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# Cell type difference in influenza a viral mRNA nuclear export

Sean William Larsen

# CELL TYPE DIFFERENCE IN INFLUENZA A VIRAL mRNA NUCLEAR EXPORT

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A Thesis

Presented to the

Faculty of

California State University,

San Bernardino

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Biology

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by

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ABSTRACT

Influenza transcribes viral mRNA in the host cell nucleus and the viral mRNAs must be exported for translation in the cytoplasm. Most cellular mRNA nuclear export is mediated by Nxf1 while select mRNAs utilize Crm1. Published results in kidney cells reveal no dependence on Crm1 but show select influenza mRNA nuclear export is dependent on Nxf1. Influenza infects cells of the respiratory tract; thus A549 human lung cells are likely a better model for influenza infection than HEK 293T or MDCK cells. I utilized expression of Dominant Negative Nxf1 protein (DN-Nxf1) to inhibit Nxf1 nuclear export (Kang, 1999) in both kidney cells (HEK 293T) and lung cells (A549). In agreement with published data using siRNA to down-regulate Nxf1, expression of DN-Nxf1 resulted in inhibition of HA, NS1, M1, and M2, but not NP and PB2 mRNA nuclear export in 293T cells. Quantitative PCR showed that DN-Nxf1 expression resulted in 43%, 74%, 18%, and 8% reductions in cytoplasmic HA, NA, M, and NS mRNAs. Interestingly, expression of DN-Nxf1 in A549 cells inhibited both NP and HA mRNA nuclear export, revealing a cell type difference for influenza NP mRNA nuclear export. Quantitative PCR showed that DN-Nxf1 expression resulted in 13%, 30%, 74%, 30%, and 16% reductions in cytoplasmic HA, NP, NA, M, and NS mRNAs. Similar to results obtained with 293T cells, polymerase-encoding mRNAs showed only minimal dependence on Nxf1. Quantitative PCR confirmed no significant difference in cytoplasmic PB2 mRNA in both A549 and 293T cells. To strengthen the evidence that Nxf1 is utilized for NP and HA mRNA

nuclear export, but not polymerase encoding mRNAs, Nxf1 was immunopurified along with associated mRNAs in A549 cells. NP, NA, HA, NS1, NS2, M1, and M2 mRNAs were found specifically associated with Nxf1 but PB1, PB2, and PA mRNAs were not. These results support the hypothesis that some influenza mRNAs utilize atypical mRNA nuclear export; defining this pathway may lead to discovery of novel antiviral targets. Furthermore, our data reveal a cell type difference in the role of host Nxf1 for influenza NP mRNA nuclear export.

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# DEDICATION

I dedicate this thesis to my parents Colleen and Brian, as well as my brothers and sisters, Siobhan, Aidan, Alannah, and Quinn whose constant support and sarcasm keep me moving forward. I would also like to dedicate this to my fellow Good Vibe Tribe family members, keep on keepin on!

TABLE OF CONTENTS

LIST OF FIGURES

# CHAPTER ONE

# BACKGROUND AND SIGNIFICANCE

## Societal Effects of Influenza

Every year influenza epidemics are responsible for three to five million hospitalizations and 250,000 to 500,000 deaths throughout the world (CDC). Epidemics are characterized by sustained, widespread person-person transmission within a circumscribed area (Bouvier & Palese, 2008). Epidemics peak in the winter season, and are estimated to be responsible for $10 billion in medical costs and a total economical burden of $87 billion each year (Molinari et al., 2007). Three times during the 20th century the world experienced influenza pandemics: the 1918 Spanish flu, the 1957 Asian flu, and the 1968 Hong Kong flu. Pandemics are characteristically similar to epidemics, except the transmission is worldwide instead of within a circumscribed area (Cox & Subbaro, 2000). The first pandemic experienced in the 21st century was the 2009 novel H1N1 also known as the Swine flu (WHO). The Swine flu was an estimated burden of $3 trillion on the global economy (Lagacé-Wiens et al., 2010).

Symptoms of an influenza infection involve quickly developing high fever, dry cough, headache, muscle pain, general fatigue, sore throat, and a runny nose (WHO). Influenza spreads when an infected person coughs and droplets containing the virus are expelled into the air or onto the infected

person's hand, potentially breathed in by others or spread by contact (WHO). The virus has an incubation period of roughly two days, and usually victims recover from symptoms within a week without medical attention. However, those at high risk, such as children under the age of two, elderly older than 65 years, and those with medical conditions involving the heart, lungs, liver, blood, kidneys, or immunologically compromised, can go on to develop severe illness possibly resulting in death (WHO).

## Virion

The Influenza virus is a member of the Orthomyxoviridae family; a family of enveloped, segmented, negative sense, single stranded RNA (ssRNA) viruses (Resa-Infante et al., 2010). There are three types of Influenza, Influenza A, B, and C (Bouvier & Palese, 2008). All three types share a common ancestry, but diverged genetically, preventing intra-type re-assortment (Bouvier & Palese, 2008). Influenza A viruses are responsible for all known human pandemics and are the focus of this research. Influenza A is further classified by the subtype of the virus, which is based upon the surface glycoproteins of the virion; Hemagglutinin (HA) and Neuraminidase (NA) (Bouvier & Palese, 2008). There are 16 and 9 subtypes of the HA and NA glycoproteins, respectively (Bouvier & Palese, 2008). Only three HA subtypes (H1, H2, and H3) and two NA subtypes (N1 and N2) have been associated with human epidemics (Bouvier & Palese, 2008). This research

utilizes the Udorn Influenza A H3N2 lab strain virus to investigate influenza mRNA nuclear export.

Infectious Influenza A virions consist of three major structures, the core, the matrix, and the envelope. The core of the virion consists of structures referred to as the viral ribonucleoprotein (vRNP) (Martin-Benito et al., 2001). Each vRNP consists of one negative sense ssRNA segment encapsulated by Nucleoprotein (NP), and bound at both the 3' and 5' ends by the viral RNA-dependent RNA polymerase (RdRP). The RdRP is made up of two basic proteins (PB1 and PB2) as well as an acidic protein (PA), which together form the active RdRP, a heterotrimer (Resa-Infante et al., 2010, Bouvier & Palese, 2008). The positively charged pocket of NP allows for interaction with the negatively charged phosphate backbone of the RNA (Bouvier & Palese, 2008). Of the 7 proteins found in the virion, NP is found in the most abundance, as its role is to nonspecifically bind the vRNA genome. It is important to note that NP does not protect the RNA from RNases (Martin-Benito et al., 2001, Bouvier & Palese, 2008). The vRNP in the core is responsible for viral transcription and replication.

Surrounding the vRNPs is a matrix of M1 proteins, which is further surrounded by a host-derived lipid bilayer envelope containing three transmembrane proteins: HA and NA, which are external, and an ion channel (M2), which is internal (Nayak et al., 2009, Bouvier & Palese, 2008). Because the envelope is host derived, it contains cholesterol-enriched lipid rafts as well

as non-raft lipids (Nayak et al., 2009). The HA and NA proteins are raft anchored on the surface and play intricate roles in the infection and budding process respectively. The M2 protein is loosely associated with lipid rafts through a cholesterol binding protein, and plays a large role in the fusion of the virion with the endosome (Nayak et al., 2009). The envelope surrounds the virion and the shape can vary (Figure 1).

The influenza RNA genome is segmented and consists of eight individual segments, which code for at least 11 different proteins (Bouvier & Palese, 2008). Similar to cellular transcription, influenza transcription also occurs in the nucleus so that host-splicing machinery can be utilized. Influenza genes can be separated into three distinct categories; intronless (segments 1-6; PB2, PB1, PA, HA, NP, and NA), intron containing (segments 7 and 8; M1 and NS1), and fully spliced (segments 7 and 8; M2 and NS2) (Read & Digard, 2010, Bouvier & Palese, 2008). The genes can also be categorized into Early (PB2, PB1, PA, NP and NS1), and Late (HA, NA, M1, M2, and NS2), based simply upon the timing of protein expression (Hatada et al, 1989).

## Infection

### Attachment and Entry

The target cells of choice for Influenza A virus are respiratory epithelial cells. Influenza A viruses recognize their target cells by N-acetylneuraminic

(sialic) acid residues on the cell surface (Bouvier & Palese, 2008). Once there is viral attachment to the sialic acid residue on the cell surface, the virion is internalized via receptor-mediated endocytosis (Cros et al., 2005). Due to the low pH environment of the endosome, the acidity causes a conformational change in the HA protein (Cros et al., 2005, Bouvier & Palese, 2008). The conformational change in HA exposes a fusion peptide, which facilitates the fusion of the viral and endosomal membranes, creating a pore through which vRNPs can escape into the cytoplasm (Cros et al., 2005, Bouvier & Palese, 2008). Hydrogen ions are pumped into the virion via the M2 ion channel and leads to acidification of the inside of the virion, which causes disruption of protein-protein interactions; mainly dissociation of the vRNP from the M1 protein matrix (Cros et al., 2005, Bouvier & Palese, 2008). This is a critical step for proper release of the vRNPs to the cytoplasm. The Nuclear Localization Signals (NLSs) found on vRNP proteins allows vRNP nuclear localization, the site of Influenza A replication and transcription (Cros et al., 2005, Resa- Infante, Martin-Benito et al., 2001) (Figure 2).

Replication and Transcription

Once the vRNP reaches the nucleus, viral transcription (formation of mRNAs which are destined to be translated into proteins) begins. Both cellular and viral mRNAs consists of the genetic information flanked by a cap-structure at the 5' end and a poly A tail at the 3' end. Cellular mRNAs obtain a 5' cap via Capping Enzyme complex, which is found associated with RNA

Polymerase II. The poly-A tail is added via Poly-A polymerase, which adds adenine residues to terminated mRNA transcripts in a template independent manner, resulting in poly-adenylated mRNAs. In contrast, Influenza mRNAs obtain their 5' cap by stealing the caps off host pre-mRNAs. PB2, an 82 kDa protein, and PA, a 78 kDa protein, are both involved in the cap-snatching process due to their cap binding and endonucleolytic properties, respectively. These stolen caps act as primers for the transcription of the viral mRNA by PB1, an 83 kDa protein, which possesses the polymerase activity. Influenza mRNAs obtain their poly-A tail via a stuttering mechanism, by the viral RdRP, triggered by a short stretch of Uracils, usually 5-7, at the 5' end of the vRNA template, resulting in polymerization of multiple adenine residues or a poly-A tail (Bouvier & Palese, 2008). Once capped and poly-adenylated, the viral mRNAs visually look the same as host mRNAs (Figure 3).

After viral protein synthesis and accumulation of viral NP and RdRP proteins, replication (formation of progeny vRNA) will begin. Replication is unprimed and involves the formation of a full-length positive sense copy RNA molecule, cRNA, which is encapsulated by free NP and bound at the ends by viral polymerase proteins. In the second step in the replication process, the cRNA is used as template by the RdRP to polymerize the end replication product, vRNAs, which are also encapsulated by free NP and bound by the viral RdRP to form vRNPs. The new vRNPs can be used for assembly into

new virions, repeated rounds of replication, or transcription to amplify viral gene expression (Figure 3).

Splicing and Translation

Splicing plays an important role in influenza infection. As mentioned above, each virion contains 8 segments, but its genome encodes for at least 11 different proteins. Segments 7 and 8 encode two proteins each, M1 and NS1 which are intron containing viral mRNAs, and M2 and NS2 which are fully spliced viral mRNAs. UAP56, a cellular splicing factor, and member of the DEAD Box family of RNA helicases, was found to interact with influenza NP (Naito et al., 2007, Nagata et al., 2008). This seems to suggest that the splicing of influenza mRNAs may occur via canonical host splicing machinery. However, when UAP56 is downregulated via siRNA, there is either slight or no decrease in total viral load and no change in ratio of spliced mRNAs to their respective intron containing mRNAs (Unpublished Newcomb Lab, Nagata et al., 2008, Read & Digard, 2010). Therefore, while it is likely that host-splicing factors such as RNA helicases are involved are involved in viral mRNA splicing, it is not proven that viral mRNA splicing is occurring through a typical host mechanism.

Cellular mRNA splicing is connected to mRNA nuclear export via interactions between splicing factors and the nuclear export machinery, specifically Nxf1 or Nuclear Export Factor 1. The bulk of cellular mRNAs exit the nucleus via Nxf1 (Carmody & Wente, 2009). It appears that only select

influenza viral mRNAs exit the nucleus though Nxf1 although published data on influenza mRNA export remains sparse and conflicting. The focus of my thesis research was to clarify the role of host Nxf1 in influenza viral mRNA nuclear export and therefore nuclear trafficking will be discussed in detail in Chapter 2. After nuclear export, the viral mRNAs are translated by host ribosomes. Viral mRNAs encoding envelope proteins, HA, NA, and M2, are translated by membrane bound ribosomes.

