Regulation of hnRNP A1 Cellular Localization by Protein Kinases and its Biological Impact

By

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Abstract

Human Rhinoviruses (HRVs) utilize Internal Ribosome Entry Sites (IRES) to drive viral protein synthesis. IRESs are specialized RNA elements present within the 5' UTR of mRNAs that recruit ribosomes independently of the 5' m^7G cap structure. hnRNP A1 (heterogeneous nuclear ribonucleoprotein A1), a multifunctional RNA binding protein, is required for the IRES-dependent translation of many specific RNAs within the cell cytoplasm. The phosphorylation of hnRNP A1 is required for its cytoplasmic accumulation. I have identified and validated the role of HK2 in hnRNP A1 cellular localization by immunofluorescence microscopy, by analysis of HRV infection and by siRNA-based screening. These studies show that decreased HK2 protein levels lead to decreased cytoplasmic accumulation of hnRNPA1 during osmotic shock and HRV infection, to a decrease in HRV-infected cells and to decreased caspase activation in osmotically stressed and HRV-infected cells. Thus, HK2 may regulate hnRNP A1 cytoplasmic localization following HRV infection.

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List of Abbreviations

Akt	Protein kinase B (PKB)
Apaf-1	Apoptotic protease activating factor-1
APS	Ammonium persulfate
ARK5	AMPK-related protein kinase 5
ATP	Adenosine triphosphate
Bax/Bak	Bcl2-like protein 4/Bcl-2 homologous antagonist killer
Bcl-XL	B-cell lymphoma extra large
3-BrPa	3-Bromopyruvic acid; 3-Bromopyruvate
BSA	Bovine serum albumin
Casp3/7	Caspases 3 and 7
CD4	Cluster of differentiation 4
Clk	CDC-like kinase
c-myc	Cellular myelocytomatosis oncogene
CO_2	Carbon dioxide
Co-IP	Co-immunoprecipitation
COPD	Chronic obstructive pulmonary disease
Ct	Cycle threshold
DAPI	4',6-diamidino-2-phenylindole
ddH_2O	Double-distilled water
DMEM	Dulbeccos's modified eagle's medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	Ethylenedietholaminetetra-acetic acid
EGR2	Early growth response 2
eIF	Eukaryotic initiation factor
ERK8	Extracellular signal-regulated kinase 8
EV71	Enterovirus 71
FBS	Foetal bovine serum
FGF2	Fibroblast growth factor 2

FITC	Fluorescein isothiocyanate
FLAG	FLAG octapeptide protein tag
FRAP	Fluorescence recovery after photobleaching
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescence protein
GSK3β	Glycogen synthase kinase 3 beta
GST	Glutathione-S-transferase
HCV	Hepatitis C virus
HeLa	Henrietta Lacks cell line
HeLa T4+	T4 cell-activated HeLa cell line
HindIII	*Haemophilus influenzae* Restriction enzyme 3
His	Histidine protein tag
HIV	Human immunodeficiency virus
HK	Hexokinase
hnRNP	Heterogeneous nuclear ribonucleoprotein
Hoechst	Trihydrochloride trihydrate
HRP	Horseradish peroxidase
HRV	Human rhinovirus
ICAM	Intercellular adhesion molecule 1
IRES	Internal ribosome entry site
ITAF	IRES trans-acting factor
kDa	Kilodalton
KOD	*Thermococcus kodakaeraensis* polymerase
LB	Luria-Bertani
LDLR	Low-density lipoprotein receptor
mA	Milliamps
MAPK	Mitogen-activated protein kinase
MALDI-TOF	Matrix-assisted laser desorption/ionization-time-of-flight
MBP	Myelin basic protein
MNK1/2	MAPK interacting Ser/Thr kinase 1/2
MOI	Multiplicity of infection
mRNA	Messenger RNA

NLS	Nuclear localization signal
Ns	Non-significant
NTPase	Nucleotide tri-phosphate protease
OSM	Osmotic shock
PABP	Poly-A binding protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	PBS/Tween 20 (0.1%)
PCBP2	Poly(rC) binding protein 2
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
Poly-A	Poly-adenylated
pre-mRNA	Precursor messenger RNA
PTB	Polypyrimidine tract binding protein
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RPM	Revolutions per minute
RRM	RNA-recognition motif
RT-PCR	Reverse-transcription PCR
SDS	Sodium dodecyl sulfate
siControl/ siC	Non-targeting siRNA
γ-^{32}P-ATP	Radio-labeled ATP at the γ phosphate position
siHK2	HK2-targeting siRNA
siRNA	Short-interfering RNA
SR	Serine/arginine proteins
SRPK	Serine/threonine protein kinase
ssRNA	Single-stranded RNA
TCID50	Tissue culture infectious dose
TEMED	Tetramethylethylenediamine
Tris-Cl	Tris-base/hydrogen chloride mixture

TRITC	Tetramethylrhodamine isothiocyanate
U2OS	Cultured osteosarcoma cells
µCi	microCuries
Unr	Upstream of N-ras
UT	Un-transfected
UTR	Un-translated region
UVC	Ultra-violet C rays
VDAC	Voltage dependent anion channel
VPg	Viral protein genome-linked
XIAP	X-linked inhibitor of apoptosis protein

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Preamble

The health and survival of an organism is greatly dependent on its ability to adapt to stresses such as disease and environmental changes. This remains true at the cellular level, where cells wield various mechanisms to efficiently detect, prepare and adapt to a multitude of stresses. Even today, researchers continue to uncover these mechanisms, of which some remain poorly characterized, to understand and possibly correct faulty stress responses from cells in disease conditions. In this study, mechanisms involving the RNA-binding protein hnRNP A1 are investigated in the context of osmotic shock and of Human Rhinovirus infection in mammalian cells. The functions of hnRNP A1 are now well known; however, the understanding of its regulation, more specifically of the regulation of its location in the cell during cellular stress, remains inadequate. The characterization of this essential protein's regulation will provide a greater comprehension of cellular stress response mechanisms.

Chapter 1: Introduction

1.1 Human Rhinovirus

Human Rhinovirus (HRV) is a positive-sense single-stranded RNA (ssRNA+) virus from the family *picornaviridae*. This family of viruses also includes Poliovirus, Enterovirus 71 (EV71) and Hepatitis A virus, among many others, which share similar structure and replication mechanisms [Lin et al., 2009 (review) and in Chase and Semler, 2012]. Like other ssRNA+ viruses, HRV has been shown to utilize hnRNP A1 to promote viral RNA translation via IRES [Cammas et al., 2007]; however, the mechanism by which hnRNP A1 is localized to the cell cytoplasm and recruited to viral IRESs during viral infection remains

uncharacterized. In the following sections, HRV attributes and impact on the human cell will be addressed.

1.1.1 Clinical Implications

Rhinoviruses are the primary cause of the common cold. They infect the upper respiratory tract of millions of individuals in North America each year, causing unpleasant disease conditions, upper respiratory tract complications and costing millions of dollars in over-the-counter medications and in missed school and work days [Fendrick et al., 2003, Roelen et al., 2011]. Colds affect children more often than adults; in Canada, it is estimated that children get upper respiratory tract infections, which are primarily caused by Rhinoviruses, 3 to 8 times a year [Thomas, 2010]. This translates to an average of more than 30 million upper respiratory tract infections in Canadian children alone each year.

Adults tend to downplay the negative effects of rhinovirus infections and continue attending their workplace, further spreading the disease. For the majority of people, common colds have little more symptoms than sore throat, fatigue and malaise; however, for some individuals a "meagre" cold can cause serious complications, especially in people suffering from immunodeficiency, COPD and asthma. Also, many rhinovirus infections lead to doctor's visits where physicians are unable to provide relief from the infection and have their valuable time wasted during those visits.

These observations advocate for a great need for research in this field. Understanding the molecular mechanism of infection of rhinoviruses would not only improve the treatment of infections, cutting major costs on pharmaceuticals, doctors' visits and missed school and

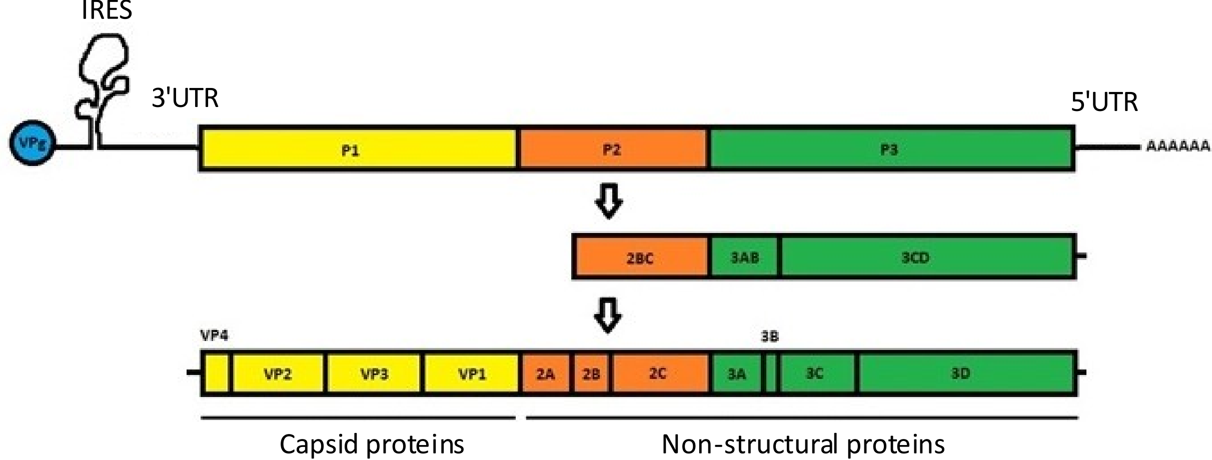
work days, but also translate to similar therapeutic approaches to other Picornaviruses as they tend to share analogous mechanisms of replication and viral protein synthesis.

1.1.2 Replication mechanisms

HRV is composed of a single-stranded positive-sense RNA genome encapsulated within an icosahedral-shaped capsid. Its genome encodes a polyprotein which is made up of the different structural and non-structural viral proteins essential for HRV formation (Figure 1.1). This single-stranded RNA (ssRNA) does not contain a 5'cap to promote its translation but rather contains an IRES and a protective VPg protein in its 5'UTR which support the synthesis of the viral polyprotein. [Lin et al., 2009 (review), Tapparel et al., 2007]

HRV, like other Picornaviruses, starts its journey into the host cell by interacting with a host membrane receptor in order to enter the cell by endocytosis. Rhinoviruses can be classified into two groups, Major Group and Minor Group, based on the receptors they bind to [Tuthill et al., 2010 (review)]. Most Major Group HRVs bind to the Intercellular Adhesion Molecule 1 (ICAM-1) and Minor Group HRVs bind to the Low Density Lipoprotein Receptor (LDLR) family of proteins. CD4 is also a known receptor of HRV and other viruses such as HIV [Tuthill et al., 2010 (review), Olson et al., 1993].

Following the interaction with specific receptors, HRV enters the cell by endocytosis. Once the virus gains access to the host cell cytoplasm, its genome is released into the cytoplasm and is efficiently translated into its corresponding polyprotein *via* its IRES. As the viral RNA is positive-sense, it is translated at a higher speed compared to



IRES
3'UTR
5'UTR
VPg
P1
P2
P3
AAAAAA
2BC
3AB
3CD
VP4
3B
VP2
VP3
VP1
2A
2B
2C
3A
3C
3D
Capsid proteins
Non-structural proteins

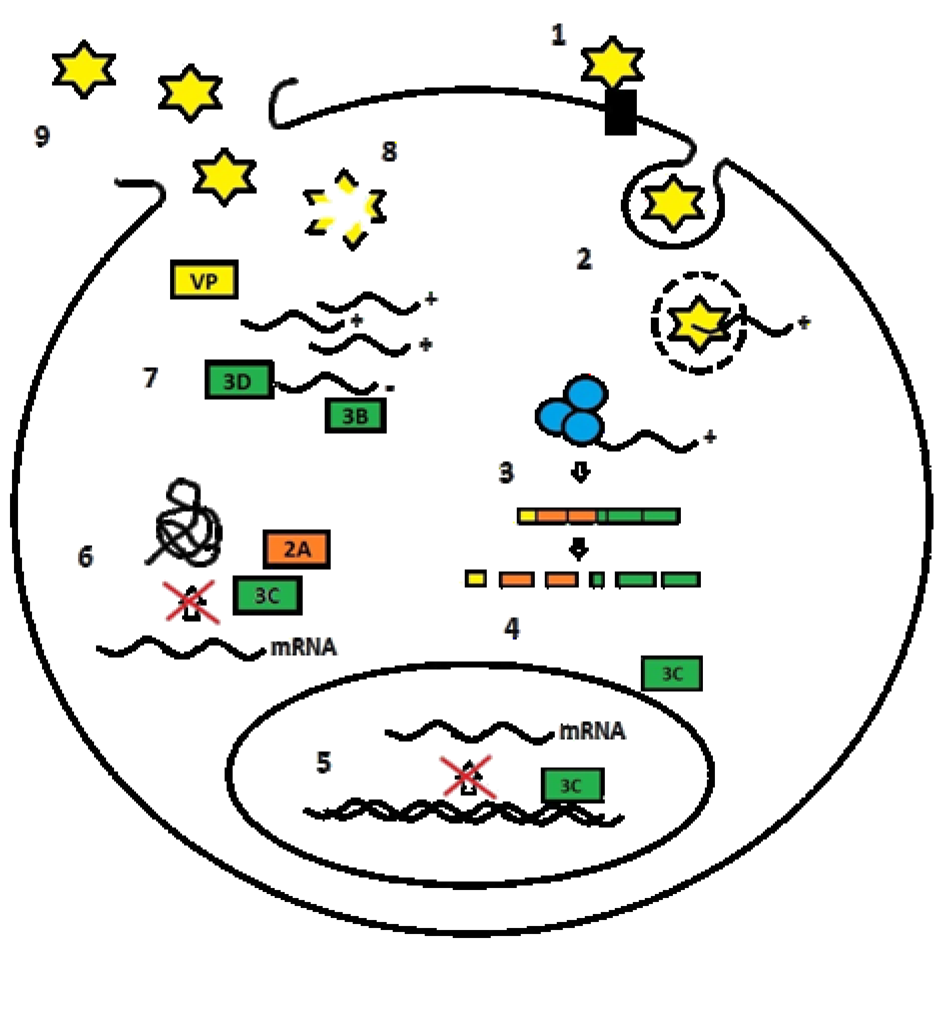
Figure 1.1. **Representation of HRV RNA genome and subsequent peptides. Top:** HRV genome illustrated as initially translated polyprotein. **Middle:** HRV genome illustrated as intermediate cleaved proteins. **Bottom:** HRV genome illustrated as individual cleaved proteins, 3'poly-A tail, 5'UTR containing IRES and VPg protein. Modified from [Lin et al., 2009]

negative-sense RNAs, as the translation machinery can directly synthesize the resulting protein. The viral polyprotein is cleaved into its functional proteins: capsid proteins VP1 to VP4 and non-structural proteins 2A, B, C and 3A, B, C and D. Proteins 2A and 3C are proteases that participate in the shut-off of cap-dependent host translation. They do so quickly following infection in order to promote a higher rate of IRES-mediated translation. Cap-dependent translation is inhibited by the cleavage of eIF4G [Chase and Semler, 2012] by 2A protease, of eIF4A1 by 3C protease, and of PABP by both 2A and 3C proteases. 3C protease also enters the nucleus of the host cell and inhibits gene transcription [Lin et al., 2009]. In parallel, ITAFs such as hnRNP A1, PTB and Unr among others, are recruited to the viral IRES [Lin et al., 2009].

Picornavirus IRESs can be classified in four groups, Type I, II, III and IV [Lin et al., 2009 (review)]. The IRESs within the same group have well conserved structures and sequences, unlike IRESs from different groups which differ from each other, suggesting that viruses from the same group utilize the same types of ITAFs to drive viral protein synthesis. All picornavirus IRESs contain an AUG codon downstream of a pyrimidine-rich tract. In most viruses, this AUG will directly bind to the recruited ribosome; however, in poliovirus this AUG does not participate in translation initiation. Rather, an AUG codon 160 nucleotides downstream of the typical initiation codon binds to the ribosomal subunits [Hellen and Sarnow, 2001]. This shows that even similarly-structured IRESs can vary in function between different viruses. In fact, picornaviruses all require certain ITAFs: HRV IRES for instance, requires PTB and unr while poliovirus IRES, belonging to the same group as HRV, is not activated by unr [Hellen and Sarnow, 2001].

