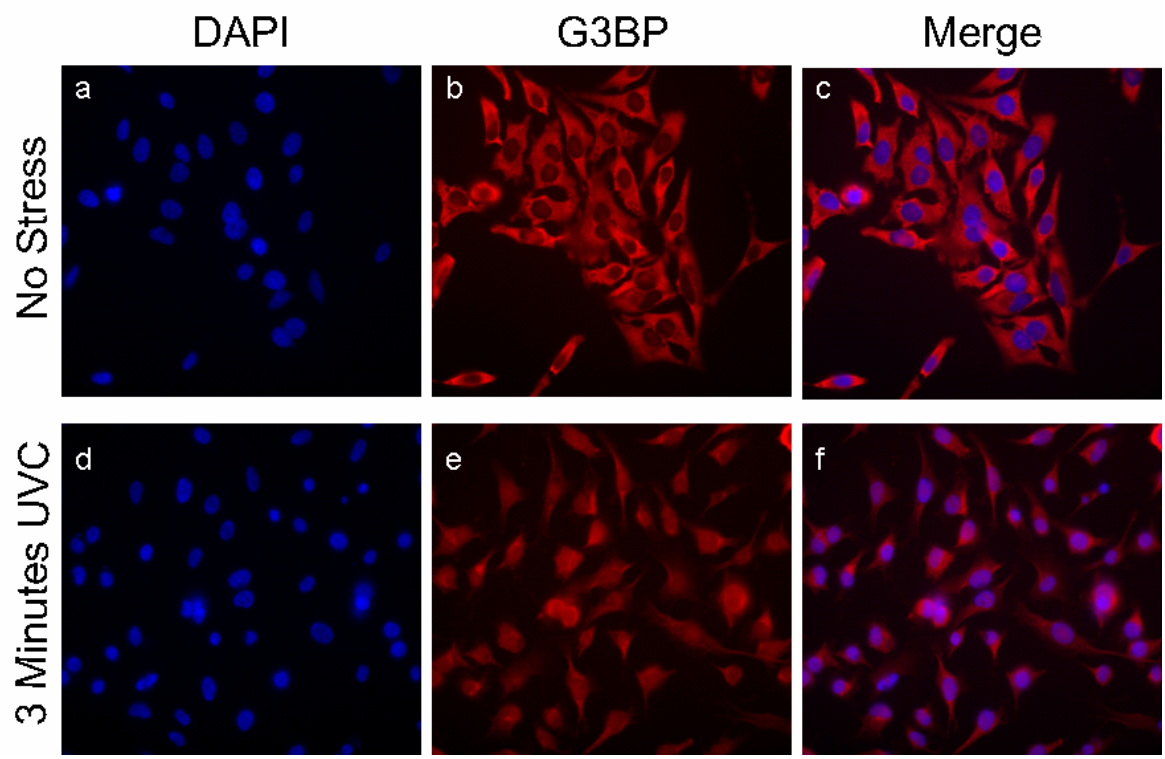


Figure 2. Elevated UV exposure moves G3BP from the cytoplasm to the nucleus. In non-stressed cells (panels a to c), G3BP is distributed roughly evenly throughout the cytoplasm. Treatment of cells with an elevated UV exposure of 3 minutes (0.72 J/cm^2) results in G3BP nuclear localization, as demonstrated by its co-localization with DNA (Merge DAPI/G3BP staining, panel f). Nuclear localization appeared maximal 1 hour after UV treatment. UV-irradiated cells are notably shrunken and apoptotic in appearance.

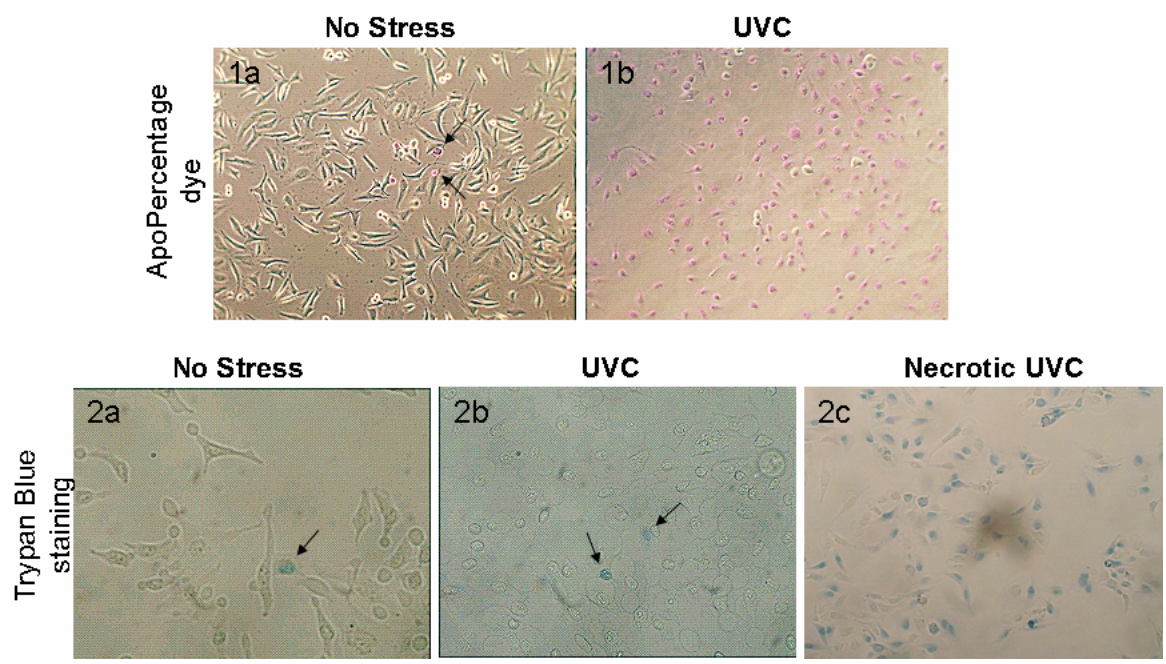


Figure 3. UV exposure produces apoptotic cells. The ApoPercentage apoptosis assay noted the induction of apoptosis upon a UV treatment of 0.72 J/cm^2 (panels 1a to 1b). Virtually no cells are labelled with the indicator dye in the Non-stressed sample (1a, arrows indicates minimal population that displays positive staining). Over 95% of the cell population displays positive staining 30 minutes after UV exposure (1b). Trypan Blue exclusion indicates that the experimental UV exposure does not produce necrotic cell death (panels 2a to 2c). The population of necrotic cells between the UV- and non-stressed samples are roughly equivalent (compare 2a with 2b, arrows indicates minimal population that displays positive staining). As a positive control for UV-induced necrotic cell death (2c), cells were irradiated with more than double the experimental dose of UV (1.68 J/cm^2).

with the APOP*ercentage*™ apoptosis assay, which labels specifically apoptotic, and not viable or necrotic, cells (BiocolorLTD. 2004). In brief, the assay capitalizes on the apoptotic phenomena of loss of asymmetry of phospholipids in the plasma membrane through the induction of 'floppases' (Zhou et al 1997), which result in the flipping of the substrate, phosphatidylserine, normally exclusively on the inner leaflet of the membrane, to the outer leaflet. This 'scrambling' of the plasma membrane allows for unidirectional cellular uptake of the purple labelling dye. The dye cannot be retained within necrotic cells (BiocolorLTD. 2004) for they no longer maintain membrane integrity. As demonstrated in Figure 3, panel 1b, virtually 100% of the cell population acquired positive staining 30 minutes post UVC treatment, while the converse is observed with the non-stressed sample (Figure 3, panel 1a). The second analysis was performed by Trypan Blue staining which specifically labels necrotic, and not viable or apoptotic, cells. In short, Trypan Blue specifically labels nuclear lamins and cannot permeate the plasma membrane, thus, positive staining is obtained only when there is loss of membrane integrity. The quantity of necrotic cells 1 hour post our conventional UVC treatment were comparable with the non-stressed sample (Figure 3, panels 2a and 2b). As a positive control, we attempted to induce necrotic cell death by exposing cells to an excessive UVC treatment of 1.68 J/cm^2 (7 minutes, more than double our conventional UV treatment). As expected, this population shows virtually 90% positive staining (Figure 3, panel 2c). In closing, G3BP accumulates in the nucleus in cells stressed with apoptotic levels of UV.

Cellular responses to environmental stress are dependant on the dose of the stress, where low amounts induce biochemical pathways that are distinct to those induced under high amounts of stress (Adler et al 1996b; Latonen et al 2001). Our current observations show that low UV treatment recruits G3BP to stress granules (which confer a protective

element to cells), while apoptotic UV treatment results in its nuclear localization. These observations become more significant when placed in perspective of a recently published work by Cande and colleagues (2004). They demonstrated that stress granule formation, and G3BP's ability to assemble these granules, is inhibited by the activation of the protein, Apoptosis Inducing Factor (AIF). Conclusions drawn from this work is that there are opposing forces in cellular responses to environmental stress — to heal or to die — and the induction of apoptosis inhibits biochemical pathways akin to cellular repair and survival. These conclusions had us question whether G3BP nuclear localization would be *dominant* or *favoured* over its recruitment to stress granules. If so, this might suggest that G3BP's nuclear localization is a phenomenon connected specifically to the process of apoptosis and serves some dominant role that is distinct to that of its function in stress granules. To address this question, we sequentially stressed cells with excessive heat (heat shock) and UV (Figure 4). Heat shock (45^{o}C, 30 minutes) followed by UV-irradiation (0.72 J/cm2, recovery 1 hour) localizes G3BP in both stress granules and the nucleus, as observed by its co-localization with HuR and DNA, respectively (Figure 4, b to d). Interestingly, prior UV-irradiation to heat shock produces solely G3BP nuclear localization, despite their remaining a significant amount of G3BP in the cytoplasm (Figure 4, f to h). It appears that initiating G3BP's nuclear localization prevents it from assembling stress granules. Since its nuclear localization occurs under apoptotic UV, these observations coincide with those of Cande et al. (2004). To conclude, under apoptotic conditions G3BP nuclear localization is favoured while being inhibited from forming 'pro-survival' stress granules.

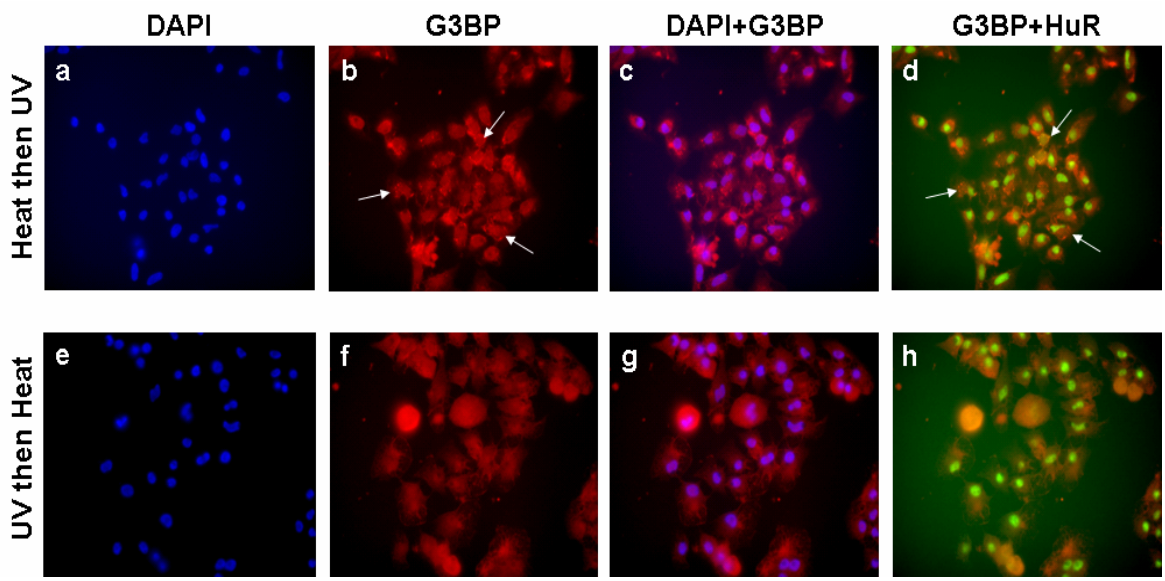


Figure 4. G3BP's nuclear localization is dominant over its recruitment to stress granules. Cells that are initially heat shocked at 45°C for 30 minutes and then UV irradiated (0.72 J/cm^2, 1 hour recovery) have G3BP localized to both cytoplasmic stress granules and the nucleus (panel b). Nuclear and stress granule localization is confirmed by the observed co-localization with DNA (DAPI) and HuR, respectively (panels c and d, respectively). Arrows indicate typical cells displaying both stress granules and nuclear localization. Conversely, cells that are UV irradiated initially (with 1 hour recovery), then heat shocked, produce only nuclear G3BP (panel f). No stress granules are observed despite their remaining a significant amount of G3BP in the cytoplasm (panel f and g).

To further characterize the experimental dose of UVC, we addressed whether if G3BP's nuclear accumulation shows tissue specificity or occurs in a more degenerate fashion. A variety of both murine, like NIH 3T3, NIH^{scr}, C2C12, MEF (data not shown) and human HEK 293, 293T cell (data not shown) were UV stressed and visualized according to our experimental methods developed with the HeLa cell line[‡‡]. These cell lines responded to UV treatment in a roughly equivalent fashion, where G3BP's nuclear accumulation appeared maximal 1 hour after the exposure. It now appears evident that G3BP's nuclear localization with our experimental conditions is not tissue specific.