Assembly and Exit

In order for a virion to be infectious, it must contain all 8 vRNP segments, thus once the 8 vRNP segments are replicated to abundant levels, assembly and budding can commence (Nayak et al., 2009). Assembly and budding occurs at the cell membrane, and is triggered by an accumulation of vRNP bound M1 protein on the cytoplasmic side of the lipid bilayer (Bouvier & Palese, 2008). HA will continually bind the virions to the sialic acid residues until budding is complete, at which time the sialidase activity of NA will cleave the sialic acid residues, releasing the progeny virions (Bouvier & Palese, 2008). Additionally, NA will remove sialic acid residues from the virions, preventing binding of new progeny to each other and therefore leading to enhanced infectivity (Bouvier & Palese, 2008). It is interesting to note that NA is also believed to have the ability to break down respiratory tract secretions, facilitating easier access to respiratory cells (Bouvier & Palese, 2008).

## Antigenic Variation

Influenza is difficult to control due to rapid genetic mutation resulting in genetic variation. There are two methods by which influenza introduces antigenic variation to the viral population, antigenic drift and antigenic shift. Antigenic drift is caused by mutations during genome replication because the RdRP has no proofreading abilities, resulting in genetic variation. This produces viruses with mutations in the amino acid coding sequence of all segments; however, HA/NA variable regions can best tolerate the changes and even benefit from them by enabling the virus to evade the immune system. Because neutralizing antibodies against influenza are HA/NA specific, these antigenic changes may render a body's previously established antibody defenses against influenza subtypes useless. The second method by which influenza introduces antigenic variation to its population is by means of antigenic shift. Antigenic shift is characterized by a major change, which results in an entirely new HA subtype introduced into the human population. This can be paired with or without a change in the NA variant. There are two ways by which antigenic shift can occur. The first involves transmission of an influenza virus directly from an animal reservoir to humans without re-assortment, resulting in a new HA variant introduced to humans. The second involves re-assortment of human and animal (i.e. swine, avian) viruses resulting in a new HA subtype introduced, once again with or without a new NA subtype (Cox & Subbaro, 2000). A new HA subtype will have no antibody

defense throughout the population, thus antigenic shift results in more effective changes than antigenic drift.

HA interacts with the host cell through sialic acid residues on the cell surface. There are two types of sialic acid residues on cells as the terminal 2-carbon of the sialic acid residue can bind either the 3-carbon or 6-carbon of galactose, forming either α2-3 or α2-6 linkages, resulting in different steric configurations of the terminal sialic acid (Bouvier & Palese, 2008). Different HA subtypes exhibit preference for binding either α2-3 or α2-6 configurations (Bouvier & Palese, 2008, Matrosovich, 2004). In human tracheal epithelial cells, the α2-6 configurations tend to dominate, however, once the lower respiratory tract is reached, α2-3 configurations can be found more frequently than in the upper respiratory tract. With avian respiratory tract cells, α2-3 tends to dominate (Matrosovich, 2004). Avian influenza viruses prefer infecting cells with α2,3 linkages, and because these linkages are found in minimal amounts in the mammalian upper respiratory tract, infection of humans with highly virulent H5N1, known as the bird flu, is very rarely reported (Cox & Subbaro, 2000, Matrosovich, 2004). However, in instances of high exposure to H5N1, such as working on a farm with infected birds or swimming in a virus laden pond/lake, humans have been know to be infected (Adams & Sandrock, 2010). This is because α2,3 linkages can be found in increased numbers in the lower respiratory region of humans (Ito et al., 2000). The number of recorded human H5N1 infections as of 2009 was 433 and of

these 433 infections, 262 cases resulted in death (Adams & Sandrock, 2010). Previously it was believed that H5N1 would have to acquire one or more mammalian influenza genes, however, newer publications have shown that only 4 mutations are necessary (Cox & Subbaro, 2000, Imai et al., 2012).

In contrast to humans, the tracheal epithelium of pigs contains both $\alpha$2,3 and $\alpha$2,6 linkages, rendering them susceptible to viruses of mammalian as well as avian origin (Cox & Subbaro, 2000). This fact places pigs in a very unique position because if a pig were to be infected simultaneously with viruses of human as well as avian origin, it would act as a mixing vessel for influenza re-assortment (Cox & Subbaro, 2000). The result of which would be unique influenza viruses. This is thought to be the mechanism by which the 2009 triple re-assortment novel H1N1 virus emerged (CDC Swine).

## Current Methods to Control Influenza Infection

Currently there are two preferred methods of controlling influenza infection. The first method is by means of vaccination. This prevents infection by production of antibodies against the specific NA/HA subtypes that are predicted to be in circulation during that year's epidemic, providing immunity. Immunization is currently achieved through inactive virus vaccines or live attenuated virus vaccines. Inactivated virus vaccines involves the subcutaneous injection of a killed/inactivated virus, and live attenuated virus vaccines are live viruses that encode viral proteins altered to diminish their

pathogenic properties yet still elicit the desired immune response. Influenza vaccines are solely based upon current HA/NA subtypes in circulation and therefore predicted to be found in the next year's epidemic. Because these vaccines are based upon predictions, if the HA/NA subtype used in the vaccine does not match that of the virus in circulation, the vaccine is rendered useless. Additionally, due to the rapid evolution of the virus new vaccines are required each succeeding year. Also of importance, is that current time required for seasonal influenza vaccine production is roughly six months (CDC). Thus, in times of pandemic, this time frame renders vaccines ineffective. The most time consuming step of creating a vaccine is the generation of a reference virus (Fauci, 2005). With the advent of reverse genetics, efforts by the NIH to shorten vaccine production time have cut the reference virus generation time down to a few weeks. An H5N1 vaccine made utilizing this method is now in phase I clinical trials (Fauci, 2005).

Antiviral medications combat influenza by preventing viral spread. There are two varieties of antiviral drugs aimed at influenza which target either the activity of the M2 ion channel or the NA protein. Adamantanes, specifically amantadine and rimantadine, are antiviral drugs that target the M2 ion channel (Pubbaraju et al., 2010). These two drugs inhibit viral replication by blocking the ion-channel activity of the M2 protein, preventing the dissociation of the vRNP from the M1 matrix (Pubbaraju et al., 2010, Cros et al., 2005, Bouvier & Palese, 2008). Some subtypes such as H3N2 and the

H1N1 subtype causing the 2009 pandemic have been reported to be completely resistant to adamantane antivirals (Pubbaraju et al., 2010). Neuraminidase inhibitors, specifically, oseltamivir and zanamivir, block the function of the NA protein, thereby inhibiting the release of progeny virions (Bouvier & Palese, 2008). Recent studies and clinical records have indicated a rise in H1N1's resistance to oseltamivir.

Nearly all strains of seasonal H1N1 found to be in circulation during 2008-2009 were resistant to oseltamivir treatment but only 1% of H1N1 from the 2009 pandemic was found to be resistant to the treatment (Thorlund et al., 2011, Okomo-Adhiambo, 2010). With 2009 H1N1 pandemic strains, it had been believed there is no known resistance to zanamivir (Thorlund et al., 2011, Okomo-Adhiambo, 2010). However, there was a recent discovery of a 2009 H1N1 pandemic strain with neuraminidase I223R mutation that showed cross-resistance to all three available neuraminidase inhibitors, oseltamivir, zanamivir and peramivir (Van der Vries et al., 2011). An infection with this strain would leave a physician with no possible antiviral options to combat influenza spread, since H1N1 was also shown resistant to adamantanes.

With the long wait times involved in the development and production of vaccines as well as the continued development of antiviral resistance; there is a need for new antiviral targets. Because influenza transcribes and replicates in the host nucleus, mRNA nuclear export could potentially provide an additional target to halt influenza spread. My study aims to define the nuclear

mRNA export pathways of influenza viral mRNA. If viral mRNAs are discovered to utilize neither known mRNA nuclear export pathway this would indicate the mRNA exports via undefined mRNA nuclear export pathway. This might be an atypical pathway not utilized by many host mRNAs and thus might reveal novel antiviral target.

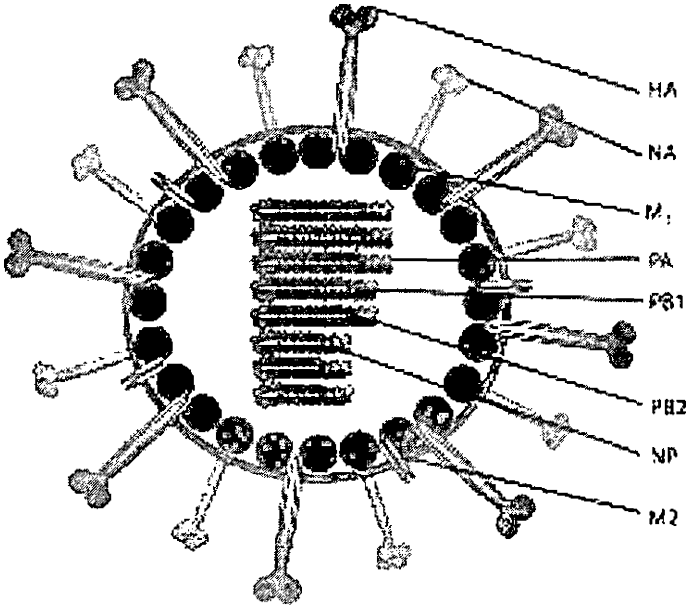


Figure 1. Influenza A Virion.

Consists of 8 vRNP segments [(-) ssRNA bound at both ends by the RdRP, and encapsulated by NP], surrounded by a matrix of M1 proteins, and a host derived lipid bilayer envelope which contains three transmembrane proteins [M2, HA, and NA].

Credit: Adams, S, and Sandrock, C. (2010). Avian Influenza: Update. *Medical Principles and Practice* 19(6), 421-32.

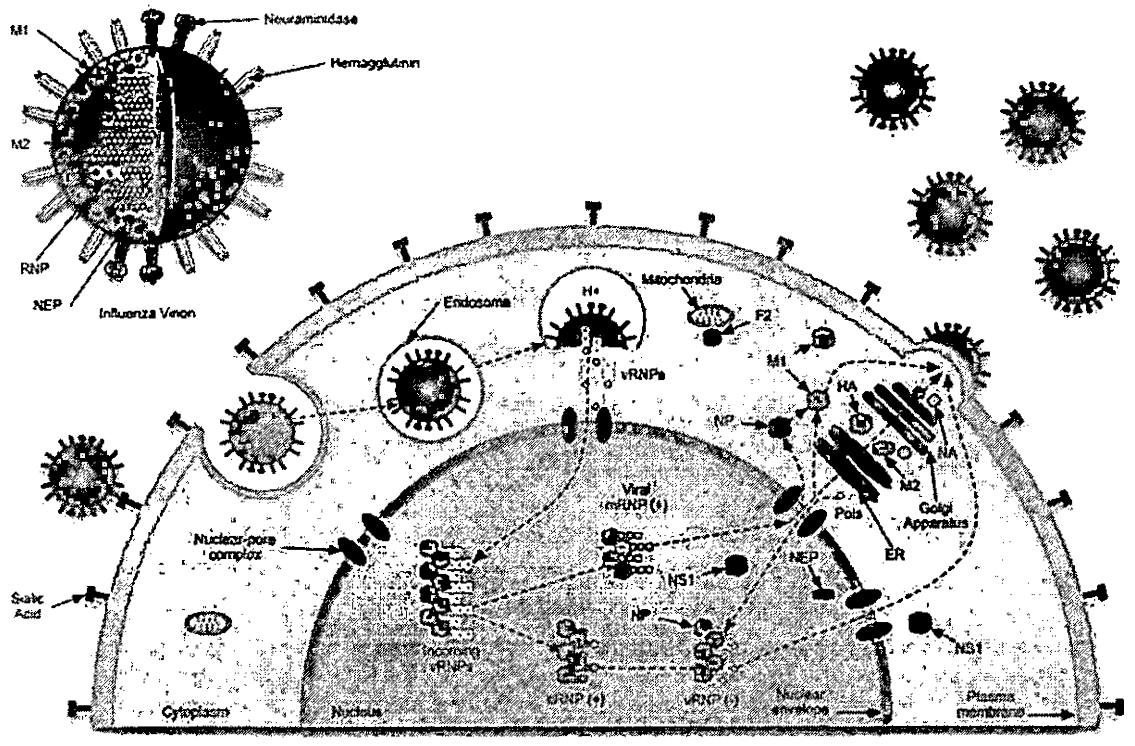


Figure 2. Influenza A Life Cycle.

Virion is internalized via receptor mediated endocytosis. vRNPs are released to the cytoplasm, where NLSs found on vRNP proteins localize the vRNPs to the nucleus, the site of influenza transcription and replication.