As viral translation is underway through IRES activation, the other resulting viral proteins efficiently replicate the viral genome, package the genome into an assembled capsid and lyse the cell in order to infect neighbouring cells. To do so, protein 2B increases the host cell membrane permeability, protein 2C, an NTPase, promotes vesicle formation, proteins 2A, 3A and 3C inhibit the intracellular transport of proteins and protein 3B primes RNA synthesis in order for protein 3D, an RNA polymerase, to efficiently replicate the positive-stranded RNA into negative-strands. RNA synthesis is commenced following the inhibition of viral protein synthesis: viral proteinases 3C and 3CD (an intermediate cleavage product of the viral polyprotein, see Figure 1.1) cleave PCBP2, an essential ITAF in order to halt IRES-mediated translation. The newly cleaved PCBP2 is assembled into a replication complex along with the required viral proteins which drive RNA replication. The subsequent negative-stranded RNA is replicated into functional positive-stranded genomes to be packaged into the newly forming viruses. (See Figure 1.2 for a schematization of the HRV life cycle) [Lin et al., 2009 (review)]

The last step of HRV life cycle is its release into the extracellular environment through the lytic cycle of the host cell. As the virus spreads to new host cells, it requires the infected host cell to burst, after which the virus can emerge and infect new cells. Although this mechanism is not completely understood, it has been shown that HRV infection leads to the activation of caspase 9 and subsequently of caspase 3, which leads to apoptosis of the host cell and promotes the release of virus progeny [Deszcz et al., 2005]. In this model, cytochrome c can leak into the cytoplasm from the mitochondrial membrane following apoptotic signals and cause the activation of caspases, which will destroy the cell. Caspase 8 is not activated, which suggests that this mechanism does not involve the


1
9
8
2
VP
+
+
+
+
7
3D
-
3B
+
3
6
2A
3C
mRNA
4
3C
mRNA
5
3C

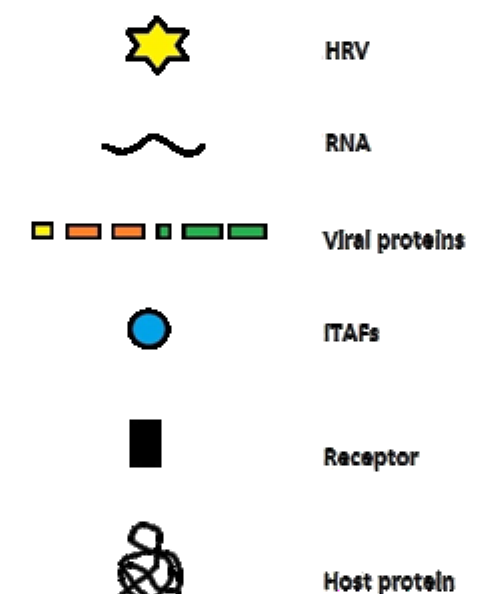

HRV
RNA
Viral proteins
ITAFs
Receptor
Host protein

Figure 1.2. **Representation of HRV life cycle in the host cell**. **1.** HRV capsid proteins interact with host cell membrane receptor. **2.** HRV enters the host cell by endocytosis; membrane is disintegrated as viral RNA is expulsed into the host cell cytoplasm. **3.** Host proteins translate viral RNA into HRV polyprotein via IRES. **4.** Polyprotein is cleaved into functional proteins. **5.** Viral proteases arrest host transcription in the cell nucleus and **6.** arrest host translation mechanisms. **7.** HRV RNA is replicated from positive-strand to negative-strand, to final positive-stranded RNA. **8.** Virions are formed from viral capsid proteins and replicated RNA. **9.** Lysis of host cell; newly formed HRV viruses emerge from cell and spread to neighbouring host cells to repeat life cycle.

extrinsic receptor-mediated pathway of apoptosis but utilizes only the intrinsic pathway. This model is also supported by the findings that HRV infection also leads to the cleavage of high molecular weight DNA, which is another indication of apoptosis in the cell [Kerr et al., 1972].It has been shown by [Cammas et al., 2007] that hnRNP A1 promotes HRV IRES translation in host cells. This group showed that when hnRNP A1 protein levels are decreased in the cell, HRV IRES activity also decreases. They also show that hnRNP A1 accumulates in the cytoplasm of infected cells and physically binds to the HRV IRES. In this study, the mechanism by which hnRNP A1 accumulates in the cell cytoplasm following HRV infection was investigated.

1.2 RNA-binding protein hnRNP A1

Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) is an RNA-binding protein which belongs to a large family of hnRNP proteins and is involved in pre-mRNA splicing regulation [Mayeda and Krainer, 1992] as well as mRNA export [Piñol-Roma and Dreyfuss, 1992], telomere maintenance [Ting et al., 2009], and mRNA stability [Zhao et al., 2009]. HnRNP A1 is primarily located in the cell nucleus, but also shuttles to the cytoplasm to export processed mRNAs. This has been suggested by the Dreyfuss group following experiments showing hnRNP A1 is bound to RNA even when it shuttles to the cytoplasm, supporting its role as a transporter of mRNAs [Piñol-Roma and Dreyfuss, 1992].

In stress conditions such as osmotic shock and UVC irradiation, hnRNP A1 localizes to the cell cytoplasm and fulfills other functions: it behaves as a regulator of the cap-independent synthesis of specific proteins and affects the stability of specific mRNAs. This

role of hnRNP A1 in cell stress is the main focus of this study and will be discussed further in the next sections.

1.2.1 Nuclear functions

HnRNPs are one of many types of proteins which regulate pre-mRNA splicing. They are most often inhibitors of splicing at the specific site where they would bind the pre-mRNA, although many exceptions exist where an hnRNP would enhance the splicing at a certain site [Zhu et al., 2001, Tange et al., 2001, House and Lynch, 2006, Coelho and Smith, 2012]. Given their mainly inhibitory properties, hnRNPs are often recruited by silencing splicing elements on the pre-mRNA. HnRNPs contain RRM (RNA recognition motif) which enables these proteins to recognize the polypurine tracts of unprocessed mRNAs, which have a high affinity for many splicing factors such as hnRNP A1, hnRNP F and SR proteins [Cléry and Allain, 2012].

HnRNP A1 is a splicing regulator that inhibits splicing by competing with other factors [Cléry and Allain, 2012]. It can dimerize as it contains two RRM domains that can bind together, which enables it to assist in alternative exon excision or in the splicing of a large intron [Ding et al., 1999, Blanchette and Chabot, 1999]. A mechanism by which these nuclear functions may be blocked is by preventing the localization of hnRNPs to the nucleus through post-transcriptional modifications. Many hnRNPs as well as SR proteins may be phosphorylated by certain protein kinases thus preventing their localization from the nucleus, causing them to accumulate into the cell cytoplasm, away from splicing events. For example, Clk and SRPK kinases have been shown to regulate SR protein localization and p38 MAPK

kinase [van der Houven van Oordt et al., 2000] as well as its downstream MNK1/2 kinase [Guil et al., 2006] have been shown to regulate the localization of hnRNP A1 following osmotic stress to the cell. These regulation mechanisms offer more control over splicing patterns during specific cellular events as they do not alter protein levels or sequences, making them easily reversible.

1.2.2 Cytoplasmic functions

Stress granules

Following various cell stresses, hnRNP A1 has been shown to not only localize from the cell nucleus to the cytoplasm, but also to accumulate into cytoplasmic stress granules [Guil et al., 2006]. Stress granules are aggregates of RNPs and mRNAs that are formed following the arrest of translation initiation in the cell, most commonly as a result of stress factors. These are thought to affect mRNA stability and translation as well as mediate specific biochemical interactions within the cell [Buchan and Parker, 2009]. As the accumulation of biomolecules into stress granules leads to a concentrated punctate area of proteins and RNA, and concomitantly to a less-concentrated cytosol, it is not unlikely that stress granules are vehicles for preventing specific interactions (as biomolecules become "trapped" within the granules) or for promoting certain reactions between RNA and RNP molecules.

Taking this into account, the accumulation of hnRNP A1 into stress granules could facilitate its function during cell stress, which is to mediate cap-independent translation. Guil et al. show using FRAP (fluorescence recovery after photobleaching) assays that hnRNP A1

inhabits these granules very actively, constantly shuttling in and out of the observed stress granules [Guil et al., 2006]. This group also shows that the hyperphosphorylation of hnRNP A1 that is required for its cytoplasmic localization is not required for its accumulation within stress granules; however, the binding of hnRNP A1 to mRNA molecules is required for both stress granule accumulation and hyperphosphorylation [Guil et al., 2006].

Translation regulation

As a response to stress, the cell attenuates global protein synthesis that uses cap-dependent mechanisms and turns to the cap-independent translation of select RNAs [Graber and Holcik, 2007 (review)]. This allows for a selective synthesis of proteins that participate in the stress response mechanisms of the cell, such as anti- and pro-apoptosis proteins [Xu et al., 2005 (review)] and heat-shock proteins [Santoro, 2000 (review)] among others. Many of these proteins are translated via Internal Ribosome Entry Sites (IRES) located in the 5'UTR of their mRNA sequence, which form secondary RNA structures capable of interacting with protein factors and recruiting ribosomes to the mRNA without the presence of canonical translation initiation factors. During canonical protein synthesis, mRNAs, which contain a 5'-cap composed of a methylated guanosine, undergo translation initiation, elongation and termination. Translation initiation is the step that most efficiently regulates protein synthesis, as much more energy and resources are required to stop the synthesis reaction or change a protein's components once it is synthesized. When a cell requires the translation of only a select number of specific RNAs, it shuts-off global translation by preventing the initiation step through the 5'-cap of mRNAs. To do so, the binding of the canonical initiation factor eIF4 is inhibited and the ribosomal 40S subunit is unable to proceed with the scanning of the mRNA in order to initiate protein synthesis [Poulin and Sonenberg, 2000 (review)].

Following the arrest of global protein synthesis, the cell requires specific proteins to be translated in order to adapt and survive; therefore, the translation mechanism switches to cap-independent mechanisms, such as IRES, to initiate this translation process.

IRESs are essential for the synthesis of proteins when global protein synthesis is shut-down, such as during the cell cycle progression into G_0 and M phase and following cell stresses [Hellen and Sarnow, 2001 (review)]. Although translation *via* IRES may not require canonical translation initiation factors, it does require other factors termed IRES trans-acting factors (ITAF). The exact functions of the ITAFs that are recruited to specific IRESs remain unclear; however evidence suggests that the cellular localization of ITAFs play an important role in regulating their activity [Lewis et al., 2007].

As mentioned, hnRNP A1 has been shown to localize to the cytoplasm of cells following cells stresses such as osmotic shock [Allemand et al., 2005, Bevilacqua et al., 2010, Lewis et al., 2007], UVC irradiation [Cammas et al., 2007], Actinomycin D treatment [Piñol-Roma and Dreyfuss, 1992] and viral infection [Cammas et al., 2007]. During these stresses, hnRNP A1 functions as an ITAF and has been shown to physically bind to and modulate the activity of the IRES of various cellular mRNAs such as XIAP [Lewis et al., 2007], Bcl-xL [Bevilacqua et al., 2010], FGF2 [Bonnal et al., 2005], EGR2 [Rübsamen et al., 2012], cyclin D1 [Jo et al., 2008], c-myc [Jo et al., 2008] and APAF1 [Cammas et al., 2007] as well as IRESs of positive-sense ssRNA viruses such as Human Rhinovirus [Cammas et al., 2007], Enterovirus 71 [Lin et al., 2009 (2)], Sindbis virus [Lin et al., 2009 (2)] and Hepatitis C virus [Kim et al., 2007] among others. Interestingly, while some IRESs are negatively regulated by hnRNP A1, viral, FGF2, cyclin D1 and c-myc IRESs are positively regulated.

Regulation of localization

It has been shown that a non-canonical nuclear localization signal (NLS) is present in the C-terminus of hnRNP A1 protein which interacts with transportin proteins in the nuclear membrane pores. This NLS, termed M9, is a 37 amino acid-long motif that is required for the nuclear import hnRNP A1 [Pollard et al., 1996]. This has been shown by the fusion of this motif to Glutathione S-transferase (GST) protein, which in turn resulted in a nuclear localization of this typically cytoplasmic protein [Fridell et al., 1997]. The deletion of the M9 motif from hnRNP A1 results in a cytoplasm-restricted protein [Izaurralde et al., 1997]. Another region of hnRNP A1 that has been shown to be essential for its cytoplasmic localization is the F-peptide, of which the phosphorylation is necessary for hnRNP A1 to accumulate to the cytoplasm (Figure 1.3). A phospho-mimetic version of this peptide, of which 6 consecutive serine residues (4 of which are phosphorylated during cellular stress) are exchanged for aspartic acid residues has been shown to accumulate to the cytoplasm in the absence of cell stress; in contrast, a phospho-null variant, where these 6 key serine residues are exchanged for alanine residues, is restricted to the nucleus, even during cell stress [Allemand et al., 2005]. As Allemand et al. propose, it is possible that the hyperphosphorylation of the F peptide during cell stress prevents the binding of the M9 motif to transportin proteins, resulting in the inability of hnRNP A1 to enter the cell nucleus. This could happen through a conformational change in the C-terminus of the protein, causing a steric hindrance within the M9 motif, thus preventing proper interactions with transportins.

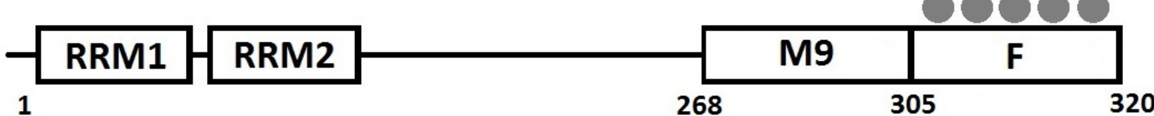

RRM1
RRM2
M9
F
1
268
305
320

Figure 1.3. **Representation of hnRNP A1 domains**. RRM: RNA recognition motif. M9: C-terminal M9 motif. F: C-terminal F-peptide. Gray dots represent six consecutive serine residues that are phosphorylated during cell stress. Number of amino acids is indicated at bottom of map. Modified from [Allemand et al., 2005]

As mentioned above, MNK1/2 protein kinase has been shown to phosphorylate hnRNP A1, resulting in its cytoplasmic localization [Guil et al., 2006]. Others have shown that eIF2α phosphorylation also mediates hnRNP A1 localization in the cell following stress. Bevilacqua et al. have inhibited the phosphorylation of eIF2α *in vivo* by mutating its serine residue at position 51 to an alanine residue, which in turn prevents the arrest of cap-dependent translation of RNAs following cell stress. In this system during cell stress, hnRNP A1 does not accumulate in the cytoplasm [Bevilacqua et al., 2010]. This suggests that either the arrest of cap-dependent translation in response to cell stress, or the phosphorylation of eIF2α alone is required for hnRNPA1 to accumulate in the cell cytoplasm and fulfill its cytoplasmic functions. In addition to the localization of hnRNP A1 being affected in this model, its function as an ITAF is also compromised as Bcl-XL and XIAP protein levels are higher, and downstream caspase 3 activation is nonexistent in cells expressing the mutant eIF2α. This defect can be rescued by the expression of Bcl-XL siRNA, following which caspase 3 is cleaved and cell death is more prominent. HnRNP A1 cytoplasmic localization can also be rescued with the expression of the cytoplasm-restricted phospho-mimetic hnRNP A1 mutant, which results in a decrease in BcL-XL protein levels and an increase in caspase 3 activation in stressed cells [Bevilacqua et al., 2010], thus suggesting that the function of hnRNP A1 is dependent on its localization in the cytoplasm.

In summary, evidence shows that, in response to stress, infection and cell cycle progression, cells utilize IRES-mediated protein synthesis in order to efficiently and selectively control the protein population within in order to adapt to environmental changes. To do so, many ITAFs are recruited to their respective IRES through their cellular localization and participate in the translation of specific mRNAs.

Preliminary data from the Holcik laboratory suggest that, along with a few other protein kinases, HK2 protein levels affect hnRNP A1 localization in cells during stress response. A siRNA-based screen of mammalian protein kinases in osmotically stressed U2OS cells was performed in order to identify which kinases are required for hnRNP A1 to localize to the cell cytoplasm during this stress. When HK2 protein levels were knocked-down using a pool of siRNA, hnRNP A1 remained localized in the cell nuclei during osmotic shock [Crasto and Holcik, unpublished data]. This preliminary data strongly suggests that multiple protein kinases may be involved in the regulation of hnRNP A1 localization through phosphorylation and provides a foundation for this study.

1.3 Hexokinase 2

Hexokinase 2 (HK2) is a widely studied protein whose well-known role is to phosphorylate glucose in order to initiate the glycolysis chain of reactions. Its activity is carefully regulated by the cells' need for ATP: in a catalytic state, HK2 is active in driving glycolysis while in an anabolic state, HK2 activity is reduced in order to conserve energy. This negative feedback is often deregulated in cancer cells. In this study, I propose a new function for HK2 in the cell, one that involves cell stress response and the regulation of hnRNP A1's function as an ITAF.

Function in glycolysis

As mentioned, HK2 phosphorylates glucose in the cell cytosol in order to produce glucose-6-phosphate in an ATP-dependent manner. This nearly irreversible reaction is required for glucose molecules to be processed through the cell's metabolism into ATP, metabolites and CO_2.

HK2 is located both onto the outer mitochondria membrane (OMM) as bound to VDAC proteins and within the cell cytosol. Mitochondria-bound HK2 is more efficient at driving glycolysis as it is physically closer to ATP molecules emerging from the mitochondria and the resulting glucose-6-phosphate can be directly incorporated into the downstream glycolysis and oxidative phosphorylation reactions. [Robey and Hay, 2006 (review)] In conditions of glucose starvation, glucose-6-phosphate accumulates in the cytosol as the cell converts to an anabolic state and promotes the dissociation of HK2 from the VDAC proteins. If this state is sustained for too long, the Bax/Bak protein heterodimer (a pro-apoptotic protein complex) will bind to the VDAC proteins to then promote the release of cytochrome c from the mitochondria's inter-membrane space and the cell undergoes apoptosis [Pastorino et al., 2002].

HK2 is a member of a family of four different hexokinases: HK1, HK2, HK3 and HK4. Although they all share the main function of phosphorylating glucose molecules, hexokinases differ in key characteristics. For example, HK4 also known as glucokinase, has the lowest affinity for glucose of all hexokinases, while HK3 lacks the capacity to bind to VDAC proteins and HKI has only one catalytic domain to phosphorylate glucose. In contrast, HK2 has two catalytic domains, has the highest affinity to glucose, and binds to VDAC proteins on the mitochondria, making it the most efficient and up-regulated hexokinase in cancer cell metabolism.