Phosphorylation mutants of G3BP demonstrate that forcing its nuclear localization appears to induce apoptosis

It had been previously shown that the phosphorylation status of G3BP mediates its endoribonuclease activity and is relevant to changes in its sub-cellular localization under conditions of stress. Specifically, phosphorylation of its serine residue 149 appears to promote its nuclear localization under conditions of serum starvation (Gallouzi et al 1998; Tourriere et al 2001) while the absence of phosphorylation at this residue appears to regulate its ability to assemble stress granules (Tourriere et al 2003). We wished to address whether the phosphorylation status of G3BP was pertinent to our observations with UV, and such, utilized a series of previously characterized (Tourriere et al 2001) plasmid constructs of eukaryotic Green Fluorescent Protein (GFP) tagged- G3BP1 with amino acid substitutions in residues known to be phosphorylated. For these constructs, the amino acid substitutions are of the residues serine 149 and serine 232. These residues are replaced with either an alanine (A), which mimics a non-phosphorylated serine, or a

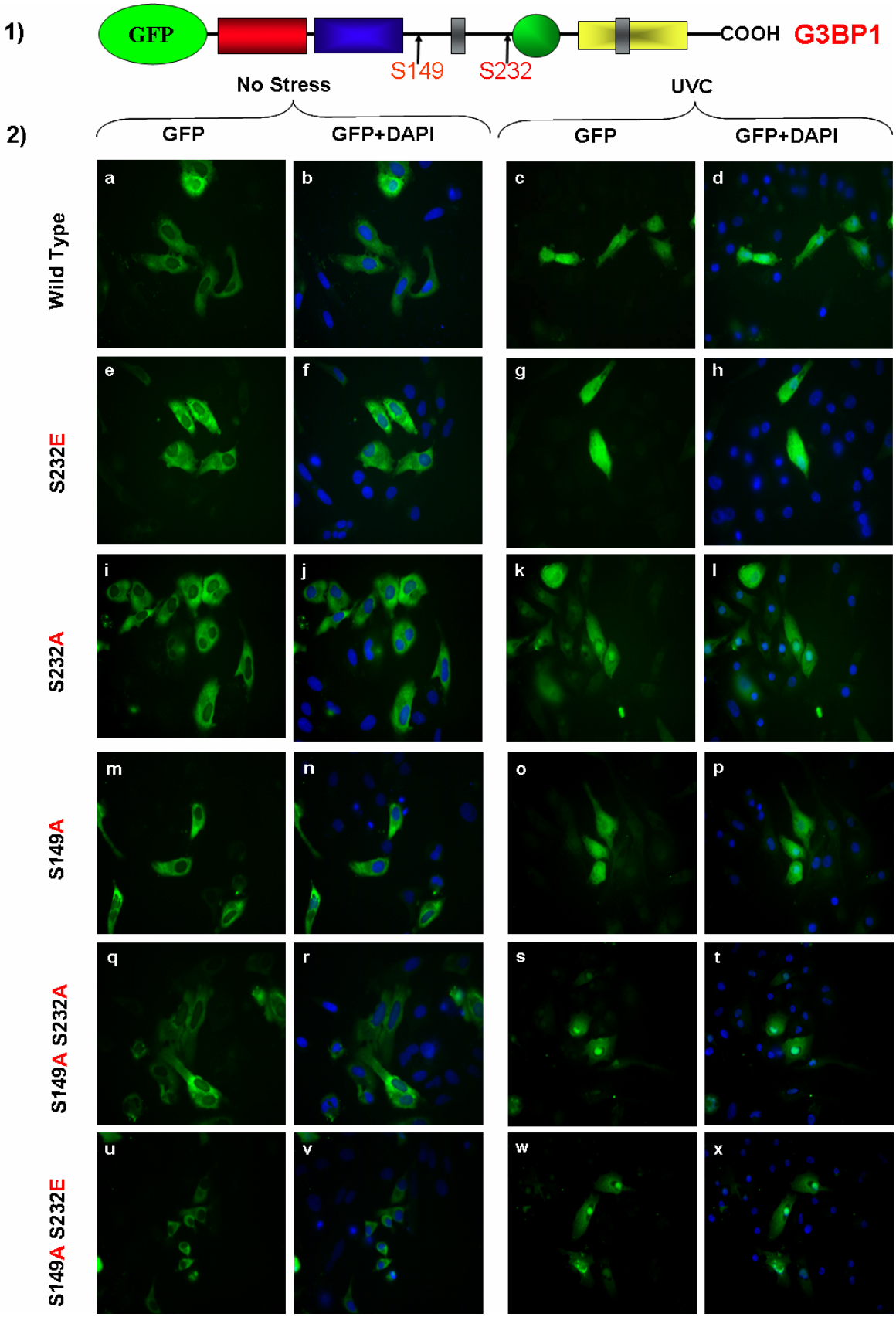
[‡‡] For additional examples of human tissues tested, refer to figures 8 and 11.

glutamate (E), which mimics a constitutively phosphorylated serine. Transfection of HeLa cells with these plasmid constructs and subsequent visualization of the GFP-fused protein "brought to light" many interesting observations (Figure 5).

To begin, approximately 0.5 μg of each plasmid was used per transfection for a 35 mm culture dish. We note that the amount of plasmid transfected was initially optimized to a quantity that was sufficient for adequate expression but did not produce a vast over-expression of the protein, since over-expression of G3BP induces stress granule formation in the absence of stress (Tourriere et al 2003). Cells, non-stressed or UV irradiated, were fixed for visualization 45 hours post-transfection (emphasis placed).

In general, G3BP's subcellular localization is unaffected by amino acid substitutions at serine residue 232, which has been described previously (Tourriere et al 2001). More specifically, these constructs behaved like wild-type GFP-G3BP, being localized to the cytoplasm (Figure 5, No-Stress column, panels a to j). Subsequent UV irradiation, all of these constructs display nuclear localization (Figure 5, UVC column, panels c to l). The same results were obtained with constructs where the serine residue 149 is substituted with alanine. As previously described, these constructs localize to the cytoplasm (Figure 5, No-Stress column, panels m to v), and like the previous constructs, acquire a nuclear localization following UV irradiation (Figure 5, UVC column, panels o to x).

Substitution of serine residue 149 with glutamate was previously shown to induce the constitutive nuclear localization of this protein construct (Tourriere et al 2001). When we performed transfections — repeatedly — with all the constructs containing the S149E substitution, very few GFP-expressing cells were obtained and the few that did did not appear viable or of similar morphology to the GFP-expressing cells obtained with the other plasmid constructs (Figure 5, panels y to jj). We initially assumed this problem was


1)
GFP
COOH
G3BP1
S149
S232
No Stress
UVC
2)
GFP
GFP+DAPI
GFP
GFP+DAPI
Wild Type
S232E
S232A
S149A
S149A S232A
S149A S232E
a
b
c
d
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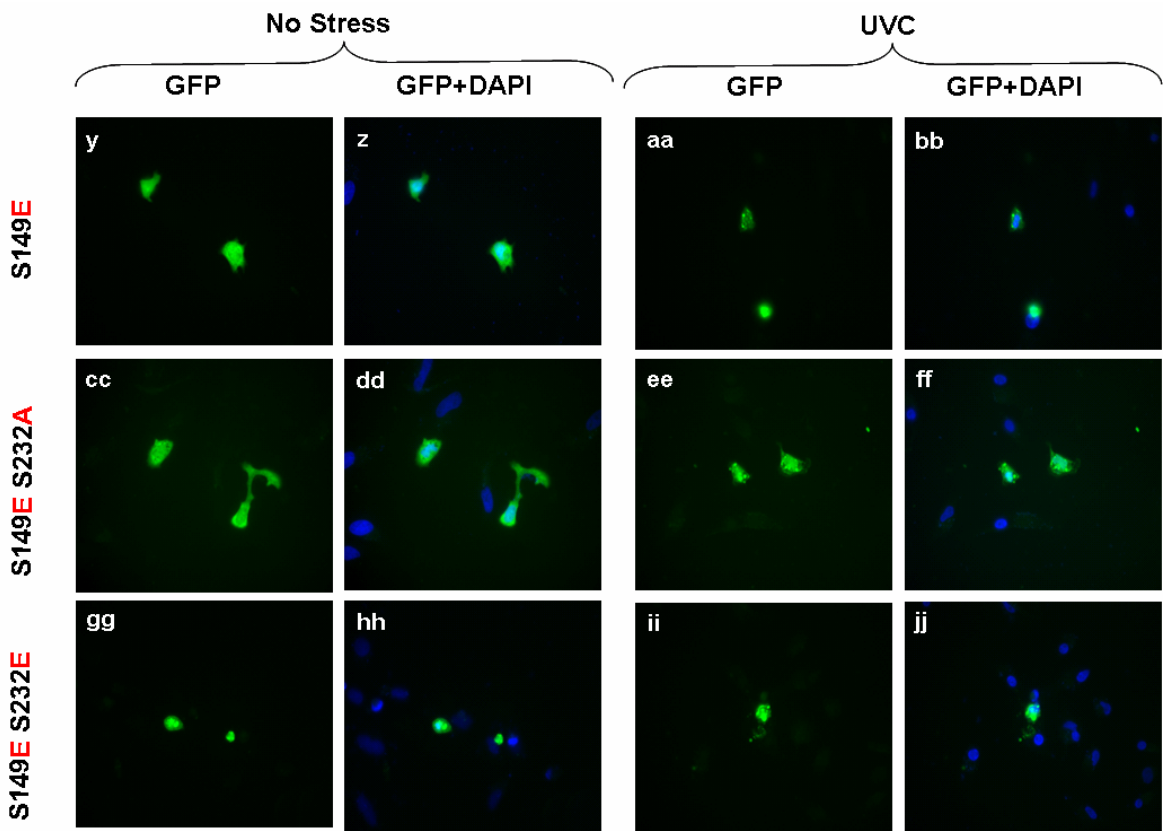


Figure 5. Localization of GFP-tagged phosphorylation mutants of G3BP. 1) Diagram of N-terminal GFP-tagged G3BP constructs. Previously characterized phosphorylation sites, serine residues 149 and 232, are highlighted. These residues are replaced with either the phospho-mimetic residue of glutamate (E) or the non-phosphorylable residue of alanine (A). 2) HeLa cells were transfected with 0.5 µg of each plasmid per 35 mm culture plate. Non-stressed and UV irradiated cells were fixed for fluorescence microscopy approximately 45 hours post transfection. As previously described, constructs containing mutations in serine 232 behave like wild type G3BP, being localized to the cytoplasm in the absence of stress (No-Stress column, panels a to j). Post UV irradiation, all of these constructs localize to the nucleus, as observed by their co-localization with DNA (UVC column, panels c to l). Replacing serine residue 149 with alanine mimics the results with serine 232, being cytoplasmic in the absence of stress (No-Stress column, panels m to v), and nuclear post UV irradiation (UVC column, panels o to x). Replacing serine residue 149 with glutamate was reported previously to induce G3BP nuclear localization in the absence of stress. We observe that along with its nuclear localization, it induces cell death that is irrelevant to UV irradiation (panels y to jj). S: serine; E: glutamate; A: alanine.

due to low transfection efficiency. This did not make sense since the transfections were performed in tandem with the other plasmid constructs and the supposed low transfection efficiency occurred with three distinct plasmids (S149E, S149E S232A, and S149E S232E). Upon closer examination of the cells, we realized that these cells were apoptotic in appearance, namely, they had a shrunken cytoplasmic volume (pyknosis), ruffled plasma membrane, and nuclear ruffling and fragmentation (karyorrhexis) (Figure 6, panel d to f). We questioned why previous work with the S149E construct only noted its nuclear localization and did not note cells that were apoptotic in appearance (Tourriere et al 2001). This is explained by the fact that previous work with the S149E construct visualized the transfected cells 20 hours post transfection, far ahead of our time point of 45 hours. When we repeated the transfection of the S149E construct and fixed the cells for visualization 20 hours post transfection, the majority of GFP-expressing cells appeared viable and displayed GFP nuclear localization (Figure 6, panels a to c).

Enticed by these findings, we decided to quantify the amount of GFP-expressing cells displaying apoptotic morphology for a comparative analysis between the wild type, S149E, and S149A constructs. We repeated the transfections and fixed the cells 30 hours post transfection— a mid time point that would allow for a sufficient population of GFP-expressing cells that are not too advanced in apoptosis, which results in loss of the GFP-expressing population (in the case of the S149E construct). All GFP-expressing cells were counted by eye up to a population of approximately 150, where cells that had aspects of cellular shrinkage and nuclear blebbing were defined as apoptotic in appearance (Figure 7). From three independent trials, it is observed that the percentage of GFP-expressing