Credit: Palese, P, Shaw, ML. *Orthomyxoviridae: The Viruses and Their Replication* 2001 Fields Virology, 5th edition D.M. Knipe and P.M. Howley, Editors. 2006, Lippencott Williams and Wilkins: Philadelphia ISBN-10:0-7817-6060-7

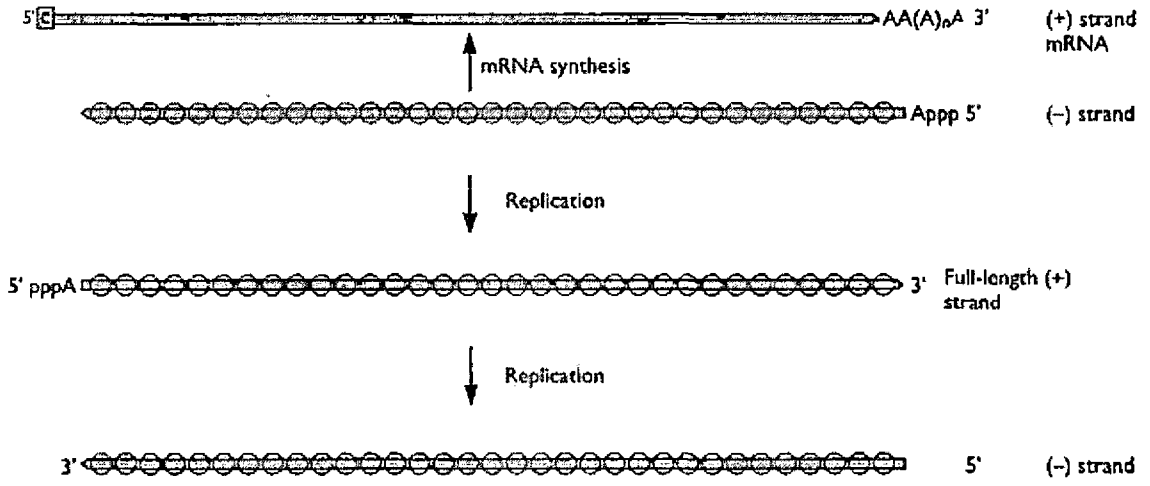


Figure 3. Influenza A Transcription And Replication.

Transcription results in a (+) sense mRNA that has 5' cap and host sequences and terminates early and stuttering to generate poly A tale. Replication results in a (-) sense vRNA via a full length unmodified (+) sense cRNA intermediate.

Credit: Racaniello, V. Influenza Viral RNA Synthesis. *Virology Blog — About Viruses and Viral Disease*. Web. 29 Nov. 2011. <http://www.virology.ws/2009/05/08/influenza-viral-rna-synthesis/>.

# CHAPTER TWO

# NUCLEAR TRAFFICKING

## Cellular Nuclear Trafficking

### Nuclear Pore Complex

The major structure involved in nuclear trafficking is an assembly of nucleoporins located within the nuclear envelope. This assembly of nucleoporins is termed the Nuclear Pore Complex (NPC) (Durairaj et al., 2009). Forming the bulk of the NPC is a central spoke complex, with rotational symmetry surrounding the central transporter channel. In addition, a ring forms the channel's borders with the cytoplasm and nucleus (Durairaj et al., 2009). Extending from each ring are eight fibrils, which are free at the cytoplasmic side, but come together to form a basket structure at the nuclear side (Durairaj et al., 2009) (Figure 4).

Molecules with a molecular weight less than 40,000 to 50,000 are understood to freely diffuse through the NPC. However, larger molecules require a nuclear localization signal (NLS) or a nuclear export signal (NES). These epitopes allow proteins to be actively transported into or out of the nucleus, through interaction with components of the NPC (Cros et al., 2005). RNAs are transported by interaction with proteins in ribonucleoprotein complexes or RNPs.

### Export Pathways

Human mRNA nuclear export through the NPC is regulated by the interaction of proteins, generally termed Nuclear Export Factors (NXF), and mRNA (Durairaj et al., 2009). Nuclear Export Factor 1 (NXF1/TAP) is the primary human cellular mRNA nuclear export factor, shuttling mRNAs from the nucleus to the cytoplasm (Carmody & Wente, 2009) (Figure 5). NXF1 has low affinity for mRNAs themselves, and interaction is strengthened through adaptor proteins, termed heterogenous nuclear ribonucleoproteins (hnRNPs) (Durairaj et al., 2009). Nuclear mRNA export is coupled to mRNA transcription and processing events such as splicing. UAP56 is a DEAD-box RNA helicase required for proper mRNA processing and transcriptional elongation. UAP56 interacts with the tetrameric THO complex; consisting of Hpr1, Thp2, Tho2, and Mft1. The THO complex is implicated to play an essential role in transcriptional elongation (Jimeno et al., 2002). Together, the THO complex and UAP56 are referred to as the transcription/export (TREX) complex (Durairaj et al., 2009). UAP56 recruits Aly, a hnRNP which then recruits NXF1 to the mRNA by strengthening NXF1-mRNA interaction. Aly is displaced by p15, NXF1's cofactor, allowing NXF1/p15 to directly bind the mRNA (Hautbergue et al., 2008). The NXF1/p15 complex will directly interact with the Phenylalanine/Glycine repeats in the NPC nucleoporins, facilitating the nuclear export process (Durairaj et al., 2009). As the mRNP moves through the NPC, RNA helicase DBP5, which is activated by Gle1, a

cytoplasmic protein, removes P15 from NXF1, ensuring unidirectional transport (Stewart, 2007).

In addition to the NXF1 pathway, a small subset of cellular mRNAs, cellular RNAs, and some viral mRNAs are known to exit the nucleus through a Ran-GTP dependent mechanism. This involves exportin, nuclear export transport receptors, binding the RNA along with Ran-GTP. The RNA-exportin-Ran-GTP complex is translocated to the cytoplasm, where GTP is hydrolyzed to GDP and the complex dissociates releasing the cargo (Serpeloni et al., 2011, Bogerd). A cellular mRNA export pathway that is Ran-GTP dependent is the Crm1 Pathway, which is responsible for the export of Cyclin D1 mRNA (Carmody & Wente, 2009). Crm1 is not a RNA binding protein, thus Crm1-mediated export of endogenous mRNAs must occur through adaptor proteins (Carmody & Wente, 2009). The adaptor protein for Cyclin D1 export is eukaryotic translation initiation factor 4e (eIF4e) (Carmody & Wente, 2009). An example of a virus that utilizes this pathway is the Human Immunodeficiency Virus (HIV). In this pathway, Rev, a viral protein, binds the Rev Responsive Element within the intron of viral mRNAs, and interacts with Crm1 via a leucine rich NES, facilitating the export of unspliced and partially spliced viral mRNAs (Cros et al., 2005, Yi et al., 2002). Two other types of RNAs that are shuttled between the nucleus and cytoplasm in a Ran-GTP dependent manner are tRNAs and miRNAs. The specific exportins involved in the transport of miRNA and tRNA are exportin-5 and exportin-t,

respectively (Figure 5) (Serpeloni et al., 2011). To date no mRNAs have been shown to export via exportin-t or exportin-5

## Influenza Nuclear Trafficking

### Influenza vRNP Nuclear Import and Export

Influenza vRNPs are much larger than 50kDa, preventing them from freely diffusing through the NPC. Therefore, it was hypothesized that at least one component must contain a NLS. All vRNP proteins (NP, PB1, PB2, and PA) were found to contain an NLS (Cros et al., 2005, Bullido et al., 2000, Neumann et al., 1997, Wang et al., 1997, Akkina et al., 1987, Jones et al., 1986, Nieto et al., 1994). Interestingly, the import of vRNA was demonstrated to be dependent upon NP protein (O'Neil et al., 1998), and multiple studies demonstrated that NP contains at least three NLSs, but that only the NLS found in the N-terminus 13 amino acids of the protein was essential for nuclear localization (Cros et al., 2005, Bullido et al., 2000, Neumann et al., 1997, Wang et al., 1997). The vRNPs are translocated to the nucleus through interaction of vRNP NLSs with karyopherin alpha in a Ran-GTP dependent manner (Cros et al., 2005, O'Neil 1995).

Once viral replication has been completed, vRNPs must exit the nucleus to reach the cell membrane, the site of virion assembly and packaging. Initial investigations implicated M1 in vRNP export, and later it was found that NS2 bound M1 to export vRNPs from the nucleus (Huang, X

et al. 2001., Yasuda et al., 1993). NS2 contains a leucine-rich Nuclear Export Signal (NES), which could functionally play the role that the HIV Rev protein does and interact with the Crm1 pathway (O'Neil et al., 1998). Deletion of the NES in NS2 resulted in inhibition of vRNP export (Neumann et al., 2000). It is believed that the defect is not due to inability of NS2 to interact with Crm1, but rather the formation of the Crm1-NS2-vRNP-Ran-GTP complex (Neumann et al., 2000).

Influenza mRNA Nuclear Export

Influenza mRNA nuclear export is less understood. One study had suggested RdRP proteins bind the mRNA in not only the nucleus but also in the cytoplasm, and functionally replace the export and translation factor eIF4e (Burgui et al., 2007). However, the RdRP was not found to co-immunopurify with viral mRNAs in the cytoplasm (Bier et al., 2011). Therefore it is presumed that influenza mRNAs are associated with host proteins for mRNA nuclear export. However, there is conflicting data regarding which host pathway is utilized and the exact host proteins involved (Wang et al., 2008, Read & Digard, 2010, Satterly et al., 2006).

An investigation by Satterly et. al. into host shut off properties of the influenza virus found that at 6 hours post infection (h.p.i.) there was NS1-NXF1 interaction, which led to inhibition of NXF1-mediated export of antiviral related genes such as interferon. The investigators were led to conclude that influenza mRNAs must export via Crm1. While they reference a paper to

defend their conclusion, the citation examined influenza vRNP export (Neumann et al., 2000). This conclusion is in stark contrast to the results of Elton et. al., as well as more recent studies, which have found that Crm1 plays no role in Influenza mRNA nuclear export (Elton et al., 2001, Read & Digard, 2010, Wang et al., 2008). Elton et al, found that blocking of the Crm1 pathway via Leptomycin B (LMB) treatment resulted in reduced virion production as vRNPs were blocked from exiting the nucleus during assembly. However, the treatment had no implication upon viral protein expression, thus no impact upon viral mRNA nuclear export.

A study by Wang et. al. found that LMB treatment had no effect at all on PB1, M1, and NA mRNA export. Interestingly, they found that both NS1 and NXF1 co-localize with intronless, intron containing, and fully spliced influenza mRNAs, confirming the association Satterly et. al. identified. However, they found that PB1, NA, and M mRNAs co-immunoprecipitate with NXF1, supporting use of the NXF1 pathway. Unfortunately, the immunoprecipitation experiment lacked a positive control to detect a percentage of the total RNA. This control is crucial because during infection viral transcripts are abundantly produced. Without a positive control representing 1 to 10% of total RNA, a false interaction could be easily detected because RT-PCR is a very sensitive method. The mRNA-protein interaction is dynamic and if 10% of the RNA can be specifically detected in

the immunoprecipitate, but not the negative control, it is very likely that this interaction is occurring, thus confirming pathway interaction.

A third study by Read and Digard, found that while inhibition of Crm1 did not alter influenza mRNA export, inhibition of the NXF1 pathway resulted in nuclear retention of HA, M, and NS but not NP or PB2 mRNAs. These results further confirm those of Wang et. al. and Elton et. al. in that influenza mRNA does not utilize Crm1-mediated export and that at least some influenza mRNAs utilize Nxf1-mediated export. This report was the first to identify viral mRNAs utilize neither defined mRNA nuclear export pathway. Interestingly, they found that PB2 mRNA was not dependent upon NXF1, and assumed that since PB2 did not appear to be dependent upon NXF1, then PB1 and PA would not be as well because they are all mRNAs which are expressed early and encode subunits of the viral RdRP. However, in the Wang et. Al. investigation, PB1 was found associated with NXF1. Yet, Read and Digard did not confirm pathway inhibition through analysis of cellular mRNA nuclear export. Furthermore, they did not look at direct interaction of Nxf1 with viral mRNAs, a necessary experiment to rule out indirect effects observed with down regulation of NXF1.

All these investigations into influenza mRNA nuclear export have another potentially major flaw, in the choice of cell types chosen. According to August Krough, with each scientific question, there is an ideal model to be used to answer that question (Krough, 1929). While these studies were all

performed in kidney cells, either Madin Darby Canine Kidney (MDCK) and/or 293T human kidney embryonic cells as their model, influenza infects the respiratory tract, thus kidney cells are not the best models. My thesis aimed to determine which influenza viral mRNAs utilize the Nxf1 mRNA nuclear export pathways in human lung epithelial cells (A549). To accomplish this I employed two different methods to inhibit the NXF1 pathway and analyze viral mRNA export, and directly assess viral mRNA association with Nxf1. Viral mRNAs whose nuclear export is not mediated by NXF1 must utilize a novel as yet undiscovered pathway, or another RNA export pathway such as exportin-t or exportin-5, which do not typically export mRNAs. Identification of an atypical mRNA nuclear export pathway utilized by influenza to export its mRNAs out of the nucleus may reveal a novel antiviral target.