I hypothesize that HK2 regulates the localization of hnRNP A1 through its phosphorylation during cell stress and during Human Rhinovirus infection. In order to test this hypothesis, this study was conducted addressing four main objectives: 1. to validate the findings from the siRNA-based screen in osmotically shocked cells; 2. to investigate the

effect of HK2 knock-down on the HRV life cycle; 3. to investigate the effects of hnRNP A1 localization on the HRV life cycle, and 4. to elucidate the phosphorylation mechanism by which hnRNP A1 localization is regulated.

Chapter 2: Materials and Methods

2.1 Cell Culture and Transfection

HeLa T4+ cells were cultured in HyClone™ High-Glucose Dulbecco's Modified Eagle's Medium (Thermo Scientific) containing 4500 mg/L Glucose, 1.0584% L-Glutamine, 100 000 U/L Penicillin, 100 µg/L Streptomycin, and 1% Heat-inactivated Foetal Bovine Serum (referred to as "Complete DMEM" throughout this report) at 37°C and 5% CO_2. For knock-down experiments, 5.0E04 cells were seeded in a 12-well plate in Complete DMEM containing no antibiotics and grown for 24 hours. Cells were transfected with 20nM scrambled-sequence siRNA (negative control, GE Healthcare Dharmacon Inc.) or siHK1 (Silencer® Select siRNA, Ambion® Life Technologies Inc.) or siHK2 (Silencer® Select siRNA, Ambion® Life Technologies Inc.) using Lipofectamine® RNAiMAX transfection reagent (Invitrogen) following manufacturer's protocol for forward transfection and grown for 48 hours. For over-expression experiments, 1.0E05 cells were seeded in a 12-well plate in Complete DMEM containing no antibiotics grown for 24 hours. Cells were transfected with 0.5 µg (GFP-HK1) or 1.5µg (hnRNP A1) plasmid DNA using Lipofectamine® 2000 transfection reagent (Invitrogen) following manufacturer's protocol and grown for 24 hours. GFP-HK1 plasmid was purchased through Addgene database (Addgene plasmid 21917, [Sun et al., 2008]), hnRNP A1 F2 and ΔM9 mutant plasmids were generously provided by Dr Stephen Lewis and by Dr Maria Hatzoglou, respectively; both were previously described [Allemand et al., 2005, Izaurralde et al., 1997].

2.2 Cloning

To create FLAG-HK2 construct, PCR was performed using GFP-HK2 construct (Addgene Plasmid 21920, [Sun et al., 2008]) as a template, primers containing FLAG sequence, EcoRI and HindIII target sequences (See Appendix), Novagen® Hot-Start KOD RNA polymerase kit (EMD Chemicals Inc.) and manufacturer's protocol. PCR product was sub-cloned into pcDNA3.1+ vector by digesting both PCR product and vector (500 ng of DNA) with EcoRI and HindIII restriction enzymes (Invitrogen), 10 X Restriction buffer M (Invitrogen) and ddH2O for 2 hours at 37°C. Digested DNA was run on 0.8% agarose gel (0.8% agarose and 0.006% ethidium bromide in 1 X TAE Buffer (0.04M Tris Base, 0.001M EDTA pH 8.0, 0.114% Acetic Acid)) and purified from gel using UltraClean® 15 DNA purification kit (MO BIO Laboratories, Inc.). PCR product and vector were ligated in 1:1, 1:3, 1:5 and 1:10 vector: insert ratios using T4 DNA Ligase (Invitrogen) and manufacturer's protocol. DNA ligations were transformed into competent DH5α *E.coli* by incubating them on ice for 30 minutes, at 42°C for 45 seconds on ice for 2 minutes before the addition of LB broth without antibiotic. Bacterial culture was incubated at 37°C for 1 hour at 200 RPM, and spread onto plates of LB agar containing 100 μg/mL ampicillin and incubated at 37°C overnight. Colonies from agar plates were picked and grown into a 4-mL culture in LB broth containing 100 μg/mL ampicillin. Plasmid DNA was isolated from bacterial culture using QIAprep® Spin miniprep kit (QIAGEN) and digested with EcoRI and HindIII following protocol described above. Digested DNA was run on a 0.8% agarose gel and plasmids containing insert DNA were sequenced for confirmation of presence of HK2 sequence (see Appendix for sequencing primer information).

2.3 HRV Infection and Osmotic Shock

For Human Rhinovirus (HRV) infection, 4.0E05 cells in 12-well culture plate were infected by removing culture medium from cells, rinsing cells once with PBS buffer, adding 100 µL of stock HRV (original HRV aliquot generously provided as a gift from Dr Nahum Sonenberg [Goodman Cancer Centre, McGill University, Montreal, Canada]) or 100 µL PBS buffer (negative control) to cells. Plate was incubated at 37°C and 5% CO_2 for 1 hour with gentle swirling every 15 minutes. 1.0 mL of Complete DMEM was added to cells and infection was carried-out for 4 hours, 6 hours, 10 hours or 24 hours. For kinetic cell imaging experiments (see Kinetic Cell Imaging), Complete DMEM was supplemented with 1:5000 Caspase 3/7 dye (Essen Biosciences). Comparable amounts of HRV were used throughout experiments as determined by Caspase 3/7 activity. For osmotic shock experiments, 1.2E06 cells in a 6-well culture plate were osmotically shocked by removing culture medium from cells and replacing it with 2.0 mL warmed Complete DMEM containing 0.3M or 0.4M D-sorbitol (Sigma-Aldrich Co.) or with 2.0 mL Complete DMEM (negative control). Cells were replaced in 37°C and 5% CO_2 incubator for 2 hours. For kinetic cell imaging experiments (see Kinetic Cell Imaging), Complete DMEM was supplemented with 1:5000 Caspase 3/7 dye (Essen Biosciences).

2.4 Protein Extraction and Western Blot Assay

For protein extractions, cultured cells were harvested by rinsing twice with PBS buffer and scraping with Corning® cell lifter, centrifuging at 13 000 RPM for 1 minute. Supernatant was removed and cell pellet was re-suspended in 50-100 µL of RIPA lysis buffer (50 mM Tris-Cl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.5% SDS, 0.5% sodium

deoxycholate, 1% PMSF, 1% Leupeptin). Cell lysate was incubated on ice for 15 minutes and centrifuged at 4°C at 13 000 RPM for 15 minutes. Supernatant (whole cell lysate) was used in subsequent experiments or stored at -80°C. For western blot assays, whole cell lysates were quantified using Bio-Rad protein assay colorimetric assay kit and manufacturer's protocol (Bio-Rad Laboratories Inc.). Appropriate volume of lysate and ddH_2O were mixed with 5 X Laemmli Buffer (0.3M Tris-Cl/SDS pH 6.8, 1% SDS, 50% Glycerol, 0.0125% Bromophenol Blue, 10% β-Mercaptoethanol) and heated in boiling H_2O for 5 minutes to prepare for SDS-PAGE. Lysates were loaded onto 10% polyacrylamide gel (Stacking: 4% Acrylamide/Bis 37.5:1, 0.125M Tris-Cl pH 6.8, 0.1% SDS, 0.05% APS, 0.001% TEMED. Resolving: 10% Acrylamide/Bis 37.5:1, 0.375M Tris-Cl pH 8.8, 0.1% SDS, 0.05% APS, 0.001% TEMED). Electrophoresis was performedat a constant voltage of 125V for 1.5 hours. Gel content was transferred to 0.2µm PVDF membrane at constant amperage of 300mA for 1.25 hours using wet transfer apparatus. Membrane was blocked for 45 minutes with 5% skim milk in PBST buffer (1 X PBS, 0.1% Tween-20) or with Odyssey® Blocking Buffer (LI-COR Biosciences) and incubated at 4°C with primary antibody dilution (See Appendix) in PBST overnight. The next day, membrane was incubated for 1 hour at room temperature with secondary antibody dilution (See Appendix). For HRP-linked secondary antibodies, membrane was incubated with ECL solution (Pierce® ECL Western blotting substrate, Thermo Scientific; Amersham™ ECL™ Prime Western Blotting Detection Reagent, GE Healthcare) and exposed to X-Ray film (HyBlot CL® Autoradiography film, Denville Scientific, Inc.; Amersham Hyperfilm™ ECL, GE Healthcare Ltd.) for 1 second to 15 minutes. The film was developed using Kodak X-OMAT 2000A processor (Eastman Kodak Company). For fluorescent secondary antibodies,

membrane was imaged directly using LI-COR Odyssey® imaging system and Image Studio version 2.0.38 software (LI-COR Biosciences).

2.5 RNA Extraction and RT-qPCR

RNA extraction was done using Absolutely RNA miniprep kit (Agilent Technologies) and manufacturer's protocol. RNA was quantified using NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). RT-PCR was performed from 300ng RNA using qScript™ cDNA Supermix (Quanta Biosciences) and manufacturer's protocol. qPCR was performed using PerfeCTa® SYBR® Green FastMix® (Quanta Biosciences) following manufacturer's protocol (see Appendix for primer information). Optimal annealing temperature was determined by temperature gradient using Mastercycler® ep *realplex* real-time PCR system (Eppendorf). Standard curve range was determined by the Ct value at optimal annealing temperature (see formula in Appendix). Standards (1:1 ratio of control and treated RNA samples) were diluted appropriately and standard curve was obtained following qPCR protocol (PerfeCTa® SYBR® Green FastMix® and Mastercycler® ep *realplex* real-time PCR system and manufacturers' protocols). qPCR of samples of interest was performed following qPCR protocol described above. Ct values obtained were converted to relative quantities of RNA using standard curve. For HRV qPCR experiment, HRV RNA levels were normalized to GAPDH RNA levels.

2.6 In-vitro *Kinase Assay and FLAG Purification*

To perform *in vitro* kinase assay, 2μg of recombinant purified substrate protein (See Appendix for His-tagged protein information, see FLAG purification protocol below for FLAG-tagged protein information) and 300ng of recombinant purified active kinase (see

Appendix) were mixed with 10 X Kinase Buffer (200mM Tris-Cl pH 7.5, 50mM β-Glycerophosphate, 2mM $NaVO_4$, 5mM DTT), 10 X Mg + Mn/ATP mix (300 µM ATP, 66mM $MgCl_2$, 33mM $MnCl_2$), 2ug of myelin binding protein (MBP) and 5-10 µCi γ-^{32}P-ATP are added to 1 X solution. Mixture was incubated at 30°C for 20 minutes. 5 X Laemmli Buffer was added and PAGE was performed. Gel content was transferred as described in Western blotting protocol. Membrane was directly exposed to X-Ray film overnight at -80°C and developed as described in western blotting protocol.

To purify FLAG-tagged proteins from cells for use in kinase assay, cells (grown in 6-well culture plate) were transfected for 24 hours with 2µg of plasmid DNA expressing FLAG-tagged protein of interest following transfection protocol described above. Cells were collected and lysed with Co-IP lysis buffer (25mM Tris-Cl pH 7.6, 150mM NaCl, 50mM NaF, 0.5mM EDTA pH 8.0, 0.5% Triton-X 100, 5mM β-glycerophosphate, 5% glycerol, 1mM PMSF, 1mM $NaVO_3$) by sonication at 8% for 10 seconds. Samples were centrifuged at 13 000 RPM for 15 minutes and lysate was incubated with ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich Co.) for 2 hours. Affinity gel was washed with Co-IP buffer three times, with 1 X kinase buffer once. Buffer was removed and affinity gel was used in kinase assay.

2.7 Immunofluorescence and Confocal Imaging

Cells were seeded onto square coverslips (22 X 22 mm, #1 thickness, VWR®) in 6-well culture plate as described in Cell Culture protocol. At end-point of cell culture experiment, coverslips were rinsed 3 times with PBS, and cells were fixed using 3.7% formaldehyde diluted in PBS for 15 minutes. Coverslips were rinsed once with PBS and incubated with 0.2% Triton-X 100-PBS buffer for 5 minutes. Coverslips were rinsed 3 times with PBS

buffer and blocked with FBS blocking buffer (0.1% FBS, 0.2% BSA, 0.004% Triton-X 100, diluted in PBS buffer) for 15 minutes.Coverslips were incubated with primary antibody (1:300 dilution in Triton-X 100/BSA buffer: 0.2% BSA, 0.004% Triton-X 100, diluted in PBS buffer) for 1 hour. Coverslips were rinsed 3 times for 5 minutes each time with Triton-X 100/BSA buffer. Secondary antibody (Alexa Fluor anti-mouse 488 or anti-rabbit 594, 1:1000 dilution in Triton-X 100/BSA buffer) was added to coverslips for 1 hour. From this point on, culture plate containing treated coverslips were shielded from light using aluminum foil cover. Coverslips were rinsed 3 times for 5 minutes each time with Triton-X 100/BSA buffer. 1 μg/mL Hoechst 33342 solution (trihydrochloride trihydrate, Invitrogen) diluted in PBS was added to coverslips for 5 minutes. Finally, coverslips were rinsed 3 times for 5 minutes each time with PBS, after which they were mounted onto glass slides using Dako Fluorescent Mounting Medium (Dako North America, Inc.) Note: all steps described in procedure were done on rocking platform at room temperature, except for cell culture. Cells were imaged using Olympus Fluoview FV-1000 Laser Confocal Microscope and software with 40X or 60X objectives.

2.8 Kinetic Cell Imaging

Cells seeded into 6-well or 12-well culture plates were imaged periodically, every hour for 24 to 48 hours, using the IncuCyte™ Zoom Live Content Kinetic Imaging System (Essen BioScience) and software. Fluorescence from Caspase 3/7 dye and cell confluence was analyzed from images using IncuCyte™ Zoom software and subsequent data was exported to GraphPad Prism 5 software for graph analyses.

2.9 HK2 inhibitor

3-Bromopyruvate (3-BrPa) (Calbiochem®) reconstituted in ddH_2O was added directly to cell culture medium at desired time at a concentration of 100 μM. Sterile ddH_2O was added to cells as a negative control. For kinetic cell imaging studies of osmotically shocked cells, 3-BrPa was added to cells immediately following the addition of D-sorbitol-supplemented medium to cells (see Osmotic Shock protocol).

2.10 Statistical Analyses

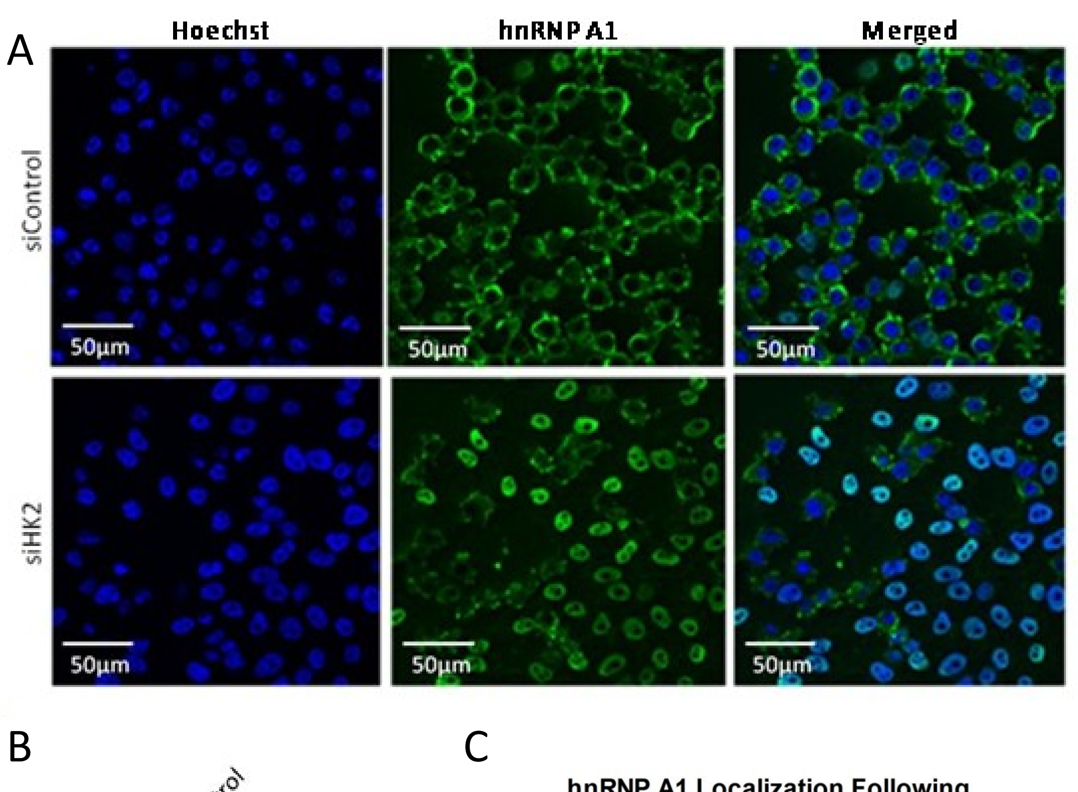
Student's T-test was performed using GraphPad Prism 5 software to determine p-value in repeated experiments. Data was collected from confocal microscopy images by cell counting in multiple randomized fields. Numbers of replicates consist of number of images from which cells were counted; error bars represent standard deviation. For kinetic cell imaging experiments, data was collected using IncuCyte Zoom software. Numbers of replicates consist of number of fields imaged per well in the culture dish; error bars represent standard error as calculated by the software. For qRT-PCR experiments, average RNA expression is calculated using data collected from three biological replicates and three technical replicates for each biological replicate. Error bars represent standard deviation.