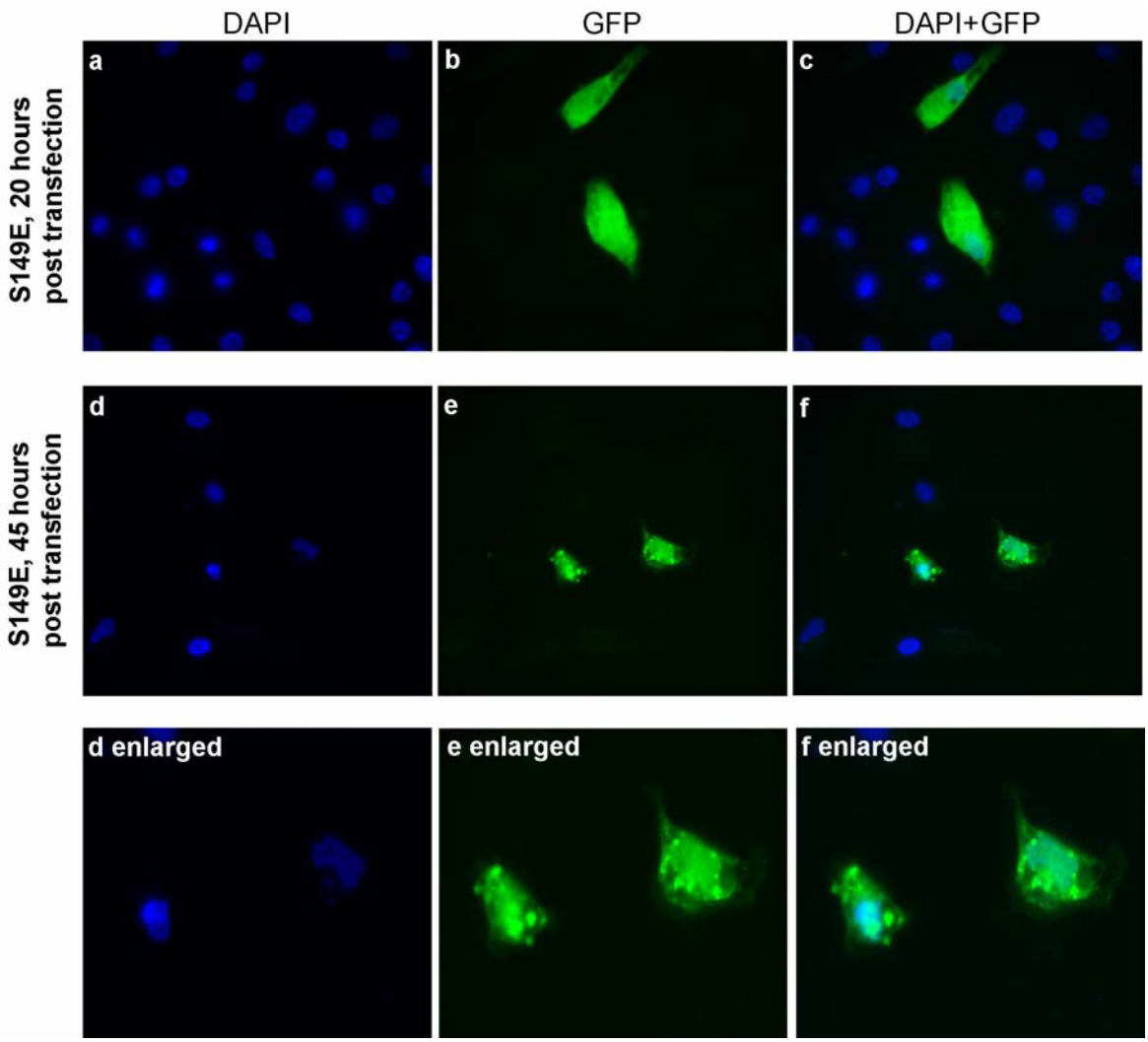


Figure 6. The GFP-G3BP S149E construct produces cells apoptotic in appearance late after transfection. HeLa cells were transfected with 0.5 µg of GFP-G3BP S149E plasmid per 35 mm culture plate and were fixed for visualization 20 or 45 hours post transfection. 20 hours post transfection, GFP-expressing cells appear viable in appearance and display GFP fluorescence within the nucleus (panels a to c). Conversely, 45 hours post transfection, GFP-expressing cells have apoptotic morphology of cellular shrinkage and nuclear fragmentation and blebbing.

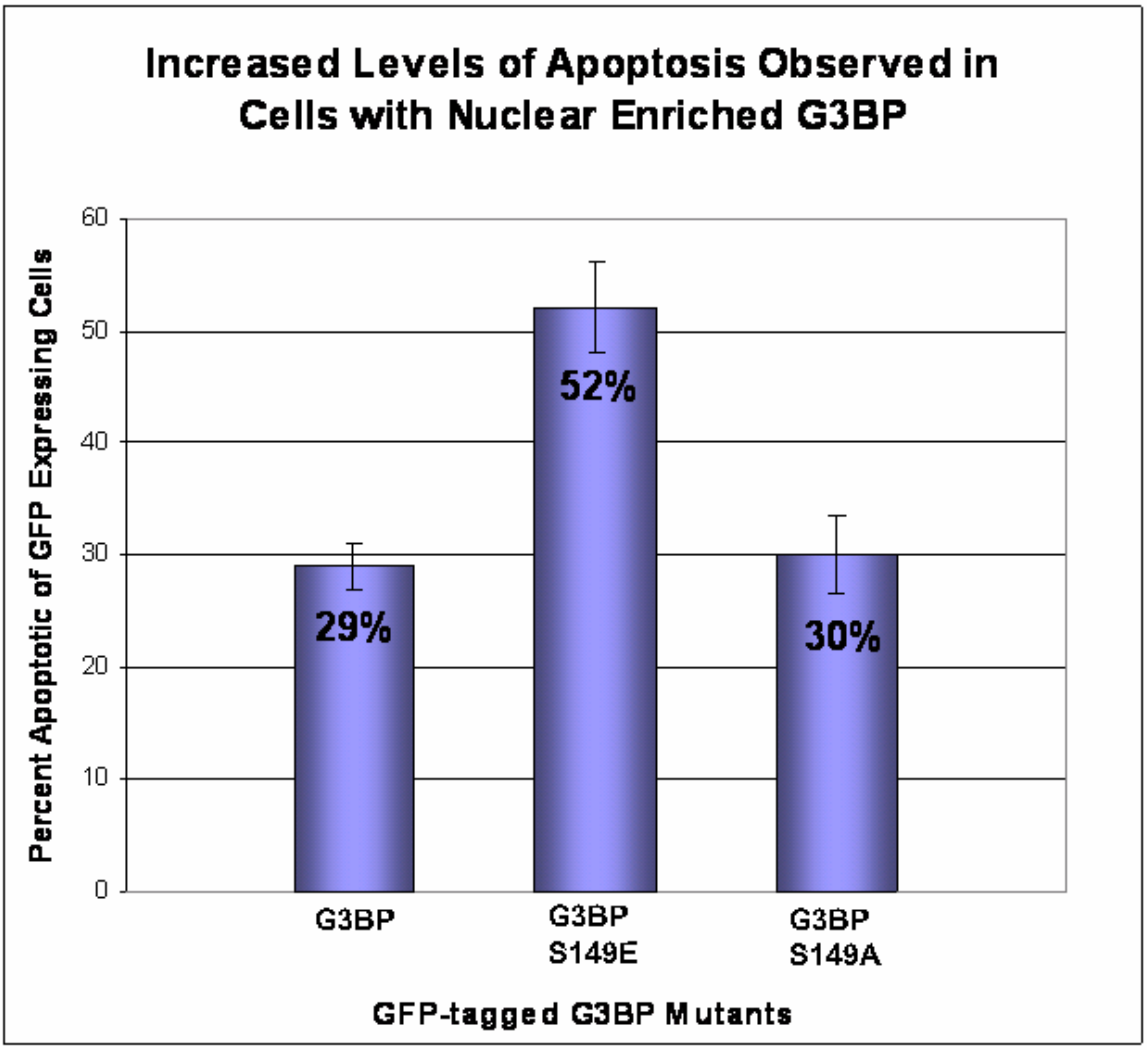


Figure 7. Quantification of cells apoptotic in appearance for specific GFP-G3BP constructs. HeLa cells were transfected with 0.5 µg of GFP-G3BP (wild type), S149E, S149A plasmids per 35 mm culture plate and were fixed for visualization 30 hours post transfection. GFP-expressing cells were counted (total population approximately 150), where cells that had aspects of cellular shrinkage and nuclear blebbing were defined as apoptotic in appearance. About 30% of the population of cells expressing the GFP-G3BP and S149A constructs have apoptotic morphology. About half the population of cells expressing the S149E construct have apoptotic morphology. Error bars are representative of the standard deviation (SD) from three independent trials.

cells displaying apoptotic morphology between the wild type and S149A construct is roughly equivalent, being approximately 30%. The percentage of the population with the S149E construct is significantly higher at 52%.

Several interesting results were obtained when we employed GFP-G3BP plasmid constructs containing amino acid substitutions at two characterized phosphorylation sites. For one, all of these constructs are observed to acquire a nuclear localization following UV irradiation. Since the dual substitution of S149A S232A, mimicking a hypo-phosphorylated G3BP, can acquire a nuclear localization, we conclude that phosphorylation of G3BP is not required for its nuclear localization under UV stress. Transfections with the phospho-mimetic G3BP S149E construct, previously shown to produce a protein that localizes to nucleus, was observed to be toxic to cells 45 hours post-transfection. The morphology of these cells appears to be apoptotic in nature. Though we have not confirmed that these cells are in fact apoptotic, we observed that this constitutively nuclear construct produces quantities of cells of apoptotic morphology that are significantly greater than that of wild type G3BP or the hypo-phosphorylated homologue, G3BP S149A. The conclusions we make from these results are that it appears that G3BP nuclear localization is linked to cellular apoptosis. Since forcing its nuclear localization in the absence of stress appears to be deleterious to cells, its nuclear localization subsequent apoptotic doses of UV might serve to promote UV-induced apoptosis. Thus, our succeeding experiments focussed on identifying if metabolic pathways integral to UV-induced apoptosis could play a role in the mechanism for G3BP nuclear localization.

G3BP's UV-induced nuclear localization is not dependant on the key apoptotic gatekeeper, p53

Our observations up to this point strongly associate G3BP nuclear localization with the process of UV-induced apoptosis. From these observations, our research interest veered towards identifying if principle metabolic pathways integral to apoptosis could play a role in the nuclear localization of G3BP. Our investigations commenced with addressing if the key apoptotic regulator (Ko & Prives 1996; Levine 1997), p53, could be associated with our findings. In relation to UV-stress specifically, p53 becomes active as a transcription factor, that in turn induces cell cycle arrest and apoptosis, from DNA damage and the activity cytosolic stress-activated protein kinases (Bartek & Lukas 2001; Bulavin et al 1999; Latonen et al 2001).

In order to define whether the activity of p53 was relevant to our investigations, we assessed if G3BP nuclear localization would be perturbed in cells lacking p53. We therefore irradiated a p53-null cell line, H1299 lung adenocarcinoma cells, with our experimentally-determined UV treatment. Remarkably, unlike all other cells lines tested, the p53-null cells did not show G3BP nuclear localization one hour post-UV treatment (Figure 8). Rather, G3BP nuclear accumulation was significantly delayed, where maximal nuclear accumulation appeared 2 hours after applying the stress (Figure 8, compare panel e with h). It is interesting to note that G3BP's nuclear localization appears to occur once the cells acquire the apoptotic morphology of cellular and nuclear shrinkage (Figure 8, compare panel e with h).

The delay in G3BP nuclear localization would be a particularly interesting observation if it were indeed tied to p53 metabolic pathways. To affirm if the delay in the kinetics of G3BP's nuclear localization was due to the absence of p53, we re-introduced

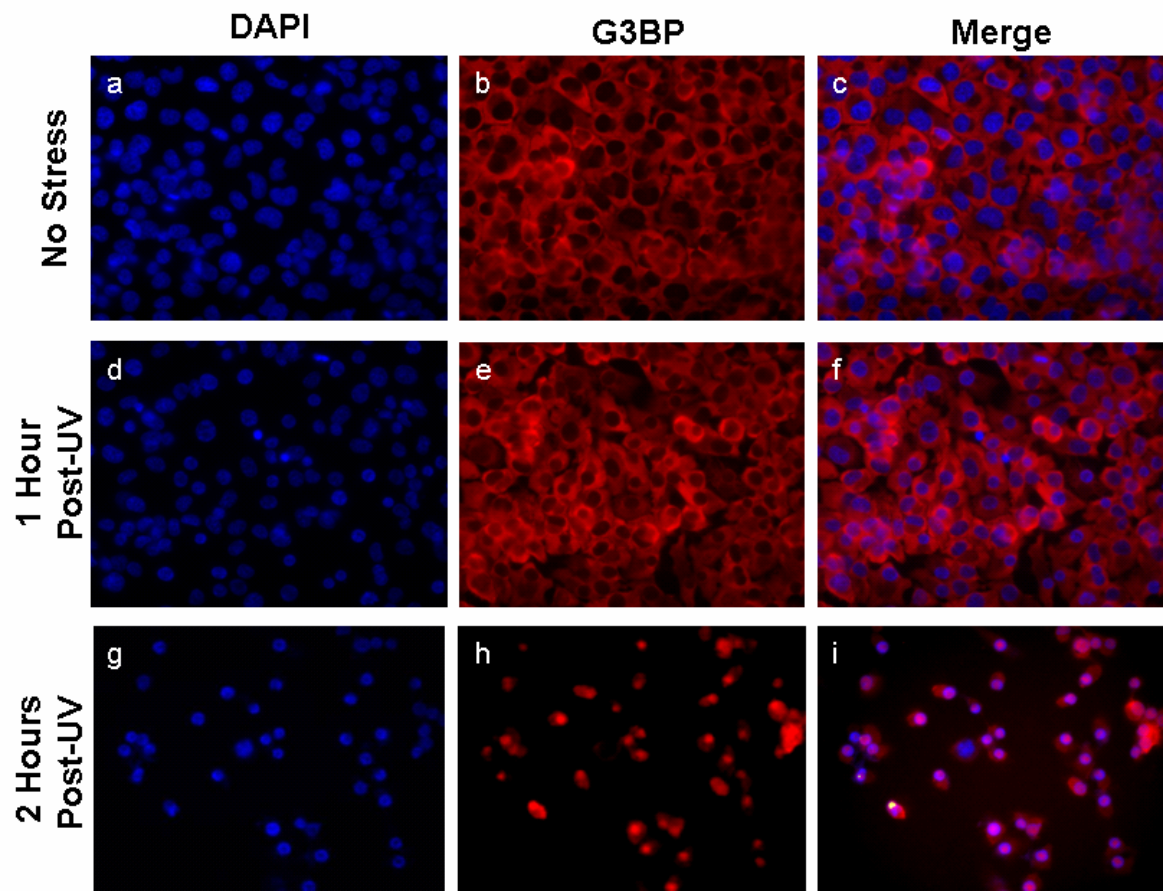


Figure 8. G3BP nuclear localization is delayed in the p53-null cell line, H1299. With the experimentally determined UV treatment (0.72 J/cm^2), G3BP nuclear accumulation is maximal approximately one hour after applying the stress in all tissues tested. One exception is the p53-null cell line, H1299, where nuclear levels of G3BP appear equivalent to that of the non-stressed sample one hour after the UV treatment (compare panel b with e). Yet, G3BP does eventually accumulate in the nucleus of this cell line, though this is observed in twice the amount of time relative to other tissues (panels h and i).

its corresponding gene, and thus protein expression, via adenovirus-mediated gene transfer (Figure 9). Re-introduction of p53 protein levels produced no change in the kinetics of G3BP nuclear localization upon UV treatment in comparison to the p53-null cells (Figure 9, compare panel c with g). We note that the level of expression of andenovirus-p53 appears to be relatively high, as observed by intense immunofluorescence staining (Figure 9, panel f). Additionally, p53 undergoes transient stabilization and nuclear localization after UV-stress (Takekawa et al 2000), which is observed in our UV-irradiated adenoviral infected H1299 cells. To assess whether the andenoviral-introduced p53 protein was functional, we resolved cellular extracts of UV-irradiated, adenoviral infected H1299 cells by SDS-PAGE and probed for its ability to induce the expression of one of its target transcripts, p21/WAF1/CIP1. Subsequent UV stress, p53 becomes active as a transcription factor and induces the transcription of p21/WAF1/CIP1 gene, resulting in elevated p21/WAF1/CIP1 protein levels (Gottlieb & Oren 1996). Blotting for both p53 and p21 indicates that the adenoviral gene transfer results in re-establishing p53 expression in H1299 cells and that this protein is functional by the observed induced expression of p21 (Figure 10).