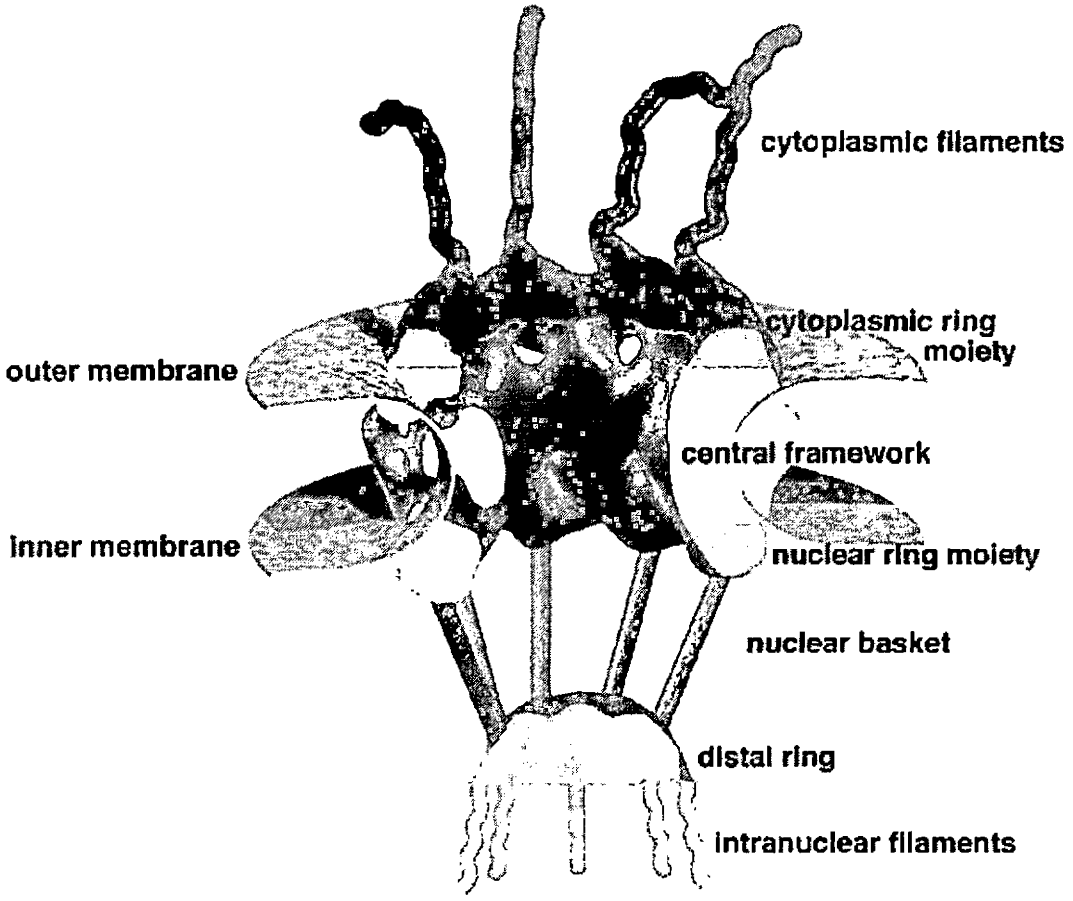


Figure 4. The Nuclear Pore Complex.

Composed of a central transporter channel surrounded by a central framework. Ring moieties form borders with the nucleus and cytoplasm. Extending from each ring are eight fibrils, which are free in the cytoplasm and come together at a distal ring on the nuclear side to from a nuclear basket.

Credit: Koser, J, Maco, B, Aebi, U, and Fahrenkrog, B, (2005). The Nuclear Pore Complex Becomes Alive: New Insights into Its Dynamics and Involvement in Different Cellular Processes. *Atlas of Genetics and Cytogenetics in Oncology and Haematology.*

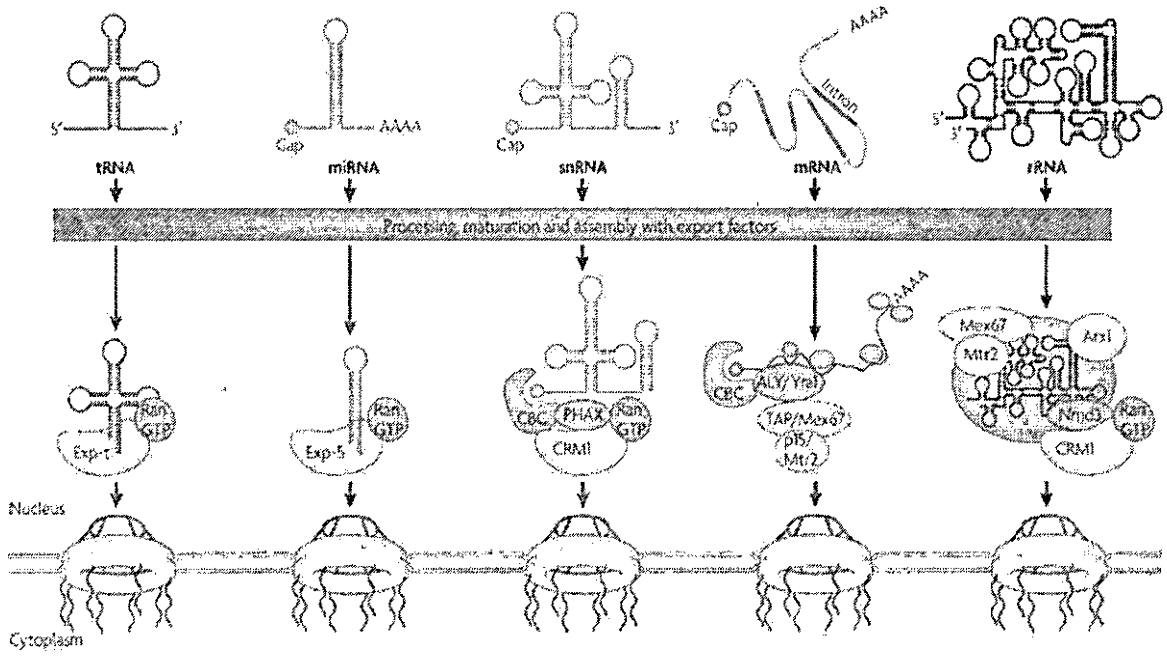


Figure 5. Known Cellular mRNA Nuclear Export Pathways.

The majority of cellular mRNAs exit the nucleus in a NXF1 dependent manner. tRNA and miRNA exit in a Ran-GTP dependent manner via exportin-t and exportin-5 respectively. A small subset of mRNAs as well as rRNAs exit in a Ran-GTP dependent manner using Crm1.

Credit: Kohler,A and Hunt, E, (2007). Exporting RNA From the Nucleus to the Cytoplasm. *Nature*, 8, 761-773.

# CHAPTER THREE

# SELECTIVE INHIBITION OF NUCLEAR EXPORT PATHWAYS

## Background

To examine the pathways used by influenza viral mRNAs to exit the nucleus, I used two different methods to block the Nxf1 mRNA nuclear export pathway. The first method was by expression of a dominant negative NXF1 (DN-NXF1) variant lacking a nuclear export signal, leading to inhibition of the NXF1 pathway (Kang & Cullen, 1999). The expression of this protein allows NXF1 to bind mRNA but does not allow the export, leading to pathway inhibition (Kang & Cullen, 1999). The second method was by expression of siRNAs targeting specific export factors. Transfection with siRNA specific to NXF1 and UAP56 mRNA inhibits expression of both Nxf1 and UAP56, and inhibition of both nuclear export pathways. Treatment with siRNA would be expected to completely inhibit the pathway, because siRNA is an efficient method to down regulate target gene expression, whereas expression of DN-NXF1 may not cause complete inhibition unless expression of the dominant negative form was to exceed that of endogenous.

To confirm inhibition of each pathway, host mRNA nuclear export was assessed. Beta-actin is known to exit the nucleus via the NXF1 pathway thus analysis of this host mRNA will confirm pathway inhibition (Carmody & Wente, 2009, Huang, Y et al., 2004). I primarily examined A549 cells. However, in

order to confirm and expand upon previously published work (Read & Digard, 2010), I also examined nuclear export in 293T cells.

## Results

Plasmid DNA encoding DN-NXF1, or vector alone (CMV) with eGFP to monitor transfection efficiency, were transfected into 293T or A549 cells. The cells were allowed to proliferate for 48 hours before one set was infected with Influenza A Udorn at high multiplicity of infection (MOI). At 3.5 hours post infection (hpi), cytoplasmic and nuclear RNAs and proteins were isolated. The clarity of the cytoplasmic/nuclear fractionations was assessed via SDS-PAGE and western blotting techniques (Figure 6 & 7). Both 293T and A549 cellular fractionation was successful, as TAT-SF1 and SP1, nuclear proteins, are solely observed in nuclear samples, while Tubulin and Hsp90, cytoplasmic proteins, are only observed in cytoplasmic fractions.

Total polyadenylated RNA was reverse transcribed into cDNAs using oligo dT. Influenza virus results in host shut off of gene expression and thus the uninfected samples were assessed for inhibition of nuclear export pathway using PCR with gene specific primers to analyze host mRNA nuclear export. Beta-actin is known to exit the nucleus via the NXF1 pathway (Huang, Y et al., 2004). Analysis of Beta-actin mRNAs confirmed that expression of the dominant negative proteins resulted in NXF1 pathway inhibition (Figure 8).

RNA from the cytoplasm of infected cells was assessed using RT with oligo dT and PCR with gene specific primers. With 293T cells, semi-quantitative PCR reveals a clear decrease in cytoplasmic HA, NA, M1, and NS1 but no change in NP, PB2 mRNAs (Figure 9). Interestingly, cytoplasmic NS2 mRNA increased with DN treatment (Figure 9). Quantitative PCR allows us to determine the relative inhibition of different viral mRNAs. Results showed cytoplasmic NA, HA, M, and NS mRNAs in the DN treated cells were 26%, 66%, 82%, and 92% of the control cells, with t-tests indicating all differences to be statistically significant (Figures 12 & 13). However, total cytoplasmic NP and PB2 mRNAs were found to be 102% and 107% of the control cells, with t-tests indicating no significant difference between the two treatment groups. Our results confirm Read and Digard's conclusions that the nuclear export of NP and polymerase mRNAs is not mediated by NXF1 in 293T cells.

In A549 cells, semi-quantitative PCR reveals a clear decrease in cytoplasmic NP, NA, and HA, but no detectable decrease in cytoplasmic PA, PB1 or PB2 mRNAs when DN-NXF1 is expressed (Figures 10 & 11). qPCR results show the total cytoplasmic HA, NP, NA, M, and NS mRNAs in the DN treated cells to be 87%, 70%, 27%, 70%, and 84% of the control cells, with t-tests indicating that all differences were statistically significant (Figures 12 & 13). Conversely, total cytoplasmic PB2 mRNA was found to be 97.22% of the control cells, with t-test indicating no significant difference between the two

treatment groups. Our results in A549 cells reveal cell type difference for NP mRNA export compared to 293T cells.

The second method used to target the major nuclear export pathways was through siRNA to target NXF1 and another host export factor, UAP56. A549 cells were transfected with siRNA targeting Nxf1, UAP56, or scrambled control for 48hrs. UAP56 is a DEAD box RNA helicase thought to be upstream of both Nxf1 and Crm1 mediated mRNA nuclear export (Carmody & Wente, 2009). Cells were either mock or infected for 3 hours, and then proteins and RNA from the cytoplasm and nucleus were collected. NXF1 and UAP56 protein expression were successfully down regulated by their respective siRNA treatment in A549 cells (Figure 14).

Down-regulation of NXF1 led to a decrease in cytoplasmic Beta-actin mRNAs (Figure 15). We further found that all influenza mRNAs examined were at decreased levels in the cytoplasm, including NP and PA mRNAs (Figure 16). This differed from the results obtained with expression of DN-Nxf1 to inhibit Nxf1-mediated nuclear export, and led us to further analyze nuclear RNA. We found there was a decrease in influenza nuclear mRNA in cells with NXF1 down-regulated (Figure 17), indicating indirect inhibition of viral transcription. Thus any changes observed in influenza mRNA export cannot be claimed as due to inhibition of the nuclear export pathway, but rather due to indirect effects on viral mRNA synthesis. Therefore we did not further pursue inhibition of NXF1 by siRNA.

Down-regulation of UAP56 led to no decrease in cytoplasmic Beta-actin mRNAs (Figure 15). This is likely due to the fact that there are other RNA helicases, which have overlapping functions, such as the highly similar URH49 (Pryor et al., 2004). However, down-regulation of UAP56 led to a decrease in cytoplasmic Cyclin D1 mRNAs which is expected because UAP56 is also implicated upstream of the Crm1 nuclear export pathway (Figure 15). We found that the down-regulation of UAP56 led to no changes in cytoplasmic influenza mRNA levels (Figure 16). This agrees with data in kidney cells that Crm1 is likely not involved with influenza A viral mRNA nuclear export.

In summary, siRNA against UAP56 suggests that UAP56 is essential for the Crm1 pathway and confirms that influenza does not utilize this pathway. siRNA against Nxf1 lead to indirect inhibition of viral mRNA expression and therefore we could not use this approach to assess the role of Nxf1 in viral mRNA nuclear export. Our experiments with DN-Nxf1 expression were more fruitful and revealed a cell type difference between NP mRNA export in 293T and A549 cells.