Chapter 3: Results

3.1 HK2 knock-down affects hnRNP A1 cellular localization during osmotic shock

As mentioned in Chapter 1, HK2 was identified in RNAi-based kinome screen as a kinase whose knockdown resulted in a nuclear localization of hnRNP A1 during osmotic shock in U2OS cells. To validate these findings, HK2 knockdown was repeated in HeLa T4+ cells in a 6-well culture plate format using HK2-targeting siRNA different from the ones used in the RNAi-based kinome screen. Results show that HK2 knockdown (confirmed by western blot in Figure 3.1 B) does in fact prevent hnRNP A1 cytoplasmic accumulation during osmotic shock compared to control siRNA-transfected cells (Figure 3.1 A) when endogenous hnRNP A1 protein is visualized by immunofluorescence. Cells were counted to quantify the number of cytoplasmic hnRNP A1-expressing cells during the stress in both HK2 knockdown and control siRNA-transfected cells (Figure 3.1 C). While 93.3% of osmotically shocked siControl cells show cytoplasmic accumulation of hnRNP A1, only 32.8% of osmotically shocked siHK2 cells show cytoplasmic hnRNP A1 (Figure 3.1 C, $p=0.0031$).

To verify that the HK2-targeting siRNA is specific to HK2 mRNA and does not target HK1 mRNA (which was not identified as a regulating kinase in the RNAi-based kinome screen), I over-expressed either GFP-tagged HK1 or FLAG-tagged HK2 in cells where HK1 or HK2 were knocked down. Results from this experiment show that siHK1 effectively down-regulates expression of GFP-HK1 but does not target FLAG-HK2, and conversely, that HK2 knockdown effectively down-regulates expression of FLAG-HK2 but does not target GFP-HK1 (Figure 3.1 D). These experiments confirm the specificity of HK2 targeting by siRNA.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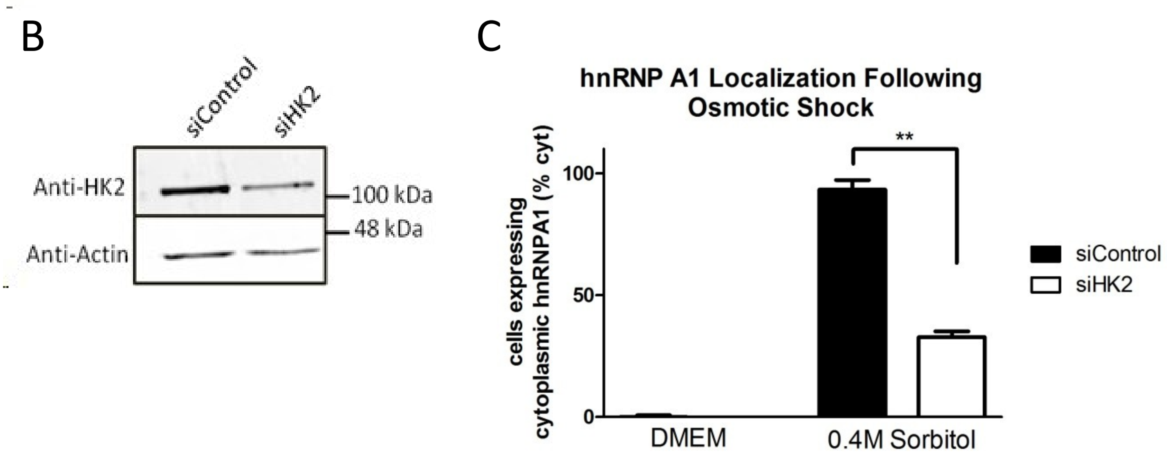
hnRNP A1
Merged
siControl
siHK2
50µm


B
siControl
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100 kDa
48 kDa
C
hnRNP A1 Localization Following Osmotic Shock
cells expressing cytoplasmic hnRNPA1 (% cyt)
100
50
0
**
DMEM
0.4M Sorbitol
siControl
siHK2

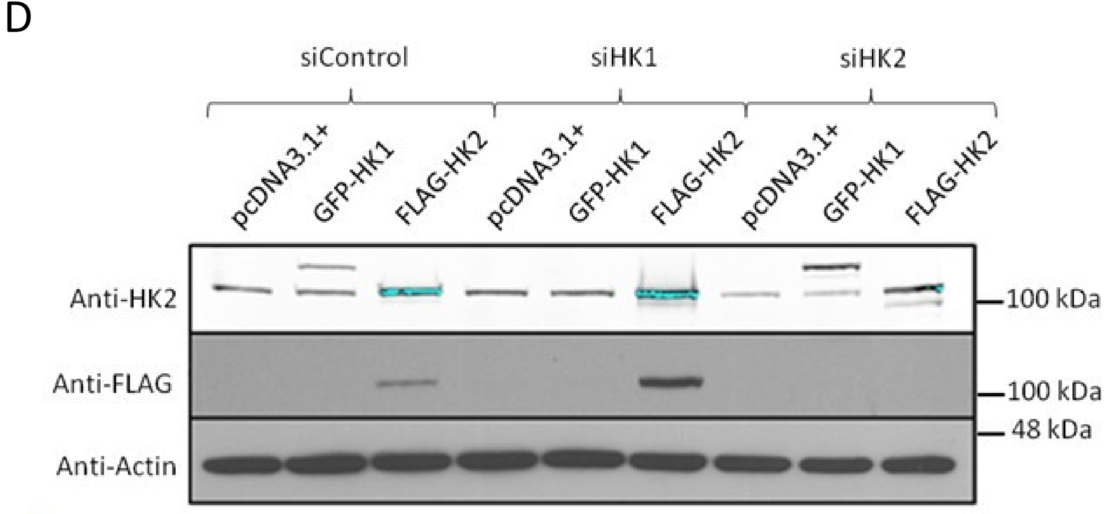

D
siControl
siHK1
siHK2
pcDNA3.1+
GFP-HK1
FLAG-HK2
Anti-HK2
Anti-FLAG
Anti-Actin
100 kDa
100 kDa
48 kDa

Figure 3.1. **Hexokinase 2 knockdown prevents cytoplasmic accumulation of hnRNP A1 in HeLa T4+ cells treated with sorbitol. A)** Representative confocal images of immunofluorescence analysis of cellular hnRNP A1 in sorbitol-treated cells. Top row images represent control siRNA-transfected cells and bottom row images represent siHK2-transfected cells. All cells were treated with 0.4M D-sorbitol for 2 hours. Images were taken with 60X oil-immersion objective; Hoechst DNA stain was visualized through DAPI filter, hnRNP A1 was visualized though FITC filter. See Figure S1 (Appendix) for images taken in control conditions **B)** Western blot of HK2 in control siRNA-transfected and siHK2-transfected HeLa T4+ cells (whole cell lysates). β-actin is shown as loading control. **C)** Quantification of HeLa T4+ cells expressing cytoplasmic hnRNP A1 following osmotic shock from immunofluorescence analysis. 3 images were analyzed per condition, where approx. 85 cells per image were counted. Error bars represent average +/- standard deviation; n=3, p=0.0031) **D)** Western blot of HK1 and HK2 knockdown in cells over-expressing GFP-HK1 or FLAG-HK2. HK2 antibody is used to visualize both HK1 and HK2; FLAG antibody is used to confirm presence of FLAG-HK2 construct and β-actin is/was used as a loading control.

3.2 Inhibition or depletion of HK2 protein affects cell response to osmotic shock

To investigate whether the effects of HK2 knockdown on hnRNP A1 localization and function are due to HK2 protein levels or loss of function and are independent of siRNA transfection, a HK2 inhibitor, 3-BrPa, was added to osmotically shocked cells in which hnRNP A1 was visualized by immunofluorescence. Results show that HK2 inhibition by 3-BrPa prevents hnRNP A1 cytoplasmic accumulation during osmotic shock as compared to siControl-treated cells (Figure 3.2 A). Cells were counted to quantify the number of cytoplasmic hnRNP A1-expressing cells during the stress in both 3-BrPa-treated and control siRNA-transfected cells (Figure 3.2 B). While 54.2% of osmotically shocked siControl cells show cytoplasmic accumulation of hnRNP A1, only 3.75% of osmotically shocked siHK2 cells show cytoplasmic hnRNP A1 (Figure 3.2 B, $p=0.0128$).

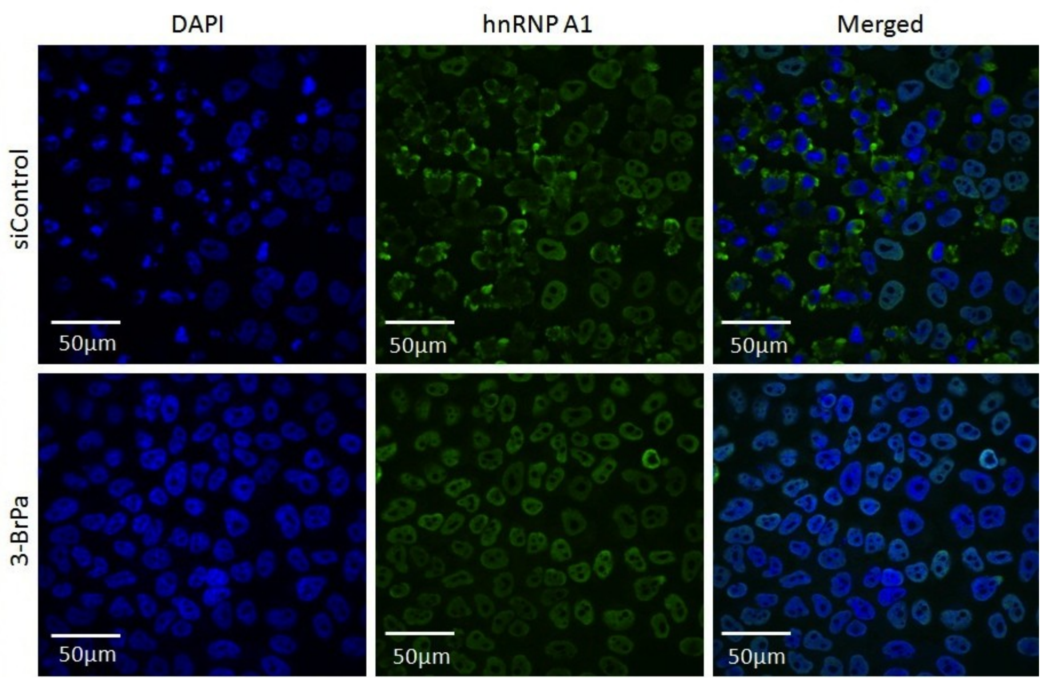
While performing these experiments, it was observed that HK2 knockdown not only prevents hnRNP A1 cytoplasmic accumulation but also prevents cell death from osmotic shock. This was investigated further by analysis of caspase 3 and 7 activation through the course of 6 hours after 0.3M D-sorbitol treatment in cells treated with siControl, siHK2 or 3-BrPa using live cell imaging. These results show that caspase activation is completely attenuated when HK2 is knocked down or inhibited whereas control siRNA-transfected osmotically shocked cells undergo caspase activation and apoptosis in a short period of time (Figure 3.3 A).

To investigate whether the cytoplasmic functions of hnRNP A1 are impaired along with its localization in HK2 knockdown cells, the levels of anti-apoptotic protein Bcl-XL, a known translational target of hnRNP A1,werestudied in osmotically shocked HeLa T4+ cells treated with siControl, siHK2 or 3-BrPa. Results show that Bcl-XL protein levels slightly decreased

following 2-hour osmotic shock treatment in siControl-treated cells (which is consistent with previously published data [Bevilacqua et al., 2010]); in contrast, Bcl-XL protein levels were increased in cells treated with siHK2 or with 3-BrPa compared to siControl-treated cells irrespective of the osmotic shock (Figure 3.3 B).

In summary, these results show that, in addition to HK2 knockdown, inhibition of HK2 activity also affects hnRNP A1 localization and function in osmotically shocked HeLa T4+ cells. This not only confirms the involvement of HK2 in hnRNP A1-mediated stress response mechanisms, but shows that HK2 enzymatic activity is required in this process. In un-transfected cells, osmotic shock leads to the cytoplasmic accumulation of hnRNPA1, subsequent decrease in BcL-XL protein levels and to the activation of caspases 3 and 7 which ultimately leads to cell death. siHK2 transfection or 3-BrPa treatment in cells prevents this chain of events by preventing cytoplasmic accumulation of hnRNP A1, which is unable to down-regulate BcL-XL expression, and this subsequently results in an inhibition of caspase activation and blocking of cell death.

A



B

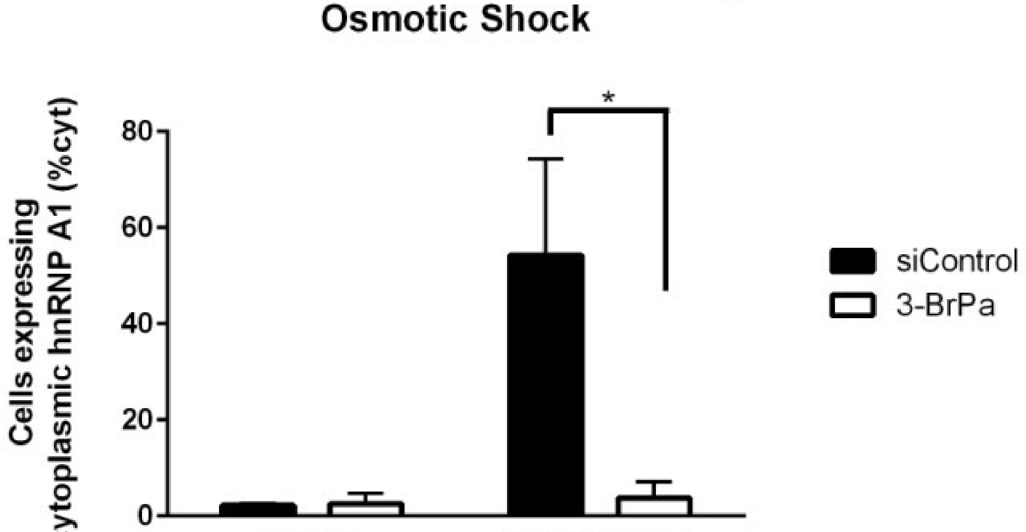
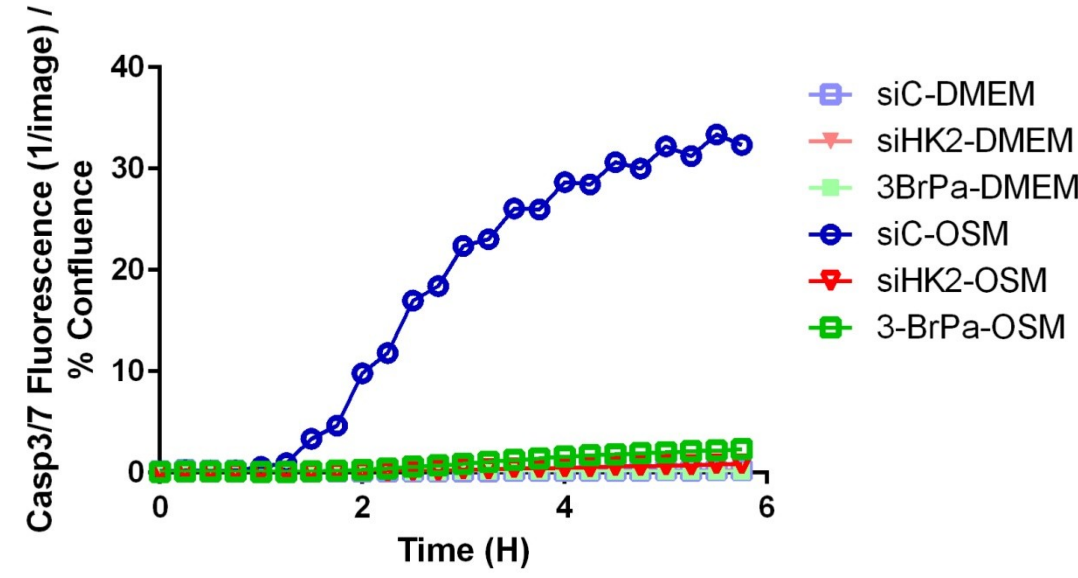


Figure 3.2. HK2 inhibition prevents hnRNP A1 cytoplasmic accumulation following osmotic shock. A) Representative confocal images of immunofluorescence analysis of cellular hnRNP A1 in sorbitol-treated cells. Top row images represent control siRNA-transfected cells and bottom row images represent 3-BrPa-treated cells. All cells were treated with 0.4M D-sorbitol for 2 hours. Images were taken with 60X oil-immersion objective; Hoechst DNA stain was visualized through DAPI filter, hnRNP A1 was visualized though FITC filter. See Figure S1 (Appendix) for images taken in control conditions **B)** Quantification of HeLa T4+ cells expressing cytoplasmic hnRNP A1 following osmotic shock from immunofluorescence analysis. 3 images were analyzed, where approx. 2000 total cells were counted per condition. Error bars represent average +/- standard deviation; n=3, p=0.0128.