The above analyses had us conclude that p53 is unlikely to be involved in the mechanism for G3BP nuclear localization following UV irradiation. The reason as to why there is a delay in the kinetics of G3BP nuclear localization in these cells, specifically, remains to be determined.

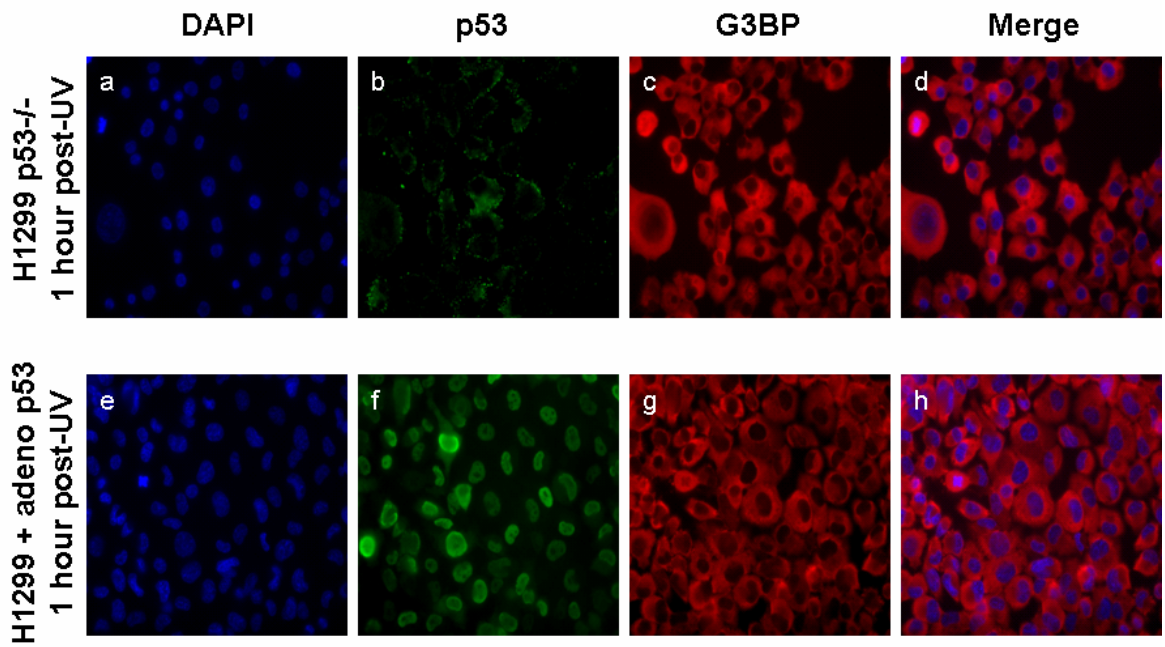


Figure 9. Re-introduction of p53 has no effect on the kinetics of G3BP nuclear localization. H1299 cells were UV irradiated and immuno-stained for p53 and G3BP. These cells display background fluorescence emissions for p53 (panel b) and cytosolic G3BP one hour post-UV treatment (panel c). Re-introduction of the p53 gene via adenoviral gene transfer results in elevated p53 protein expression, which localizes primarily in the nucleus (panel f). Even in presence of elevated p53, these cells display no difference in the kinetics of G3BP nuclear localization post-UV treatment relative to the p53-null variety of cells (compare panels c to g).

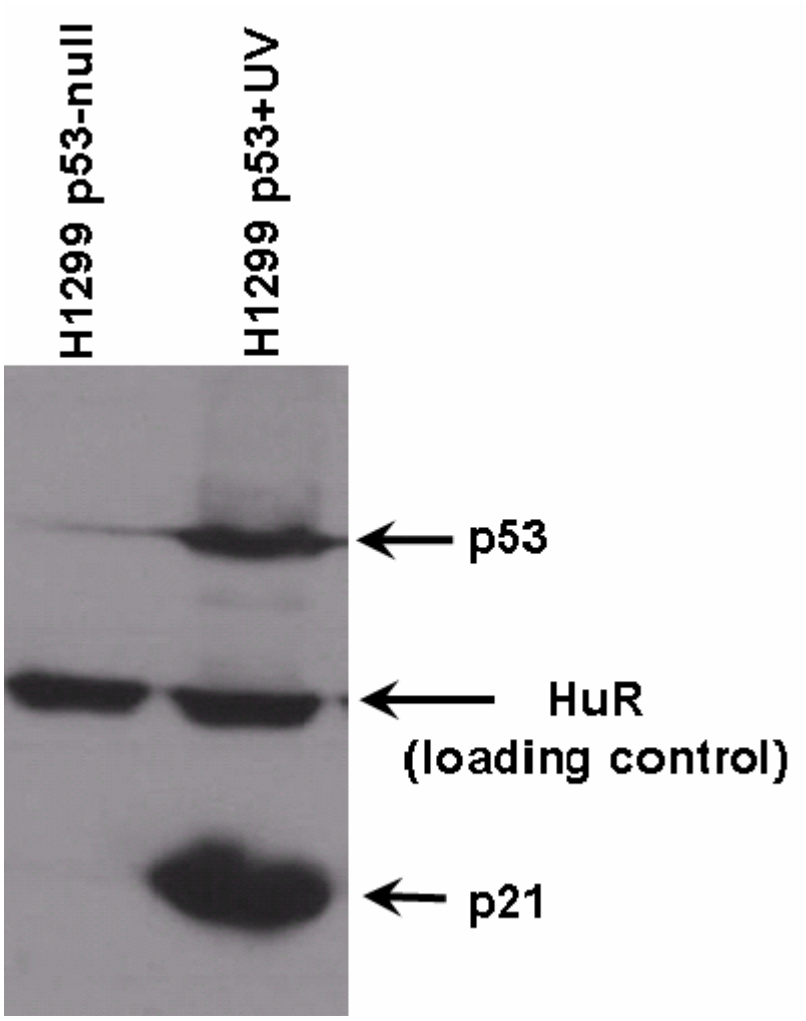


Figure 10. Introduction of p53 gene via adenoviral infection produces functional p53 protein. One 10 cm plate of H1299 cells was collected for each analysis. Cells were lysed in lysis buffer and 10 µg of protein was resolved on a 10 % gel for analysis. Equal loading was confirmed by probing for HuR as a loading control. H1299 cells display an absence of p53 protein and its down-stream transcription target, p21. Adenoviral-introduction of the p53 gene results in p53 protein expression, which was confirmed to be functional through the induction of p21 protein expression subsequent to UV irradiation.

G3BP nuclear localization appears to be specific to UV-induced apoptosis and not to genotoxicity

One fascinating recently published work prompted us to investigate whether G3BP's nuclear localization was due to UV specifically or to its more general genotoxic effects. By use of cDNA microarrays, Amundson et al. (2005) identified stress-specific gene expression profiles that led to the observation that stresses may be categorized, or grouped, by eliciting the induction of common, signature, mRNAs. Numerous stresses were assayed and each was placed in one of four derived categories: Oxidative, Ionizing, Nongenotoxic, and Genotoxic. Of the Genotoxic class were a) the compound Methyl methanesulfonate, b) the chemotherapeutics Cisplatin, Doxorubicin, Camptothesin, and c) Ultraviolet Radiation. All of these agents incur DNA damage by modifying nucleotides and/or producing single strand breaks (Amundson et al 2005). We asked the question whether if G3BP's UV-induced nuclear localization might be caused by the more general influence of metabolic pathways excited by genotoxicity. Therefore, we assessed G3BP localization after treatment of proliferating IDH4[§§] cells with Cisplatin, Doxorubicin, and Camptothesin (Figure 11). We employed a non-genotoxic chemotherapeutic, Vinblastin (whose primary mode of action is its ability to inhibit microtubule polymerization (Kovacs & Csaba 2006)), as a negative control to our inquiry. As displayed in figure 11, the genotoxic compounds behaved as did the non-genotoxic Vinblastin, whereby G3BP remained primarily in the cytoplasm. The functionality of the drugs was proven by observing the induction and stabilization of p53 by Western blot analysis (Figure 12). Once again, genotoxic compounds result in the transient stabilization of p53 protein,

[§§] We were advised to use a primary cell line, rather than the cancer cells, HeLa, for they respond better to chemotherapeutics and one can assess their functionality by p53 induction (personal communication, Tremblay lab members of McGill University).

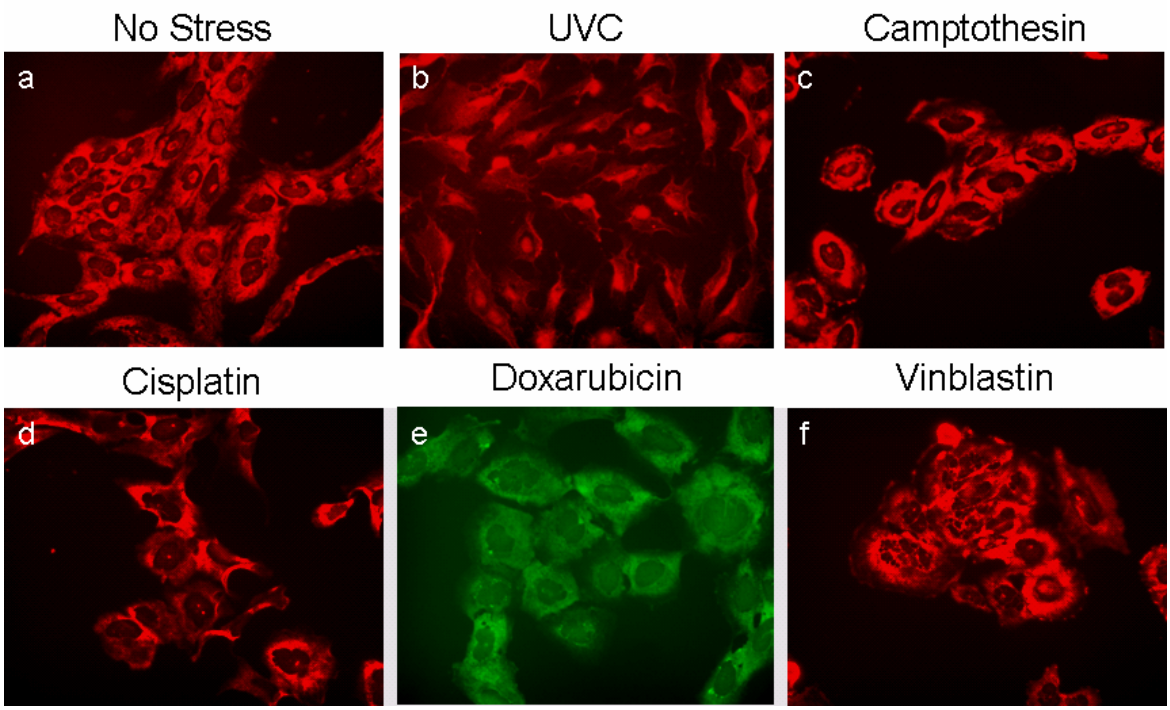


Figure 11. G3BP's subcellular localization is not influenced by genotoxic chemotherapeutics. G3BP's subcellular localization in proliferating IDH4 cells was visualized through immunofluorescence after UV irradiation and 8 hours post-treatment with the genotoxic chemotherapeutics Camptothesin, Cisplatin, and Doxarubicin (panels b to e). Unlike after UV irradiation, cells treated with the genotoxic compounds have G3BP localized to the cytoplasm, as is the case with the untreated negative control (panel a). Treatment of cells with the non-genotoxic toxic chemotherapeutic, vinblastin, also display G3BP in the cytoplasm. G3BP was visualized by immunofluorescence with a rhodamine conjugated secondary antibody for all assays except that involving doxarubicin. Like rhodamine, doxorubicin emits in the red wavelength spectrum. Thus, a FITC conjugated secondary antibody, which emits in the green wavelength spectrum, was employed in this analysis.