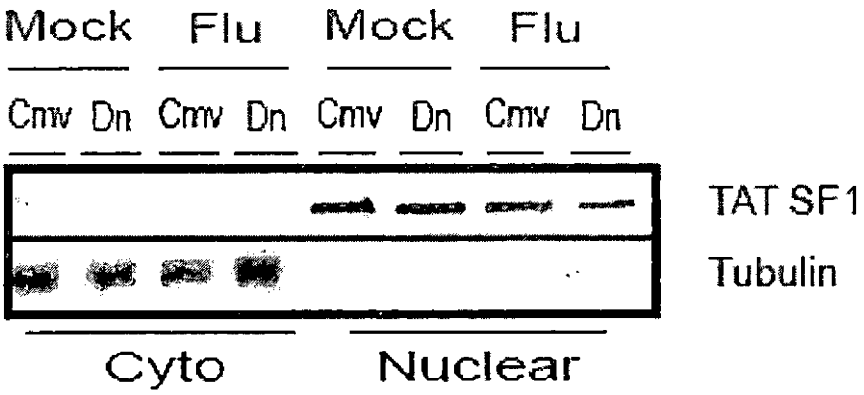


Figure 6. Western Blot of Mock Infected and Infected 293T Kidney Cells.

293T cells were transfected with plasmids expressing dominant negative Nxf1 or vector control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection or mock infected. At 3 hours post infection, cells were collected and cytoplasmic and nuclear RNA was isolated and analyzed by western analysis. α-tubulin was used to detect tubulin, a protein localized to the cytoplasm. α-TAT-SF1 was used to detect TAT-SF1, a protein localized to the nucleus.

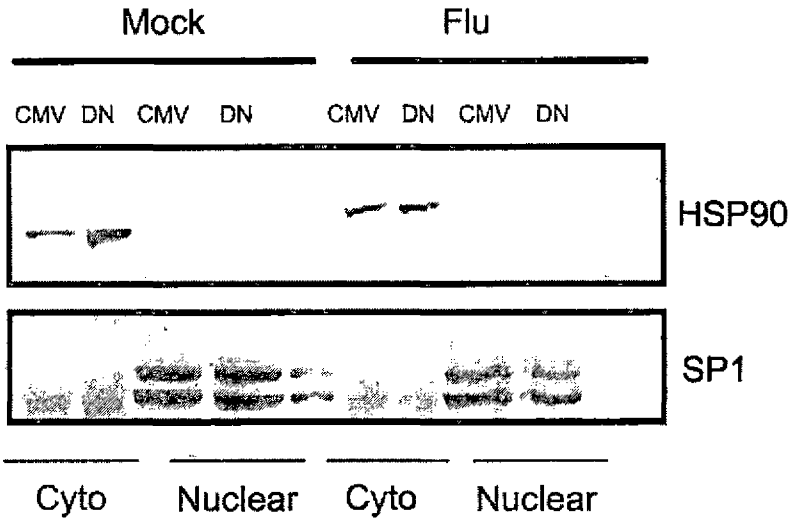


Figure 7. Western Blot of Mock Infected and Infected A549 Lung Cells.

A549 cells were transfected with plasmids expressing dominant negative Nxf1 or vector control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection or mock infected. At 3 hours post infection, cells were collected and cytoplasmic and nuclear RNA was isolated and analyzed by western analysis. α-HSP90 was used to detect HSP90, a protein localized to the cytoplasm. α-SP1 was used to detect SP1, a protein localized to the nucleus.

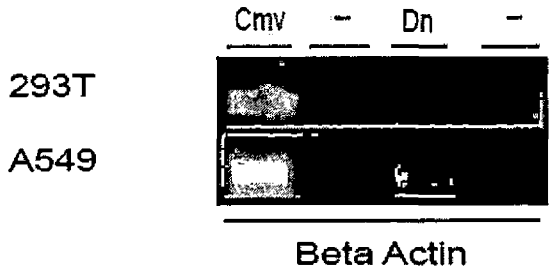


Figure 8. RT-PCR of Host Beta-Actin mRNA in 293T Kidney and A549 Lung Mock infected Cells.

A549 and 293T cells were transfected with plasmid expressing Dn-Nxf1(Dn) or vector as control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection or mock infected. At 3 hours post infection, cells were collected and cytoplasmic and nuclear RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and PCR with gene specific primers as indicated. Host beta actin mRNAs were decreased in the cytoplasm of both A549 and 293T cells expressing dominant negative Nxf1, confirming inhibition of Nxf1-mediated mRNA nuclear export.

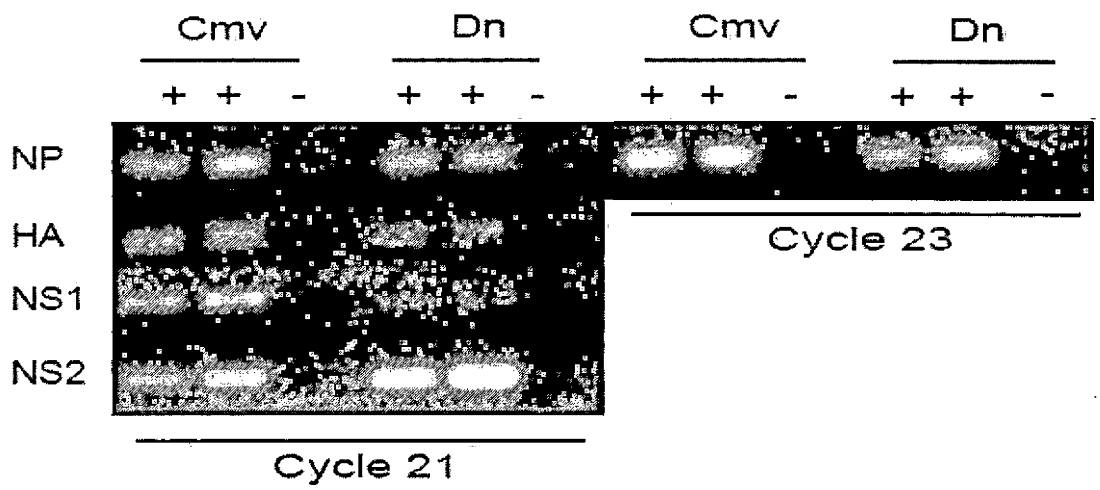


Figure 9. RT-PCR of Influenza Genes in 293T Kidney Cells.

293T cells were transfected with plasmid expressing Dn-Nxf1(Dn) or vector as control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection or mock infected. At 3 hours post infection, cells were collected and cytoplasmic and nuclear RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and PCR with gene specific primers as indicated. Influenza HA and NS1 mRNAs were found in decreased amounts in the cytoplasm of cells expressing Dn. Influenza NP and NS2 mRNAs were not found in decreased amounts.

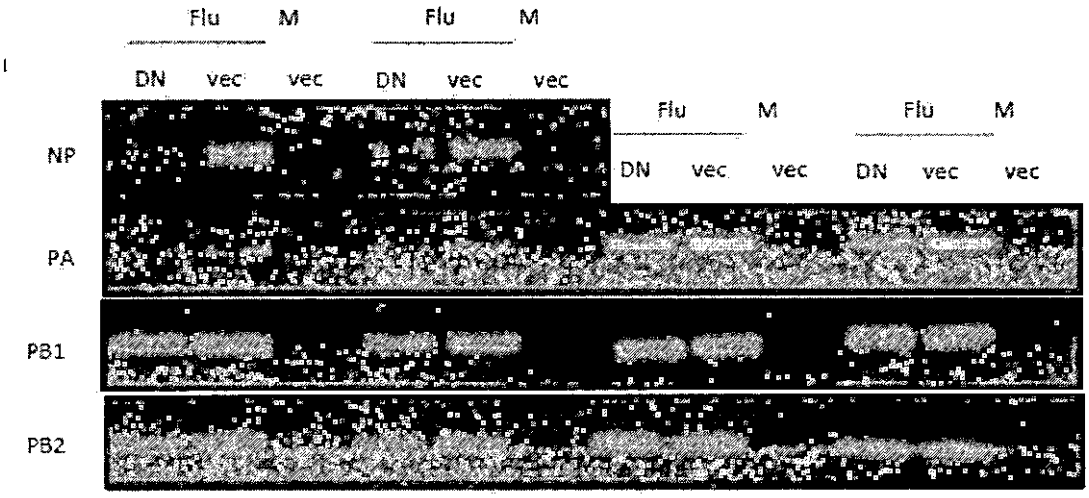


Figure 10. RT-PCR of Influenza Genes in A549 Lung Cells.

A549 cells were transfected with plasmid expressing Dn-Nxf1(Dn) or vector as control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection or mock infected. At 3 hours post infection, cells were collected and cytoplasmic and nuclear RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and PCR with gene specific primers as indicated. PA, PB1, and PB2 mRNAs were not found in decreased amounts in the cytoplasm of cells expressing Dn. However, NP mRNA was.

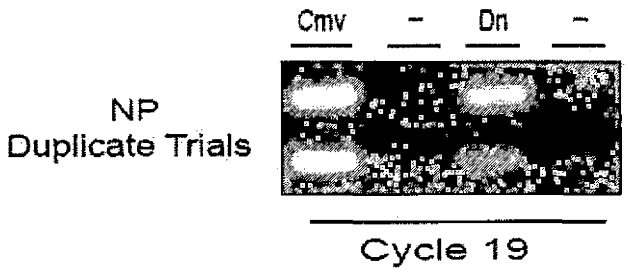


Figure 11. RT-PCR of Influenza NP in A549 Lung Cells.

A549 cells were transfected with plasmid expressing Dn-Nxf1(Dn) or vector as control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection or mock infected. At 3 hours post infection, cells were collected and cytoplasmic and nuclear RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and PCR with gene specific primers as indicated. Duplicate trials show NP mRNAs found in lower amounts in the cytoplasm of cells expressing Dn.

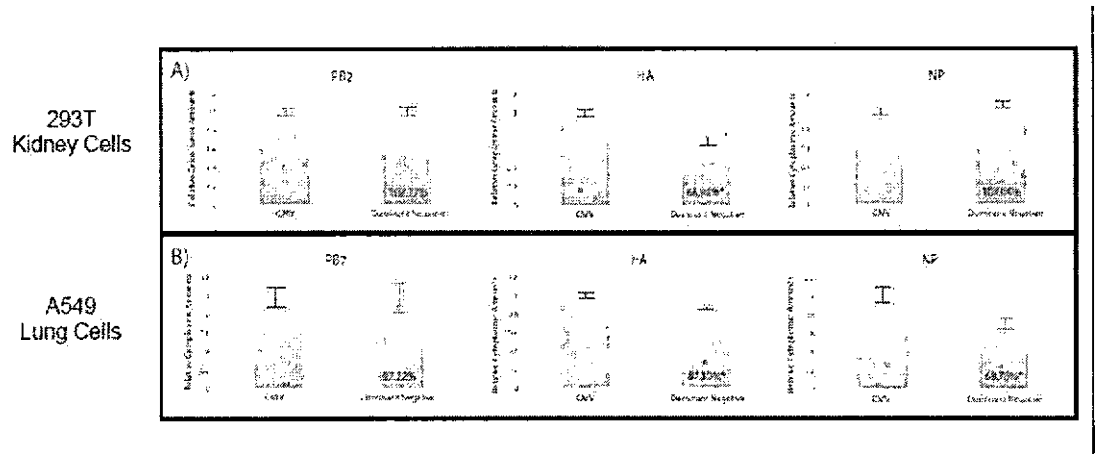


Figure 12. qPCR of Influenza Genes in A549 Lung and 293T Kidney Cells.

A549 and 293T cells were transfected with plasmid expressing Dn-Nxf1(Dn) or vector as control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection or mock infected. At 3 hours post infection, cells were collected and cytoplasmic and nuclear RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and qPCR with gene specific primers as indicated. A) 293T cells, B) A549 cells. Influenza HA mRNAs show a defect in cytoplasmic levels when dominant negative Nxf1 is expressed in both 293T and A549 cells. PB2 mRNAs show no defect in cytoplasmic levels in both 293T and A549 cells when dominant negative Nxf1 is expressed. NP mRNA shows a defect in cytoplasmic levels in A549 cells expressing dominant negative Nxf1, but not in 293T cells. * (P-Value <.05)

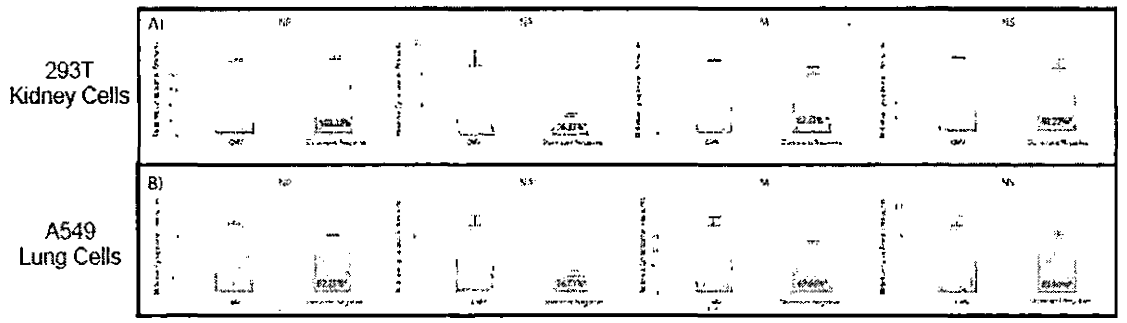


Figure 13. qPCR of Influenza Genes in A549 Lung and 293T Kidney Cells.