A



B

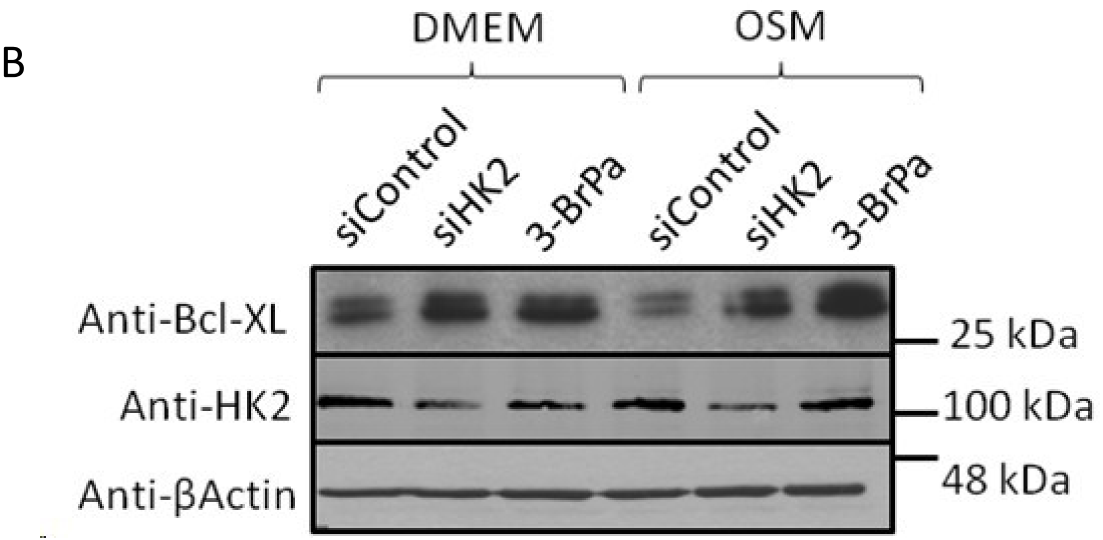


Figure 3.3. HK2 inhibition or knockdown prevents apoptosis of cells following osmotic shock. A) Caspase 3 and caspase 7 activity was measured in control siRNA-transfected, HK2 siRNA-transfected or 3-BrPa-treated HeLa T4+ cells which were subsequently treated with 0.3 M sorbitol for 2 hours. Y-axis represents number of fluorescent cells per image normalized to cell confluence. Error bars represent average +/- standard deviation; n=3 (3-BrPa treatment), n=4 (siRNA treatment). Calculated using IncuCyte ZOOM imaging software. **B)** Western blot of Bcl-xL in control siRNA-transfected, HK2 siRNA-transfected or 3-BrPa-treated HeLa T4+ cells treated with 0.4M D-sorbitol for 2 hours. β-actin is shown as a loading control.

3.3 hnRNP A1 phosphorylation by HK2

HnRNP A1 phosphorylation has been reported to be required for its accumulation into the cytoplasm of the cell during cell stress [Allemand et al., 2005]. To further investigate if HK2 directly phosphorylates hnRNP A1, an *in vitro* kinase assay was performed using recombinant His-hnRNP A1, FLAG-hnRNP A1, His-HK2 and FLAG-HK2. Results show that FLAG-HK2 can directly phosphorylate recombinant His-hnRNP A1 (Figure 3.4). In addition to this finding, this *in vitro* assay shows that His-HK2 does not phosphorylate His-hnRNP A1and only phosphorylates FLAG-hnRNP A1, although much less efficiently than FLAG-HK2. Interestingly, when comparing the levels of phosphorylation of FLAG-hnRNP A1 and FLAG-hnRNP A1ΔM9, hnRNP A1ΔM9 is phosphorylated to a much lesser extent by HK2, suggesting that the M9 motif of hnRNP A1 is required for its phosphorylation. These results also show that FLAG-HK2 does not phosphorylate FLAG-hnRNP A1 or FLAG-hnRNP A1ΔM9 when compared to GST control.

In summary, these results show that in certain conditions, HK2 can phosphorylate hnRNP A1 *in vitro*. Mammalian cell-purified kinase is required in order for this phosphorylation to occur, suggesting the need for an active kinase and/or co-factors that are only available in mammalian cells. Also, His-HK2 seems to phosphorylate hnRNP A1 more efficiently than hnRNP A1ΔM9, which suggests that this motif is required for phosphorylation by HK2.

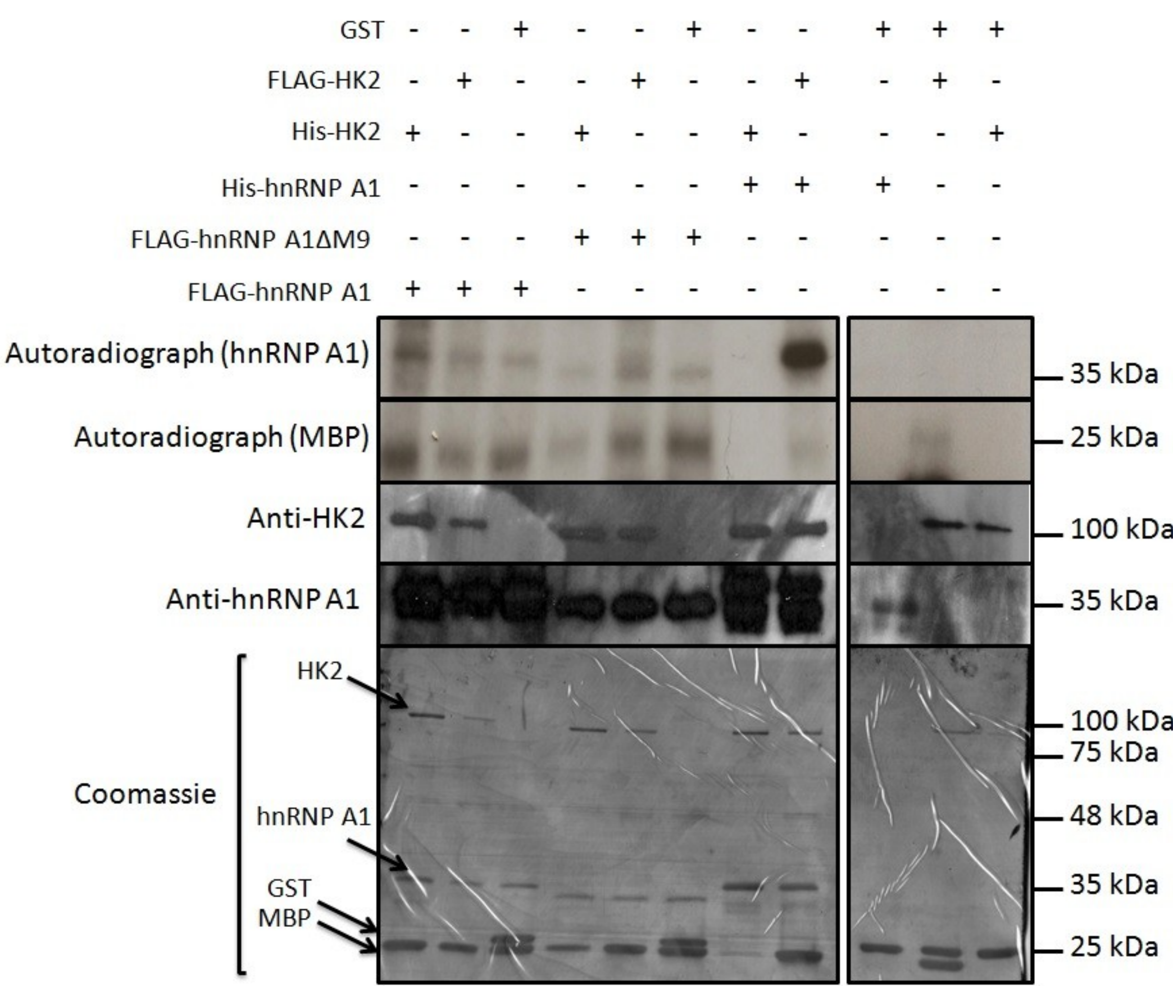

GST
FLAG-HK2
His-HK2
His-hnRNP A1
FLAG-hnRNP A1ΔM9
FLAG-hnRNP A1
Autoradiograph (hnRNP A1)
Autoradiograph (MBP)
Anti-HK2
Anti-hnRNP A1
Coomassie
HK2
hnRNP A1
GST
MBP
35 kDa
25 kDa
100 kDa
35 kDa
100 kDa
75 kDa
48 kDa
35 kDa
25 kDa

Figure 3.4. **HK2 potentially phosphorylates hnRNP A1 *in vitro*. Top panel:** autoradiography of *in vitro* kinase assay of either FLAG-hnRNP A1 (wt and ΔM9) or His-hnRNP A1 and either FLAG-HK2 or His-HK2 in the presence of ^{32}P-γATP. FLAG-tagged proteins were incubated with assay reagents and components while bound to Anti-FLAG M2 agarose beads. 2 μg of His-hnRNP A1 and 500ng of His-HK2 were used. **Lower panels:** Western blot analysis of HK2 and hnRNP A1 from *in vitro* kinase assay and Coomassie stain of assay membrane. Arrows indicate proteins on Coomassie-stained membranes.

3.4 HK2 knock-down affects cell response to HRV infection

HnRNP A1 was previously shown to positively regulate HRV IRES-mediated translation [Cammas et al., 2007]. In my analyses, hnRNP A1 and HRV proteins were visualized by immunofluorescence in siHK2-treated HRV-infected cells. Results show that similarly to osmotic shock conditions, HK2 knockdown prevents hnRNP A1 cytoplasmic accumulation in response to HRV infection as compared to siControl-treated cells; in infected cells, hnRNP A1 effectively accumulates into the cell cytoplasm (Figure 3.5). Also, results show that along with a lower number of cells expressing cytoplasmic hnRNP A1, HK2 knockdown leads to a lower number of infected cells (Figure 3.6 A). In fact, while 84.9% of control cells express HRV proteins, only 48.3% of HK2 knockdown cells express viral proteins. In addition to immunofluorescence analysis of HRV proteins in HeLa T4+ cells, qRT-PCR of HRV RNA was also performed in order to monitor the effect of HK2 knockdown on HRV replication. Results show a significant decrease in accumulation of HRV RNA in infected HK2-knocked-down cells as compared to control siRNA-treated cells at both 4 hours and 6 hours post-infection (Figure 3.6 B). To further investigate the effect of HK2 knockdown on HRV life cycle, HeLa T4+ cells were treated with siHK2 and infected with HRV, after which caspase 3 activation was measured for 24 hours. Results show that caspase 3 activation in HRV-infected cells is delayed consistently by approximately 8 hours when HK2 is knocked-down from cells (Figure 3.6 C).

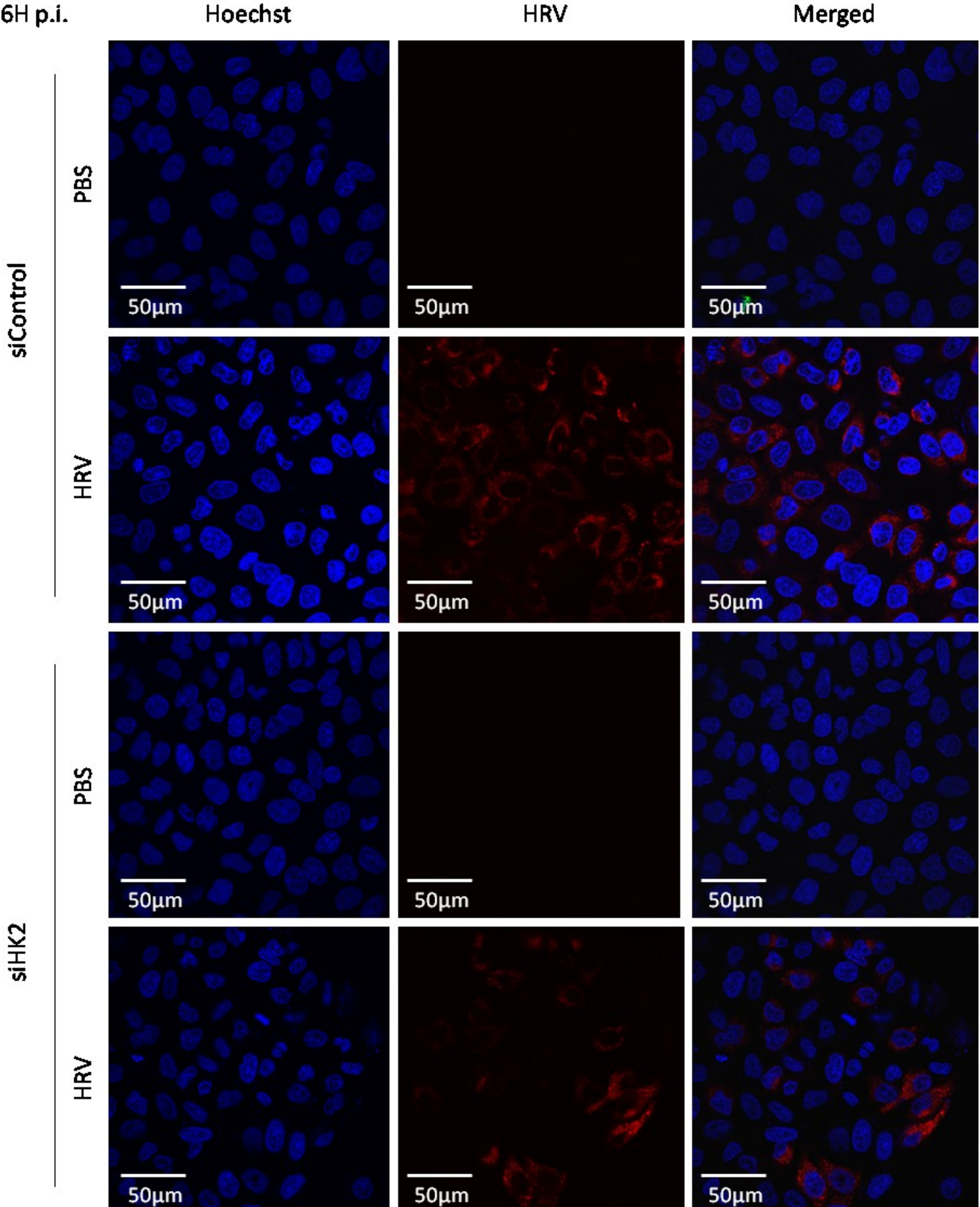
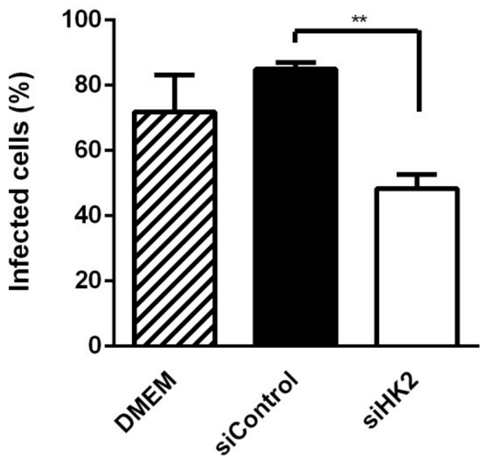
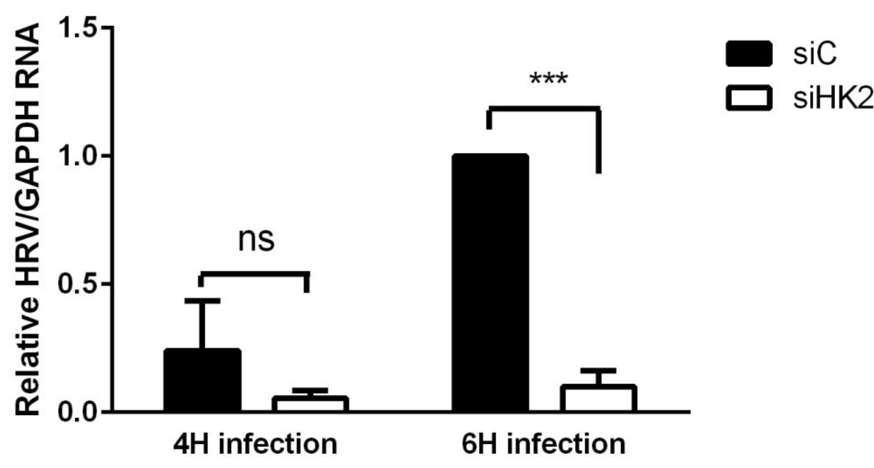

6H p.i.
Hoechst
HRV
Merged
siControl
PBS
HRV
siHK2
PBS
HRV
50µm

Figure 3.5. HK2 knockdown attenuates **Human Rhinovirus infection in HeLa T4+ cells.** Representative confocal images of an immunofluorescence analysis of HRV-infected or PBS-treated control siRNA or HK2 siRNA-transfected HeLa T4+ cells. Top two rows represent control siRNA transfected cells and bottom two rows represent HK2 siRNA transfected cells. Cells were either treated with PBS or infected with HRV for 6 hours. Hoechst DNA stain was visualized using DAPI filter; HRV capsid proteins were visualized through TRITC filter. Images were taken with 60X oil-immersion objective.