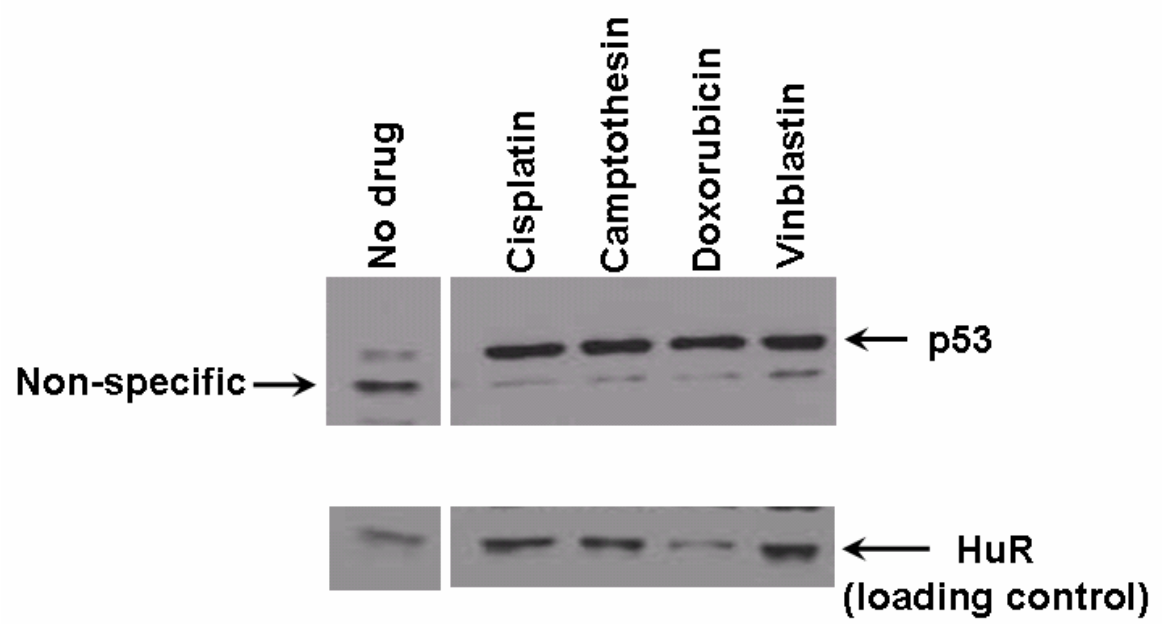


Figure 12. Functionality of chemotherapeutics confirmed by the induction and stabilization of p53 protein. Proliferating IDH4 cells were incubated in the presence of the above chemotherapeutics for 8 hours. Cells were lysed in lysis buffer and 15 µg of protein was resolved on a 10 % gel for analysis. Equal loading was confirmed by probing for HuR as a loading control. Cells cultured in the absence of chemotherapeutics contain basal levels of p53 protein. Cells cultured in the presence of each chemotherapeutic contain p53 protein levels that are several fold higher relative to basal levels.

which results in increasing the cellular levels of this protein (Takekawa et al 2000). In comparison to untreated proliferating IDH4 cells, which contain basal amounts of p53, cells treated for 8 hours with all the chemotherapeutics contain elevated p53 protein expression.

These observations appear to show that G3BP's UV-induced nuclear localization is due to UV specifically and not to cellular responses to DNA damage or induction of apoptosis by chemotherapeutics. Additionally, analysis with the non-genotoxic chemotherapeutic, vinblastin, also suggests that G3BP nuclear localization is specific to UV irradiation and does not appear to be linked to the induction of apoptosis via chemotherapeutics in general. Because of these findings, we decided to focus our subsequent analyses on metabolic pathways induced by apoptotic levels of UV that are less or not affiliated with those associated with DNA damage. More specifically, we placed interest on biochemical pathways that localize to the cytoplasm and shied away from those that originate from the nucleus.

G3BP nuclear localization appears to be down stream of plasma membrane signalling

Aside from inducing stress-activated metabolic pathways that originate from DNA damage, ultraviolet radiation also causes damage to biochemical factors outside of the nucleus. For example, UV irradiation denatures plasma membrane bound receptors, which in turn forces their oligomerization and activation even in the absence of their complementary ligand (ligands typically being mitogenic stimuli). To elaborate, UV can force the activation of receptors for epidermal growth factor, tumour necrosis factor,

interleukin-1, and CD95 (Fas/APO-1) (Aragane et al 1998; Rehemtulla et al 1997; Rosette & Karin 1996). The activation of these plasma membrane receptors results in the stimulation of downstream signalling cascades, which culminate in the activation of stress-activated protein kinases like JNK and p38 MAPK (Bode & Dong 2003). The activation of these signalling cascades appear to be mediated through GTP-binding protein family members like Cdc42, Rac, and Ras (Coleman et al 2004).

Addressing whether plasma membrane signalling could influence G3BP UV-induced nuclear localization became the focus of our investigations. We were particularly interested in addressing this possibility since previous studies concerning changes in G3BP's subcellular localization due to environmental stress had implicated Ras-signalling in this process (Tourriere et al 2003; Tourriere et al 2001). One mean to inhibit plasma membrane signalling in general is to treat cells with low concentrations of detergent. To elaborate, treatment of cells with non-inonic detergents slightly solubilizes cellular plasma membranes, yet they retain their integrity (Adler et al 1995). This partial solubilization prevents plasma membrane bound receptors from multimerizing, thus inhibiting their activation (Carlin et al 1994; Gates & King 1993; Spaargaren et al 1991). In relation to UV stress specifically, Triton X-100 pre-treatment was shown to inhibit UV-induced Ras-signalling and the down stream activation of stress activated protein kinases (Adler et al 1995; Devary et al 1992). We chose to inhibit plasma membrane signalling prior to UV treatment by pre-treating cells with 0.032% Triton X-100, which was dissolved in culture media. A 5 minute pre-treatment with the detergent (also present in the media during the 1 hour recovery period after UV stress) was observed to inhibit G3BP nuclear localization subsequent UV irradiation in comparison to conventionally UV-stressed cells (Figure 13). The presence of the detergent did not appear to inhibit

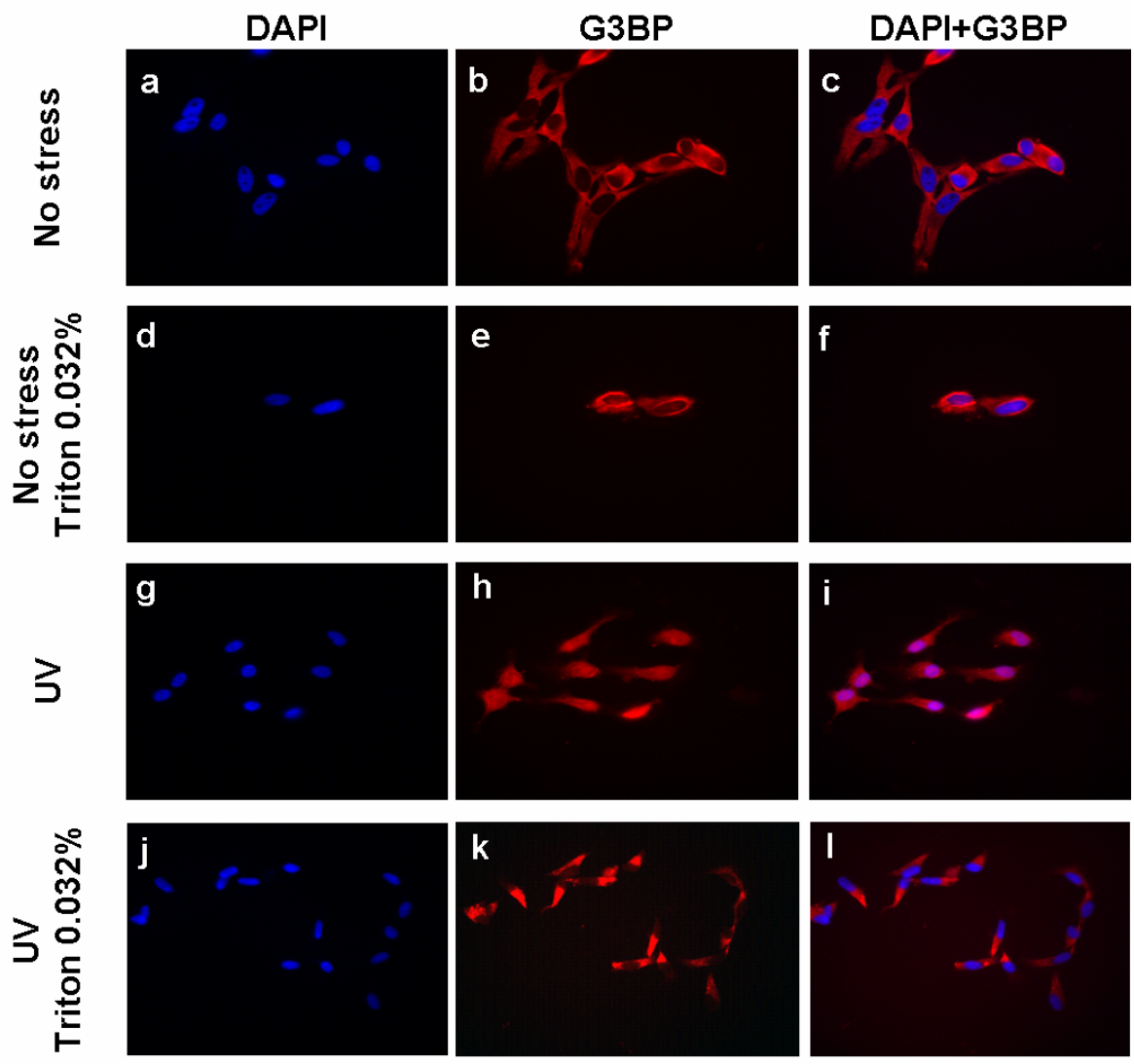


Figure 13. Pre-treatment of cells with Triton X-100 inhibits G3BP nuclear localization. Incubation of HeLa cells in media containing 0.032% Triton X-100 5 minutes before UV irradiation, and during the 1 hour recovery period, inhibits G3BP's UV-induced nuclear localization (compare panel h to k). The presence of the detergent does not appear to inhibit apoptosis in general since the cells still acquire apoptotic morphology subsequent UV (compare panel k to h). G3BP's subcellular localization in the presence of the detergent, without stress, is equivalent to that of the No Stress negative control (compare panels b to e).

apoptosis in general subsequent to the UV treatment for the cells display cellular and nuclear shrinkage (Figure 13, panels j to l). The detergent itself had no effect on G3BP localization (Figure 13, panels e and f).

In summation, pre-treatment of cells with low concentration of Triton X-100 inhibits G3BP nuclear localization after UV irradiation. We assume this effect is due to inhibiting plasma membrane signalling but this was not proven for means to test for the inhibition of plasma membrane receptor activation were unavailable at the time of this analysis. Thus, we claim that plasma membrane signalling is *implicated* and preliminary, but not affirmed, in the mechanism for G3BP nuclear localization from UV irradiation.

Preliminary implication of the stress activated protein kinases, JNK and p38 MAPK, and active CASPases in G3BP's UV-induced nuclear localization

Following our preliminary observations that implicated plasma membrane signalling, we focussed our investigations on addressing what downstream signal transduction cascades might play a role in our observations with G3BP nuclear localization from UV stress. We immediately scrutinized the stress activated protein kinases, p38 MAPK and JNK. These kinases become active by UV stress through DNA damage and plasma membrane signalling by the activation of membrane bound receptors (Adler et al 1996a; Adler et al 1995; Bode & Dong 2003; Chen et al 1996; Seo et al 2004; Takekawa et al 2000). Once active, these kinases serve to arrest the cell cycle and induce apoptosis (if the stress is severe) by regulating the activity of numerous cellular targets like p53. Ras-signal transduction cascades partly orchestrate their activation in relation to plasma membrane signalling mechanisms. To reiterate, Ras-signalling pathways were previously implicated in playing a role in altering G3BP's subcellular localization under

conditions of stress, specifically its recruitment to stress granules by arsenite treatment and its nuclear localization upon serum starvation (Tourriere et al 2003; Tourriere et al 2001).

Tangential to stress activated protein kinases, we were also interested in assessing if the activity of CASPases could be linked to G3BP nuclear localization from UV stress. Aside from the general importance of CASPases in conducing apoptosis, we hypothesized that these proteases could be related to our observations from results obtained with the p53-null cell line, H1299 (Figure 8). In these cells, G3BP nuclear localization is delayed, but eventually accumulates in the nucleus only once the cells begin to acquire apoptotic morphology. CASPases coordinate multiple apoptotic processes, one being the acquisition of apoptotic cellular morphology of pyknosis and karyorrhexis (Hacker 2000). To conclude G3BP nuclear localization appears to occur when CASPases are active and have commenced altering cellular morphology.

To address whether the stress activated protein kinases and active CASPases might play a role in our observations, we decided to inhibit the activity of these enzymes with selective inhibitors. Available to us was the kinase inhibitor CNI 1493 and the broad CASPase inhibitor ZVAD-fmk (ZVAD). CNI 1493 is an effective inhibitor of both p38 MAPK and JNK (Cohen et al 1997; Hommes et al 2002). Incubation of cells with either of the aforementioned inhibitors 2 ½ hours before UV irradiation, and during the 1 hour recovery period, inhibited G3BP nuclear localization (Figure 14). Incubation of cells with the inhibitor in the absence of stress had no effect on G3BP localization, where it remained a primarily cytosolic entity (data not shown). We note that while G3BP remains in the cytoplasm in the presence of these inhibitors, the UV-stressed cells still display early apoptotic morphology of cellular shrinkage (Figure 14, compare panel b to h and k).