A549 and 293T cells were transfected with plasmid expressing Dn-Nxf1(Dn) or vector as control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection or mock infected. At 3 hours post infection, cells were collected and cytoplasmic and nuclear RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and qPCR with gene specific primers as indicated. A) 293T cells, B) A549 cells. Influenza NA, M, and NS mRNAs show a defect in cytoplasmic levels when dominant negative Nxf1 is expressed in both 293T and A549 cells. NP mRNA shows a defect in cytoplasmic levels in A549 cells expressing dominant negative Nxf1, but not in 293T cells. * (P-Value <.05).

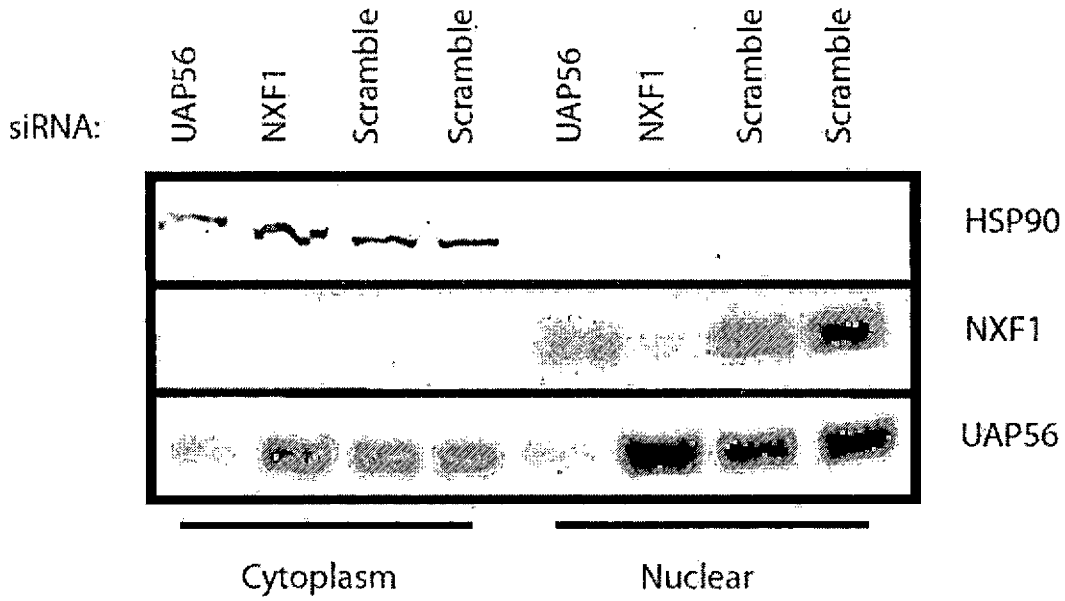


Figure 14. Western Blot of Cells Treated with siRNA Against NXF1 and UAP56 in A549 Lung Cells.

A549 cells were transfected with 2nM siRNA targeting Nxf1, UAP56, or scramble as control. At 48 hours post transfection cells were infected with Influenza A Udorn at high multiplicity of infection. At 3 hours post infection, cells were collected and nuclear and cytoplasmic extracts were prepared. RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and PCR with gene specific primers as indicated. Western blot confirms down-regulation of target genes. Hsp90 is a cytoplasmic localized protein and while Nxf1 is nuclear localized and UAP56 is present in both cytoplasm and nucleus.

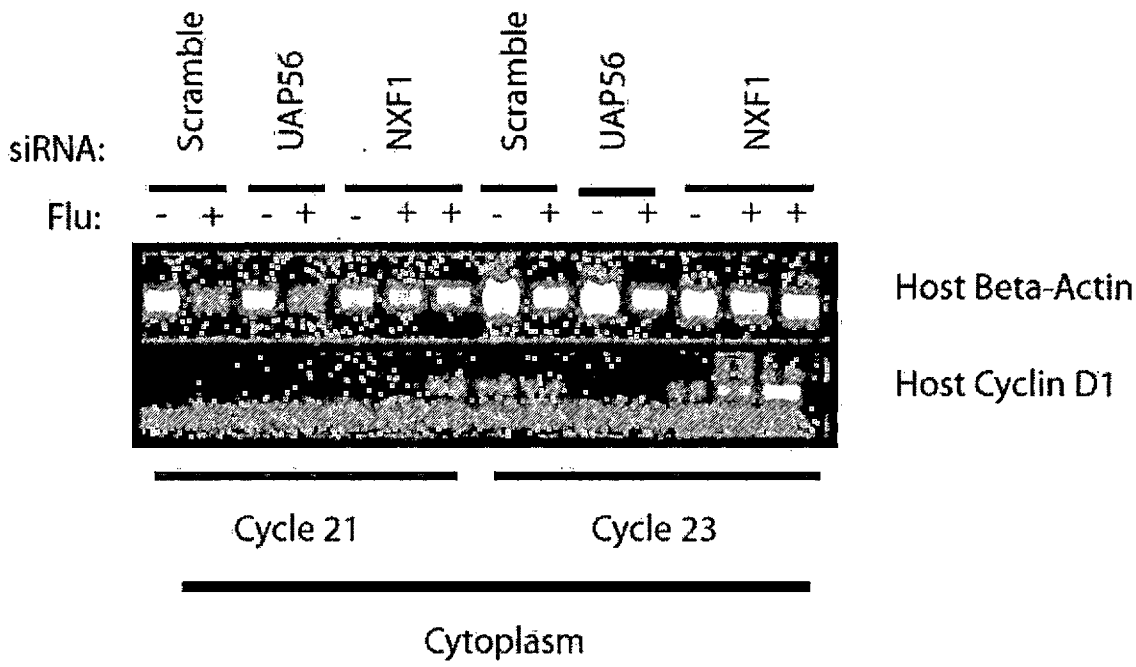


Figure 15. RT-PCR of Host Genes in Cytoplasmic Fraction of NXF1 and UAP56 siRNA Treated A549 Lung Cells.

A549 cells were transfected with 2nM siRNA targeting Nxf1, UAP56, or scramble as control. At 48 hours post transfection cells were infected with Influenza A Udorn at high multiplicity of infection (Flu +) or mock infected (Flu -). At 3 hours post infection, cells were collected and nuclear and cytoplasmic extracts were prepared. RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and PCR with gene specific primers as indicated.

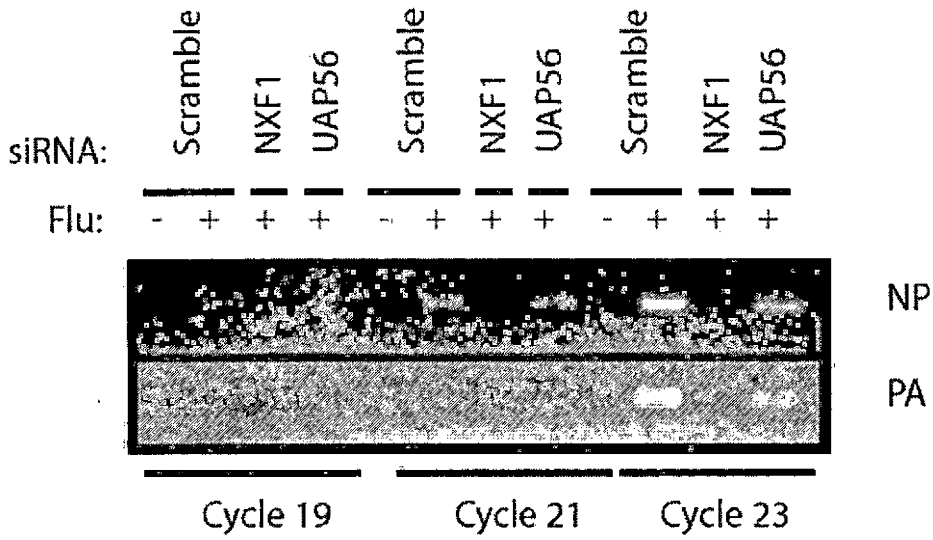


Figure 16. RT-PCR of Infuenza Genes in Cytoplasmic Fraction of NXF1 and UAP56 siRNA Treated A549 Lung Cells.

A549 cells were transfected with 2nM siRNA targeting Nxf1, UAP56, or scramble as control. At 48 hours post transfection cells were infected with Influenza A Udorn at high multiplicity of infection (Flu +) or mock infected (Flu -). At 3 hours post infection, cells were collected and nuclear and cytoplasmic extracts were prepared. RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and PCR with gene specific primers as indicated. Down-regulation of Nxf1 leads to decreased influenza NP, PA, and M mRNA in cytoplasm.

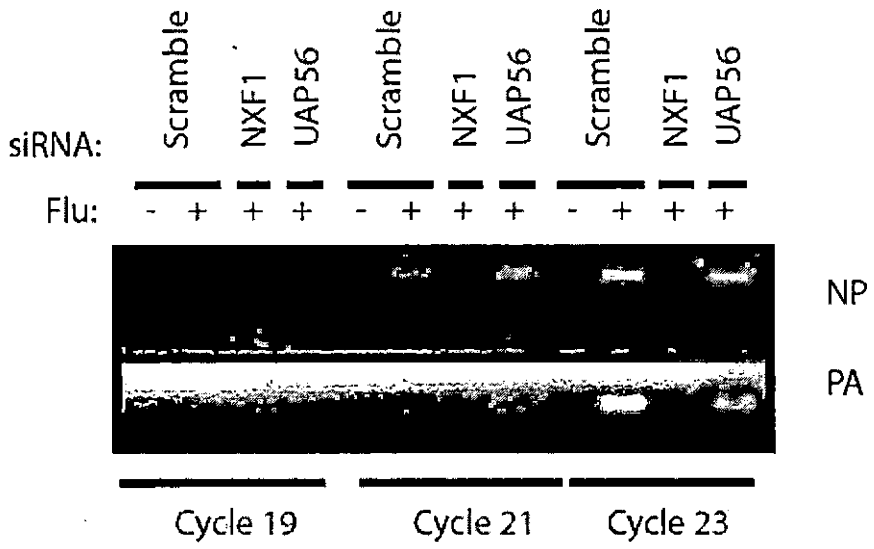


Figure 17. RT-PCR of Infuenza Genes in Nuclear Fraction of NXF1 and UAP56 siRNA Treated A549 Lung Cells.

A549 cells were transfected with 2nM siRNA targeting Nxf1, UAP56, or scramble as control. At 48 hours post transfection cells were infected with Influenza A Udorn at high multiplicity of infection (Flu +) or mock infected (Flu -). At 3 hours post infection, cells were collected and nuclear and cytoplasmic extracts were prepared. RNA was isolated, quantified, and equal concentrations were subjected to reverse transcription with oligo dT and PCR with gene specific primers as indicated. Down-regulation of Nxf1 leads to decreased influenza NP, PA, and M mRNA in nucleus.

# CHAPTER FOUR

# INTERACTION WITH NUCLEAR EXPORT FACTOR NXF1

## Background

Inhibition of mRNA nuclear export through either expression of a dominant negative protein or siRNA treatment will lead to the inhibition of the export and expression of many genes. Some of these genes may encode proteins involved in the transcription and/or export of influenza mRNAs, thus inhibiting influenza mRNA nuclear export via indirect effects. Several host factors, such as Hsp-90 and hCLE have been shown to play a role in the transcriptional activity of the influenza virus (Huarte et al., 2001, Naito et al., 2007). NXF1 is responsible for the nuclear export of the majority of cellular mRNAs. Complete shutdown of this pathway, as with siRNA, likely prevents the export of a host factor necessary for viral replication. Our siRNA results (Chapter 3) support this idea. Additionally, a previous investigation by another lab, found significantly decreased cell viability with siRNA treatment for NXF1 at 48 hours post transfection (Johnson et al., 2009).

In order to alleviate concerns over indirect effects, interaction of Influenza mRNAs with the NXF1 pathway should be confirmed. If influenza mRNAs are to utilize the NXF1 pathway, they must be associated with the NXF1 protein while undergoing the export process.

## Results

Plasmid DNA encoding FLAG-Nxf1 or Flag alone (vector) were transfected into A549 cells. At 48 hours post transfection, the cells were infected at a high MOI for 7 hours with Influenza A Udorn. Total protein was isolated and immunopurified with anti-FLAG antibody under conditions to limit disrupting interactions with influenza mRNAs. The FLAG alone extract serves as negative control. Pull down of Nxf1 was verified via western blot with anti-FLAG (Figure 18). Total and Nxf1 associated mRNAs were reverse transcribed into cDNAs with oligo dT and analyzed via gene specific PCR for identification of influenza mRNAs associated with FLAG -Nxf1. The positive control was 10% of total RNA. NP, M, NS, and HA mRNAs were found to co-immunopurify with the NXF1 protein but not the vector, confirming specific interaction of Nxf1 with the viral mRNAs (Figure 20). The interaction of NP mRNA with FLAG-Nxf1 in A549 has been replicated more than five times. Approximately 13% of the total HA mRNAs were found to be associated with FLAG-Nxf1 via qPCR (Data not shown). Since interaction with the export factor is dynamic, it was expected that only a fraction of the viral mRNAs would be found associated with Nxf1. No PB1, PB2, or PA mRNAs were found associated with NXF1 (Figure 19). These negative results were confirmed with each experiment that showed a positive pull down of NP mRNA. Our results confirm the dependence of NP mRNA on the NXF1 pathway in A549 lung cells, the subject of the cell type difference.