A



B



C

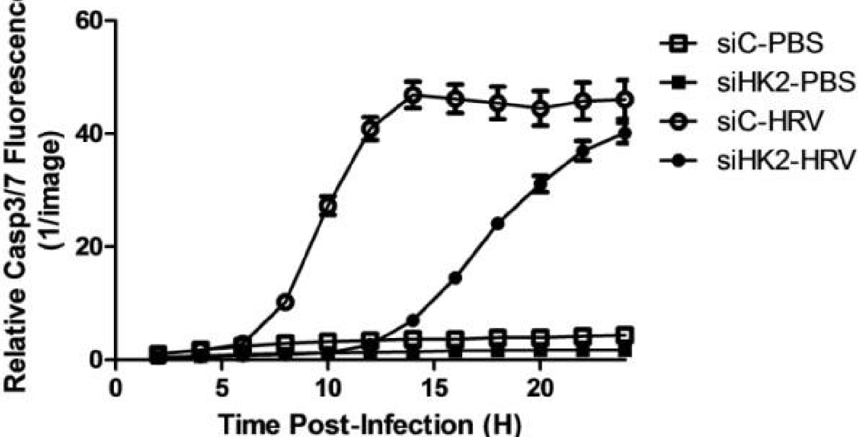


Figure 3.6. **HK2 knockdown leads to a decrease in HRV infection in HeLa T4+ cells. A)** Quantification of number of infected HeLa T4+ cells from immunofluorescence assay. 3 images were analyzed, where approx. 85 cells per image were counted. Error bars represent average +/- standard deviation; n=3, p=0.0002. **B)** HRV RNA levels in HRV-infected control siRNA-transfected or HK2 siRNA-transfected HeLa T4+ cells, measured by qRT-PCR. Absolute amount of HRV RNA normalized to GAPDH mRNA levels is shown. Cells were infected for either 4 hours or for 6 hours. Error bars represent average +/- standard deviation; n=3, p=0001 **C)** Caspase 3/7 activity in HRV-infected control siRNA-transfected or siHK2-transfected HeLa T4+ cells. Y-axis represents number of fluorescent cells per image, normalized to cell confluence (in %). Error bars represent average +/- standard deviation; n=3. Calculated using IncuCyte ZOOM imaging software.

3.5 Rescuing HRV infection in HK2-depleted cells using mutant hnRNP A1 protein

To confirm that the effect of HK2 knockdown in HRV-infected cells is due to the defective hnRNP A1 cellular localization, a cytoplasm-restricted mutant hnRNP A1 protein (FLAG-hnRNP A1ΔM9) and a nucleus-restricted mutant hnRNP A1 protein (FLAG-hnRNP A1 F2) were over-expressed in HRV-infected cells where HK2 was knocked-down. FLAG and HRV proteins were visualized in cells using immunofluorescence methods in order to visualize the extent of HRV infection in these cells as well as investigate whether the expression of exogenous hnRNP A1 protein leads to an increase in HRV proteins in transfected cells (Figure 3.7). Compared to hnRNP A1 F2, hnRNP A1ΔM9 expression in cells leads to an increase in HRV proteins in FLAG-expressing cells (Figure 3.8 A, p=0014). In fact, in HK2-knockdown cells, expression of hnRNP A1ΔM9 results in the same number of infected cells as hnRNP A1-transfected siControl cells; as seen previously, HK2 knockdown leads to a lower amount of HRV-infected cells as 47.8% of un-transfected and 29.6% of hnRNP A1 F2-transfected siControl cells are infected while only 10.91% of un-transfected and 9.73% of hnRNP A1 F2-transfected siHK2 cells are infected. Transfection of hnRNP A1 ΔM9 leads to an infection in 31.42% of siControl cells and 39.17% of siHK2 cells (Figure 3.8 A).

Western blot analysis was also performed on whole cell lysates of HRV-infected HK2 knockdown cells expressing hnRNP A1 mutant proteins. Results show that HK2 knockdown does not affect the levels of HRV proteins in cells compared to siControl-treated cells but that cells expressing mutant hnRNP A1 protein express HRV proteins at lower levels than in un-transfected cells (labelled “UT”, Figure 3.8 B). HnRNP A1 F2 and hnRNP A1ΔM9-expressing cells do not seem to express different levels of HRV proteins when infected (Figure 3.8 B).

In Summary, these results show that a less-prominent HRV infection in HK2 knockdown cells can be rescued by transfection of an exogenous mutant hnRNP A1 protein localized exclusively within the cell cytoplasm. The number of infected cells in HK2 knockdown cells transfected with hnRNP A1 ΔM9 is significantly higher than in cells transfected with a nucleus-localized hnRNP A1 mutant, F2; however, there is no difference seen in HRV protein levels in these cells.

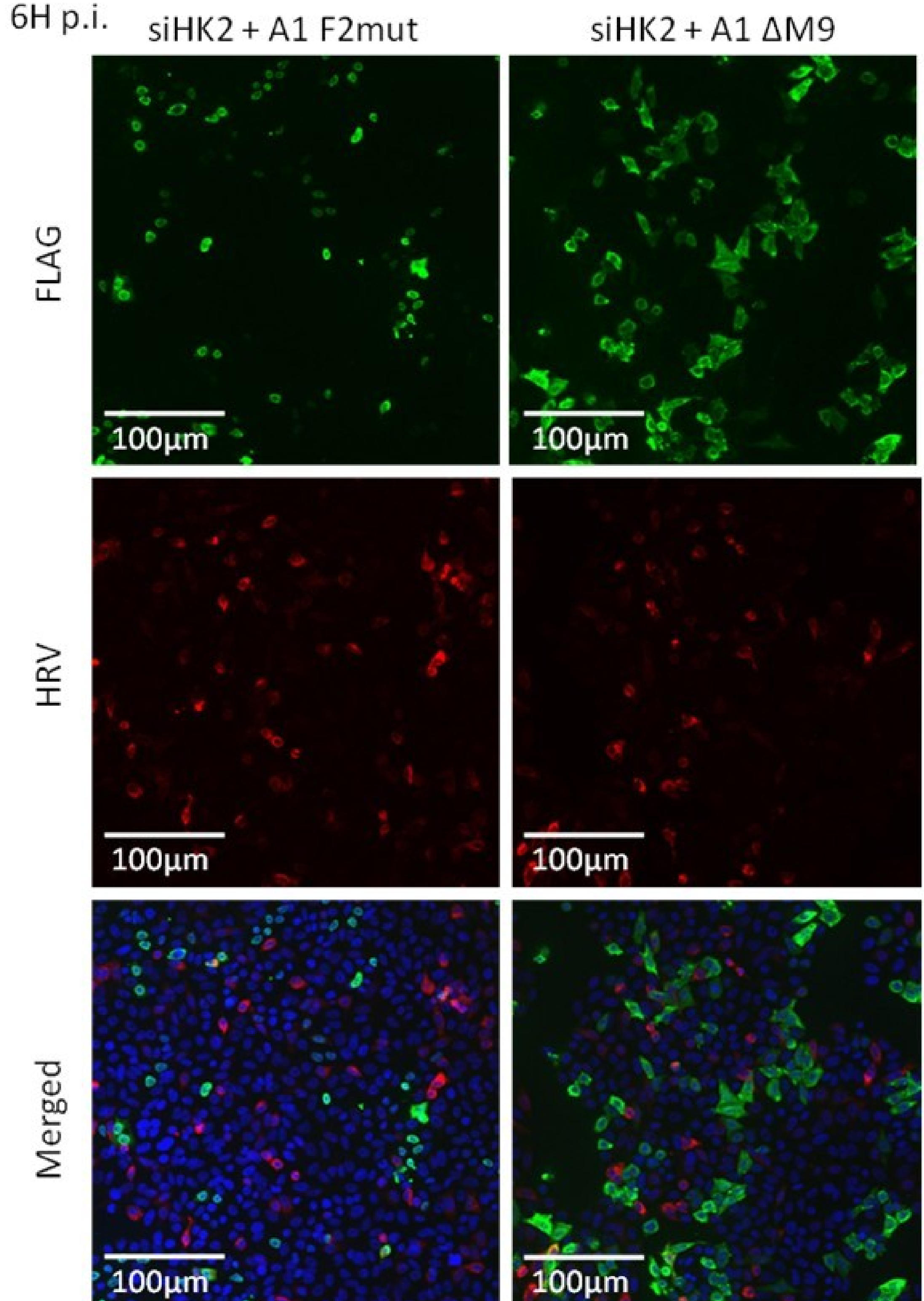
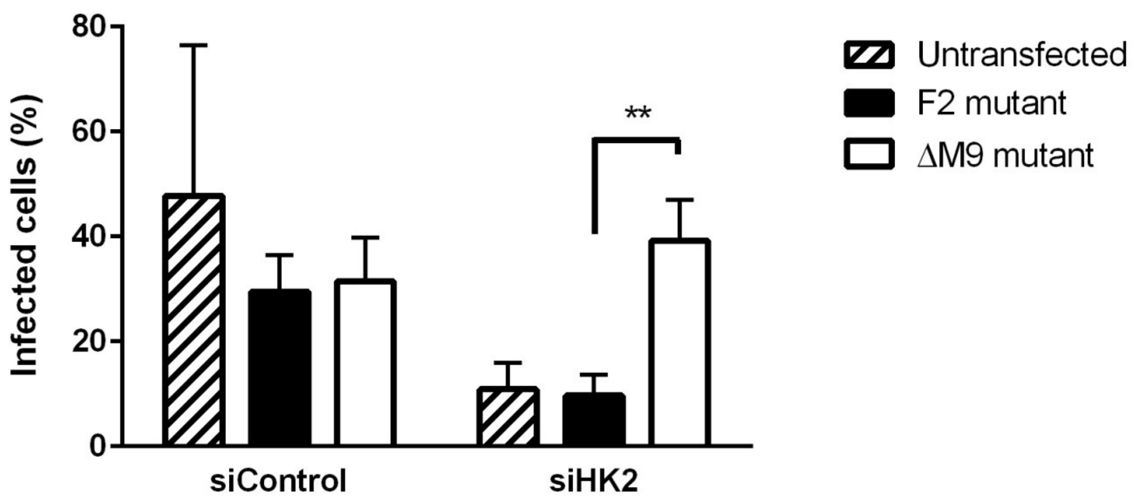
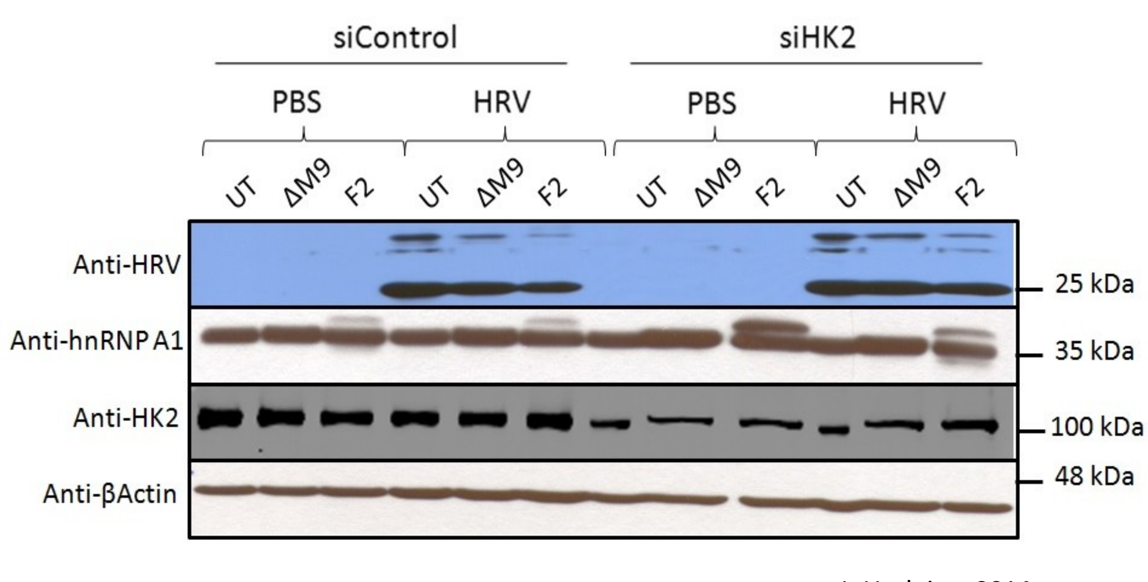

6H p.i.
siHK2 + A1 F2mut
siHK2 + A1 ΔM9
FLAG
HRV
Merged
100µm

Figure 3.7. Expression of exogenous hnRNP A1 protein in HK2-knockdown HRV-infected cells. Representative confocal images of an immunofluorescence analysis of HRV-infected HK2 siRNA-transfected HeLa T4+ cells. Left column represents cells transfected with FLAG-hnRNP A1 F2 construct and right column represents cells transfected with FLAG-hnRNP A1ΔM9 construct. All cells were infected with HRV for 6 hours. Hoechst DNA stain was visualized using DAPI filter, HRV capsid proteins were visualized through TRITC filter; FLAG-tag was visualized through FITC filter. Images taken with 60X oil-immersion objective.

A



B



J. Hodgins, 2014

Figure 3.8. Forced expression of cytoplasmic hnRNP A1 in HRV-infected siHK2-transfected HeLa T4+ cells leads to a higher expression of HRV proteins. A) Quantification from immunofluorescence assay (performed by Jonathan Hodgins, BSc candidate) of number of infected cells in each sample. 5 images were analyzed for FLAG-hnRNP A1 F2-expressing cells, where approx. Images from 3 biological replicates were analyzed, where approx. 70 cells per image were counted. Error bars represent average +/- standard deviation; n=3, p=0.0014. **B)** Western blot (performed by Jonathan Hodgins, BSc candidate) of HRV proteins, hnRNP A1 and HK2 in control siRNA-or HK2 siRNA-transfected HeLa T4+ cells either un-transfected or transfected with either FLAG-hnRNP A1 F2 or FLAG-hnRNP A1ΔM9 construct and treated with PBS (control) or HRV for 6 hours. β-actin is shown as loading control. Whole cell lysates were collected from the same cell cultures as those analyzed by immunofluorescence.

Chapter 4: Discussion

The cellular localization of hnRNP A1 changes from nuclear to cytoplasmic during certain cell stresses and participates in the translation regulation of mRNAs in the cell cytoplasm. Past studies show that phosphorylation of hnRNP A1, within its C-terminus, is needed for this cytoplasmic localization to occur during stress [Allemand et al., 2005]; however, the scope of this localization regulation is poorly understood. This knowledge sets the rationale for this study: to investigate the role of a specific kinase, HK2, in hnRNP A1 cytoplasmic localization. This study further investigates the hypothesis that the HK2-mediated regulation of hnRNP A1 localization influences the cytoplasm-located functions of hnRNP A1 during osmotic shock and Human Rhinovirus infection. Results suggest that HK2 indeed mediates hnRNP A1 localization in these conditions and subsequently affect hnRNP A1 cytoplasmic functions.

Following previous experiments conducted in this field, notably the RNAi-based kinome screen performed by J. Crasto and M. Holcik [Crasto and Holcik, unpublished data], I investigated if HK2 knockdown indeed attenuates hnRNP A1 cellular localization during cell stress. Results show that the specific knock-down of HK2 protein levels using a different siRNA sequence than that used in the RNAi-based screen, leads to hnRNP A1 being mainly localized in the nucleus of osmotically stressed cells (Figure 3.1 A, C). To exclude the involvement of another hexokinase in hnRNP A1 localization mechanisms, HK1 levels were monitored in siHK2-treated cells. Results show that siHK2 selectively knocks-down HK2 protein levels, leaving HK1 protein levels unchanged (Figure 3.1 D). In addition to this finding, none of the other hexokinases were identified by the siRNA-based kinase screen [Crasto and Holcik, unpublished data]. These results strongly suggest that HK2 is

specifically involved in hnRNP A1 localization mechanisms. Along with data collected from the RNAi-based kinome screen, these data show that the presence of HK2 is specifically required for the cytoplasmic localization of hnRNP A1 during osmotic shock.

In addition to the siRNA knock-down of HK2 as a method to validate its involvement in hnRNP A1 localization regulation, 3-BrPa-mediated inhibition of HK2 activity is also utilized in this study. This inhibitor, which has been shown to kill cancer cells through the inactivation of HK2's ability to phosphorylate glucose and drive glycolysis, functions by dissociating HK2 from VDAC proteins on the mitochondrial membrane [Chen et al., 2009]. The results from siRNA-based and inhibitor-based experiments are very different in their mechanisms of action, and offer insight into the effects of both HK2 protein levels as well as its activity on hnRNP A1 biology. As shown in Figures 3.1, 3.2 and 3.3, both siRNA and 3-BrPa treatments lead to an impaired cytoplasmic accumulation of hnRNP A1, to increased cell survival and to an increased expression of Bcl-XL following osmotic shock. This not only confirms that HK2 is involved in hnRNP A1 localization regulation, but that it does so specifically through its activity or its location on the mitochondrial membrane. These results also support previous claims that hnRNP A1's cellular localization is important for its location-specific functions [Lewis et al., 2007].

In fact, results show that following a 2-hour osmotic shock in cells, Bcl-XL protein levels are higher when HK2 is knocked-down or inhibited when compared to stressed cells that are treated with siControl (Figure 3.3 B). This suggests that hnRNP A1 is not available in the cytoplasm of the HK2-knockdown cells to negatively regulate Bcl-XL mRNA via its IRES as it has been previously shown to do [Bevilacqua et al., 2010]. Perhaps this increase in Bcl-XL levels contributes to the increased cell survival seen during osmotic shock (Figure

3.3 A): results of a kinetic cell imaging assay performed show that caspases 3 and 7 are not activated when HK2 is knocked down or inhibited, whereas control stressed cells display high levels of caspase activation. The effect of hnRNP A1 localization on cell survival has been previously explored: hnRNP A1 cytoplasmic localization during stress was inhibited by preventing the phosphorylation of eIF2a [Bevilacqua et al., 2010] and was enhanced by the expression of a cytoplasm-restricted hnRNP A1 mutant [Lewis et al., 2008]. These groups have shown that Caspase 3 is inactive and Bcl-XL levels are higher during osmotic shock when hnRNP A1 does not accumulate into the cytoplasm [Bevilacqua et al., 2010]. Also, XIAP, another inhibitor of apoptosis protein, has been shown to be repressed through its IRES by cytoplasmic hnRNP A1 [Lewis et al., 2008]. The results presented in this study further support these previous observations in a different cell model, using a different mechanism of restricting hnRNP A1 to the nucleus during osmotic shock. Together, these findings strongly suggest that the prevention of hnRNP A1 cytoplasmic accumulation during cell stress leads to increased cell survival.