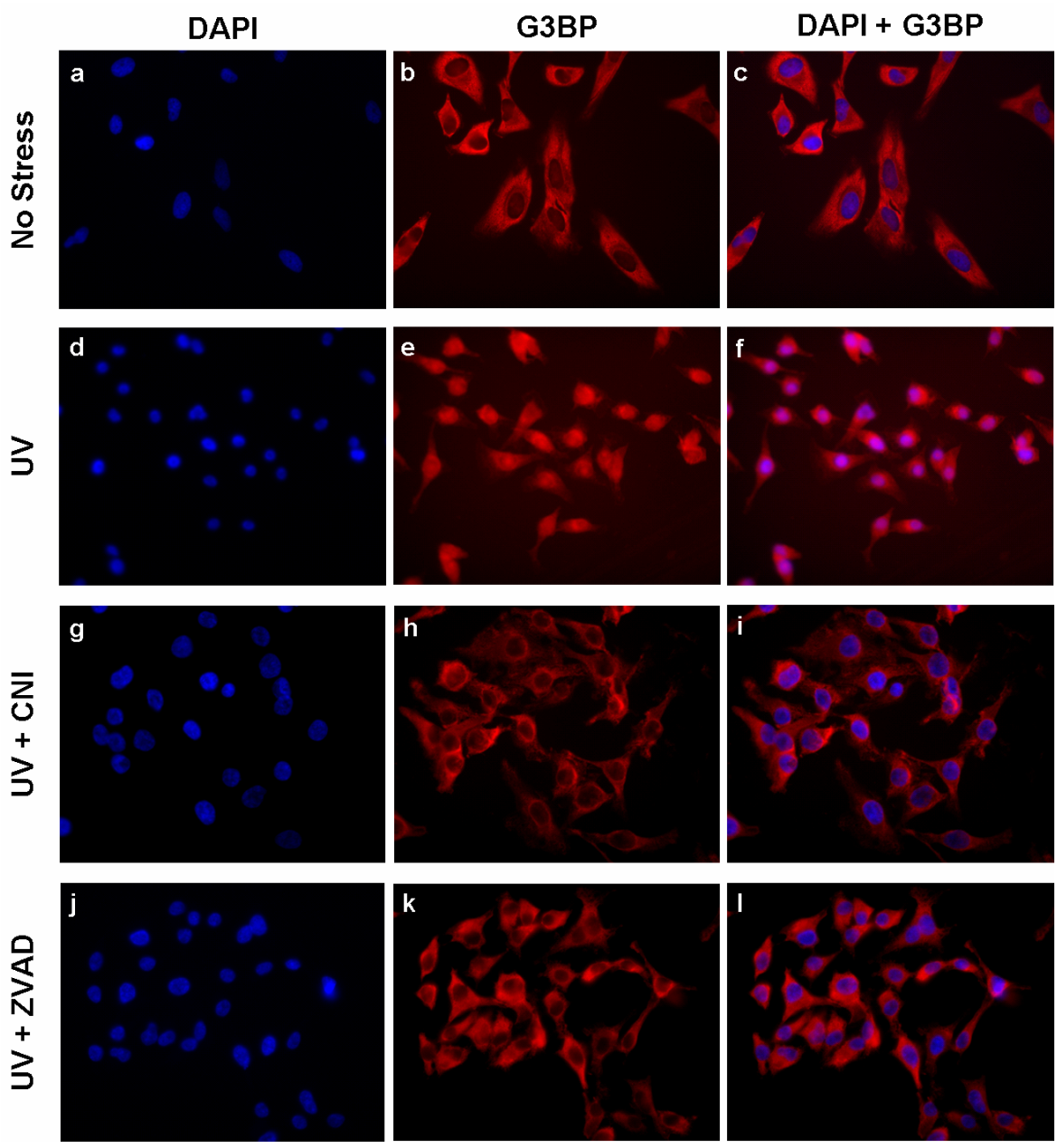


Figure 14. Pre-treatment of cells with the inhibitors CNI1493 or ZVAD prevents G3BP's UV-induced nuclear localization. Pre-treatment of cells with the broad kinase inhibitor, CNI1493 (1μM final concentration), prevents G3BP nuclear localization following UV stress (compare panels e to h). Cells UV-irradiated in the presence of CNI1493 appear apoptotic in appearance, where they display aspects of cellular and nuclear shrinkage (compare panels b to h). Pre-treatment of cells with the broad CASPase inhibitor, ZVAD (20μM final concentration), mimics results obtained with CNI1493, such that G3BP nuclear localization is inhibited following UV irradiation (compare panels e to k). Cells UV-irradiated in the presence of CNI1493 and ZVAD display early apoptotic morphology of the beginnings of cellular shrinkage (compare panels b to k).

We conclude this section with the belief that the stress activated protein kinases, p38 MAPK and JNK, and active CASPases, *might* play a role in the mechanism for G3BP nuclear localization. Controls to assess the effectiveness of the inhibitors employed were not performed during the course of this thesis due to time constraints. Yet, the results displayed in figure 14 are representative of three independent trials. Thus, we claim that stress activated protein kinases and active CASPases are *implicated* and preliminary, but not affirmed, in the mechanism for G3BP nuclear localization from UV irradiation.

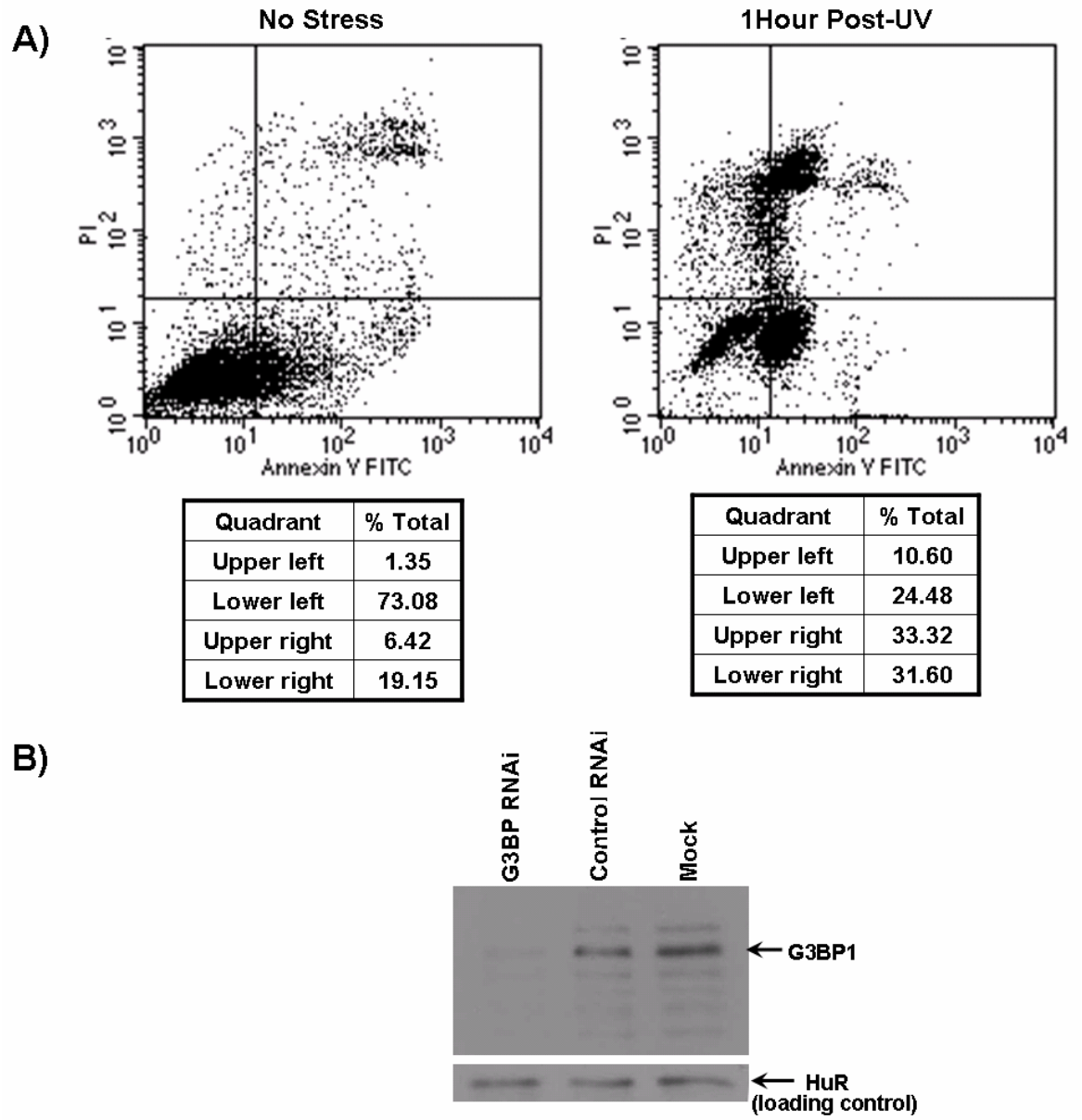
G3BP's role in promoting UV-induced apoptosis remains to be determined: Optimization of FACS analysis to follow the induction of apoptosis

G3BP is a multifunctional protein that functions in a variety of cellular processes (Table 2). Its multifunctional nature creates somewhat of a conundrum when it comes to defining a specific cellular utility for this entity. Our observations up to now solidify the idea that this RNA-binding protein plays a significant role in mammalian cellular responses to environmental stress. In relation to cellular responses to elevated UV exposure, we believe that G3BP's induced nuclear localization might serve to promote the advancement of apoptosis. This conviction mainly arises from our results displayed in figures 5 to 7, where forcing G3BP nuclear localization by use of the phopho-mimetic S149E construct produces cells with apoptotic characteristics in the absence of stress.

In order to link formally G3BP with a role in promoting apoptosis, we planned to deplete the protein from cells and assess whether the quantity, or the rate, of apoptosis induction would be perturbed following UV irradiation. The results presented in this section are the initial trials performed that serve as an optimization process to perform this analysis. To begin, our chosen strategy to quantify the induction of apoptosis

following our UV treatment is the use of the technique of flow cytometry, also known as Fluorescence Assisted Cell Sorting (FACS). This technique quantifies the number of cells, in suspension, that are labelled with fluorescent moieties. For analyses related to apoptosis, these moieties are commonly Propidium Iodide (PI) and FITC-conjugated α–Annexin V antibodies. PI is impermeable to the plasma membrane and chealates DNA. Therefore, cells with high PI labelling have permeable membranes; this being primarily a trait of necrotic cells but is also characteristic of cells in the late stage of apoptosis. Annexin V— similar to the phosphatidylserine molecule that we described for figure 3— is normally located on the inner leaflet of plasma membrane and becomes flipped to the extracellular side during apoptosis induction. Once at the extracellular face, its corresponding antibody may bind, labelling the cell with the fluorescent moiety conjugated to the antibody. Therefore, cells with high Annexin V labelling have plasma membrane asymmetry, which is indicative of cells in early and late stages of apoptosis.

We UV irradiated cells and analyzed by FACS the population distribution of apoptotic cells after a series of recovery periods post-UV stress. We aimed to determine a period after applying the stress where the majority would be in the stages of apoptosis. We observed that this optimal population distribution was obtained one hour after UV irradiation (Figure 15a). Interestingly, this time point after UV stress coincides with the time where G3BP nuclear localization is maximal (Figure 2). Cells populations determined by FACS analysis for a non-stressed sample is distributed as follows: 1% display high PI and low Annexin V staining (necrotic cell population), 73% display low staining for both labels (viable cell population), 19% display high Annexin V and low PI staining (early apoptotic cell population), and 6% display high staining for both labels (late apoptotic cell population). One hour post-UV irradiation, the population shifts to the



Quadrant	% Total
Upper left	1.35
Lower left	73.08
Upper right	6.42
Lower right	19.15

Quadrant	% Total
Upper left	10.60
Lower left	24.48
Upper right	33.32
Lower right	31.60

Figure 15. Optimization of parameters to follow the induction of apoptosis.
A) FACS analysis of cells UV irradiated with our experimentally determined conditions indicate that more than 60% of the total cell population is apoptotic one hour following UV stress. The upper left quadrant is indicative of necrotic cell population, lower left is viable cells, lower right is early apoptotic cells, upper right is late apoptotic cells.
B) Transfection of 60pmole siRNA (per well of 24 well plate at 40% confluency) targeting G3BP effectively knocks-down G3BP protein expression in comparison to samples of a mock transfection or transfection with control siRNA. Cell samples were lysed in lysis buffer 40 hours post-transfection and 10µg of protein was resolved on a 10% gel for analysis. PI: Propidium Iodide

following distribution: 10% necrotic cell population, 24% viable cell population, 32% early apoptotic cell population, and 33% late apoptotic cell population. Thus, more than 60% of cell population are apoptotic with these experimental parameters.

Our chosen strategy to deplete cellular levels of G3BP is by use of small interfering synthetic RNA transcripts (siRNA). These small segments of RNA correspond to an antisense sequence to a region of a specific mRNA transcript. Association of the two RNA molecules produces a double stranded RNA species that is perceived to be foreign by the cell. The cell responds by silencing the translation of the mRNA transcript and targets it for degradation. This in turn results in abolishing the protein expression of the entity encoded by the mRNA transcript. We utilized a sequence for siRNA previously described in literature for a G3BP knock-down procedure (Rahmouni et al 2005). Replicating transfections with siRNA targeting G3BP in HeLa cells does effectively knock-down G3BP protein levels in comparison to the controls of a mock transfection (no siRNA) and transfection with a control siRNA whose sequence is not antisense to any cellular mRNA transcript (Figure 15b).

The following results are optimized parameters determined for a subsequent analysis that aims to assess whether depletion of G3BP might delay the quantity or rate of apoptosis induction following UV stress. From this foundation, we determined that the subsequent analysis would be to replicate the knock-down of G3BP and quantify the apoptotic population of these cells by FACS analysis one hour after UV stress. If the population were markedly lower in comparison to non-transfected cells, this would be indicative that G3BP functions in the progression of apoptosis induced by UV stress.

Materials and Methods

Cell culture and growth conditions

All cells were cultured at a temperature of 37^{o}C in a humidified atmosphere of 5% CO_2 composition. Hela cells (ATCC) were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Gibco). Cells were split at about 80% confluence.