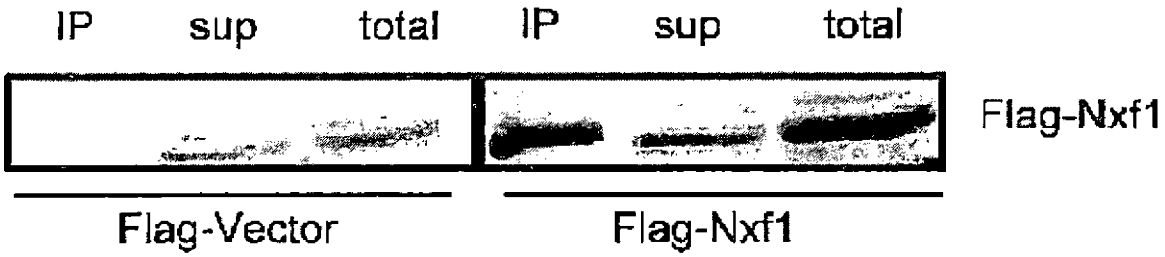


Figure 18. Western Blot of Co-Immunopurification Samples.

A549 cells were transfected with plasmid to express FLAG-Nxf1 or FLAG-vector as negative control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection. At 7 hours post infection, total cell extracts were prepared and subject to immunopurification with anti-FLAG antibody and analyzed by western analysis. α-NXF1 was used to detect the FLAG tagged NXF1 protein. FLAG-Nxf1 was found in the samples expressing FLAG-Nxf1, confirming successful Co-immunopurification.

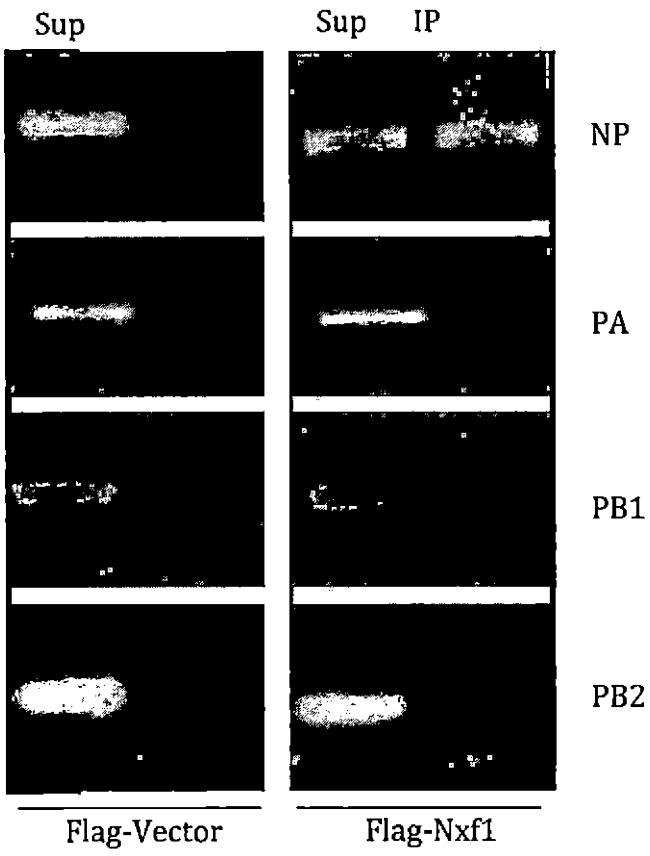


Figure 19. RT-PCR of Influenza Polymerase mRNAs Co-Immunopurified with FLAG-NXF1 and FLAG-Vector.

A549 cells were transfected wth plasmid to express FLAG-Nxf1 or FLAG-vector as negative control. At 48 hours post transfection, cells were infected with Influenza A Udorn at high multiplicity of infection. At 7 hours post infection, total cell extracts were prepared and subject to immunopurification with anti-FLAG antibody. RNA from both the immunopurified complexes (IP) and total cell extracts were quantified and equal concentrations subject to reverse transcription using olido dT and PCR with influenza gene specific primers as indicated. 10% of the RNA from the total RNA samples is used for PCR as positive control. PA, PB1, and PB2 mRNAs were not found associated with FLAG-Nxf1.

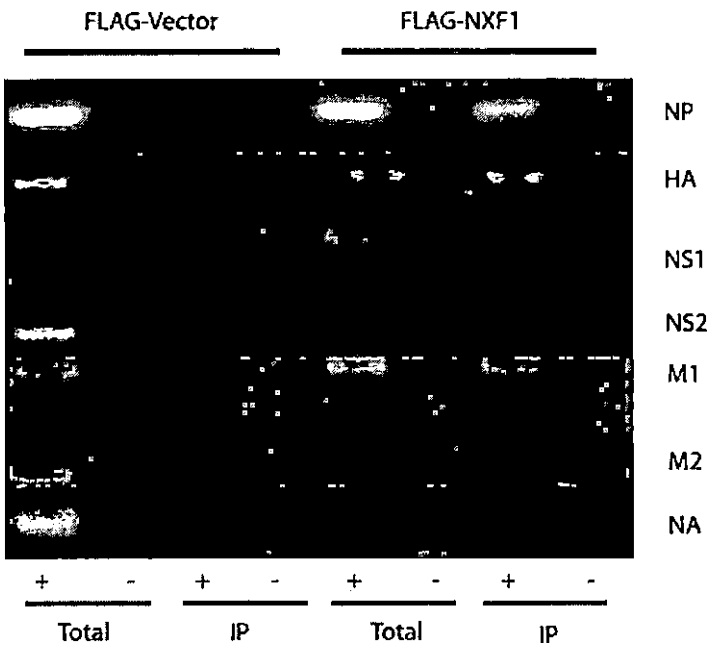


Figure 20. RT-PCR of Influenza Polymerase mRNAs Co-Immunopurified with FLAG-NXF1 and FLAG-Vector.

A549 cells were transfected with plasmid to express FLAG-Nxf1 or FLAG-vector as negative control. At 48 hours post transfection, cells were infected with Influenza A·Udorn at high multiplicity of infection. At 7 hours post infection, total cell extracts were prepared and subject to immunopurification with anti-FLAG antibody. RNA from both the immunopurified complexes (IP) and total cell extracts were quantified and equal concentrations subject to reverse transcription using olido dT and PCR with influenza gene specific primers as indicated. Equal concentration of RNA from the total RNA samples is used for PCR as positive control. NP, HA, NS1, NS2, M1, M2, and NA mRNAs specifically immunopurify with anti-flag antibody when FLAG-Nxf1 is expressed but not in FLAG-vector control.

| Gene | Read and Digard | DN- 293T Kidney | siRNA | DN- A549 Lung | IP- A549 Lung |
|---|---|---|---|---|---|
| *HA* | Nxf1 | Nxf1 | ---------- | Nxf1 | Associated with Nxf1 |
| *NA* | ---------- | Nxf1 | ---------- | Nxf1 | Associated with Nxf1 |
| *NP* | Not Nxf1 | Not Nxf1 | ---------- | Nxf1 | Associated with Nxf1 |
| *M* | Nxf1 | Nxf1 | ---------- | Nxf1 | Associated with Nxf1 |
| *NS* | Nxf1 | Nxf1' | ---------- | Nxf1 | Associated with Nxf1 |
| *PB1* | ---------- | ---------- | ---------- | Not Nxf1 | N/A with Nxf1 |
| *PB2* | Not Nxf1 | Not Nxf1 | ---------- | Not Nxf1 | N/A with Nxf1 |
| *PA* | ---------- | ---------- | ---------- | Not Nxf1 | N/A with Nxf1 |
| | **No use of Crm1** | | **No use of Crm1** | | |

Figure 21. Conclusion Table.

Table summary of DN-NXF1 and co-Immunopurification experiments as well as Read and Digard's results.

# CHAPTER FIVE
# MATERIALS AND METHODS

## Cells

293T (Human embryonic kidney cells) and A549 (Human lung epithelial) cells were purchased from ATCC American tissue culture collection and maintained at 37°C with 5% $CO_2$ in DMEM with 10% FBS.

## Plasmids

Plasmids encoding DN Nxf1 was provided by B. Cullen and plasmid encoding FLAG-NXF1 was provided by J. Steitz (Steitz, Kang & Cullen, 1999). DNA was purified using QIAGEN maxi prep kit per manufacturers protocol.

## DNA Transfection

293T or A549 cells were grown to approximately 70% confluency in 100 mm dishes. The pcDNA plasmids encoding FLAG-NXF1 (10 μg) and FLAG-Vector/eGFP (1 μg), or DN-Nxf1 (10 μg) and CMV (9 μg) with eGFP (1μg) was transfected into cells using Mirus transfection reagent at a ratio of 1:3 (293T cells) or 1:2 (A549 cells), DNA to reagent as specified in manufacturers protocol. eGFP was used to monitor transfection efficiency. 4 8 hrs post transfection one set was mock infected and the other infected at a high MOI (2-5 MOI). Cells were collected 3.5 hrs post infection in Pathway inhibition experiments, and 6.5 hrs post infection in Co-Immunopurification experiments.

SiRNA Transfection

A549 cells were transfected with 2nM SMART pool siRNA (Dharmacon) targeting Nxf1, UAP56, or scramble as control using Dharmacon transfection reagent per manufacturers protocol. At 48 hours post transfection cells were infected with Influenza A Udorn at high multiplicity of infection (Flu +) or mock infected (Flu -). At 3 hours post infection, cells were collected.

Cytoplasmic/Nuclear RNA and Protein Isolation

Cells pellet was washed in 5X volume of cell pellet with Reticulate Standard Buffer (RSB: 10mM Tris HCl pH7.5, 10mM KCl, 1.5mM $MgCl_2$) with protease and RNase inhibitors. Cells were then re-suspended in RSB at 10X the volume of the cell pellet and incubated on ice for 10 minutes. NP-40 was added to a final concentration of 0.2% to disrupt plasma membranes. Visual inspection of the cells before and after addition of NP-40 ensured burst plasma membranes and intact nuclei. Nuclei were pelleted by centrifugation at 300xg for 8 minutes at 4°C. The cytoplasmic extract was collected and the nuclear pellet was re-suspended in Dignam Buffer C without glycerol (20mM HEPES pH 7.9, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA), plus protease and RNase inhibitors, to release nuclear molecules. Both nuclear and cytoplasmic extracts were clarified from debris by high-speed centrifugation for 10 minutes at 4°C. An equal amount of 20mM HEPES pH 7.9, 0.2mM

EDTA was added to the nuclear extract to reduce the total NaCl and $MgCl_2$ concentrations. Some volume was saved for protein analysis. Equal volume of Phenol/Chloroform/Isoamyl alcohol (25:24:1) is added to the cytoplasmic and nuclear fractions. Sample was vortexed 4 times for 10 seconds and placed on ice in between. Samples were centrifuged at 13,000 RPM for 10 minutes at 4°C. Aqueous layer was collected and 0.5 volume $NH_4OAc$ (7.5 M) and 2X volume 100% EtOH was added and RNA was allowed to precipitate overnight at -80°C. Samples were centrifuged at 13,000 RPM for 20 minutes at 4°C. Pellet was washed in 75% EtOH and centrifuged at 13,000 RPM for 5 minutes at 4°C. EtOH was removed and pellet was allowed to air dry for 10 minutes and resuspended in 10 mM Tris in DEPC $H_2O$.

## SDS-PAGE and Western Blot

Protein extracts were separated by SDS-10% PAGE. Proteins were transferred to nitrocellulose using Fisher semi-dry blot apparatus and probed with primary and HRP-conjugated secondary antibodies as indicated. Pierce ECL reagents were used to detect HRP conjugated secondary antibody. Blots were developed using the Chemi-Hi setting on the ChemiDoc™ XRS (BioRad) system and digital images were obtained using Quantity One software.

## Antibodies

α-TAT-SF1, α-Tubulin, α-SP1, α-HSP90, αNXF1, and α-FLAG were purchased from Abcam and used per manufacturers instructions. Secondary HRP coupled α-Mouse and α-Rabbit were purchased from Pierce and used per manufacturer's instructions.

## RT-PCR

RNA was first treated with RNase-free DNase (Promega) to degrade any contaminating DNA. 1µg cytoplasmic and .5µg nuclear RNA were subject to reverse transcription using Promega reverse transcription system per manufacturer's protocol with oligo dT as primer.