To further investigate the effects of HK2 on hnRNP A1 cellular localization, a different target of hnRNP A1 was studied: the Human Rhinovirus (HRV). Although this is a completely different biological system than osmotic shock in cells, it remained very relevant in studying hnRNP A1 localization as HRV also depends on the cytoplasmic localization of the protein to promote viral IRES translation. [Gustin and Sarnow, 2002] have shown that many nuclear-localized proteins accumulate in the cytoplasm following poliovirus infection, and suggest this also happens with other members of the Enteroviridae genus. [Walker et al., 2013] This is mainly due to the cleavage of nucleoporin proteins on the nuclear membrane of the host cell. Nucleoporins Nup 153l, Nup 62 and Nup98 are degraded by viral 2A and 3C

proteases, which results in the cytoplasmic localization of many nuclear proteins. In contrast, it is presumed that hnRNP A1 does not localize to the cell cytoplasm through this pathway as it interacts with transportin nuclear membrane proteins, not nucleoporins. This suggests hnRNP A1 shuttles through a different mechanism during HRV infection than most proteins, possibly the same as during osmotic shock.

Results show that HK2 knockdown in HRV-infected cells leads to a decrease in the number of cells infected and delays caspase 3 activation in response to HRV (Figure 3.6 A, C). In addition, HK2 knockdown prevents hnRNP A1 cytoplasmic localization significantly in HRV-infected cells. This data shows that HK2 protein levels in the cell greatly affects HRV infection in cells as well as the cell response to infection; in addition to leading to less cytoplasmic localization of hnRNPA1, the majority of infected cells express cytoplasmic hnRNP A1 whereas non-infected cells mostly express only nuclear hnRNP A1. These results are accompanied by other findings showing a decrease in HRV RNA levels when HK2 is knocked-down (Figure 3.6 B); however there is no evidence of HRV proteins being similarly deregulated (Figure 3.8 B). This unexpected result can be explained by the fact that RNA samples are diluted extensively prior to qRT-PCR analysis in order to compare with an adequate standard curve; in contrast, protein samples from whole-cell lysates are not diluted before SDS-PAGE analysis, leading to a more saturated amount of HRV particles. This saturation could have prevented the detection of any differences between HRV protein samples. The findings that HK2 knockdown leads to a decrease in HRV RNA in infected cells also raises the possibility that hnRNP A1 could be involved in HRV RNA replication in addition to translation. This speculation is supported by the fact that PCBP2, which is

another essential factor in the IRES translation of HRV proteins, is also utilized by the virus replication machinery to promote the activity of HRV RNA polymerase [Lin et al., 2009].

To further confirm that hnRNP A1's function as an ITAF is essential for HRV replication, a rescue experiment was performed by over-expressing mutant hnRNP A1 proteins, expressed exclusively in the cytoplasm (hnRNP A1ΔM9) or in the nucleus (hnRNP A1 F2mut) of HRV-infected cells. The effects of HK2 knockdown were studied in this system. As expected, results show that expression of hnRNP A1ΔM9 in HK2 knockdown cells rescue HRV infection when compared to control (hnRNP A1 F2) cells by allowing hnRNP A1 to regulate the translation of HRV RNA in the cell cytoplasm. Another group [Cammas et al., 2007] showed through a bi-cistronic assay that hnRNP A1ΔM9 can drive HRV IRES translation when the cells were transfected with the construct in question. Although their experiments do not elucidate on the complete HRV replication mechanism in which hnRNP A1ΔM9 plays a role, this publication along with the results presented here, strongly support the hypothesis that cytoplasm-located hnRNP A1 is required for proper HRV protein synthesis and subsequent life cycle progression.

To investigate if HK2 can directly phosphorylate hnRNP A1, I conducted an *in vitro* kinase assay using purified recombinant hnRNP A1 and HK2 proteins. It seems that post-translational modifications, additional proteins or other factors are needed with HK2 in order to promote hnRNP A1 phosphorylation, as only mammalian-cell-FLAG-purified HK2 lead to a successful phosphorylation reaction (Figure 3.4). The possibility that VDAC proteins are being co-purified with HK2 in this sample and are required for HK2 in vitro kinase activity should be explored, as VDAC are essential for the efficient activity of HK2 *in vivo*. It remains unknown if this lack of phosphorylation of hnRNP A1 by His-tagged HK2 is

due to the absence of proteins or factors such as VDAC, or if HK2 simply does not phosphorylate hnRNP A1. A limitation that arises when studying the activity of HK2 in a kinase assay is not having a positive control; in fact, only glucose has ever been shown to be phosphorylated by HK2. Glucose cannot be utilized in this technique as a control as it cannot be analyzed by SDS-PAGE.

Another major observation from this experiment is that FLAG-HK2 does not phosphorylate FLAG-tagged hnRNP A1. This could possibly be due to technical difficulties as FLAG-purified proteins remain bound to agarose beads, preventing optimal interaction between the two proteins. Another possible reason for this result is the co-purification of an inhibitor of phosphorylation within the FLAG-purified hnRNP A1 protein, either an inhibitory protein or modification, preventing HK2 from phosphorylating hnRNP A1 in this sample.

In summary, these results suggest that HK2 might be capable of phosphorylating hnRNP A1 *in vitro* under specific conditions. They also show the possibility that HK2 interacts with another protein kinase, which is co-purified with HK2 in this assay and directly phosphorylates hnRNP A1 *in vitro*. Regardless of which protein kinase(s) is/are involved in hnRNP A1 phosphorylation, I propose that the phosphorylation of the F-peptide by HK2 causes a sort of conformational change or steric hindrance within hnRNP A1 which blocks the M9 motif from interacting with transportin proteins. As a result, during cell stress, hnRNP A1 is unable to re-locate to the nucleus. This idea is based on what is currently shown in the literature, such as the M9 motif being a nuclear localization signal [Pollard et al., 1996], and the F-peptide, being conveniently located beside the M9 motif, which is hyper-phosphorylated following a cell stress signal [Allemand et al., 2005]. Previously

published findings identified protein kinases p38 MAPK and MNK1/2 as being required for hnRNP A1 cytoplasmic accumulation during osmotic shock [van der Houven van Oordt et al., 2000, Guil et al., 2006]. For the first time, HK2 is identified as being involved in hnRNP A1 localization mechanisms during osmotic shock.

A limitation of this study comes from the required use of HeLa T4+ cells. These cells, like most cancer cells, express an abnormally high amount of HK2, mostly bound to the mitochondria, as previously mentioned in the Introduction section. This increase in mitochondrial-bound HK2 enables multiple cancer-phenotypic conditions: first, mitochondrial-bound HK2 is protected from its inactivation by glucose-6-phosphate, thus promoting an active glycolysis in the cell even in the abundance of the HK2 product [Mathupala et al., 2009]. Second, the abundance of VDAC-bound HK2 competes with the binding of the pro-apoptotic protein complex Bax/Bak, thus preventing the cell from undergoing apoptosis through the Bax/Bak pathway [Pastorino et al, 2002]. Third, mitochondria-bound HK2 is presented with ATP more efficiently, as mentioned, and can utilize readily-available ATP molecules to drive its enzymatic reaction. Finally, the over-expression of HK2 leads to an increase in aerobic glycolysis, resulting in an increase in lactate concentration in the cell. This promotes an acidic environment within and around the cell, protecting it from activated immune cells [Fischer et al., 2007]. The phenomenon of cancer cells preferably undergoing glycolysis even in the presence of oxygen in the environment is called the Warburg Effect, named after Otto Warburg who first described it in 1924 [Vander Heiden et al. 2009]. The main component of the Warburg effect is HK2, which is over-expressed in cancer cells making it a major contributor to these cells' particular metabolism. This high amount of HK2 in the cells could present a different mechanism of

hnRNP A1 localization that is not present in non-cancerous cells. It could also be the reason why only HK2 is involved in this process and other hexokinases are not.

As these studies in HeLa T4+ cells have uncovered a connection between HK2 and hnRNP A1 and HRV, further studies should be conducted *in vivo* in order to investigate the involvement of HK2 in a more accurate HRV infection system. There exist a few mouse models which show similar symptoms to HRV infection as humans do [Bartlett et al., 2008], and these could be utilized to study the effect of HK2 inactivation in infected epithelial cells. This could be done by localizing HK2 inhibitor drugs, such as 3-BrPa, to the respiratory system by inhalation, avoiding exposure of the drug to the entire organism.

Further investigations should also include the identification of protein kinases which directly phosphorylate hnRNP A1. This could be done by further investigating if HK2 directly phosphorylates hnRNP A1; proteins should be identified within the FLAG-purification sample of HK2 to determine if another kinase is present or if other proteins allow the activation of HK2. This could be done by co-immunoprecipitation of FLAG-HK2 and other bound proteins, followed by SDS-PAGE and sequencing of the fractionated proteins by MALDI-TOF spectrometry. Also, as the results presented here and previous findings [van der Houven van Oordt et al., 2000, Guil et al., 2006] show, there are different protein kinases capable of phosphorylating hnRNP A1; therefore further studies should investigate a broader number of kinases which phosphorylate the M9 peptide of hnRNP A1, such as MNK1/2 and p38 MAPK, and that may play a role in its cellular localization. Perhaps by conducting a phosphorylation screen, other undiscovered involved kinases would surface and help uncover hnRNP A1 regulation mechanisms. These kinases might not directly affect hnRNP A1 cytoplasmic localization during cell stress, but may participate

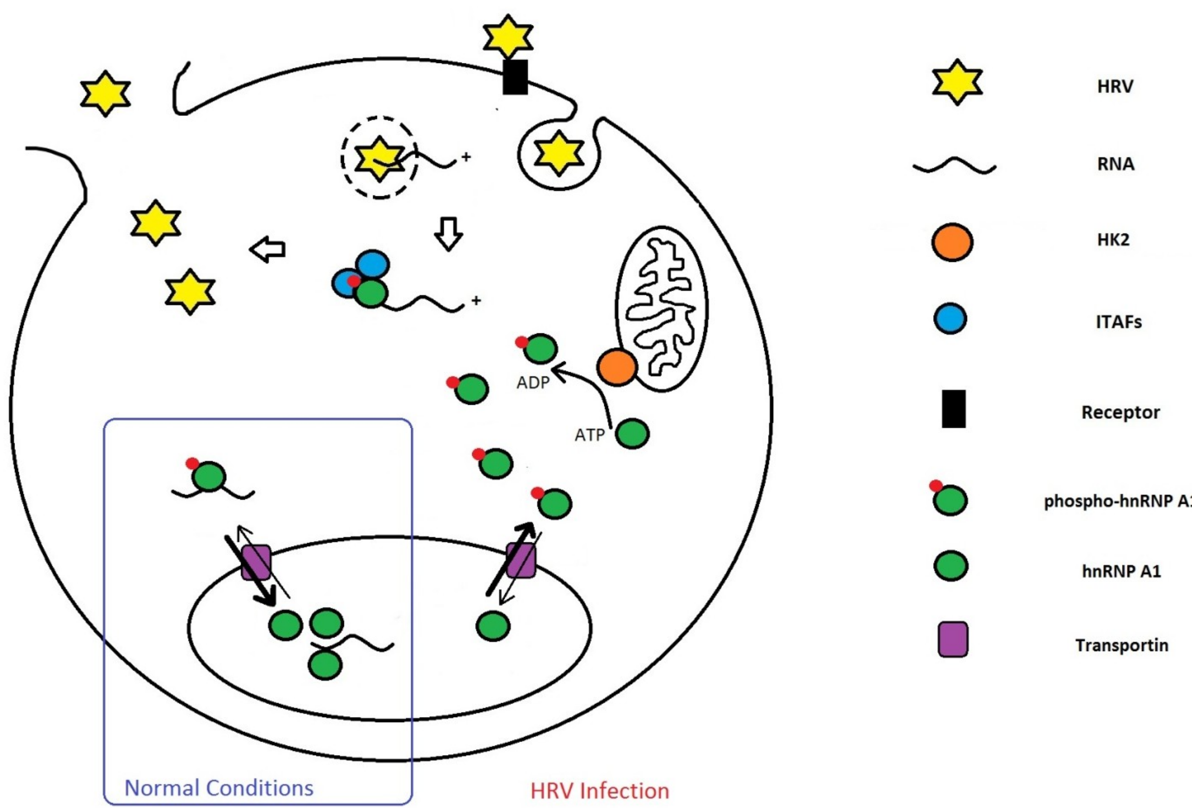
alongside HK2 in a more complex pathway; perhaps other kinases are involved in activating signalling pathways following the stress signal

This study is relevant to the broad field of molecular biology in many ways. As most Enteroviruses replicate via similar mechanisms, studying one particular strain of virus can help understand the biology of the entire genus of Enteroviridae viruses which include poliovirus and coxsackievirus, among many others. Also, other hnRNPs, such as hnRNP C, have also been shown to be involved in the translation of other picornavirus. [Brunner et al., 2010] have shown that hnRNP C localizes to the cell cytoplasm following poliovirus infection, that a decrease in hnRNP C protein levels leads to a delay in poliovirus replication in cells, and that an over-expression of hnRNP C leads to a significant increase in the rate of poliovirus production.

Since HRV replicates in cells which have an excess of HK2, such as cancer cells, more effectively than in cells which have a lower level of HK2, I speculate that HRV may have oncolytic properties. Supporting this claim is the fact that a few other members of the picornaviridae family have been shown to be oncolytic viruses such as poliovirus, senecavirus and ECHO (Enteric Cytopathic Human Orphan) virus, the latter being the first non-genetically-modified virus approved in an anti-cancer therapy which is available to patients as Rigvir® [Chumakov et al., 2012 (review)]. Interestingly, oncolytic poliovirus in clinical trials is modified by substitution of its IRES for the HRV2 IRES in order to decrease its neurotropism [Gromeier et al., 2000].

In summary of the results presented here, I show that HK2 is a component of the hnRNP A1 cellular localization mechanism and influences downstream hnRNP A1

cytoplasm-based functions. This further supports that hnRNP A1 localization is a crucial part of its functions as the expression of a cytoplasm-exclusive protein rescues its functions. This is particularly interesting in the context of HRV as it enhances our knowledge on some of its replication mechanisms. A model is proposed (Figure 4.1) to illustrate the mechanism by which hnRNP A1 accumulates into the cell cytoplasm following HRV infection.



+
+
ADP
ATP
Normal Conditions
HRV Infection
HRV
RNA
HK2
ITAFs
Receptor
phospho-hnRNP A1
hnRNP A1
Transportin

Figure 4.1. **Model of hnRNP A1 cytoplasmic accumulation following HRV infection**. HRV infects the host cell, leading to the accumulation of hnRNP A1 in the cytoplasm following its phosphorylation by mitochondria-bound HK2. HRV RNA is translated , following hnRNP A1 binding to HRV IRES.

Conclusion

In conclusion, this research project highlights key events that occur during cell stress, such as osmotic shock and HRV infection. I have shown that the knock-down or inactivation of HK2 leads to an impaired cytoplasmic accumulation of hnRNP A1 following osmotic shock or HRV infection. I have also shown that HRV infection can be rescued with the expression of a cytoplasm-exclusive hnRNP A1 protein. In addition, HK2 knock-down and inactivation were also shown to lead to decreased cell death, possibly through the translation regulation of Bcl-XL. Finally, the possibility that HK2 can directly phosphorylate hnRNP A1 was explored and discussed. These results advance our knowledge of hnRNP A1 cellular localization mechanisms as well as its implications in HRV infection and osmotic shock. This study also opens the door to further studies in picornavirus biology and in the regulation of hnRNP A1 in the cell.

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Contribution of collaborators

HRV rescue experiments were conducted by Jonathan Hodgins, BSc candidate, as part of an internship in the Holcik laboratory.

Protocols and techniques for kinase assays, protein purification and confocal microscopy were provided by Dr Urszula Liwak.

Infectious HRV samples were generously provided by Dr Nahum Sonenberg and HRV antibodies were generously provided by Dr. Wai-Ming Lee, University of Wisconsin.

hnRNP A1 F2 and ΔM9 mutant plasmids were generously provided by Dr Stephen Lewis and by Dr Maria Hatzoglou, respectively.