H1299 cells (a generous provision by the Branton laboratory of McGill University) were cultured in alpha minimum essential medium (alpha-MEM, Gibco) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were split at about 80% confluence.

Proliferative IDH4 cells (a generous gift from the Myriam Gorospe Laboratory of Cellular and Molecular Biology, National Institute on Aging-IRP, National Institutes of Health; Baltimore, Maryland USA) were cultured as previously described (Wang et al 2001). In brief, cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.4 μg/ml of Dexamethasone (Sigma). Cells were split at about 80% confluence.

Cell cultures were periodically assessed (minimum once every two weeks) for mycoplasma contamination through DAPI staining and visualization with fluorescence microscopy.

Environmental stress and inhibitors protocols

<u>UV stress</u>

Culture medium was removed and cells were washed with PBS. Not all of the PBS solution was removed in order to prevent desiccation of the cells during the irradiation

process. With covers removed, cell plates were placed in the centre of a Stratalinker® UV Crosslinker (Model 2400, Stratagene), approximately 15 centimetres from the emission source, at the standard emission setting of 120 000μJ. The wavelength of the emission source was 254 nm, which corresponds to UVC radiation. The short UV exposure of 2 seconds corresponds to $3x10^{-3}$ J/cm^2, while the apoptotic exposure of 3 minutes corresponds to 0.72 J/cm^2, while the necrosis exposure of 7 minutes corresponds to 1.68 J/cm^2. Cells were allowed to recover at the aforementioned culture conditions (37^{o}C) in culture media for the stated durations: 6 hours with $3x10^{-3}$ J/cm^2 UV for stress granule formation, 1 hour with 0.72 J/cm^2 UV for nuclear localization and apoptosis, and 1 hour with 1.68 J/cm^2 UV for necrotic cells death.

Osmotic stress

Within the cell culture plate, culture media was removed and replaced with media containing 0.5 M of sorbitol. Cells were incubated in this media for 25 minutes at 37^{o}C for stress granule formation.

Heat shock

Within their culture plates containing culture media, cells were floated in a circulating hot water bath set to 45^{o}C. Cells were maintained at this temperature for 30 minutes for stress granule formation.

Arsenite oxidative stress

Within their culture plate, culture media was removed and replaced with culture media containing arsenite. Arsenite (Sigma) stock solution was dissolved in culture media to final concentration of 0.5 mM. Cells were incubated in this solution for 30 minutes at 37^{o}C for stress granule formation.

Genotoxic chemotherapeutic treatments

Within their culture plate, culture media was removed and replaced with culture media containing chemotherapeutics. Chemotherapeutics were dissolved in culture media to the following concentrations: Doxorubicin 0.5μg/μL (provided by the Tremblay laboratory of McGill University), Vinblastin 1ng/μL (Sigma, St. Quentin Fallavier, France), Camptothesin 14 000nM (Sigma), and Cisplatin 30μM (provided by the Tremblay laboratory of McGill University). Cells were incubated in this solution for 8 hours at 37^{o}C to induce genotoxic stress.

Triton X-100 inhibition of plasma membrane signalling

Within their culture plate, culture media was removed and replaced with culture media containing 0.032% Triton X-100 (Sigma) by volume. This concentration was previously characterized by Adler et al (1995) to inhibit plasma membrane signalling and subsequent JNK activity. Cells were UV irradiated as described above but the culture media that the cells were incubated in during the 1 hour recovery period contained 0.032% Triton X-100.

ZVAD inhibition of active CASPases

Within their culture plate, culture media was removed and replaced with culture media containing the ZVAD broad CASPase inhibitor. The ZVAD-fmk (Sigma) stock solution was dissolved to final concentration of 20μM in culture media. Cells were incubated in the presence of the inhibitor for 2 ½ hours prior to UV irradiation and during the 1 hour recovery period subsequent UV irradiation.

CNI 1493 inhibition of SAPK's

Within their culture plate, culture media was removed and replaced with culture media containing the CNI 1493 kinase inhibitor. CNI 1493 (Cytokine PharmaSciences) was initially dissolved 1:100 in a sterile solution of 5% dextrose. This solution was added

to culture media to have 1μM final concentration of the inhibitor. Cells were incubated in the presence of the inhibitor for 2 ½ hours prior to UV irradiation and during the 1 hour recovery period subsequent UV irradiation.

Plasmid and siRNA transfections and p53-adenovirus infections

Plasmid transfections

Hela cells were plasmid transfected in 6-well plates at 60% confluence according to the LipofectAMINE and Plus Reagent transfection protocol (for one well: 5μL LipofectAMINE Reagent, 5μL Plus Reagent, Invitrogen). Cells were assessed for protein expression 20, 30, and 45 hours post-transfection as described in the Results section.
0.5μg of each plasmid were used for transfection per 35mm well. pEGFP-G3BP and its phosphorylation mutants were previously described (Tourriere et al 2001).

Adenovirus infections

H1299 cells were adenovirus infected in 6-well plates at 80% confluence (the adenovirus, at a concentration of $2.3x10^{10}$ pfu/mL, was a generous gift from the Branton Laboratory of McGill University). The viral stock solution was diluted in culture media at amounts so that approximately 50 pfu/cell of viral particles were available for infection. Cells were incubated at $37^{o}C$ in the presence of the virus for 1 hour, with gentle rocking every 20 minutes. Media was replaced with regular culture media and cells were assessed for p53 expression 24 hours post-infection. The adenovirus is a derivative of vectors described by Bacchetti and Graham (1993).

siRNA transfections

siRNA transfections were performed in 6-well culture plates on HeLa cells at approximately 60% confluency. 180 pmol of siRNA was used for each transfection. For all transfections, LipofectAMINE Plus (Invitrogen) was used, following the

manufacturer's protocol. The siRNA oligonucleotide against G3BP1 was equivalent to that used by Rahmouni et al (2005) and the control siRNA was equivalent to that used by van der Giessen et al (2003). siRNA oligonucleotides were obtained from Dharmacon RNA technologies. 'Mock' refers to samples treated with transfection reagents in the absence of siRNA. Cells were assessed for protein knock-down 40 hours post-transfection.

Immunofluorescence and visualization of GFP-tagged proteins

Immunofluorescence manipulations were done as previously described (Fan & Steitz 1998). Cells grown on glass microscope slide cover slips were washed twice with PBS and fixed in 3% paraformaldehyde/PBS for 20 minutes. Cells were washed with PBS and then permeabilized with a solution of 0.5% Triton X-100/1% normal goat serum in PBS for 15 minutes. After washing, cover slips were incubated with primary antibody for 1 hour with gentle rocking. The following dilutions for primary antibodies were used: 1:1500 for α-GST-G3BP 3387 polyclonal sera (previously characterized in Roudaia 2004), 1:1500 α-HuR monoclonal 3A2 Ab, 1:100 α-P53 monoclonal 1801 (a generous gift from the Branton Laboratory of McGill University). After washing, cover slips were subsequently incubated with secondary antibody for 1 hour with gentle rocking. The following dilutions for secondary antibodies were used: 1:500 for Alexa Fluor 594 rhodamine goat α-rabbit Ab (Molecular Probes), 1:500 for Alexa Fluor 594 FITC goat α-rabbit Ab (Molecular Probes), 1:500 for Alexa Fluor 594 goat α-mouse Ab (Molecular Probes). Prior to final washings (3 total: twice with PBS, final with water), cover slips were incubated with DAPI stain (1:20000 dilution of 1mg/ml stock) for 5 minutes. Anti-quenching solution (Vectashield, Vector Laboratories Inc.) was applied before final mounting of the cover slips. Cells were visualized with a Zeiss Axiovision 3.1

microscope using 40x oil objective and an Axiocam HR (Zeiss) digital camera was used for photography. Cells transfected with GFP-G3BP constructs were prepared (fixed, permeabilized, and DAPI stained) and visualized as stated above.

Western blots

Cells were collected and incubated for 15 minutes, on ice, in lysis buffer (50mM HEPES, 150mM NaCl, 10% Glycerol, 1% Triton X-100, 10mM sodium pyrophosphate, 100mM NaF, 1mM EGTA, 1.5mM $MgCl_2$, Complete EDTA-free Protease Inhibitor cocktail (Roche Diagnostics), 1mM orthovanadate, 0.01mM PMSF). Protein concentrations were determined spectrophotometrically with the BioRad assay according to the manufacturer's instructions (Bio-Rad Laboratories Inc.). Western blotting was performed as previously described (Brennan et al 2000). Blots were probed with the following primary antibodies at these concentrations: α-GST-G3BP 3387 polyclonal sera (1:1000), α-HuR monoclonal 3A2 Ab (1:15000), α-p53 monoclonal 1801 (1:1000), α-p21 polyclonal (1:500, Santa Cruz). Blots were subsequently probed with the following secondary antibodies at these concentrations: α-rabbit HRP-conjugated (1:15000, Jackson ImmunoResearch Laboratories), and α-mouse HRP-conjugated (1:10000, Jackson ImmunoResearch Laboratories).

Apoptosis and necrosis assays

Trypan blue necrosis assay

Cell plates were UV-irradiated as described above. After the 1 hour recovery period, media in the plates was replaced with PBS/Trypan Blue dye solution (1:25 dilution of 0.4% Trypan Blue stock, Invitrogen). After 5 minutes, the Trypan Blue solution was removed and cells were washed twice with ice-cold PBS. Cells were visualized with a

Zeiss Axiovert 25 microscope using 10X objective, 10X ocular lenses. A Sony Cyber Shot digital camera was used for photography.

APOPercentage apoptosis assay

Cells were labelled with the apoptosis indicator dye according to manufacturer's protocol (BiocolorLTD. 2004). In brief, cell plates were UV-irradiated as described above. After the 1 hour recovery period, media in the plates was replaced with ice-cold culture media containing the labelling dye (1:20 dilution of dye to media). Plates were incubated in the presence of the dye for 30 minutes, on ice, and were subsequently washed 5 times with ice-cold PBS. Cells were visualized with a Zeiss Axiovert 25 microscope using 10X objective, 10X ocular lenses. A Sony Cyber Shot digital camera was used for photography.

Apoptotic cell quantification of cells transfected with GFP-tagged G3BP constructs

HeLa cells were transfected with 0.5µg of each plasmid (GFP-G3BP, GFP-G3BP S149E, and GFP-G3BP S149A). Cells were fixed 30 hours post-transfection and prepared for fluorescence imaging as described above with the following note to methodology: All washings and removal of solutions were performed gently and with great care in order to avoid removing apoptotic cells, which do not adhere readily to the glass cover slip. Cover slips were scanned by eye (40X oil objective) where any cell displaying green fluorescence was counted up to a population of approximately 150. Any fluorescent cell displaying traits of overt cellular shrinkage, or cellular shrinkage and nuclear ruffling and fragmentation, where qualified as apoptotic.

Fluorescence assisted cell sorting (FACS) analysis

10 cm culture plates of HeLa cells at 85% confluency were collected by trypsination and later pelleted by centrifugation (non-stressed and UV-stressed 1hour recovery

samples). The cellular density of cell pellets for each sample were quantified and normalized so that each sample contained a roughly equivalent number of cells to be subsequently labelled with PI and α-annexin V antibodies. Apoptotic and necrotic cells were identified by annexinV-FITC and propidium iodide staining, respectively, through flow cytometery (Martin et al 1995).

Discussion

Do there exist many worlds, or is there but a single world? This is one of the most noble and exalted questions in the study of Nature.

- Albert Magnus, thirteenth century

The family of G3BP proteins were first isolated through studies that identified protein binding partners to the SH3 domain of RasGAP (Kennedy et al 2002; Parker et al 1996). Subsequent characterization of these proteins demonstrated that they are phosphorylation-dependant endoribonucleases. This finding marked the first mechanism whereby the stability of specific mRNA transcripts show regulation by mitogenic signals that convey biochemical directives through Ras-signalling pathways. Since then, implicated cellular roles for these proteins has expanded far beyond that of post-transcriptional regulation of mRNA expression (Table 2). G3BP proteins may become the interest of numerous investigators in a variety of specializations for they are linked to biochemical processes ranging from Ras-signalling, tissue development, cancer progression, and cellular responses to environmental stress. The following thesis dissertation aimed at solidifying the initial conviction that G3BP1 could be an integral cellular entity in one of these aforementioned processes, that being its role in mammalian cellular responses to environmental stress.

Our strategy for initially classifying G3BP in stress responses was to observe changes in its subcellular localization via immunofluorescence imaging subsequent to a variety of environmental stresses. Like with the previously characterized stresses of elevated heat and treatment with arsenite (Mazroui et al 2006; Tourriere et al 2003), we observe G3BP's recruitment to cytoplasmic stress granules through conditions of osmotic stress and low doses of UV (Figure 1). Of greatest interest, we identified that G3BP rapidly accumulates in the nucleus following apoptotic doses UV (Figures 2 and 3). Its

nuclear accumulation appears maximal one hour after applying the stress, a period that mimics G3BP nuclear accumulation observed upon treatment of breast cancer cells with the growth factor, Heregulin (Barnes et al 2002). Our observations are the second characterization of this cytosolic RNA-binding protein translocating to the nucleus under conditions of stress, the first being observed with the stress of serum starvation (Tourriere et al 2001). We note that, up to now, accumulative evidence now affirms the fact that G3BP alters its subcellular localization in a *stress-specific* manner. Aside from the fact that our observations indicate that G3BP nuclear localization appears specific to UV and not to genotoxic stress in general, treatment of cells with chemotherapeutic stressors does not appear to induce a change in this protein's subcellular localization (Figure 11). Lastly, our investigations suggest that changes in G3BP's subcellular localization from stress are mutually exclusive, where its nuclear localization from apoptotic doses of UV is dominant over its recruitment to cytoplasmic stress granules (Figure 4). This observation coincides with assessments made by Cande and colleagues (2004), where they note G3BP's function in assembling stress granules is inhibited in the presence of the apoptosis-promoting factor, AIF. The observations of G3BP being localized to both stress granules and the nucleus following heat *then* UV treatment is interesting when placed into perspective of work done by Wu et al (2005). They observed that pre-treatment of cells with arsenite would protect these cells from undergoing apoptosis from UV irradiation. We question whether this 'protective' effect of arsenite might be related partly to G3BP. Arsenite sequesters G3BP in stress granules. Arsenite pre-treatment might be sequestering G3BP in stress granules such that there is little free cytosolic G3BP available to translocate to the nucleus from UV stress, thus impeding G3BP's possible function in the progression of apoptosis.