## PCR Primers

Gene specific primers for semi-quantitative PCR are as follows:

For NP (5' NP- CCAGAAGAAGTGTCCTTCCG, 3'NP-CGTACTCCTCTGCATTGTCTCC), Beta-Actin (E3Fb-GGACGACATGGAGAAATCTGGCACC, E4Rb-GCGGAACCGCTCATTGCCAATGG), PB1 (5' PB1-CCCCTGAATCCATTTGTCAGCCATA, 3'PB1-ATGAAGGACAAGCTAAATTG),
HA (5'HA- GCTCTGGAGAACCAACATACAA, 3'HA-ACAAGGGTGTTTTTAATTACTAATA), NS (5' NS1/NS2-

GGATTCCAACACTGTG, 3'NS2- GCCATCTTATCTCTTCG), M (5'M-GCCTTCTGACCGAGGTCG, 3'M- CCCAATGATACTCGCAGC), PB2 (5' PB2- CCACCCAGATAATAAAGCTTCTCCCC, 3'PB2-GTCAGTAAGTATGCTAGAGTCCCG), PA (5'PA-ATGACCAAAGAGTTTTTTGAGAATA, 3'PA-GTATGGATAGCAAATAGTAGCATTG), and Cyclin D1 (5'UTRCyclin-CGGTCACCTAGCAAGCTGCC, 3'UTRCyclin-CAGGACAGACTCCGCTGTGA).

## Immunopurification

Total protein extracts were incubated with α-FLAG antibody (Stratagene) (1:50) and protease inhibitors for 1 hour at 4° C. Extracts were then incubated with PA/G sepharose beads with protease inhibitors at 4° C overnight. The samples were then spun for 8 seconds at 13,000 RPM at 4° C. Supernatant was collected. Beads were washed 3 times in 1 mL sonication buffer with RNase inhibitors. With each wash samples were then centrifuged for 8 seconds at 13,000 RPM at 4° C. 1/3 of beads from last wash is saved for protein analysis, and 2/3 of supernatant is saved for RNA isolation.

## RNA Isolation

Equal volume (RNA containing resuspended beads) of Phenol/Chloroform/Isoamyl alcohol (25:24:1) is added to the resuspended bead immunopurification sample and total extract (for the positive control). Sample was vortexed 4 times for 10 seconds and placed on ice in between. Samples were centrifuged at 13,000 RPM for 10 minutes at 4°C. Aqueous layer was collected and 0.5 volume $NH_4OAc$ (7.5 M) and 2X volume 100% EtOH was added and RNA was allowed to precipitate overnight at -80°C. Samples were centrifuged at 13,000 RPM for 20 minutes at 4°C. Pellet was washed in 75% EtOH and centrifuged at 13,000 RPM for 5 minutes at 4°C. EtOH was removed and pellet was allowed to air dry for 10 minutes and resuspended in 10 mM Tris in DEPC $H_2O$.

## Total Protein Isolation

Cell pellets were resuspended in 1 mL of Sonication Buffer (100mM Tris HCl pH 7.5, 100mM NaCl, 2.5 mM $MgCl_2$, 0.5% Triton x-100) with protease and RNase inhibitors. Cells were lysed using Fisher Scientific Sonic Dismembrator for 30 pulses at 30%, output 3-4. Sonicated materials were loaded onto a 30% sucrose cushion (30% Sucrose, 10 mM Tris HCl pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$) and centrifuged at 4000 RPM for 15 minutes at 4°C to clarify total protein extract.

## Quantitative PCR: Protocol and Primers

Quantitative PCR reactions were run in the Applied Biosystems StepOnePlus Real Time PCR system using Sybr Green Master mix (Applied Biosystems)

Gene specific primers PCR are as follows:

NA(Forward-TGTGTGCTCAGGGCTTGTTG, Reverse-CTCCGTGATTCCCTTTCTCATT), HA(Forward-ACTGAAGTCAGGATACAAAGACTGGAT, Reverse-CCCCAGCAAAACAACACAAA), NP(Forward-GTGTGCAACCTGCATTTTCTGT, Reverse-TCTGAGGTTCTTCCCTCCGTATT), PA(Forward-GGACAAATGGAACATCAAAGATTAAA, Reverse-CAGAAGACTCGGCTTCAATCATG), PB1(Forward-GGGAAAGGATACATGTTCGAAAGT, Reverse-ACTTTAGGTCAATGCTTGCTAGCA), PB2(Forward-AATAAAGCTTCTCCCCTTTGCA, Reverse-CCCTCACATTCACAGTCAATGAA)

## Statistical Analysis of QPCR Data

Raw CT values were analyzed in Microsoft Excel using the ΔCt/ΔCt formula of $2^{CT_{\text{average Control sample}}}/2^{CT_{\text{average Treated sample}}}$. Two Tailed T-Test was conducted in Microsoft Excel, and any p value greater than .05 was judged to be an insignificant difference. Error bars were obtained by

calculating the standard deviation of the sample set divided by the square root of the sample set size

# CHAPTER SIX

# DISCUSSION AND FUTURE RESEARCH

## Summary

A critical step for Influenza A infection is the localization of the vRNPs to the host cell nucleus, the site of virus replication and transcription (Cros et al., 2005, Resa- Infante, Martin-Benito et al., 2001) (Figure 2). Once the viral transcripts are expressed, M and NS can undergo splicing, and all the mRNAs must exit the nucleus in order to be translated in the cytoplasm. The major structure involved in nuclear trafficking is an assembly of nucleoporins located within the nuclear envelope. This assembly of nucleoporins is termed the Nuclear Pore Complex (NPC) (Durairaj et al., 2009). Molecules with a molecular weight less than 40,000 to 50,000 are understood to freely diffuse through the NPC. However, larger molecules require a nuclear localization signal (NLS) or a nuclear export signal (NES). These epitopes allow proteins to be actively transported into or out of the nucleus, through interaction with components of the NPC (Cros et al., 2005). RNAs are transported by interaction with proteins in ribonucleoprotein complexes or RNPs.

Human mRNA nuclear export through the NPC is regulated by the interaction of proteins, generally termed Nuclear Export Factors (NXF), and mRNA (Durairaj et al., 2009). Nuclear Export Factor 1 (NXF1/TAP) is the primary human cellular mRNA nuclear export factor, shuttling mRNAs from

the nucleus to the cytoplasm (Carmody & Wente, 2009) (Figure 5). It is presumed that influenza mRNAs are associated with host proteins for mRNA nuclear export. However, there is conflicting data regarding which host pathway is utilized and the exact host proteins involved (Wang et al., 2008, Read & Digard, 2010, Satterly et al., 2006).

The Satterly study and the Wang study both showed that influenza NS1 protein interacts with host Nxf1 in kidney cells. Our lab has also confirmed this interaction in A549 cells (Data not shown). While the Satterly lab concluded the interaction with NS1 was to inhibit host mRNA nuclear export, the Wang paper concluded that NS1 may be involved in viral mRNA nuclear export because their study suggested some viral mRNAs export through Nxf1. The role of the NS1-Nxf1 interaction remains undefined, but it is clear that the Nxf1 nuclear export is not inhibited during infection as the Wang study, the Read and Digard study, and my own research conclude many viral mRNAs depend on expression of Nxf1 for export from the nucleus.

The Read and Digard study was the first to show evidence that not all viral mRNAs utilize Nxf1. Coupled with evidence from multiple studies showing Crm1 is not involved in viral mRNA nuclear export, these results indicate that some viral mRNAs utilize an undefined mRNA nuclear export pathway. Our data make a significant contribution to the published literature. First, we confirm that Crm1 is not utilized for viral mRNA export, as exemplified by inhibition of the upstream protein, UAP56, which specifically

inhibits cyclin D1 mRNA nuclear export but no viral mRNAs. Second, we confirm that some viral mRNAs utilize Nxf1 and others do not rely on either Nxf1 or Crm1 (as seen by inhibition of UAP56) (Figures 9,10, 12,13, 16,17, 19 & 20). This confirms that some viral mRNAs utilize an as yet undefined nuclear mRNA export pathway and identification of this pathway may reveal potential novel antiviral targets. Importantly, our data reveal a cell type difference in use of Nxf1. This finding has implications for interpretation of previous studies, as kidney cells may not be the best model for studying the influenza A virus.

This research is important is because it adds to our understanding of the influenza virus life cycle and supports the existence of an undefined mRNA nuclear export pathway. Identification of this atypical pathway utilized by influenza may reveal new insights into cellular biology and provide novel antiviral targets to fight influenza.

## Discussion

Most pertinent to this study is the Read and Digard study which found that the siRNA inhibition of the NXF1 pathway, in 293T cells, resulted in nuclear retention of HA, M, and NS but not NP or PB2 mRNAs (Read & Digard, 2010). Additionally, Read and Digard and Wang et. al. both concluded that the inhibition of Crm1 did not alter influenza mRNA export (Wang et al., 2008, Read & Digard, 2010). According to August Krough, a Nobel laureate

physiologist, with each scientific question, there is an ideal model to be used to answer that question (Krough, 1929). Previous studies investigating the use of the host mRNA nuclear export pathways by the influenza virus have used 293T and MDCK cells as the model of choice. Influenza infects the upper respiratory tract, and thus kidney cells may not be the best choice for characterizing influenza infection.

It is also difficult to understand how Read and Digard were able to obtain results using siRNA to inhibit Nxf1. Using identical conditions, we found that influenza viral mRNA expression is inhibited under this condition. siRNA is an extremely efficient method to down regulate target gene expression, resulting in nearly complete inhibition of the pathway. NXF1 is responsible for the nuclear export of the majority of cellular mRNAs. Complete shutdown of this pathway, as with siRNA, likely prevents the export of a host factor necessary for viral replication. Our findings echo a previous investigation, which found significantly decreased cell viability with siRNA treatment for NXF1 at 48 hours post transfection (Johnson et al., 2009).

Previous studies in 293T cells have shown that PB2 and NP do not exhibit dependence upon the Nxf1 pathway, but that HA, M, and NS do (Read & Digard, 2010). DN experiments in 293T cells not only confirmed previous findings, but expanded upon them by showing that not only was M, NS, and HA mRNA dependent upon Nxf1, but NA mRNA was as well. In agreement with Read and Digard, NP and PB2 mRNAs were not dependent upon Nxf1.

Interestingly, when we examine nuclear export in A549 lung cells, we see that not only are NA and HA mRNAs dependent upon Nxf1 but so is NP mRNA. These results reveal an important difference between the two cell types. This is the first report of a cell type difference in influenza A mRNA nuclear export.

Our method of inhibition varied from the siRNA approach used in the Read and Digard study, and we replicated their results, in the same cell type (293T cells). While our methods varied, our conclusions were the same. Since inhibition of the Nxf1 pathway in DN experiments is dependent upon efficiency of transfection, as well as the fraction of DN-Nxf1 compared to wild type Nxf1, the range of inhibition can vary from experiment to experiment. Despite this, the pattern remains the same, and we do not see variance in which mRNAs show dependence upon Nxf1 export.

If influenza mRNAs are to utilize the NXF1 pathway, it is assumed they must be associated with the NXF1 protein while undergoing the export process. Read and Digard did not confirm this interaction, but the Wang study did, and concluded that NA, M and PB1 mRNAs co-immunopurified with NXF1. In A549 lung cells, NP, NA, HA, M, and NS mRNAs all showed dependence upon the Nxf1 pathway in DN experiments and thus we should find them associated with the Nxf1 protein. This is indeed what we find. Also confirming previous findings, as well as our own experiments, the polymerase (PB1, PB2, and PA) mRNAs were not found to co-immunopurify with FLAG-Nxf1, and therefore we conclude not dependent upon the Nxf1 pathway. The

only difference is where we do pull down NS2 mRNA with the NXF1 protein, but do not find NS2 mRNA to be dependent upon NXF1, in 293T kidney cells, the DN-NXF1 experiments. This leaves the verdict on NS mRNA nuclear export to be determined.

Taken as a whole, the DN and co-immunopurification experiments provide compelling evidence that cell type differences exist and should be taken into consideration when interpreting studies examining influenza infection dynamics. Knowing that influenza infects the respiratory tract and armed with knowledge that there is a cell type difference at play, at least for viral NP mRNA nuclear export. For this reason, A549 cells appear to be the better cell model.

## Future Directions

Future direction of this study should be to examine the role of other nuclear export pathways in influenza A mRNA nuclear export. UAP56 is known to be upstream of both NXF1 and Crm1, and siRNA data supports the conclusion that Crm1 does not play a role in influenza mRNA nuclear export (Figure15). However, Leptomyocin B treatment to block Crm1 export in both A549 and 293T cells, would confirm this pathway is not used in A549 cells as well as previous findings in 293T cells. The next step would be to determine whether or not exportin-t or exportin-5 play roles in influenza A mRNA nuclear export. This would involve co-immunopurification experiments for each, as

well as pathway inhibition experiments. Beyond these experiments, if the polymerase mRNAs are not dependent upon Nxf1, Crm1, exportin-t, or exportin-5 mediated mRNA nuclear export, which protein is responsible for their export? Is it host or viral? Regardless of its origin, if it is responsible for the nuclear export of RdRP mRNAs, then it should be interacting with the mRNA. The viral mRNAs can be used to biochemically or genetically screen for interacting proteins. For example, a yeast three hybrid, or a biochemical pull down of modified mRNAs. The answer to these questions could provide very exciting revelations in our understanding of the human cell, or even provide novel influenza antiviral targets. My thesis lays the groundwork for future revelations.

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