Appendix

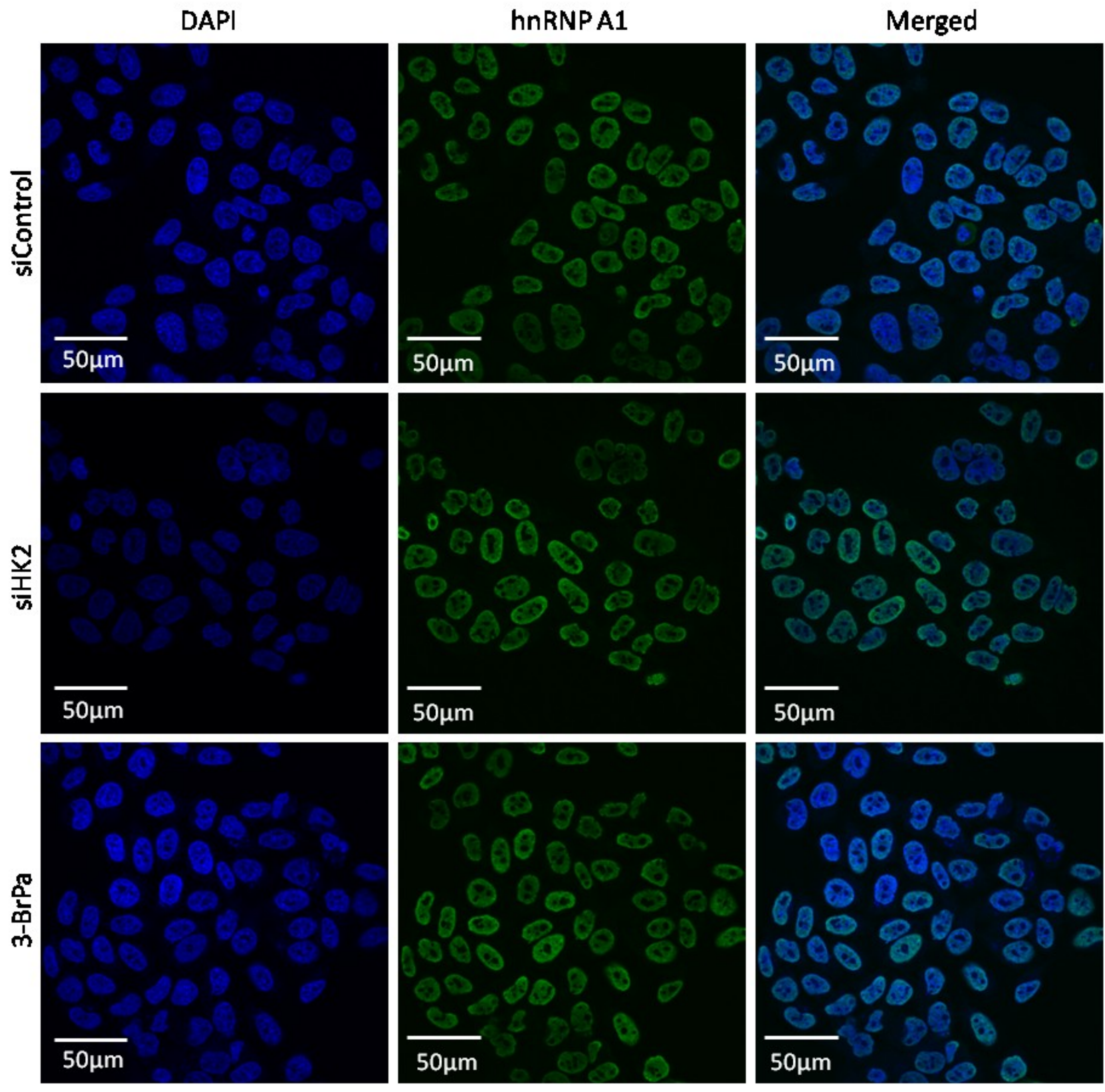


Figure S1. siRNA-transfected and 3-BrPa-treated HeLa T4+ cells in control growth conditions Representative confocal images of immunofluorescence analysis of cellular hnRNP A1 in HeLa T4+ cells grown in DMEM. Top row images represent control siRNA-transfected cells, second row images represent siHK2-transfected cells and bottom row images represent 3-BrPa-treated cells. Images were taken with 60X oil-immersion objective; Hoechst DNA stain was visualized through DAPI filter, hnRNP A1 was visualized though FITC filter.

siRNA sequences (5' to 3'):

siControl: UUCUCCGAACGUGUCACGUdTdT
siHK1: GGAAGGAGAUGAAGAAUGGtt
siHK2: GGAUGAAGGUAGAAAUGGAtt

Primer sequences (5' to 3'):

HK2 Fwd cloning:
CACAAAGCTTATGGATTACAAGGACGACGACGATAAGACCAAGTGCAGAAGGTTGAC
HK2 Rev cloning: TGTGGAATTCCTATCGCTGTCCAGCCTCAC
HK2 sequencing: TCTACTATTGGGGTCGACGG
HRV Fwd: GATCAGGTGGATTTTCCCTC
HRV Rev: GTGATTGACCAGCTGATGATG
GAPDH Fwd: ACAGTCAGCCGCATCTTCTT
GAPDH Rev: ACGACCAAATCCGTTGACTC

Restriction enzyme target sequences:

EcoRI: GAATTC
HindIII: AAGCTT

qPCR formula and standard curves:

$$2^{\frac{35-Ct\,@\,optimal\ temp.}{8}}$$

HRV: y=-3.463x + 11.88
GAPDH: y=-2.83x + 18.7

Recombinant protein information:

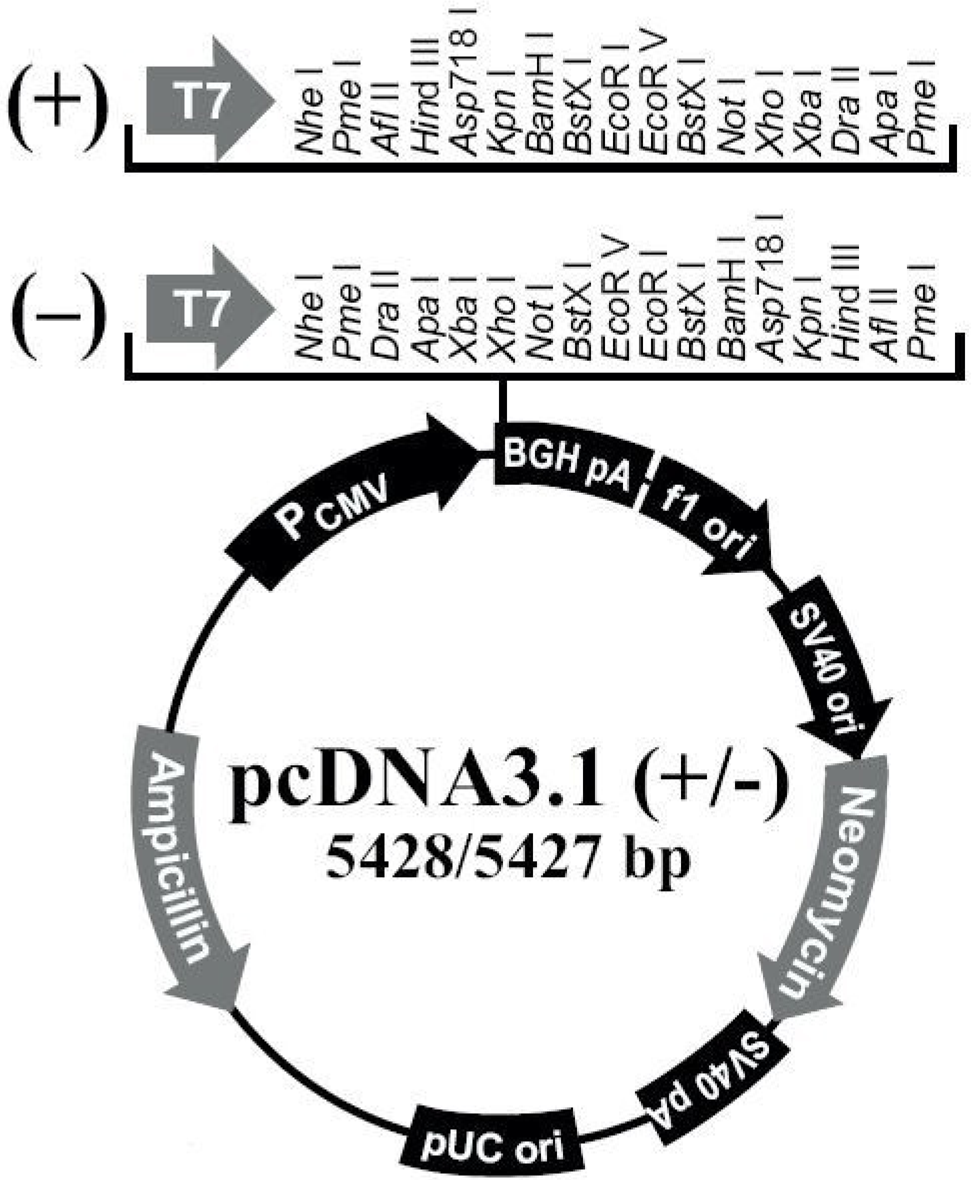
His-HK2: Hexokinase 2 Human, His tag, E.coli; ATGen, Cat No. HXK0703
His-hnRNP A1: HnRNP A1 Human, His tag, E.coli; ATGen, Cat No. ATGP1411

Antibody dilutions:

hnRNP A1 (Sigma): 1:5000 in PBST
HRV: 1:5000 in PBST
HK2 (Cell Signalling): 1:1000 in 50% Odyssey blocking buffer, 50% PBST
Bcl-XL (Cell Signalling): 1:500 in PBST
b-Actin (Abcam): 1:10000 in PBST
FLAG (Sigma): 1:5000 in PBST
Mouse, HRP-linked (Cell Signalling): 1:5000 in PBST
Rabbit, HRP-linked (Cell Signalling): 1:5000 in PBST
Mouse, Alexa Fluor 700 (Invitrogen): 1:10000 in PBST

Plasmid information:

pcDNA3.1+: Invitrogen, Cat No. V790-20



Curriculum Vitae

Languages: English, French

Education:

2012-2014 M.Sc. Biochemistry
Expected date of graduation: November 2014
University of Ottawa
Ottawa, Ontario, Canada

2010-2012 B.Sc. Specialisation in biochemistry
Degree obtained: May 26, 2012
Université de Moncton
Moncton, New-Brunswick, Canada

Laboratory Experience:

2012-2014 **Masters Thesis**

Supervisor: Martin Holcik, PhD

Project Title: Regulation of hnRNP A1 Localization by Protein Kinases and its Biological Impact

2011-2012 **Initiation à la recherche (Honour's Thesis)**

Supervisor: Pier Jr Morin, PhD

Project Title: The implication of miR-34 and miR-284 in freeze-tolerant *Eurosta solidaginis* and *Epiblema scudderiana*

2011 **Undergraduate Research Internship**

Supervisor: Pier Jr Morin, PhD

Project Title: The implication of miRNAs in the freezing processes of *Eurosta solidaginis*

Laboratory skills:

Bioinformatics: NCBI Gene, Protein and PubMed databases, Primer 3 Imput v. 0.4.0, DIANA Lab miRNA database, MirBase miRNA database, Primer Designer, DNAMan, ChemSketch (ACDLABS 12.0), Serial Cloner, Columbus imaging software (Perkin-Elmer Inc.), IncuCyte imaging software (Essen Bioscience)

Molecular biology: Isolation and purification of proteins, Isolation of total RNA, Isolation of miRNAs, Isolation of total DNA, cloning and analyses, RT-PCR, qPCR, agarose gel electrophoresis, SDS gel electrophoresis, Western blotting, ODYSSEY fluorescent imaging, basic cell cultures, RNAi analyses, viral manipulations and analyses

Microbiology: Various bacterial and fungal cultures, DNA transformation

Biochemistry: Immunofluorescence, Confocal microscopy, In vitro phosphorylation assays, ^{32}P-ATP manipulation, Column chromatography, spectrophotometry, dialysis, ELISA immunoassay, lyophilisation, Bradford and BCA protein assays

Work experience:

2014 **Children's Hospital of Eastern Ontario (CHEO) Research Institute**

Research assistant– CHEO Inflammatory Bowel Disease Research Centre

Responsibilities: Register eligible patients for participation in diverse clinical research studies. Collect patient samples and process for analysis. Follow-up with patients in hospital clinic throughout the course of the studies. Keep accurate paper and electronic records of data collected.

Supervisor: Ruth Singleton, R.N.

2011 **Université de Moncton**

Laboratory assistant– Laboratoire de techniques chimiques (CHIM 2072)

Responsibilities: Guide students through multiple analytical chemistry techniques, assist the laboratory professor in protocol setups, in teaching proper techniques and in reviewing student laboratory reports

Supervisor: Katy Beaulieu

2010/2012 **Greater Moncton Pest Control Commission**

Petitcodiac River Dyke surveyor, mosquito control technician

Responsibilities: Survey and record surface water changes in the Petitcodiac River area following a dyke construction plan to study the effects on the proportion and species variation in local mosquito populations

Employer: Louis Lapierre, PhD

2009 **Greater Moncton Pest Control Commission**

Mosquito control technician, pesticide applicator

Responsibilities: Locate local mosquito breeding grounds, biological pesticide application, record effects on mosquito larvae population proportions, operate specialised all-terrain vehicles for pesticide application

Employer: Louis Lapierre, PhD

2009 - 2011:

Montana's Cookhouse, Server

Responsibilities: Serve quality food to guests in a timely and professional manner, provide comfortable atmosphere through good customer relations, understand and comply to provincial health and food safety regulations

2008 - 2011:

Apple-A-Day, cashier

Responsibilities: organize and mount produce display stands, manipulate cash register, promote good customer relations

2008:

Société historique de Memramcook, Historic guide

Responsibilities: Guide visitors through a historic exhibit, manipulate a computer program to present the genealogic history of the visitors

2007 **Village de Memramcook**

Water and sewage treatment assistant

Responsibilities: perform daily colorimetric testing and chemical mineral testing for iron and manganese on local public drinking water, collect treated sewage water for laboratory testing

Supervisor: Martin Leblanc

Volunteering:

2013-2014: Volunteer, Let's Talk Science Education Program

2013: Telephone operator, Children's Hospital of Eastern Ontario (CHEO) Annual Telethon

2012: Demonstrator/Educator, Children's Hospital of Eastern Ontario Research Day

2008 - 2010: Fundraiser organization for the Tree of Hope Foundation (Moncton, N.-B.)

2008 : Promotion of the Salon Carrière 2008 at Université de Moncton

2008 : 55+ Canada Games (Université de Moncton)

Extracurricular Activities:

2013-2014 Laboratory Safety Coach, Children's Hospital of Eastern Ontario Research Institute

2011 Tutor, corrector, Department of Chemistry and Biochemistry, Université de Moncton Faculty of Science

2010 President, The Tree of Hope Foundation Committee of the Université de Moncton Faculty of Science

2010 President, Université de Moncton Faculty of Science Banquet Organization Committee

2009-2010 Secretary, Student council of the Université de Moncton Faculty of Science Student Association (AEFSUM Inc.)

2009-2010 Active member, Friends of Doctors Without Borders Université de Moncton

2008-2009 1st year representative, Special Programs student council Université de Moncton Faculty of Science

Publications:

Lynn A. Courteau, Kenneth B. Storey, Pier Jr Morin. Differential expression of microRNA species in a freeze-tolerant insect, *Eurosta solidaginis*. Cryobiology. 2012. Vol. 65 No. 3 p.210-214

Pierre J. Lyons, Julie J. Poitras, **Lynn A. Courteau**, Kenneth B. Storey and Pier Jr Morin. Identification of differentially regulated microRNAs in cold-hardy insects. *Cryoletters*. 2013. Vol. 34 No. 1 p. 83-89

Awards:

2013-2014 Queen Elizabeth II Graduate Scholarship in Science and Technology: Ontario Graduate Scholarships (15,000.00$)

2013-2014 Excellence Scholarship: University of Ottawa (15,000.00$)

2012-2013 Admission Scholarship: University of Ottawa (15,000.00$)

2011 Scholarship award: Caisse Populaire Dieppe-Memramcook (500.00$)

2009 Scholarship award: Fondation des Caisses Populaires (1,000.00$)

2009 Millennium excellence scholarship award (4,500.00$)

2008 Scholarship award: Université de Moncton (1,000.00$)

2008 Scholarship award: Université de Moncton (1,000.00$)

2008 Scholarship award: Patrimoine canadien - Recrutement (1,000.00$)

2008 Scholarship award: Religieux Sainte-Croix d'Acadie (1,000.00$)

2008 Scholarship award: Caisse Populaire de Memramcook (250.00$)

Conferences and Presentations:

2011 **Lynn A**. **Courteau** et Pier Jr Morin. November 2011, Moncton, Canada. microRNAs: A “cool” family of ribonucleic acids. NBHRF 3rd Annual Health Research Conference

2012 Pierre Lyons, **Lynn A. Courteau**, and Pier Jr. Morin, May 2012, Cap Breton, Canada. microRNAs: A “cool” family of ribonucleic acids. ChemCon 2012.

2012 **Lynn A. Courteau**, Pierre Lyons and Pier Jr. Morin. May 2012, Sackville, Canada. microRNAs: A “cool” family of ribonucleic acids. 51st Meeting of the Canadian Society of Zoologists.

2012 **Lynn A. Courteau**, Kenneth B. Storey and Pier Jr Morin. June 2012, Rosario, Argentina. Characterization of a cold associated miRNA signature in a freeze tolerant insect. CRYO 2012

2013 **Lynn A. Courteau**, Jack Crasto and Martin Holcik. May 2013, Ottawa, Canada. Regulation of hnRNP A1 Localization by Protein Kinases and its Biological Impact. University of Ottawa Department of Biochemistry, Microbiology and Immunology Poster Day.

2013 **Lynn A. Courteau**, Jack Crasto and Martin Holcik. October 2013, Ottawa, Canada. Regulation of hnRNP A1 Localization by Protein Kinases and its Biological Impact. Children's Hospital of Eastern Ontario Research Day.

2014 **Lynn A. Courteau**. March 2014, Ottawa, Canada. Regulation of hnRNP A1 Localization by Protein Kinases and its Biological Impact. University of Ottawa Department of Biochemistry, Microbiology and Immunology Seminar Day.