Our subsequent research goals focussed on determining possible mechanisms and functions for G3BP's observed nuclear accumulation from UV-induced apoptosis. The results of our main findings will now be summarized (Figure D). Before we begin, we would like to assert the fact that the presumed mechanisms and functions expressed here are preliminary and should not be construed as confirmed findings. Rather, the final purpose of this thesis work represents a foundation that subsequent research initiatives can build upon, where specific cellular processes have been implicated through our research such that subsequent analyses should aim to confirm these conclusions.

Through the use of GFP-tagged constructs of G3BP containing amino acid substitutions in two of its characterized phosphorylation sites, studies have demonstrated that the phosphorylation status of serine residue 149 regulates changes in G3BP's subcellular localization from stress. Specifically, the absence of phosphorylation at this site is linked to its ability to assemble stress granules while the phosphorylation of this residue may regulate it nuclear localization induced by serum starvation (Tourriere et al 2003; Tourriere et al 2001). Replicated transfections of these constructs demonstrated that all acquire a nuclear localization following our experimentally determined UV treatment (Figure 5). Since the S149A S232A construct— mimicking a hypophosphorylated G3BP protein— acquires a nuclear localization, we believe that phosphorylation of G3BP may not be a prerequisite for its nuclear localization under UV stress. Thus, it appears that the regulation process for this cellular movement might be distinct under conditions of serum starvation and UV irradiation.

Our most interesting observations arose from transfections of GFP-G3BP constructs that contain the amino acid substitution of S149E (Figure 5). These constructs were previously reported to be constitutively localized to the nucleus, where these observations

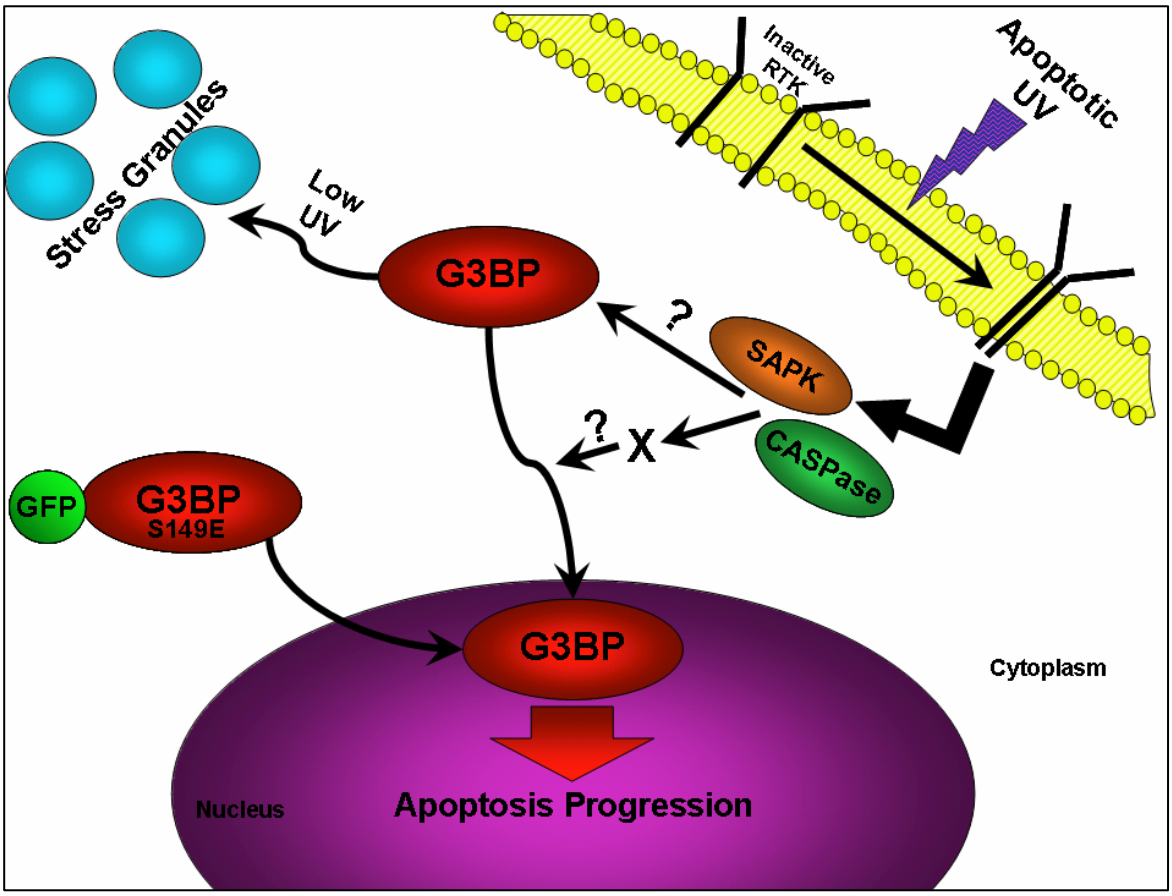


Figure D. Hypothetical metabolic scheme and function for G3BP's alterations in subcellular localization from UV stress. Low doses of UV stress promote G3BP's assembly of the pro-survival cellular structures of cytoplasmic stress granules. Apoptotic doses of UV stress promote G3BP's translocation to the nucleus. The nuclear accumulation of G3BP appears to serve a role in the process of apoptosis since forcing its nuclear accumulation, as observed with the GFP-tagged S149E construct, produces cells with apoptotic morphology. The induction of plasma membrane signalling and subsequent activation of stress activated protein kinases (SAPKs) and CASPases are implicated in the mechanism for G3BP nuclear localization. It is unknown whether the activity of SAPKs and CASPases directly target G3BP or if they target another cellular intermediate involved in the process of this cellular protein movement.

were made 20 hours after the transfection of this construct (Tourriere et al 2001). We observe that this constitutively nuclear version of G3BP appears to be cytotoxic and produces cells with apoptotic morphology when the construct is expressed in cells for periods over 20 hours (Figures 5 to 7). We did not perform analyses to confirm whether these GFP-expressing cells are in fact apoptotic. Therefore, we propose that subsequent analyses should replicate the data presented in figure 6, but with techniques that do not reside in subjective cell counting as was performed in this work. One appropriate technique would be to isolate the GFP-expressing cells by flow cytometry and analyze their apoptotic population distribution as is displayed in our FACS analysis presented in figure 15. Regardless, we feel that our preliminary results with the S149E construct suggests that G3BP nuclear localization is some how linked with the induction or promotion of apoptosis.

G3BP stress-induced nuclear localization and its link to the process of apoptosis is an interesting possibility. Subsequent investigations should focus on determining the specific functions G3BP partakes in within the nucleus. When reviewing the past literature on G3BP, one sees that specific enzymatic functions and biochemical processes are associated with this protein within this subcellular locale. For one, Costa and colleagues (1999) classified G3BP as a non-canonical helicase that effectively unwinds DNA, RNA, and DNA/RNA double helices. It would be of interest to isolate nuclear extracts following UV irradiation and assess whether this nuclear G3BP is active as a helicase through use of *in-vitro* helicase assays that were employed by Costa et al (1999). Secondly, G3BP appears to function as a transcription factor, but this function has only been formally linked to the very specific process of vaccinia virus replication (Katsafanas & Moss 2004). Yet, its function as a transcription factor might be broader in scope. It has

been previously shown that when G3BP acquires a nuclear localization, by Heregulin treatment of breast cancer cells, it co-localized specifically with acetylated histone H3 (Barnes et al 2002). Acetylated histone H3 is a well documented marker of sites of active transcription (Kruhlak et al 2001). Whether G3BP functions in the process of transcription following UV stress merits examination. It would be of interest to assess differences in mRNA expression subsequent UV stress comparatively between wild type cells and G3BP-null cells, possibly by employing cDNA microarray analysis. Similar analyses have been described previously by Zekri and colleagues (2005) when they characterized differences in mRNA expression levels between wild type and $G3BP^{-/-}$ mouse embryonic fibroblasts. Identification of G3BP acting as a transcription factor following UV stress would be a particularly interesting finding. Most notably, G3BP would mark an impressive interconnection between Ras-signalling pathways, post-transcriptional regulation and mRNA stability, and then, stress induced gene expression. In conclusion, the specific function G3BP serves within the nucleus of stressed cells remains to be determined, but previous observations suggest it might play a role in genetic expression through its helicase activity or its ability to contribute to the process of transcription.

Previous studies have implicated Ras-signalling as playing a role in mediating changes in G3BP's subcellular localization from stress. Constitutively active Ras-signalling increases the kinetics by which G3BP assembles stress granules from arsenite treatment (Tourriere et al 2003). G3BP nuclear localization occurs when Ras-signalling is silenced by serum depravation, whereby the presence of RasGAP within the cell is required for this nuclear localization to occur (Tourriere et al 2001). Our preliminary experimentation with inhibiting plasma membrane signalling with low concentrations of

Triton X-100 allude that G3BP nuclear localization following UV stress might also be mediated by Ras-signalling pathways (Figure 13). Our results are only suggestive since appropriate controls that demonstrate inhibition of plasma membrane signalling and the subsequent attenuation of Ras-signalling were not performed. The analysis with Triton X-100 should be replicated where the activity of plasma membrane receptors are assessed. This could be done by assessing the degree of phosphorylation of the epidermal growth factor receptor (EGFR) by western blotting. EGFR is characterized to become active by auto, cross-phosphorylation following UV irradiation, even in the absence of its complementary ligand (Rosette & Karin 1996).

By use of the inhibitors CNI 1493 and ZVAD, we have preliminary premonitions that the stress activated protein kinases (SAPKs), p38 MAPK and JNK, and active CASPases might play a role in the mechanism of G3BP's UV-induced nuclear localization (Figure 14). Controls are needed to assess the degree of inhibition by these drugs. Therefore, Western blotting analysis should be employed to observe the attenuation of CASPase cleavage of one of its targets, like the protein, spectrin, and the attenuation of phosphorylation of SAPKs targets, like c-jun. Though not proven, the implication of SAPKs and CASPases with our observations merits discussion.

For one, it would be of great interest to determine whether the activity of SAPKs and CASPases has a direct or indirect effect on G3BP. It is unknown if G3BP is a target for CASPase cleavage, but G3BP is known to change its degree of phosphorylation induced by serum starvation (Gallouzi et al 1998; Tourriere et al 2001). Could SAPKs have G3BP in their list of proteins they target for phosphorylation during responses to UV stress? An interesting possibility that should be addressed in future investigations. In reference to CASPases, we note that G3BP's main protein binding partner, RasGAP, is a

target for CASPase cleavage. RasGAP is known to be cleaved into three segments by CASPase-3 upon the induction of apoptosis (Yang & Widmann 2001). These CASPase cleavage sites are within the N-terminal region and flank the SH3-domain known to be the docking region for G3BP. Could inhibiting the cleavage of the RasGAP binding region occupied by G3BP sequester it within the cytoplasm? An interesting possibility that should be investigated. In all, we do not particularly favour the idea that SAPKs and CASPases play an active role in the mechanism for G3BP nuclear localization. Our observations that implicate SAPKs and CASPases in the process of G3BP nuclear localization confront us with a 'chicken or the egg' paradigm. Is the presumed inhibition of these proteins directly inhibiting G3BP cellular movement, or are we merely observing a general delay in the advancement of apoptosis? A caveat that will need to be addressed in future investigations.

The observation that the kinetics of G3BP nuclear localization is twice as lengthy specifically in the p53-null H1299 cell line is a bit of a curiosity. Since re-introduction of p53 protein had no effect on the kinetics of G3BP nuclear localization, we deem that the reason for this delay is disconnected to metabolic pathways that originate from the nexus of p53 activation. One should note that this cell line is an advanced metastatic tumour that is well characterized for genetic deletions in the p53 gene. One can only wonder what other innumerable chromosomal abnormalities might reside in these cells, any of which might deregulate the metabolic mechanism for G3BP nuclear localization following UV irradiation.

To conclude our thoughts on G3BP's nuclear localization upon UV-induced apoptosis we will address the broader implications of this observation. A review written on the G3BP proteins aptly described them as being biochemically 'promiscuous' since

they have been associated with a multitude of cellular processes (Irvine et al 2004). Our observations both complement – and conflict – with the notion that G3BP proteins have a function in a variety of metabolic processes that do not appear to be interrelated. Our observations *complement* this notion since we have associated G3BP with the novel process of UV-induced cellular apoptosis. Our observations *conflict* this notion for they can be linked to the previous findings that G3BP's subcellular localization is influenced by plasma membrane signalling and environmental stress. Our experiments add UV to the list of environmental stresses that influence G3BP's subcellular localization, where high and low UV exposures cause its migration to distinct subcellular locales (Figure C and D).

As a final thought, the preliminary evidence that suggests that forcing G3BP's nuclear accumulation results in cell death is particularly thrilling from a therapeutic perspective. It is known that the overexpression of G3BP proteins is a general trait for numerous tumour types (Barnes et al 2002; French et al 2002; Guitard et al 2001). Thus, the induction of the metabolic pathway responsible for G3BP's nuclear localization with a specific agonist could possibly be a tactic that would favour the death of tumour cells over healthy tissue. Therefore, the metabolic process and regulation of G3BP nuclear localization merits further investigations, for this understanding could hold promising ramifications for the treatment of cancer.

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